CHARACTERIZATION AND LETHALITY OF 4,5',8-TRIMETHYLPSORALEN AND 5-METHYLISOPSORALEN ADDUCTS ON VIRAL DNA IN PROKARYOTIC AND EUKARYOTIC CELLS

by

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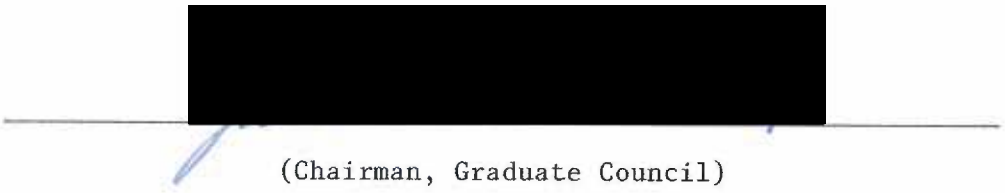

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

human fibroblasts with MV40, MV40-4, and human xenotropic murine leukemia virus.

ABBREVIATIONS

Ad 2	Adenovirus Type 2
AGMK	African green monkey kidney
AMT	4'-Aminomethyl-4,5',8-trimethylpsoralen hydrochloride
ATCC	American Type Culture Collection
bp	Base Pairs
3-CP	3-Carbethoxypsoralen
cis-pt (II)	Cis-diamminedichloroplatinum (II)
DEB	Diepoxybutane
4,4'-DMA	4,4'-Dimethylangelicin or 4,4'-Dimethylisopsoralen
4,5'-DMA	4,5'-Dimethylangelicin or 4,5'-Dimethylisopsoralen
DMC	Decarbamoyl mitomycin C
DMEM	Dulbecco's modified Eagle's medium
EMS	Ethylmethane sulfonate
FA	Fanconi's anemia
FCS	Fetal calf serum
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HGMCR	Human genetic mutant cell repository
HI-FCS	Heat inactivated fetal calf serum
HMT	4'-Hydroxymethyl-4,5',8-trimethylpsoralen
HN2	Nitrogen mustard
HPLC	High-performance liquid chromatography
HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
λ	Lambda bacteriophage

5-MA	5-Methylangelicin or 5-Methylisopsoralen
MEM	Minimal essential medium, Eagle
MEME	Minimal essential medium (Eagle's)
5-MIP	5-Methylisopsoralen or 5-Methylangelicin
MMC	Mitomycin C
MMS	Methylmethanesulfonate
m.o.i.	Multiplicity of infection
5-MOP	5-Methoxypsoralen
8-MOP	8-Methoxypsoralen
MULV	Murine leukemia virus
MV40	Moloney murine leukemia-simian virus 40 recombinant virus
NEAA	Nonessential amino acids
NM	Nitrogen mustard
4NQO	4-Nitroquinoline-1-oxide
PFUs	Plage forming units
PUVA	8-Methoxypsoralen plus UVA
SCE	Sister chromatid exchange
SV40	Simian virus 40
4,4',6-TMA	4,4',6-Trimethylangelicin or 4,4',6-Trimethylisopsoralen
TMP	4,5',8-Trimethylpsoralen
UDS	Unscheduled DNA synthesis
UV	Ultraviolet light (254 nm)
UVA	Long-wavelength ultraviolet light (320 nm to 400 nm)
XP	Xeroderma pigmentosum

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ABSTRACT

The monofunctional and bifunctional furocoumarins 5-methylisopsoralen (5-MIP) and 4,5',8-trimethylpsoralen (TMP), respectively, have been employed to characterize the ability of xeroderma pigmentosum (XP) and Fanconi's anemia (FA) fibroblasts to repair photoinactivated herpes simplex virus type 1 (HSV-1) by host cell reactivation. XP fibroblasts were generally found to have reduced levels of 5-MIP monoadduct repair but were shown to repair TMP crosslinks at similar levels to those observed for repair proficient human fibroblasts. FA fibroblasts rescued 5-MIP and TMP photoinactivated HSV-1 at levels equivalent to repair proficient human fibroblasts regardless of the cell age in relation to cell senescence. One FA fibroblast strain was shown to have reduced colony forming ability after exposure to TMP. HSV-1 photoinactivation kinetics produced by 5-MIP provide a reliable method for characterizing DNA repair deficiencies in XP fibroblasts, while such a method did not detect a deficiency in FA crosslink repair. This apparent discrepancy may be explained by the fact that one crosslink is lethal for HSV-1 in repair proficient mammalian cells. However, the 5-MIP monoadduct level required for a lethal event is considerably higher and therefore might detect different levels of DNA repair deficiencies in XP cells.

Quantitation of the 5-MIP and TMP levels and characterization of the TMP-nucleic acid adducts formed during photoinacitvation of simian virus 40 (SV40) and lambda (λ) bacteriophage showed that the photoinactivation kinetics do not accurately describe the relationship between adduct level

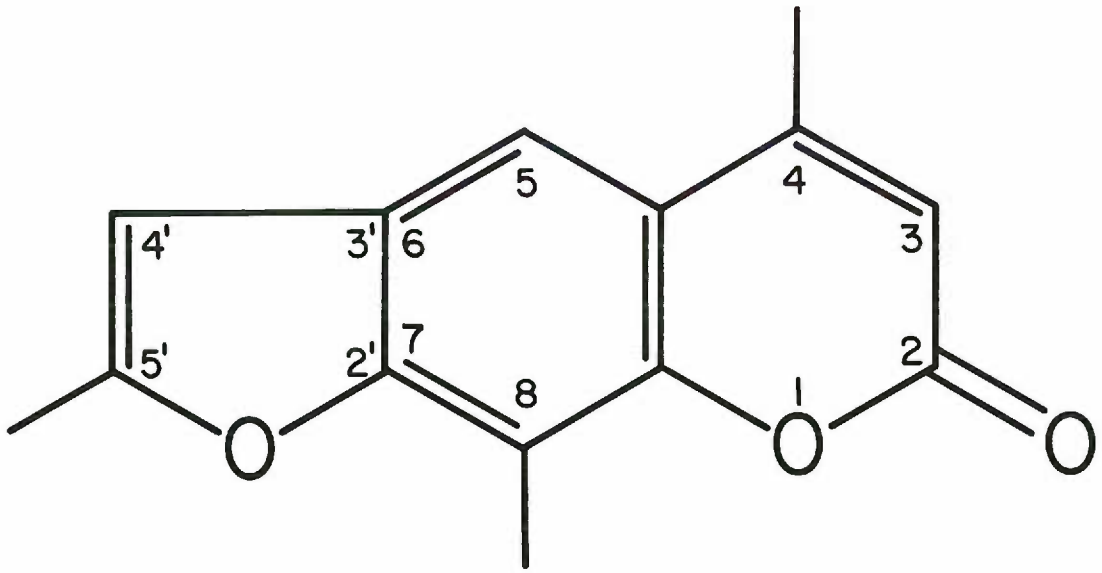
and lethality. Photoinactivation kinetics suggested that λ was more sensitive than SV40 and HSV-1 to both furocoumarins. However, when the surviving fractions were correlated with photoaddition based on adduct levels per viral genome it suggested that λ was considerably less sensitive than either SV40 or HSV-1. Although the eukaryotic viruses HSV-1 and SV40 have extremely different genome sizes, they were approximately equal in their sensitivities to 5-MIP and TMP. Correlation of surviving fractions with TMP and 5-MIP adduct levels per 1,000 base pairs (bp) eliminates the differences in photoaddition rates and emphasizes the effect of target size. The order of sensitivity to adduct level per 1,000 bp is HSV-1 > λ > SV40 for 5-MIP and TMP. The TMP-nucleic acid adducts present on the λ and SV40 DNAs for various points during the photoinactivation kinetics were characterized by enzymatic hydrolysis and high-performance liquid chromatography to determine the level of crosslinks and monoadducts per viral genome. TMP photoinactivation of λ and SV40 followed by dialysis to remove unbound molecules and a second long-wavelength ultraviolet irradiation (UVA) showed a reduction in virus titer which corresponded to an increase in crosslink levels per λ and SV40 genomes at the expense of furan-side monoadducts. Thus continued killing of λ and SV40 by UVA irradiation in the absences of unbound TMP occurs due to the formation of crosslinks. In the absence of multiplicity reactivation, one TMP crosslink represents a lethal event for SV40 while λ appears to have an increasing tolerance to crosslinks as the total TMP adduct level increases per genome. The level of 5-MIP monoadducts per viral genome that produces a lethal event is considerably higher for λ than for SV40 or HSV-1.

I. Introduction and Statement of the Problem

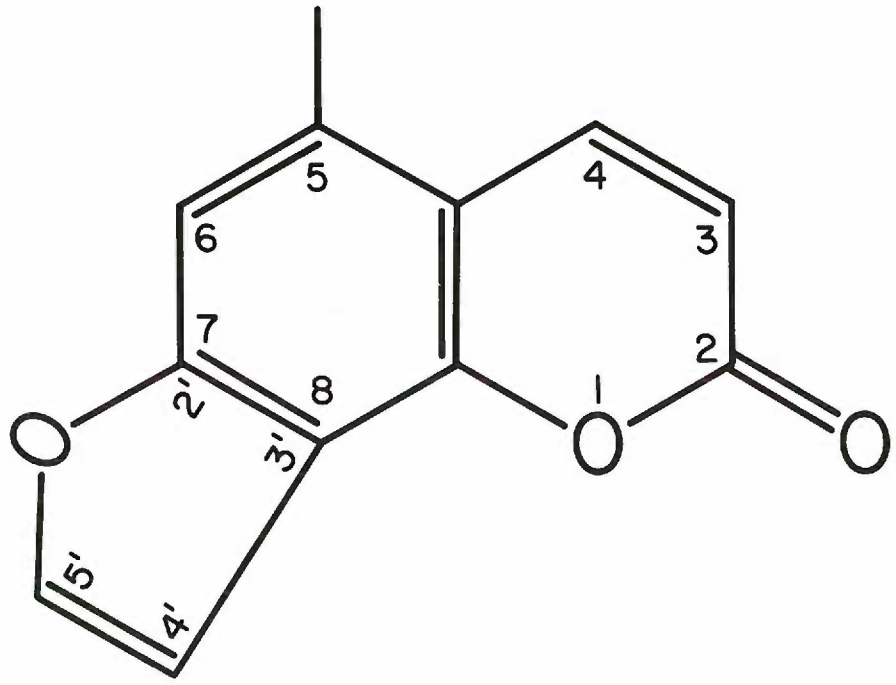
The long range objective of this research is to ultilize psoralen photodamaged viruses as a probe to understand eukaryotic repair pathways. This thesis project consists of two areas of research. One approach is to characterize the ability of Xeroderma pigmentosum (XP) and Fanconi's anemia (FA) cells to rescue psoralen photoinactivated herpes simplex virus (HSV) for two psoralen derivatives: 5-methylisopsoralen (5-MIP) and 4,5',8-trimethylsporalen (TMP) (Fig. 1). The former is an isopsoralen that only produces monoadducts while the latter psoralen derivative forms crosslinks efficiently. This will allow a comparison of two distinct repair pathways: the excision repair system that has been studied primarily by UV-inactivation and would be expected to repair monoadducts, and a more complex pathway such as a recombination or post-replication pathway that would be expected to repair crosslinks, if in fact they can be repaired at all. The second approach will consist of an examination of what constitutes a lethal event in psoralen photoinactivation of viruses. Again, a comparison of 5-MIP and TMP photoinactivation kinetics will be determined, in this case for the prokaryotic lambda bacteriophage as well as the eukaryotic papovavirus Simian Virus 40 (SV40). In conjunction with these photoinactivation kinetics the adduct level for each psoralen compound will be determined for each virus as a function of viability. This investiagation will include an examination of the effect of converting TMP monoadducts to crosslinks on the lethality of the adduct to each virus.

Figure 1. The chemical structures of TMP and 5-MIP.

4,5',8–TRIMETHYLPSORALEN (TMP)



5–METHYLISOPSORALEN (5–MIP)



Furocoumarins (psoralen and its derivatives) have been used to photoinactivate a variety of viruses and cell types. Intact cells and viruses are readily permeable to these compounds, which intercalate into the stacked bases of the DNA without resulting in permanent damage. In the presence of long-wavelength ultraviolet light (UVA, 320 to 420nm) furocoumarins bind covalently with pyrimidine residues via a cyclobutane ring. A second photoevent allows pyrimidines located on the opposite DNA strand in an adjacent base pair to react, forming a crosslink. Although there are approximately two to three times more monoadducts than crosslinks, crosslinks are thought to be responsible for virus inactivation. Experiments utilizing psoralen derivatives such as 5-MIP, an isopsoralen that does not produce crosslinks, also implicate monoadducts in virus inactivation, although psoralen crosslinking derivatives (e.g. TMP) are more effective in virus inactivation.

Furocoumarins provide distinct advantages over other methods of studying DNA repair mechanisms. One is that they allow comparison of the DNA repair of two different types of adducts produced by related compounds (i.e. 5-MIP and TMP) under similar conditions. This eliminates the problem of comparing two distinctly different types of DNA damaging compounds that produce monoadducts and crosslinks under dissimilar conditions and may be repaired by different or varied DNA repair pathway(s). Another advantage is that furocoumarins can be radioactively labelled. This allows for relatively easy determination of adduct level per viral or host genome along with the comparison of the lethality of monoadducts and crosslinks by photoinactivation kinetics. Ultimately it also provides an assay to develop <u>in vitro</u> repair systems by monitoring the removal of radioactive psoralen from the DNA. Lastly, since TMP can

produce both monoadducts and crosslinks, the percentage of both types of adducts as well as the distribution of various monoadduct stereoisomers can be determined by high pressure liquid chromatography (HPLC) and correlated with lethality in virus photoinactivation.

Human fibroblasts from patients with DNA repair defects provide a valuable tool for elucidation of eukaryotic DNA repair pathways. XP and FA are two autosomal recessive diseases correlated with DNA repair deficiencies. Genetic studies with XP fibroblasts and cell fusion techniques indicate that at least nine complementation groups (A-I) and one variant group exist. Fibroblasts from all complementation groups except the variant exhibit reduced levels of excision repair after UV irradiation. XP variants have near normal or normal levels of excision repair of pyrimidine dimers, but are defective in the postreplication repair pathway. FA fibroblasts exhibit an increased susceptibility to bifunctional compounds such as the crosslinking agents mitomycin C (MMC) and nitrogen mustard (NM). Recent literature however, has presented conflicting reports on the sensitivity of FA cells to bifunctional compounds. In one report, FA cell senescence has been correlated with defective removal of NM crosslinks, while cells in early passage repaired NM crosslinks. Fibroblasts from several XP complementation groups and and one variant will be studied by examining the photoinactivation kinetics of HSV for TMP and 5-MIP to determine whether the DNA repair defect of the cells affects the removal of psoralen crosslinks (TMP) or monoadducts (5-MIP). Similar experiments will be done for FA cells to determine their sensitivity to monoadducts and crosslinks at both early and late cell generations. By comparing the repair of 5-MIP and TMP in late generation cells one can ask if the loss of TMP repair is a specific

loss of the repair of crosslinks or a total loss of any type of DNA repair, a question that has not been addressed in the literature.

The ability to transform and immortalize XP fibroblasts will be investigated by infecting cells with a xenotropic murine leukemia virus (MuLV) enveloped retroviral vector containing the early region of SV40 (MV40). Cell transformation will be tested by the focus forming transformation assay. XP immortalization by MV40 will involve the growth of both transformed and untransformed fibroblasts in culture until cell senescence and/or continuous growth is observed. XP fibroblast transformation will provide a valuable tool for biochemical investigation of their DNA repair pathways, since transformed cells have shorter generation times and increased cell densities in tissue culture over their untransformed counterparts. This will facilitate biochemical investigations of DNA repair due to an increased abundance of biological material for isolation of biological molecules and in vitro repair studies. Immortalization of XP fibroblasts will ensure that specific cell strains that have already been extensively characterized for their defects in DNA repair are not lost due to death of the patient or finite generations of human fibroblasts.

The lethal event in psoralen photoinactivation of lambda and SV40 will be investigated in two ways. One is to establish the 5-MIP photoinactivation kinetics of each virus with radiolabelled isopsoralen. At specific times on the inactivation curve samples of each virus will be removed and the viral DNA isolated. Subsequently the adduct level per genome will be determined for each time point. This approach will allow a correlation of monoadduct level per virus genome with the amount of biological killing observed on the photoinactivation kinetic curves. A

second approach will be to examine the photoinactivation kinetics of TMP on both viruses in order to assess the lethality of TMP crosslinks. Both viruses will be photoinactivated with radiolabelled TMP to establish the photoinactivation kinetics. Samples will be taken at various times and dialyzed to remove any unbound TMP. Following dialysis, samples taken at early times will be reirradiated, and then subsequently assayed for biological activity. Since TMP has the ability to form both monoadducts and crosslinks this experiment is designed so that in the absence of any unbound TMP the loss of biological activity should be due to the conversion of monoadducts to crosslinks that occurs following a second photochemical event. Isolation of the viral DNA from both the initial irradiation time point and the corresponding reirradiated samples will provide several pieces of information. One is that it will correlate the biological killing to the adduct level per virus genome for each irradiation time. It will also ensure that no free TMP is being added or bound TMP lost during the reirradiation steps, since the adduct level of the reirradiated samples should be the same as for the initial time points from which it was derived. Again, since TMP can form two types of adducts, each DNA sample from the inital and reirradiated times will be digested to the nucleoside level followed by separation of monoadducts and crosslinks on HPLC for each time point. Determination of the percentages of monoadducts and crosslinks at various adduct levels for each sample will allow correlation of the crosslink level with biological activity. Also by knowing the percentage of each type of adduct formed by TMP, a comparison to the 5-MIP photoinactivation kinetics and adduct levels can be made in order to assess the difference if any between psoralen and isopsoralen monoadducts. It has been assumed in the

literature, but by no means demonstrated, that the two are biologically equivalent.

Although both lambda and SV40 contain duplexed DNA within an icosahedral capsid the two viral DNAs are packaged in association with different proteins. While lambda DNA is packaged with polyamines, SV40 DNA is found in a nucleosomal structure associated with host histone proteins similar to that found in eukaryotic chromatin. The morphological differences in the two virus structures coupled with the two different hosts provide a comparison of two distinctly different systems to investigate the effect of psoralen monoadduct and crosslink repair.

A. Specific Aims

I. Investigate human DNA repair pathways by examining the ability of seven complementation groups and one variant of XP fibroblasts to rescue psoralen photoinactivated HSV (Manuscript 2 and Appendix A).

1. Determine 5-MIP photoinactivation kinetics of HSV treated with 2.0 μg/ml 5-MIP and an average light intensity of 2.5 mW/cm^2 on eleven fibroblast strains.

2. Determine TMP photoinactivation kinetics of HSV treated with 0.1 μg/ml TMP and an average light intensity of 0.9 mW/cm^2 on eleven fibroblast strains.

3. Determine TMP photoinactivation kinetics of HSV treated with 0.01 μg/ml TMP and an average light intensity of 2.5 mW/cm^2 on eleven fibroblast strains.

II. Investigate the effect of cell senescence on human DNA repair pathways of several FA fibroblasts by quantitating the rescue of psoralen inactivated HSV treated with concentrations of 2.0 ug/ml 5-MIP and 0.01 ug/ml TMP and an average light intensity of 2.5 mW/cm^2 (Manuscript 3).

1. Determine 5-MIP and TMP photoinactivation kinetics of HSV on early generation FA fibroblasts.

2. Determine 5-MIP and TMP photoinactivation kinetics of HSV on FA fibroblasts of approximately 45 generations.

III. Compare 5-MIP and TMP photoinactivation kinetics of HSV as rescued by a representative of XP complementation group A fibroblasts transformed by SV40 to those rescued by non-transformed fibroblasts (Manuscript 2 and Appendix A).

IV. Determine the ability of a murine retroviral vector containing the early region of SV40 (MV40) to transform and immortalize XP fibroblasts (Appendix B).

1. Compare the ability of ecotropic and xenotropic MuLV enveloped MV40 to transform XP and normal fibroblasts by the focus forming transformation assay.

2. Examine the ability of xenotropic MV40 to immortalize XP cells.

3. Determine the ability of xenotropic MV40 containing both large T and little t or large T only to transform cells by the focus forming assay.

V. Characterize the lethal event of lambda and SV40 psoralen photoinactivation (Manuscript 4).

1. Establish lambda and SV40 photoinactivation kinetics for 5-MIP and TMP.

2. Correlate the photoinactivation kinetics of lambda and SV40 with their viral DNA adduct levels of 5-MIP and TMP.

3. Examine the effect of TMP crosslinks on photoinactivation of lambda and SV40. This involves the irradiation of both viruses in the presence of TMP followed by removal of unbound drug by dialysis. Early irradiation times are then reirradiated for given periods to convert TMP monoadducts to crosslinks. These reirradiated time points are then assayed biologically and their TMP adduct levels compared to the original photoinactivation curve and adduct levels.

4. Digest lambda and SV40 DNA isolated from the above TMP reirradiation experiments to nucleosides and separate the thymine crosslinks, and furan-side and pyrone-side monoadducts on HPLC to determine the percentage of crosslinks and monoadducts per time point sampled on the photoinactivation curve.

VI. Development of optimal conditions for titration of SV40 by the plaque assay method (Manuscript 1).

1. Determine the optimal adsorption volume for detecting the maximum number of plaque forming units (PFUs).

2. Identify the optimal adsorption time of virus to cell monolayers for maximum titer.

3. Examine the effect of fetal calf serum concentration and agar overlay volume on plaquing efficiency.

4. Compare plaque number and size under four different overlay media and agar feeding protocols.

5. Determine the postincubation period when maximum virus titers are obtained.

6. Examine the effect of DEAE-Dextran, dextran sulfate, and DMSO supplements to agar overlay medium on plaque size, number, and incubation period required for maximum virus titer detection.

II. Literature Review

A. Psoralens

1. Introduction

Psoralens have provided a strong tool for investigation of nucleic acid structure and function and have been extensively reviewed (Cimino et al., 1985). Briefly, psoralens have been utilized to examine the nucleic acid structures and functions of DNA and RNA found in prokaryotes, eukaryotes, and viruses of each system. Psoralens have been applied to the study of chromatin structure, cruciform formation, secondary-structure in single-stranded DNA, secondary structure of ribosomal RNA, DNA torsional tension, secondary structure of transfer RNA, secondary structures in heterogeneous and small nuclear RNAs, fixation of nucleic acid complexes, secondary structure in viral RNA genomes, and tertiary interactions in nucleoprotein complexes (Cimino et al., 1985). Since the basis of this thesis deals with the biological response to psoralens and isopsoralen a review of the literature will be confined to the biological consequences of these compounds. Psoralen photochemistry and its reaction with DNA has also been extensively reviewed (Hearst et al., 1984; Hearst, 1981).

The primary advantage of psoralens is that they readily enter and exit cells and viruses and in the absence of light, generally do not cause any damage to the biological entity. Upon entry of psoralens into cells and viruses, psoralens photoreact with nucleic acids by three sequential events. Intercalation of psoralens between the stacked DNA

base pairs is termed dark binding. The second event results in psoralen monoadduct formation that covalently binds the psoralen molecule to DNA by photoexcitation with long-wavelength ultraviolet light (UVA, 320 to 400nm). This produces formation of a cyclobutane ring between the 5,6 double bond of an adjacent pyrimidine (primarily thymine) and either the 4',5' double bond of the furan ring or the 3,4 double bond of the pyrone ring of the psoralen molecule (Fig. 1). Furan monoadducts retain an absorbance between 320 to 380nm and can be excited by a second photon to form a second cyclobutane ring between the psoralen pyrone double bond and the adjacent 5,6 double bond of the pyrimidine. This crosslink formation within the DNA helix represents the third event (Hearst et al., 1984).

Although it is clear that the biological consequences of bifunctional isopsoralen plus UVA may be lethal or mutagenic, the mechanism by which crosslinking derivatives do so is controversial. This controversy arises from the observation that UVA irradiation of bifunctional psoralens results in a mixture of monoadducts and crosslinks with a continuously changing ratio during their photoreaction. Therefore kinetic analysis of survival curves and mutation frequencies is difficult to interpret, although a concensus has been reached concerning the biological consequences of both types of adducts. Exposure of bifunctional psoralens to low UVA light intensity over an extended period of time results in a biological response similar to monofunctional psoralens plus UVA, while irradiation of bifunctional psoralens with the same UVA dose administered at a high light intensity over a shorter time interval confers an increased lethality due to an increase in crosslinks (Averbeck, 1982).

Psoralens have achieved clinical significance as photosensitizing chemicals efficient in the treatment of certain dermatological diseases. They have an important role in photochemotherapy (psoralen plus UVA or PUVA) of vitiligo (Lerner et al., 1953; Arora et al., 1976), psoriasis (Parrish et al., 1974; Melski et al., 1977; Roenigk, 1979; Vella Briffa et al., 1981) and to a lesser extent diseases such as parapsoriasis, mycosis fungoides (Gilchrist et al., 1976; Roenigk, 1984; DuVivier and Vollum, 1980), atopic dermatitis, polymorphous light reaction, lichen planus, and pityriasis (Song P.-S. and Tapley, 1979). Although the efficacy of PUVA therapy of psoriasis approaches 90 percent and is cost-effective as well as being acceptable by the patient relative to other forms of therapy (Stern et al., 1981; Roenigk, 1981), it is not without significant acute and potential chronic side effects. Nausea, insomnia, depression, localized blistering, pruritis (Wolff, 1977) and cataract formation (Freeman and Troll, 1969) have been observed. Biological changes due to PUVA include mutagenesis (Vella Briffa and Warin, 1979; Bridges and Strauss, 1980), carcinogenesis (Stern et al., 1979; Halprin, 1980; Roenigk and Caro, 1981), skin hyperpigmentation (Parrish et al., 1971), and immunological alterations that include inhibition of cell-mediated immunity (Morhenn et al., 1980), temporary impairment of cutaneous delayed cellular hypersensitivity (Bridges et al., 1981) contact allergy to psoralen (Weissmann et al., 1980), immunosuppression of contact hypersensitivity (Kripke et al., 1983), and the production of somatic mutations in circulating lymphocytes in situ (Bridges et al., 1981).

Furocoumarins (psoralens and its derivatives) are found naturally in over two dozen plant sources including Rutaceae (e.g. bergamot, lime,

clove, other citrus fruits), Umbelliferae (e.g. celery, parsnip, carrot, Ammi majus), Leguminosae (e.g. pea bean, peanut, Psoralea corylifolia) and Moraeceae (e.g. fig) (Pathak et al., 1962, Benedetto, 1977; Pathak et al., 1974). Early medical writings have dated the use of psoralen photochemotherapy for treatment of vitiligo back to approximately 1200-2000 B.C. (Benedetto, 1977; Fitzpatrick and Pathak, 1984). Physicians, herbalists, and Hindu priests used boiled extracts of seeds from Ammi majus in Egypt or seeds from Psoralea corylifolia (scurfy pea or Bavachee) in India to treat vitiligo. These extracts were either applied to the patient's skin topically or ingested orally followed by exposure of the skin to the intense sunlight. This form of therapy is still in current use in the late twentieth century in India, Pakistan, China, and the Far East (Pathak et al., 1984).

Modern psoralen research began in 1938 when a phytophotodermatitis of the skin was investigated and found to occur when the skin came into contact with certain psoralen containing plants and UV light (Kuske, 1939). Artificial light sources were found to stimulate melanogenesis. Successful repigmentation of vitiligo had been recorded 5 years earlier (Uhlmann, 1934). In the late 1940's the three psoralens 8-methoxypsoralen (8-MOP, ammoidin, xanthotoxin or methoxalen), 5-methoxypsoralen (5-MOP, bergapten or majudin), and 8-isoamylenoxypsoralen (ammidin) were isolated in crystalline form from the powdered seeds of Ammi majus (Fahmy and Abu-Shady, 1947). One year later crystalline 8-MOP followed by exposure to sunlight was used for the treatment of vitiligo (El Mofty, 1948).

2. Erythemal Response, Hyperpigmentation, and Carcinogenesis.

The theraputic benefits of furocoumarins in dermatology is well recognized. They are currently used in the repigmentation of leukodermic spots characteristic of vitiligo, for treatment of psoriasis and other skin diseases, and in cosmetic preparations, for increased suntanning and subsequent tolerance to sunlight. However, these compounds are not without adverse acute side effects including phototoxicity (erythema and edema) and inflammatory changes, as well as the chronic conditions of skin cancer and aging of the skin. Two families of furocoumarins exist: the psoralens and the angelicins. The bifunctional linear parent furocoumarin psoralen has the ability to form DNA monoadducts and crosslinks while the monofunctional angular parent furocoumarin angelicin (isopsoralen) can only form monoadducts. Information is accumulating that psoralen crosslinks are mainly responsible for these adverse side effects whereas monofunctional furocoumarins appear to be less harmful. The following discussion in this section and subsequent sections will compare the biological consequences of monofunctional and bifunctional furocoumarins.

The cellular responses of both erythema and hyperpigmentation to psoralens plus UVA result in an increase in: a) the number of melanocytes; b) the number of melanosomes synthesized by proliferating melanocytes; c) the amount of melanization in melanosomes due to increased tyrosinase activity in melanocytes; and d) the number of melanosomes transferred to keratinocytes (Rodighiero, 1985).

Ranked in the order of highest to lowest degree of erythemal response to bifunctional furocoumarins are psoralen > TMP > 8-MOP > 5-methoxypsoralen (5-MOP). This is the same pattern as seen for hyperpigmentation induction by bifunctional furocoumarins (Pathak and

Fitzpatrick, 1959). Monofunctional furocoumarins such as 4,5'-dimethylangelicin (4,5'-DMA), 5-MIP, 3-carbethoxypsoralen (3-CP), isopsoralen, and 4,4',6-trimethylangelicin (4,4',6-TMA) are nonerythematogenic (Baccichetti et al., 1984; Baccichetti et al., 1981; Wolff and Honigsmann, 1984; Mullen et al., 1984). 4,4'-Dimethylangelicin (4,4'-DMA), a monofunctional furocoumarin, causes erythema formation but only in the presence of concentrations and light intensity 5 and 4 times greater, respectively, than 8-MOP (Baccichetti et al., 1984; Baccichetti et al., 1981).

Comparison of the monoadduct furocoumarins, pyrido (3,4-C) psoralen and 7-methyl (3,4-C) psoralen, to 8-MOP and 5-MOP at the same concentrations and UVA light intensity, demonstrated that the pyridopsoralens produced a slight erythema and limited hyperpigmentation compared to the severe phototoxicity of the bifunctional furocoumarins (Dubertret et al., 1985). Monofunctional derivatives of isopsoralens have been demonstrated to cause moderate hyperpigmentation (Rodighiero, 1985), although this has not been observed for 3-CP (Dubertret et al., 1978). In general, monofunctional furocoumarins result in moderate or no erythema production and hyperpigmentation relative to the high levels produced by bifunctional furocoumarins.

Both monofunctional and bifunctional furocoumarins have been found to be carcinogenic to mice. However, it was observed that 4,5'-DMA and 5-MIP were more carcinogenic than 8-MOP (Mullen et al., 1984). No relationship has been found between 8-MOP DNA crosslinks and tumor incidence in mice (Grube et al., 1977). Pyridopsoralens are 3 to 4 times less carcinogenic than 8-MOP under identical experimental conditions

(Dubertret et al., 1985). Methylisopsoralen is weakly carcinogenic and 3-CP is noncarcinogenic (Mullen et al., 1984; Dubertret et al., 1978).

Although 8-MOP is most widely used for treatment of psoriasis, both 5-MOP and TMP are also effective in treatment (Wolff and Honigmann, 1984). The monoadduct producing pyridopsoralens were slightly more efficient than 8-MOP in the treatment of psoriasis under identical conditions (Dubertret et al., 1985). The monofunctional furocoumarins gave poor clinical results in the treatment of psoriasis and use of 3-CP has produced conflicting data on its efficacy (Wolff and Honigsmann, 1984; Dubertret et al., 1978).

The literature so far concludes that bifunctional psoralen derivatives are clinically effective in the treatment of psoriasis because of their ability to form DNA crosslinks that are considered the essential molecular lesion in PUVA (Wolff and Honigsmann, 1984). However, two lines of evidence argue against this. Crosslinks in the epidermal DNA of patients treated with psoralens have only been detected after psoralens were administered in high doses and not in doses used in the PUVA clinical treatment (Lerche et al., 1979; Cech et al., 1979). Secondly, it has been demonstrated that a second UVA dose after removal of nonbound psoralen resulted in the conversion of DNA monoadducts to crosslinks in cultured Chinese hamster cells and was correlated with increased cell killing (Ben-Hur and Elkind, 1973a). This reirradiation approach in PUVA treatment of psoriasis however has shown no better response than regular PUVA therapy (Wolff and Honigsmann, 1984).

Results from 4 prospective studies of PUVA treated patients indicates a significant and dose-related increase in the risk of squamous cell carcinoma (Stern, 1984). Although differences in study populations,

methodologies, and levels of psoralens plus UVA (PUVA) occurred between the 4 studies, the conclusion was that premalignant and malignant lesions were more frequent in patients with higher total exposure to PUVA. A higer incidence of skin tumors was found in patients having prior exposure to carcinogenic treatments before use of PUVA (Stern, 1984; Halprin et al., 1984). This observation gives rise to the hypothesis that PUVA acts as a pseudopromoter or cocarcinogen, allowing expression of preexisting potentially malignant cells when the immune control system is blocked by PUVA (Bridges et al., 1981). The observed squamous cell carcinomas produced by PUVA have rarely metastasized and in this manner resemble sunlight induced squamous cell carcinomas. No significant relationship between PUVA exposure and increased risk of biologically aggressive squamous cell carcinomas, malignant melanomas, or lymphoreticular neoplasms has been observed (Stern, 1984). Other studies have not found an increased incidence in skin cancer of PUVA treated patients without prior exposure to a carcinogen (Roenigk and Caro, 1981; Honigsmann et al., 1980; Lassus et al., 1981; Halprin et al., 1984; Hensler and Christophers, 1984). This discrepancy in skin cancer incidence may be related to the differences in PUVA doses (Halprin et al., 1984; Stern, 1984).

3. Chromosomal Aberrations and Sister Chromatid Exchange

Chromosomal aberrations in human cells were only induced by isopsoralen at high doses of UVA whereas low levels of light were sufficient to cause breakage with 8-MOP and 5-MOP (Natarajan et al., 1981). Induced aberrations were predominantly of the chromatid type.

8-MOP induced chromosomal aberrations in Chinese hamster ovary cells occurred at significantly lower concentrations than khellin, a furochromone that infrequently produces crosslinks (Abeysekera et al., 1983). Use of the reirradiation protocol demonstrated that 8-MOP DNA crosslinks in L5178Y mouse lymphoma cells are at least 10 to 20 fold more effective in producing chromosomal aberrations than the monoadducts from which they are produced (Hook et al., 1983; Liu-Lee et al., 1984b). It was also observed that crosslinks induced new aberrations in second-division cells due to lesions that survived the first round of replication. Dicentric aberrations were observed in first division cells while chromatid deletions and exchanges occurred in second division cells (Hook et al., 1983). Lymphocytes from psoriasis patients treated first with PUVA did not exhibit any chromosomal aberrations or sister chromatid exchanges (SCE) (Brogger et al., 1978). However, when lymphocytes were removed from the blood of patients, treated by the PUVA protocol but not exposed to UVA, and then irradiated in vitro, chromosomal aberrations were induced (Swanbeck et al., 1975). This indicates that the UVA dose in vivo is substantially less than that in vitro and it alone does not result in chromosomal aberrations.

SCE levels in Chinese hamster ovary cells and human diploid fibroblasts induced by 8-MOP and 5-MOP gave similar results, although the human cells are generally more sensitive. Isopsoralen yielded the lowest level of SCE in both Chinese hamster ovary cells and human diploid fibroblasts, with the latter being more sensitive (Ashwood-Smith et al., 1982). By either increasing the concentration of 8-MOP, 5-MOP or 5-MIP or the amount of UVA a linear increase in Chinese hamster ovary cell SCE occurred. 5-MIP gave the lowest level of induction, whereas 3-CP

required a 20 fold higher concentration before an increase in SCE occurred (Loveday and Donahue, 1984). The ratio of induced mutations to induced SCE was similar for the linear furocoumarins 8-MOP, 5-MOP and 3-CP while it was much higher for the angular furocoumarin 5-MIP (Loveday and Donahue, 1984). Again it was demonstrated that on an equal molar basis 8-MOP was a more important inducer of SCE than isopsoralen, but this difference was correlated with the fact that 8-MOP is more efficient in forming monoadducts than isopsoralen indicating that monoadducts are responsible for SCE in Chinese hamster ovary cells (Linnainmaa and Wolff, 1982). It was also demonstrated that 8-MOP produced lesions of short duration that induced SCE in the first round of DNA replication and that isopsoralen lesions of longer duration lasted for more than one round of replication. Although TMP is a more potent inducer of SCE than 8-MOP (Carter et al., 1982), the hypersensitivity of the X-linked genodermatosis disease, dyskeratosis congenita, to SCE induction by TMP is controversial (Carter et al., 1979; Kano and Fujiwara, 1982).

While evidence has been presented that monoadducts are responsible for SCE (Linnainmaa and Wolff, 1982), experiments in human cells using PUVA treatment followed by a second UVA irradiation showed a marked increase in SCE (Bredberg and Lambert, 1983). It should be noted that PUVA treatment alone did not produce a change in the frequency of SCE observed in control cells. Another report has also concluded that 8-MOP monoadducts in Chinese hamster ovary cells produced by laser light are equally effective as crosslinks in inducing SCE (Sahar et al., 1981), but caution should be taken in comparing human and Chinese hamster ovary cells. The monofunctional linear furocoumarin 5,7-dimethoxycoumarin induced almost the same number of SCE per unit dose of UVA in Chinese

hamster ovary cells as 8-MOP (Ashwood-Smith et al., 1983). Yield of SCE per human fibroblast at equal molar concentrations of furocoumarins and increasing UVA produced an order of effectiveness for the linear part of the induction curves of 7-methyl pyrido [3,4-c] psoralen > pyrido [3,4-c] psoralen > 8-MOP >> 3-CP (Billardon et al., 1984). The monofunctional furocoumarins 3-CP and 7-methyl pyrido [3,4-c] psoralen induced a very slight increase in SCE during the dark reaction (without UVA) (Billardon et al., 1984).

Evidence generally suggests that crosslinks are more potent inducers of chromosomal aberrations than monoadducts. It is not yet clear what role monoadducts or crosslinks have in SCE induction. However, no attempt has been made to correlate the level of chromosomal aberrations and SCE with the number of crosslinks and monoadducts per genome after treatment with mono- and bifunctional furocoumarins that have different DNA binding constants.

4. Mutagenesis and Genetic Recombination

While it is clear that furocoumarins plus UVA are mutagenic to prokaryotes and eukaryotes, several reports have described furocoumarin induced mutagenesis during dark binding (without UVA). 8-MOP has been reported to be a frameshift mutagen in Escherichia coli and Salmonella typhimurium (Bridges and Mottershead, 1977; Clarke and Wade, 1975). Further studies have substantiated this frameshift mutagenesis in E. coli and S. thyphimurium for the bifunctional furocoumarins 8-MOP, 5-MOP, TMP, psoralen and the monofunctional furocoumarins pyrido [3,4-c] psoralen, 7-methyl pyrido [3,4-c] psoralen, 5-MIP, and 5,7-dimethoxycoumarin

(Lecointe, 1984; Quinto et al., 1984; Ashwood-Smith, 1978; Ashwood-Smith et al., 1980; Monti-Bragadin et al., 1981; Ashwood-Smith et al., 1983). Frameshift mutagenesis has been shown to be recA$^+$ dependent (Bridges and Mottershead, 1977; Lecointe, 1984).

When mutation frequencies in E. coli produced by furocoumarins plus UVA are expressed as a function of survival, mutagenic efficiency was ranked in the following order: 8-MOP > psoralen > 4-methylsporalen > 4-methyl-4',5'-dihydropsoralen (Fujita, 1984). The monofunctional dihydropsoralen required much higher UVA doses to induce mutations than the bifunctional psoralens. Other monofunctional furocoumarins such as 4,4',6-TMA and 4,4'-DMA had reduced mutagenic activity, while 4,5'-DMA was almost nonmutagenic when compared to the bifunctional psoralen 8-MOP and psoralen (Baccichetti et al., 1981; Baccichetti et al., 1984).

Utilizing 8-MOP and the reirradiation protocol it was demonstrated that E. coli deficient in excision repair (uvrA$^-$) and killed by one crosslink per genome resulted in a reduced mutation frequency due to the increase in lethal crosslinks, while in repair proficient E. coli, crosslinks are more mutagenic than monoadducts (Bridges et al., 1981).

8-MOP and 8-isoamylenoxypsoralen were capable of producing about the same number of revertants in Chlamydomonas reinhardii while the angular monofunctional 5-MIP showed weak mutagenic activity (Abel and Schimmer, 1981). Both monoadducts and crosslinks produced by 8-MOP are capable of inducing mutations in Aspergillus nidulans (Scott and Maley, 1981).

Monoadducts of isopsoralen and crosslinks of 8-MOP both induce nuclear mutations in wild type Saccharomyces cerevisiae although the latter produces a higher induction frequency (Grant et al., 1979). A radiation sensitive S. cerevisiae strain, that is excision repair

deficient, is more sensitive to mutation induction by both furocoumarins. The two monofunctional pyridopsoralens, pyrido [3,4-c] psoralen and 7-methyl pyrido [3,4-c] psoralen, produce fewer nuclear mutations in S. cerevisiae than the monofunctional compound 3-CP which was less effective than 8-MOP (Averbeck et al., 1985; Dubertret et al., 1978). Examination of the ability of furocoumarins to induce cytoplasmic "petite" mutations involving damage to S. cerevisiae mitochondrial DNA where excision repair is nonexistent (Waters and Moustacchi, 1974; Magana-Schwencke et al, 1982), gives the following order of highest to lowest inducers per unit UVA dose and equimolar furocoumarin concentration: TMP > 5-MOP > 3-CP > 8-MOP > 4,5'-DMA (Averbeck et al., 1984) and 7-methyl pyrido [3,4-c] psoralen > pyrido [3,4-c] psoralen > 3-CP > 8-MOP (Averbeck et al., 1985; Dubertret et al., 1978). However, if one examines the order of furocoumarin nuclear mutation induction a different pattern is observed: TMP > 5-MOP > 8-MOP > 4,5'-DMA > 3-CP (Averbeck et al., 1984). The monofunctional furocoumarin 3-carbethoxyhomopsoralen produces higher levels of cytoplasmic "petite" mutations and was similar in nuclear mutation induction to 3-CP in S. cerevisiae (Averbeck et al., 1985).

Mutation induction in Chinese hamster ovary cells was greatest when exposed to 5-MOP, followed by 8-MOP and 5-MIP, with the lowest inducer being 3-CP (Loveday and Donahue, 1984). Using the reirradiation protocol and 8-MOP it was demonstrated that crosslinks resulted in an increased induction of mutations in Chinese hamster ovary cells over their respective monoadducts (Babudri et al., 1981). In the absence of UVA, 8-MOP did not cause mutations in Chinese hamster cells, nor in the presence of UVA were mutations detectable in mouse fibroblasts (Uwaifo et al., 1983). Mutation induction in Chinese hamster ovary cells was higher

for 4,5'-DMA than 8-MOP when either the UVA radiation or furocoumarin concentration was varied while the other remained constant (Swart et al., 1983). The comparison of several different monofunctional and bifunctional furocoumarins to produce mutations in Chinese hamster ovary cells from highest to lowest inducing capacity was 7-methyl pyrido [3,4-c] psoralen > pyrido [e,4-c] psoralen > 8-MOP > 3-CP (Dubertret et al., 1985; Averbeck et al., 1984). Again by either varying the UVA dose or furocoumarin concentration while keeping the other parameter constant, the monofunctional 4,5'-DMA was more mutagenic in human skin fibroblasts than 8-MOP (Swart et al., 1983).

Although it appears that both monoadducts and crosslinks are mutagenic the number of each type of lesion per genome that constitutes a mutagenic event has not been determined and therefore may be leading to inappropriate conclusions. It is also suggested that different eukaryotic or prokaryotic cells may respond differently to mutation induction by mono- and bifunctional furocoumarins, making it difficult to formulate generalized conclusions.

5. Furocoumarin Transformation

8-Methoxypsoralen in the presence of UVA will transform established mouse fibroblast cell lines (Burger and Simons, 1978; Uwaifo et al., 1983). The transforming ability of several furocoumarins on mouse embryonic cells demonstrated that 7-methyl pyrido [3,4-c] psoralen > 8-MOP > pyrido [3,4-c] psoralen as a function of UVA dose, and 8-MOP > 7-methyl pyrido [3,4-c] psoralen > pyrido [3,4-c] psoralen as a function

of cell survival for induction of the transformed state (Dubertret et al., 1985).

6. Viral Mutagenesis, Photoinactivation, and Induction

A 7 fold increase in lambda bacteriophage (λ) mutation frequency is observed using the reirradiation protocol and 8-MOP, suggesting that crosslinks have a higher mutagenic effect than monoadducts (Belogurov and Zavilgelsky, 1981). Pretreatment of bacterial host cells with UV irradiation enhances the survival and mutation frequencies of UV irradiated bacteriophages and is termed Weigle reactivation (Weigle, 1953). Weigle reactivation of λ exposed to 8-MOP and isopsoralen resulted in increased mutation frequencies (Yasui et al., 1981; Belogurov and Zavilgelsky, 1981), with mutations in the host recA, lexA, and uvrA genes suppressing this effect (Belogurov et al., 1976; Belogurov and Zavilgelsky, 1978). Increased mutation frequencies were higher for 8-MOP than isopsoralen which is attributed to the fact that crosslink repair occurs by an error-prone pathway (Belogurov and Zavilgelsky, 1978; Sinden and Cole, 1978), whereas monoadducts are repaired by error-free excision and recombination (Belogurov et al., 1976; Belogurov and Zavilgelsky, 1978).

8-MOP mutagenesis of bacteriophage T4 is dependent upon the presence of the wild type T4 gene 43 DNA polymerase. In the presence of an antimutator polymerase allele, mutagenesis is inhibited (Yarosh et al., 1980). Mutations in T4 produced by psoralen generally resemble transversions rather than transitions (Drake and McGuire, 1967). Host cell repair of 8-MOP adducts on T3, T5, and T7 bacteriophages are

dependent upon excisional and recombinational host enzymes (Godsell et al., 1973). Irradiation of M13 mp10 phage DNA in the presence of the bifunctional furocoumarin 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) at wavelengths above 320nm and 390nm produces approximately 65 percent crosslinks and 85 percent monoadducts respectively (Piette et al., 1985). Upon transfection of this DNA into E. coli it was observed that crosslinks were both more lethal and more mutagenic than monoadducts. Phage mutants predominantly contained transversions, although single base pair frameshifts and transitions were detected (Piette et al., 1985).

Weigle reactivation of λ photoreacted with 8-MOP and isopsoralen in wild type E. coli has enhanced survival (Yasui et al., 1981; Belogurov and Zavilgelsky, 1981). 8-MOP crosslinks are predominantly covalently bound in the left half of λ intraphage DNA, which codes for the structural genes of the phage, while native lambda DNA did not exhibit this crosslink asymmetry (Shurdov and Popova, 1982).

8-MOP and angelicin both inhibit injection of λ DNA into E. coli (Kittler et al., 1980). It was suggested that this inhibition of λ DNA injection was due to unique crosslinks formed between the tightly packaged DNA strands within the phage head by angelicin and 8-MOP. It has been reported that angelicin produces a small but reproducible amount of λ DNA interstrand crosslinks (Lown and Sim, 1978). However, this observation has not been confirmed by other investigators (Ashwood-Smith and Grant, 1977).

Prophage λ induction is observed in the presence of 8-MOP without UVA (Ellenberger, 1981). The bifunctional furocoumarins 4-methylpsoralen, 8-MOP and psoralen produced a maximum λ prophage fraction greater than 20 percent while the monofunctional furocoumarin

4-methyl-4',5'-dihydropsoralen was only 2 percent (Fujita, 1984). 8-MOP has also been found to be a potent inducer of prophages in S. typhimurim (Wheeler et al., 1983). Psoralen is able to induce the S2 prophage in wild type, uvr-1 and uvr-2 (UV light sensitive strains), and PS01 and PS07 (psoralen sensitive mutants) but not from the recombinational deficient strain, rec-1 of Haemophilus influenzae (George and Notani, 1980). Prophage induction occurred in the absence of protein synthesis.

The bifunctional furocoumarins HMT, 4'-methoxymethyl-4,5',8-trimethylpsoralen, and 4'-aminomethyl-4,5',8-trimethylpsoralen hydrochloride (AMT) photoinactivated the single-stranded RNA virus, vesicular stomatitis virus, 1,000 fold more rapidly than TMP (Hearst and Thiry, 1977). The double-stranded DNA viruses, herpes simplex virus type 2 and vaccinia are 1,000 times more sensitive than vesicular stomatitis virus to photoinactivation by AMT. DNA viruses such as pseudorabies, infectious canine hepatitis, and fowl pox are extremely sensitive to psoralen photoinactivation while the RNA viruses of Teschen disease, New Castle disease, and foot and mouth disease are relatively insensitive to photoinactivation (Musajo et al., 1965). Bifunctional furocoumarin photoinactivation of the RNA virus, western equine encephalitis is similar at the same concentrations of TMP and AMT hydrochloride while a 10 fold increase in psoralen concentration was needed to produce similar inactivation kinetics (Hanson et al., 1978). Herpes simplex virus has a greater sensitivity and different response to photoinactivation by bifunctional furocoumarins than western equine encephalitis virus. On a molar basis herpes simplex virus photoinactivation by TMP is 10 and 100

fold more sensitive than AMT and psoralen, respectively (Hanson et al., 1978).

Graffi leukemia cells irradiated in the presence of psoralen, 8-MOP or TMP lose their ability to produce tumors when transplanted into adult mice but produce late onset leukemia in newborn mice (Musajo and Baccichetti, 1972; Bordin and Baccichetti, 1974). The ongenic RNA tumor virus, contained within and produced by Graffi cells, only produces late onset leukemias in newborn and not adult mice. Irradiation of cell free supernatants from Graffi leukemia cells in the presence of the three bifunctional furocoumarins did not inhibit production of leukemia when injected into newborn mice (Brodin and Baccichetti, 1974).

Treatment of adenovirus-2 with TMP plus UVA indicated that the surviving viral molecules correlated with the fraction of noncrosslinked adenovirus DNA molecules and suggested that human fibroblasts cannot repair a crosslink in viral DNA when a single virus infects a cell (Day et al., 1975).

8-MOP treatment of cutaneous herpes simplex virus type 2 in the experimental guinea pig model inhibited development of existing vesicles, accelerated healing of the lesions, and significantly reduced the virus titer (Oill et al., 1978). Without UVA irradiation 5(w-diethylaminopropyloxy)psoralen hydrochloride and 8(w-diethylaminopropyloxy)psoralen hydorchloride reduced plaque formation by 50 percent at concentrations of 3.5 and 24.5 μg/ml, respectively (Palu et al., 1984). HSV-1 yield in African green monkey kidney cells treated with 8-MOP and isopsoralen indicated that the bifunctional furocoumarin is 7.5 times more efficient than isopsoralen for inhibiting virus production (Coppey et al., 1979). Unirradiated HSV-1 growth in African

green monkey kidney cells was inhibited in cells treated with 8-MOP and UV irradiated between 302 to 370 nm but not 232 to 297 nm, which is consistent with the 8-MOP adsorption spectrum (Coohill and James, 1979). African green monkey kidney cells irradiated at 320 and 327 nm UV wavelengths in the presence of 8-MOP resulted in approximately the same inhibition to support growth of HSV while little inhibition occurred in the presence of 365 nm of UV radiation (Bocksthaler et al., 1982). Radiation enhanced reactivation of HSV-1 irradiated at 265 nm and assayed on African monkey kidney cells exposed to 8-MOP with irradiation at wavelengths between 240 to 400 nm has a peak response above 311 nm (James and Coohill, 1979).

Transformation of baby hamster kidney cells by polyoma virus is increased 30 fold if the cells are pretreated with 8-MOP plus UVA (Morhenn and Kaye, 1979). Quanitation of infectious SV40 induced from Syrian hamster kidney cells transformed with SV40 by 8-MOP and isopsoralen resulted in the former being more effective at low UVA irradiations, while the latter would induce the same levels of virus as the UVA irradiation was increased (Moore et al., 1983). This suggests that the induction of SV40 is associated with the repair of monoadducts. Induction of SV40 by 8-MOP from SV40 transformed syrian hamster cells occurs at maximal levels when the UV radiation wavelength is in the region of 302 to 365 nm (Moore and Coohill, 1981). Simian virus 40 DNA isolated from intracellular chromatin or extracellular virus after photoaddition of TMP indicated that DNA was crosslinked preferentially at sites susceptible to digestion by micrococcal nuclease digestion and that the protected sites were spaced between the crosslinks in a 200 base pair repeating pattern such as that protected by nucleosomes (Hallick et al.,

1978). SV40 intracellular minichromosomes photoreacted *in vivo* with HMT have a 400 base pair region around the origin of replication that is free of nucleosomes and preferentially bound by this furocoumarin (Robinson and Hallick, 1982). Extracellular SV40 however is not preferentially accessible to HMT indicating that it does not contain a nucleosome free origin of replication (Kondoleon et al., 1983).

Five different bifunctional furocoumarins at concentrations 500 times greater than that required to inactivate viruses did not inhibit scrapie agent titers in partially purified preparations from murine spleens and hamster preparations (McKinley et al., 1983). It was suggested by McKinley et al. (1983) that scrapie agent may not contain nucleic acids or that furocoumarins cannot penetrate the protein coat.

It is clear that a considerable amount of information has accumulated concerning virus inactivation and induction in transformed cells. However, no systematic study of virus inactivation with mono- and bifunctional furocoumarins at known adduct levels is available.

7. Furocoumarin Photoinactivation, Biochemistry, and Repair in Prokaryotic and Eukaryotic Cells.

It was first shown that *E. coli* in the presence of 8-MOP and UVA resulted in cell killing that depended on both the furocoumarin concentration and the fluence of UVA (Oginsky et al., 1959). A single 8-MOP monoadduct is lethal to *E. coli uvrA recA*, deficient in both excision and postreplication repair, while *E. coli uvrA* may be inactivated by one crosslink (Bridges et al., 1981). Repair proficient *E. coli* survive large numbers of both monoadducts and crosslinks,

although the latter are more lethal. Partially purified extracts from E. coli containing uvrA$^+$ or both uvrB$^+$ and uvrC$^+$ gene products are only active as a mixture for endonuclease activity on 8-MOP damaged ColE1 DNA (Seeberg, 1981). Break formation in 8-MOP treated ColE1 by the endonuclease had a preference for monoadducts, but also occurred at crosslinks with a lower efficiency. Micrococcus luteus UV endonuclease, which removes UV induced DNA pyrimidine dimers, does not remove 8-MOP adducts (Seeberg, 1981).

5-MOP plus UVA exposure of E. coli repair deficient mutants showed that survival was least in a uvrA$^-$ recA$^-$ strain while survival was greater for the uvrA$^-$ strain whereas survival of wild type repair proficient E. coli was not affected (Pool et al., 1982). E. coli arcA mutants, which have been suggested to have an increased permeability in their cell envelopes, are more sensitive to TMP photoinactivation than wild type E. coli (Hansen, 1982). However, it was observed that AMT photobinding to RNA was identical in wild type and arcA mutants while the binding to arcA DNA was much increased over wild type, thus indicating that there was a specific alteration in the immediate environment of the arcA cellular DNA. By using the reirradiation protocol but allowing a period of incubation between the second UVA radiation it has been demonstrated that E. coli uvrA$^-$ mutants possess a DNA repair pathway for certain 8-MOP monoadducts which is observed by a substantial decrease in lethality (Bridges and Stannard, 1982). This repair pathway was postulated to be prereplicative, unaffected by chloramphenicol or caffeine, and inhibited at 4°C. It was unaffected by a rep mutation but blocked by a polA mutation, suggesting involvement of DNA strand breakage and some exonuclease activity.

A new pathway for repair of 8-MOP crosslinks in E. coli has been proposed that does not involve both an excision step (dependent upon the uvrA and uvrB genes) and a recombination step (dependent upon the recA gene) (Bridges and von Wright, 1981). This pathway is independent of the uvrA gene but was inactive in rep (specifying a DNA-unwinding enzyme) mutants (Bridges, 1984). This minor pathway is dependent upon the $recA^+$ gene where it is more active in minimal than nutrient growth media. In minimal media, approximately 7 to 8 crosslinks are repaired for every lethal crosslink, while for the richer media this number is 5 crosslinks (Bridges, 1984).

The efficiency of lethality for E. coli was ranked in the following order: 4-methylpsoralen > psoralen > 8-MOP >> the monofunctional 4-methyl-4,5'-dihydropsoralen (Fujita, 1984). DNA synthesis in E. coli was inhibited to approximately the same extent when the adduct levels of psoralen and isopsoralen (monofunctional furocoumarin) where equal but a UVA dose of 2.7 times higher was required by isopsoralen than psoralen to result in 37 percent survival of the bacteria (Bordin et al., 1976).

To achieve 90 percent lethality in E. coli with isopsoralen required 5.5 times more UVA irradiation than with psoralen (Ashwood-Smith and Grant, 1977). The monofunctional furocoumarin 4,4'-DMA is more lethal to wild type E. coli than psoralen while 4,5'-DMA has limited lethality. Use of the reirradiation protocol demonstrated additional killing due to the formation of psoralen crosslinks, but no additional killing by 4,4'-DMA since it is unable to form crosslinks (Baccichetti et al., 1981). At equal molar concentrations the monofunctional furocoumarin 4,4',6-TMA is more lethal than 8-MOP to E. coli $uvrA^-$ (Baccichetti et al., 1984). E. coli $umuC^-$ mutants (specific genes essential for mutation

induction) are inactivated 4 times more rapidly than umuC$^+$ bacteria by isopsoralen while the two bifunctional furocoumarins, 8-MOP and TMP, had slight or undectable differences (Miller and Eisenstadt, 1985).

The double mutants uvrA$^-$ recA$^-$ in E. coli and Bacillus subtilis indicate that a single furocoumarin monoadduct or UV induced pyrimidine dimer per genome represents a lethal hit (Bridges et al., 1979; Harter et al., 1976). The presence of TMP crosslinks in recipient B. subtilis interfere both with initiation of recombination and with the completion of heteroduplex formation of donor marker transforming DNA (Mooibroek et al., 1982). Micrococcus radiodurans mtcA$^-$ mutants (hypersensitive to mitomycin C) inactivated by TMP crosslinks but not monoadducts can be rescued by further addition of ionizing radiation or UV (254 nm) light. Rescue of inactivated cells is prevented by the presence of chloramphenicol (Hansen, 1982). This alternative pathway for repair of furocoumarin crosslinks can only be induced by the more abundant lesions produced by ionizing radiation.

The spacing of replicons in pea root meristems during synchronized entry into S phase from arrest at the G1/S boundary after pretreatment with TMP produced a marked shortening of the replicon spacing, suggesting that there is a premature arrest in the replication fork, and resulted in recruitment of additional initiation points within a given replicon family (Francis et al., 1985). The repair of 8-MOP crosslinks in Tetrahymena thermophila is divided into 3 phases: a protein DNA complexing phase, a DNA incision phase, and last, a DNA ligation phase (Nielson and Kober, 1985). Novobicin, nalidixic acid, n-butyrate, and cycloheximide inhibited the incision but not the protein DNA complexing step, and ligation was partly inhibited by nalidixic acid. It was

suggested that topoisomerases were involved in the repair of crosslinks. 5-MOP crosslinks are more lethal to the two protozoans Tetrahymena pyriformis, Paramecium caudatum, and the yeast Candida albicans than 8-MOP crosslinks (Young and Barth, 1982).

Radiation sensitive S. cerevisiae mutants rad 2-20 and rad 9-4 are deficient in excision and recombinational repair, respectively, both of which are more sensitive to 8-MOP photoadducts than wild type cells. Double mutants are more sensitive than single mutants (Averbeck and Moustacchi, 1975). Damage and repair of nuclear and mitochondrial genetic functions can be studied together in S. cerevisiae and imply that a respiratory function is required for expression of the excision repair enzymes (Ben-Hur and Song, 1984). A third repair pathway that is involved in some steps in recombinational repair encompasses rad 50 through rad 57 mutants. With the exception of rad 53, these mutants have an increased sensitivity to mono- and bifunctional furocoumarins (Henriques and Moustacchi, 1980a). This is especially pronounced in the exponential phase growth of S. cerevisiae. Three epistatic groups have been used to classify the rad mutants whose genes in these groups affect three distinct modes of DNA repair, which are named for a prominent locus in each. The RAD 3 (rad 1 through 4,7,10,14,16,23), RAD 6 (rad 5,6,8,9,15,18), and RAD 52 (rad 50 through 57) groups are involved in the excision of UV induced pyrimidine dimers, mutagenic error-prone repair (recombinational repair) of UV and X-ray induced lesions, and DNA double-stranded break repair plus recombinational repair of X-rays, respectively. S. cerevisiae mutants pso1-1, pso2-1, and pso3-1 have increased sensitivity to 8-MOP and 3-CP (Henriques and Moustacchi, 1980b). The pso1-1 mutant has a cross sensitivity to UV and γ-rays

whereas pso2-1 and pso3-1 participate only in the repair of furocoumarin monoadducts and crosslinks. Mutant pso1-1 has been assigned to the RAD 6 epistatic group (Henriques and Moustacchi, 1981). Further characterization of the gene products $PSO1^+$, $PSO2^+$, and $PSO3^+$, indicate that they act on a spectrum of DNA lesions, predominantly necessary for the repair of crosslinks irrespective of their molecular nature, and specifically act on furocoumarin sublethal lesions (Cassier and Moustacchi, 1981). Removal of TMP from a large number of mutants in the S. cerevisiae epistatic group RAD 3 demonstrated that the majority of mutants had little or no nicking of crosslinked DNA, and where nicking occurred the mutants were much less efficient than the RAD^+ strain in removing crosslinks (Miller et al., 1982a; Miller et al., 1982b).

Excision dificient S. cerevisiae have been shown to tolerate 8-MOP monoadducts for at least one round of DNA replication (Chanet et al., 1983). S. cerevisiae rad1, rad2, and rad4 excision mutants are defective in the incision of both TMP monoadducts and crosslinks, while the rad3 mutant is proficient in the incision of monoadducts but not crosslinks (Miller et al., 1984). Equal adduct levels of the monofunctional 7-methyl pyrido (3,4-c) psoralen and 8-MOP results in a rapid ligation during excision repair for the latter furocoumarin, whereas the former is only partially resealed (Magana-Schwencke and Moustacchi, 1985). The tolerance of 8-MOP monoadducts in excision deficient S. cerevisiae is controlled by the mutagenic ($RAD6^+$ gene product) and possibly the recombinogenic ($RAD2^+$ gene product) repair pathways, while 8-MOP crosslink repair is controlled by the $RAD2^+$ (excision pathway), $RAD6^+$ (mutagenic pathway), $RAD52^+$ (recombinogenic pathway), $PSO1^+$ (mutagenic pathway), and $PSO2^+$ (mutagenic and recombinogenic pathways) gene products

(Chanet et al., 1985). A minor, alternate error prone crosslink repair pathway that is independent of the recombinational pathway occurs in S. cerevisiae where RAD6 and PSO2 gene products interfere with this repair process (Cassier et al., 1985).

The two monofunctional furocoumarins 7-methyl pyrido (3,4-c) psoralen and pyrido (3,4-c) psoralen are more lethal than the monofunctional 3-CP and bifunctional 8-MOP for wild type S. cerevisiae (Averbeck et al., 1985a; Dubertret et al., 1985). Monofunctional 3-carbethoxypyranocoumarin is less lethal than 3-CP, which is significantly less lethal than 8-MOP to S. cerevisiae on an equimolar basis (Averbeck et al., 1985b).

The exposure of mouse L cells to psoralen and AMT showed that the intracellular concentrations reached a plateau within 2 min. by microspectrofluorometry, while a period of 20 min. was required for TMP to penetrate the cells and achieve maximal intracellular concentration. Washing of these cells in medium without furocoumarins resulted in only a partial removal with an equal concentration distribution of all three furocoumarins between the cell nucleus and cytoplasm. Inhibition of DNA synthesis in mouse Ehrlich ascites tumor cells shows that the methyl derivatives of isopsoralen (angelicin) are more potent inhibitors than their parental compound or two bifunctional furocoumarins, which gave the following inhibition order: 4,4',6-TMA > 4,4'-DMA > 4,5'-DMA > psoralen and 8-MOP > angelicin (Baccichetti et al., 1981; Baccichetti et al., 1984).

The relative photobiological activities of several furocoumarins investigated in L1210 mouse leukemia cells by cell viability, DNA synthesis inhibition, furocoumarin-DNA photoaddition, and DNA interstrand

crosslinking all gave the same ranking order of: TMP > HMT > AMT > 8-MOP (Nielson and Bohr, 1983a). A direct correlation between phototoxicity, DNA interstrand crosslinks, and inhibition of DNA synthesis was indicated. Monofunctional angelicin and 3-CP do not form crosslinks in mouse embryo fibroblasts while crosslink formation by psoralen was UVA dose dependent (Szafarz et al., 1983). After a 1 hour dark postincubation period no psoralen adducts were repaired, while 41% of the 3-CP adducts were removed. The phototoxic action of 8-MOP was primarily due to inhibition of DNA synthesis in mouse L1210 cells (Nielson and Linnane, 1983b). At a concentration of 200 ng/ml 8-MOP, 95% of L1210 DNA synthesis was inhibited within 2 hours while only 40% RNA synthesis inhibition occurred. A ten fold increase in 8-MOP concentration inhibits RNA synthesis by 90%, but only caused a moderate inhibition in protein synthesis. Approximately 70% of the 8-MOP monoadducts were repaired in L5178Y mouse lymphoma cells by 6 hours and were unavailable to form crosslinks upon a second UVA exposure (Liu-Lee et al., 1984a).

DNA fragments containing the putative origins of replication have been isolated from mouse Ehrlich ascites tumor cells due to the fact that TMP crosslinks allowed initiation of DNA replication to occur but inhibited elongation (Russev and Vassilev, 1982). This approach has shown that several nonhistone proteins bind to the putative mouse origin of replication (Anachkova and Russev, 1983). In mouse FM3A cells treated with 8-MOP, the inhibition and recovery of DNA synthesis was correlated with a halt in the G2 phase, followed by resumption of the cell cycle (Hyodo et al., 1982a). RNA and protein synthesis in FM3A cells is stimulated by low doses of 8-MOP and UVA but strongly inhibited by high doses. Further studies with FM3A cells and 8-MOP showed that the

decrease in DNA synthesis was not due to the rate of DNA elongation but due to a change in the frequency of initiation of replication (Hyodo et al., 1982b). It was suggested that some crosslinks may be repaired during prolonged incubation, but the majority of crosslinks persisted after 24 hours incubation. Treatment of murine melanoma cells with HMT that produces one molecule per 10^6 base pairs of DNA caused the cells to accumulate predominantly in the G2 phase, while 2 to 3 HMT molecules per base pair blocked cells in the S and G1 phases (Varga et al., 1982).

V79 Chinese hamster cell killing was due to 8-MOP crosslinks and not monoadducts (Babudri et al., 1981). At sublethal doses of 8-MOP and 5-MOP, only the 8-MOP forms crosslinks, although both furocoumarins decreased the rate of DNA synthesis (Weniger, 1981). Repair of low adduct levels of 5- and 8-MOP adducts is not completed by 24 hours postirradiation. The first use of the reirradiation protocol concluded that approximately 2,000 TMP crosslinks per Chinese hamster cell genome was a lethal event (Ben-Hur and Elkind, 1973b). It was also observed that about 90 percent of all TMP adducts could be removed. The authors conclude that TMP crosslinks can be removed, since they represented approximately 11 percent of the total population of adducts. Cultured skin fibroblasts from young male guinea pigs treated with isopsoralen plus UVA or 8-MOP plus 395 nm UVA (forming primarily monoadducts) resulted in equal levels of DNA synthesis inhibition (Pohl and Christophers, 1980). Isopsoralen was required at a concentration 39 times higher than 8-MOP to achieve equal inhibition. Recovery of DNA synthesis was observed for both isopsoralen and 8-MOP (UVA, 395 nm), while cells treated with 8-MOP plus 365 nm UVA (primarily forms crosslinks) inhibited DNA synthesis for more than 4 days.

Although it has been observed that repair of UV induced pyrimidine dimers is similar in highly repeated α and bulk DNA, AMT and isopsoralen repair in α DNA was 30 percent of that in bulk DNA (Zolan et al., 1982a). Repair of both furocoumarins in α and bulk DNA had the same kinetics, repair patch sizes, and adduct levels, which did not account for the reduced repair in α DNA. It was concluded that the two diastereomers of the furan thymine monoadducts of HMT were formed in the same relative amounts in α and bulk DNA and were removed at the same relative rates (Zolan et al., 1984). However, the conversion of these monoadducts to crosslinks was markedly reduced in α DNA relative to bulk DNA, suggesting a possible conformational constraint in the internucleosomal DNA in α chromatin which might account for the repair deficiency.

While monofunctional 3-carbethoxyhomopsoralen produced a stronger DNA synthesis inhibition in human fibroblasts than 8-MOP and monofunctional 3-CP at low doses of UVA, these cells recovered more rapidly from DNA synthesis and growth inhibition when treated with 3-carbethoxyhomopsoralen than with 8-MOP (Averbeck et al., 1985b). It has also been observed that the two monofunctional pyridopsoralens (7-methyl pyrido (3,4-c) psoralen and pyrido (3,4-c) psoralen) have a stronger inhibition of DNA synthesis in human cells than 8-MOP (Dubertret et al., 1985). Human epidermal cells exposed to 8-MOP concentrations equivalent to those found in the serum of psoriatic patients produced slower cell growth and lower cell yields compared to the controls (West and Faed, 1983). Use of the reirradiation protocol demonstrated that TMP crosslinks are more efficient in inhibiting ornithine decarboxylase induction and RNA synthesis than monoadducts in human breast carcinoma cells (Prager et al., 1983).

In a comparison of the alkaline elution and electron microscopy techniques to measure 8-MOP and TMP crosslink removal in human lymphocytes, repair was detected only by the former technique (Bohr and Nielsen, 1984). Exposure of human lymphocytes and skin fibroblasts to a single clinical dose of PUVA (8-MOP + UVA) resulted in DNA damage. Analysis of DNA from skin fibroblasts derived from psoriatic patients after 5 years of PUVA treatment by alkaline elution technique suggested the presence of crosslinks (Bredberg et al., 1983b). Human lymphocyte survival declines exponentially as a function of the relative number of 8-MOP crosslinks which correlated the phototoxic effect with the presence of crosslinks (Cohen et al., 1981). UVA irradiation alone induced DNA strand breaks in human lymphocytes that are closed at a slower rate than UV (100 nm to 280 nm) generated strand breaks (Holmberg et al., 1985). Pretreatment of lymphocytes with UVA delays closure of the intermediate strand breaks formed by the repair of UV induced DNA pyrimidine dimers. Human polymorphonuclear neutrophils treated with monofunctional 4,5'-DMA plus UVA or UVA alone are more sensitive to UVA irradiation than similarly treated lymphocytes (Walther et al., 1985). Isopsoralen produced less phototoxicity than its 4,5'-dimethyl derivative in neutrophils. 4,5'-DMA without UVA causes a concentration dependent lethality to polymorphonuclear neutrophils (Walther et al., 1985) which was also observed for human erythrocytes exposed to monofunctional 3-CP but not bifunctional 8-MOP (Muller-Runkel and Grossweiner, 1981). Human thymus derived lymphocytes normally proliferate when cultured with lymphocytes or epidermal cells from unrelated individuals, since their HLA-D antigens are recognized as foreign by the thymus derived lymphocytes. This response can be inhibited if the responder or

stimulator cells are first treated with 8-MOP plus UVA (Morhenn et al., 1980). The majority of epidermal cells were insensitive to 8-MOP concentrations lethal to lymphocytes and monocytes, suggesting that PUVA may exert its beneficial effect by inhibiting the cell mediated immune responses.

While UV induced pyrimidine dimers are randomly distributed in the chromatin (Williams and Friedberg, 1979), furocoumarins are found primarily in the linker regions between the nucleosomes (Cech and Pardue, 1977; Wiesenhahn et al., 1977). Using isoposralen treated human cells, it was demonstrated that the distribution of the transiently sensitive staphylococcal nuclease repair patches is independent of the initial distribution of linker specific isopsoralen lesions in the chromatin (Zolan et al., 1982b). Repair patches produced by the linker specific isopsoralen become randomly distributed in the chromatin, implying that nucleosomes do not necessarily return to their original sites on the DNA strand after repair. Both normal and XP repair deficient cells showed identical inhibition of scheduled DNA synthesis after exposure to 8-MOP plus UVA, although the latter was more sensitive to killing than the former (Baden et al., 1972). Repair synthesis of 8-MOP lesions was demonstrated in normal cells but not in XP fibroblasts. Cells treated with 8-MOP plus UVA have much lower levels of DNA repair synthesis relative to the degree of scheduled DNA synthesis inhibition than cells exposed to UV (254 nm) (Baden et al., 1972).

Human diploid fibroblasts had similar repair times for both bifunctional 8-MOP and monofunctional isopsoralen when the same concentrations and UVA doses were used (Kaye et al., 1980). No repair replication was observed for either furocoumarin in XP fibroblasts.

Partial crosslink removal occurs in normal, XP variant, and FA fibroblasts but not in XP fibroblasts (Kaye et al., 1980). UVA irradiation of normal human fibroblasts without furocoumarins induced DNA breaks that are rapidly resealed (Bredberg et al., 1982). PUVA induced monoadducts in human fibroblasts remained in the DNA for at least 7 days postirradiation and were converted to crosslinks by a second dose of UVA. Crosslinks induced by the combination of PUVA plus UVA is repaired in normal human fibroblasts by the formation of DNA breaks, but was not observed in XP fibroblasts of complementation group A (Bredberg et al., 1982). DNA polymerase α functions in the repair of UV and 5-MIP lesions in normal and XP fibroblasts (Cleaver et al., 1984a). XP fibroblasts of different complementation groups had the same relative efficiency for excision repair (A<C<D) for both types of DNA induced lesions, indicating a close resemblance between UV and 5-MIP adducts. At high doses, UV pyrimidine dimers were repaired at a maximum rate twice that for 5-MIP (Cleaver et al., 1984a).

Normal human fibroblasts rapidly exicsed approximately 80 to 90% of the isopsoralen monoadducts and 65% of the 5-MIP monoadducts within 24 hours postexposure (Cleaver and Gruenert, 1984b). XP fibroblasts of complementation groups A, D, and E excised approximately 20, 55-60, and 80% respectively of isopsoralen monoadducts which resembled the excision of pyrimidine dimers in these complementation groups, except for group D. Since group D fibroblasts were more proficient in repairing isopsoralen monoadducts than pyrimidine dimers, it was suggested that these fibroblasts lack a gene product that is required for the repair of pyrimidine dimers to a greater extent than for isopsoralen adducts (Cleaver and Gruenert, 1984b). After a second UVA irradiation of normal

and XP complementation group E fibroblasts initially exposed to 8-MOP plus UVA, a marked increase in DNA strand breakage occured postirradiation. This was not observed in XP fibroblasts of complementation groups A and D (Bredberg and Soderhall, 1985). This suggested that the repair deficient gene product in group E cells is specific to pyrimidine dimers and not furocoumarin adducts. When furocoumarin adducts are formed at low frequencies, such that there is a high survival of normal and XP fibroblasts, they are found in both linker and core regions of chromatin. However, there is a preference for linker regions (Cleaver, 1985b). Isopsoralen, 5-MIP, 8-MOP and TMP adducts were enriched 2 to 3 fold per nucleotide in linker DNA relative to core DNA. 5-MIP monoadducts were initially in linker DNA, but slowly became randomized over a 12 hour growth period. This suggested a slow lateral movement of nucleosomes within the DNA that is independent of DNA, RNA, protein, or poly (ADP-ribose) synthesis and independent of DNA repair. Excision repair of 5-MIP monoadducts however resulted in a rapid but transient local rearrangement in nucleosomal conformation, with an initial increase in staplylococcal nuclease sensitivity that reverts to the sensitivity of bulk chromatin in less than 1 hour (Cleaver, 1985b). In vivo TMP crosslinking of XP DNA allowed isolation of nascent DNA chains that contained the DNA replication origins which are enriched 5 to 10 fold for the short dispersed repetitive sequences that were members of the human Alu family (Anachkova et al., 1985).

Human fibroblasts derived from young (3 days to 3 years) and old (84 to 94 years) donors repair UV (254 nm) induced pyrimidine dimers and TMP crosslinks with equal efficiency when measured by colony forming ability or HSV host cell reactivation, which suggested that human DNA repair

capacities do not decline with age (Hall et al., 1982). Human fibroblasts that contained DNA interstrand TMP crosslinks which inhibited 99.5% of the replicative DNA synthesis were used to accurately measure the rate of DNA repair synthesis induced by other DNA damaging agents through the incorporation of radioactive thymidine without interference from semiconservative DNA synthesis (Heimer et al., 1983). This method provides a simple and rapid technique over previous methods to inhibit replicative DNA synthesis for DNA repair studies.

From the proceeding discussion it is clear that mono- and bifunctional furocoumarins provide a powerful tool to study the DNA repair pathways in prokaryotic and eukaryotic cells. Since furocoumarins covalently bind to DNA in the same manner, it allows a direct comparison to be made between the repair of two structurally similar molecules. Furthermore, the use of XP and FA fibroblasts, which are sensitive to monoadducts and crosslinks, respectively, provides two systems that are somewhat analagous to the E. coli repair mutants for the elucidation of eukaryotic DNA repair pathways. It is evident that there are differences in the eukaryotic repair pathways for removal of mono- and bifunctional furocoumarins, although more information is required concerning the repair of known levels of crosslinks and monoadducts per genome. The technique of alkaline gel electrophoresis has been utilized to determine the level of furocoumarin crosslinks per genome (Cech, 1981). However, this technique allows only an approximation of the crosslink level, and it does not allow quantitation of the different types and levels of furocoumarin monoadducts per genome, nor does it permit an analysis of their conversion to crosslinks upon reirradiation in the absence of unbound furocoumarin. Such information is readily obtainable by

high-performance liquid chromatography (HPLC) (Kanne et al., 1982) which will therefore make it possible to assess the number of crosslinks that constitute a lethal event in the presence of a known level of monoadducts.

B. Xeroderma Pigmentosum

1. Clinical Characteristics

XP is an autosomal recessive skin disease which is characterized by early onset of skin and eye photosensitivity to sunlight in homozygotes that show a high incidence of skin cancers and frequent neurological abnormalities (Reed et al., 1969; Robbins et al., 1974). Heterozygotes are generally found to be asymptomatic (Swift and Chase, 1979; Cleaver et al., 1981). A high predominance of patients are siblings of consanguine marriages, especially in Egypt (Hashem et al., 1980), Israel (Berlin and Tager, 1962), and Japan (Takebe et al., 1977). XP has a general worldwide distribution in all racial groups of a frequency of 1 in 250,000 (Cleaver, 1983) but is found to be 1 in 40,000 to 1 in 25,000 in Japan (Takebe et al., 1977) and Egypt (Hashem et al., 1980), respectively.

Herba and Kaposi (1874) first described the disease and its possible relationship to inheritance and sunlight. The genetic background and amount of sunlight exposure results in widely varying nature and severity of cutaneous, ocular, and neurological symptoms (Rook, 1979; Robbins et al., 1974). An abnormal erythemal response usually occurs in the first years of life. This lower than normal minimal erythemal dose is used in early diagnosis prior to the appearance of other skin changes (Ramsay and Giannelli, 1975). Skin changes develope to varying degrees in different patients later in life and include excessive freckling, keratoses, and cancers. Diagnosis is based on characteristic changes in the skin pigment due to alteration in the melanocyte structure and function

(Guerrier et al., 1973) and recurring cancers in areas of the skin exposed to sunlight that include squamous and basal cell carcinomas, keratochanthomas, angiomas, malignant melanomas, fibromas, and sarcomas (Robbins et al., 1974; Lynch et al., 1967). Photophobia and mild conjunctivitis are observed in minimal ocular abnormalities (Robbins et al., 1974). XP variant or pigmented xerodermoid patients present a spectrum of the above cutaneous manifestations and ocular abnormalities but have a normal erythemal response (Ramsay and Giannelli, 1975; Burk et al., 1971). Such patients can only be diagnosed biochemically (Cleaver et al., 1980; Lehmann et al., 1975).

The association of neurological disorders with XP was first described by deSantis and Cacchione (1932). Few XP patients exhibit all the symptoms of deSanctis-Cacchione syndrome (xerodermic idiocy) that includes microcephaly with progressive mental deficiency, sensorineural deafness, ataxia, retarded skeletal development, chorioathetosis, and eventual quadriparesis (de Sanctis and Cacchione, 1932; Reed et al., 1965; Reed et al., 1969). Common neurologic abnormalities in XP patients include microcephaly, mental deficiency, and areflexia that is associated with a nonspecific neuronal loss and not specific morphological or cellular abnormalities (Reed et al., 1969; Thrush et al., 1974). Neuronal loss may be due to arrested development in association with microcephaly or generalized atrophy.

A deficiency in reporting and the early death of some XP patients due to the consequences of their skin disease has not provided adequate information on the frequency of nonskin types of cancers other than those already described for the skin (Cleaver, 1983). Although nonskin cancers

have been reported for XP patients, the frequency of such cancers has not been determined in relation to control patients of identical age.

2. Cellular Properties

Fibroblast cultures established from XP patients exhibit life spans and generation rates similar to that of fibroblast cultures derived from normal individuals (Cleaver et al., 1981). Their nutritional requirements are not different from that of normal fibroblasts (Cleaver, 1983).

a. Chromosomal Aberrations and Sister Chromatid Exchange

Although there has been a few reports of abnormal karyotypes in fibroblast cultures established from XP patients, in general there is no distinct karyotypic changes as observed in Down's and Bloom's syndromes that are associated with a high incidence of cancer (Waltimo et al., 1967; German, 1972; Stefanini et al., 1980). A normal frequency of spontaneous SCE is found in XP cells (Wolff et al., 1975), although induction of SCE by UV and other carcinogens results in a higher increased frequency than detected in normal cells (Wolff et al., 1977; DeWeerd-Kastelein et al., 1977). XP cells also undergo higher levels of chromosomal aberrations after exposure to UV and carcinogens than normal cells (Parrington et al., 1971; Sasaki, 1973; Stich et al., 1973).

b. Colony Forming Ability

Gatler (1964) first demonstrated that XP cells were more sensitive to UV than normal cells based on the observation that total protein in XP cultures was substantially less than that of normal cultures after irradiation. It has subsequently been found that cells from most XP patients had a decreased ability to form colonies after UV irradiation relative to normal cells (Cleaver, 1970; Goldstein, 1971; Takebe et al., 1972; Stich et al., 1973; Andrews et al., 1978). Fibroblasts derived from patients exhibiting neurological abnormalities are generally more sensitive in the colony forming ability assay (Andrews et al., 1978). Although XP cells have increased sensitivity to 4-nitroquinoline-1-oxide, benz[a]anthracene and various aromatic amides, all of which generally form bulky adducts and mimic UV radiation, they are normal in their response to N-methyl-N'-nitro-N-nitrosoguanidine (Takebe et al., 1972; Stich et al., 1973; Stich and San, 1973, Maher et al., 1976). Several investigators have reported the ability to isolate phenotypic revertants resistant to UV irradiation (Royer-Pokora and Haseltine, 1984; Thielmann et al., 1983; Ganesan et al., 1982). A simple back mutation within the gene responsible for reversion is thought to be responsible for the appearance of UV resistant XP cells but experiments so far have not excluded other possible mechanisms of reversion.

XP variant cells from pigmented xerodermoid patients do not have any neurological involvement and demonstrate only a slight increased UV sensitivity (Burk et al., 1971; Cleaver, 1972; Andrews et al., 1978; Stich and San, 1973). Addition of caffeine postirradiation to XP variant fibroblasts greatly increases their killing while XP and normal cells remain unaffected (Painter, 1980).

c. Mutagenesis

Examination of the surviving XP and XP variant cells following UV irradiation or exposure to chemical carcinogens demonstrates an increased frequency of resistance to 6-thioguanine, ovabain, and diptheria toxin relative to normal cells (Maher et al., 1976; Maher et al., 1979; Glover et al., 1979). This suggests that the genetic defects in all XP cells results in an increased mutability which is in contrast to the variable response observed in the colony forming ability assay after exposure to UV irradiation, indicating that there is a generalized defect in XP cell repair that introduces errors resulting in mutations after UV irradiation (Arlett and Harcourt, 1983). This is also true of the XP variant cells that retain the capacity to repair UV damage and exhibit normal survival but still demonstrate high mutation frequencies due to loss of the repair system fidelity (Cleaver, 1983).

d. Viral Host Cell Reactivation

Reduced survival of the UV irradiated DNA viruses vaccinia (Zavadova, 1971; Lytle et al., 1972), HSV (Lytle, 1972; Rabson, 1969; Hall, 1980; Boorstein et al., 1983), adenovirus (Day, 1974a; Day, 1974b), and SV40 (Aaronson and Lytle, 1970) have been reported for host cell reactivation in XP cells when compared to normal cells. The D_{37} (UV dose required to produce 37% virus survival) of UV irradiated adenovirus and HSV is approximately 20 and 3 times less, respectively when assayed in XP cells versus normal cells (Day, 1974a; Day, 1974b; Lytle et al., 1972; Rabson et al., 1969). It has been estimated that approximately one

pyrimidine dimer per adenovirus genome equals the D_{37} when grown in XP cells. UV irradiated adenovirus when assayed on XP variant cells had a D_{37} that was approximately 2 times less than normal cells. This correlates well with the XP variant colony forming ability (Day, 1975). Although SV40 extracellular virus and free DNA have reduced survival after UV irradiation in XP cells (Aaronson and Lytle, 1970; Abrahams and Eb, 1976) only free RNA from encephalomyocarditis virus and not extracellular virus exhibited reduced survival after damage in XP cells (Zavadova, 1971). Host cell reactivation of UV irradiated HSV and SV40 DNA by a SV40 transformed XP UV resistant revertant cell line was comparable to a normal transformed cell line and distinctly different from the parental transformed XP cell line from which the UV resistant cell lines were derived (Royer-Pokora and Haseltine, 1984).

Enhanced reactivation occurs for UV irradiated adenovirus (Jeeves and Rainbow, 1983) and HSV (Abrahams et al., 1984) in UV irradiated XP cells. Optimal viral enhanced reactivation was observed to occur at lower UV doses for both the virus and XP cells in comparison to normal cells. HSV optimal enhanced reactivation in normal and XP variant cells occurred at UV doses that were equal for the cells and equal for the virus (Abrahams et al., 1984). Adenovirus enhanced reactivation also occurred at lower viral UV doses in XP cells than normal cells with XP variant cells being substantially lower than the control cells (Jeeves and Rainbow, 1983). Maximal enhanced reactivation and enhanced mutagenesis of HSV have similar kinetics of appearance in normal and XP cells. However for XP variant cells the maximal expression of enhanced mutagenesis was significantly delayed with respect to enhanced reactivation (Abrahams et al., 1984). Enhanced reactivation and enhanced

mutagenesis are suggested to be separate processes that occur independently of each other. At a given UV dose for HSV, UV stimulated recombination was higher in XP cells than normal cells which were slightly higher than XP variant cells (Dasgupta and Summers, 1980). Host cell reactivation of UV and γ irradiated adenovirus 2 by XP heterozygotic fibroblasts resulted in a minor reduction in viral structural antigen production in relation to normal fibroblasts (Rainbow, 1980). Fibroblasts derived from XP homozygous sibs of the heterozygous patients had a severe reduction in adenovirus host cell reactivation.

e. Viral and Carcinogenic Transformation

Numerous attempts have been made to establish immortalized cell lines of XP cells by various viral and carcinogenic agents with little success. Two reports have described the transformation and immortalization of two strains of XP cells by SV40 extracellular virus. They only undergo immortalization after a crisis period that coincided with the cell senescence period of their corresponding nontransformed cells (Takebe et al., 1974; Yagi and Takebe, 1983). After a period of nonproliferation during the crisis period, SV40 transformed XP cells started active proliferation. This is consistent with other reports on transformation of human cells (Girardi et al., 1965; Topp et al., 1980; Sack, 1981). Both XP cell lines retained the same phenotypic defect in repair of UV induced damage as their parental cells. It has been suggested that XP cells have a higher frequency of transformation by SV40 than (Veldhuisen and Pouwels, 1970) observed for FA and Down's syndrome (Aaronson and Todaro, 1968; Todaro et al., 1966), diseases associated

with a high incidence of cancer. This increased susceptibility to SV40 transformation has not been confirmed by several other investigations (Aaronson and Lytle, 1970; Parrington et al., 1971). Transformed XP cells have been demonstrated to shed infectious SV40 (Lomax et al., 1978).

UV irradiation of XP cells prior to transformation with SV40 indicated little or no increase in the frequency of transformation (Key and Todaro, 1974; Hall, 1981). Another investigation demonstrated an increased transformation frequency in both XP cells (Hagedorn et al., 1983) and in normal human cells (Hagedorn et al., 1983; Hall, 1981) following UV irradiation. Transformation frequencies of normal and heterozygous XP cells with UV irradiated or unirradiated SV40 showed no differences while XP variant cells exhibited an increased transformation frequency with irradiated SV40 (Hall and Tokuno, 1979). XP variant cells were also demonstrated to produce lower than normal quantities of infectious SV40 although repair of UV damaged SV40 was similar in normal and XP variant cells.

In general cells derived from XP homozygous and heterozygous patients showed higher susceptibility than normal cells to transformation by feline sarcoma virus and Kirsten murine sarcoma virus (Chang, 1976). Preexposure of XP cells to 4-nitroquinoline-1-oxide enhanced their susceptibility to transformation by feline sarcoma virus and Kirsten murine sarcoma virus, whereas this effect was not observed in normal human cells. An XP cell strain derived from a 14 year old patient (who did not exhibit any skin tumors at the time of biopsy) underwent spontaneous transformation in vitro without a limited life span (Thielmann et al., 1983). Comparison of this immortalized XP cell line to the parental cell strain from which it was derived demonstrated that

the spontaneous transformant grew faster, formed colonies in methylcellulose, gave rise to spontaneous fibrosarcomas in nude mice, had a greatly enhanced repair capacity, and exhibited abnormal cytogenetic and ultrastructural morphologies not detected in the parental XP cells. Attempts to transform the parental XP cells *in vitro* by UV and chemical carcinogens were not successful.

Immortalized lymphoblastoid cell lines have been established by transformation of lymphocytes derived from XP patients with Epstein-Barr virus (Andrews et al., 1974; Henderson, 1978; Tohda et al., 1978). It has been demonstrated that transformed lymphocytes and untransformed fibroblasts derived from the same XP patient exhibit the same reduced levels of DNA repair (Tohda et al., 1978). UV irradiation of lymphocytes or Epstein-Barr virus prior to transformation resulted in a greatly reduced transformation frequency in XP lymphocytes in relation to similar levels in XP heterozygous and normal human lymphocytes (Henderson, 1978).

An SV40 immortalized cell line has been shown to be hypersensitive to ethyl methanesulfonate when measured by SCE and colony forming ability, but retained the same sensitivity to killing by UV irradiation and deficiency in repair replication as other XP cells in its complementation group (Heddle and Arlett, 1980). Enzyme typing for the primary cell strain and the transformed line derived from it contained the same phenotypes. This same transformed XP line has been reported to be deficient in the excision of 0^6-alkylguanine (Goth-Goldstein, 1970). It is generally considered that SV40 immortalization of XP and normal human cells does not alter their UV repair capacities relative to nontransformed cells (Bootsma et al., 1970; Smith and Hanawalt, 1976; Smith and Hanawalt, 1978). However, evidence has shown that several SV40

transformed human skin fibroblast strains are unable to repair N-methyl-N'-nitro-N-nitrosoguanidine in contrast to their nontransformed fibroblasts that were repair proficient (Day et al., 1980). This increased sensitivity of transformed human fibroblasts to certain chemical carcinogens may either represent an alteration due to viral transformation or may indicate that the transformation process enhances selection for a primary fibroblast that is already sensitive to these chemical carcinogens but was not detectable in a heterogeneous primary fibroblast population. One case of hypersensitivity to ethyl methanesulfonate induced SCE has been demonstrated for primary lymphocytes derived from one XP patient as compared to control lymphocytes (Evans, 1977).

Replicons in XP SV40 transformed cells were at least twice the size of their untransformed counterpart, although DNA fork displacement rates were only slightly increased in the transformed cells (Kapp et al., 1979). Repair of DNA protein crosslinks in SV40 transformed XP cells was nearly equal to that of normal human skin fibroblasts while its untransformed XP cell equivalent was greatly reduced and similar to another XP cell strain of the same complementation group (Gantt et al., 1984). Prior UV irradiation of a chimeric plasmid resulted in a UV dose-dependent increase in the yield of transformants when introduced into repair proficient human and SV40 transformed XP cells by the calcium phosphate coprecipitation method (Spivak et al., 1984). Approximately the same UV-dependent increase in transformants occurred in the XP and normal cells.

Exposure of XP cells to the tumor promoter, 12-0-tetradecanoylphorbol-13-acetate, induced similar changes to those

observed in normal cells but did not result in anchorage-independent growth or immortalization for either XP or normal cells (Antecol and Mukherjee, 1982). This indicates that cells derived from XP patients having a genetic disorder associated with a high risk of cancer do not exist in a preneoplastic or initiated state susceptible to oncogenic transformation by a tumor promoter. N-Methyl-N'-nitro-N-nitrosoguanidine was found to transform XP cells but not heterozygous XP or normal cells. The transformed cells proved to be nonmalignant when transplanted into athymic nude mice (Shimada et al., 1976).

3. Biochemical Characteristics

a. Excision Repair

Cleaver (1968; 1969) first demonstrated that XP cells derived from XP patients who have been diagnosed as having the common or neurological forms of XP are defective in their ability to perform excision repair of UV irradiated DNA. It is thought that the defect in the XP excision repair process occur at the early step of DNA incision (Friedberg et al., 1979). Strand breaks in the DNA of UV irradiated normal human cells occur very rapidly postirradiation giving rise to an increase in low molecular weight single-stranded DNA (Kohn et al., 1981). A gradual return to normal molecular weight DNA is observed upon completion of excision repair (Fornace et al., 1976). XP cells fail to accumulate any significant fraction of breaks postirradiation, thus giving rise to the hypothesis that XP cells are deficient in DNA incision (Fornace et al., 1976). Further evidence for this idea is observed when

the purified T4 bacteriophage pyrimidine dimer DNA glycosylase-apurinic endonuclease is added to permeabilized UV irradiated XP cells. This technique restores normal rates of DNA repair synthesis (Tanaka et al., 1975). XP cells representing complementation group F only had partial restoration of this unscheduled DNA synthesis following addition of the T4 enzyme. Although XP complementation group A,B,C,D, and G are severely defective in incision, group E and F cells appear to be leaky in their incision defect when tested by the endonuclease-sensitive-site assay (Paterson et al., 1981; Zelle and Lohman, 1979) utilizing T4 or Micrococcus luteus purified enzymes that specifically recognize pyrimidine dimers. Although these enzymes specifically recognize the pyrimidine dimer, analogous enzymes have not been found in uninfected E. coli or in eukaryotic cells (Demple and Linn, 1980; Cleaver, 1983).

DNA polymerase β and possibly polymerase α function in the polymerization step of excision repair prior to ligation (Hubscher et al., 1979; Ciarrocchi et al., 1979). Excision repair must relax the nucleosomal structure so that damaged DNA regions are more accessible to repair enzymes (Bodell, 1977; Cleaver, 1977; Smerdon et al., 1978). This relaxation process is thought to be dependent upon poly(adenosine diphosphoribose)$_n$ synthesis that is defective in XP cells (Durkacz et al., 1980; Berger et al., 1980). Excision of pyrimidine dimers can remove approximately 100 bases (Cleaver, 1974a; Cleaver, 1968; Regan et al., 1971; Edenberg and Hanawalt, 1972) including both pyrimidines and purines (Cleaver, 1973). Continuous exonucleolytic degradation of the DNA strands is thought to occur by excision past the excised pyrimidine dimer (Cleaver, 1983).

It has been observed that the number of sites (single strand breaks) involved in excision repair at any instant is less than 1 percent of the total number of pyrimidine dimers in normal eukaryotic cells (Ben-Hur and Ben-Ishai, 1971; Cleaver et al., 1972; Setlow et al., 1969). This leads to the idea that in excision repair a steady state exists. Repair enzymes move from one pyrimidine dimer to another in which strand incision, excision, polymerization and ligation occurs in rapid succession (Cleaver, 1974a). This continuous excision of pyrimidine dimers, with subsequent base polymerizations, accounts for the very low frequency of single strand breaks (Fornace et al., 1976; Kleijer et al., 1973; Dingman and Kakunaga, 1976). It has been estimated that in normal eukaryotic cells, less than or equal to 1 site in 2×10^8 daltons of DNA is involved in excision repair at a given time (Cleaver, 1974b; Dunn and Regan, 1979). Recently, it has been demonstrated that one UV pyrimidine dimer in plasmid vectors will inactivate a target size of approximately 2 kilobases, the size of the putative chloramphenicol acetyltransferase transcriptional unit, in XP cells but not repair-proficient cells (Protic'-Sabljic and Kraemer, 1985). This inactivation is independent of plasmid size or of different promoters when UV-irradiated plasmid vectors were transfected into XP and normal cells and assayed by host cell reactivation.

Experiments conducted in vivo on XP patients and in vitro with XP cells demonstrate that excision of pyrimidine dimers and repair replication occur at rates between 0 to 90 percent of normal cells (Bootsma et al., 1970; Epstein et al., 1970). Although the most severly repair deficient XP cells were thought to have little if any residual repair capacity (Kraemer, 1980) it has been shown that they contain a low

residual level of DNA excision repair (Fornace and Seres, 1983). Normal levels of repair replication and pyrimidine dimer excision are observed in XP variant cells (Burk et al., 1971; Cleaver, 1972; Cleaver et al., 1970; Lehmann et al., 1975). Deficiencies in XP repair replication have been shown to occur in vitro in fibroblasts, liver cells, and peripheral lymphocytes (Burk et al., 1971; Dupuy et al., 1974; Cleaver, 1968).

b. Repair of UV-like and X-ray-like Mutagens and Carcinogens

XP cells repair x-ray damaged DNA at levels similar to that observed in normal cells (Cleaver, 1973). Agents that are repaired at normal levels in XP cells are considered x-ray-like, examples of which are methyl methane sulfonate (Cleaver, 1971) and N-methyl-N'-nitro-N-nitrosoguanidine (Cleaver, 1971; Stich et al., 1973). X-ray-like chemicals are repaired by short excision repair patches of 1 to 3 nucleotides while UV-like chemicals have long excision repair patches of up to 100 nucleotides (Friedberg et al., 1979).

4. Genetic Heterogeneity

On the basis of complementation studies in heterokaryons and of the different levels of reduced excision repair in XP cell strains, at least 9 different complementation groups (A thru I) have been described (de Weerd-Kastelein et al., 1972; Kraemer et al., 1975; Arase et al., 1979; Keijzer et al., 1979; Robbins et al., 1983; Fischer et al., 1985). The clinical and biochemical characteristics of each complementation group have been reviewed extensively (Cleaver, 1985;

Fischer et al., 1985; Friedberg et al., 1979). The number of complementation groups is more intriguing since it points to the possibility of several different genetic functions involved in the incision of UV irradiated human DNA. Three gene products have been found to be required for incision of UV irradiated E. coli DNA (Sancor and Rupp, 1983). The lower eukaryotic S. cerevisiae is dependent upon at least five RAD genes for incision in vivo of UV irradiated DNA (Friedberg et al., 1983). Two models have been proposed for the requirement of multiple gene products for incision of UV damaged DNA. One model suggests that these gene products are part of a large multiprotein complex required for the specific cutting of DNA in association with excision of UV damaged bases and conformational changes that occur on the chromosome and make the damaged DNA accessible to repair enzymes. The other model proposes that the incision activity is part of a smaller multi-enzyme complex that functions not only in incision, but repair synthesis and DNA ligation. Inactivation of one component of this multi-enzyme complex would result in defective excision repair. It is considered that this second model is in question since the levels of DNA polymerase and DNA ligase activities are not significantly different in XP and normal human cells (Pedrini et al., 1974; Parker and Lieberman, 1977; Bertazzoni et al., 1977).

Support of the multiprotein complex comes indirectly from several observations. It has been clearly shown that extracts from normal human cells excise pyrimidine dimers from their endogenous chromatin or exogenously purified DNA. However extracts from XP cells are defective in removal of UV damage from their chromatin but not from purified DNA (Mortelmans et al., 1976; Friedberg et al., 1977; Kano and Fujiwara,

1983; Fujiwara and Kano, 1983; Mansbridge and Hanawalt, 1983). Extracts from normal human cells undergo DNA excision repair in XP chromatin, thus indicating that a defect does not exist at the level of the chromatin structure itself. Further support for this model was found in studies using calf thymus extracts which indicated that a high molecular weight protein is responsible for incision of pyrimidine dimers (Waldstein et al., 1979).

Further indirect evidence for a multiprotein complex in normal human cells for excision repair of pyrimidine dimers is provided by the observation that following UV irradiation, the addition of methyl methanesulfonate inhibits repair (Gruenert and Cleaver, 1981; Park et al., 1981; Cleaver, 1982). Since XP cells have normal levels of methyl methanesulfonate repair (Friedberg et al., 1979), it is felt that the repair pathways of UV and alkylating damage are separate. Inhibition of excision repair by methyl methanesulfonate is suggested to be due to alkylation of a protein target.

One approach to understanding excision repair has been the attempt to molecularly clone repair genes. One report has recorded the ability of DNA from repair proficient human cells to complement the phenotype of XP cells by transfection (Takano et al., 1972). However, this report is questionable since similar studies have reported a high frequency of phenotypic reversion in XP cells (Protic-Sabljic et al., 1983; Ganesan et al., 1983).

Microinjection of cell extracts from repair proficient human cells have been found to increase levels of unscheduled DNA synthesis temporarily, while extracts from XP cells injected into XP cells of the same complementation group fail to complement repair (Hoeijmakers et al.,

1983; DeJonge et al., 1983). Cell extracts have been demonstrated to be of protein origin since they are inactivated by proteases. This genetic complexity of XP cells may not only represent a defect in excision repair but also a defect in the necessity for chromosomal modification prior to incision of the pyrimidine dimers (Friedberg, 1985).

A newly proposed genetic model termed co-recessive inheritance has been proposed for XP in which the excision deficient trait is expressed when the individual is homozygous or hemizygous for defective alleles at more than one of a specific set of loci (Lambert and Lambert, 1985). This model, Lambert and Lambert (1985) state, accounts for the paradoxes in XP that include a large number of complementation groups in the presence of a biochemically limited DNA repair defect, the co-existance of XP and Cockaynes' syndrome in complementation groups B and H, siblings from one XP family with markedly different degrees of DNA excision repair defects, transmission of the disease in an X-linked manner in another family, existence of individuals with marked DNA repair defects without clinical XP, and the paradox of XP not associated with an increase in incidence of internal cancer.

Lambert and Lambert (1985) suggest that this co-recessive inheritance model will be difficult to prove and say that one must choose the correct individuals or cell strains to study. They also suggest that it may not apply to all XP complementation groups. However, it should be noted that the specific DNA repair defect(s) in XP have not been identified and until cloned probes exist that can identify the defective alleles, this co-recessive hypothesis will remain untested. Only after these defective alleles are identified can it be shown that XP is expressed in a homozygous or hemizygous and not heterozygous patients.

Evidence has also accumulated that suggests that the repair defects in XP cells may not be entirely associated with the removal of pyrimidine dimers, but involve steps prior to their removal. Therefore, if a number of gene products are required prior to excision repair, a defect in any one of these genes would be manifested as a defect in excision repair of pyrimidine dimers and be clinically diagnosed as XP. As yet, the repair steps required prior to dimer removal have not been elucidated.

C. Fanconi's Anemia

1. Clinical Characteristics

FA is an autosomal recessive disorder clinically characterized by a progressive pancytopenia (Fanconi, 1967). This disorder was first described by Fanconi (1927) as a lethal familial anemia in three brothers between the ages of 5 and 7 years (Fanconi, 1927). The patients were first diagnosed as having pernicious anemia because of the presence of hyperchromic erythrocytes, but upon autopsy, the bone marrow was atrophic and yellow with few foci of hematopoesis, while in pernicious anemia the bone marrow is hyperactive and red (Fanconi, 1967). FA patients usually die at an early age from hemorrhage, infection, or leukemia. Variability in the FA phenotype makes the diagnosis difficult and unreliable when based on clinical manifestations alone. A summary of 152 cases of FA correlated the combination of panmyelopathy with diverse congenital abnormalities (Gmyrek and Syllm-Rapoport, 1964). This progressive pancytopenia is first observed as a thrombocytopenia, followed by leukopenia and finally, aplastic anemia (Gmyrek and Syllm-Rapoport, 1964). In early stages of this disease the bone marrow may be hyperactive but it later becomes hypoplastic with the tendency of bleeding following an infectious illness. Skeletal, renal, and cardiac malformations, short stature, and abnormal pigmentation that includes cafe-au-lait spots and hyper- and hypopigmentation may be observed in the FA phenotype (Alter and Potter, 1983). FA is found in all ethnic groups where the carrier frequency is approximately 1 in 300. This estimate is subject to controversy due to problems of accurate diagnosis.

Hypopigmentation, vitiligo of the skin, stunted growth at birth, along with subnormal birth weight are indicative of abnormal development in utero (Friedberg et al., 1979). Of 82 FA cases reviewed, only 13 patients had no malformations or abnormalities while 20 percent were mentally retarded (Nilsson, 1960). Over 60 percent of all cases involve males where its onset occurs within the first 4 to 7 years of life while it is generally first observed in females around 6 to 10 years of life (Nilsson, 1960; Gmyrek and Syllm-Rapoport, 1964). This preference for males must be viewed by the fact that this sex difference is also observed among the unaffected siblings and is not statistically different (Schroeder et al., 1976). Evidence for the genetic origin of this disease is seen in the observation that FA has high familial occurrence (Nilsson, 1960) and high incidence of consanguinity (Gmyrek and Syllm-Rapoport, 1964). Although FA has been proposed to have an autosomal recessive mode of inheritance (Reinhold et al., 1952; Swift and Hirschhorn, 1966; Schroeder et al., 1976) the high ratio of 1 to 1.17 of affected to unaffected siblings (Fanconi, 1967) along with the small number of known patients may require further genetic considerations for this order. Due to the many different types of malformations, the occurrence of hypoplasia without malformations, and similar types of malformation in non-sibling relatives may be indicative of a multigenetic disorder that accounts for this disease (Fanconi, 1967; Nilsson, 1960). The number and severity of malformations may point to genetic heterogeneity as observed in XP (Schroeder et al., 1976).

A high incidence of leukemia and several other malignant neoplasms are found in both FA patients and their heterozygous relatives (Garriga and Crosby, 1959; Swift and Hirschhorn, 1966; Gmyrek et al., 1967; Swift,

1971; Schroeder and Kurth, 1971; German, 1972). Although neoplasia does not contribute significantly to the death of FA patients due to their short life spans, heterozygous relatives have a three times higher risk of dying from malignant neoplasms than the general population (Swift, 1971; Swift, 1976). However, when this study was expanded to include a larger number of FA families from more diverse backgrounds, no excess of cancer(s) or deaths due to cancer was detected in blood relatives or among the heterozygotes (Swift et al., 1980; Caldwell et al., 1979). An increased incidence of diabetes has been associated with FA heterozygous females (Swift et al., 1972; Swift, 1976).

Prenatal and postnatal diagnosis of FA is done by exposing homozygous blood lymphocytes, amniotic fluid cells, and heterozygous blood lymphocytes to the bifunctional alkylating agent diepoxybutane (Auerbach et al., 1981). Homozygous cells have a 175 fold increase in chromosome breakage characterized by multiple complex chromatid exchanges, while heterzygous cells have a 4 fold increase in chromosomal breakage when compared to control cells. FA homozygous, heterozygous, and normal fibroblasts can also be distinguished from each other by this technique (Auerbach and Wolman, 1976; Auerbach and Wolman, 1978; Auerbach et al., 1979; Auerbach and Wolman, 1979).

2. Cellular Properties

a. Cell Physiology and Growth

Fibroblast cultures derived from skin biopsies of patients with FA have doubling times significantly longer than normal control

cells (Elmore and Swift, 1975), decreased plating efficiency, and accumulation of mitotic cells in culture (Weksberg et al., 1979). FA fibroblasts maintained in a nonproliferating state for 14 to 27 days increased the cumulated population doublings by 15 cell generations and remained in culture 210 days longer than FA cells not arrested (Diatloff-Zito and Macieira-Coelho, 1982). Cells arrested at later passages produced the same results. A spontaneous chromosomally abnormal clone, identified cytogenetically in a FA cell strain, rapidly became the only cell type in the culture after the normal cell population underwent senescence by 18 population doublings (Auerbach et al., 1980). This abnormal clone showed signs of cell senescence after 46.5 population doublings.

Lymphocytes derived from FA patients had a significant slowing of their cell cycle compared to controls, which was also observed to a lesser degree in heterozygous cells (Dutrillaux et al., 1982; Sasaki, 1975; Dutrillaux and Fosse, 1976). The slow cell cycle is mostly due to an extended G2 phase and represents a subpopulation of faster growing cells from the same patient which was correlated with increased chromatid anomalies (Dutrillaux et al., 1982). One FA fibroblast strain examined had a reduced in vitro longevity (Thompson and Holliday, 1983) which correlated with the appearance of a significant fraction of heat labile glucose-6-phosphate dehydrogenase that was not apparent in long lifespan control cells until they become senescent (Holliday and Thompson, 1983). Similar results were observed in fibroblasts derived from Werner's syndrome, ataxia telangiectasia, Bloom's syndrome, Cockaynes syndrome, and progeria, syndromes that are associated with either reduced DNA repair or chromosomal instability. These observations suggest that for

maximum *in vitro* lifespan, repair processes are necessary, thus giving support for the error theory rather than the programmed theory of aging (Thompson and Holliday, 1983).

b. Chromosomal Abberrations and Sister Chromatid Exchange

While it is now generally believed that SCE and chromosomal aberrations are two different molecular mechanisms for the responses of mutagen induced DNA damage (Gebhart, 1981), the SCE test is regarded as a sensitive method of detecting DNA damaging agents (Kato and Shimado, 1975; Perry and Evans, 1975; Evans, 1977). The classical mutagen testing by cytogenetic analysis of chromosomal aberrations is associated in most cases with cell death, whereas SCE's are representative of events compatible with cell survival and mutagenesis (Wolff, 1977). It has been postulated that the majority of SCE are genetically neutral due to equal exchanges of sister chromatids while some unequal exchanges occur leading to deletions, insertions, or frameshift mutagenesis. Untreated FA lymphocytes and fibroblasts have an increased level of chromosomal aberrations but exhibit normal SCE frequencies similar to that of normal control cells (Chaganti et al., 1974; Kato and Stich, 1976; Latt et al., 1975; Novotna et al., 1979; Sasaki, 1980; Berger et al., 1980; Kano and Fujiwara, 1981; Cerevenka et al., 1981). It should be noted however that slight but significant spontaneous levels of SCE were observed in peripherial lymphocytes from FA patients (Porfirio et al., 1983). Induction of SCE by chemicals that crosslink DNA has been reported to suppress, elevate, or produce normal responses, depending upon the experimental details. Reduced SCE is

observed in peripheral lymphocytes exposed to mitomycin C during the preharvest replication cycle (Latt et al., 1975; Latt et al., 1983; Cerevenka et al., 1981; Latt et al., 1982). Two reports demonstrated that FA lymphocytes respond normally to mitomycin C and polyfunctional triethylene phosphoramide (Sasaki, 1980; Novotna et al., 1979), while nitrogen mustard is a hyperinducer of SCE (Berger et al., 1980). Diepoxybutane is ineffective in inducing SCE in FA lymphocytes (Porfirio et al., 1983). Mitomycin C induction of SCE in FA fibroblasts is normal if the cells are exposed for approximately one cell cycle (Latt et al., 1982), but is elevated if the mitomycin C exposure is only for one hour (Kano and Fujiwara, 1981). However, it was shown that FA lymphocytes had about 1.4 times higher SCE frequencies than normal cells in both mitomycin C treated and untreated cultures (Miura et al., 1983). Similar results of increased SCE in FA fibroblasts were obtained following exposure for one hour to bifunctional TMP plus UVA, diepoxybutane, and cis-diamminedichloroplatinum (II) (Fujiwara et al., 1984). The mitotic index of FA cells is generally low (German et al., 1978; Weksberg et al., 1979). Slight variations in cell culture condition thus might cause a disproportionate change in the types of cells completing two cycles of replication before collection at metaphase which would account for some of the interstudy variation that has been reported (Latt et al., 1983).

FA and control cells have very similar frequencies of SCE after exposure to monofunctional agents. SCE in FA lymphocytes induced by ethylmethane sulfonate was slightly less than in normal cells (Latt et al., 1975) but was reported to be normal for monofunctional decarbamoyl mitomycin C (Kano and Fujiwara, 1981).

Increased levels of chromosomal aberrations occurrs when FA lymphocytes are exposed to the crosslinking agents mitomycin C, diepoxybutane, nitrogen mustard, and 8-MOP plus UVA, but very low levels occur in normal lymphocytes (Sasaki and Tonomura, 1973; Sasaki, 1978; Cohen et al., 1982). Chromosomal aberration formation induced by mitomycin C in FA cells is most pronounced in the G1 phase and decreased in yields as the S phase progresses (Sasaki, 1975). Cis-diamminetetrachloroplatinum (IV) and cis-diamminedichloroplatinum (II) both form DNA crosslinks and increase chromosomal aberrations in FA lymphocytes relative to normal cells (Poll et al., 1982).

FA and normal cells have similar levels of chromosomal aberrations when induced by the monofunctional agents methylmethanesulfonate, N-methyl-N'-nitro-N-nitrosoguanidine, 4-nitroguinoline-1-oxide, and the DNA protein crosslinker trans-diamminetetrachloroplatinum (IV) (Sasaki and Tonomura, 1973; Poll et al., 1982; Sasaki, 1978). Radiation by gamma-rays does not increase the level of induced chromosomal aberrations in FA cells over normal cells (Sasaki and Tonomura, 1973; Sasaki, 1978), while irradiation by ultraviolet (254 nm) and X-rays increase the levels above normal cells (Sasaki and Tonomura, 1973; Sasaki, 1978; Duckworth-Rysiecki and Taylor, 1985). It has been reported though that the two monofunctional agents ethylmethane sulfonate and decarbamoyl mitomycin C increase chromosomal aberrations in FA lymphocytes and fibroblasts (Auerbach and Wolman, 1976; Sasaki, 1975; Sasaki and Tonomura, 1973), as does the antitumor glycopeptide bleomycin (Cohen et al., 1982; Duckworth-Rysiecki and Taylor, 1985). No difference in the induction of chromosomal aberrations by mitomycin C and triethylene

phosphoramide between FA and normal cells has been reported (Novotna et al., 1979).

It has also been reported that lymphocyte cultures from two patients diagnosed with FA contained only a 20 and 40 percent fraction respectively that exhibited increased chromosomal aberrations by bifunctional alkylating agents, while the remaining cells responded like the controls in failing to show any damage (Kwee et al., 1983; Auerbach et al., 1981).

c. Colony Forming Ability

It is evident that the decrease in cell survival of FA fibroblasts and lymphocytes is hypersensitive to the DNA crosslinking agents mitomycin C, nitrogen mustard, and diepoxybutane as compared to controls (Fujiwara et al., 1984; Fornace et al., 1979; Arlett and Harcourt, 1978; Fujiwara et al., 1977; Finkelberg et al., 1974; Ishide and Buchwald, 1982; Poll et al., 1984a). This increased sensitivity to FA cell killing by mitomycin C indicates that their are differences in susceptibility to killing by different cell strains (Poll et al., 1984a; Ishida and Buchwald, 1982; Fujiwara et al., 1977). Other DNA crosslinking agents such as TMP plus UVA, cis-diamminedichloroplatinum (II), 8-MOP plus UVA, cyclophosphamide, 1,3-butadine diepoxide, and 4-[Bis(2-chloroethyl)amino]-L-phenylalanine (melphalan) result in reduced FA cell survival in relation to control cells but are less effective than mitomycin C (Poll et al., 1984a, Ishida and Buchwald, 1982; Wunder and Fleischer-Reischmann, 1983; Poll et al., 1984b; Fujiwara et al., 1984). Three nitrosourea analogs which crosslink DNA are more toxic to FA cells

than normal cells, but their is heterogeneity in sensitivity among both types of cells (Ishida and Buchwald, 1982).

The colony forming ability of FA cells exposed to monofunctional agents such as ethylmethane sulfonate and decarbamoyl mitomycin C appears to be generally no different from normal (Arlett and Harcourt, 1978; Finkelberg et al., 1974; Fujiwara et al., 1977). However, heterogeneity in response to cell survival of both FA and normal cells has been reported for the monofunctional alkylating agents ethylmethane sulfonate, N-methyl-N'-nitro-N-nitrosoguanidine, and methylmethanesulfonate (Ishida and Buchwald, 1982).

FA cells are no more sensitive than normal cells to bleomycin which causes DNA strand breaks, the release of bases, and disruption of the deoxyribose moiety similar to X irradiation or 4-nitroguinoline-1-oxide, which binds to DNA and has a mechanism of action similar to UV (254 nm) (Ishida and Buchwald, 1982). Several FA cell strains have normal survival to UV (254 nm) (Fujiwara et al., 1977; Fornace et al., 1979; Smith and Paterson, 1981) although one strain is hypersensitive to both 313 nm UV and 254 nm UV (Smith and Paterson, 1981). Radiation of FA and normal cells by X- and gamma-rays showed the same degree of colony survival (Fornace et al., 1979, Duckworth-Rysiecki and Taylor, 1985; Finkelberg et al., 1974). One FA fibroblast strain was resistant to killing by ethidium bromide as were several normal cell strains (Gupta and Goldstein, 1982).

d. Mutagenesis

The mutation frequencies of FA cells by ethylmethane sulfonate and mitomycin C in the generation of ovabain (autosomal dominate marker) resistance is one to two orders of magnitude lower than controls as is thioguanine (X-linked recessive marker) resistance, indicating that the generation of somatic mutations are impaired in FA cells (Finkelberg et al., 1977). No significant difference was observed between one FA fibroblast strain and normal fibroblasts in the rate of N-methyl-N'-nitro-N-nitrosoguanidine mutation to diphtheria toxin resistance (Gupta and Goldstein, 1980).

e. Viral Host Cell Reactivation

No difference in the survival of SV40 DNA crosslinked with cis-diamminedichloroplatinum (II) was found between FA and control cells, although FA cells are more sensitive to the cytotoxic action than controls in the clonogenic cell survival assay (Poll et al., 1984b). UV (254 nm) irradiated adenovirus type 2 and 12 have reduced host cell reactivation in several different FA fibroblast strains relative to normal cells (Rainbow and Howes, 1977a; Rainbow and Howes, 1977b). Reduced repair of gamma irradiated adenovirus type 2 is also observed in one FA fibroblast strain (Rainbow and Howe, 1977b).

f. Viral and Carcinogenic Transformation

Transformation of seven different strains of normal fibroblasts with SV40 gave similar transformation frequencies of approximately 0.03 percent while two FA fibroblast strains had transformation frequencies

more than ten times higher. Two strains heterozygous for FA were considerably more susceptible to viral transformation (Todaro et al., 1966). The transformation frequency is directly related to the fraction of SV40 T antigen producing cells observed in an acute infection (Aaronson and Todaro, 1968). Susceptible FA strains have an efficiency of T antigen production per plaque forming unit of 1 in 250 while in normal resistant strains it is 1 in 10,000. After T antigen is formed each cell have the probability of 1 in 250 of giving rise to transformed colonies. It appears that the difference in susceptibility within human cell strains involves a step prior to or associated with SV40 T antigen induction (Aaronson and Todaro, 1968). Further research indicated that FA fibroblasts did not have an increased SV40 sensitivity but that the differences in transformation frequency among cell strains by extracellular virus was eliminated by use of SV40 DNA (Aaronson, 1970). This suggests that the resistance of some cell strains to extracellular virus is due to a block at an early point in infection.

Fibroblasts from both parents of two siblings diagnosed with FA not only demonstrated chromosomal abnormalities but also had increased numbers of SV40 transformed colonies, a result that was also observed in the one sibling tested (Dosik et al., 1970). However not all unaffected parents or siblings of patients with FA have increased frequencies of transformation by SV40, although no data on the cytogenetics of each cell strain used was given (Young, 1971). The elevated expression of SV40 T antigen in FA fibroblasts from homozygous and heterozygous patients is also observed in fibroblasts derived from patients with thrombocytopenia-absent radius syndrome and related conditions. This elevated T antigen expression also occurred in their normal relatives

fibroblasts, which are all at high risk for acute nonlymphocytic leukemia, but not in other patients fibroblasts with sporadic aplastic anemia that do not have a predisposition to leukemia (Lubiniecki et al., 1977; Lubiniecki et al., 1980). FA fibroblasts apparently do not exist in a preneoplastic or initiated state since chronic application of the tumor promoter 12-0-tetradecanoylphorbol-13-acetate did not result in anchorage independent growth or unlimited growth potential in culture (Antecol and Mukherjee, 1982).

3. Biochemical Characteristics

a. Repair of DNA Crosslinking Agents

Determination of the sedimentation velocity of DNA isolated from FA fibroblasts crosslinked with mitomycin C in alkaline sucrose gradients showed no evidence for single strand cutting and sedimented 2.5 times faster than that of the untreated control (Fujiwara et al., 1977). The fast sedimenting DNA remained unaltered during postincubation of the cells due to lack of half excision repair (a double nick, one on either side of a crosslink) of crosslinks. Hydroxyapatite chromatography reveals the reversible double stranded property of mitomycin C crosslinked DNA after denaturation and demonstrated that 3 FA fibroblast strains had 2 to 8 fold reduced rates of crosslink removal relative to normal fibroblasts (Fujiwara et al., 1977). However, further studies utilizing mitomycin C and the alkaline elution method found that at equal levels of DNA crosslinking in FA and normal fibroblasts, a gradual decrease of crosslinking was detected postincubation, where the

rate of repair was same for both FA and normal fibroblasts (Fornace et al., 1979).

Repair of nitrogen mustard and mitomycin C crosslinks by the alkaline elution technique showed that early passsage FA fibroblasts could repair crosslinks. However, after the passage number of the fibroblasts increased, their was a corresponding decrease in their ability to repair nitrogen mustard crosslinks, their repair capacity was nearly eliminated in late passage cells (Sognier and Hittelman, 1983). Mitomycin C induced SCE frequency in normal fibroblasts was reduced in a biphasic manner with a repair incubation time which corresponded to the molecular kinetics of crosslink and monoadduct removal (Fujiwara et al., 1984). The first decline has a $t_{\frac{1}{2}}$ equal to 2 hours and the second of $t_{\frac{1}{2}}$ equal to 14 to 18 hours. However, FA fibroblasts lack the first half excision repair process which corresponds to their observed absence of the first SCE component (Fujiwara et al., 1984). This slowly declining component was observed with a higher SCE frequency detected 24 to 48 hours after exposure to mitomycin C. Further studies using mitomycin C sensitive FA fibroblasts failed to show any difference in the kinetics of the unhooking step for mitomycin C or 8-MOP crosslink repair relative to control FA fibroblasts by the alkaline sucrose sedimentation gradient and alkaline denaturation/renaturation hydroxyapatite chromatography techniques for the two crosslinking agents, respectively (Poll et al., 1984a). Another FA fibroblast strain was no different than control fibroblasts in removal of 8-MOP crosslinks by the heat denaturation/renaturation method followed by digestion with single stranded specific S_1 nuclease digestion (Kay et al., 1980).

Normal and FA fibroblasts were treated with 8-MOP plus UVA and then tested by the alkaline elution technique for removal of crosslinks at various times up to 7 days postirradiation. The FA elution pattern was unaltered in relation to normal fibroblasts (Bredberg and Soderhall, 1985). After 1 to 7 days postirradiation both FA and normal fibroblasts were exposed to a second UVA dose and tested for crosslink removal by alkaline elution. The elution rate increased in normal fibroblasts while FA cells had increased filter retention which suggested that another crosslink reaction occured upon further irradiation during the repair process in FA fibroblasts (Bredberg and Soderhall, 1985).

b. Repair of DNA Monofunctional Agents

FA fibroblasts have the same ability as normal fibroblasts to repair monofunction decarbamoyl mitomycin C when measured by the alkaline sucrose sedimentation gradient technique (Fujiwara et al., 1977). Normal and FA fibroblasts both remove approximately 50 percent of the bound N-acetoxy-2-acetylaminofluorene within 48 hours (Amacher and Lieberman, 1977).

c. Repair of Radiation Induced DNA Damage

UV (254 nm) irradiated FA cells appear to produce single strand incisions in response to pyrimidine dimer induction and are able to polymerize nucleotides into the new complementary strand to replace the damaged DNA. However, they are deficient in an exonuclease function that removes the damaged DNA strand after the endonucleolytic incision

(Poon et al., 1974). Irradiation of normal and FA fibroblasts with UV (254 nm) induced approximately 21 to 85 x 10^5 pyrimidine dimers per cell. Less than 10 percent of the pyrimidine dimers lost by 30 hours, were lost by 6 hours postirradiation from both FA and normal fibroblasts (Ehmann et al., 1978). Both FA and normal fibroblasts had a half maximal loss of dimers between 12 to 20 hours after irradiation and a maximal loss per cell of 12 to 20 x 10^5 pyrimidine dimers, within 20 to 30 hours, after which no further loss occurred. Rejoining of DNA single stranded breaks after X-irradiation and the formation of excision breaks after UV (254 nm) irradiation were normal in both FA and normal fibroblast when determined by the alkaline elution technique (Fornace et al., 1979). Whole cell homogenates and nuclear sonicates from two out of five FA fibroblast strains were substantially below normal in excision repair of the gamma-ray induced 5,6-dihydroxydihydrothymine products in bacteriophage DNA (Remson and Cerutti, 1976).

d. Other Aspects of DNA Repair

Bifunctional 8-MOP plus UVA causes a greater inhibition of DNA synthesis than monofunctional isopsoralen plus UVA in normal and FA cells (Gruenert et al., 1985). Replicon initiation was inhibited in normal but not FA cells when exposed to UVA without any furocoumarins, while in the presence of isopsoralen or 8-MOP replicon initiation was inhibited in both cell strains (Gruenert et al., 1985). DNA replication fork displacement rates of FA fibroblasts and fibroblasts from a variety of other human cell strains is remarkably constant, with an average for all cell strains of 0.53 $\pm$ 0.08 μm/min. (Kapp and Painter, 1981).

Replication preceeds bidirectionally from a central origin and adjacent origins initiate synchronously in normal and FA cells (Hand, 1977).

It has been noted that the addition of superoxide dismutase or catalase decreases the frequency of chromosomal aberrations in FA lymphocytes (Nordenson, 1977), and that there is a correlation between the increasing frequency of aberrations with increasing concentrations of oxygen which is not observed in normal cells (Joenje et al., 1981). However, upon exposure to high concentrations of oxygen no evidence of DNA single strand breaks or DNA damage in FA fibroblasts was obtained when analyzed by alkaline elution or endonuclease sensitivity (Seres and Fornace, 1982). FA fibroblasts exposed to mitomycin C have a greater accumulation in the G2 + M phases than normal cells. FA cells were found to be blocked prior to mitosis (Kaiser et al., 1982; Latt et al., 1982). Exposure of FA and normal fibroblasts to the monofunctional agent, ethylmethane sulfonate resulted in similar, but smaller accumulations in the G2 + M phases (Kaiser et al., 1982).

Although FA cells are sensitive to gamma-ray and alkylating agents, which are both capable of producing apurinic and apyrimidinic sites, the levels of apurinic DNA endonuclease activities in cell extracts are normal (Teebor and Duker, 1975; Moses and Beaudet, 1978). The specific activities of DNA polymerases α, β, and γ from FA fibroblasts were within the limits observed for normal human fibroblasts and do not have altered sedimentation properties (Bertazzoni et al., 1978; Parker and Lieberman, 1977). While caffeine inhibits some DNA repair processes it has no effect on all three DNA polymerases (Parker and Lieberman, 1977). The activity of DNA topoisomerase I, a nicking-closing enzyme, has 88 to 100 percent of its total cellular activity in the nuclear extracts with a

minor proportion of the enzyme (up to 12 percent) randomly present in some of the cytoplasmic extracts of both FA and normal fibroblasts (Auerbach et al., 1982). It was concluded that FA is not due to a maldistribution of topoisomerase I between the nuclei and cytoplasm. However, another report found no topoisomerase I activity in the cytoplasmic fraction of normal human fibroblasts and normal placentas while examination of the same tissues derived from FA patients revealed enzyme activity in the cytoplasmic fraction (Wunder, 1984). The cytoplasmic topoisomerase I activity from one FA fibroblast strain constituted 6.5 percent of the whole cellular activity leading to the conclusion that this enzyme is maldistributed within the FA cell. The differences in conclusions may be caused by different experimental methodology or the difference between a rapidly growing culture versus a stationary culture (Wunder, 1984). The activity of the chromatin bound poly(ADP-ribose) polymerase is markedly stimulated by DNA strand breaks (Durkacz et al., 1980; Berger and Sikorski, 1981) and catalyzes the synthesis of poly(ADP-ribose) from NAD^+. FA cells have lower NAD^+ levels than normal cells and while both cell types have an increase in poly(ADP-ribose) polymerase activity following exposure to UV (254 nm) or N-methy-N'-nitro-N-nitrosoguanidine the FA cells have less enzyme activity (Berger et al., 1982). NAD^+ levels also decrease in both normal and Fanconi's anemia cells in response to DNA damage but it was observed that the initial levels of NAD^+ were lower in FA cells than cells from normal donors (Berger et al., 1982).

4. Genetic Heterogeneity

The extensive genetic heterogeneity in two repair deficient human disorders, XP and ataxia teleangiectasia has been proven by complementation studies through heterokaryon analyses. This approach concluded that intergenic heterogeneity is of less importance in FA (Zakrzewski and Sperling, 1982). Moreover, evidence has been presented that complementation does not occur between FA strains in which different biochemical lesions have been postulated (Zakrzewski et al., 1983). It should be noted however that the inability to find complementation groups in FA may arise from the fact that some patients only contain a subpopulation of cells that respond to clastogens with the remainder responding like normal cells. In one out of four patients with FA, it was observed that only 40 percent of the cultured cells demonstrated increased chromosomal aberrations when exposed to mitomycin C, diepoxybutane, or cis-diamminedichloroplatinum (II), while the remaining 60 percent responded like normal donor cells (Kwee et al., 1983). The other three FA patients showed 100 percent involvement of all cells in the induction of chromosomal aberrations by the three clastogens. Another FA patient has been reported whose lymphocytes only showed 20 percent diepoxybutane responsive cells (Auerbach et al., 1981).

It is evident that FA cells have increased chromosomal aberrations and reduced colony forming ability after exposure to crosslinking agents. However, it is not celar that all FA cells have a defect in crosslink removal and normal levels of crosslink induced SCE. Since FA cells have not been as extensively characterized as XP cells, this controversy in crosslink repair may just represent a lack of a thorough understanding of the heterogeneity involved in FA.

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Manuscript 1.

Optimal Conditions for Titration of SV40 by the Plaque Assay Method

Summary. The parameters of the Simian Virus 40 (SV40) plaque assay on African green monkey kidney cells were optimized for reproducibility and maximum plaquing efficiency. Plaques were visible as early as 8 days postinfection; maximum titers were obtained with a 10- to 11-day incubation period. Titers read 12-16 days postinfection were not significantly higher than those observed after 10-11 days. Adsorption volumes > 0.1 ml/60 mm Petri dish decreased plaque forming units (PFUs) detected. Times > 60 min for adsorption of virus to the cell monolayer did not significantly increase the titer; adsorption times < 60 min resulted in decreased titers. Under standard conditions, 3 ml of overlay medium containing 0.8% agar was applied following virus adsorption and again on days 5 and 10. Concentrations of fetal calf serum (FCS) in the overlay medium of 2.5 to 7.5% gave equal plaque formation. FCS concentrations of 1 and 10% resulted in slightly decreased and increased plaquing efficiencies respectively. Of the reagents tested, agar or agarose containing overlay media produced plaques of maximum number and size. An overlay of methyl cellulose resulted in the same number of plaques, but their size was reduced by approximately 70% relative to those observed in agar; thus longer incubation times were required. Gum tragacanth overlay medium was actually inhibitory to plaque development. DEAE-dextran, dextran sulfate, or DMSO added to agar overlay medium did not enhance plaque number or size, nor did they shorten the incubation period required for their detection.

INTRODUCTION

Since the original report by Stinebaugh and Melnick (1962) that Simian Virus 40 (SV40) was capable of producing plaques when inoculated onto African green monkey kidney (AGMK) cells, no systematic approach to determine the optimal conditions for the SV40 plaque assay has been reported. In spite of the fact that this virus is one of the most widely studied animal viruses from a molecular standpoint, reliable quantitation of viable particles has remained a troublesome aspect of many investigations. Titration of SV40 using the plaque assay has the disadvantage of requiring the maintenance of cells for approximately 2 to 6 weeks before plaques can be enumerated (Black et al., 1964; Riggs and Lennette, 1965; Takemoto et al, 1966; Ravid et al., 1968; Rosenberg et al., 1981), and assays that fail to produce plaques or that yield highly variable titers are not uncommon. Although SV40 produces a lytic infection in permissive cells, there appears to be no specific mechanism for release of progeny virions (Acheson, 1980), and a large fraction of the virus particles remain cell associated (Granboulan et al., 1963). The relatively small numbers of extracellular virions that are observed, especially at early times after infection, may be the result of increased plasma membrane permeability (Norkin, 1977). Membrane association has also been reported for BK and polyoma viruses (Sechafer et al., 1975; Sechafer et al., 1978).

Several techniques have been applied to decrease the incubation time of the SV40 plaque assay. Rapid titration of SV40 by an immunofluorescent focus assay has been reported to reduce the assay time to 3 to 6 days (Ravid, et al., 1968; Aaronson and Todaro, 1970).

Pretreatment or addition of dimethyl sulfoxide (DMSO) to the agar overlay medium using AGMK cells has been reported to reduce the SV40 plaque assay time as well as to increase the plaque number (Cleaver, 1974). Enhancement of polyoma plaque formation has been observed upon addition of dexamethasone to the agar overlay medium (Morhenn et al., 1973). Cell transformation with polyoma virus is improved by addition of DMSO (Kisch, 1969). Quantitation of infectivity by immunoperoxidase staining for T antigen-producing cells 3 days postinfection was in agreement with the number of plaques observed with conventional assay methods (D'Alisa and Gershey, 1978). Finally, an agar overlay medium supplemented with dextran sulfate has been reported to decrease the length of incubation required for the plaque assay from 21 to 7 days (D'Alisa and Gershey, 1978). Thus, techniques have been reported that shorten the time required to quantitate SV40 and/or increase plaque size; however, no detailed examination of the basic steps in the plaque assay has been described which defines the optimum conditions for plaque formation. In this report the effect of viral adsorption time, adsorption volume, concentration of fetal calf serum (FCS) in the overlay medium, and the incubation time of the plaque assay on the final SV40 titer are examined. Other factors influencing the plaque assay are also discussed.

MATERIALS AND METHODS

Cells and Virus Stocks

African green monkey kidney cell lines CV-1 and CV-1P were obtained from Paul Berg. The latter cell line is a subclone of CV-1 cells. SV40 strain 776 was obtained from Kathy Danna. Cells were cultured in

Dulbecco's Modified Eagle Medium (Gibco) supplemented with 44 mM $NaHCO_3$, 100 units Penicillin, 50 µg/ml Streptomycin and 5% FCS. All experiments were done with stock virus prepared by infection of CV-1 cells with plaque-purified SV40 at a multiplicity of infection of 0.01 plaque-forming units (PFUs) per cell. CV-1P cells were used in all plaque assays.

Standard Plaque Assay Method

The protocol for the plaque assay was adapted from that of Mertz and Berg (1974) and then subsequently modified and optimized as described. Assays were performed in 60 mm plastic petri dishes (Corning Glass Works) with monolayers of CV-1P cells that were confluent within 24 hr after seeding of the dishes. Ten-fold serial dilutions of virus were made in Minimum Essential Medium, Eagle (MEM) (Flow Laboratories, Inc.) supplemented with 2.0 mM L-Glutamine, 8.9 mM $NaHCO_3$, 100 units Penicillin, 50 µg/ml Streptomycin, and 2.5% FCS (MEM-2.5). For each dilution, 0.2 ml was adsorbed at 37°C for 1 hr unless otherwise stated. Each petri dish was agitated every 15 min during the adsorption period. Unless otherwise indicated, overlay medium consisting of 5 ml 0.8% (w/v) Difco Bacto-Agar (Difco Laboratories) in MEM-2.5 per petri dish was then added. Plaque assays were supplemented with 5 ml of overlay medium every four days. Agar at a concentration of 1.6% (w/v) was made fresh each day and mixed with an equal volume of 2X MEM-5 for every initial overlay and feeding. Incubation of the assay was for 14 days at 37°C except where noted.

Cells were stained by a modification of the procedure of Holland and McLaren (1959) as described by Dobos (1976). Agar overlay medium was

removed at the end of the incubation period and the cell monolayer fixed with a fixing solution containing final concentrations of 25% formaldehyde, 10% ethyl alcohol, and 5% glacial acetic acid for 1 hr. The solution was then removed by washing with water and 1% crystal violet in fixing solution was added for 30 min to stain the cells (Fig. 1). Adding the fixative directly to the agar overlay results in dark-staining plaques that appear to be clumps of dead cells. By removing the agar before fixation, the plaques are seen as clear holes in the stain and are more readily counted. In the latter case, it is assumed that the dead cells are removed with the agar. All viral dilutions were assayed in quadruplicate; the brackets in Figures 2-5 represent the standard error of the mean. All experiments reported in this paper were carried out at least twice, with similar results.

Biochemicals and SV40 Antiserum

Dimethyl sulfoxide (Fischer Scientific Co.) was added to a final concentration of 0.1% (v/v) to the 0.8% agar-MEM-2.5 overlay medium to test its effect on plaque size and time of appearance. Similar experiments were done using DEAE-Dextran and dextran sulfate (Sigma Chemical Co.), both at a final concentration of 0.1 mg/ml in the overlay medium. Alternative overlay mediums were tested for their ability to enhance plaque production when made in the final concentrations of 0.7% (w/v) methyl cellulose (4000 centipoise) (Fischer Scientific Co.), 0.8% (w/v) gum tragacanth (Fischer Scientific Co.), and 0.8% (w/v) Sea Plaque agarose (FMC Corp.) in MEM-2.5. Where indicated, neutralizing SV40 horse serum (Flow Laboratories, Inc.) with a minimum titer of 1:320 was serially diluted ten-fold in MEM-2.5 and overlayed at concentrations of

10^{-2} to 10^{-6} in 5 ml volumes as a substitute for overlay medium. All alternative overlay media were added every 4 days as described for the standard plaque assay method. Neutralizing SV40 antiserum medium was removed every 4 days and fresh medium containing antiserum was added.

RESULTS

Adsorption Times and Volumes

Virus stocks were plaqued by the standard assay method with the exception that the adsorption volume was varied from 0.1 to 0.5 ml in 0.1 ml increments. Adsorption medium was removed prior to addition of the overlay medium. Adsorption volumes of 0.1 ml resulted in a titer 2-3 fold greater than that obtained with 0.5 ml (Fig. 2). In this experiment the absolute number of PFU added was increased with adsorption volume, and the titer was then computed. Similar results were observed when 0.1 ml of each virus stock was diluted individually in MEM-2.5 to give the described range of adsorption volumes, each with the same number of PFUs (data not shown). Both experiments dramatically illustrate that as the adsorption volume increases, the number of plaques or titer on an individual petri dish decreases. The fact that the negative influence of layer volumes is not merely a dilution effect (the concentration of virus remained constant in the experiment in Fig. 2) is somewhat surprising. Perhaps the meniscus effect allows adsorption of virus to the plastic sides of the dish, or the decreased volume stresses the cell sheet in such a way that virus is more likely to adsorb.

The effect of adsorption time on the observed titer of SV40 is shown in Figure 3. Virus titers were found to steadily increase up to the 60 min adsorption period, and then to rise more gradually to a plateau by 90-120 minutes depending upon the experiment. In order to minimize problems with evaporation, standard conditions of 60 min and 0.1 ml were chosen.

Overlay Medium: Volume and FCS Concentration

The volume of agar overlay medium in each addition was varied from 1 to 5 ml in the standard assay. No change of SV40 titer was observed from 2 to 5 mls; use of only 1 ml resulted in death of the cells. However, the average plaque diameter (determined for 40 to 50 plaques) did vary somewhat; a volume of 3 ml produced the largest plaques (data not shown).

The effect of FCS concentration in the overlay medium on SV40 titer and plaque size was investigated. Results depicted in Figure 4 show that concentrations of FCS below 2.5% resulted in a reduced titer while FCS concentrations of 2.5, 5, and 7.5% showed essentially the same titers. Ten percent FCS produced the highest titer. The average plaque diameter was linearly related to FCS concentration, especially between 2.5 and 10% FCS (data not shown).

Development of SV40 Titer with Time

Replicate plaque assays were initiated at the same time and overlayed with 3 ml 0.8% agar in MEM-2.5. Starting with day 8 postinfection and every day thereafter for 9 days, dishes were fixed and stained for plaque enumeration. By day 11 the maximum SV40 titer had been obtained, with no further titer increase observed upon incubation for up to 16 days (Fig. 5). Results of two other experiments showed maximum titers were achieved by days 10 and 11, respectively. By counting on day 10 when the plaques are still quite small, up to 35 plaques can readily be counted on a 60 mm dish. As can be seen in Fig. 1, longer incubation times simply increase the plaque size.

Comparison of Different Overlay Media and Agar Feeding Times

No difference in virus titer was observed when plaque assays were overlayed with 5 ml of 0.7% methyl cellulose, 0.8% agar, or 0.8% agarose. Plaques produced under methyl cellulose were 66 to 76% smaller in size than those produced under agar or agarose and therefore require 14 days for development. The latter two overlay media resulted in the formation of equal sized plaques on day 11. Although gum tragacanth has been reported to increase the efficiency of plaque formation in some virus systems (Mirchamsy and Rapp, 1968), we found it to be inhibitory to SV40 plaque formation. Titers were approximately 10% of those observed with the other three overlay media and the diffuse pinpoint plaques that resulted were difficult to count.

Triplicate plaque assays were performed simultaneously but overlayed at different times after the initial overlay. One assay was overlayed with 3 ml 0.8% agar-MEM-2.5 four times according to the standard assay method of an initial overlay and three feedings. A second assay was overlayed with the same volume but only three times (initial, day 5, and day 10). The third assay was initially overlayed with 6 ml and fed on day 7 with the same volume. The three different overlay protocols resulted in plaques of the same size (data not shown); the plaque assay overlayed 3 times with 3 ml produced slightly higher titers. Plaque assays that were not fed during the incubation time did not produce open plaques, but were observed as foci of infection that were difficult to enumerate. Their cell monolayers did not appear as healthy as those fed during the incubation period (data not shown).

Effect of Biochemical Additives in the Overlay Medium

Dimethyl sulfoxide added to the agar overlay medium at a final concentration of 0.1% (v/v) increased neither plaque numbers nor plaque size. Dextran sulfate or DEAE-Dextran (both at final concentrations of 0.1 mg/ml) added to the agar overlay medium had no effect on the time at which plaques first appeared. Dextran sulfate at the above concentration resulted not only in a decrease in titer over the control but also in non-specific cytopathic effect of the entire cell monolayer for both control and virus inoculated petri dishes.

Substitution of Overlay Medium with Anti-SV40 Serum Containing MEM

SV40 antiserum dilutions in MEM-2.5 were tested as an alternative to semisolid or solid overlay mediums. A dilution of 10^{-2} was found to be toxic to the cells whereas a 10^{-3} dilution was merely inhibitory to all plaque formation. Antisera dilutions of 10^{-4} and 10^{-5} resulted in titers similar to the agar controls. However, the diffuse pinpoint plaques formed at these dilutions were difficult to enumerate. Titers for antisera diluted to 10^{-5} were always found to be slightly higher than the agar controls. A 10^{-6} antiserum concentration had no effect on plaque formation and exhibited extensive viral CPE.

DISCUSSION

Adsorption volumes of 0.1 ml per 60 mm diameter petri dish, rather than the commonly used adsorption volume of 0.5 ml, resulted in the maximum number of PFUs. Similar results were reported for Infectious Hematopoietic Necrosis Virus in which a decrease in titer was observed

when volumes of medium greater than 0.15 ml were adsorbed to cells (Burke and Mulcahy, 1980). In the same study, it was shown that adsoprtion times over 60 min did not increase plaque numbers, while shorter times decreased plaque numbers. In this study, periods of time greater than 60 min for adsorption of 0.1 ml volume provided only a small increase in titer. Agar overlay volumes of 3 ml were found to be optimum for plaque development. The assay was further enhanced by feeding with this same volume on days 5 and 10, although the precise feeding schedule is not particularly critical. Fetal calf serum concentrations less than 2.5% in the overlay medium were inhibitory to plaque formation and in fact to cell maintenance. Concentrations higher than 2.5% in the overlay medium showed no titer increase until a concentration of 10%. Although plaque size increased directly with increases of FCS concentration it did not alter the titer nor shorten the time period for the plaque assay sufficiently to warrant use of concentrations greater than 2.5%.

Agar and agarose overlay media were equally effective in plaque formation. Methyl cellulose overlay produced the same titer as agar and agarose but required a 14 day incubation period for enumeration of plaques. Agar, which is considerably less expensive than agarose, was chosen for all plaque assays.

Dimethyl sulfoxide has been reported to enhance plaque formation and number for the SV40 (Strain SP-1)-CV-1 system (Cleaver, 1974), but had no effect on the SV40 (Strain 776)-CV-1P plaquing protocol described in this report. Dextran sulfate has been shown to enhance plaque formation for variant poliovirus (Takemoto and Leibhaver, 1962) and SV40 (Strain VA 45-54) (D'Alisa and Gershey, 1978) in HeLa and CV-1 cells respectively. In our hands, the recommended concentration of dextran sulfate was

actually toxic and limited plaque formation in CV-1P cells. Medium containing SV40 antiserum produced diffuse plaques that required 14 days for development.

Immunological methods have been developed that result in titration of SV40 within 3 to 6 days (Ravid et al., 1968; Aaronson and Todaro, 1970; D'Alisa and Gershey, 1978). Although these techniques are rapid they become very labor intensive when dealing with a large number of plaque assays and are always subject to control checks of the immunological reagents.

In summary, the optimization of the SV40 plaque assay in terms of plaquing efficiency and time has resulted in a reliable assay that produces a linear dose-response curve which can be completed in 10 to 11 days with a minimum of handling and materials. The reproducibility of this assay has allowed the utilization of SV40 for psoralen repair studies that require a careful determination of inactivation kinetics (Kondoleon et al., 1982).

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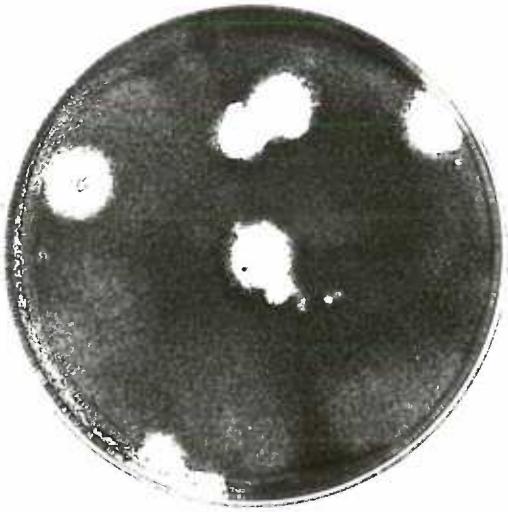
FIGURE LEGENDS

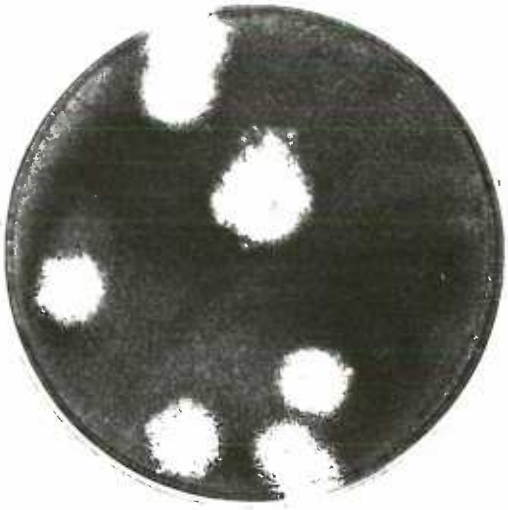
Figure 1. SV40 plaque formation on CV-1P cells.

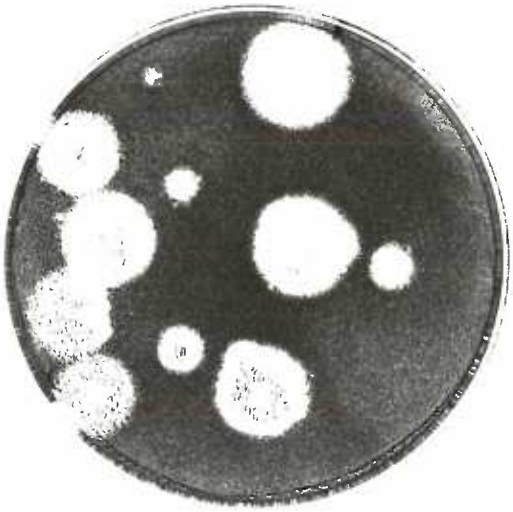
CV-1P monolayers were fixed and stained on days 11, 12, 13, and 14 postinfection as described in the Materials and Methods section. The plaque assay was performed by adsorbing 0.1 ml of the appropriate viral dilution for 60 min followed by addition of 3.0 ml agar overlay medium containing 2.5% FCS. Each plate was overlayed in the same manner on days 5 and 10 postinfection. All four monolayers were infected with an equal concentration of SV40.

DAY 11

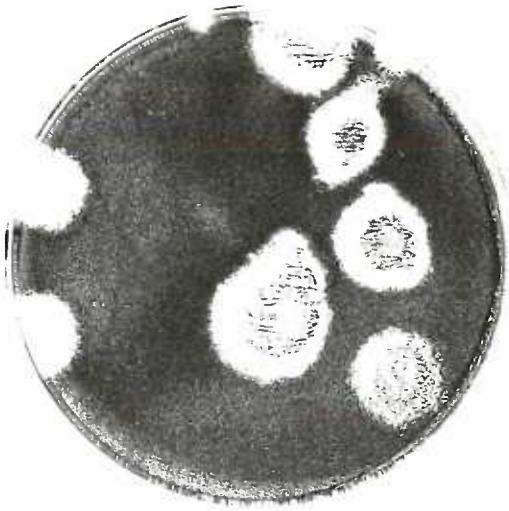


DAY 12





DAY 13



DAY 14

Figure 2. Effect of adsorption volume on SV40 titer in the plaque assay. Plaque assay conditions were as described in Materials and Methods. SV40 serially diluted ten-fold in MEM-2.5 was inoculated onto separate quadruplicate monolayers for each adsorption volume tested. Adsorption volumes ranged from 0.1 to 0.5 ml in 0.1 ml increments for each viral dilution. Thus the absolute number of PFU innoculated per plate (and not the concentration of virus) was varied over a 5-fold range. Adsorption media was aspirated from each monolayer prior to addition of the agar overlay media.

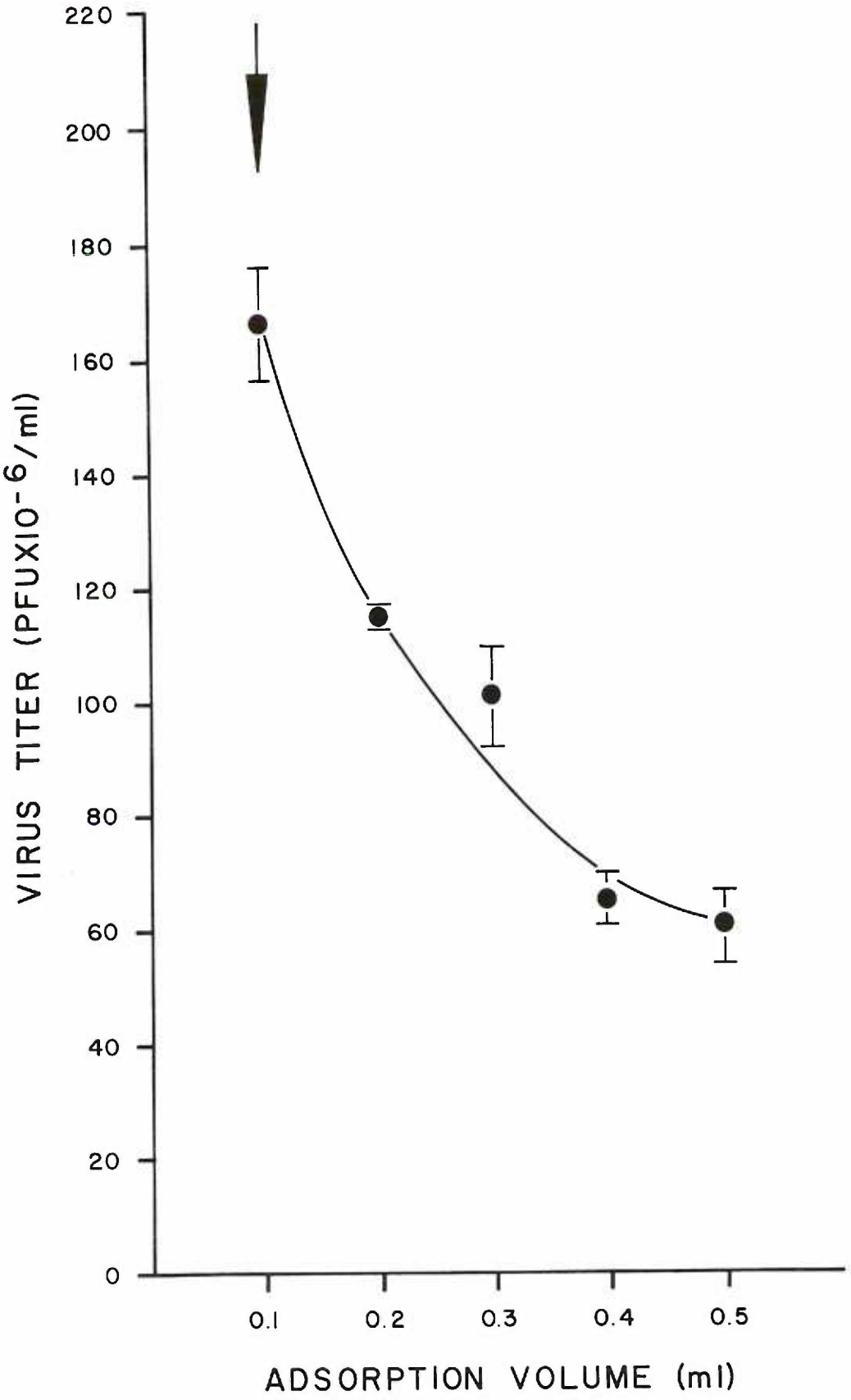

220
200
180
160
140
120
100
80
60
40
20
0
VIRUS TITER (PFUX10-6/ml)
0.1
0.2
0.3
0.4
0.5
ADSORPTION VOLUME (ml)

Figure 3. Effect of adsorption time on SV40 titer in the plaque assay. Conditions for the plaque assay were as described in Materials and Methods. Each viral dilution tested was adsorbed in quadruplicate monolayers of CV-1P cells for the adsorption time shown.

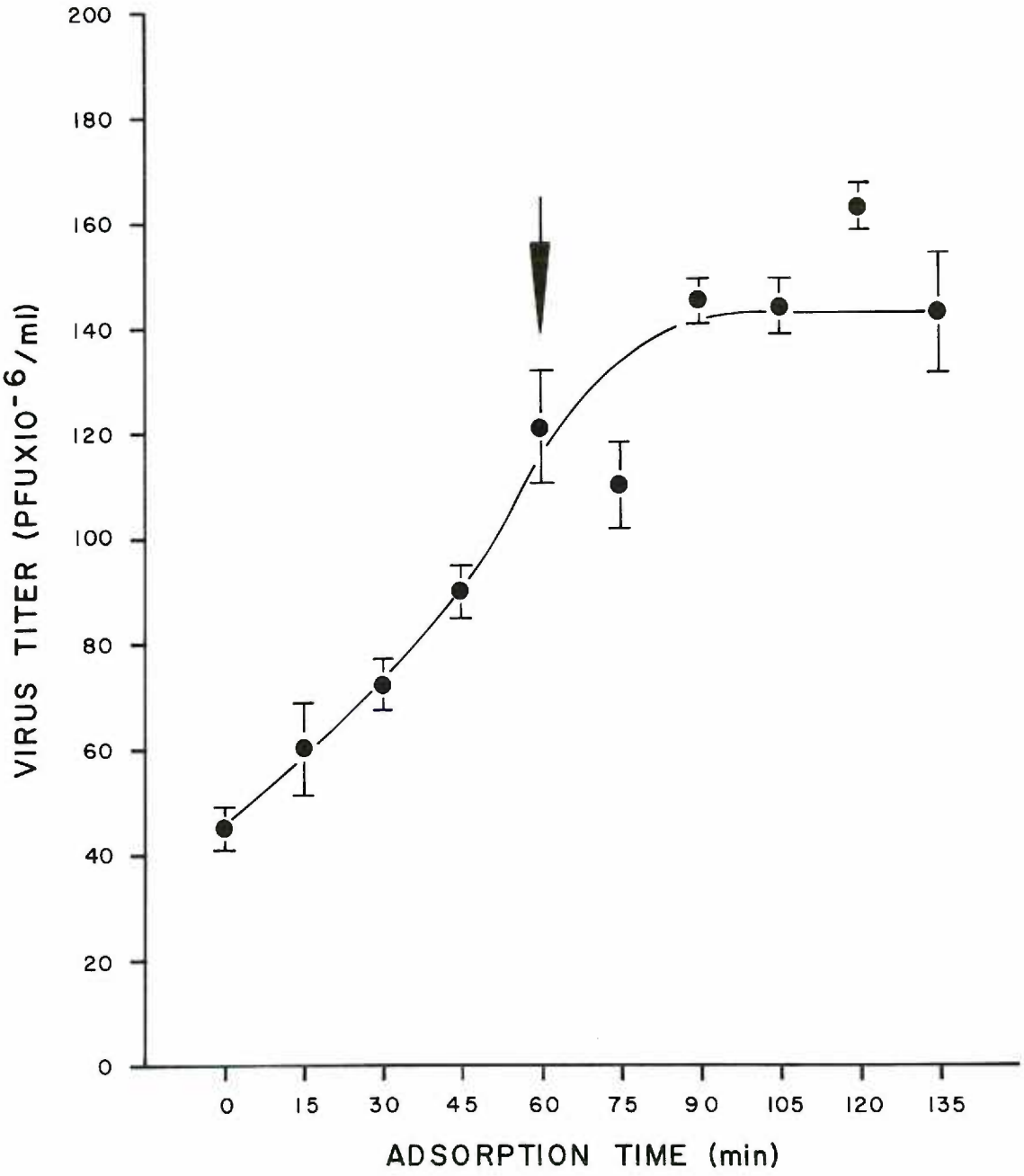

200
180
160
140
120
100
80
60
40
20
0
VIRUS TITER (PFUX10-6/ml)
0
15
30
45
60
75
90
105
120
135
ADSORPTION TIME (min)

Figure 4. Effect of fetal calf serum concentrations in the agar overlay medium on SV40 titer in the plaque assay. SV40 titers were determined by the plaque assay method described in the Materials and Methods section. Each viral dilution was overlayed with 0.8% (w/v) agar medium containing the indicated concentration of FCS.

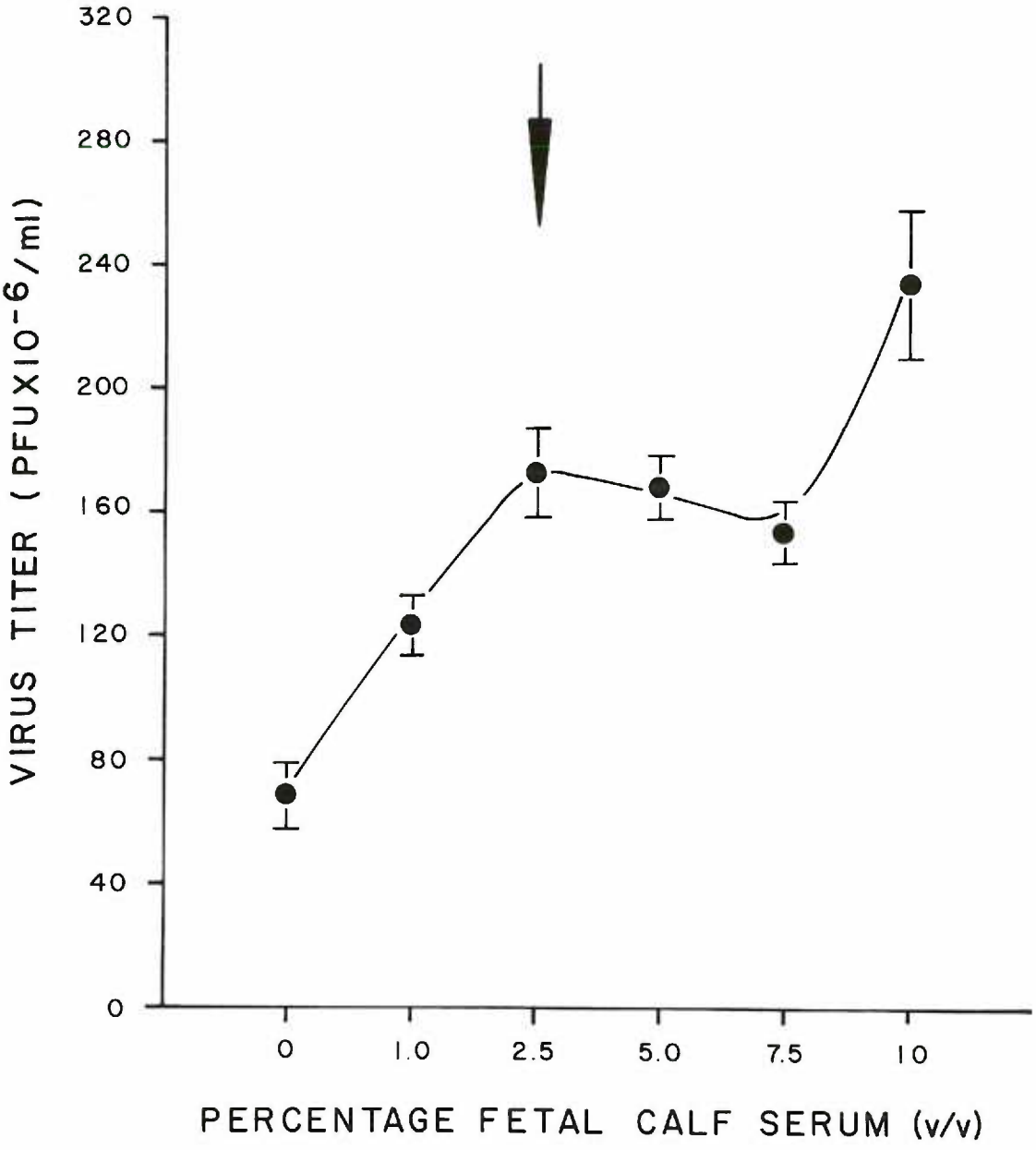
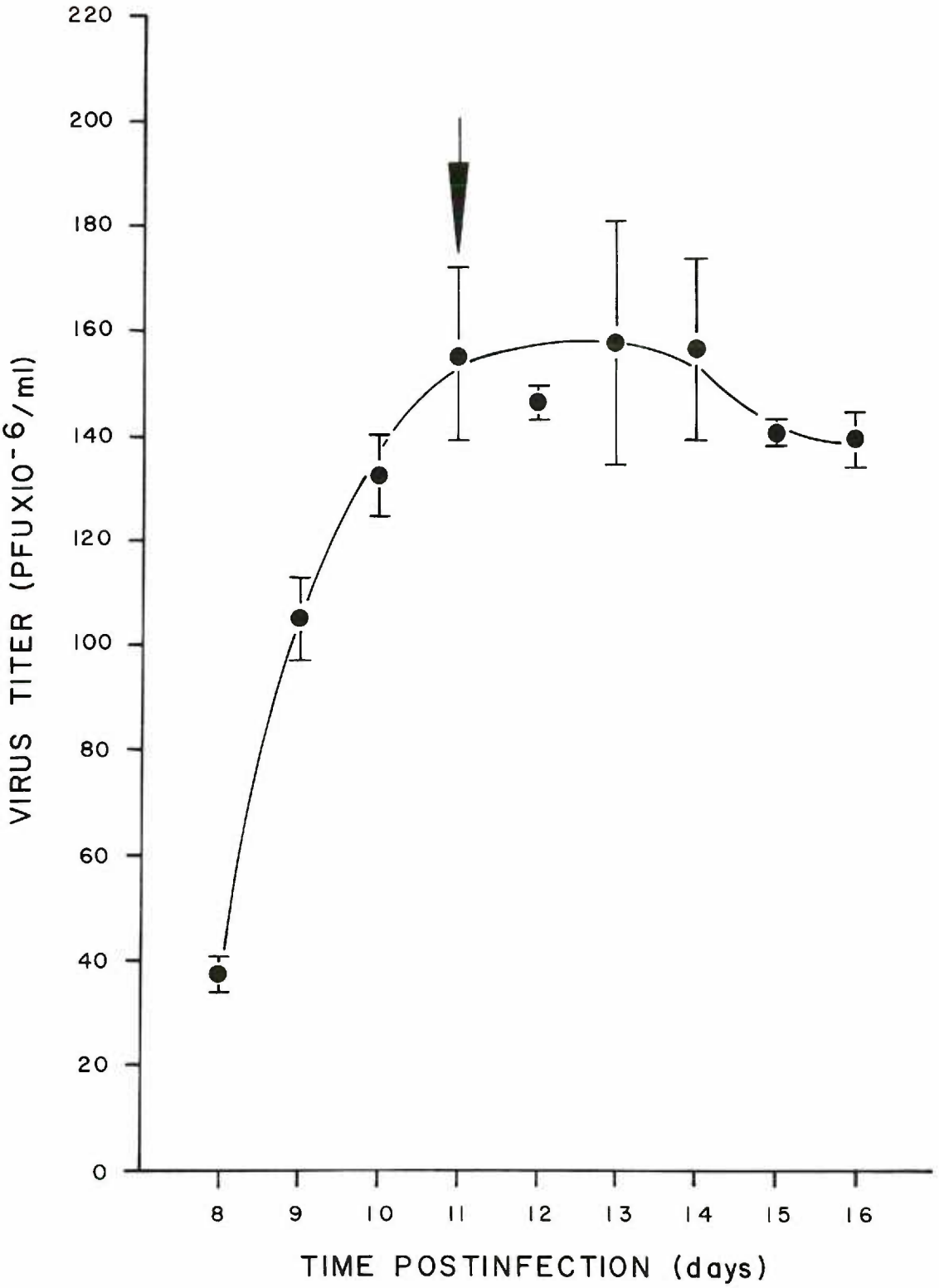

320
280
240
200
160
120
80
40
0
VIRUS TITER (PFUX10-6/ml)
0
1.0
2.5
5.0
7.5
10
PERCENTAGE FETAL CALF SERUM (v/v)

Figure 5. Effect of total incubation time on SV40 titer in the plaque assay. All plaque assays were done on the same day from the same viral dilutions as described in Materials and Methods. Quadruplicate plates were fixed and stained for plaque enumeration starting on day 8 postinfection and continued every 24 hr thereafter, as shown.


220
200
180
160
140
120
100
80
60
40
20
0
VIRUS TITER (PFUX10-6/ml)
8
9
10
11
12
13
14
15
16
TIME POSTINFECTION (days)

Manuscript 2.

Psoralen Photoinactivation of Herpes Simplex Virus: Monoadduct and Cross-link Repair by Xeroderma Pigmentosum and Fanconi's Anemia Cells

ABSTRACT

Furocoumarins (psoralen and its derivatives) are used to photoinactivate a variety of viruses and cell types. In the presence of long wavelength UV light (UVA), furocoumarins bind covalently with pyrimidine residues via a cyclobutane ring. A second photoevent allows pyrimidines located on the opposite DNA strand in an adjacent base pair to react, forming a cross-link. In the experiments described in this report, psoralen photoinactivation is employed to investigate human DNA repair pathways by analyzing the ability of Xeroderma pigmentosum (XP) and Fanconi's anemia (FA) cells to rescue psoralen inactivated Herpes Simplex Virus (HSV). Comparison of several XP complementation groups and one XP variant with normal human fibroblasts demonstrates that the ability of all cells to repair damage by 4,5',8-trimethylpsoralen (TMP), a derivative which forms cross-links efficiently, is similar. However, HSV photochemically reacted with 5-methylangelicin (5-MA), an isopsoralen which forms only monoadducts, is repaired at significantly lower levels in several XP complementation groups than in control fibroblast cells, which indicates that the XP repair deficiency resides in the removal of monoadducts and not of cross-links in these cell lines. Surprisingly, the FA cells rescue both TMP and 5-MA-treated virus with slightly greater efficiency than that observed in normal human fibroblasts.

INTRODUCTION

Human fibroblasts from patients with DNA repair defects provide a valuable tool to the elucidation of eukaryotic DNA repair pathways (1, 2). Xeroderma pigmentosum (XP) and Fanconi's anemia (FA) are two autosomal recessive diseases correlated with DNA repair deficiencies. The XP causes severe photosensitivity of exposed regions and a high incidence of skin cancer (basal cell carcinomas, squamous carcinomas, and malignant melanomas) (2, 3). FA is a progressive pancytopenia with early onset of thrombocytopenia followed by leukopenia and finally aplastic anemia (2).

Genetic studies with XP fibroblasts and cell fusion techniques indicate that at least seven complementation groups (A-G) and one variant group exist (4-7). Fibroblasts from all complementation groups except the variant exhibit reduced levels of excision repair after UV irradiation (2). XP variants have near normal or normal levels of excision repair of pyrimidine dimers but are defective in the postreplication repair pathway (7). Virus inactivation kinetics of UV irradiated Simian Virus 40 (SV40), Herpes Simplex Virus Type 1 (HSV-1), and Adenovirus in XP fibroblasts are decreased relative to normal fibroblasts (8-11). Survival of XP fibroblasts to ionizing radiation is normal or slightly reduced when compared to normal fibroblasts (2), although the survival of gamma-irradiated adenovirus was reduced in XP fibroblasts (12). XP fibroblasts are hypersensitive to certain chemicals that form large adducts to DNA such as acetylaminofluorene and nitroquinoline oxide. In contrast, they demonstrate a normal response to small alkylating agents like N-methyl-N-nitro-N-nitrosoguanidine, methyl methanesulphonate, ethyl methane sulfonate and the cross-linking agent

mitomycin C (MMC) (2,13). However, it was reported that adenovirus treated with nitrous acid, 4,5',8-trimethylpsoralen (TMP) or chloropromazine resulted in decreased survival in XP cells (14-16).

Fibroblasts from patients with Fanconi's anemia exhibit an increased susceptibility to chromosomal breakage when exposed to a variety of bifunctional compounds such a MMC, nitrogen mustard (NM), and 8-methoxypsoralen (8-MOP) (2). Survival curves consistently indicate an increased sensitivity to the cross-linking agent MMC (17, 18). In contrast, reports concerning the removal of cross-links by FA fibroblasts have presented conflicting results. One group reported a two to eight fold reduced rate of cross-link repair for three FA fibroblast cell lines (FA9TO, FA14TO, FA12TO) (17), while two other groups using two different FA fibroblast cell lines (HG-261 and CRL-1196) found that there was no defect in their ability to remove the bifunctional compounds MMC and 8-MOP respectively (18, 19). In the second report, however, the short irradiation time employed may have resulted in 8-MOP binding primarily as monoadduct. Recently it was reported that FA cell senescence may play a role in whether or not NM cross-links are repaired. Early passage cultures of FA fibroblasts (FA9, FA18) previously reported to be defective in the removal of MMC cross-links were found to repair NM cross-links while the same FA fibroblasts grown to late passages lost most of this capacity (20).

This paper will present results comparing the ability of XP and FA fibroblasts to repair DNA damage induced by furocoumarins plus UVA. Furocoumarins readily intercalate between the stacked DNA bases of intact cells or viruses without resulting in DNA damage. Once bound and in the presence of UVA (320-420 nm) these drug molecules react covalently with

pyrimidine residues to form monoadducts or crosslinks (21-23). Although there are approximately two to three times more monoadducts than crosslinks, (24, Wiesehahn and Hallick, unpublished observations), cross-links are thought to be responsible for virus inactivation, cell killing, mutagenesis, loss of tumorigenicity in transformed cells, and increased rates of recombination in phage-prophage crosses (21, 25-29). Experiments utilizing psoralen derivatives such as angelicin, an isopsoralen which does not produce cross-links, and khellin, which forms them infrequently, also implicate monoadducts in cell killing and virus inactivation although they are considerably less effective then psoralen cross-linking derivatives (21, 30).

During the past few years severe cases of psoriasis have been treated with considerable success by the combination of 8-MOP and UVA (the PUVA treatment) (21). It was suggested by Dr. Hanawalt at these meetings three years ago that the treatment of PUVA patients with drugs that efficiently cross-link DNA, perhaps with an additional irradiation in the absence of drug, might enhance the ability to kill (psoriatic) cells with cross-links and minimize the probability of monoadduct repair via an "error-prone" pathway, thereby decreasing the possibility of malignant transformation. We subsequently examined the feasibility of this suggestion by analyzing the photoinactivation kinetics of bacteriophage lambda, which is extremely sensitive to psoralen killing, and the papovavirus SV40, which is relatively resistant to psoralen photoinactivation (29). Results from the two virus-host systems were quite different. In experiments designed to measure the effects of crosslinks, aliquots of psoralen-treated virus were reirradiated in the absence of drug. In the case of lambda, reirradiation resulted in an

inactivation rate virtually identical to that observed in the continuously irradiated samples. This indicates that cross-link formation can account for nearly all lambda inactivation observed in this region of the curve. However, when the same approach was extended to SV40, the resulting rate of inactivation was considerably less in the reirradiated samples than in those treated continuously in the presence of drug. This relative resistance may imply that monoadduct formation is rate-limiting in this system, perhaps due to protection of the DNA afforded by histones in the virion (31, 32). Alternatively, the repair capacity of the eukaryotic host cell may differ markedly from that of the prokaryotic host Escherichia coli.

Although many chemical agents, including the furocoumarin TMP, have been utilized to study the defective repair mechanisms of XP and FA fibroblasts, none compare the removal of monoadducts and cross-links, particularly with compounds that bind to DNA by similar mechanisms. These experiments were designed to examine the kinetics of HSV-photoinactivation by the cross-linking psoralen derivative TMP and by the isopsoralen derivative 5-methylangelicin (5-MA), which only forms monoadducts. The ability of normal human, XP, and FA fibrolasts to repair viral DNA damaged by psoralen cross-links or monoadducts was assessed by titering the inactivated virus in parallel on each cell type. One normal human cell line was examined along with one represenatative of each of the XP complementation groups A-G, the XP variant and one FA cell line. Results indicate that all XP cell types examined repair TMP adducts in the viral DNA as efficiently as the normal control cells. Surprisingly, it was observed that the FA cell line is better able to repair TMP photoadducts than normal fibroblasts. Kinetics of 5-MA

inactivation of HSV-1 demonstrated that many representatives of the XP complementation groups were less capable of monoadduct repair of viral DNA than the normal fibroblasts or XP variant cells. While several XP cell strains were very sensitive to 5-MA addition, consistent with the reported decrease in unscheduled DNA synthesis (UDS) after UV irradiation, other XP fibroblasts did not show a corresponding decrease. The overall repair pathway or some step(s) in the pathway may not occur at the same rate for the removal of pyrimidine dimers as for that of 5-MA monoadducts. Both FA cells and the XP2RO cells representing complementation group E were found to repair 5-MA monoadducts more efficiently than normal cells. The reason for this is unclear, but group E has been reported to have a 40-60% capacity for excision repair relative to normal control cells. HSV-1 photoreacted with TMP appeared to undergo multiplicity of reactivation when plaqued on all XP and FA cells. This phenomenon was not evident for HSV-1 photoreacted with 5-MA and plaqued on XP or FA cells.

MATERIALS AND METHODS

Human Cell Lines and Growth Medium

Xeroderma pigmentosum primary cells XP10BE (CRL 1204), XP6BE (CRL 1157), XP4BE (CRL 1162), XP12BE (CRL 1223), normal human control skin fibroblast CCD-25SK (CRL 1474), and Fanconi's anemia cells HG-261 (CCL 122) were obtained from the American Type Culture Collection. Xeroderma pigmentosum primary cells XP3YO (GM 3542), XP2B1 (GM 3021A), and GM 1854 were purchased from N. I. G. M. S. Human Genetic Mutant Cell Repository. Primary cells XP10BE, XP6BE, XP12BE, and XP4BE were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 44mM

$NaHCO_3$ and 10% fetal calf serum (FCS). Minimum Essential Medium Eagle (MEME) (Flow Laboratories, Inc.) supplemented with 2.0 mM L-glutamine, 23.8 mM $NaHCO_3$, and 2X concentration of Non-Essential Amino Acids (NEAA), Amino Acids, and Vitamin solutions, plus 10% FCS was used for the culture of primary cells XP2B1, XP3YO, GM 1854, XP2RO, and HG-261. CCD-25SK was cultured in MEME supplemented with 1X NEAA, 2.0 mM L-glutamine, 23.8 mM $NaHCO_3$, and 10% FCS. The SV40 transformed XP12BE Xeroderma pigmentosum cell line derived from XP12BE (ATCC CRL 1223) primary cell line was provided by W. Summers and cultured in DMEM-10% FCS and 44 mM $NaHCO_3$. All culture media contained penicillin and streptomycin in final concentrations of 100U/ml and 50µg/ml respectively.

Virus Stocks and Furocoumarins

Herpes Simplex Virus Type I (CL101 (HSV-1) was kindly provided by W. Summers. HSV-1 was propagated in Vero (African Green Monkey Kidney) cells at a multiplicity of infection of 0.01 plaque-forming units (PFUs) per cell. A single virus stock stored at -70°C was utilized in all experiments.

4,5'8-trimethylpsoralen (TMP) (33) and 5-methylangelicin (5-MA) (34) were generously provided by S. Isaacs and J. Hearst. Stock solutions in ethanol were stored at room temperature in the dark. Drug concentration was calculated from the extinction coefficient by measuring the absorbance at 249 nm in aqueous solution.

Irradiation Experiments

Stock HSV-1 in MEME-2% FCS was adjusted to a final concentration of 0.01 µg/ml TMP, 0.10 µg/ml TMP or 2.0 µg/ml 5-MA. Drug-virus solutions were held at 4°C in the dark for 20 min. or longer. All irradiations were done on a single bank of two General Electric F15T8BLB fluorescent

bulbs at the average incident light intensity indicated in the figure legends. The degree of incident intensity was controlled by use of plexiglass shields. Virus-drug samples were irradiated in separate 35mm tissue culture dishes (Corning) in 100 µl aliquots for each time point. Controls for all experiments consisted of virus plus drug without irradiation (0 min) and virus with UVA alone for the maximum time used in each experiment.

Each time point was serially diluted in MEME-2% FCS and adsorbed to cell monolayers in 35mm tissue culture dishes for 60 min at 37°C. Monolayers were then overlayed with 0.5% (W/V) methyl cellulose in MEME-5% FCS and incubated at 37°C for 4 days. At this time all plates were fixed and stained for plaque enumeration. All graphs depicting kinetic photoinactivation of HSV-1 have been normalized to the control samples containing TMP or 5-MA without irradiation (0 min). The experiments depicited in Figures 2-4 have been repeated two to three times with similar results; for ease of presentation only one complete experiment is shown.

RESULTS

The Effects of Psoralen Monoadducts and Cross-links on HSV-1 viability when assayed on XP Cells

In order to compare the repair pathways of XP cells with those of normal cells, HSV-1 was photoinactived with both TMP and 5-MA at relatively high light intensities. The results are depicted in Figure 1. As shown in Figure 1a, the effect of TMP on the inactivation kinetics of HSV-1 in the repair deficient human cell types XP12BE (A), XP2RO (E), XP4BE (Variant, V), and CCD-25SK (Normal Control, NC) is similar. There

may be a slight defect in host-cell reactivation in cells of complementation group D (XP6BE).

In contrast, a marked difference in the titers of 5-MA treated HSV (Figure 1b) is seen when assayed on XP12BE (A) or XP6BE (D) cells implying a defect in monoadduct repair. XP2RO (E) and XP4BE (V) cells are similar to CCD-25SK (NC).

TMP Photoinactivation Kinetics of HSV-1

The observations described above were extended to all of the XP complementation groups and to cells from a patient with Fanconi's anemia. In addition, higher initial virus titers were employed to enable us to follow the kinetics over a greater time period. Published data concerning the ability of XP and FA fibroblasts to repair DNA damage produced by various cross-linking agents has not consistently demonstrated a defect in these cell types (2, 13, 15, 17-19). A detailed investigation of the kinetics of TMP photoinactivation of HSV-1 was undertaken in XP and FA cells. The levels of UV-induced UDS reported for these cell types is summarized in Table 1. The inactivation curves of such experiments are depicted in Figure 2. All cell lines tested demonstrate that repair of TMP-damaged virion DNA promotes virus multiplicity reactivation (nonlinear dilution series at multiplicities greater than 2-10). This phenomenon was not observed in the experiments presented in Figure 1, presumably due to the lower initial virus titer. FA cell line HG-261 was consistently found to have a slightly increased ability to repair TMP adducts over the normal control cell line CCD-25SK. In most experiments, the ability of different XP complementation groups to repair TMP adducts appeared equivalent to that of normal control cells. Differences at the late time points (40 and 60 min) are difficult

to interpret because of the apparent ability of HSV-1 to be reactivated by recombination at high multiplicities. This point will be examined in more detail in the Discussion.

5-MA Photoinactivation Kinetics of HSV-1

Photoinactivation kinetics of HSV-1 by 5-MA are depicted in Figures 3 and 4. Cell lines HG-261 (FA), XP2RO (E), and GM 1854 (Heterozygous, B-H) repair 5-MA monoadducts as efficiently or slightly better than the CCD-25SK (NC) and XP4BE (V) cells (Figure 3). Although differences in HSV-1 inactivation rates are slight, the results with the HG-261, XP2RO, and GM 1854 cells were consistently observed to fall above that of CCD-25SK. All cell lines depicted in Figure 3 demonstrate a linear response to 5-MA photoinactivation of HSV-1. Photoinactivation kinetics of HSV-1 by 5-MA when assayed on XP12BE (A), and XP12BE-SV40 (transformed, A-SV40), XP10BE (C), XP6BE (D), XP3YO (F), and XP2B1 (G) are depicted in Figure 4. The data from normal fibroblasts is repeated from Figure 3. All experiments in Figures 3 and 4 were conducted at the same time, but they are plotted separately for ease of presentation. Two observations can be made from these results. First, XP12BE (A), XP12BE-SV40 (A-SV40), and XP2B1 (G) are the most sensitive to 5-MA damage of HSV-1 and demonstrate linear inactivation kinetics. Secondly, cell lines XP10BE (C), XP6BE (D) and XP3YO (F) exhibit intermediate sensitivity to 5-MA damage of HSV-1 when compared to the normal control cells and the more repair deficient cells of complementation groups A and G. In addition, HSV-1 inactivation kinetics assayed on this group (C, D and F, Fig. 4) consistently demonstrate a break in the linear curve between 30 and 50 min of irradiation. No evidence of multiplicity

reactivation of 5-MA inactivated HSV-1 was observed on any of the cells studied.

DISCUSSION

The kinetics of HSV-1 photoinactivation demonstrate that all XP cells tested repair TMP cross-links to approximately the same extent as the normal control cell line CCD-25SK (Figures 1 and 2). In contrast to these observations, the XP cell types from the complementation groups tested exhibit quite different 5-MA photoinactivation kinetics of HSV-1. Cell lines XP12BE (A), XP12BE-SV40 (A-SV40), and XP2B1 (G) are the most deficient in monoadduct repair, an observation consistent with their low level of UV-induced UDS (see Table 1). Monoadduct repair by fibroblasts XP10BE (C), XP6BE (D), and XP3YO (F) is moderately deficient and compares to the reported levels of 10-50% UV-induced UDS. The same rate of HSV-1 reactivation is observed on the XP4BE (V) and CCD-25SK (NC) cells. Complementation group B heterozygous cells GM 1854 (B-H) repairs 5-MA monoadducts as efficiently or better than the normal control cell line CCD-25SK. XP2RO (E) exhibits a 40-60% UV-induced UDS (2,36) yet has consistently resulted in a slightly higher rate of host-cell virus reactivation when compared to CCD-25SK (NC).

Reports in the literature suggest that the repair of cross-links generated by 8-MOP does not occur in XP12BE (A) (19) and XP1 (XP1LO, A) cells (38), although results of TMP photoinactivation of HSV-1 show that the ability of all XP cells examined to repair damaged virus is comparable to that seen in the normal control cells (CCD-25SK). Initial experiments using high concentrations of TMP (1.0 μg/ml) and a high incident light intensity (2.7mW/cm^2) resulted in a 5.0 log decrease in

HSV-1 titer within 5 min. In such an experiment a difference in HSV-1 titers of 1-2 logs is observed between XP12BE (A) and CCD-25SK (NC). At such short irradiation times, a high percentage of the bound drug molecules are probably still monoadducts, as has been demonstrated in the lambda system by reirradiation experiments (29). Thus these data support the conclusion that the repair defect in XP cells resides in the excision repair of psoralen monoadducts (15, 39) while the repair of cross-links is limited and most likely represents a lethal event in eukaryotes (26, 39-40).

HSV-1 treated with either 5-MA or TMP consistently demonstrates a greater sensitivity to photoinactivation when assayed on the normal control cell line CCD-25SK than when titered on FA cells. This does not merely reflect differences in plaquing efficiency as the data are normalized at the 0 time point. Although current literature as well as this report present conflicting evidence as to the ability of FA cells to repair cross-linking agents, several explanations may account for these apparent discrepancies (17-20). It is possible that a virus-coded recombination pathway and/or the non-nucleosomal structure of intracellular HSV DNA may facilitate repair of both monoadducts and cross-links (41-43). This explanation is consistent with our observation that TMP induces a high degree of virus multiplicity reactivation in normal control, FA, and XP human cells. Surprisingly, it is not observed in VERO cells (data not shown). However, we were unable to detect any multiplicity of reactivation of 5-MA photoinactivated HSV-1 plaqued on FA, XP, or normal control cells. An alternative explantion is that the FA cell line HG-261 reported in this paper is not defective in TMP cross-link removal in spite of its sensitivity to MMC cross-links (18).

It is interesting to note that the FA cell line reported in this paper was tested between passages 42 to 45, which is well past passage numbers reported to be defective in NM cross-link removal from cellular DNA (20).

In conclusion, the repair of TMP adducts on HSV-1 DNA by all XP complementation groups and the XP variant examined occurs with an efficiency similar to that of the normal control cells and may involve HSV-1 recombination. Several XP complementation groups tested are defective to varying degrees in 5-MA repair although XP2RO (E) cells are able to repair monoadducts as efficiently as the normal cells. The FA cells (HG-261) examined are able to repair both 5-MA and TMP photoinactivated HSV-1 somewhat more efficiently than the normal cells used in this study.

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FIGURE LEGENDS

Fig. 1 Comparison of TMP and 5-MA photoinactivation kinetics of HSV-1 at an average incident light intensity of 2.8mW/cm^2 when assayed on normal human and Xeroderma pigmentosum cells. Virus suspensions were treated with TMP or 5-MA plus UVA for varying periods of time up to 60 min. Titration of HSV-1 was determined by the plaque assay method for each time point using the following cell lines: normal human control CCD-25SK (NC,●), XP14BE Variant (V,□), XP12BE (A,X), XP6BE (D,■), XP2RO (E,◆). a) Photoinactivation kinetics of HSV-1 exposed to 0.01 μg/ml TMP plus UVA. The normalized value indicated as less than (<) for XP12BE cell line at 30 min irradiation time represents the limit of detection by the plaque assay. b) Photoinactivation kinetics of HSV-1 exposed to 2.0 μg/ml 5-MA plus UVA. Normalized values indicated as less than (<) for XP6BE and XP12BE cell lines at 20 min irradiation time represent the limit of detection by the plaque assay.

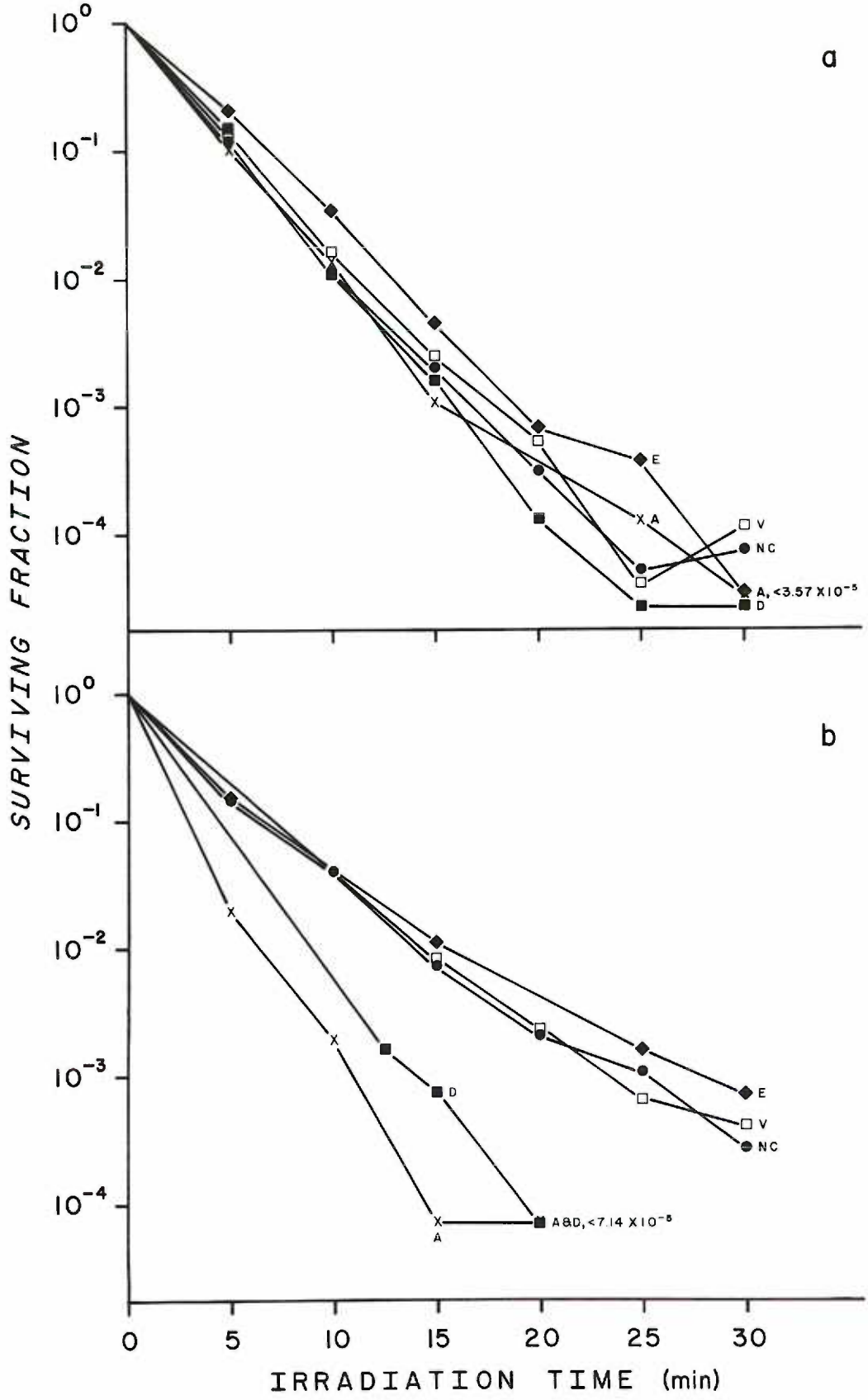
a
b
SURVIVING FRACTION
IRRADIATION TIME (min)
E
V
NC
A, <3.57 X10⁻⁵
D
A&D, <7.14 X10⁻⁵
A
0
5
10
15
20
25
30

Fig. 2 Comparison of TMP photoinactivation kinetics of HSV-1 assayed on normal human control, Xeroderma pigmentosum, and Fanconi's anemia cells. Virus suspensions were exposed to 0.1 μg/ml TMP plus UVA for varying periods of time up to 60 min. HSV-1 was quantitated by the plaque assay method for each time point using the following cells: normal human control CCD-25SK (NC,●), XP4BE Variant (V,□), XP12BE-SV40 (A-SV40,0), GM 1854 (B-H,Δ), XP10BE (C,◇), XP6BE (D,■), XP2RO (E,◆), XP3YO (F,▲), XP2B1 (G,▼) and HG-261 Fanconi's anemia (FA,▽). Photoinactivation kinetic curves for all cell strains are only depicted to 40 min irradiation time. At later times multiplicity reactivation hindered virus titration. Normalized time points indicated as less than (<) for XP6BE, XP3YO, and XP2B1 cells represent the limit of detection by the plaque assay method due to multiplicity of reactivation. Average light intensity was 0.9 mW/cm^2.

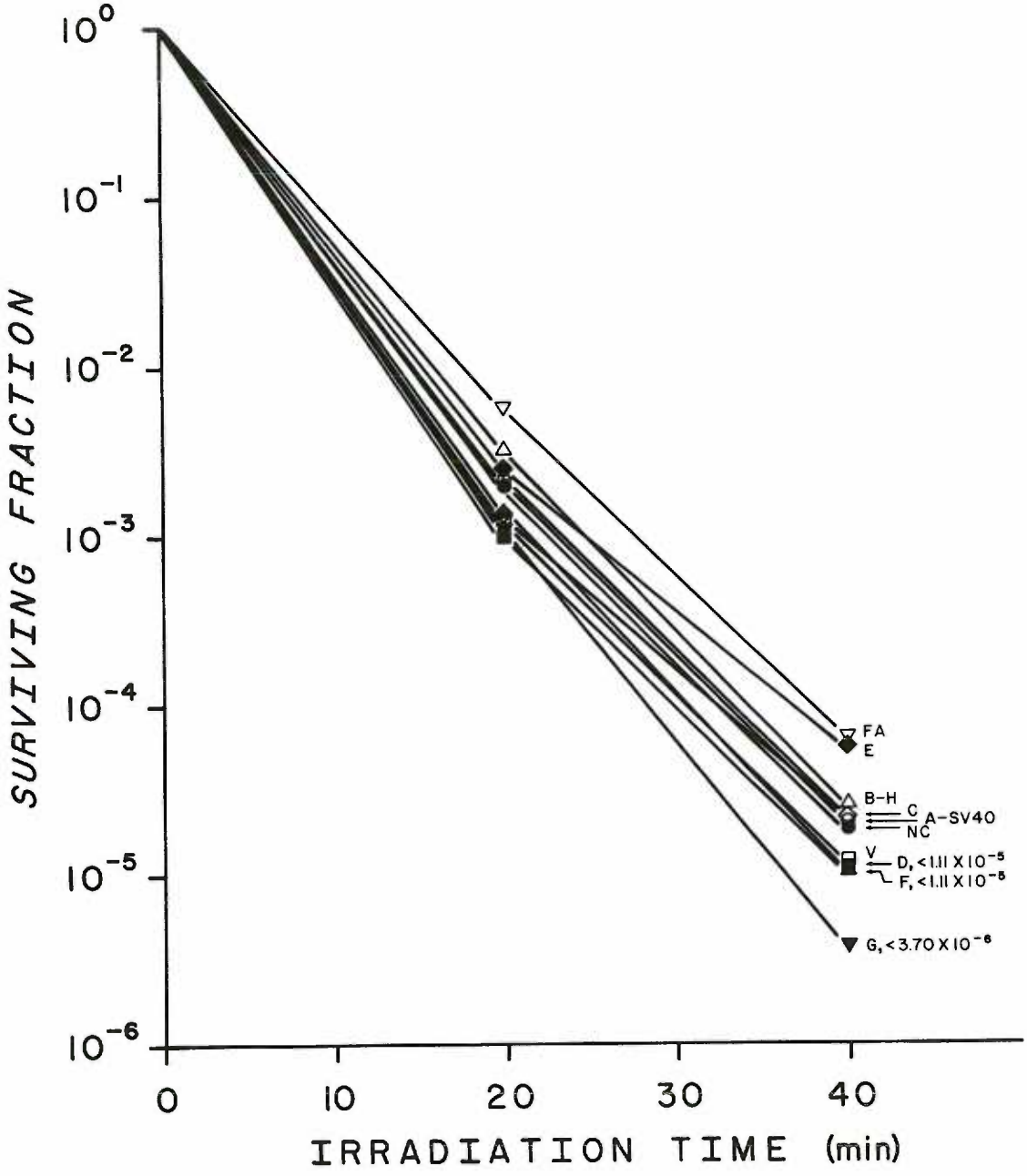
SURVIVING FRACTION
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
10
20
30
40
IRRADIATION TIME (min)
FA
E
B-H
C
NC
A-SV40
V
D, <1.11 X 10⁻⁵
F, <1.11 X 10⁻⁵
G, <3.70 X 10⁻⁶

Fig. 3 Comparison of 5-MA photoinactivation kinetics of HSV-1 assayed on cells with no apparent repair deficiencies. Virus suspension were exposed to 2.0 μg/ml 5-MA plus UVA for varying periods of time up to 60 min. HSV-1 infectivity was determined by the plaque assay method for each time point using the following cells: normal human control CCD-25SK (NC,●), XP4BE Variant (V,□), GM1854 (B-H,Δ) XP2RO (E,◆) and HG-261 Fanconi's anemia (FA,▽). Average light intensity was 2.3 mW/cm^2.

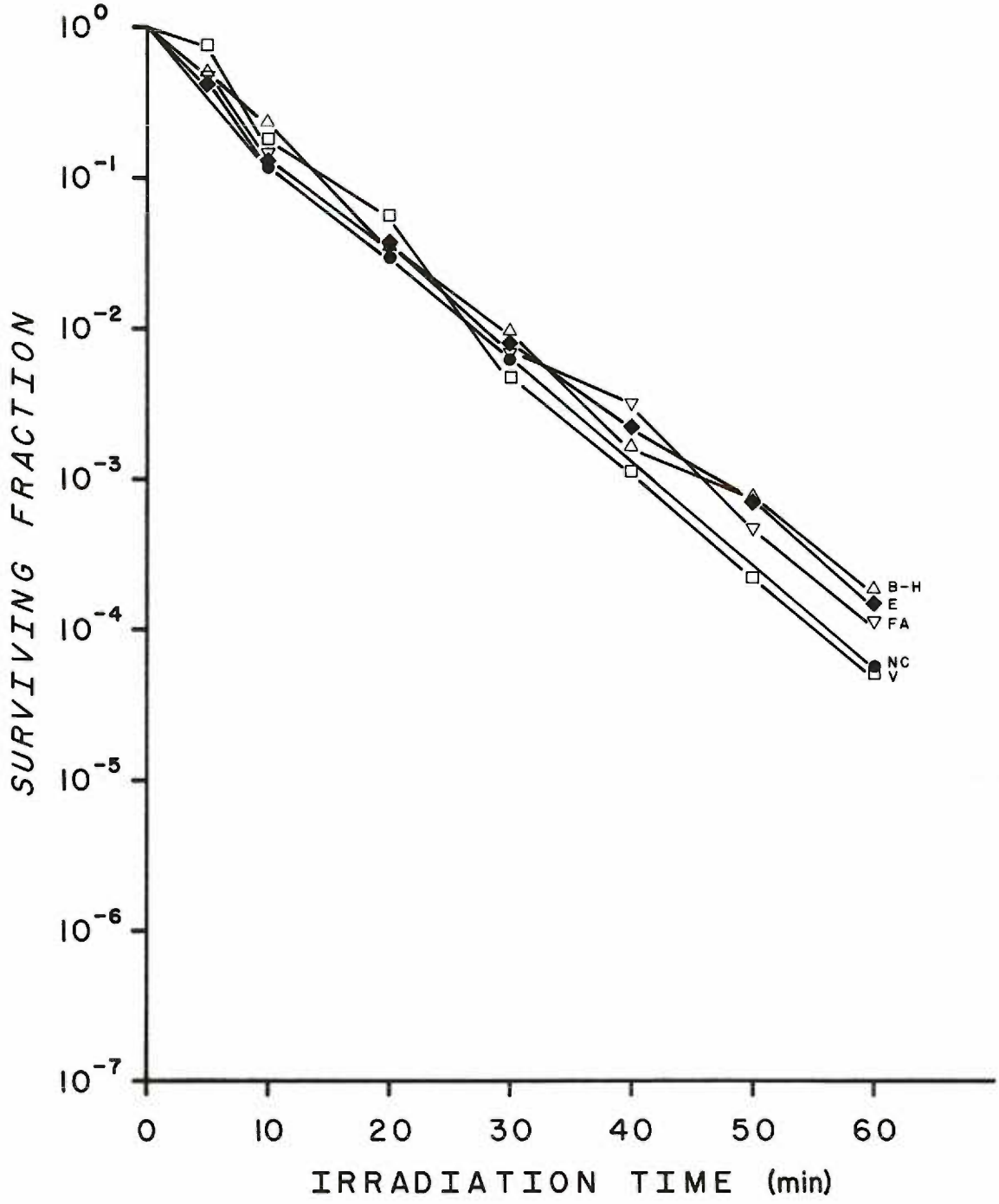
SURVIVING FRACTION
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
0
10
20
30
40
50
60
IRRADIATION TIME (min)
B-H
E
FA
NC
V

Fig. 4 Comparison of 5-MA photoinactivation kinetics of HSV-1 assayed on normal human control cells and Xeroderma pigmentosum cells deficient in monoadduct repair. Virus suspensions were exposed to 2.0 μg/ml 5-MA plus UVA for varying periods of time up to 60 min. HSV-1 was quantitated by the plaque assay method for each time point on the following cells: normal human control CCD-25SK (NC,●), XP12BE (A, X), XP12BE-SV40 (A-SV40,o), XP10BE (C,◇), XP6BE (D,■), XP3YO (F,▲) and XP2B1 (G,▼). Normalized values indicated as less than (<) for XP12BE, XP10BE and XP2B1 cells at the 60 min. irradiation time represent the limits of detection by the plaque assay method. Average light intensity was 2.3 mW/cm^2.

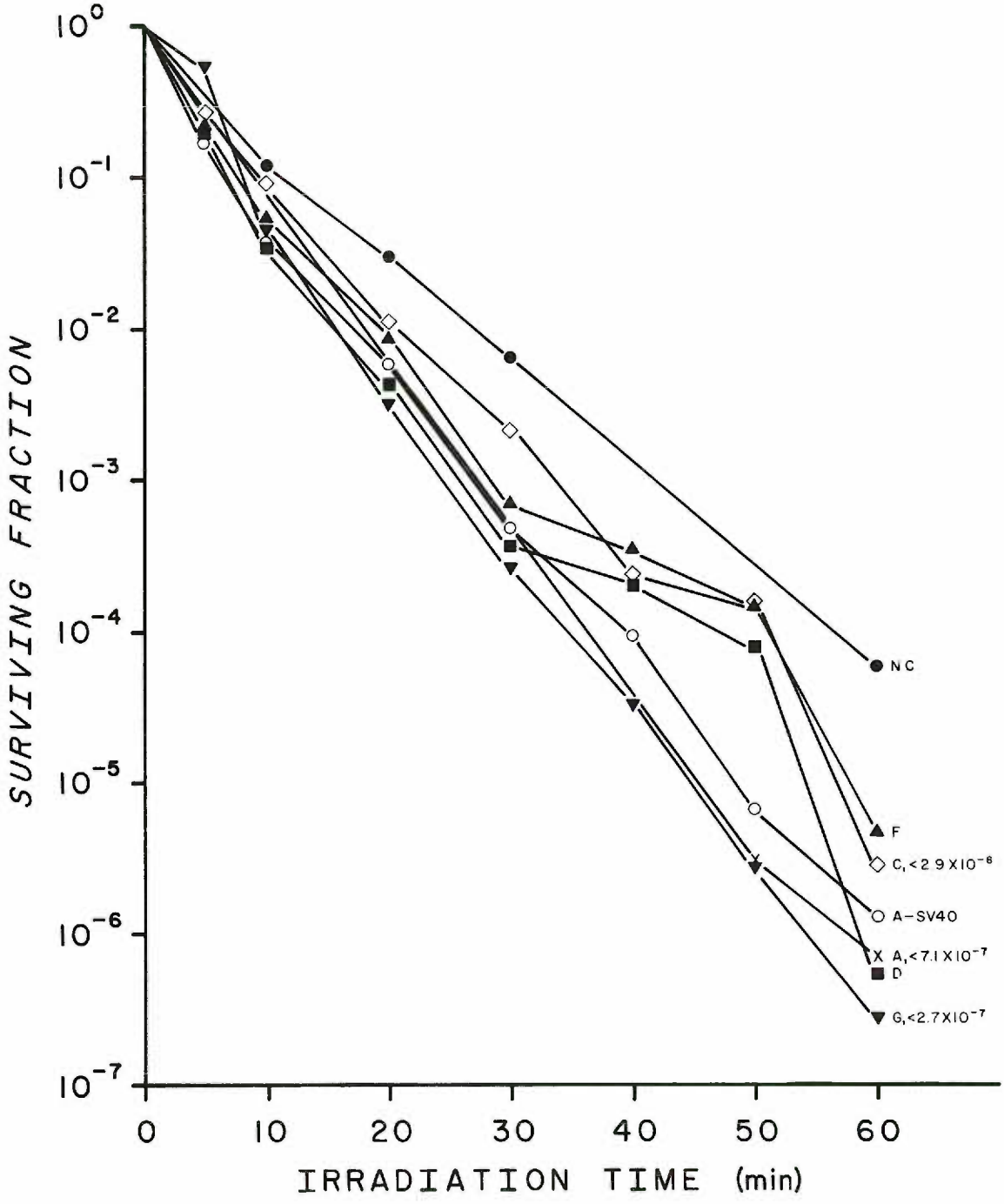
SURVIVING FRACTION
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
0
10
20
30
40
50
60
IRRADIATION TIME (min)
NC
F
C, $< 2.9 \times 10^{-6}$
A-SV40
A, $< 7.1 \times 10^{-7}$
D
G, $< 2.7 \times 10^{-7}$

TABLE 1

Table 1. Levels of UV-induced unscheduled DNA synthesis in normal, Xeroderma pigmentosum, and Fanconi's anemia fibroblast cell lines

Cell Line	Complementation Group	% UDS[a]	References
CCD-25SK[b]	NC	100	
XP4BE[c]	V	100	2
XP12BE	A	<2	2,35
XP12BE-SV40[d]	A-SV40		
GM 1854[e]	B-H	100	2,35
XP10BE	C	10-20	2,3
XP6BE	D	25-50	2,35
XP2RO	E	40-60	2,36
XP3YO	F	10	37
XP2B1	G	<2	2,6
HG-261	FA[f]	100	17

a. Percent UV-induced unscheduled DNA synthesis

b. Normal human control skin fibroblast cells

c. Fibroblast cells from a patient with diagnosed XP that exhibit normal UDS but defective postreplication repair

d. SV40 transformed XP12BE fibroblast cells

e. Heterozygous complimentation group B fibroblast cells

f. Fanconi's anemia fibroblast cells

Manuscript 3.

Fanconi's anemia fibroblasts are not deficient in the repair of psoralen crosslinks or monoadducts on viral DNA

Summary

Host cell reactivation of 4,5',8-trimethylpsoralen (TMP) and 5-methylisopsoralen (5-MIP) photoinactivated Herpes simplex virus (HSV) was investigated using seven Fanconi's anemia (FA) and one normal human fibroblast cell strain at low and high cell population doublings. TMP forms a mixture of monoadducts and diadducts (crosslinks) on DNA, whereas 5-MIP forms only monoadducts. No reduced survival of TMP or 5-MIP photoinactivated HSV was observed when assayed on FA relative to normal fibroblasts regardless of whether the fibroblasts were at low or high cell population doublings. In fact enhanced host cell reactivation of photoinactivated HSV was demonstrated in one FA fibroblast strain for TMP and in three strains for 5-MIP. Multiplicity reactivation was observed for TMP-inactivated HSV under conditions that favor a low ratio of crosslinks to monoadducts. However, it did not occur in 5-MIP photoinactivated virus, nor in HSV inactivated under conditions that result in a lower overall level of photoadducts per virion but a higher ratio of crosslinks to monoadducts. Cell survival after long-wavelength ultraviolet light (UVA) irradiation in the presence of TMP was also monitored for three of the FA strains relative to that of normal fibroblasts. In addition, the sensitivity of one FA and one fibroblast strain to 5-MIP was examined. No difference in colony forming ability was detected between the FA and the normal fibroblast strain after exposure to 5-MIP plus UVA, and only one FA fibroblast strain was found to have significantly reduced cell survival after exposure to TMP plus UVA. In conclusion, no defect in the host cell reactivation of psoralen crosslinks or monoadducts was observed in the seven FA strains tested.

However, cell survival was reduced in one FA strain after treatment with TMP.

Fanconi's anemia (FA) is a rare autosomal recessive disease characterized by a progressive hypoplastic pancytopenia (Nilsson, 1960). Growth retardation, several congenital abnormalities (Nilsson, 1960), chromosomal instability and an increased incidence of leukemia (Schroeder and Kurth, 1971) and malignant solid tumors (Swift et al., 1971) are associated with this disease. Although direct evidence for the nature of the molecular defect in FA has not been established (Schroeder, 1982; Cleaver, 1980), investigations utilizing cells from FA patients indicate that they may be deficient to various degrees in the repair of DNA interstrand crosslinks (Cleaver, 1980).

FA cells have been reported to possess an increased sensitivity to several DNA crosslinking agents. When compared to normal cells they showed decreased cell survival following exposure to mitomycin C (MMC) (Fujiwara et al., 1977; Nagasawa and Little, 1983; Fornace et al., 1979); 4,5',8-trimethylpsoralen (TMP) (Fujiwara et al., 1984; Poll et al., 1984a) or 8-methoxypsoralen (8-MOP) plus long-wavelength ultraviolet light (UVA) (Wunder and Fleischer-Reischmann, 1983; Sasaki, 1978); diepoxybutane (DEB) (Ishida and Buchwald, 1982); nitrogen mustard (HN2) (Dean and Fox, 1983; Sasaki, 1978); tetramethylene bis-methanesulfonate (busulfan) (Sasaki, 1978) and cis-diamminedichloroplatinum (II) [cis-Pt (II)] (Fujiwara et al., 1984).

Normal and FA cells have been reported to respond similarly in cell survival experiments to the monofunctional agents decarbamoyl mitomycin C (DMC) (Kano and Fujiwara, 1981) and irradiation by X-rays (Fornace et al., 1979), ultraviolet light (UV, 254 nm) (Smith and Paterson, 1981) and gamma-rays (Sasaki, 1978; Duckworth-Rysiecki and Taylor, 1985). However, other reports have demonstrated some FA strains to have a slightly

increased sensitivity to UV (254 nm) irradiation (Fornace et al., 1979; Sasaki, 1978; Smith and Paterson, 1981) and to the monofunctional agents methylmethanesulfonate (MMS) (Sasaki, 1978), 4-nitroquinoline-1-oxide (4NQO) (Sasaki et al., 1977), ethylmethane sulfonate (EMS) (Sasaki, 1978), and DMC (Sasaki et al., 1977; Fujiwara et al., 1977) when assayed for colony forming ability.

A cellular defect in the repair mechanism(s) of DNA interstrand crosslinks has been proposed to explain the hypersensitivity of FA cells to MMC (Sasaki and Tonomura, 1973; Sasaki, 1975). While it has been reported that FA cells are unable to make the first excision of an MMC-induced DNA interstrand crosslink (Fujiwara and Tatsumi, 1975; Fujiwara et al., 1977; Fujiwara, 1982), others have been unable to confirm this observation with MMC (Fornace et al., 1979; Poll et al., 1984a) or 8-MOP (Poll et al., 1984a; Kaye et al., 1980). It has been suggested that the ability to repair DNA interstrand crosslinks is associated with FA cell culture age (Sognier and Hittleman, 1983) and genetic heterogeneity (Sasaki, 1978; Duckworth-Rysiecki et al., 1985). Normal cells exhibit a delayed S phase following exposure to HN2 whereas FA cells do not. The implication is that this delay allows DNA repair prior to completion of DNA synthesis and that its absence in FA cells may contribute to their sensitivity to bifunctional alkylating agents (Dean and Fox, 1983).

Host cell reactivation of damaged viruses has also been utilized to examine the DNA repair defect(s) in FA cells. Reduced host cell reactivation of UV and gamma irradiated adenovirus type 2 (Ad 2) has been observed in FA fibroblasts relative to normal fibroblasts (Rainbow and Howes, 1977; Rainbow, 1978). However, FA fibroblasts are repair

proficient for methylnitrosoguanidine damaged Ad 5 (Day et al., 1980). Enhanced host cell reactivation of gamma irradiated herpes simplex virus (HSV) was found in one strain of FA fibroblasts while a second strain showed a level equal to that of normal fibroblasts (Zamansky and Little, 1982). Normal and FA fibroblast strains have also been shown to have no difference in their ability to reactivate cis-Pt(II) damaged simian virus 40 (SV40) DNA (Poll et al., 1984b). Exposure of Ad 2 to the bifunctional furocoumarin TMP resulted in equal levels of host cell reactivation by FA and normal fibroblasts (Day et al., 1975), while photoinactivation of HSV by TMP and the monofunctional reagent 5-methylisopsoralen (5-MIP) demonstrated enhanced host cell reactivation by FA fibroblasts (Fendrick and Hallick, 1984).

Furocoumarins (psoralen and its derivatives) have been used extensively to study DNA repair and inactivation due to their affinity for nucleic acids (Brendel and Ruhland, 1984). Three sequential events occur when furocoumarins photoreact with nucleic acids (for review, see Hearst et al., 1984). In the first step, defined as dark binding, psoralens intercalate between the stacked base pairs of the DNA helix. Photoexcitation of the intercalated psoralens by UVA (320 to 400 nm) produces monoadducts covalently joined by cyclobutane rings between the furan or pyrone psoralen rings and adjacent pyrimidines (primarily thymines). Photoexcitation of the furan-side monoadducts represents the third event and results in DNA interstrand crosslinks by formation of a second cyclobutane ring between the pyrone psoralen rings and adjacent pyrimidines.

We previously reported that host cell reactivation of photoinactivated HSV-1 by either TMP or 5-MIP in one FA fibroblast strain

was equal to or greater than that of normal fibroblasts (Fendrick and Hallick, 1984). TMP photoinactivation kinetics of HSV have now been extended to several FA fibroblast strains to determine their ability to repair furocoumarin crosslinks by host cell reactivation. HSV photoinactivation by TMP was compared at two different UVA light intensities, that was suggested to form different ratios of TMP crosslinks to monoadducts. The effect of cell aging on the ability of FA fibroblasts to repair either a mixture of TMP monoadducts and crosslinks or 5-MIP monoadducts was also examined. In all cases, no significant differences in host cell reactivation of TMP or 5-MIP photoinactivated HSV were observed between the seven FA strains tested and normal fibroblasts.

Materials and Methods

Human fibroblast strains. Normal and FA fibroblast strains listed in Table 1 were purchased from the American Type Culture Collection (ATCC) and N.I.G.M.S. Human Genetic Mutant Cell Repository (HGMCR) as indicated. CRL 1196 fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 44 mM $NaHCO_3$ and 10% (v/v) fetal calf serum (FCS). Minimum Essential Medium, Eagle (MEME) supplemented with 2 mM L-Glutamine, 23.8 mM $NaHCO_3$, and a 1X concentration of nonessential amino acids (NEAA) solution plus 10% (v/v) FCS was used for culture of CCD-25Sk normal human fibroblasts. The remaining fibroblast strains were cultured in MEME supplemented with 2 mM L-Glutamine, 23.8 mM $NaHCO_3$, 10% (v/v) FCS, and 2X concentrations of NEAA, essential amino acids, and vitamin solutions. All culture media was supplemented with 100u penicillin and 50 µg/ml streptomycin.

Virus Stocks. Herpes simplex virus type 1 clone 101 (HSV-1) was propagated in VERO (African green monkey kidney) cells at a multiplicity of infection equal to 0.01 plaque-forming units (PFUs) per cell. Two virus stocks stored at -70°C were utilized for the experiments.

Furocoumarins. TMP (4,5',8-trimethylpsoralen) (Isaacs et al., 1977) and 5-methylisopsoralen (5-MIP) (Isaacs et al., 1984) were purchased from HRI Associates, Inc. Emeryville, CA. Stock psoralen concentrations in 100% ethanol were calculated from their extinction coefficients of 3.1 x 10^4 L $M^{-1}cm^{-1}$ (TMP, 249 nm) (Wiesehahn et al., 1977) and 2.4 x 10^4 L $M^{-1}cm^{-1}$ (5-MIP, 249 nm) (Isaacs et al, 1984).

Irradiation experiments. Stock HSV-1 in MEME containing 2% (v/v) FCS was adjusted to a final concentration of 0.01 μg/ml TMP, 0.10 μg/ml TMP, or 2.0 μg/ml 5-MIP as indicated in the figure legends. Solutions of HSV-1 plus furocoumarins were held in the dark for 20 min or longer 1t 4°C. A single bank of two General Electric F15T8BLB flourescent bulbs was used for all irradiations. The average incident light intensity for each experiment was adjusted with plexiglass shields and is shown in the figure legends. Aliquots of 200 μl HSV-1 plus furocoumarins were irradiated in separate 35 mm plastic tissue culture dishes for each time point. Experimental controls consisted of HSV-1 plus furocoumarin without UVA (0 min) and HSV-1 with UVA only for the maximum irradiation time for each experiment.

Determination of the HSV-1 surviving fraction by the plaque assay method was previously described (Fendrick and Hallick, 1984). This method was modified by using fibroblast monolayers in 60 mm tissue culture dishes. Photoinactivation kinetics for HSV-1 by 0.01 μg/ml TMP and 2 μg/ml 5-MIP were determined on both low and high generation fibroblasts while kinetics for HSV-1 photoinactivation by 0.10 μg/ml TMP was from low generation fibroblasts (Table 2). We define fibroblast generations by the number of cell population doublings.

Measurement of colony forming ability. FA HG 261, GM1309, and GM2053 fibroblast strains were used at 44, 45, and 45 cell population doublings, respectively for all experiments. CCD-25Sk normal fibroblasts were used at 38 (Fig. 4A) and 18 (Fig. 4B) cell population doublings. Fibroblasts were seeded at a density of 500 cells/cm^2 in 150mm (Fig. 4A) dishes or 100 cells/cm^2 in 100mm dishes (Fig. 4B) and cultured in growth

medium for approximately 24 h at 37°C. Growth medium was then removed from each plate and replaced with 15 ml (Fig. 4A) or 5 ml (Fig. 4B) irradiation medium consisting of growth medium without FCS, supplemented with 15mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH7.2, and either 1 ng/ml TMP or 0.2 μg/ml 5-MIP (Fig. 4A) or 0.5 ng/ml TMP (Fig. 4B). All plates were equilibrated for 30 min at 4°C in the dark prior to UVA irradiation at the average incident light intensity shown in the figure legend. After irradiation each plate was washed twice with 15 ml irradiation medium without TMP or 5-MIP, allowing each plate to equilibrate at 20°C for 20 min prior to removal of wash medium. Each plate was overlayed with growth medium and incubated at 37°C for 3 weeks with weekly medium changes. After 3 weeks each plate was fixed and stained. Colonies were counted for each irradiation time point and normalized to their respective 0 min control without UVA to determine surviving fractions. A second control of 30 min UVA irradiation in the absence of furocoumarin showed no decrease in colony forming ability.

Results

Photoinactivation kinetics of HSV-1 with both TMP and 5-MIP were determined on one normal and several FA fibroblast strains at both low and high generations. HG 261 fibroblasts were not tested for HSV-1 host cell reactivation at low CPDs, since they were purchased at approximately 35 generations. Figures 1A and 1B illustrate TMP photoinactivation on low and high passage cells respectively. Although the extent of HSV photoinactivation as determined on low generation FA cells was somewhat greater than that for high generation FA cells in these experiments, no reduced levels of host cell reactivation were observed between any of the FA strains and normal fibroblasts in either case. The slight overall difference between the two cell populations may be due to the fact that the virus stock used for irradiation experiments with high FA generations had a higher initial titer. The concentration of HSV-1 will influence the number of adducts bound per genome and consequently the ultimate adduct killing effect. Since experiments designed to measure adduct levels indicated that TMP is the rate limiting reagent under similar irradiation conditions (unpublished results).

Similarly, there was no evidence for defective repair by FA cells of 5-MIP monoadducts (Figs. 2A and 2B). Photoinactivation kinetics of HSV-1 by 5-MIP for both low and high generations were very similar. As previously described for TMP, the HSV-1 concentration may have resulted in slightly less killing in the experiment with cells of high generations, although in this case 5-MIP is present in large excess.

Fig. 3 depicts the TMP photoinactivation kinetics of HSV-1 irradiated under conditions designed to achieve the same level of killing

seen in Fig. 1 at a lower ratio of crosslinks to monoadducts. This is accomplished by irradiating the virus in the presence of a higher concentration of TMP and a lower UVA intensity (0.1 μg/ml TMP at 0.9 mW/cm^2 rather than the 0.01 μg/ml TMP at 2.5 mW/cm^2 used in Fig. 1). Again, no reduced host cell reactivation is observed for any of the FA strains. Surviving fractions for all fibroblast strains could not be determined for the 60 min irradiation period since no clear HSV-1 plaques were detectable at the lowest dilution possible for this experiment. At this dilution a diffuse viral cytopathic effect (CPE) or viral toxicity was observed that affected from 20 to 50% of the cell monolayer. The next dilution did not exhibit any viral toxicity, nor were there any plaques. Thus, multiplicity reactivation may account for the observed CPE in the sample. It was never observed with 5-MIP or for irradiation conditions with higher light intensity and lower TMP concentration, even when the killing level was adjusted so that the same multiplicity of infection could be used in the assay.

Controls for all HSV-1 photoinactivation experiments consisted of HSV-1 plus furocoumarin (0 min time point) and HSV-1 plus UVA irradiation for 60 min in the absence of furocoumarin. No significant loss in HSV-1 titer was observed for either of these controls. It was observed that HCR of 5-MIP reacted virus by the FA fibroblast strains HG 261, GM1309, and GM2053 was always higher than that of the normal strain (Figs. 2A and 2B). This was also true for HG 261 but not GM1309 and GM2053 when HSV-1 was irradiated with TMP (Figs. 1 and 3). This modest enhancement of host cell reactivation was reproducible and does not reflect differences in plaquing efficiencies since the data are normalized at the 0 irradiation time.

FA HG 261 and normal CCD-25Sk fibroblasts were examined for their colony forming ability when irradiated in the presence of 1 ng/ml TMP and 0.2 μg/ml 5-MIP (Fig. 4A). No difference was observed in the colony forming ability of either fibroblast strains regardless of the furocoumarin derivative used. Both fibroblast strains were more sensitive to TMP than to 5-MIP, even though the 5-MIP concentration was 200-fold higher than that of TMP. As shown in Fig. 4B, the TMP photoinactivation was repeated and extended to the GM1309 and GM2053 FA fibroblast strains. Only the GM1309 FA fibroblast strain was observed to have substantially reduced cell survival relative to normal cells when exposed to 0.5 ng/ml TMP plus UVA. In contrast, HG 261, GM1309 and GM2053 FA fibroblast strains were more sensitive to MMC inactivation than the normal CCD-25Sk fibroblast strain when tested by colony forming ability (data not shown).

DISCUSSION

No reduction in host cell reactivation of HSV-1 by FA fibroblasts was demonstrated for photoinactivation of HSV-1 by TMP or 5-MIP when assayed on either low or high generation fibroblasts (Figs. 1 & 2). This observation was also extended to the colony forming ability of FA HG 261 and GM2053 fibroblast strains at high generations (Fig. 4). Only the FA GM1309 fibroblast strain tested at high generations had a markedly reduced cell survival after treatment with TMP plus UVA (Fig. 4B). Although FA GM1309 fibroblasts were not tested at low generations for TMP killing, we did observe that low generation GM1309 fibroblasts were the most sensitive of the three tested to MMC killing (see discussion below). Although it has been reported that the ability to repair HN2 crosslinks has been lost in FA fibroblasts of late culture age (Sognier and Hittleman, 1983), this was not the case for HCR of TMP photoinactivated HSV-1.

The colony forming ability of FA fibroblasts HG 261, GM1309 and GM2053 upon exposure to MMC has been reported to be reduced when compared to normal fibroblasts (Nagasawa and Little, 1983; Fornace et al., 1979). Decreased colony forming ability has also been observed for GM2053 upon exposure to HN2 (Dean and Fox, 1983). We have tested the sensitivity of these three FA fibroblast strains in our laboratory and confirmed their reduced levels of colony forming ability after exposure to MMC when compared to our normal fibroblast strain (data not shown). Reduced FA cell survival after exposure to 8-MOP (Poll et al., 1984a) and increased chromosomal abberrations upon exposure to 8-MOP (Sasaki and Tonomura, 1973) may represent a situation in which genetic heterogeneity

(Duckworth-Rysiecki et al., 1985) influences the response to DNA interstrand crosslinking agents. While it has been reported that FA fibroblast GM1309 is appreciably more sensitive than GM2053 to MMC when measured by colony forming ability (Nagasawa and Little, 1983), in these experiments no difference in host cell reactivation of TMP (0.01 and 0.1 μg/ml) photoinactivated HSV-1 was observed. GM1309 cell survival however, was considerably more sensitive to TMP than it was for the GM2053 or HG 261 strains. It has also been reported that the removal of 8-MOP crosslinks from CRL 1196 FA fibroblasts was no different from that of normal fibroblasts (Kaye et al., 1980).

When measured by either xeroderma pigmentosum (XP) host cell reactivation of 5-MIP photoinactivated HSV-1 (Fendrick and Hallick, 1984) or by the ability of XP fibroblasts relative to normal fibroblasts to remove angelicin or 5-MIP monoadducts from the cellular DNA (Cleaver and Gruenert, 1984), similar conclusions were reached concerning the ability of XP fibroblasts from several different complementation groups to repair monoadducts produced by monofunctional furocoumarins. Such comparisons in many systems have led to the assumption that the host cell reactivation assay is a reliable method for studying cellular defects in DNA monoadduct repair.

However, this may not be the case for the repair of DNA crosslinks. The kinetics of host cell reactivation for TMP photoinactivated HSV-1 did not detect the apparent reduced ability to repair TMP crosslinks in the FA GM1309 cell strain that was detected in the cell survival experiment. Two possibilities may account for the inability of TMP photoinactivated HSV-1 to detect a deficiency in FA crosslink repair by host cell reactivation. First, it is known that in the absence of multiplicity

reactivation, one TMP crosslink per viral genome represents a lethal event for HSV-1 and SV40 (Fendrick, Isaacs and Hallick, in preparation; Hall, 1982), while Chinese hamster cells require approximately 2,000 TMP crosslinks per genome to produce one lethal event (Ben-Hur and Elkind, 1973). Even in the presence of multiplicity reactivation approximately two TMP crosslinks per SV40 viral genome represents a lethal event (unpublished data). Thus, if the HSV-1 host cell reactivation assay is sensitive to an extremely low level of TMP crosslinks per HSV-1 genome, it may not be possible to detect a TMP crosslink repair deficiency in FA fibroblasts. Perhaps even in normal cells a crosslink cannot be repaired within the time constraints of the plaque assay.

Secondly, while no direct comparison has been made between the number of MMC and TMP crosslinks per FA genome required for a lethal event, it is known that FA GM1309 fibroblast survival is markedly lower than that of FA GM2053 and normal fibroblasts (Nagasawa and Little, 1983) with both agents. Normal, GM2053, and GM1309 fibroblast strains exhibited a 10 percent survival after exposure to 0.75 μg/ml, 0.5 μg/ml, and 0.05 μg/ml MMC respectively (Nagasawa and Little, 1983). We observed the FA HG 261 strain to be intermediate in sensitivity to MMC killing when compared to the more sensitive FA GM2053 and less sensitive normal CCD-25Sk strains (data not shown). The TMP crosslink level per cellular genome of the moderately MMC sensitive FA strains HG 261 and GM2053 may not be sufficient to produce the reduced cell survival observed for the extremely MMC sensitive FA GM1309 strain (Fig. 4B). It has been suggested that the variation in FA fibroblast strain survival after exposure to MMC and TMP can be explained by the existence of two different complementation groups (Duckworth-Rysiecki et al., 1985).

Photoreaction of HSV-1 with TMP (for two different experimental conditions) or with 5-MIP (Figs. 1-3) demonstrated that FA and normal fibroblasts have equal levels of host cell reactivation. This has also been observed for FA reactivation of cis-Pt (II) damaged SV40 DNA (Poll et al., 1984b) and TMP-UVA treated Ad 2 (Day et al., 1975). We have consistently observed slightly enhanced host cell reactivation by HG 261, GM1309 or GM2053 fibroblasts for 5-MIP photoinactivated HSV-1 (Fig. 2), a phenomenom also reported for gamma irradiated HSV when assayed on GM1309 FA fibroblasts (Zamansky and Little, 1982). HSV-1 irradiated with UVA in the presence of TMP demonstrated enhanced host cell reactivation only on HG 261 but not on GM1309 or GM2053 fibroblasts (Fig. 1 and 3). In the TMP experiments there is a mixture of furocoumarin monoadducts and crosslinks, whereas the 5-MIP reaction results only in monoadducts. Similar TMP and 5-MIP adduct levels per HSV-1 genome are observed with these irradiation conditions for 5-MIP and for TMP at 0.1 μg/ml (approximately 40 adducts per genome), although the levels are 4 to 10 fold lower for 0.01 μg/ml TMP (unpublished data). The lethality of TMP crosslinks appears to be sufficient to overcome any enhanced host cell reactivation of HSV-1 resulting from monoadduct damage for GM1309 and GM2053 FA fibroblasts, but not for HG 261 FA fibroblasts. It should also be noted that HSV-1 has a lower plaquing efficiency on HG 261 than on GM1309 and GM2053 FA fibroblasts (Table 2).

It is also apparent that the extent of TMP photoinactivation of HSV-1 depicted in Fig. 3 was greater than that observed with either 5-MIP (Fig. 2) or under the conditions shown in Fig. 1 which favored a higher ratio of TMP crosslinks to monoadducts. The apparent paradox between the TMP results is readily explained by adduct load. In separate experiments

under similar conditions, we have determined the actual adduct levels per viral genome at various time points. For HSV-1 exposed to TMP under the conditions employed in Figs. 1 and 3, the resulting adduct levels are 5 and 34 respectively for 40 min UVA irradiations. For 5-MIP, the adduct level corresponding to the 40 min time point of Fig. 2 is 38 molecules per genome. This indicates that a mixture of monoadducts and crosslinks is more lethal than approximately the same 5-MIP monoadduct level for HSV-1 (Figs. 2 and 3), whereas a considerably lower level of TMP adducts (but with a higher ratio of crosslinks to monoadducts) inactivates HSV-1 at nearly the same rate as 5-MIP (Figs. 1 and 2).

No titer could be obtained at the 60 min time point for HSV-1 photoinactivated in the presence of 0.1 μg/ml TMP (Fig. 3) due to a diffuse CPE or viral toxicity (no visible plaque formation) on all cell strains at the lowest viral dilution. A normal cell monolayer but no plaques were observed at the next 10-fold dilution. Multiplicity reactivation of HSV by genetic recombination has been reported to play a role in the repair of UV irradiated HSV (Hall et al., 1980; Selsky et al., 1979) and TMP photoinactivated HSV (Hall and Scherer, 1981). The fact that multiplicity reactivation in our hands was not observed with 5-MIP monoadducts or with the lower level but presumably higher crosslink ratio of TMP adducts may imply that a threshold level of crosslink damage is required to stimulate the recombination process.

We conclude that the FA fibroblast strains tested in this paper demonstrate no defect in the repair of either TMP monoadducts plus crosslinks or of 5-MIP monoadducts on HSV-1 DNA as determined by host cell reactivation. It is also apparent that FA fibroblast age does not influence their ability to repair TMP or 5-MIP photoinactivated HSV-1.

Similar conclusions can be made for the ability of HG 261 fibroblasts to repair TMP or 5-MIP adducts and GM2053 FA fibroblasts to repair TMP adducts on the cellular DNA as measured by colony forming ability. Only in GM1309 fibroblasts directly exposed to TMP was a defect in repair observed. Whether this is influenced by cell age is not known since this strain was tested for cell survival at high generations for TMP. It seems unlikely since it was sensitive to MMC at low generations (data not shown; Nagasawa and Little, 1983). These results indicate that caution must be exercised when host cell reactivation data is used to monitor cellular repair deficiencies. Further, all "crosslinks" may not be removed by the same cellular pathway. Deficiencies in the repair of mitomycin damage do not necessarily correlate with deficiencies in the repair of psoralen crosslinking agents.

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TABLE I

FANCONI'S ANEMIA FIBROBLAST STRAINS USED.

Strains	Source[a]	Description	Sensitivity to Mitomycin C[b]	References
CCD-25Sk	ATCC	Normal	N[c]	This paper
HG 261	ATCC	FA	S	Fornace et al., 1979
CRL 1196	ATCC	FA	S	Raj and Heddle, 1980
GM0368	HGMCR	FA	S	Raj and Heddle, 1980
GM0449A	HGMCR	FA	S	Latt et al., 1975
GM0646	HGMCR	FA	S	HGMCR
GM1309	HGMCR	FA	S	Nagasawa and Little, 1983
GM2053	HGMCR	FA	S	Nagasawa and Little, 1983

a. ATCC, American Type Culture Collection, Rockville, MD; HGMCR, N.I.G.M.S. Human Genetic Mutant Cell Repository, Camden, NJ.

b. Sensitivity determined by colony forming ability or increased chromosomal abberrations.

c. S = Sensitive, N = Normal.

TABLE II
FIBROBLAST GENERATION NUMBERS FOR HSV-1 PHOTOINACTIVATION EXPERIMENTS.

Fibroblast Strains	Fibroblast Cell Population Doublings[a]					% Plaquing Efficiency[b]	
	0.01 µg/ml TMP		0.1 µg/ml TMP	2.0 µg/ml 5-MIP			
	Low	High	Low	Low	High	Low	High
CCD-25Sk	22	38	22	23	38	100 ± 4	100 ± 1
HG 261	N.T.[c]	50	48	N.T.	50	N.T.	31 ± 3
CRL 1196	21	44	20	21	44	79 ± 5	97 ± 2
GM0368	18	33[d]	19	19	26[d]	218 ± 8	107 ± 2
GM0499A	33	45	31	31	45	123 ± 3	235 ± 5
GM0646	24	45	21	21	45	140 ± 4	179 ± 2
GM1309	17	44	15	17	44	166 ± 3	90 ± 1
GM2053	19	44	15	18	44	180 ± 4	268 ± 4

a. Age of the cells is given by the number of cell population doublings at the time of the indicated experiment.

b. Plaquing efficiency in the absence of photoinactivation is shown relative to unirradiated HSV-1 plaqued on CCD-25Sk. The numbers respresent an average of two or more assays ± standard error.

Table 2 (cont.)

c. N.T., not tested.

d. FA fibroblasts GM0368 were considered to be at high CPDs by generation 33 and 26 because this strain undergoes cell senescence at approximately 35 CPDs.

Figure Legends

Fig. 1. Comparison of TMP photoinactivation kinetics of HSV-1 when assayed on normal and FA fibroblast strains at low (A) and high (B) generations. HSV-1 was exposed to 0.01 μg/ml TMP plus UVA at an average incident intensity of 2.5 mW/cm^2. Normal CCD-25Sk, ●; FA HG 261, ■; FA CRL 1196, ▲; FA GM0368, ◆; FA GM0449A, 0; FA GM0646, □; FA GM1309, Δ; FA GM2053, ◇ .

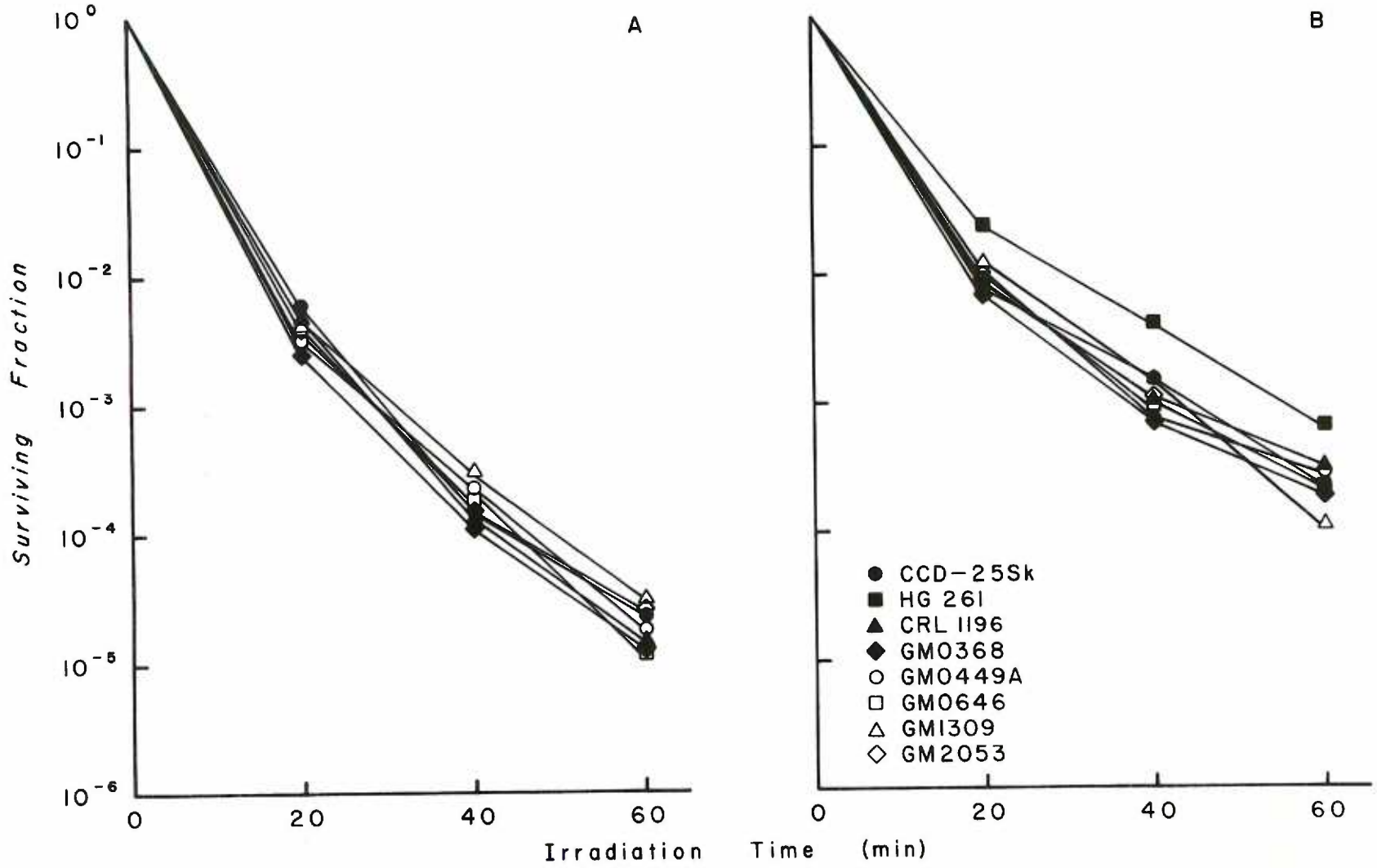

A
B
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
20
40
60
Irradiation Time (min)
CCD-25Sk
HG 261
CRL 1196
GM0368
GM0449A
GM0646
GM1309
GM2053

Fig. 2. Comparison of 5-MIP photoinactivation kinetics of HSV-1 when assayed on normal and FA fibroblast strains at low (A) and high (B) generations. HSV-1 was exposed to 2 μg/ml 5-MIP plus UVA at an average incident intensity of 2.5 mW/cm^2. Fibroblast strain designations are the same as in the legend to Fig. 1.

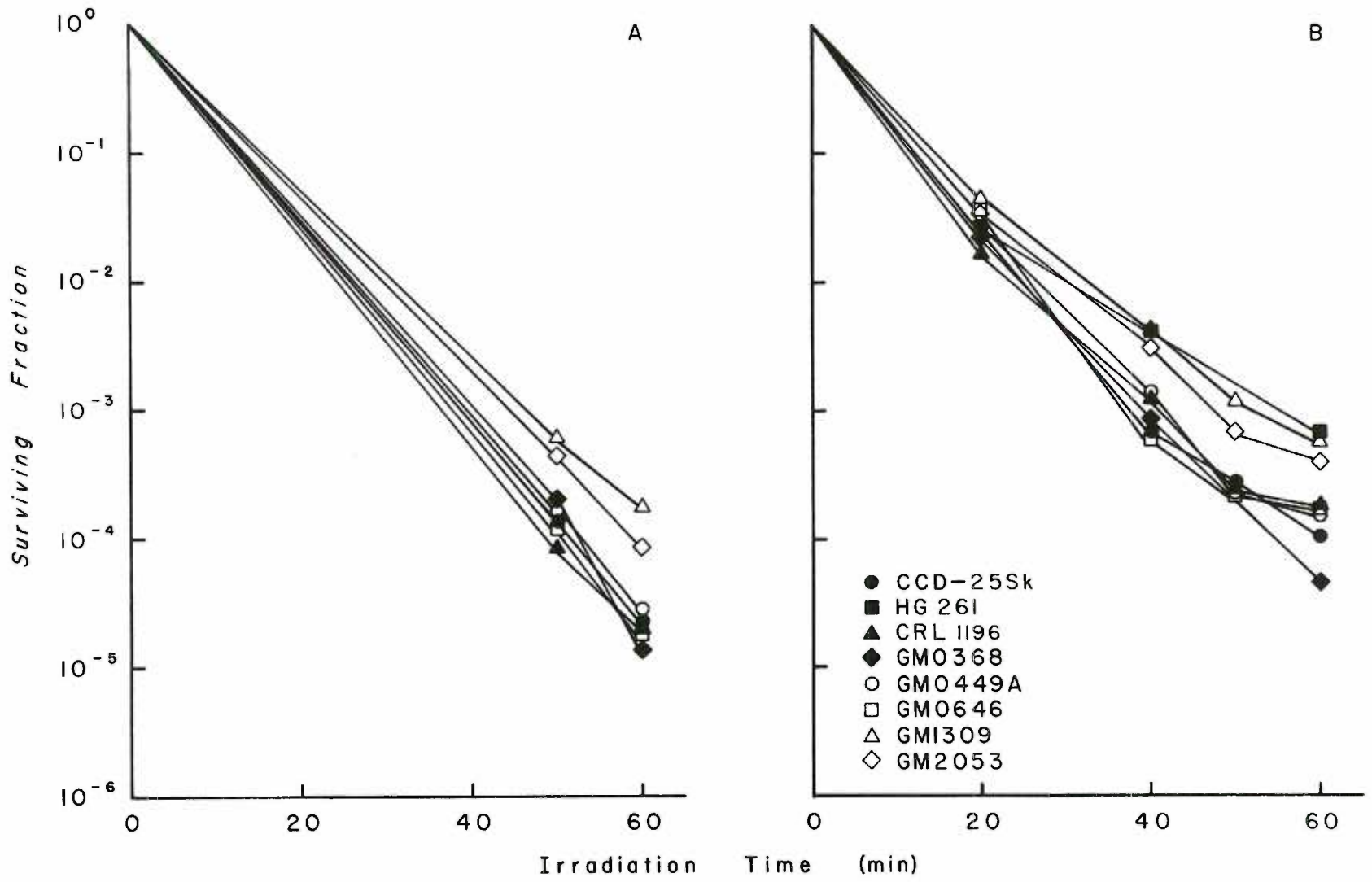

A
B
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
20
40
60
Irradiation Time (min)
CCD-25Sk
HG 261
CRL 1196
GM0368
GM0449A
GM0646
GM1309
GM2053

Fig. 3. TMP photoinactivation kinetics of HSV-1 when assayed on normal and FA fibroblast strains. HSV-1 was exposed to 0.1 μg/ml TMP plus UVA at an average incident intensity of 0.9 mW/cm^2. Fibroblast strain designations are the same as in the legend to Fig. 1. Normalized values indicated as less than (<) for GM0368 at 40 min irradiation time represents the limit of detection by the plaque assay.

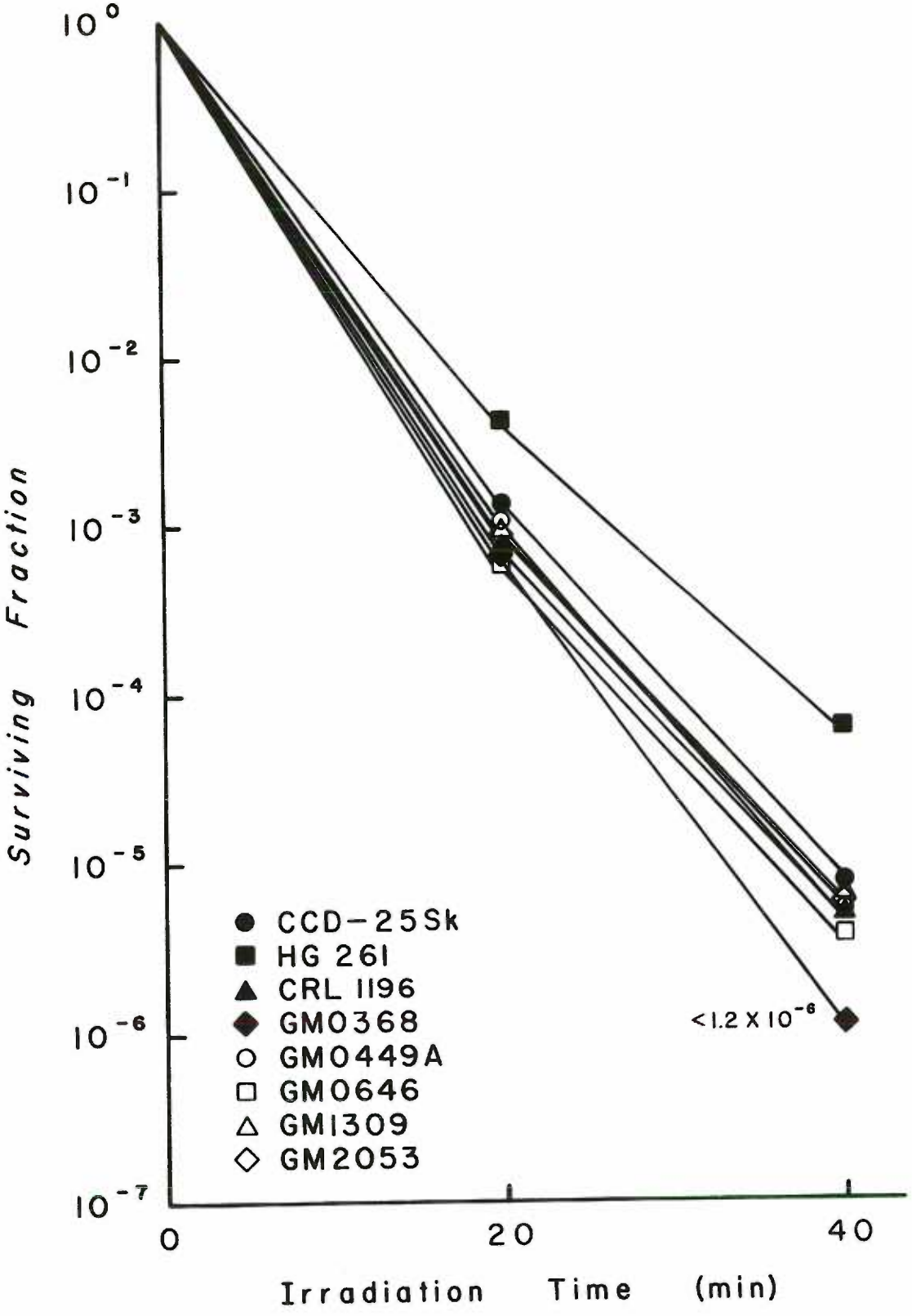
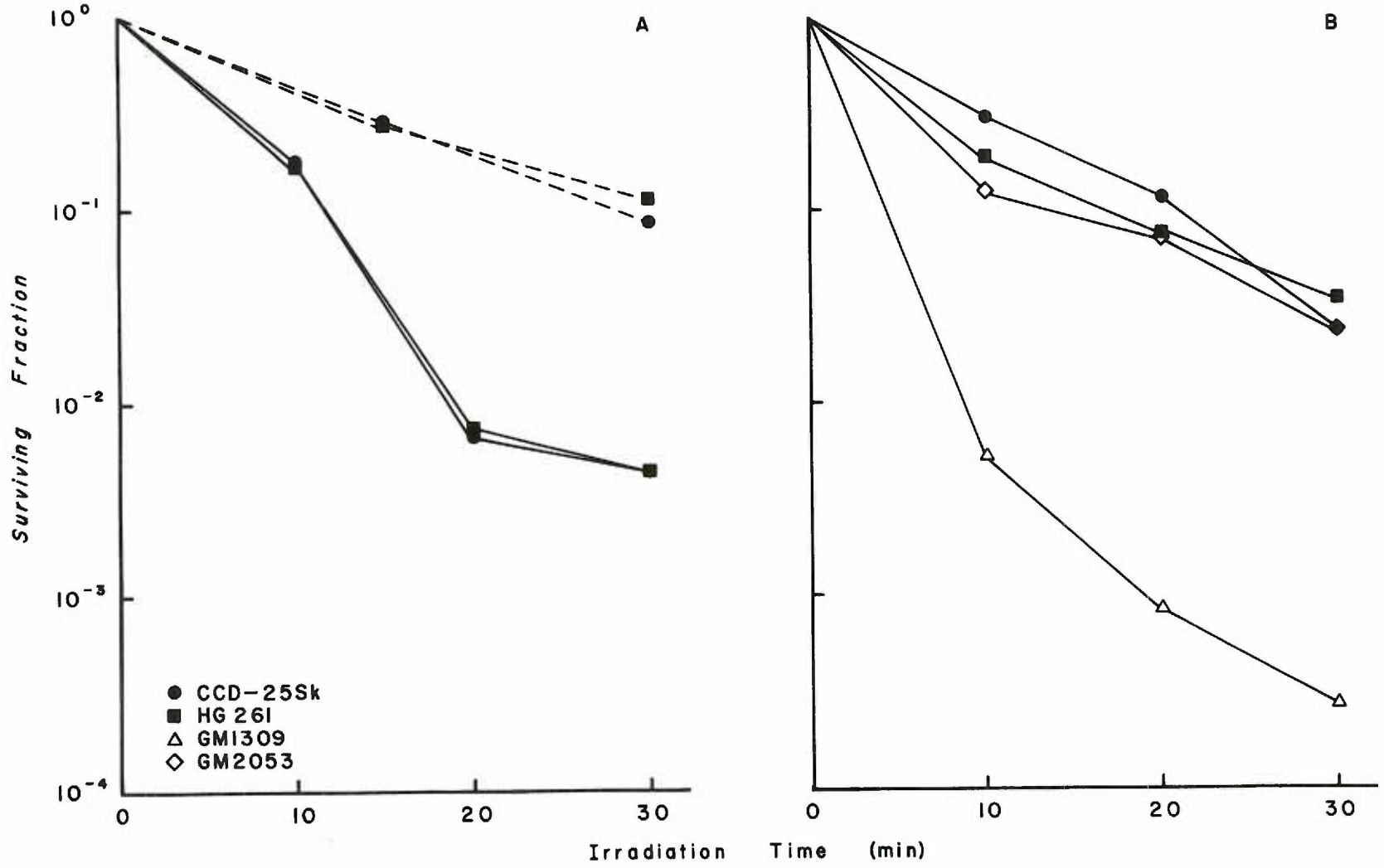

Surviving Fraction
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
0
20
40
Irradiation Time (min)
CCD-25Sk
HG 261
CRL 1196
GM0368
GM0449A
GM0646
GM1309
GM2053
<1.2 X 10^-6

Fig. 4. Cell killing of normal human and FA fibroblasts by TMP and 5-MIP. Survival of normal CCD-25Sk and FA HG 261 fibroblast strains after exposure to 1 ng/ml TMP (————) and 0.2 μg/ml 5-MIP (------) plus UVA at an average incident intensity of 4.6 mW/cm^2(A). Survival of normal CCD-25Sk, FA HG 261, GM1309, and GM2053 fibroblast strains after exposure to 0.5 ng/ml TMP plus UVA at an average incident intensity of 3.8 mW/cm^2(B). Fibroblast strain designations are the same as in the legend to Fig. 1.


A
B
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Surviving Fraction
CCD-25Sk
HG 261
GM1309
GM2053
0
10
20
30
Irradiation Time (min)

Manuscript 4.

Chemical Structure of Psoralen-Nucleic Acid Photoadducts in DNA Virus Inactivation

ABSTRACT

The kinetics of photoinactivation by 5-methylisopsoralen (5-MIP) and 4,5',8-trimethylpsoralen (TMP) were determined for the prokaryotic virus bacteriophage lambda (λ) and the two eukaryotic viruses, herpes simplex virus type 1 (HSV-1) and simian virus 40 (SV40). The angular isopsoralen 5-MIP only forms DNA monoadducts, while the linear psoralen derivative TMP forms both DNA monoadducts and crosslinks. Photoinactivation kinetics indicated an apparent order of sensitivity of λ > HSV-1 > SV40 and demonstrated that all three viruses were more sensitive to TMP than to 5-MIP photoaddition. Tritium labelled 5-MIP and TMP were utilized to determine the adduct levels at various times of irradiation. Expression of the surviving fraction as a function of adduct level per viral genome demonstrated that in fact λ was considerably less sensitive than either SV40 or HSV-1 to both 5-MIP and TMP. The eukaryotic viruses HSV-1 and SV40 were approximately equal in their sensitivities to 5-MIP and TMP in spite of their extremely different genome sizes. In order to characterize the chemical nature of the lethal event, experiments were performed in which λ and SV40 were irradiated in the presence of TMP, dialzyed to remove unbound molecules, and reirradiated in the absence of additional TMP. DNA was isolated from the inactivated virus samples and analyzed by enzymatic hydrolysis and high-performance liquid chromatography (HPLC) of the resulting nucleosides to characterize the chemical structure and level of the TMP-nucleic acid photoadducts. It was demonstrated that the additional photoinactivation observed upon reirradiation was accompanied by the conversion of TMP furan-side thymidine monoadducts to thymidine-thymidine crosslinks for both viruses.

In the absence of multiplicity reactivation, one TMP crosslink per SV40 genome was a lethal event. However, the number of TMP crosslinks associated with a lethal event in the λ *E. coli* system was suggested to increase as the total number of adducts per genome increase.

INTRODUCTION

Psoralens have advanced the understanding of nucleic acid structure and function as well as the biochemistry of DNA mutagenesis and repair through their use as photoprobes in vitro and in vivo (Cimino et al., 1985; Brendel and Ruhland, 1984). Furocoumarins (psoralen and its derivatives) photoreact with nucleic acids in three distinct but sequential events. Dark binding occurs first, by intercalation of psoralens between the stacked DNA base pairs. Psoralen photoexcitation by long-wavelength ultraviolet light (UVA, 320 to 400 nm) results in a second event (monoadduct formation) in which the molecule is covalently bound to the DNA. A cyclobutane ring is formed between the 5,6 double bond of an adjacent pyrimidine (primarily thymine) and either the 4',5' double bond of the furan ring or the 3,4 double bond of the pyrone ring. Photoexcitation of the furan-side monoadducts, which retain an absorbance between 320 and 380 nm, can result in the formation of a second cyclobutane ring between the psoralen pyrone double bond and the 5,6 double bond of an adjacent pyrimidine. This third event forms a crosslink between the strands of the DNA helix (Hearst et al., 1984).

Furocoumarins have the advantage that they reversibly enter cells and viruses and, in the absence of light, generally do not cause any damage. Although it is clear that the biological consequences of both mono- and bifunctional furocoumarins plus UVA may be lethal or mutagenic, the mechanism by which crosslinking derivatives do so is controversial. Irradiation with bifunctional furocoumarins results in a mixture of monoadducts and crosslinks, the ratio of which changes continuously

during the photoreaction. Thus a single kinetic analysis of survival curves is difficult to interpret. For example, the biological response to bifunctional furocoumarins plus low doses of UVA administered over a prolonged time period (i.e., low UVA intensity) has been reported to resemble the response to a monofunctional furocoumarin plus UVA, while the same UVA dose given in a shorter time interval to bifunctional furocoumarins conferred a greater lethality, presumably due to an increase in the ratio of crosslinks to monoadducts (Averbeck, 1982).

The biological effect of bifunctional furocoumarins plus UVA has indicated that crosslinked DNA is lethal to both prokaryotic (Cole, 1971) and eukaryotic (Ben-Hur and Elkind, 1973a) cells. Wild type (wt) Escherichia coli can survive 53 to 71 crosslinks of 4,5',8-trimethylsporalen (TMP) per chromosome (Sinden and Cole, 1978); approximately 120 8-methoxypsoralen (8-MOP) crosslinks per wt Saccharomyces cerevisiae genome were lethal (Magana-Schwencke et al., 1982); and Chinese hamster cell killing required approximately 2,000 TMP crosslinks per genome (Ben-Hur and Elkind, 1973b). Recombination deficient E. coli strains and the S. cerevisiae pso 2-1 mutant were killed by one or two crosslinks per genome (Sinden and Cole, 1978; Magana-Schwencke et al., 1982). Monofunctional furocoumarins have also proven to be lethal to prokaryotic and eukaryotic cells although they are much less toxic than bifunctional furocoumarins (Grossweiner and Smith, 1981; Papadopoulo et al., 1983).

Viruses have provided another approach to understanding the biological consequences of furocoumarins plus UVA. It has been reported that a single TMP crosslink is sufficient to inactivate both lambda bacteriophage (λ) (Cole, 1971) and adenovirus type 2 (Day et al., 1975)

in the absence of multiplicity reactivation. TMP photoinactivated simian virus 40 (SV40) and herpes simplex virus (HSV) have been shown to be repaired by multiplicity reactivation, whereas approximately one crosslink is lethal to SV40 in the absence of multiplicity reactivation (Hall, 1982; Hall and Scherer, 1981). TMP and khellin crosslinks were more effective than khellin monoadducts in both the induction of λ phage-prophage genetic recombinaton and λ killing (Cassuto et al., 1977). The presence of TMP crosslinks, but not monoadducts, in λ infecting E. coli (λ) lysogens increased genetic recombination that was dependent upon the host uvrA$^+$ and recA$^+$ gene products (Lin et al., 1977). Ross and Howard-Flanders (1977a,b) showed that TMP crosslinks promoted the "cutting in trans" of undamaged covalently closed circular λ DNA, but not heterologous phage 186 DNA molecules in uvrA$^+$ recA$^+$ E. coli (λ) lysogens. TMP lesions on the infectious λ DNA did not inhibit injection or circularization. A 1.8 fold increase in UVA dose is required to produce an equal killing of λ by 8-MOP when assayed on UV-induced E. coli relative to unirradiated cells (Weigle reactivation, Yasui et al., 1981), implicating the inducible SOS system in psoralen repair.

The biological responses to monoadducts and crosslinks have been compared in several virus systems. Monofunctional 4,5'-dimethylisopsoralen and psoralen photoinactivation kinetics of extracellular T2 phage were similar (Baccichetti et al., 1979). However, vegetative T2 phage was more resistant to killing by 4,5'-dimethylisopsoralen than psoralen. Baccichetti et al. (1979) suggested that the highly folded packaged phage DNA in the extracellular phage contained unique lesions, presumably crosslinks produced by 4,5'-dimethylisopsoralen. It has been shown that phage DNA injection and

replication, but not phage adsorption, are reduced by 8-MOP and isopsoralen photoinactivation of extracellular λ (Hradecna and Kittler, 1982). It was proposed that λ DNA injection was inhibited by the formation of unique crosslinks at adjacent sites on the tightly folded helix within the phage head by both furocoumarins, whereas these unique crosslinks would not occur in the vegetative DNA helix (Hradecna and Kittler, 1982).

Weigle reactivation of λ phage photoreacted with isopsoralen was greatly enhanced in E. coli uvrA$^-$ relative to wt E. coli, whereas it was somewhat less than that in wt cells for λ damaged with 8-MOP (Lichtenberg and Yasui, 1983). Taken together with the absolute level of virus survival in the absence of Weigle reactivation, their results imply that isopsoralen monoadducts can be removed efficiently by the repair induced in the absence of the UVRA gene products, whereas those of 8-MOP (presumably crosslinks) cannot. The relatively small Weigle reactivation of isopsoralen in wt E. coli may reflect the efficiency of constitutive repair systems. In the same study, a reduced Weigle reactivation was also observed when either recB or recF E. coli was infected with λ photoreacted with 8-MOP, although the effect was much more dramatic for recF cells. Since the recF mutation had little effect on phage survival per se, it was concluded that it actually reduced the induction of Weigle reactivation. A similar effect of recF could be seen for isopsoralen damaged virus in uvrA cells, but not in wt E. coli, again presumably because it was masked by high levels of constitutive repair.

No difference was observed in the TMP photoinactivation kinetics of HSV when assayed on normal, xeroderma pigmentosum (XP) and Fanconi's anemia fibroblasts (FA); however, reduced survival of HSV photoreacted

with 5-methylisopsoralen (5-MIP) was detected for several XP complementation groups (Fendrick and Hallick, 1984). Thus, the excision pathways believed to be defective in XP cells appear to be involved in the repair of monoadducts but not that of crosslinks. Interestingly, the photoinactivation kinetics of both TMP and 5-MIP reacted HSV-1 on several FA strains are not reduced relative to normal fibroblasts (Fendrick, Logan, Polonoff and Hallick, in preparation). Since FA cells exhibit enhanced sensitivity to mitomycin C, they are thought to be defective in crosslink repair (Fujiwara et al., 1984), but such a defect cannot be demonstrated for psoralen crosslinked viral DNA.

Another approach to understanding the biological consequences of monoadducts and crosslinks on viruses produced by bifunctional furocoumarins has been to examine the response to UVA irradiation following removal of the noncovalently bound furocoumarins after an initial irradiation. Such a reirradiation protocol allows the conversion of monoadducts to crosslinks, thereby increasing the proportion of crosslinks for a given adduct level (Ben-Hur and Elkind, 1973a). Reirradiation experiments have implicated TMP crosslinks as the primary lethal event in the photoinactivation of λ, whereas in the case of SV40, it was concluded that a combination of TMP monoadducts and crosslinks may be lethal or monoadduct formation may be rate limiting (Kondoleon et al., 1982). From a similar protocol, lethality in E. coli and T7 phage has been attributed mostly to 8-MOP crosslinks, whereas increased mutation frequencies are to 8-MOP monoadducts (Seki et al., 1978). Psoralen damage on T3 phage is repaired by the host systems, whereas survival of T4 does not appear to be affected by host mechanisms (Strike et al., 1981). 8-MOP crosslinks were observed to be the major lethal event in T3

photoinactivation, but 8-MOP monoadducts were also shown to contribute to the lethality of T4. Multiplicity reactivation was able to repair monoadducts but was ineffective in the rescue of crosslinked T4 DNA (Strike, 1981).

A critical limitation for many of these studies reported to date is the absence of quantitative information on furocoumarin adduct levels and types as a function of viral photoinactivation. We report here the photoinactivation kinetics of λ, HSV-1 and SV40 by 5-MIP and TMP, a monofunctional and bifunctional furocoumarin, respectively. Photoadduct levels of the two furocoumarins are correlated with the inactivation kinetics of all three viruses. UVA reirradiation with TMP is utilized to investigate the effect of crosslink lethality on λ and SV40. Photoinactivation kinetics for such a reirradiation protocol are correlated with the chemical structure of the furocoumarin adducts present on the viral DNA as determined by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Mammalian Cells, Bacteria and Viruses. MA-134 and VERO (African green monkey kidney) cells were cultured in Minimum Essential Medium, Eagle (MEM) supplemented with 23.8mM $NaHCO_3$, 2.0mM L-glutamine, and 10% (v/v) heat inactivated fetal calf serum (HI-FCS). Dulbecco's Modified Eagle Medium supplemented with 44.0mM $NaHCO_3$ and 5% (v/v) HI-FCS was used for culture of CV-1P cells, a subclone of CV-1 cells. All culture media was supplemented with 100U penicillin and 50µg/ml streptomycin. Strain SV40 776 was propagated in MA-134 cells at a multiplicity of infection (m.o.i.) of 10 plaque-forming units (PFUs) per cell for virus production. Extracellular SV40 was purified as previously described by Konodoleon et al. (1983). A single stock of purified SV40 at a titer of 5×10^{10} PFUs ml^{-1} was utilized in all experiments. SV40 plaque assays were performed according to the method of Fendrick and Hallick (1983).

HSV-1 (CL101) was propogated in VERO cells at a multiplicity of infection of 0.01 PFUs per cell. Virus and cells were collected 96 h postinfection and frozen once at -70°C. Cells were concentrated by centrifugation and then sonicated with a Model W-225R sonicator cell disruptor and special (stepped) microtip (Heat Systems-Ultrasonic Inc., N.Y.). Sonicated cells were then added back to the culture medium. Cell debris and nuclei were pelleted by centrifugation at 1,000 g for 10 min. at 4°C. Supernatant was collected and centrifuged a second time at 20,000 g for 20 min. at 4°C to remove additional cell debris and mitochondria. HSV-1 was concentrated by pelleting onto a 50% (w/v) sucrose cushion in TS (0.15 M NaCl, 0.02M Trizma, pH 7.5) at 112,500 g for 120 min. at 4°C. Discontinuous sucrose gradients composed of 20%,

35%, and 50% (w/v) sucrose in TS pH 7.5 were centrifuged at 112,500 g for 60 min. at 4°C to band the virus. HSV-1 bands were collected and dialyzed against TS, pH 7.5. Virus suspensions were made 2% HI-FCS and stored at -70°C. A single stock of purified HSV-1 at a titer of 2.5 x 10^8 PFUs ml^{-1} was utilized in all experiments.

HSV-1 was plaqued on VERO cells with a 0.5% (w/v) methyl-cellulose overlay in MEM-5% HI-FCS. A single λ cI^- bacteriophage stock with a titer of 2 x 10^{12} PFUs ml^{-1}, produced by infecting E. coli QD and purifying as previously described (Kondoleon et al., 1982), was utilized for all experiments.

Psoralen Derivatives. 4,5',8-[^{3}H] Trimethylpsoralen (TMP) and [^{3}H] 5-methylisopsoralen (5-MIP) were purchased from HRI Associates, Inc. (Emeryville, CA) (Isaacs et al., 1977; Isaacs et al., 1984). Stock psoralen concentrations in 100% ethanol were calculated by extinction coefficients of 3.1 x 10^4 $LM^{-1}cm^{-1}$ (TMP, 249nm) (Wiesehahn et al., 1977) and 2.4 x 10^4 $LM^{-1}cm^{-1}$ (5-MIP, 249nm) (Isaacs et al., 1984). Specific activities of [^{3}H]-TMP and [^{3}H]5-MIP were determined to be 0.67 and 0.99 Ci/mM, respectively.

Irradiation Experiments. SV40 and lambda were irradiated in TBS (0.15 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Trizma, pH7.4, 0.5% (v/v) HI-FCS) and TMG (50 mM Trizma, pH7.4, 10 mM $MgSO_4$, 0.01% (w/v) Gelatin) buffers, respectively. Virus solutions were adjusted to a final concentration of 2.0 μg/ml and 1.0 μg/ml TMP for SV40 and λ, respectively, or 10.0 μg/ml 5-MIP for both viruses. HSV-1 solutions were adjusted to a final concentration of 2.0 μg/ml 5-MIP and 0.01 μg/ml or 0.10 μg/ml TMP as indicated. Virus-psoralen solutions were held at 4°C in the dark for 20 min or longer prior to aliquoting for

irradiation. A single bank of two General Electric F15T8BLB fluorescent bulbs were used at an average incident intensity of 0.9 mW/cm^2 or 2.5 mW/cm^2 as indicated. The degree of incident intensity was controlled by use of plastic shields. Virus-psoralen samples were irradiated in separate 60 mm tissue culture dishes in aliquots of 1.0 ml or less for each sample to prevent each aliquot from coming into contact with the vertical sides of the dish. Controls for all experiments consisted of virus plus psoralen without irradiation (0 min) and virus with UVA alone for the maximum irradiation time used in each experiment.

Photoinactivation kinetics for SV40 and λ with TMP and 5-MIP were determined by plaque titration for each time point. Each sample was then dialyzed against TBS (SV40) and TMG (λ) to remove unreacted psoralen. Following dialysis, photoinactivation kinetics were reenumerated for each time point as a control for loss of viability. No significant difference was observed between the pre- and post-dialysis photoinactivation for all experiments. Specific time points (post-dialysis) for the TMP irradiation experiments of SV40 and λ were then divided into two equal aliquots, one of which was reirradiated at the same average incident intensity as the initial irradiation. Each initial irradiated and reirradiated time point was assayed as previously described to determine photoinactivation kinetics. All graphs depicting photoinactivation kinetics of SV40 and lambda have been normalized to the control samples containing TMP or 5-MIP without irradiation (0 min).

Virus Adduct Level Determination. Each irradiation time point for all experiments was adjusted to a final concentration of 0.5mg/ml proteinase K (Sigma), 1.0% (v/v) SDS (sodium lauryl sulfate), 0.04 M EDTA (etheylenediaminetetraacetic acid), pH8.0 and digested at 37°C for

approximately 6 h. A second aliquot of proteinase K equal to the initial volume was added to each time point and digested for another 12 h. All time points were adjusted to a final concentration of 0.5M NaCl and extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), followed by three extractions with buffered ether. Extracted time points were adjusted to a final concentration of 0.3 M sodium acetate and precipitated with three volumes cold 100% ethanol. The isolated DNA pellets were dried in vacuo and redissolved in 15 mM NaOAc pH5.0 (sodium acetate) or TE, pH8.0 (0.01 M Trizma, pH8.0, 0.001 M EDTA) for TMP and 5-MIP photoreacted time points, respectively. The optical density and amount of radioactivity for each DNA-furocoumarin mixture was then determined in order to calculate adduct level.

Adduct Isolation and Characterization. TMP-modified DNA was hydrolyzed for high-performance liquid chromatography (HPLC) analysis of adduct products according to the method of Kanne et al (1982). DNA was hydrolyzed by incubation at 37°C with 80 units of DNase II (EC 3.1.22.1) per mg of DNA. After 12 h, a second volume of DNase II was aliquoted into each sample and hydrolysis continued for another 12 h. Each sample was adjusted to pH7.0 followed by addition of 0.2 units of phosphodiesterase II (EC 3.1.4.18) per mg of DNA and incubated for 24 h, at which time an additional 0.2 units of enzyme was added and incubation continued for another 24 h. DNA solutions were then adjusted to pH8.0, 0.2 units of alkaline phosphotase (EC 3.1.3.1) per mg DNA were added and the reaction was incubated for an additional 24 h. After a total hydrolysis time of 96 h, the digestion was adjusted to a pH of 2.2 by addition of 3 M H_3PO_4 and held at room temperature for 30 min. Samples were passed through 0.2μ Nylon-66 filters (Rainin) and then analyzed by

HPLC with a Beckman Model 332 Gradient Liquid Chromatograph System. Filtered lysates were injected onto a reverse-phase octadecylsilane (ODS) column (5μ Ultrasphere; 4.6 mm x 25 cm; Beckman) and gradient eluted in 20 mM KH_2PO_4,pH2/methanol at a flow rate of 4 ml min^{-1}. Scintallation counting was employed to detect the presence of ^3H in the column effluent.

RESULTS

Furocoumarin photoinactivation kinetics of DNA viruses. Photoinactivation kinetics of λ and SV40 viruses clearly indicate that both viruses are more readily inactivated by TMP than 5-MIP during continuous UVA irradiation (Fig. 1A), even when the initial 5-MIP concentration was 5-10 fold greater than that of TMP. Similar results were obtained for HSV-1 at lower concentrations of both derivatives (0.1 μg/ml TMP and 2.0 μg/ml 5-MIP) (Fig. 1A).

When λ and SV40 virus samples are removed at various times of irradiation, dialyzed to remove unbound TMP, and subsequently reirradiated, the killing of both viruses is enhanced (Fig. 1B). This additional inactivation following reirradiation has been attributed to the conversion of psoralen monoadducts to crosslinks. Although psoralen adduct levels per genome can be quantitated by the use of radioactive labelled psoralens and the crosslink yield estimated, the actual distribution of crosslinks and monoadducts per genome has not been previously reported for viral photoinactivation.

Relationship of TMP adduct photoaddition to viral photoinactivation kinetics. HPLC analysis of covalently bound radioactive psoralen adducts provides a powerful tool to directly quantitate the number of monoadducts and crosslinks per genome. It is also possible to distinguish furan- and pyrone-side thymidine monoadducts, as well as the furan-side cytidine monoadducts. The number and type of psoralen adducts can then be correlated with the photoinactivation kinetics produced by continuous irradiation, and the conversion of monoadducts to crosslinks after reirradiation in the absence of unbound psoralen can be monitored.

Viral DNA was isolated from λ and SV40 virus samples for each of the TMP photoinactivation time points shown in Fig. 1B. The total TMP adduct level per λ and SV40 viral genome was quantitated for each sample (Table 1). The TMP modified viral DNAs were then enzymatically hydrolyzed to produce a mixture of TMP modified nucleosides and a much larger amount of unmodified nucleosides. The different types of TMP modified nucleosides were separated from this mixture by HPLC and quantitated by liquid scintillation counting (Table 1 and Figure 2).

The kinetics of addition suggest that λ DNA is more accessible than SV40 DNA to TMP covalent binding. However, because of differences in the target sizes, light intensities and the initial ratio of TMP to DNA, such comparisons are difficult to interpret. By relating the surviving fraction to the absolute level and type of photodamage per genome (Fig. 3), the biological consequences of psoralen photoaddition can be more precisely described.

Analysis of λ and SV40 TMP adducts formed during viral photoinactivation suggests that the additional viral inactivation upon reirradiation does occur due to the conversion of monoadducts to crosslinks (Figs. 2 and 3). Reirradiation of the λ 10 min. sample for another 20 min. increases the crosslink level from approximately 4 to 15 crosslinks per genome which results in a 100-fold decrease in viability in the absence of TMP addition to the viral DNA (Table 1 and Figs. 2A and 3A). Similar results are observed for SV40 when the 10 min. irradiation is reirradiated for an additional 20 min. The TMP crosslink level increased from 1.6 to 4 crosslinks per SV40 genome, with a corresponding 3-fold decrease in viability (Table 1 and Figs. 2B and 3B). Total TMP adduct levels for λ and SV40 remained very similar between the initial

irradiation and the reirradiation indicating that the increases in viral inactivation result only from the formation of new TMP crosslinks from monoadducts and not the addition or loss of TMP adducts already covalently bound to the viral DNA (Table 1).

For both viruses the increase in crosslinks upon reirradiation is primarily at the expense of furan-side thymidine monoadducts, the species that would be expected to be the immediate precursor to a diadduct (Fig. 2). There also appears to be a greater decrease in the level of furan-side deoxyuridine (dU) TMP monoadducts on λ DNA than on SV40 DNA (Table 1 and Fig. 2). The enzymatic hydrolysis of TMP modified DNA results in the deamination of furan-side deoxycytidine to form dU. A 20 min. reirradiation of the λ 10 min. sample resulted in a decrease of dU monoadducts from 9.8 to 2.8 while the same reirradiation time for the SV40 20 min. irradiation point exhibited a minor increase from (1.2 and 1.1 dU monoadducts per genome). The level of pyrone-side monoadducts did not change appreciably upon reirradiation.

The photoaddition kinetics of total TMP adducts per HSV-1 genome are shown in Table 2. The HPLC analysis of the different types of TMP adducts was not performed for HSV-1. At a concentration of 0.01 μg/ml TMP the photoaddition kinetics exhibit a plateau at approximately 5 adducts per genome although the photoinactivation kinetics indicate that HSV-1 continues to be inactivated after this time (data not shown). This is presumed to occur due to the conversion of bound TMP monoadducts to crosslinks.

Relationship of 5-MIP photoaddition and viral photoinactivation kinetics. While 5-MIP can covalently bind to the DNA by either the pyrone or furan reactive sites of the molecule, it is considered a

monofunctional furocoumarin due to its angular nature which sterically inhibits DNA crosslinking. Photoaddition kinetics clearly show that on a per genome basis, 5-MIP is covalently bound more rapidly to λ than to SV40 DNA under identical irradiation conditions (Table 3). However, when corrected for target size, the photoaddition of 5-MIP occurred more rapidly to SV40 DNA. Once again, the different input ratios of psoralen to DNA make it difficult to draw precise conclusions regarding the relative accessibility of the two viral DNAs to 5-MIP.

The higher rate of 5-MIP photoaddition per λ virion corresponds with the increased rate of λ inactivation relative to the lower level of SV40 photoaddition and inactivation. Continued photoaddition of 5-MIP results in linear photoinactivation kinetics of λ over a range of 120 min. irradiation (Fig. 1A). 5-MIP photoinactivation kinetics of SV40 shows a high level of virus inactivation from 0 to 45 min. after which the kinetics appear to reach a plateau (Fig. 1A). This two step 5-MIP photoinactivation curve for SV40 does not correlate with any substantial change in the rate of 5-MIP photoaddition over the 120 min. irradiation (Table 3). The lower rate of SV40 inactivation from 60 to 120 min. is believed to be the result of multiplicity reactivation, as indicated by nonlinear dilution series (data not shown).

Lethality of TMP crosslinks and monoadducts. In order to better define the actual lethal event, the surviving fraction has been expressed as a function of each type of adduct level per genome. This was done by extrapolating the various parameters on the reirradiation kinetic curves (Fig 3) back to that of the continuous irradiation (Table 4). At reirradiation levels of 6.7 and 14.8 TMP crosslinks per λ genome the surviving fractions were 1.9×10^{-3} and 2.9×10^{-4} respectively. The same

crosslink levels on the continuous irradiation show higher surviving fractions of $1.9x10^{-2}$ and $5.6x10^{-4}$ even though the corresponding TMP monoadduct levels have increased two fold from 31 and 42 to 61 and 83 monoadducts per λ genome respectively (Table 4, panel B). This suggests that when the same number of crosslinks are associated with a low level of monoadducts they are more lethal to λ than when monoadducts are present at a two fold higher level (Table 4). This effect is most pronounced for the earlier λ time point, and was not observed for SV40 (Table 4, panel D).

Continuous irradiation conditions that would be predicted to result in 31 and 42 TMP monoadducts per λ genome, the same level as observed for the reirradiated, samples, should result in crosslink levels of 1.8 and 2.9 per genome, in contrast to the levels of 6.7 and 14.8 observed in the reirradiation protocol (Table 4, panel B). Thus, with a constant level of monoadducts, the lower crosslink level per λ genome produced during continuous irradiation results in an almost 3 fold higher surviving fraction. In other words to achieve the same extent of killing with a continuous irradiation protocol as that seen in the reirradiated samples a considerably higher adduct load is required for both viruses, presumably because of the lower ratio or crosslinks to monoadducts.

The Poisson distribution can be used to predict the number of crosslinks that constitute a lethal event by comparing the surviving titer to the probability of a virus receiving no (or 1 or 2, etc.) "hits" at a given average density of crosslinks per genome. Approximately 0 to 1 and 3 to 4 TMP crosslinks per λ genome represent a lethal event for 6.7 and 14.8 crosslinks per λ genome, respectively, regardless of whether they are produced during continuous irradiation or reirradiation (Table

5). In other words the number of TMP crosslinks representing a lethal event for λ increases with the increase in crosslink density.

The number of TMP crosslinks or 5-MIP monoadducts per genome that constitutes a lethal event for SV40 are quite different than for λ. Approximately one crosslink per SV40 genome is lethal at all irradiation times in the absence of multiplicity reactivation (Table 5). At the 15 min. irradiation time, approximately 13 to 14 5-MIP monoadducts per SV40 genome result in one lethal event, whereas one lethal hit per λ genome requires a seven-fold higher 5-MIP adduct load per virus (data not shown). Therefore, the effect of monoadducts, including those of TMP, on SV40 may be more pronounced than was observed for λ. If the TMP crosslink levels of 4 and 5.2 per SV40 genome of the reirradiated samples are extrapolated to the continuous irradiation curve, the monoadducts increase from the reirradiation level of 7.6 and 11.8 to values of 16.9 and 17.1 for continuous irradiation (Table 4, panel D). In both situations, this increase in TMP monoadducts per SV40 genome results in a decreased surviving fraction (Tables 1 and 4).

Photoinactivation kinetics as a function of adduct level. By expressing the surviving fraction for λ, SV40, and HSV-1 as a function of adducts per 1000 base pairs (bp), the differences in the rates of photoaddition are eliminated and the effect of target size is emphasized (Fig. 4A). The relative order of sensitivity to adduct level per nucleotide is HSV-1 > λ > SV40 for both derivatives. All three viruses are much more sensitive to TMP than to 5-MIP, even when the lower photoefficiency of 5-MIP is eliminated by considering adduct level rather than irradiation time. Perhaps the most revealing analysis is to express the surviving fraction as a function of adduct level per genome (Fig.

4B). It is evident that λ is much more resistant to 5-MIP inactivation than HSV-1 and SV40. A surviving fraction of $1.0x10^{-2}$ extrapolates to approximately 167 5-MIP monoadducts per λ genome but only about 40 monoadducts per SV40 and HSV-1 genome (Fig. 5). Remarkably, the curves for SV40 and HSV-1 5-MIP photoaddition kinetics are virtually identical. This implies that even though there is a large difference in their target sizes they are equally sensitive to the same 5-MIP monoadduct levels per viral genome. The apparent "biphasic" nature of the SV40 curve for 5-MIP coincides with an apparent multiplicity reactivation (data not shown). Thus, this relative resistance to inactivation may be due to recombinational rescue.

Total TMP adducts per viral genome again show that λ is much more resistant to photoinactivation than HSV-1 or SV40 (Fig. 4B). At a surviving fraction of $1.0x10^{-3}$ there are approximately 93 TMP adducts per λ genome and only 2.1 and 2.5 per HSV-1 and SV40 genomes, respectively. Again the sensitivity of SV40 and HSV-1 to TMP level are identical in spite of a 30-fold difference in genome size.

DISCUSSION

Results from the photoinactivation kinetics of λ, SV40 and HSV-1 by the bifunctional and monofunctional furocoumarins TMP and 5-MIP are consistent with the longstanding assumption that a crosslink is more lethal than a monoadduct. To address the lethality of TMP crosslinks, the reirradiation protocol (Ben-Hur and Elkind, 1973a) was utilized in conjunction with photoinactivation kinetics, adduct level determinations and characterization of the TMP-DNA adduct products by HPLC. Such an analysis proved that the decrease in surviving fraction of λ and SV40 upon reirradiation in the absence of psoralen correlates with the increase of TMP crosslinks and the decrease of monoadducts. This conversion of monoadducts to crosslinks in SV40 occurs primarily at the expense of the furan-side thymidine monoadduct, whereas both cytidine and thymidine monoadducts may contribute to crosslink formation in λ (Table 1). The role of cytidine adducts in λ photoinactivation is not clear. It is possible that a TC or a CC crosslink contributes to the lethality of TMP for λ. In fact, the decrease in dU monoadducts is accompanied by the appearance of a small peak whose mobility is slightly faster than that of the T-T crosslink (Fig. 2), but it has not yet been characterized separately.

Comparison of the TMP monoadduct and crosslink levels for continuous irradiation and reirradiation clearly show that the surviving fractions of λ and SV40 respond very differently (Table 4). When the λ TMP crosslink levels are equal for continuous irradiation and reirradiation, not only is the monoadduct level for continuous irradiation 2 fold higher, but the surviving fraction is 10 fold higher than that after

reirradiation. In contrast, when SV40 TMP crosslink levels are the same for continuous irradiation and reirradiation, that increase in monoadducts for continuous irradiation is correlated with a reduction in the surviving fraction. This observation may be the result of two different findings. First, it was shown that at higher levels of TMP crosslinks for λ, more crosslinks may be required to constitute a lethal event (Table 5). Secondly, a very high level of 5-MIP monoadducts per λ genome is required to produce a lethal event (Table 3 and Fig. 1). Thus, a two fold increase in TMP monoadducts might not constitute a lethal event when the crosslink levels were constant and therefore would not reduce the surviving fraction. However, if during repair of this higher TMP monoadduct level there was a higher probability of crosslinks being excised from one of the λ DNA strands through repair of the monoadduct, then the resulting surviving fraction might increase. This would not occur or would have a limited effect for SV40 since one TMP crosslink represents a lethal event at any crosslink level and a lower level of 5-MIP monoadducts are lethal.

Characterization of TMP adducts on the HSV-1 genome was not determined. However, it is clear from a comparison of the photoinactivation kinetics (Fig. 1A) and the determination of adduct levels (Table 2) that treatment with appropriate concentrations of TMP and 5-MIP can result in a 1000 fold greater inactivation by TMP than by 5-MIP at comparable numbers of adducts per genome, presumably due to the presence of crosslinking.

SV40 and HSV-1 are killed by approximately the same number of 5-MIP adducts per genome (Fig. 4B), whereas λ is the least sensitive to 5-MIP photoaddition. Not only is the prokaryotic virus λ more resistant to TMP

and 5-MIP photoaddition than HSV-1 or SV40, it is also suggested that it may be able to tolerate an increasing number of TMP crosslinks (Fig. 4B, Table 5) even in the absence of multiplicity reactivation. This is in disagreement with the previously reported observation that a single TMP crosslink was lethal to λ (Cole, 1971). It should be noted that Cole (1971) only examined very early irradiation times. Under similar irradiation conditions, we also observed one TMP crosslink to be lethal (Table 5). Whether λ is able to tolerate a higher number of TMP crosslinks for irradiations greater than 20 min. is not known, although we were not at the limit of detection of our plaque assay. However, since the number of TMP crosslinks constituting a lethal event for λ was statistically determined, some caution must be used in making a definite conclusion. More information is required concerning the lethality of TMP crosslinks to λ at different levels of monoadducts or total adducts. It has been reported that wt E. coli can survive 53 to 71 TMP crosslinks, but defects in the repair genes dramatically reduce that tolerance, implying that cellular host functions can repair TMP crosslinks (Sinden and Cole, 1978).

The adduct level determinations for TMP led to the rather surprising conclusion that the eukaryotic viruses, HSV-1 and SV40 are also approximately equal in sensitivity to TMP adduct levels (Fig. 4B). SV40 is photoinactivated by one TMP crosslink per genome in the absence of multiplicity reactivation suggesting that HSV-1 is also inactivated by one crosslink per genome. This is in agreement with the reports that one TMP crosslink is lethal to Ad 2 and SV40 (Day et al., 1975; Hall, 1982).

The surprising fact shown by the photoaddition kinetics is that the two eukaryotic viruses (HSV-1 and SV40) with very different target sizes,

are equally sensitive to psoralen photoadducts. HSV-1 and SV40 photoinactivation kinetics were determined by plaque assay on similar cell lines, both of which were derived from African green monkey kidney cells. The data imply that at least in these cells, a much lower level of psoralen damage is tolerated than in E. coli cells. This sensitivity may imply that the eukaryotic repair processes are much less efficient than the prokaryotic systems for both monoadducts, presumed to be repaired by an excision pathway, and for crosslinks.

In summary we suggest that the eukaryotic viruses SV40 and HSV-1 are inactivated by one TMP crosslink per genome, whereas the prokaryotic λ virus may tolerate a higher level of crosslinks at least at high adduct loads, perhaps due to the induction of the SOS systems by the damaged viral DNA. λ is also considerably more resistant than SV40 and HSV-1 to the total level of both TMP and 5-MIP photoadducts, implying either that the replication and transcription enzymes of E. coli are more tolerant of these photoadducts or that the repair pathways in this eukaryotic system are much less efficient than those of E. coli. It should be pointed out that no report to date has shown the biological consequences of 5-MIP monoadducts and the monoadducts of TMP to be identical, and in fact in vitro data from the relative inhibition of restriction enzymes by 5-MIP and the TMP derivatives, 4'-hydroxymethyl-TMP (HMT), imply that HMT monoadducts may be considerably more harmful (Ostrander et al., 1986).

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Figure Legends

Fig. 1 TMP and 5-MIP photoinactivation kinetics of HSV-1, λ and SV40. (A) Continuous irradiation photoinactivation kinetics of HSV-1, λ and SV40. (B) Effect of reirradiation protocol on λ and SV40 photoinactivation kinetics. (o) λ, 1.0 μg/ml TMP; (□) SV40 2.0 μg/ml TMP; (Δ) HSV-1, 0.1 μg/ml TMP; (●) λ, 10 μg/ml 5-MIP; (■) SV40, 10 μg/ml 5-MIP; (▲) HSV-1, 2.0 μg/ml 5-MIP. Irradiation and reirradiation protocols are described in materials and methods. The TMP photoinactivation of λ and HSV-1 were carried out at an average incident UVA intensity of 0.9 mW/cm^2. All other irradiations were done with a light intensity of 2.5 mW/cm^2.

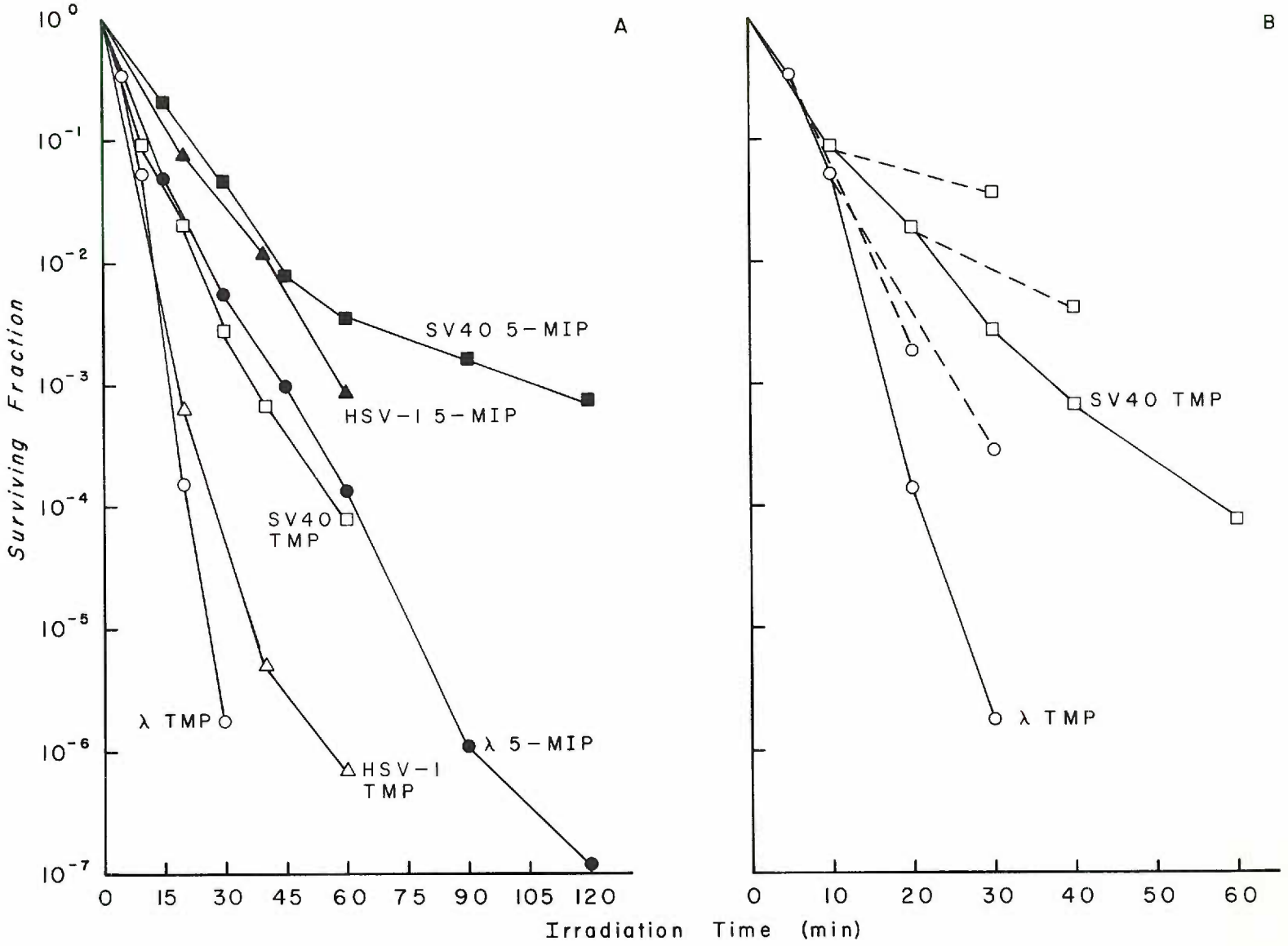
A
B
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
SV40 5-MIP
HSV-1 5-MIP
SV40 TMP
λ TMP
λ 5-MIP
HSV-1 TMP
0 15 30 45 60 75 90 105 120
Irradiation Time (min)
SV40 TMP
λ TMP
0 10 20 30 40 50 60

Fig. 2 Representative HPLC profiles of hydrolyzed DNA for TMP photoinactivated λ and SV40. TMP-modified viral DNAs were enzymatically hydrolyzed and then analyzed by HPLC to separate TMP crosslink (XL), furan-side cytidine monoadducts (dU), furan-side thymidine monoadducts (F), and pyrone-side thymidine monoadducts (Py) as described in the materials and methods. The HPLC gradient elution profiles for λ (A) and SV40 (B) represents the unmodified data directly obtained by scintillation counting for the detection of ^{3}H in the HPLC column effluent. Approximately 41 μg, 39 μg, and 15 μg λ DNA were analyzed for the 10, 10(20), and 30 min. λ irradiations, respectively. Approximately 39 μg, 36 μg, and 24 μg SV40 DNA were analyzed for the 10, 10(20), and 30 min. SV40 irradiations, respectively. Numbers in brackets indicate irradiation times after dialysis. The HPLC column effluent was fractionated into eighty 4ml samples. Radioactivity was determined by scintillation counting of 1ml per sample. Recovery of ^{3}H after HPLC analysis ranged from 65 to 73% and 70 to 77% for λ and SV40 DNA's, respectively.

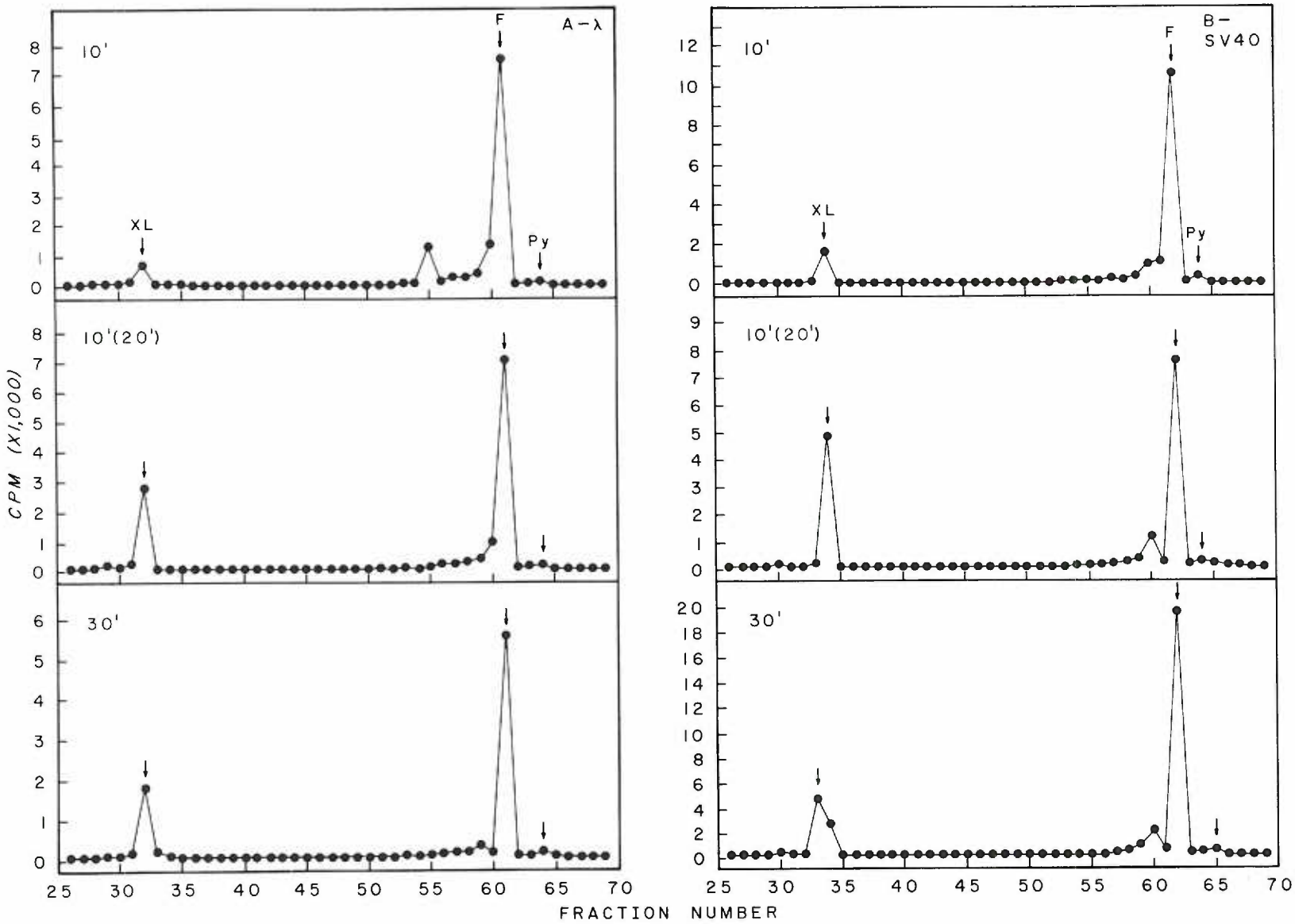
A–λ
B–
SV40
10'
10'(20')
30'
F
XL
Py
CPM (X1,000)
FRACTION NUMBER

Fig. 3 Surviving fraction of λ (A) and SV40 (B) as a function of the number of TMP crosslinks, monoadducts, and total adducts per genome. The symbols for λ represent: Crosslinks (X); monoadducts (o): total adducts (●). The symbols for SV40 represent: Crosslinks (X); monoadducts (□); total adducts (■). Irradiation and reirradiation protocols are described in the materials and methods. Solid lines represent continuous irradiation and dashed lines represent reirradiations for λ and SV40. The data are calculated from Table 1.

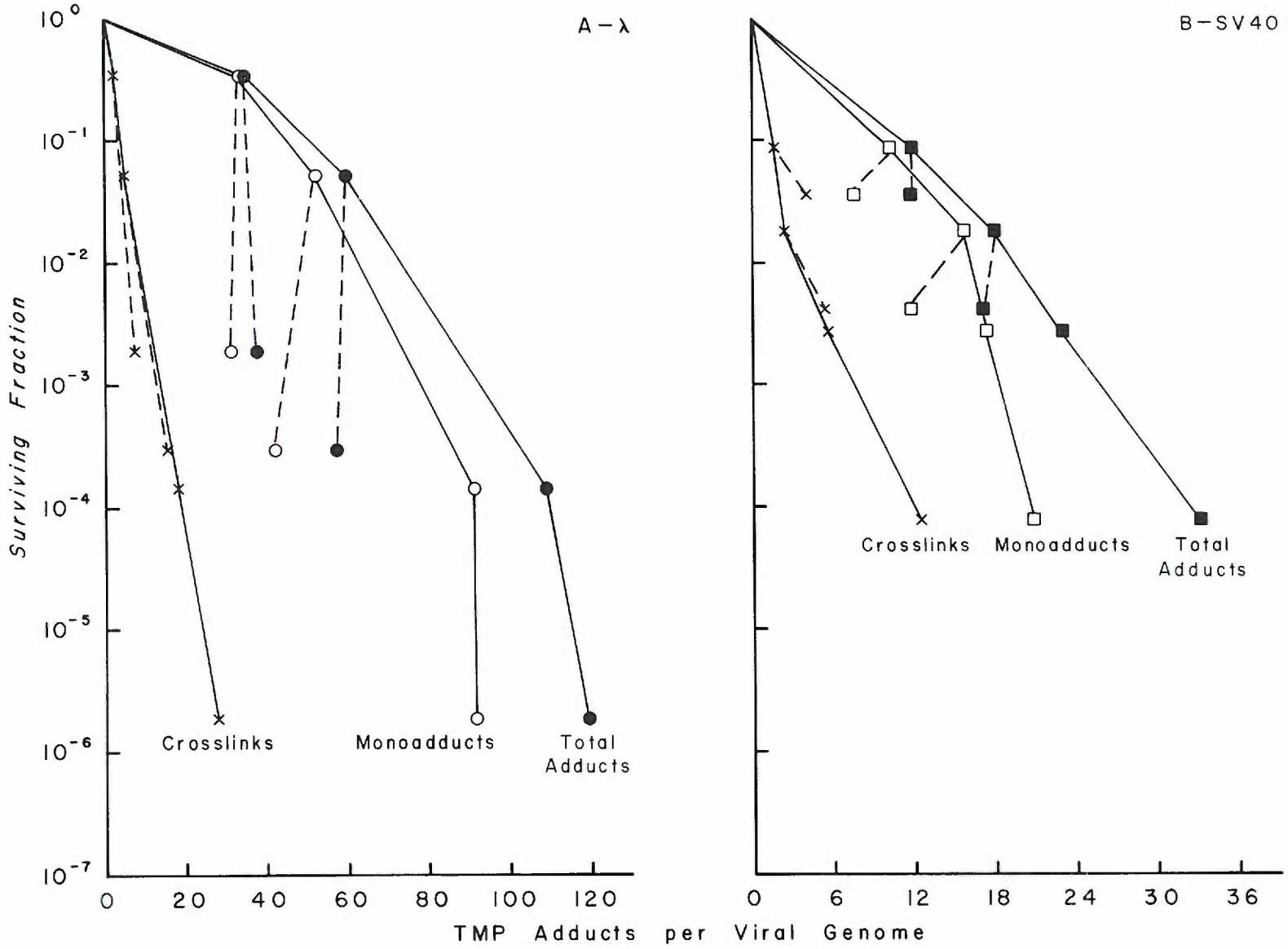
A – λ
B – SV40
Surviving Fraction
10 °
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
10 -7
Crosslinks
Monoadducts
Total Adducts
0 20 40 60 80 100 120
0 6 12 18 24 30 36
TMP Adducts per Viral Genome

Fig. 4 Surviving fraction of λ, SV40 and HSV-1 as a function of the number of TMP or 5-MIP adducts per 1000 bp (A) or genome (B). Symbols are the same as described in Fig. 1. The data are calculated from Fig. 1 and Table 1.

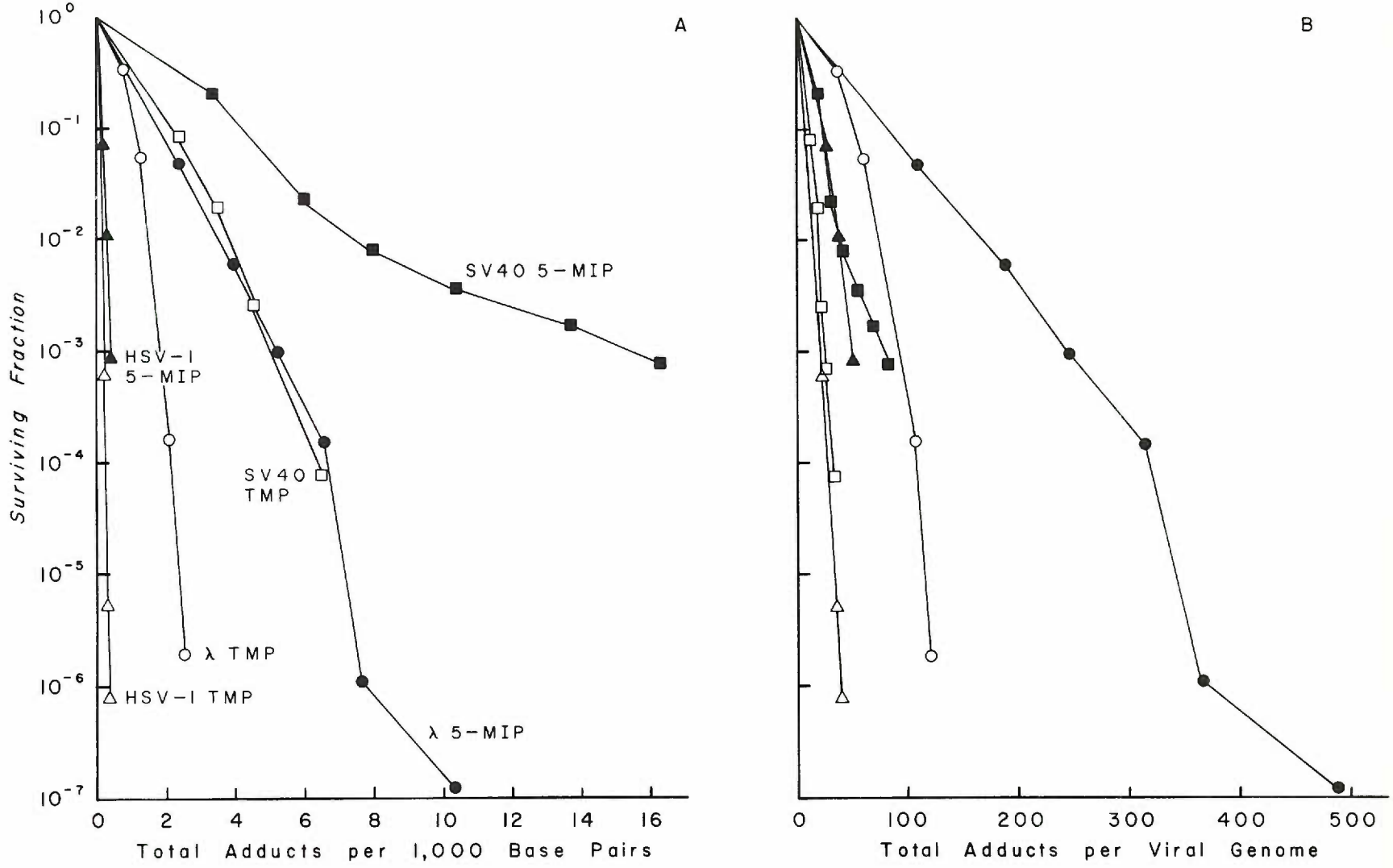
A
B
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
SV40 5-MIP
HSV-I 5-MIP
SV40 TMP
λ TMP
HSV-I TMP
λ 5-MIP
0 2 4 6 8 10 12 14 16
Total Adducts per 1,000 Base Pairs
0 100 200 300 400 500
Total Adducts per Viral Genome

Table 1

HPLC Analysis of the Different Types of Viral DNA-TMP Adducts.

Irradiation Time (min.)	Number of Adducts per Genome					
	Total Adducts	Crosslinks	Total Monoadducts	dU[a]	F[b]	Py[c]
A. Photoinactivation of λ by 1.0 μg/ml TMP and 0.9 mW/cm^2 UVA.						
Continuous Irradiation.						
0	1.6					
5	34.4	1.9	32.4	4.2	27.2	1.1
10	59.1	4.3	54.8	9.8	43.7	1.3
20	108.6	17.9	90.7	9.6	78.6	2.3
30	119.2	27.9	91.3	10.5	77.4	3.3
Reirradiation.						
5(15)[d]	37.2	6.7	30.5	2.3	26.7	1.4
10(20)	56.5	14.8	41.7	2.8	36.9	1.9
B. Photoinactivation of SV40 by 2.0 μg/ml TMP and 2.5 mW/cm^2 UVA.						
Continuous Irradiation.						
0	1.5					
10	11.8	1.6	10.2	0.4	9.4	0.4
20	18.0	2.3	15.7	1.2	14.2	0.3
30	23.1	5.7	17.4	2.7	14.0	0.7
40	24.6	7.4	17.1	2.8	13.7	0.6
60	33.2	12.4	20.8	3.1	17.0	0.6
Reirradiation.						
10(20)	11.6	4.0	7.6	1.5	5.7	0.4
20(20)	17.0	5.2	11.8	1.1	10.4	0.3

Table 1 (cont.)

a. Furan-side deoxyuridine monoadducts formed by deamination of cytidine during enzymatic hydrolysis.

b. Furan-side thymidine monoadducts.

c. Pyrone-side thymidine monoadducts.

d. Number in brackets indicates irradiation time after dialysis.

Table 2

TMP and 5-MIP Adduct Levels per HSV-1 Genome

Irradiation Time (min.)	Adducts/Genome		
	0.01μg/ml TMP[a]	0.1μg/ml TMP[b]	2.0μg/ml 5-MIP[a]
0	0.2	2	1
20	4.6	22	26
40	5.4	34	38
60	5.1	41	51

a. Irradiation was performed at an average incident light intensity of 2.5 mW/cm^2.

b. Irradiation was performed at an average incident light intensity of 0.9 mW/cm^2.

Table 3

5-MIP Adduct Levels per λ and SV40 DNA Genomes.

Irradiation Time (min.)	λ 10ug/ml 5-MIP	SV40 10μg/ml 5-MIP
0	1	2
15	112	18
30	188	31
45	249	41
60	316	54
90	369	71
120	493	84

Table 4

Experimental and Extrapolated TMP Adduct Levels per λ and SV40 Genome.

Irradiation Time (min.)	Total Crosslinks	Total Monoadducts	Surviving Fraction
A. Experimental TMP Adduct Levels per λ Genome[a].			
Continuous Irradiation.			
5	1.9	32.4	3.4×10^{-1}
10	4.3	54.8	5.2×10^{-2}
20	17.9	90.7	1.6×10^{-4}
30	27.9	91.3	1.8×10^{-6}
Reirradiation.			
5(15)	6.7	30.5	1.9×10^{-3}
10(20)	14.8	41.7	2.9×10^{-4}
B. Extrapolated TMP Adduct Levels per λ Genome During Continuous Irradiation[b].			
Crosslink Level per Genome Equal.			
11.7	6.7	61.1	1.9×10^{-2}
17.8	14.8	82.5	5.6×10^{-4}
Total Monoadduct Level per Genome Equal.			
4.8	1.8	30.5	3.6×10^{-1}
7.0	2.9	41.7	1.6×10^{-1}
Equal Surviving Fraction.			
15.7	12.1	75.5	1.9×10^{-3}
18.9	16.4	87.0	2.9×10^{-4}
C. Experimental TMP Adduct Levels per SV40 Genome.			
Continuous Irradiation.			
10	1.6	10.2	8.9×10^{-2}
20	2.3	15.7	1.9×10^{-2}
30	5.7	17.4	2.7×10^{-3}
40	7.4	17.1	6.7×10^{-4}
60	12.4	20.8	7.6×10^{-5}
Reirradiation.			
10(20)	4.0	7.6	3.6×10^{-2}
20(20)	5.2	11.8	4.0×10^{-3}
D. Extrapolated TMP Adduct Levels per SV40 Genome During Continuous Irradiation[b].			
Crosslink Level per Genome Equal.			
24.9	4.0	16.9	7.0×10^{-3}
28.5	5.2	17.1	3.4×10^{-3}
Total Monoadduct Level per Genome Equal.			
7.3	1.2	7.6	1.6×10^{-1}
13.2	1.9	11.8	5.4×10^{-2}
Equal Surviving Fraction.			
15.8	2.1	13.3	3.6×10^{-2}
27.8	4.9	19.0	4.0×10^{-3}

Table 4 (cont.)

a. Experimental values are derived from the data in Fig. 1 and Table 1.

b. Extrapolated values are taken from the curves in Fig. 3, and are obtained with respect to the indicated parameter of the reirradiated data.

Table 5

Poisson Distribution of TMP Crosslink per λ and SV40 PFU.

Irradiation Time (min.)	No. Crosslinks per PFU	Irradiation Titer(PFU/ml)[a]	m.o.i.[b]	No. Crosslinks that are lethal	Cumulative Surviving Titer[c]	m.o.i.[d]
A. Photoinactivation of λ by 1.0 μg/ml TMP and 0.9 mW/cm^2 UVA.						
Continuous Irradiation.						
0	N.D.	9.2×10^{11}	3.1×10^{-7}	N.D.	N.D.	6.1×10^{-7}
5	1.9	3.1×10^{10}	2.4×10^{-6}	0-1	3.9×10^{11}	4.9×10^{-7}
10	4.3	4.8×10^{8}	2.2×10^{-3}	0-1	6.7×10^{10}	4.4×10^{-6}
20	17.9	1.4×10^{6}	1.7×10^{-3}	4-5	3.2×10^{8}	3.5×10^{-3}
30	27.9	1.6×10^{6}	0.13	6-7	2.4×10^{6}	0.25
Reirradiation.						
5(15)	6.7	1.8×10^{9}	1.9×10^{-6}	0-1	8.7×10^{9}	3.8×10^{-6}
10(20)	14.8	2.7×10^{8}	9.4×10^{-5}	3-4	8.9×10^{8}	1.9×10^{-4}
B. Photoinactivation of SV40 by 2.0 μg/ml TMP plus 2.5 mW/cm^2 UVA.						
Continuous Irradiation.						
0	N.D.	1.2×10^{10}	4.9×10^{-6}	N.D.	N.D.	1.8×10^{-3}
10	1.6	1.1×10^{9}	1.6×10^{-5}	0	2.3×10^{9}	5.9×10^{-3}
20	2.3	2.3×10^{8}	1.4×10^{-4}	0	1.2×10^{9}	5.3×10^{-2}
30	5.7	3.3×10^{7}	1.3×10^{-3}	0	4.1×10^{7}	0.47
40	7.4	8.0×10^{6}	0.01	0-1	6.2×10^{7}	3.9
60	12.4	9.1×10^{5}	0.08	1-2	4.5×10^{6}	30
Reirradiation						
10(20)	4.0	4.3×10^{8}	9.2×10^{-5}	0-1	1.1×10^{9}	2.9×10^{-2}
20(20)	5.2	4.8×10^{7}	6.6×10^{-4}	0	6.6×10^{7}	0.22

Table 5 (cont.)

a. Irradiation titer equals the surviving viral titer after exposure of TMP plus UVA for the indicated irradiation time.

b. Determination of m.o.i. is calculated from the initial λ and SV40 titer and the viral dilutions used to determine the surviving titer for each irradiation time.

c. Poisson distribution of TMP crosslinks per PFU that yielded a surviving titer equal to or greater than the irradiation titer. Range of crosslinks indicates that the cumulative surviving titer equal to the irradiation titer is between the range of crosslink valves.

d. Determination of m.o.i. based on the total number of virus particles in the initial λ and SV40 titer.

e. N.D., not determined.

IV. Discussion and Summary

Less than two decades after Cleaver (1968) first demonstrated that XP cells were defective in the repair of UV (254 nm) induced pyrimidine dimers, a vast amount of literature has accumulated on the excision repair of DNA damage (Kraemer, 1985). This enormous amount of literature has been focused on several principal areas of investigation. One area has been to compare the cellular response or defect in DNA excision repair of UV or monofunctional chemicals to that of bifunctional chemicals. UV or monofunctional chemicals cause DNA lesions in one strand of the DNA helix, whereas the bifunctional chemicals form interstrand crosslinks.

In conjunction with these studies, extensive characterization of the different XP complementation groups has occurred. The currently known excision repair deficient XP patients comprise nine complementation groups, classified A-I. Assignment of fibroblast strains to a complementation group is based on the restoration of normal rates of UV induced unscheduled DNA synthesis by fusion of a fibroblast strain from one XP patient with the fibroblast strain from another XP patient. XP fibroblast strains are classified into two categories. Nucleotide excision repair deficient fibroblasts constitute the XP complementation groups and the nucleotide excision repair proficient fibroblasts represent the XP variant category. XP variant fibroblasts have normal rates of unscheduled DNA synthesis.

This interest in the different XP complementation groups is derived from the fact that each group has a different level of UV induced unscheduled DNA synthesis (Robbins, 1983). Normal fibroblasts have 100

percent unscheduled DNA synthesis. XP complementation groups range from a low of 0.4 to 1.3 percent for group A to a high of 25 to 50 percent unscheduled DNA synthesis for group D. It has been hypothesized that each complementation group represents a different repair gene in eukaryotic cells (Lambert and Lambert, 1985), somewhat analagous to the uvr mutants of E. coli (Sancar and Rupp, 1983). Several of the repair steps have been analyzed in XP fibroblasts including: strand incision, excision, gap-filling DNA synthesis, strand ligation, and structural transitions of chromatin at damage sites (Cleaver, 1983). Incision, however, has been the only step identified as being defective. It is possible that the products of nine and perhaps more genes are involved in the XP excision repair pathway up to the incision step (Hoeijmakers et al., 1983).

Another approach to understanding XP repair has been through enzymological investigations. Due to the possibility of low levels of these enzymes, little success has occurred. Low constitutive levels of the uvr gene products in E. coli have also hindered their isolation and purification for mechanistic studies (Grossman, 1983). An alternative approach to enzyme purification has been the microinjection of crude extracts from repair proficient human and non-complementing XP cells, inducing a temporary restoration of DNA repair in XP cells (Hoeijmakers et al., 1983). Similar experiments with crude extracts from E. coli uvrABC and yeast did not stimulate XP repair. While crude cellular extracts from XP complementation groups A, C, and G are unable to excise pyrimidine dimers from their homologous chromatin, group D extracts were proficient in dimer excision from their native chromatin (Fujiwara and Kano, 1983). However, when the loosely bound nonhistone proteins were

removed from the native chromatin of groups A, C, and G, their homologous extracts excised dimers with the same efficiency as that for purified DNA. These results suggest that XP groups A, C, and G are not deficient in excision repair.

Since the early 1980s extensive effort has been directed toward the cloning of XP DNA repair genes. This type of approach received its impetus from the report that transfection of XP group A cells with normal human DNA conferred UV resistance (Takano et al., 1982). Most investigators now agree that this successful published report should be disregarded due to a uniform failure in transfecting UV resistance into XP cells (Cleaver, 1985). It has recently been shown that transfection with human DNA to UV sensitive Chinese hamster ovary cells DNA restores UV resistance (Rubin et al., 1983; Waldren et al., 1983). It has been suggested that the unsuccessful transfection studies utilizing the XP group A cells as recipients may reflect some unique feature of the group A gene, such as its organization or size that limits easy success in these experiments (Cleaver, 1983). This lack of success is not related to XP cells being incompetent recipients, since they are readily transfected with exogenous cloned DNA (Protic-Sabljic et al., 1985).

It is evident that the knowledge of mammalian DNA repair is very limited when compared to that in prokaryotes and simple eukaryotes. Recent reports have described the molecular cloning of DNA repair genes and biochemical charactization of their gene products for E. coli uvrA (Sancar and Rupp, 1979), uvrB (Sancar et al., 1981), uvrC (Sharma et al., 1981), uvrD (Arthur et al., 1982) and S. cerevisiae RAD genes (Schild et al., 1983; Calderon et al., 1983; Prakash et al., 1983).

In order to better understand eukaryotic DNA repair, XP fibroblasts were examined for their ability to repair 5-MIP monoadducts and TMP crosslinks (Manuscript 2 and Appendix A). The combination of 5-MIP and TMP have two advantages over other DNA damaging agents. One is that it allows comparison of repair between monoadducts and crosslinks that form DNA lesions by the same cyclobutane linkages. Secondly, both 5-MIP and TMP are available as radiolabelled molecules that provide for a very precise, easy, and rapid method to determine the number of DNA lesions per genome and their rate of excision from the DNA. This later point has an advantage over the three current methods used for measuring UV induced pyrimidine dimer levels and excision (Cleaver and Gruenert, 1984). All three methods are complex procedures and indicate different rates of dimer excision for the same experiment.

At the time these studies wer initiated, little information existed on the ability of XP cells to repair furocoumarin monoadducts and crosslinks. Therefore, one normal, one XP variant, one representative of XP complementation groups A through G, and one unclassified XP fibroblast strain were characterized for their ability to repair 5-MIP monoadducts and a mixture of TMP crosslinks and monoadducts (Manuscript 2 and Appendix A). XP DNA repair has been studied by the virus-host cell reactivation of UV irradiated viruses (Day, 1974; Arase et al., 1979; Keijzer et al., 1979) which correlated well with UV induced unscheduled DNA synthesis and post-UV colony forming ability (Robbin, 1983). Each fibroblast strain was therefore tested for its ability to reactivate 5-MIP and TMP photoinactivated HSV-1 (Manuscript 2 and Appendix A). HSV-1 was chosen due to its ease of quantitation by the plaque assay method.

No difference was observed in the ability of all XP and normal human fibroblast strains, except XP2RO, to repair a mixture of TMP monoadducts and crosslinks, regardless of the UVA light intensity (Manuscript 2 and Appendix A). HSV-1 was photoinactivated with TMP at a concentration of 0.1 μg/ml and 0.01 μg/ml with UVA incident light intensities of 0.9 mW/cm^2 and 2.5 mW/cm^2, respectively. It has been suggested that a lower UVA dose will favor a lower ratio of TMP crosslinks to monoadducts than a higher UVA dose that favors a higher ratio of crosslinks to monoadducts (Averbeck, 1982). Enhanced host cell reactivation of TMP photoinactivated HSV-1 was observed in the XP2RO fibroblast strain. However, 5-MIP photoinactivation kinetics of HSV-1 did demonstrate differences in the ability of XP cells to repair monoadducts (Manuscript 2 and Appendix A). Both the XP2RO and GM1854 fibroblast strains, representing complementation groups E and B respectively, had enhanced 5-MIP host cell reactivation in relation to the normal CCD-25Sk and XP4BE variant fibroblasts. These latter two fibroblast strains have similar levels of reactivation for 5-MIP inactivated HSV-1. The GM1854 fibroblast is heterozygous for XP and would therefore have normal XP repair. XP12BE fibroblasts of complementation group A and their SV40 transformed counterpart, XP12BE-SV40, were similar in their ability to rescue 5-MIP photoinactivated HSV-1. Photoinactivation kinetics for 5-MIP showed that XP fibroblast strains XP12BE (A), XP12BE-SV40 (A), XP10BE (C), XP6BE (D), XP1PO (D), XP3YO (F), and XP2BI (G), GM3614 (unclassified) have a reduced ability to repair monoadducts (Manuscript 2 and Appendix A).

Several differences were observed between UV and 5-MIP induced host cell reactivation of human viruses. UV irradiated Ad 2 host cell

reactivation by XP4BE (Variant) fibroblasts is only 70 percent of normal (Day, 1974), while 5-MIP photoinactivated HSV-1 was similar to normal CCD-25Sk. XP2BI (G) host cell reactivation of UV irradiated SV40 DNA is 21 percent of normal (Keijzer et al., 1979) whereas XP12BE (A) host cell reactivation of UV irradiated Ad 2 is 3.5 percent of normal (Day, 1974). XP2BI (G) host cell reactivation of 5-MIP photoinactivated HSV-1 is less than that for XP12BE (A) (Manuscript 2). This difference in sensitivity to UV and 5-MIP induced lesions may reflect the differences in the two different virus systems used to determine host cell reactivation of UV irradiated virus. The finding that XP2RO (E) fibroblasts had levels of host cell reactivation equal to or greater than the normal CCD-25Sk for 5-MIP photoinactivated HSV-1 suggested that the defect in DNA repair is associated with UV induced lesions and not 5-MIP. Repair of UV induced lesions by XP2RO (E) fibroblasts when measured by unscheduled DNA synthesis and virus-host cell reactivation is > 40 and 47 percent of normal, respectively, but colony forming ability is similar to normal fibroblasts (Robbins, 1983). The observation that XP2RO (E) fibroblasts are not defective in 5-MIP or TMP repair suggests that furocoumarin adduct repair is not identical to repair of pyrimidine dimers. It has since been shown that about 80 percent of the isopsoralen adducts in XP2RO (E) fibroblasts were excised, a level similar to the 85 percent excision rate for normal fibroblasts (Cleaver and Gruenert, 1984). It has also been shown that XP2RO (E) and normal fibroblasts treated with 8-MOP had a marked increase in DNA strand breakage characteristic of DNA repair, that was not observed in XP12BE (A) and XP5BE (D) fibroblasts (Bredberg and Soderhall, 1985).

Having characterized the response of several different XP fibroblast strains for their repair of 5-MIP monoadducts, two avenues of further research are readily suggested. One is to compare the photoinactivation kinetics of HSV-1 by different furocoumarins that form only monoadducts. Possible furocoumarins include isopsoralen and its derivatives, 3-CP, and the pyridopsoralens. Photoinactivation kinetics by the different monofunctional furocoumarins should be correlated with the number of adducts per genome in order to determine whether the same number of lesions per viral genome results in equal lethality. This type of approach would eliminate differences in photoinactivation kinetics due to different levels of photoaddition for the furocoumarins. Secondly, in vitro experiments could be developed to measure the ability of XP cellular extracts to excise radiolabelled 5-MIP from SV40 minichromosomes and DNA. This type of approach would serve as a precursor for research concerning the direct isolation of proteins involved in XP DNA repair.

Although it is still controversial as to whether FA fibroblasts can repair MMC crosslinks, it is clear that their colony forming ability is reduced after treatment with crosslinking agents (Fornace et al., 1979; Fujiwara et al., 1984). Since it has only recently been shown that FA is composed of at least two complementation groups (Duckworth-Rysiecki et al., 1985) it may be that the controversial findings represent heterogeneity within FA. While it was observed that seven FA fibroblast strains were not defective in the host cell reactivation of TMP crosslinked HSV-1, regardless of cell age, one FA fibroblast strain was shown to have reduced colony forming ability (Manuscript 3). This observation may be explained by the suggestion that one crosslink per HSV-1 genome represents a lethal event in the absence of multiplicity

reactivation (Manuscript 4). Therefore, if one crosslink per HSV-1 genome cannot be repaired by a normal repair proficient cell, then no deficiency in crosslink repair would be detected in FA fibroblasts, even if they had a reduced capacity to repair crosslinks. This observation would not hold true for the XP-HSV-1 system (Manuscript 2 and Appendix A) since more than one 5-MIP monoadduct represents a lethal event and therefore would allow different numbers of monoadducts to be lethal for HSV-1. Only when FA fibroblasts were directly exposed to TMP crosslinks is their shown a reduced capacity for repair (Manuscript 3), suggesting that more than one crosslink is required for a lethal event in mammalian cells (Ben-Hur and Elkind, 1973).

Further research should be directed at the ability of FA fibroblasts to remove radiolabelled TMP crosslinks to determine if this diminished colony forming ability is due to a deficiency in the excision of TMP crosslinks. Such experiments should be done at UVA wavelengths between 320 and 390 nm where predominantly TMP crosslinks would form. It has been previously shown that the HMT photoreaction results in approximately 65% crosslinks at UVA wavelengths > 320 nm, whereas monoadducts constitute approximately 85% of the total adduct population when photoreacted with UVA wavelenghts 390 nm (Piette et al., 1985). The UVA wavelengths employed in the TMP photoinactivation of FA fibroblast strains did not select for crosslink or monoadduct formation and therefore photoinactivation is produced by a mixture of both. While 5-MIP monoadducts do not kill FA fibroblasts to the same extent as TMP adducts (Manuscript 3), no information is available on how many monoadducts represent a lethal event. Therefore, by photoreacting FA fibroblasts with TMP at UVA wavelengths > 320 nm but < 390 nm, the

majority of killing would be attributed to crosslinks. Cultures treated in parellel at the higher wavelength range should contain primarily TMP monoadducts. This type of approach would help elucidate the possibility of a defect in FA fibroblast crosslink repair.

Although the MV40 recombinant virus did not immortalize ten XP and two FA fibroblast strains, it did result in transformation (Appendix B). Due to the shorter growth cycles and higher cell densities of transformed human cells, material for biochemical studies will be more readily available and in larger quantities for the elucidation of the DNA repair pathways in eukaryotic cells.

A considerable amount of information has been acquired concerning the 5-MIP and TMP photoinactivation kinetics of the two eukaryotic viruses SV40 and HSV-1, plus the prokaryotic bacteriophage λ (Manuscript 4). Results of this research has clearly demonstrated that photoinactivation kinetics produce misleading conclusions about the sensitivities of λ, SV40, and HSV-1 to 5-MIP and TMP. Only when the number of 5-MIP and TMP adducts are quantitated per viral genome can true comparisons be made between different viruses in terms of their sensitivity to photoinactivation. The development of a reliable and reproducible SV40 plaque assay has greatly facilitated the characterization of 5-MIP and TMP photoinactivation kinetics for SV40 (Manuscript 1).

5-MIP and TMP photoinactivation kinetics suggest that λ is more sensitive than SV40 and HSV-1 to inactivation by both furocoumarins (Manuscript 4). However, this type of approach does not quantitate the number of lesions per viral genome required to produce identical surviving fractions for SV40 and λ. When the surviving fraction is

correlated with the number of adducts per viral genome, it is shown that λ is more resistant than SV40 and HSV-1 to killing by 5-MIP and TMP. This is just the opposite of the conclusion from 5-MIP and TMP photoinactivation kinetics. It is of interest that HSV-1 and SV40 which represent two extremely different target sizes are inactivated by approximately the same number of 5-MIP or TMP adducts per viral genome. This suggests that photoinactivation of SV40 and HSV-1 is more dependent upon the eukaryotic repair system than viral genome size.

Surviving fractions in relation to the number of 5-MIP and TMP adducts per viral genome however, do not compare the efficiencies of adduct addition to SV40, HSV-1, and λ (Manuscript 4). When the 5-MIP and TMP surviving fractions were correlated with the number of adducts per 1,000 bp, the effect of target size is emphasized in the absence of photoaddition rates. From this type of analysis it was shown that the sensitivities of adduct level per 1,000 bp is HSV-1 > λ > SV40. All three viruses were more sensitive to TMP than 5-MIP.

TMP reirradiation experiments where λ and SV40 were irradiated in the presence of TMP, followed by dialysis to remove unbound molecules and then irradiated a second time showed additional viral inactivation (Manuscript 4). This additional inactivation for λ and SV40 was assumed to be due to the conversion of TMP monoadducts to crosslinks. Through enzymatic hydrolysis and HPLC characterization of the TMP-nucleic acid adducts on λ and SV40 DNA for the reirradiation experiments, it was shown that a second irradiation in the absence of unbound drug resulted in the conversion of TMP furan-side monoadducts to crosslinks for both λ and SV40. Therefore, it was demonstrated that the additional λ and SV40 killing upon additional irradiation is the result of crosslink formation.

By quantitating the number of crosslinks per λ and SV40 genome an estimate of the number of crosslinks that represent a lethal event was determined (Manuscript 4). SV40 was shown to be inactivated by approximately one TMP crosslink per genome. It was suggested that one crosslink per genome also represents a lethal event for HSV-1 due to the similar TMP curves where surviving fraction is correlated with adducts per viral genome. This finding is in agreement with the previous observation that SV40 is inactivated by one TMP crosslink (Hall, 1982). λ photoinactivation, however, appears to be much less sensitive to TMP crosslinks. At low TMP adduct levels, one crosslink was lethal for λ, but as the monoadduct and crosslink levels per genome increased, it was suggested that a greater number of crosslinks were required for a lethal event. This increase in TMP crosslink number per λ genome required for a lethal event may have some correlation to the high monoadduct levels in λ. While it was shown that low levels of 5-MIP monoadducts per SV40 and HSV-1 genome were lethal, much higher levels were required for λ. Therefore, if E. coli can repair a large number of TMP monoadducts per λ genome it may be that the lethal effect of one crosslink is eliminated, presumably through repair of monoadducts, and thus more than one crosslink would be required for a lethal event. Since only one TMP crosslink and a low level of monoadducts are lethal to SV40, the chance that the deliterious effect of a crosslink would be eliminated through monoadduct repair is decreased.

An alternative hypothesis for this increase in TMP crosslink levels for λ lethality may be that as the number of lesions per genome are increased there is a greater induction of repair enzymes in E. coli, such that a higher level of crosslinks can be repaired. In conclusion, TMP

crosslinks are more lethal than monoadducts for λ and SV40. The suggestion that TMP monoadduct formation in SV40 is rate limiting (Kondoleon et al., 1982) appears not to be the case since adduct determinations have shown that the rate of SV40 photoaddition is higher than λ. The assumption that one TMP crosslink is lethal for λ (Cole, 1971) is only true at low adduct levels.

Further research should be directed toward the hypothesis that increased TMP monoadduct levels allow λ to tolerate a higher level of crosslinks as a result of increased levels of repair enzyme induction in E. coli. This can be approached by the previously discussed use of UVA irradiations at different wavelengths. Thus irradiations favoring crosslink (> 320 nm UVA) or monoadduct formation (> 390 nm UVA) could be employed to alter the ratios of crosslinks to monoadducts in conjunction with photoinactivation kinetics and HPLC characterization of the TMP-nucleic acid adducts, thereby determining the number of crosslinks required for a lethal event at high and low levels of monoadducts. In conjunction with these types of experiments it would be of interest to determine the effect different ratios of TMP crosslinks to monoadducts have on the mutation frequencies of λ and SV40.

Another avenue of research that has not been adequately investigated is the relative lethality and mutagenesis of different monofunctional furocoumarins for λ and SV40. Since no comparison of the lethal event and mutation frequency of different monofunctional furocoumarins at similar adduct levels per viral genome has been determined, it would provide direct evidence as to whether monoadducts produced by different monofunctional furocoumarins have the same biological consequences. Comparisons of the photoinactivation kinetics and induction of mutations

by different monofunctional furocoumarins in the past have not been correlated with adduct levels per genome and therefore results are biased by the different DNA binding constants for the furocoumarins tested.

The information presented in this thesis concerning the response of XP and FA fibroblasts to a monofunctional and bifunctional furocoumarin has established a basic understanding of how these repair deficient fibroblast strains respond to furocoumarin lesions. By understanding the response of XP and FA fibroblasts to furocoumarin lesions, one can now begin to investigate the different DNA repair pathways involved in monoadduct and crosslink removal. This future research will be greatly facilitated by the development of the procedures to accurately quantitate the number and type of DNA lesions through the use of radiolabelled furocoumarins and HPLC characterization of the bifunctional TMP-nucleic acid adducts. To date, only gross estimations have been made concerning the level of furocoumarin crosslinks per genome, which can now be precisely quantitated. Such a characterization of bifunctional furocoumarin adducts will provide a method of examining and comparing the DNA repair pathways of eukaryotic and prokaryotic cells in response to different adduct levels and ratios of crosslinks and monoadducts. Little or no attention has been directed toward the interaction or effect of a mixture of monoadducts and crosslinks in DNA repair processes of eukaryotic and prokaryotic systems.

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Appendix A

Xeroderma Pigmentosum and Fanconi's Anemia Cell Reactivation of 4,5',8-Trimethylpsoralen and 5-Methylisopsoralen Photoinactivation of Herpes Simplex Virus.

Abstract

Herpes simplex virus photoinactivated by two different concentrations of 4,5',8-trimethylpsoralen (TMP) results in similar photoinactivation kinetics for a given concentration when assayed on several different xeroderma pigmentosum (XP) complementation groups and one normal cell strain. One Fanconi's anemia (FA) cell strain is more proficient than the control or XP cell strains in rescue of TMP photoinactivated herpes simplex virus type 1 (HSV-1). One XP complementation group D strain and another XP cell strain that has not yet been assigned to a complementation group are both severely defective in rescue of 5-methylisopsoralen (5-MIP) photoinactivated HSV-1. XP cells and at least one FA strain are not defective in repair of TMP crosslinks but the former are deficient in 5-MIP monoadduct repair.

This study was carried out as an extension of the study described in manuscript 2 of this thesis. In summary, in manuscript 2 it was observed that there was no difference in the herpes simplex virus type 1 (HSV-1) photoinactivation kinetics by the bifunctional furocoumarin TMP when assayed on a normal fibroblast strain and on several xeroderma pigmentosum (XP) fibroblast strains from different complementation groups. However, one Fanconi's anemia (FA) strain (HG 261) and one XP strain (XP2RO, Complementation Group E) are slightly more proficient in virus rescue than the normal or other XP strains. The observation that FA HG 261 fibroblasts were not defective in repair of crosslinks conflicted with a previous report that they had reduced colony survivability when crosslinked with mitomycin C but were not defective in crosslink removal when studied by the alkaline elution method (Fornace et al., 1979). Another FA strain has been reported to have reduced survival curves when treated with mitomycin C (Fujiwara et al., 1984). This FA strain that showed reduced colony survival after exposure to mitomycin C was found to lack the first half excision step for removal of crosslinks when examined by the three different techniques of hydroxyapatite chromatography, S_1 nuclease digestion of denatured-renatured DNA, and alkaline sucrose sedimentation (Fujiwara et al., 1984). The finding that XP2RO fibroblast are not deficient in repair of TMP adducts is in contrast to their reduced levels of UV induced unscheduled DNA synthesis (Friedberg et al., 1979). It however has been found that XP2RO fibroblasts treated with 8-MOP have a marked increase in DNA strand breakage similar to normal fibroblasts but no such breaks were noted in cells from XP complementation groups A and D (Bredberg and Soderhall, 1985). It was suggested that the gene product in XP2RO fibroblasts is

required for the repair of UV (254 nm) pyrimidine dimers but not psoralen crosslinks.

Since the dose of UVA can influence the ratio of crosslinks to monoadducts for bifunctional furocoumarins (Averbeck, 1982), a higher UVA light intensity that enhances crosslink formation was utilized to study the TMP photoinactivation kinetic of HSV-1. As reported in manuscript 2, virus was exposed to 0.1 μg/ml TMP plus UVA at an average light intensity of 0.9 mW/cm^2, while Figure 1A of this appendix depicts the photoinactivation kinetics of HSV -1 exposed to 0.01 μg/ml TMP plus UVA with an average light intensity of 2.5 mW/cm^2. The concentration of TMP was decreased ten-fold in order to achieve an extended virus killing over a 60 min. period. When the furocoumarin concentration is maintained at 0.1 μg/ml and the light intensity increased to 2.5 mW/cm^2 the virus is killed to below the limits of the plaque assay by 10 min. for the normal control cells (CCD-25Sk) and does not provide reliable photoinactivation kinetics. All fibroblast strains described in manuscript 2 plus three XP strains not previously screened were tested at the higher UVA dose. The three additional XP fibroblast strains were XP12BE (Complementation Group A), XP1PO (Complementation Group D), and GM3614 (Undefined for Complementation Group, but does not belong to A,C, or D groups). In addition, these three fibroblast strains were tested by the original TMP protocol described in manuscript 2, while only XP1PO and GM3614 strains were tested for host cell reactivation of HSV-1 reacted with 5-MIP as described in manuscript 2. All irradiation experiments were done as described in the Materials and Methods of manuscript 2.

The photoinactivation kinetics of HSV-1 exposed to 0.01 μg/ml TMP plus a UVA light intensity of 2.5 mW/cm^2, which favors a higher ratio of

crosslinks to monoadducts, demonstrates that all fibroblast strains rescue photoadducted virus with similar ability (Fig. 1A) as was observed in manuscript 2. It is also observed that HG 261 FA fibroblasts are still more proficient in rescue of photoadducted virus. XP2RO cells however now appear to rescue HSV-1 at a very similar efficiency to that of the other XP and normal fibroblasts, presumably due to the higher ratio of crosslinks to monoadducts. Another difference between the TMP photoinactivation kinetics at the two different UVA doses is that at the lower UVA dose (0.9 mW/cm^2, manuscript 2, and Fig. 1B this appendix) the rate of killing of HSV-1 is greater than at 2.5 mW/cm^2 UVA (Fig. 1A). This is attributed to a higher level of total TMP adducts per virus genome at the lower UVA dose than at the higher UVA dose (discussed in manuscript 4).

Fibroblast strains XP12BE, XP1PO, and GM3614 rescue HSV-1 exposed to 0.10 μg/ml TMP plus UVA at an intensity of 0.9 mW/cm^2 with the same efficiency as the normal control cells (CCD-25Sk, Fig. 1B) and exhibit similar virus photoinactivation kinetics to all cells examined in manuscript 2. These three XP cell lines also demonstrated that repair of TMP photoadducted virus promotes virus multiplicity reactivation at the 40 min. irradiation time points.

5-MIP photoinactivation kinetics of herpes simplex virus when assayed on XP1PO and GM3614 XP fibroblast strains is depicted in Figure 1B. Rescue of virus by the two fibroblast strains indicate that they are substantially deficient in DNA repair when compared to the normal control fibroblasts. Both XP1PO (Fig. 1B) and XP6BE (manuscript 2) belong to complementation group D, but XP1PO is more repair deficient than XP6BE which has intermediate sensitivity to 5-MIP damaged HSV-1 when compared

with normal cells. It has been reported that XP6BE cells only excise 28 percent of their pyrimidine dimers while XP1PO excise over 50 percent of their isopsoralen adducts. It was concluded that complementation group D cells are more proficient in repair of isopsoralen monoadducts than of pyrimidine dimers (Cleaver et al., 1984). However, when XP1PO cells were exposed to 5μM 5-MIP there was only a 34 percent cell survival, whereas when the same cells were exposed to 10μM isopsoralen there was less than a 10 percent reduction in cell survival (Cleaver et al., 1984). With this observation that isopsoralen is less toxic than 5-MIP to XP1PO cells the finding that rescue of 5-MIP damaged HSV-1 is severely deficient may indicate that 5-MIP more closely resembles pyrimidine dimers than isopsoralen. However this interpretation should be cautiously considered since no data was given to demonstrate that each cell strain contained the same levels of 5-MIP and isopsoralen adducts per cell genome nor that similar killing levels by isopsoralen and UV (254 nm) were achieved. The 5-MIP photoinactivation of HSV-1 in the experiment described in this appendix has the advantage of exposing a homogeneous population of virus to the same concentration of furocoumarin. Irradiation by normalizing the viral titers for each irradiation time point to the 0 min. time, the difference in plaquing efficiency for each fibroblast strain is eliminated. Thus the only variability of host cell reactivation should be due to their proficiency or deficiency in repair of 5-MIP monoadducts (Fig. 1B).

Irradiation of normal control fibroblasts and XP cells GM3614 and SB65 (Complementation group A) with UV (254 nm) or 5-MIP plus UVA demonstrated that both methods of inducing DNA lesions produced a large reduction in high molecular weight DNA in normal control fibroblasts

whereas UV irradiated SB65 and 5-MIP treated GM3614 fibroblasts exhibited a very small reduction in high molecular weight DNA (Cleaver and Gruenert, 1984). When similar levels of DNA lesions produced by UV (254 nm) or 5-MIP were compared in normal control cells by measuring the maximum frequency of single strand breaks, indicative of DNA repair, UV irradiation produced almost twice as many breaks as 5-methylisopsoralen. It has also been demonstrated that XP cells from complementation groups A,C, and D have similar relative repair deficiencies for UV (254 nm) and 5-MIP lesions, all of which are very low compared to normal control cells (Cleaver and Gruenert, 1984). The unclassified GM3614 fibroblasts have the lowest level of detectable DNA repair of UV induced lesions and did not have any detectable repair of 5-MIP induced lesions (Cleaver et al., 1984a). Although these repair studies have not been correlated with colony survival it does indicate that the inability of XP GM3614 fibroblasts to repair 5-MIP DNA lesions correlates well with their low levels of HSV-1 rescue.

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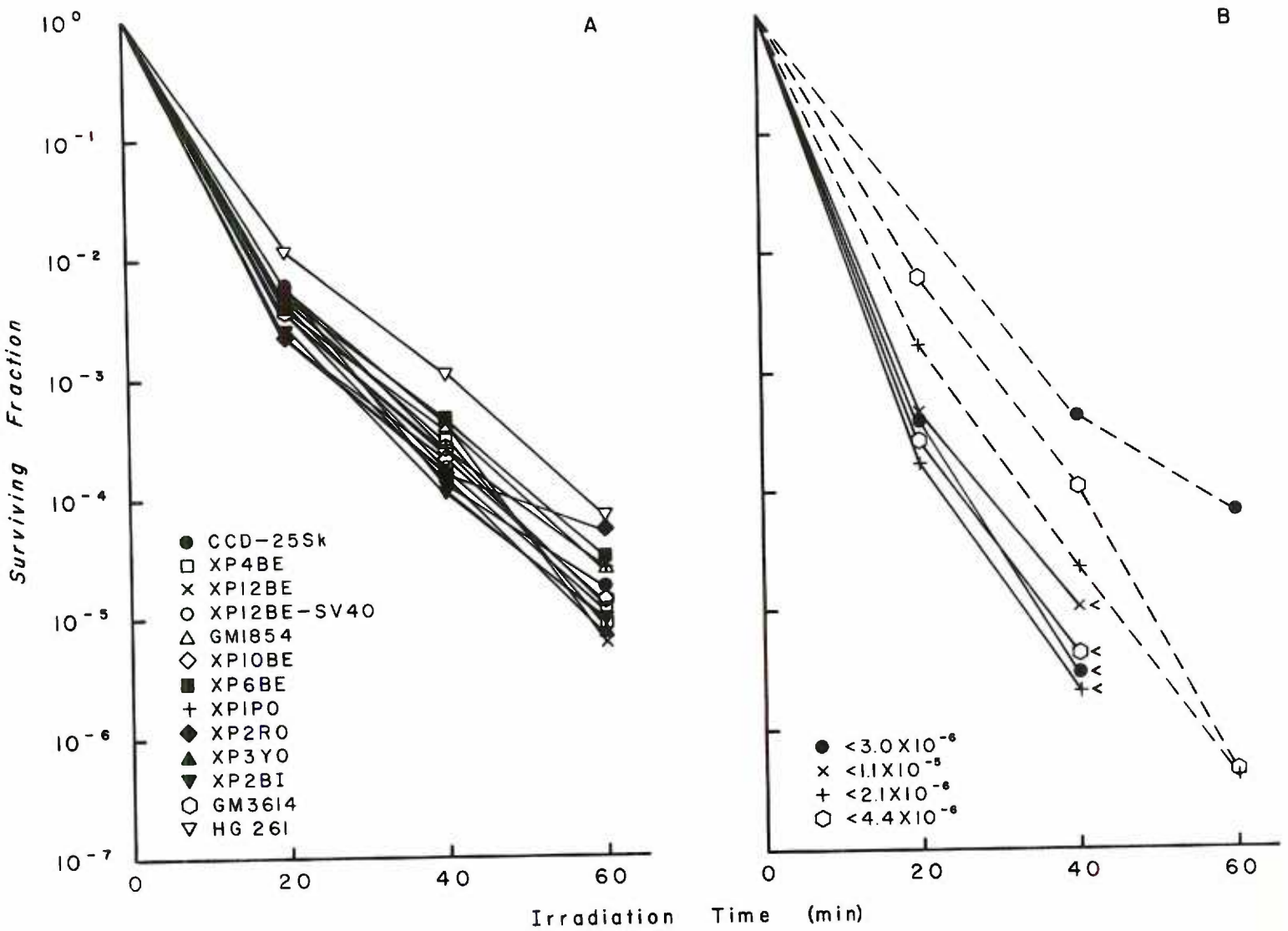
FIGURE LEGEND

Figure 1. Comparison of TMP and 5-MIP photoinactivation kinetics of HSV-1 assayed on normal human control, xeroderma pigmentosum, and Fanconi's anemia fibroblasts. Virus suspensions were exposed to 0.01 μg/ml TMP plus UVA for varying periods of time up to 60 min (A). Virus suspensions were exposed to 0.1 μg/ml TMP (——) or 2.0 μg/ml 5-MIP (----) plus UVA for varying periods of time up to 60 min (B). HSV-1 was quantitated by the plaque assay method for each time point using the following fibroblast strains: human control (C, ●), XP4BE variant (V,□), XP12BE (A,X), XP12BE-SV40 (A-SV40,0), GM1854 (B-H,Δ), XP10BE (C,◇), XP6BE (D,■), XP1PO (D,+), XP2RO (E,◆), XP3YO (F,▲), XP2BI (G,▼), GM3614 (Unclassified,○), and HG 261 FA (FA,∇). Photoinactivation kinetic curves for 0.1 μg/ml TMP (1B) for all fibroblast strains tested are depected only to 40 min. irradiation time. At later times, multiplicity reactivation hindered virus titration. Normalized time points indicated as less than (<) for CCD-25Sk, XP12BE, XP1PO, and GM3614 fibroblasts represent the limit of detection by the plaque assay method due to multiplicity reactivation. Average light intensity was 0.9 mW/cm^2 for 0.1 μg/ml TMP and 2.5 mW/cm^2 for 5-MIP and 0.01 μg/ml TMP.


A
B
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
0
20
40
60
Irradiation Time (min)
CCD-25Sk
XP4BE
XP12BE
XP12BE-SV40
GM1854
XP10BE
XP6BE
XP1P0
XP2R0
XP3Y0
XP2BI
GM3614
HG 261
$<3.0\times10^{-6}$
$<1.1\times10^{-5}$
$<2.1\times10^{-6}$
$<4.4\times10^{-6}$

Appendix B

Efficient Transformation of Normal, Xeroderma Pigmentosum, and Fanconi's Anemia Human Fibroblasts by Moloney Murine Leukemia Virus-Simian Virus 40 Recombinants (MV40).

Abstract

A minimal murine retroviral vector containing the long terminal repeats (LTRs) and cis-acting signals required for replication and packaging of infectious RNA was utilized to transform primary fibroblasts of normal and repair deficient phenotypes. The early region of simian virus 40 (SV40) was inserted between the LTRs and the resulting hybrid virus, termed MV40, was packaged by a human xenotropic murine leukemia virus. MV40 was able to transform several human primary fibroblast strains whether it contained both the SV40 large T and small t tumor antigen genes or the large T antigen alone. Of the 2 normal, 10 xeroderma pigmentosum (XP), and 7 Fanconi's anemia (FA) fibroblast strains tested for the ability to be transformed by the xenotropic MV40, only one strain, XP10BE (Complementation Group C) was not transformed. When this same MV40 virus was in the presence of an ecotropic Moloney murine leukemia helper virus it no longer transformed primary human fibroblasts. Two normal human, 10 XP, and 2 FA fibroblast strains transformed with xenotropic MV40 that encoded both large T and small t antigens were cultured until cell senescence occurred. Although the growth properties of the transformed cells were altered, none of the strains were immortalized.

Primary human fibroblast strains associated with repair deficient disorders have provided a large amount of information concerning the mechanisms of DNA repair in eukaryotes. However, the limited life span of human fibroblasts in culture (Hayflick, 1965) does not provide continuous availability of a given cell strain for extensive research and characterization. In addition, primary human fibroblasts generally have higher nutritional requirements, longer generation times, and lower cell densities at confluence (Thielmann et at., 1983). Thus, the ability to readily transform human primary fibroblasts would greatly ease the in vitro manipulation of these cells due to the increase in cell doubling times and saturation densities (Sack, 1981). Immortalization of these fibroblasts would also ensure an unlimited source of a genetically defined human cell line for extensive biochemical characterization by many researchers.

Several reports have described the transformation of xeroderma pigmentosum (XP) and Fanconi's anemia (FA) fibroblasts (Todaro et al., 1966; Aaronson and Todaro, 1968; Aaronson and Lytle, 1970; Parrington et al., 1971; Lomax et al., 1978) but only two reports have described the immortalization of XP fibroblasts with simian virus 40 (SV40) (Takebe et al., 1974; Yagi and Takebe, 1983). While human cells are semi-permissive for SV40 replication they only produce about 1 percent of the level produced by permissive African green monkey kidney cells (Ozer et al., 1981). Approximately 1 to 2 percent of the human cell population supports viral DNA replication (Zouzias et al., 1980). Levels of viral DNA replication in this subpopulation of cells is equivalent to wild type infection. Transformation and viral DNA integration occurs in a small proportion of the total cell population at a very low efficiency (Zouzias

et al., 1980). Transformation frequencies are increased by transfection with SV40 origin defective mutants (Small et al., 1982), but are still low presumably because of the inefficiency of DNA transfection.

A Moloney murine leukemia retrovirus-simian virus 40 recombinant virus (MV40) was utilized to eliminate these problems of transformation by SV40. This recombinant virus consists of the Moloney murine leukemia virus long terminal repeats and cis acting signals required for the production of an infectious RNA virus, into which was inserted the early region of SV40 which encodes the structural genes for large T antigen and small t antigen, but has eliminated the early SV40 promoter and polyadenylation sequences (Kriegler et al., 1984). Transformation with this recombinant virus, MV40, has two advantages over transformation with SV40. When the SV40 DNA linearly integrates into the genome of transformed mouse cells (Sambrook et al., 1968), the cellular DNA contains the intact early region of SV40 flanked by the late region which has been randomly broken (Botchan et al., 1976; Ketner and Kelly, 1976). Tandem duplications may be present that can include up to a complete duplication of the viral genome. The structure of the integrated SV40 DNA and/or its location in the host DNA is different in each cell line tested (Ketner and Kelly, 1976). Sequences of recombinant joints of cellular and viral DNA do not have a common integration pattern (Gutai and Nathans, 1978). Thus, it appears that integration of SV40 DNA into mouse (Ketner and Kelly, 1976), rat (Botchan et al., 1976), and human (Sack and Obie, 1981) transformed cells is a random process. It is also known that the early genes of SV40 are not sufficiently expressed to maintain morphological transformation when the viral DNA is integrated into certain positions of the cellular DNA (Kriegler and Botchan, 1983;

Scholer and Gruss, 1984), presumably because the enhancer is inactive. Although over 65% of the SV40 infected cells transiently acquire the transformed phenotype after infection (Stoker, 1968), the majority of these cells eventually lose both the transformed phenotype and viral genome (Stoker, 1968; Fluck and Benjamin, 1979). Since retrovirus replication requires a stable integration of the viral DNA, it assures stable integration (Temin, 1980; Varmus, 1982). Expression of large T and small t antigens are also controlled by the retrovirus enhancer in MV40, thus eliminating any transient or low level expression of the oncogenes that are thought to result from negative cellular control of the SV40 enhancer.

Transformation and immortalization experiments were done utilizing two normal, ten XP, and two FA fibroblast strains (Table 1). Uninfected fibroblast strains were plated at 1 x 10^5 cells per 100mm petri dish prior to infection with supernatants from the EVXπ2-xenotropic rat cell line. Development of the EVXπ2 cell line and culture conditions were previously described (Kriegler et al., 1984).

Briefly the EVXπ2 cells were developed by transforming a Rat-2 fibroblast line with MV40. The EVXπ2 cell line produced approximately equal quantities of three different MV40 viruses: a large T antigen virus, a small t antigen virus, and a virus encoding both antigens. The EVXπ2-xenotropic cells were developed by infecting EVXπ2 cells with a human xenotropic murine leukemia virus to confer human cell specificity on MV40. Tissue culture medium was harvested 24 hours after plating EVXπ2-xenotropic cells at 1 x 10^6 cells per 100mm petri dish and filtered through a 0.45 micron filter. Human fibroblast strains were pretreated with DMEM containing 4 μg/ml polybrene for 30 min. After aspiration of

the DMEM-polybrene each fibroblast strain was inoculated with the filtered medium and incubated for 24 hours, followed by removal and replacement with appropriate growth medium as described in manuscripts 1 and 3. Human fibroblast control plates were treated in the same manner but inoculated with filtered Rat-2 growth medium (DMEM). An additional control consisted of Rat-2 cells plated at 5×10^4 cells per 100mm petri dish and treated in the same fashion as MV40 infected and control human fibroblasts as a check for the presence of transforming MV40.

Human fibroblast strains were incubated for 21 to 28 days to allow the development of transformed foci. Five foci were cloned from each human fibroblast transformation plate and the remaining cells ("mixed transformed") were passaged. Control plates of each fibroblast strain were passaged on the same date as the five transformed clones were started. One control culture, five MV40 clones, and the one culture of mixed transformed and untransformed cells for each human fibroblast strain were passaged at confluence until cell senescence was reached. No transformated foci were detected in the Rat-2 or human fibroblast controls.

Two normal and two XP fibroblast strains (Table 2) were tested for the ability to be transformed by MV40 encoding both large T and small t or large T alone. In addition the efficiency of MV40 encapsidated by an ecotropic murine leukemia virus envelope was compared to that of the same virus produced with a xenotropic helper virus. A Rat-2 cell line (Rat-2-Xenotropic) producing human xenotropic murine leukemia virus only was also tested for any transforming activity. The MV40 clone producing only large T antigen was developed by transforming Rat-2-Xenotropic fibroblasts with media from MV40-4 fibroblasts producing MV40 that

contains only the large T antigen gene. MV40-4 fibroblasts were derived from NIH 3T3 fibroblasts originally transformed by MV40 from EVXπ2 fibroblasts and have been previously described (Kriegler et al., 1984). Loss of the ability to express small t antigen is presumed to have been the result of packaging a spliced large T mRNA. All experiments were done as previously described but were allowed to incubate for 32 days prior to foci enumeration.

All fibroblast strains except XP10BE were transformed by MV40-Xenotropic (produced by EVXπ2-Xenotropic cells) encoding both SV40 large T and small t SV40 antigens (Table 1). Five suspect foci were cloned from the XP10BE transformation plate and cultured. Neither the XP10BE control or "transformed" fibroblasts were positive for nuclear SV40 large T fluorescent antibody straining. The control culture of XP10BE had a similar generation number as the "transformant" cultures upon cell senescence (Table 1). Five other attempts to transform XP10BE fibroblasts were unsuccessful. It is not known why XP10BE cells were not transformed by the xenotropic MV40 or MV40-4 since they have been demonstrated to support synthesis of SV40 viral DNA (Ozer et al., 1981). It may be that they do not have a cell surface receptor for the virus glycoprotein that allows virus attachment to the cell surface. MV40-Xenotropic transformed CCD-25Sk, MRC-5, and XP6BE fibroblast strains had generation numbers increased by 17, 18, and 24, respectively, over their control cultures (Table 1). The remaining strains had similar generation numbers at the time of cell senescence for both controls and transformants. None of the 14 transformed fibroblast strains resulted in an immortalized cell line and all eventually went through cellular senescence.

MV40 that encodes both the large T and the small t antigen gene or the large T antigen gene alone will transform normal and XP fibroblasts, with the exception of XP10BE, when packaged in a xenotropic, but not an ecotropic murine leukemia virus coat (Table 2). The predominantly higher number of foci per plate in Table 2 than in the previously described experiment (Table 1) may be due to the extended incubation period, that may allow secondary infection. The result that T antigen only is capable of transforming human fibroblasts is in agreement with previous observations that small t does not confer a selective advantage for transformation (Kriegler et al., 1984; Rubin et al., 1982). This extended incubation period was used since MV40-4-Xenotropic transformation of human fibroblasts produces foci that develope at a slower rate than foci produced by MV40-Xenotropic. Other observations in this experiment include the fact that both ecotropic and xenotropic MV40 or MV40-4 transforms Rat-2 cells, and that the MRC-5 fibroblast strain transformed with MV40-Xenotropic produces higher titers of transforming virus than the EVXπ2-Xenotropic rat cell line used to transform the MRC-5 strain (data not shown).

While SV40 transformed mouse and rat cells do not undergo cell crisis, transformed human fibroblasts do undergo such a crisis period (Sack, 1981). Cellular changes that occur during crisis do not parallel abortive transformation since the transformed phenotype is maintained in cells in crisis (Gotoh et al., 1979; Sack and Obie, 1981). It is therefore assumed that a small subpopulation of transformed cells differ from the majority of cells in their ability to pass through crisis (Sack, 1981). It is not known whether this minor population represents integration of the SV40 genome into a new cellular location or selective

growth of a mutant of the original transformed population. Cell strains that do survive crisis after SV40 transformation, do so with a low frequency of 10^{-6} (Sack, 1981), may or may not produce infectious virus, and are subsequently immortalization (Gimbrone et al., 1976; Gaffney et al., 1970). It has been reported however, that normal human cells can be immortalized by SV40 without having the typical crisis and either produce infectious virus or become virus negative (Rabson et al., 1962; Santoli et al., 1975). Interestingly, it has been observed that in one immortalized human cell line which did not produce infectious virus, only 25 to 40 percent of the cell population produced large T antigen (Santoli et al., 1975).

A single SV40 particle is sufficient to initiate transformation, since the number of transformed cells is directly proportional to the multiplicity of infection. However, one transforming event usually requires 10^2 to 10^4 infectious SV40 particles and is therefore relatively inefficient (Stoker and Abel, 1963; Todaro and Green, 1966; MacPherson and Montagnier, 1964). This suggests that the crisis period followed by immortalization observed in human fibroblasts represents a mutation that occurs within the cell, either due to rearrangement of the SV40 genome within the cellular DNA or a cellular event that is mediated by SV40 but not by a direct viral DNA-cellular DNA interaction. Due to the stable integration of MV40, a mutation caused by SV40 DNA rearrangement would preclude such a mutational event leading to immortalization. Another possibility is that MV40 produces an infectious virus that may be partially cytotoxic to human cells, although the cells did not show any cytopathic effect. In conclusion, the fact that XP and FA patients have a high incidence of cancer does not predispose their fibroblasts to

transformation by the SV40 oncogenes relative to repair proficient human fibroblasts.

TABLE 1

TRANSFORMATION AND IMMORTALIZATION OF NORMAL, XERODERMA PIGMENTOSUM, AND FANCONI'S ANEMIA HUMAN FIBROBLASTS WITH MV40 ENCODING SV40 LARGE T AND LITTLE t GENES.

FIBROBLAST STRAIN	GENOTYPE[1]	GENERATION NUMBER WHEN INFECTED	GENERATION NUMBER AT CELL SENESCENCE CONTROLS	AVERAGE GENERATION NUMBER AT CELL SENESCENCE (RANGE) TRANSFORMANTS	APPROXIMATE NUMBER OF FOCI PER PLATE
MRC-5	Normal	36	66	84 ± 9[2] (77-101)	20-30
CCD-25Sk	Normal	35	43	60 ± 4 (54-64)	10-20
XP4BE	XP(V)	35	57	58 ± 10 (45-73)	5-8
XP12BE	XP(A)	32	49	59 ± 10 (49-72)	3-7
GM1854	XP(B-H)	33	39	41 ± 4 (39-49)	10-15
XP10BE	XP(C)	12	32	30 ± 2 (29-33)	None
XP6BE	XP(D)	18	30	54 ± 5 (45-61)	10-15
XP1PO	XP(D)	33	46	45 ± 3 (42-49)	50-70
XP2RO	XP(E)	38	46	49 ± 3 (45-53)	40-50
XP3YO	XP(F)	33	39	40 ± 2 (39-42)	15-20
XP2BI	XP(G)	48	57	60 ± 3 (59-64)	10-15
GM3614	XP(U)	33	44	50 ± 7 (41-60)	10-15
HG 261	FA	42	62	62 ± 4 (58-68)	15-25
GM0368	FA	21	36	42 ± 3 (38-47)	15-20

Table 1 (cont.)

1. XP=Xeroderma pigmentosum, letter in brackets indicates complementation group; V=Variant; H=heterozygous; FA=Fanconi's anemia.

2. Standard Deviation.

Table 2

TRANSFORMATION OF NORMAL AND XERODERMA PIGMENTOSUM HUMAN FIBROBLASTS WITH MV40, MV40-4, AND HUMAN XENOTROPIC MURINE LEUKEMIA VIRUS.

FIBROBLAST STRAIN	XENOTROPIC[2] MV40 (T+t)[1]	ECOTROPIC[2] MV40 (T+t)	XENOTROPIC MV40-4 (T)	ECOTROPIC MV40-4 (T)	XENOTROPIC MURINE LEUKEMIA VIRUS
MRC-5	100-150[3]	0	50-60	0	0
CCD-25Sk	5-10	0	5-10	0	0
XP10BE	0	0	0	0	0
XP6BE	40-45	0	20-25	0	0
RAT-2 CELL	50-60	20-25	40-50	50-60	0

1. Letters in brackets indicate whether MV40 encodes for both SV40 large T antigen and small t antigen genes or SV40 large T antigen gene only.

2. Indicates the tropism of the replicative competent helper MuLV.

3. Approximate number of foci per plate.

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