CHARACTERIZATION OF AN ENDOGENOUS ADP-RIBOSYL-TRANSFERASE ACTIVITY FROM PyBHK AND BEEF LIVER CELLS

by

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To my Mother and Father

for Their Gifts

of

Truth and Life

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INTRODUCTION AND STATEMENT OF THE PROBLEM

Gene expression is the process of information transfer from gene to functional protein. Such a process is regulated throughout its entire sequence, from the selection of those DNA sequences that are to be transcribed to the rate at which the final gene product is produced. It is essential for the various regulatory steps to be coordinated so that the cell can respond as a unit to changes in its environment.

To facilitate the cell to function in a coordinated manner, posttranslational modifications are used to control the activity of the enzymes mediating the various steps of gene expression. These modifications are seen at all levels of cellular organization, from DNA associated proteins, to RNA associated proteins and finally, to free cytoplasmic proteins.

ADP-ribosylation reactions are emerging as an important class of posttranslational modifications that are correlated with structural and functional changes in a wide variety of protein substrates. Although it is generally accepted that certain cellular processes are associated with ADP-ribosylation reactions, none of these reactions have been directly implicated with a specific functional role. On the other hand, bacterial ADP-ribosylating enzymes cause well characterized functional changes in eucaryotic cells. Apart from their significance in bacterial pathogenesis, these enzymes have led to speculations concerning the possible existence of analogous enzymes in uninfected eucaryotic cells.

In the case of Elongation Factor-2, the ADP-ribose acceptor of the diphtheria toxin catalyzed reaction, there are compelling reasons to believe that an endogenous EF-2 ADP-ribosylating reaction can occur. The

unique amino acid, diphthamide, serves as the site of ADP-ribosylation in EF-2. Since this amino acid has been conserved in evolution, it is likely that it serves an important function in the survival of the cell. If this is so, it would seem very unlikely that this site has been conserved only for the convenience of bacterial intoxication. Furthermore, the toxin-producing bacterial parasite has a very restricted host range. It is difficult to see the advantage conferred to the parasite by the ability of diphtheria toxin to ADP-ribosylate EF-2 from a wide variety of organisms. The most plausible explanation for the appearance of this amino acid in the native form of EF-2 is that modification of EF-2 by ADP-ribosylation within physiological limits serves to benefit the cell. The questions this thesis will address are:

1. Can an endogenous EF-2 ADP-ribosylating enzyme be found in a eucaryotic cell line?

2. If such an enzyme exists, what is its mechanism of action?

3. What is the nature of the modified product of this reaction?

4. Can this enzyme be found in other cell lines?

5. Can a model be defined for the role of an endogenous EF-2 ADP-ribosylating reaction?

REVIEW OF THE LITERATURE

Gene expression, resulting in the synthesis of a protein, can theoretically be regulated at many different levels. These include: (1) the preferential amplification of the gene; (2) transcription of the gene into RNA; (3) processing of the RNA transcript to alter its physical properties in terms of its translatability and stability; and (4) processing the protein to its final form.

Regulation of Protein Synthesis By Gene Amplification

Possible mechanisms of gene amplification include gene duplication and subsequent crossing over. The general feature of gene amplifications studied so far is that they arise to meet the requirement for an increase in a specific gene product in organisms or somatic cells where evolution has not provided for a regulatory process that increases gene transcription sufficiently. The requirement can be related to developmental needs, the presence of a growth inhibitory agent, or the existence of a catalytically defective enzyme (1). In most instances, the increased expression of a specific gene is related to an increase in transcription. In some cases, the increased amount of a protein is due to multiple copies of a particular gene. Examples of gene amplification include genes which are amplified in response to developmental regulation (2,3), and genes that are amplified in the presence of various enzyme inhibitors (4). However, the molecular mechanism of gene amplification is not yet known (5).

Regulation of Protein Synthesis at the Level of Transcription

In a mammalian cell, a gene that is selected for transcription is selected from enough DNA to code for one million genes (6). It is believed that DNA which is actively transcribed has a chromatin structure different from that of inactive chromatin. The packaging of DNA sequences into non-random characteristic chromatin structures has been reported for the histone genes of D. melanogaster (7), the heat shock genes of D. melanogaster (8), and the 5S RNA genes of Xenopus (9), suggesting that distinct chromatin arrangements must be a widespread phenomenon. It has been convincingly demonstrated that there is a dramatic increase in the relative DNAse I sensitivity of genes that are either about to be transcribed or are being transcribed (10, 11, 12, 13). A similar nuclease sensitivity is found at the 5' end of the preproinsulin II gene in tissues in which it is expressed but not in tissues that do not synthesize insulin (14). These exposed regions may reflect a preferential accessibility of the chromatin to regulatory molecules (14). It is likely that the binding of certain kinds of proteins to the DNA or posttranslational modifications of histones or both are the reasons for the hypersensitivity of DNA. For example, the level of acetylated histone H4 has been directly correlated with the DNAse I susceptibility of chromatin from trout testis (15).

1. The Transcription Process

Actively transcribed genes are found in nucleosomal structures (16, 17). The nucleosomes present in transcriptionally active chromatin are

apparently structurally different from those present in untranscribed regions as shown by differences in DNAse I sensitivity (10,11,18,19). However, the nucleosome remains intact during in vitro transcriptional initiation as evidenced by the presence of core histones in the transcription complex (20). Transcription by E. coli RNA polymerase can occur through regions of the DNA that is organized into nucleosomes (21). After transcription there is no loss of nucleosomes from the chromatin complex (21). The nucleosomes become displaced during in vitro transcription but afterwards reform on the same DNA (22).

On the other hand, regulatory proteins can recognize and bind to specific DNA sequences on nucleosome organized DNA. For instance, the lac repressor can recognize and bind specifically to the lac operator contained in restriction fragments that have been complexed with the four core histones to form artificial nucleosomes (23). Since the lac repressor can recognize and bind to nucleosome organized DNA without displacing the histone octamer, the DNA must be wound onto the core in such a way that the operator sequence is exposed. This indicates that DNA does not associate randomly with nucleosomes but adopts particular configurations such that certain sequences are exposed on the outside of the structure.

A. Mammalian RNA Polymerases.

There are three different polymerase enzymes in the nuclei of eucaryotic cells (24). These are referred to as polymerases I, II and III, although they are sometimes referred to as A, B, and C, respectively. The classification of the RNA polymerases is based on an

unequivocal criterion: the relative sensitivies to amanitin. However, classification based on order of elution from DEAE sephadex is also used (24).

The type II enzyme exists in three forms, but it is not yet known how these forms are interrelated. All three forms of this enzyme are nucleoplasmic and are responsible for the synthesis of heterogeneous nuclear RNA (hnRNA). This RNA is processed to give messenger RNA (mRNA) and some small nuclear RNA species (snRNAs).

B. Nucleotide Sequences Important for Structural Gene Transcription by RNA Polymerase II.

Studies on eucaryotic genes have concentrated on the sequences preceeding the start of transcription. Several common features are apparent. There is a sequence very similar to the "Pribnow" box sequence of bacterial mRNA coding genes approximately 25-30 bp upstream from the mRNA start sites of all as yet sequenced eucaryotic mRNA-coding genes transcribed by RNA polymerase II (25). This is usually referred to as the 'TATA' or Hogness Box. Using *in vitro* transcription systems where gene fragments with substantial amounts of 5' flanking sequence were cloned and then mutated *in vitro* by deletion of segments of sequence, the TATA box sequence has been found to ensure an accurate starting location for the initiation of transcription (26,27). These experiments have also established that sequences upstream of the TATA box are required for transcription (26,28). When these sequences are deleted, no transcripts are initiated, even if the TATA box is left intact. The nucleotide sequence as far as 500 base pairs upstream from the start of the sea urchin histone H2A gene is essential for its *in vivo* transcription (29).

This suggests a model for the binding of RNA polymerase II to active genes. In this model, the DNA immediately upstream from the actual start of transcription is organized into nucleosomes in a very precise way such that a particular conformation, recognizable by RNA polymerase, is created. This conformation exposes specific nucleotide sequences that facilitate polymerase binding and its location of the correct site for the initiation of transcription. The introduction of any protein to, or the modification of proteins already existing on these critical nucleosomes may alter the efficiency at which transcription is initiated and regulate the expression of the gene (30).

C. Other RNA Polymerases Use Different Promoter Sequences

RNA polymerases I and III transcribe RNA molecules for the ribosomal RNA components and the small nuclear RNAs, respectively. None of these genes are preceeded by the TATA box sequence found near the start of structural genes (30). In the case of the 5S ribosomal RNA gene of Xenopus, a region within the gene directs the initiation of 5S rRNA synthesis (31). This internal regulatory sequence stretches from +50 to +83 base pairs. When this sequence is intact, transcription starts approximately 50 base pairs upstream, regardless of the upstream sequence. Deleting the entire 5' flanking region of the 5S rRNA gene of Xenopus borealis gave rise to deletion mutants that were still capable of supporting 5S rRNA transcription in an oocyte nuclear extract (31). However, accurate and highly efficient transcription only occurs when the 26 base pairs immediately before the start of transcription are intact.

A specific transcription factor has been found that binds tightly to this regulatory sequence (32) which has been shown to be required for initiation of transcription (31,33). In the presence of this protein,

RNA polymerase III rapidly and accurately transcribes the gene (34). This protein also binds with 5S rRNA to form a ribonucleoprotein complex (34). Presumably, when all the transcription factor is complexed with RNA in the cytoplasm, there is none left for activation of the gene, and transcription stops. Thus, the transcription of the gene is controlled by the concentration of free transcription factor protein. 5S rRNA prevents further transcription when its concentration reaches a level that complexes all the protein.

2. The Regulation of Transcription

A. Activators of RNA polymerase.

The development of *in vitro* transcription assays established that proteins other than the RNA polymerases are required for accurate *in vitro* transcription of purified DNA templates (35,36). Both RNA polymerase II and RNA polymerase III require a specific set of factors for transcription. In general, these proteins are currently being characterized.

B. Histone Modifications and Changes in Template Availability.

Histones are conformationally unstable proteins because a high degree of covalent modification of their amino acid residues is possible. The histones have been shown to undergo phosphorylation, acetylation, and ADP-ribosylation.

i) Phosphorylation of Histones

The phosphorylation of histone proteins may play a functional role in modulating the structure of chromatin, allowing for gene expression and replication during cell division (37). There are a number of correlations between histone H1 phosphorylation and changes in chromosome organization. Ajiro et al. (38,39) have measured the extent of phosphorylation of 2 histone variants (H1A and H1B) during the cell cycle of HeLa cells. Both subtypes undergo a general increase in phosphorylation levels of ∿ 1 phosphate per histone molecule during the S phase of the cell cycle (DNA replication) and a further increase of 3-4 phosphates per molecule during mitosis. However, in the late G1 phase of the cell cycle, the level of histone H1 phosphorylation is very low. Moreover, there are differences in the location of the phosphate residues. They are located almost exclusively in the C-terminal region of the molecule during DNA replication but are located in both N and C-terminal regions during mitosis. During replication, two different C-terminal residues were found to be phosphorylated in both subtypes; one site is phosphorylated immediately before replication and the second site is phosphorylated immediately afterwards (39). These findings are in accord with the hypothesis that the H1 subtypes are functionally distinct, such that subtype specific phosphorylations contribute to the control of chromatin organization.

Histone H2A can also be phosphorylated, and there appears to be a direct correlation between the number of H2A molecules phosphorylated and the amount of constitutive heterochromatin (40), suggesting that this modification locks DNA into an inactive higher order structure.

In summary, histone phosphorylation appears to play a major role in determining the amount of DNA in the various levels of chromatin structure and in increasing the availability of the template for transcription. The nucleus contains histone specific kinases (41), indicating that this form of covalent modification may be a mechanism for the regulation of gene transcription by hormones. A calmodulin-dependent phosphoprotein phosphatase that specifically dephosphorylates histones H1, H2A, and H2B may be an essential part of this mechanism (42).

ii) Acetylation of Histones

On the other hand, histone acetylation may also play an important role in the control of gene expression. Allfrey (43) describes many examples of the correlation between increases in gene expression and increases in core histone acetylation. Increased acetylation of core histone H3 and H4 introduces alterations in nucleosome structure, seen mainly as an increased sensitivity towards DNAse I (44).

iii) ADP-ribosylation of Histones

Important novel types of modification that have attracted much attention due to their ubiquitous distribution and their implicated roles in the regulation of cell proliferation, protein synthesis, DNA and RNA metabolism are poly-and mono(ADP-ribosyl)ation reactions. Histones and other nuclear proteins were first shown to be modified by ADP-ribose groups in 1968 (45) by a chromatin associated enzyme, poly(ADP-ribose) polymerase. This enzyme is present in the nuclei of all eucaryotic cells so far examined (46). This enzyme is able to catalyze 3 enzymatic reactions: (1) the initial modification using a monomer of ADP-ribose, (2) elongation, or consecutive transfer of ADP-ribose units to result in

a homopolymer of repeating ADP-ribose units and (3) branching, where two ADP-ribose residues are both attached to a common ADP-ribose unit, forming a branched structure (47). The enzyme absolutely requires DNA for activity (48); leaving out DNA reduced enzyme activity to less than 1% of control values (49). The binding of poly(ADP-ribose) polymerase to DNA is believed to induce a conformational change of the enzyme into an active state (50).

α) Possible Functions of Histone ADP-Ribosylation

Histones were used for studies of enzyme activity since the time poly(ADP-ribosyl)ated histones were first discovered (45). Among the 5 major subgroups, histone H1 was the first demonstrated to be modified by poly(ADP-ribose) (51,52). Four other subgroups, H2A, H2B, H3 and H4 have subsequently been shown to be modified (53). The highest acceptor activity was found with histone H1; this was followed by histone H2B; histone H2A, H3 and H4 were poor acceptors (54).

Among all the proteins modified by mono and poly(ADP-ribose), histone H1 represents the most extensively studied acceptor. It has been shown to be ADP-ribosylated in isolated nuclei of several tissues *in vitro* (45,55) as well as *in vivo* (56,57). Initiation of poly-(ADP-ribosyl) histone synthesis was achieved *in vitro* using a homogeneous preparation of poly(ADP-ribose) polymerase (54). Elongation of a histone bound ADP-ribose monomer into a polymer by this enzyme has also been demonstrated (58).

Poly(ADP-ribose) synthesis is initiated by the covalent attachment of an ADP-ribose monomer, followed by elongation from the monomer. Histone and non-histone proteins including poly(ADP-ribose) polymerase itself can serve as a substrate for poly(ADP-ribosyl)ation (51). It has

been found that ADP-ribose is bound through an ester linkage to a γ-carboxyl group of a glutamic acid residue of histone H1 or H2B or the α carboxyl of a COOH-terminal lysine residue of histone H1 (59,60,61).

The biological function of this polymer is not yet fully understood (47). Although the data thus far are not conclusive, they suggest that the true catalytic function of poly(ADP-ribose) polymerase in chromatin is histone modification (62). It is proposed that poly(ADP-ribosyl)ation of the C and N terminal regions of histones lead to condensation of chromatin structure (63). It is further proposed that poly(ADP-ribosyl)ation of the termini of histone H1 will act as a bridge to neighboring histone molecules, causing compaction in a specific domain of chromatin (64). Therefore, H1-DNA interactions are sacrificed for H1-H1 interactions when H1 is poly(ADP-ribosyl)ated (46). The following sequence of events is visualized to occur which may involve poly(ADP-ribosyl)ation with a role in DNA repair and/or replication. Upon DNA strand breakage as a result of drug treatment or radiation, the enzyme quickly responds to the breaks in DNA by ADP-ribosylating histone H1. Modification of H1 in the vicinity of the breaks maintains the continuity of the DNA molecule in chromatin until the DNA is repaired or replicated. After DNA synthesis, the poly(ADP-ribose) is subsequently degraded.

Treating cells with agents that induce DNA fragmentation and DNA repair is associated with increased poly(ADP-ribose) polymerase activity (65), with elevated levels of poly(ADP-ribosyl) residues in intact cells, (66,67,68) and with a dramatic stimulation of mono(ADP-ribosyl) and poly(ADP-ribosyl) turnover (67,68).

Although the precise role of the enzyme is unknown, it is dependent on DNA for its activity and is activated by nicks in DNA (51). It has been suggested that (ADP-ribose)n biosynthesis is necessary for the repair of some types of DNA damage (69) effecting repair via activation of DNA ligase II (70). Furthermore, inhibitors of poly(ADP-ribose) polymerase appear to retard DNA repair, and they increase the cytotoxicity of alkylating agents (69).

ADP-ribosylation of chromatin proteins may also be involved in differentiation, possibly by regulating DNA recombination necessary in cell differentiation which regulates gene expression (70,71,72).

Additional evidence for an association between differentiation and poly(ADP-ribosyl)ation has been provided by studies of enzyme activity levels during differentiation in vitro. Increased poly(ADP-ribosyl) polymerase activity has been observed during the differentiation of murine erythroleukemia cells (73,71) and in a number of other differentiating cell systems (74). However, there are many exceptions. For example, poly(ADP-ribosyl) polymerase activity was found to decrease during the induced differentiation of both mouse preadipocytes (75) and the human promyelocytic cell line HL-60 (76). Inhibitors of poly(ADP-ribosyl) polymerase can also induce differentiation (77). Thus, while suggesting a role for poly(ADP-ribosyl) polymerase in differentiation, these data suggest that the mechanism may be different in the different cell lineages.

In summary, the nuclei of eucaryotic cells possess enzyme activities that can modify the interaction between histones and DNA such that the double helix can become more accessible to the transcriptional apparatus. Some modifications such as ADP-ribosylation or histone H2A

phosphorylation may move DNA into transcriptionally inactive regions of chromatin, whereas other modifications such as histone H1 phosphorylation at some sites and core histone acetylation make DNA available for transcription.

Although the function of poly(ADP-ribosyl)ation is not fully understood, many correlations between the synthesis of poly(ADP-ribose) and DNA repair or cellular differentiation have been noted. All classes of histones can be modified by ADP-ribosylation; however, histone H1 appears to be the preferred substrate, and is consequently the most extensively studied acceptor. The location of the ADP-ribosylation sites at the C and N terminal regions of histone H1 is believed to be involved in the condensation of localized regions of chromatin. It is proposed that histone H1 molecules can be linked together by poly(ADP-ribose). A decrease in the length of the ADP-ribose polymer between them will result in the formation of compact regions of chromatin. These internucleosomal histone H1 linkages may also be involved in DNA repair. Several observations have shown a positive correlation between DNA fragmentation and poly(ADP-ribose) polymerase activity. Other observations have shown a positive association between polymerase activity levels and developmental changes in many differentiating cell lines. However, there are many exceptions to these observations, suggesting that the mechanism of differentiation may be different in different cell lines.

Regulation by Post Transcriptional Modification of RNA Transcript

1. RNA Processing of Eucaryotic Cells

A. The Nature of the Primary RNA Transcript: Intervening sequences

The sequences of DNA that code for many proteins are not continuous on the eucaryotic genome (78). Non-coding sequences intervene between the coding sequences, giving rise to split genes. These non-coding sequences, introns, have been found to occur in virtually all mammalian structural genes so far examined, with the exception of the histone genes. It has now been established that the whole gene is transcribed and primary transcripts contain both coding and intervening sequences. This has been confirmed by probing heterogeneous nuclear RNA (hnRNA) with cDNA (which is complementary to cytoplasmic mRNA) and with probes containing intervening sequence DNA and showing that they both exist in primary transcript RNA. Several studies on liver and brain tissue (79) indicate the presence of transcribed sequences in the nuclei of these tissues that never appear in the cytoplasm. This implies the existence of a selection process which determines the sequences of the hnRNA to be saved for processing and exporting to the cytoplasm.

B. Splicing

Splicing is defined as the mechanism by which a single functional RNA molecule is produced by the removal of one or more internal stretches of RNA during the processing of the primary transcript, reducing it in size until all the coding sequences are contiguous (80).

Two possible splicing mechanisms have been proposed. One model requires the primary transcript to take up a thermodynamically stable

secondary structure in which the intervening sequences are looped out such that the ends to be spliced are brought together. Trapnell et. al. (81) have analyzed the sequences of 17 introns and have used computer simulations of secondary structures for a number of primary transcripts to demonstrate how highly base paired, stem loop structures could bring the sequences to be spliced into very close proximity. However, it is not clear whether all transcripts could be spliced by a mechanism depending solely on intramolecular base-pairing. An alternative processing mechanism has been proposed, independently, by a number of people, (82,83,84), that uses a small RNA molecule from a family of small nuclear RNAs (snRNAs), as a matrix upon which to align the ends of the intervening sequences so that splicing can occur. This mechanism depends upon intermolecular base pairing between the ends of intervening sequences and the snRNA. snRNAs are known to co-extract with hnRNP particles, making them likely candidates for their proposed role.

An example of this mechanism of splicing is the alignment of snRNA U1 with the 2 ends of intervening sequence G of the ovalbumin primary transcript (84). There is extensive complementarity in such a way that the intervening sequence is looped out when the two adjacent coding sequences are brought close together. After the appropriate bonds are cleaved, the coding sequences can be spliced together.

C. Splicing and the Regulation of Gene Expression

Splicing is a critical stage of gene expression in eucaryotic cells because it is the step at which a functional, information carrying molecule is generated. Splicing reactions represent mechanisms to regulate cytoplasmic mRNA levels since unspliced mRNA precursors are not

stable and are degraded before entering the cytoplasm (85,86,87,88). If the rate of processing is altered, the concentration of mature mRNA will also be altered. Moreover, if the number of splicing events is modulated, new information carrying mRNA molecules can be generated.

Direct evidence that splicing is a regulatory process exists. Splicing can combine sequences from a transcript in different ways to generate different mRNAs from one primary transcript. Several examples have been found among animal cell viruses (78,89). For example, the primary transcript of the SV40 early genes can be spliced in two different ways to produce t or T antigen, depending on whether or not the second coding sequence is eliminated from the mRNA (90). A similar example of flexibility in splicing occurs in the processing of adenovirus primary transcripts (91,92) where the same set of DNA sequences is used to code for three different proteins, a result of differential splicing.

Differential splicing has been found to occur in eucaryotic cells also and suggests a role for splicing in cellular differentiation. The enzyme α-amylase is synthesized in the cells of the parotid gland and also in the cells of the liver. The polypeptide is identical in each tissue, but the concentration of the enzyme in liver is only 1% of that in parotid cells. Surprisingly, the mRNAs coding for this identical polypeptide are completely the same (93). The parotid gland mRNA has a nontranslated leader sequence of 93 nucleotides, a coding sequence of 1540 nucleotides followed by a 33 nucleotide nontranslated 3' tail. The liver mRNA has a 204-nucleotide leader sequence of which the last nucleotides are identical to the last 48 nucleotides of the parotid leader. The coding and 3' tail sequences are also identical. There the mRNAs differ only in the sequence of their 5' leaders, a difference that

could only arise by differential splicing in the two tissues. At the present time it is not known how the difference in non-translated leader sequences affects the processing, transport, translatability and stability of the two mRNAs. Although all cells may have the same set of DNA sequences, the presence of multiple promoters coupled with differential splicing gives cells the capacity to generate new information carrying products and to generate more than one polypeptide from the same DNA sequence.

2. Structural Aspects of the RNA Transcript

A. Modifications of mRNA

The earliest detectable covalent modification to the primary transcript is the addition of a guanosine residue, via a 5'-5' sugar phosphate bond, to the 5' nucleotide of the transcript (capping). This process appears to occur soon after the initiation of transcription (94). Pulse labelling studies have shown that the cap structure is conserved during processing and appears in mature cytoplasmic mRNA (95). Capping appears to protect the 5' end of all transcripts against degradation, and its presence may also assist in the formation of the translational initiation complex at the 5' end of the molecule. However, the dependence of eucaryotic mRNA translation on the cap appears to be neither absolute nor universal (94).

A high proportion of mature mRNA molecules in the cytoplasm have a long sequence of adenyl residues attached to the 3' end, usually referred to as the poly (A) tail (96). It is now known that the tail is added to hnRNA in the cell nucleus almost immediately after the hnRNA is

transcribed (96). The length of the poly (A) tails of hnRNA varies considerably, but the size of a newly synthesized poly (A) segment is about 200 nucleotides long (97). The poly (A) tail is not synthesized by transcription; it is a post transcriptional event involving the stepwise addition of adenyl residues to the 3' end of the transcript by the enzyme poly (A) polymerase (98).

There is no consensus on the function of the poly (A) tail although 5 possible functions have been considered (99,100).

1. It is involved in transcription termination.
2. It stabilizes the 3' end of the transcript.
3. It is involved in the selection of sequences that are to be transported to the cytoplasm.
4. It plays a role in the transport mechanism.
5. It is involved in mRNA stability and translatability.

The sequence AAUAAA is found near the 3' end of virtually all primary transcripts and is believed to be the signal that is recognized by poly (A) polymerase. The highly conserved sequence AAUAAA, discovered by Proudfoot and Brownlee (101), is found 10-30 nucleotides upstream of most polyadenylation sites. Direct evidence for this has been obtained by studying deletion mutants of the SV 40 viral genome in the region of transcription termination of the later mRNAs (102). If the AAUAAA sequence is deleted, the transcripts are not polyadenylated. However, recent evidence indicate that this signal alone is not sufficient for poly (A) addition since AAUAAA is sometimes found in transcribed regions far removed from any known polyadenylation site (103).

The poly (A) tail is conserved during processing and appears in the

mature mRNA. It is possible that the poly (A) tail is involved in the transport of hnRNP particles from their site of formation to the nuclear pores. The refolding of the molecule during transport to the cytoplasm may bring the poly (A) tail into contact with internal oligo (U) sequences interspersed throughout the mRNA, providing the basis for a stabilized mRNP particle.

B. The Structural Organization of hnRNP Particles

The long primary transcript generated by RNA polymerase II is wound into a series of hnRNP particles (nuclear ribonucleoprotein complexes) linked together by spacer RNA. hnRNA is complexed with protein immediately upon synthesis (104). Purified hnRNPs are presumably composed entirely of RNA and protein. When large hnRNP complexes were isolated from sucrose gradients and examined in the electron microscope, they appeared as chains of monomers (105). The data presented were consistent with a simple polymeric model in which hnRNA is bound to a series of identical protein particles forming a beads-on-a-string structure. In some tissues there is evidence for a nonrandom phasing of the particles along the transcript (106).

A small number of proteins (Mr: 30,000 - 40,000) account for a large proportion of the mass of protein associated with these particles. Since they are found in hnRNPs from all species, they may be the structural core proteins that interact with the transcript to generate the particle.

C. Factors Which Affect the Functional Half Life of Cytoplasmic mRNA

Either during, or immediately after processing, the mRNA is translocated to a nuclear pore complex to be exported to the cytoplasm. Virtually all of the proteins associated with the mRNA prior to transport

are retained within the nucleus with the exception of one protein (Mr: 110,000) which is believed to exit the nucleus along with the mRNA (107).

Once in the cytoplasm, the mature mRNA will be distributed into distinct populations that is characteristic for each individual mRNA (108). The mRNA in the cytoplasm of eucaryotic cells appears to exist in two distinct states, as polysome bound mRNP actively engaged in protein synthesis, or as free mRNP (messenger ribonucleoprotein particles not associated with polyribosomes) (109). Some cellular mRNA species appear to be totally translationally active, while others are found in both the free mRNP and polysome compartments. Among the individual mRNAs that are found in both the free mRNP and the polysomes, there is variation in the fraction of the mRNA species found in the free mRNP state (108). It appears that there are some RNA species in these particles that are never translated, indicating that the selection of mRNAs from the mRNP pool for translation may be an important step in the regulation of gene expression (108,109).

Another way that the functional mRNA concentration can be altered is by changing the rate of degradation of the molecule. However, very little is known about this process. What is known is that there is a great heterogeneity of mRNA turnover in eucaryotic cells; some mRNAs have half lives of 15-20 min whereas others appear to be very stable.

Regulation of Eucaryotic Gene Expression at the Translational Level

1. The Mechanism of Protein Synthesis

A. Initiation

Polypeptide chain initiation in eucaryotes begins with formation of a tertiary complex consisting of equimolar amounts of eIF-2, GTP and the initiator methionyl tRNA (Met-$tRNA_i$) (110). The tertiary complex then binds to a 40S eIF-3 ribosomal subunit complex giving rise to a 40S preinitiation complex (110). In the presence of a 60S ribosome, mRNA, additional factors and ATP, an 80S initiation complex is formed, setting the stage for chain elongation. Mg^{+2} ions are required for the last two steps (110). Formation of the 80S initiation complex is accompanied by hydrolysis of the bound GTP and release of eIF-2 in the form of an eIF-2:GDP complex (111). The release of eIF-2 from GDP is catalyzed by GEF (GDP exchange factor) provided that the α subunit of eIF-2 is not phosphorylated (112). In the absence of GEF, eIF-2 would not be able to recycle and initiation would stop when the available eIF-2 is tied up as eIF-2:GDP.

B. Elongation: General Overview

Polypeptide chain elongation comprises a cyclic sequence of events. Each cycle involves the binding of AA-tRNA to the ribosome, peptide bond formation and translocation. In the binding reaction, an AA-tRNA molecule is attached to the acceptor site (A site) on the ribosome. The type of AA-tRNA to be bound is specified by the mRNA codon present at the A site. The binding of AA-tRNA is mediated by a tertiary complex involving, besides AA-tRNA, elongation factor 1 (EF-1) and GTP. The

binding of AA-tRNA is followed by peptide bond formation, i.e., the transfer of the peptidyl moiety of the peptidyl-tRNA present at a second ribosomal site (peptidyl or P site), to the amino acid of AA-tRNA at the A site. The peptidyl transferase activity resides in the structure of the ribosome and does not require the participation of any soluble protein factor. The peptidyl transfer reaction results in the formation of a new A site bound peptidyl-tRNA, the peptidyl moiety of which is now extended by one amino acid residue at its COOH end. The last step of the elongation cycle, translocation, involves the movement of the ribosome along the mRNA in the 5'-3' direction by one codon, together with the shift of the peptidyl-tRNA from the A to the P site. The translocation step thus results in the appearance of a new codon at the A site that then is available for the next incoming AA-tRNA. Translocation is catalyzed by EF-G in procaryotes and EF-2 in eucaryotes. One molecule of GTP is hydrolyzed during each step of translocation (113).

C. Termination

The termination of protein synthesis is signaled by one of the three termination codons. When UAA (Ocher), UAG (Amber), or UGA (Opal) becomes aligned with the A site of ribosome, no aminocyl-tRNA can bind. This signals the completion of elongation. The newly formed polypeptide chain is released. In eucaryotic cells there is only one releasing factor, RF-1. Termination is an energy requiring process with one molecule of GTP being hydrolyzed per polypeptide chain released. The monoribosome is released from the mRNA and becomes available for a new round of protein synthesis.

2. Eucaryotic Elongation Factors

Two elongation factors (EF-1 and EF-2) exist in the cytoplasm of the eucaryotic cell. EF-1, first isolated from reticulocytes (114), displays properties similar to those of procaryotic EF-T. EF-1 has been purified to apparent homogeneity from a variety of sources, including rabbit reticulocytes (115), wheat germ (116) and calf brain (117). EF-1 from several sources has been resolved into two functionally complementary factors (EF-1 α and EF-1 β) (118,119,120) corresponding to bacterial EF-Tu and EF-Ts. EF-2 has been purified to apparent homogeneity from many tissues, including that from rat liver (121, 122), pig liver (123), wheat germ (124), and rabbit reticulocytes (125).

A. Elongation Factor-2

Elongation factor-2 (EF-2) was first isolated by Schweet and Arlinghaus as one of two soluble factors in rabbit reticulocytes which were required for polypeptide elongation referred to as amino-acyltransferases 1 and 2 (126). Aminoacyltransferase 2 is currently referred to as Elongation Factor-2.

EF-2 was initially purified from rat liver by Galasinski and Moldave (121). Based on this procedure Raeburn et al. were able to obtain a preparation that appeared to be close to be 95% pure (122). EF-2 has been purified from a variety of tissues: rabbit reticulocytes (125), calf brain (127), hen oviduct (128), wheat germ, (129) and PyBHK hamster tumors (130). EF-2 purified from various sources appears to have many properties in common. EF-2 consists of a single polypeptide chain with a molecular weight ranging from 65,000 to 112,000, depending upon the source and the technique used to measure molecular weight. The

differences in the amino acid composition of the EF-2 isolated from rabbit reticulocyte (125) pig liver (123,131) and rat liver (132) are small.

EF-2 and EF-G have considerable similarities in their amino acid compositions (131,125,132). However, EF-2 differs from EF-G in the ability of diphtheria toxin to transfer the ADP-ribose group from NAD^+ into a covalent linkage with EF-2 (133). This causes EF-2 to lose its ability to catalyze protein synthesis (134,135).

There are four ways of measuring EF-2 content (122).

1. Polypeptide synthesis dependent on EF-2.
2. Puromycin-peptide formation using polysomes containing nascent protein chains.
3. GTP hydrolysis dependent on EF-2 and ribosomes.
4. ADP-ribosylation of EF-2 in the presence of diphtheria toxin.

B. ADP-Ribosylation of EF-2

Diphtheria toxin fragment A specifically inactivates protein synthesis elongation factor-2 from all eucaryotes by catalyzing the covalent attachment of ADP-ribose to EF-2 (134,135). In vitro studies of this reaction show that EF-2 from all eucaryotic sources tested is specifically modified by the toxin. Other proteins, including the procaryotic analogue of EF-2, elongation factor G (EF-G), are not ADP-ribosylated (136,137). The basis of this specificity was first examined by Maxwell et al., who isolated and sequenced the ADP-ribose containing tryptic peptide from rat liver EF-2 (138). Subsequently, the ADP-ribosylated amino acid was isolated in milligram quantities (139) and assigned a proposed structure (140) chemically defined as

2-[3-carboxyamido-3-(trimethyl ammonio) propyl] histidine. Since this amino acid occurs only once in EF-2 (138), it is apparent that only one ADP-ribose residue is attached to each EF-2 (132,122).

The trypsin derived sequences containing the ADP-ribosylation site have been shown to be highly conserved in yeast, wheat germ, rat and bovine liver (136), suggesting that this region serves an indispensable function in protein synthesis in addition to being the site of attack by diphtheria toxin.

C. Functional Properties of EF-2

Skogerson and Moldave first reported that rat liver EF-2 could bind to ribosomes in the presence of GTP and a sulfhydryl compound (141). In subsequent studies (142,143) these authors showed that the binding of EF-2 to ribosomes could be obtained with the nonhydrolyzable GTP analogue GDPCP as well as GDP. However, GTP was required to yield a ribosome-EF-2 complex that was active in stimulating AA-tRNA transfer to the ribosomes. These results indicate that a ribosome-EF-2 complex prepared in the presence of GTP made available an AA-tRNA binding site on the ribosome due to translocation of any peptidyl-tRNA on the ribosome from the A site to the P site. The binding of EF-2 to the ribosome requires sulfhydryl groups on both the ribosome and EF-2. It has been reported that the EF-2 binding site can be occluded by the presence of peptidyl-tRNA at the acceptor site (144). The ribosomal EF-2 binding site has been found to lie on the 60S subunit (145). EF-2 preparations from liver (144,146), reticulocytes (147) and calf brain (127) can form a complex with GTP. EF-2 can also bind to GDP (148). Equilibrium dialysis studies carried out with a homogeneous EF-2 preparation from rat liver indicate a common

binding site for GDP and GTP on EF-2 (146). The affinity of EF-2 for GDP is about five times higher than for GTP (146). ADP-ribosylated EF-2 binds guanine nucleotides equally well (149). ADP-ribosylated EF-2 is also capable of forming a tertiary complex with ribosomes and GTP (127,150). However, ADP-ribosylated EF-2 is incapable of catalyzing GTP hydrolysis and translocation (148). EF-2 requires only the 60S ribosomal particle for GTPase activity, although hydrolysis occurs more efficiently in the presence of both subunits (151). At the concentrations used in these experiments, 60S and 40S particles either alone or combined show no detectable GTPase activity in the absence of EF-2 (151). The hydrolysis of GTP is necessary for translocation to occur (152).

3. Regulation of Enzyme Synthesis at the Translational Level in Eucaryotes

Since protein synthesis is a complex process involving many different enzymes and components, it is important to define a role for each step of the protein synthetic pathway. Much of our present knowledge of translational control mechanisms is derived from studies with reticulocytes and their lysates because the control of protein synthesis in these cells is confined to translation. Fortunately, the cell free systems developed from these cells retained many of the characteristics of the cells themselves and was of immense help in elucidating the control mechanisms involved in this step.

In light of the evidence given for the importance in regulating intracellular protein concentrations by transcriptional controls, what evidence is there for translational regulation? The synthesis of specific proteins for the most part seem to be correlated with the

activation of transcription. The clearest examples of this are the induction of the synthesis of ovalbumin, conalbumin, and other egg proteins in the hen oviduct by steroid hormones (153). Studies using cloned cDNAs from mouse liver mRNA populations have shown that there is normally a good correspondence between the rate of transcription of a specific mRNA and its concentration in the cytoplasm. This suggests that the expression of most moderately abundant liver mRNAs is regulated primarily at the transcriptional level (154). However, the comparatively long half life of mRNA means that such transcriptional changes are not sufficiently sensitive to allow for the rapid adjustments in protein synthesis. Thus, the rapid initial decreases in protein synthesis seen in cells in mitosis (155) or after stimulation of cell growth by addition of hormones or growth factors (156,157,158,159) are due mainly to changes acting to regulate the rate of translation of pre-existing mRNA by pre-existing ribosomes.

Translation may be regulated at initiation or elongation steps, but in most cases studied so far, it is initiation which is the process that is primarily affected. This conclusion is supported by the fact that translational inhibition is normally accompanied by a decrease in the number and average size of polysomes and an increase in the number of inactive monoribosomes and free cytoplasmic mRNA (155).

The converse changes are usually seen after translational activation (156,157,158,159), and the ribosome transit time is normally not affected (155,160). There have been a few reports of translational controls which restrict the rate of elongation when cultured mammalian cells are deprived of serum (161,162). In these cases the activity of Elongation Factor-1 (EF-1) is inhibited whereas Elongation Factor-2 activity is

unaffected. The cells that behave in this way are all aneuploid lines with very rapid growth rates, and it may be that under adverse growth conditions the primary controls acting to restrict initiation have been lost, revealing secondary controls affecting elongation.

A. Translational Controls Acting at the Level of 40S Initiation Complexes in the Cell Free Reticulocyte Lysate

Translational control mechanisms have been elucidated mainly by studies of the rabbit reticulocyte and its corresponding cell-free system, the reticulocyte lysate.

Protein synthesis in the reticulocyte lysate is regulated by the availability of heme, as it is in whole cells (163,164). In the absence of heme, the activity of the lysate declines abruptly after a lag period to approximately 10% of its initial rate. The inhibition of protein synthesis is accompanied by polysome disaggregation and an accumulation of inactive 80S ribosomes (163,164). Consistent with this is the finding by Legon et al. that heme deficiency also causes a decline in the level of 40S initiation complexes from as early as the second minute of incubation without heme (165). Heme deficiency activates an inhibitor of protein synthesis initiation referred to as heme controlled inhibitor or HCI, from a proinhibitor of similar molecular weight (166,167,168). When purified HCI is added to a lysate that is actively synthesizing protein in the presence of hemin, it rapidly inhibits protein synthesis (169,170). The time course of translation in the presence of hemin and HCI is essentially the same as in the absence of hemin.

In both cases, the kinetics are biphasic with a normal rate for the first few minutes and an almost complete shutoff thereafter. Low concentrations of double stranded RNA (dsRNA) activate a similar

inhibitor referred to as the dsRNA activated inhibitor (DAI) (170,171). HCI and DAI are cyclic AMP-independent protein kinases that specifically phosphorylate the small α subunit of the initiation factor eIF-2, which interferes with the function of eIF-2 (170,172,173). Both HCI and DAI phosphorylate the same sites of the α subunit of eIF-2 (174). Both inhibitors are also autophosphorylated which is thought to be involved in their activation (110).

The fact that HCI and DAI are capable of inhibiting translation in hemin containing reticulocyte lysates and both can phosphorylate the α subunit of eIF-2 strongly suggests that translational inhibition by HCI and DAI is a consequence of this modification of the factor. This assumption appears to be true based on the observations that purification of these inhibitors copurifies with eIF-2 α kinase activity (170,172, 173). It is also consistent with the observation that antibody against HCI eliminates both the inhibitory and phosphorylation activities of this protein (173).

Protein kinases similar to HCI and DAI have been found to exist in a number of normal cells (175,176,177,178) including rat liver, wheat germ, and brine shrimp embryos (artemia salina), which suggests that this mechanism may be occurring widely. All of these kinases phosphorylate the small subunit of eIF-2. However, there has been no direct demonstration that the phosphorylation of eIF-2 prevents its participation in the process of initiation.

On the other hand, several observations argue against a direct causal relation between cessation of protein synthesis in a hemin - deprived lysate and the phosphorylation of eIF-2 α:

1. A decrease in eIF-2 activity due to the phosphorylation of the α subunit has not been demonstrated unambiguously (173).

2. Protein synthesis in a hemin deprived lysate can be restored to its maximum level by the addition of a protein factor (anti-inhibitor) without any decrease in the level of phosphorylation of eIF-2α (179).

3. In heme deficient lysates showing almost complete inhibition of protein synthesis, only 20-30% of the eIF-2α subunit appears to be phosphorylated (180).

These observations support the idea that phosphorylation of eIF-2α is not the direct cause of cessation of protein synthesis in rabbit reticulocyte lysates.

The role of phosphorylation became apparent following a series of breakthrough discoveries. In 1978, the search for the way in which phosphorylation of the eIF-2α subunit relates to translational inhibition led to the discovery of a new initiation factor first named ESP for eIF-2 stimulating protein (181,182). This factor is now known to catalyze the exchange of eIF-2 bound GDP for GTP, thus permiting the released eIF-2 to function in subsequent rounds of initiation. ESP is now renamed GEF for GDP exchange factor (110). Following the initial description of ESP, ESP-like factors from rabbit reticulocytes and other sources have been reported (179,183). They all share the property of stimulating in vitro ternary or 40S complex formation by intact, but not by α phosphorylated eIF-2 in the presence of Mg2+. The addition of eIF-2 or GTP to heme deficient reticulocyte lysates can restore translation (176) as can the addition of GEF or eIF-2:GEF (179,183,184). Thus, eIF-2, GTP and GEF can each restore ternary complex formation that has been inhibited by eIF-2 α

phosphorylation. These experiments show that in fact, the exchange of GTP for GDP bound to eIF-2 is inhibited following eIF-2 α phosphorylation (112,185). Phosphorylation of the α subunit of eIF-2 could modify its interaction with GEF by either decreasing or increasing their mutual binding affinity. The fact that only 20-30% of the eIF-2 appears to be α phosphorylated in fully inhibited heme-deficient lysates (180) would seem to favor the second alternative.

To test this hypothesis, experiments using only purified eIF-2 (αP):[H^3]GDP, eIF-2:[H^3]GDP, and eIF-2:GEF were performed to determine the level of exchange of [H^3]GDP with unlabelled GDP (110). Inhibition of the exchange became greater with an increasing ratio of α phosphorylated to unphosphorylated eIF-2:GDP, suggesting that translational inhibition results from immobilization of GEF in a complex with eIF-2 (αP). Any GEF in excess of that trapped by eIF-2 (αP) will function catalytically with the nonphosphorylated eIF-2 still available. Because of its high affinity for GDP, eIF-2 will not recycle if anything interferes with the activity of GEF. Phosphorylation of the eIF-2 α subunit blocks the GDP exchange and immobilizes GEF in an eIF-2 (αP):GEF complex, thus inhibiting ternary complex formation.

B. Translational Controls Acting Later in the Initiation Sequence

Although the most clearly established examples of translational control in eucaryotic systems are those which act before the 40S initiation complex is formed, there is indirect evidence of regulation after the formation of the 40S complex.

As an example, the formation of 80S initiation complexes after activation of peripheral blood lymphocytes by PHA correlates closely with

the increased rate of initiation and protein synthesis in the intact cells (186). The cytoplasm of unstimulated lymphocytes contains, in addition to a translational inhibitor which inhibits 40S initiation complex formation, a further factor which inhibits the conversion of 40S complexes to 80S complexes in the reticulocyte lysate (187).

A reduction in the level of 80S initiation complexes, even in the presence of an excess of added mRNA, is also seen in cell-free systems from Krebs ascites cells that have been incubated at 45°C (188). In this case there is no detectable defect in 40S initiation complex formation in the cell free systems, although initiation in the intact cells is strongly inhibited. These examples suggest that in nucleated cells regulation of different steps in the initiation sequence may occur, sometimes simultaneously, so that several different control mechanisms may operate together to control the rate of protein synthesis.

C. Regulation of Translation at the Elongation Step

Codon degeneracy can be used as a means of regulating the rate of translation of specific mRNAs at the elongation step because when a rare codon occurs, the system may have to wait for the appropriate aminoacyl-tRNA, which may be at a low concentration (189). These are referred to as modulating aminoacyl-tRNAs. Since the relative proportions of the isoaccepting tRNAs for each amino acid vary from tissue to tissue, the same mRNAs may be translated much more efficiently in one tissue than in another. Moreover, the relative proportions of the tRNAs change within the cells of the same tissue during differentiation and may contribute to the changes in the spectrum of proteins synthesized.

4. mRNA Structure and the Regulation of Translation

Any model for the regulation of gene expression at the level of translation must be able to explain how the rate of translation of one or a small number of mRNAs can be altered without any change in the rates of the translation of thousands of other mRNAs. All other factors being equal, the structures of individual mRNAs, especially near or at the region of initiation must influence the ability of the molecule to interact with the initiation complex.

Models for the secondary structure of the α and β globin mRNAs of mouse and rabbit have been generated by computer analysis of the products of digestion of the molecules with single and double strand specific RNAses (190). These results indicate that the AUG initiator codon in both mouse and rabbit β-globin mRNA is quite susceptible to cleavage with S1 and T1 nucleases, suggesting that it resides in a single stranded exposed region. In contrast, the AUG initiator codon in the α-globin mRNA of both species is inaccessible to cleavage, indicating that it is either buried by tertiary structure or is base paired. Since the rate of initiation of protein synthesis with β-globin mRNA in rabbit reticulocytes is faster than for α globin mRNA, these results imply a possible correlation between the differential rates of initiation with these two mRNAs and the accessibility of the respective AUG initiator codons (190). Moreover, translatability will also be affected by the proteins and RNA molecules bound to the mRNA, which may mask or expose ribosome binding sites and the AUG codon.

A. Discrimination Between mRNAs by Translational Mechanisms

There is now a great deal of evidence that translational level controls cannot only regulate the overall rate of protein synthesis but can also discriminate between different mRNAs. It has been demonstrated both by hybridization techniques and by extraction of the mRNA from the free and ribosome bound fractions and comparison of the products synthesized in cell free systems, that the distribution of individual mRNA species between the two fractions is non-random. It was concluded from studies done on mouse Taper ascites cells that these cells maintain a highly polarized distribution of certain mRNA species between the functioning (polysome) and non-functioning (free mRNP) compartments of the cytoplasm (108,109).

Typically, the same mRNA species are found in both the free mRNP and ribosome bound fractions, but the distribution between the two fractions can vary from one mRNA to another, and may also be affected by the overall rate of protein synthesis in the cell. This was first observed in reticulocytes, where α and β globin chains are normally synthesized at the same rate. When globin synthesis in the reticulocytes is reduced by starving the cells for iron, the synthesis of α globin is preferentially inhibited (191).

Lodish has proposed an explanation for this phenomenon (192). He has suggested that some mRNAs, such as the β globin mRNA, may be intrinsically more efficient at initiating than are others so that under conditions where initiation is restricted, these mRNAs will compete successfully for the initiation opportunities that are available. At low mRNA concentrations α-and β-globin mRNAs are translated in cell-free systems with approximately equal efficiency, but when competition is introduced, by raising the mRNA concentration, by the use of inhibitors

of initiation or by the use of ionic conditions suboptimal for translation, the synthesis of β-globin predominates (193,194). These results are believed to be caused by an initiation factor which preferentially translates β-globin mRNA, but is required for translation of both mRNAs.

Differences in the translational efficiency of different mRNAs have also been observed in many other types of cells such as the immunoglobulin heavy and light chains in mouse plasmacytoma cells (195), ferritin mRNA in rat liver (196) and proinsulin mRNA in pancreatic islet cells (197).

Why one mRNA should be more efficient than another at initiation is unknown, but one possibility is that the secondary structure of the mRNA may be important. Payvar and Schimke have shown that chick conalbumin mRNA is much less efficient in initiation in cell free systems than is ovalbumin mRNA, but that this difference is much reduced if the mRNAs are first denatured with methylmercury hydroxide (198).

Perhaps the most dramatic examples of translational regulation which affect the competition between different mRNAs for initiation are those responsible for the shutoff of host protein synthesis in cells infected by certain RNA viruses. Extracts of poliovirus-infected HeLa cells translate picornavirus RNAs efficiently, but are unable to utilize HeLa cell or vesicular stomatitis virus mRNAs (199,200).

Other changes in translation which are superficially rather similar to those seen after viral infection occur after a heat shock is given to Drosophila cells by raising the temperature of incubation from 25°C to 36°C (201,202). The immediate effect is for transcription of all previously active genes to cease, but the very rapid synthesis of a

number of heat shock mRNAs begin. Translation of all pre-existing mRNAs cease, although they are not degraded and their translation resume if the temperature is returned to 25°C. Lysates from normal Drosophila cells will translate both normal and heat shock mRNAs indiscriminately, but those from heat shocked cells will translate only the heat shock mRNA. The defect in the systems from heat shocked cells appears to be due to the inactivation of some factor necessary for the translation of the normal mRNA rather than to a translational inhibitor (203).

A class of small cytoplasmic RNA, about 70-90 nucleotides in size, and sedimenting as 4S in sucrose gradients are described as potent inhibitors of in vitro mRNA translation (204). This RNA, isolated from chick embryonic muscle, is able to inhibit the translation of a variety of mRNAs in a nondiscriminatory manner. The inhibitory 4S RNA (iRNA) from chick embryonic nuclei has been purified as an RNA-protein complex (10S cytoplasmic RNP) (204). The properties of iRNA indicate that it is unrelated to cytoplasmic mRNP. iRNA also appears to be distinct from small nuclear RNAs in size and base composition.

Taken together, these studies indicate that eucaryotic translational controls can discriminate among different mRNAs. Several different mechanisms appear to be involved. Changes at this level can lead to drastic alterations in the qualitative pattern of protein synthesis. It is likely that selectivity in mRNA translation is determined at the mRNP level by either the mRNA itself or by the association/dissociation or modification of various initiation factors and possibly, the effect of small cytoplasmic RNA molecules.

5. Regulation of Enzyme Activity by Posttranslational Modification

Posttranslational modification is necessary for functional or structural activation of some protein molecules. There are two kinds of posttranslational modification: proteolytic cleavage of the polypeptide chain and covalent modification of the amino acid residues of the polypeptide. Proteolytic cleavage is irreversible and is used in three different ways: liberation of an active enzyme from an inactive proenzyme or zymogen, fixation of an enzyme or protein into a cell membrane, and removal of the enzyme or protein from the cell by degrading it. Covalent modification of amino acid residues, on the other hand, is often reversible and is used to modulate the activity of the gene product.

A. Posttranslational Protein Modifications

The definition given to posttranslational modification by Finn Wold is the conversion of any of the 20 primary amino acids that have been established to be specified by the genetic code to a derivitive form, although it is still possible for the genetic code to specify more than 20 amino acids (205).

In the most general terms the types of chemical transformation involved in posttranslational modifications can be categorized as follows (205):

1) A simple reversible reaction catalyzed by a single enzyme. An example of this reaction type is the reaction by which sulfhydryls and disulfides may be reversibly interconverted in response to variations in the environmental redox potential.

2) A reversible modification reaction catalyzed by two separate enzymes. This type of modification is common; certain phosphorylation/dephosphorylation, methylation/demethylation, ADP-ribosylation/de-ADP-ribosylation and acetylation/deacetylation reactions are typical examples in this category. The reversible nature of the reactions here and in the first category are associated with a regulatory effect on the proteins modified, turning their activity on in one direction and off in the other direction.

3) An irreversible modification reaction. This is the most common reaction type; the product of this reaction is a new amino acid which is permanently associated with the functional protein.

4) Non-enzymatic modification. These reactions proceed in all living cells and are slow and spontaneous.

A new model for understanding the specificity of posttranslational modification reactions superimposes a complicated shooting gallery mechanism onto the traditional lock and key model of enzyme specificity. In this shooting gallery, the targets (proteins) passing across the line of fire (posttranslational modification) are constantly changing in color and shape, and a hit (modification) is only recorded if the proper shape and color is displayed when the shot is made, and each compartment of the cell appears to have its own set of game rules. The proteins thus have to encounter the proper enzyme, in the proper compartment, at exactly the time when they display the proper structural feature for a 'hit' to be recorded. However, the amino acid sequence is the primary specificity determinant as even the initial processing of proteins by transport, cleavage or modification is based on sequence recognition. On the other

hand, all aspects of sequence information could be included, from simple short sequences to tertiary and quaternary structures.

B. ADP-Ribosylation of Proteins as a Posttranslational Modification

ADP-ribosylation reactions occur through the transfer of the ADP-ribose portion of NAD^+ into a covalent linkage with substrates specified by the particular ADP-ribosylating enzyme. As a process which occurs to proteins after the ADP-ribose acceptor site has been incorporated into the primary structure of the polypeptide chain, such reactions can and will be considered as posttranslational modifications in this manuscript.

In these reactions, the high energy bond linking the ADP-ribose and nicotinamide moieties of β-NAD^+ is cleaved. The ADP-ribose is transferred to an appropriate acceptor, either a protein or another ADP-ribose already attached to a protein. Two general types of enzymes that catalyze ADP-ribose transfer have been identified, poly(ADP-ribose) polymerase and mono(ADP-ribosyl)transferase (206,207,208).

As mentioned previously, poly(ADP-ribose) polymerase catalyzes both the initial ADP-ribosylation of protein and the subsequent addition of ADP-ribose moieties linked through ribose-ribose bonds to form a homopolymer. This enzyme is localized primarily in the nucleus and is tightly bound to chromatin. Although the role(s) of poly(ADP-ribose) polymerase has not been precisely defined, this enzyme may be involved in DNA synthesis, cell proliferation, cell differentiation, and DNA repair.

The other type of ADP-ribosyltransferase catalyzes the convalent attachment of single ADP-ribose residues to specific protein substrates. This type of mono(ADP-ribosyl)ating enzyme can be divided into 3 groups:

bacteriophage enzymes, enzymes endogenous to animal cells and bacterial toxins that exert their effects on animal cells by catalyzing the ADP-ribosylation of specific cellular proteins.

C. Endogenous mono(ADP-ribosyl)ating Enzymes

There is evidence for the existence of mono(ADP-ribosyl)transferases in several animal tissues (207). An enzyme that catalyzes the NAD-dependent ADP-ribosylation of guanidino compounds, such as arginine and purified synthetic and cellular proteins, has been purified from the soluble fraction of turkey erythrocytes (209). Using the same purification procedure as used for the turkey erythrocyte enzyme, similar enzymes have been identified in rat liver and in human and rabbit erythrocytes (210,211).

The turkey erythrocyte ADP-ribosyltransferase (transferase A) as described by Moss (214), has an Mr of 25,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel permeation chromatography (212). The enzyme is activated in the presence of chaotropic salts which dissociate the enzyme from an inactive oligomeric form to an active protomeric form (213). In the absence of salt, enzyme activity can be stimulated by histones which can also dissociate the enzyme to an active protomeric species. The transferase can ADP-ribosylate a variety of purified proteins as well as proteins from the soluble and particulate fractions of erythrocytes. However, this enzyme does not activate turkey erythrocyte membrane adenylate cyclase and no regulatory role has been assigned to it (212).

A second ADP-ribosyltransferase (transferase B) has since been purified from the soluble fraction of turkey erythrocytes (214).

Although these enzymes catalyze the same model reactions, the ADP-ribosylation of arginine and other simple guanidino compounds, as well as the mono(ADP-ribosyl)ation of proteins, they are distinctly different in physical and kinetic properties. Transferase B can be distinguished from transferase A by the fact that its activity is independent of histones and chaotropic salts (214). The purified enzyme is seen as a single band on SDS polyacrylamide gels with an Mr of 32,000. Under experimental conditions this enzyme apparently exists as a protomer (214). The finding of two different NAD:arginine ADP-ribosyltransferases suggests that mammalian cells may contain a family of ADP-ribosyl-transferases which catalyze the ADP-ribosylation of various proteins.

In support of this, evidence for the existence of mono(ADP-ribosyl)ating enzymes from different cellular compartments have been reported. A mono(ADP-ribosyl)ating enzyme has been isolated from extracts of purified rat liver mitochondria. It appears to be different from the turkey erythrocyte ADP-ribosyltransferase as described by Moss et. al. (215), even though it can be inhibited by arginine methyl ester (209). Additionally, mono(ADP-ribosyl)ation also occurs in the 120,000 x g supernatant of rat liver cytosol and several cytosolic proteins of this fraction are modified in vitro (216). This enzyme activity is not inhibited by poly(ADP-ribose) polymerase inhibitors but can be totally inhibited by the addition of arginine methyl ester to the incubation mixture. A soluble ADP-ribosyltransferase activity in the 27,000 x g supernatant of rat liver had been described previously by Moss and Stanley (210).

These examples show that endogenous mono(ADP-ribosyl)ation reactions do occur in different compartments of the cell; however, the significance of these reactions is not known. The fact that all the endogenous reactions described so far appear to use arginine as an ADP-ribose acceptor may reflect only the way in which these enzymes have been discovered.

Evidence relating endogenous mono(ADP-ribosyl)ating reactions with regulatory functions have been circumstantial. It has been shown by Beckner and Blecher that the endogenous transferase activity localized in RL-PR-C rat hepatocyte membranes ADP-ribosylates the same 55,000 dalton membrane protein as that by cholera toxin (217). However, the endogenous ADP-ribosylation reaction, in contrast to the cholera toxin catalyzed reaction, is not competitively inhibited by added arginine. This suggests that the ADP-ribose acceptor is different in each case. Although membrane adenylate cyclase has not been reported to be activated following the endogenous reaction, it may be physiologically significant since stimulation of adenylate cyclase activity in rat hepatocytes (RL-PR-C) by isoproterenol is accompanied by enhanced ADP-ribosylation of the same 55,000 dalton protein which serves as the acceptor for both endogenous and cholera toxin catalyzed reactions (218). Another study, involving the regulation of calcium levels in rat liver mitochondria, suggests that ADP-ribosylation of an inner mitochondrial membrane protein is important for releasing calcium from mitochondria (219). However, there are compelling indirect reasons for associating an important function to the ADP-ribosylation reaction in one particular case. Elongation Factor-2 has a unique ADP-ribose acceptor site which is a posttranslationally modified histidine residue termed diphthamide. This

unique acceptor site, found only in EF-2, has been conserved throughout eucaryotic evolution (134,135,136), suggesting that it serves an essential function in cellular metabolism.

ADP-ribosylation of EF-2 is the only posttranslational modification that is known to occur at the diphthamide site. Since it appears that the diphthamide residue has been evolutionarily conserved to benefit the cell rather than providing a site for the chance intoxication by bacterial toxins, this suggests that an endogenous tranferase exists which can ADP-ribosylate EF-2 intracellularly.

D. Bacterial mono(ADP-ribosyl)ating Enzymes

There are six bacterial enzymes with ADP-ribosyltransferase activities identified to date, 5 of which are known to be toxins. These may be divided into 4 groups based on their substrate specificities. The first consists of bacterial toxins which exert their effects on cells by altering the activity of adenylate cyclase (220,221,222).

Adenylate cyclase is a membrane-bound enzyme that catalyzes the synthesis of cAMP from ATP. Its activity is physiologically regulated by hormones and neurotransmitters that act through specific receptors to either stimulate or inhibit the cyclase (223,224). The actions of both stimulatory and inhibitory signals on the catalytic unit of the cyclase system are dependent on GTP (223,224).

It is now generally believed that adenylate cyclase activity is mediated through specific regulatory proteins termed the nucleotide regulatory components (N). Two types of N units have been distinguished functionally. One mediates stimulation (termed N_s), the other inhibition (termed N_i) of adenylate cyclase activity by GTP (219). Choleragen, the

toxin responsible for the symptoms of cholera, and E. coli heat-labile enterotoxin, one of the agents involved in the pathogenesis of "traveler's diarrhea", exert their effects on cells by ADP-ribosylating a subunit of the stimulatory GTP-binding protein, N_s (225). This ADP-ribose acceptor participates in the coupling of stimulatory hormone receptors to the catalytic unit and in the activation of the catalytic unit when GTP is present (223,224). The modification produced by choleragen or E. coli LT toxin results in activation of adenylate cyclase, leading to the increase in intracellular cAMP.

The second group consists of an ADP-ribosylating toxin that has been isolated from the culture medium of Bordetella pertussis termed Islet-Activating Protein (Iap). Islet-Activating Protein enhances GTP-induced activation of membrane adenylate cyclase as a result of ADP-ribosylation of a membrane protein with an Mr of 41,000 (222). The membrane protein ADP-ribosylated by the pertussis toxin is different from that ADP-ribosylated by choleragen and E. coli heat labile enterotoxin and is believed to be a subunit of the inhibitory GTP-binding protein (N_i) of the cyclase complex (222).

Diphtheria toxin and Pseudomonas exotoxin A comprise the third group of ADP-ribosylating enzymes. They cause cell death by catalyzing the ADP-ribosylation of Elongation Factor-2 and thereby inhibiting protein synthesis (134,226). Exoenzyme S, an ADP-ribosylating enzyme produced by Pseudomonas aeruginosa that is distinct from exotoxin A, is tentatively placed in a fourth group because the ADP-ribose acceptors for this enzyme are still uncharacterized.

Different groups or classes of ADP-ribosyltransferases use different amino acids as well as different proteins as ADP-ribose acceptors. Poly-

(ADP-ribose) polymerase modifies certain specific glutamic acid residues in histones H1 and H2B (61,227). For avian erythrocyte transferases A & B, E. coli heat labile enterotoxin and choleragen, free arginine can serve as an ADP-ribose acceptor (214,228,229). The sites of ADP-ribosylation in their respective protein substrates, which have not been identified, are presumed to be arginine residues or possibly other types of guanidino groups resulting from posttranslational modification. The amino acid of the inhibitory GTP-binding protein that is modified by Bordetella pertussis toxin (Iap) is unknown. However, islet-activating protein also catalyzes the ADP-ribosylation of transducin, a guanine nucleotide-dependent regulatory protein of the retina that shares structural homology with N_i. Here, an asparagine residue has been identified as the site of ADP-ribosylation (230). This site appears to be one of two distict ADP-ribosylation sites on the protein, the other one being the site of modification by cholera toxin. Diphtheria toxin and Pseudomonas exotoxin A ADP-ribosylate a single amino acid in Elongation Factor-2, termed diphthamide (139,140). Although it has thus far been found only in Elongation Factor-2, its role in the function of that protein is unknown. The basis for its identification as a unique amino acid derivative was the fact that it is the site at which diphtheria toxin and Pseudomonas toxin A specifically incorporate an ADP-ribosyl group (231).

i) Diphtheria Toxin

Diphtheria Toxin inhibits protein synthesis in cell free systems by catalyzing the ADP-ribosylation of a single amino acid in eucaryotic Elongation Factor-2 which then becomes inactive in protein synthesis

(133,135,232,233). The toxin molecule is synthesized and released from the bacterial cell as a single polypeptide chain (234,235) with a molecular weight of approximately 62,000. It is encoded by a β phage gene (236) and is produced only by strains of C. diphtheriae lysogenic for corynebacteriophages whose genome carries the tox structural gene (134). Short treatment of diphtheria toxin with trypsin and chemical reduction yields 2 peptides: an amino terminal fragment A (24,000 daltons) and a carboxy terminal fragment B (38,000 daltons) (234,235). Fragment A is responsible for the enzymatic activities of the toxin (237,238). Fragment B is thought to be involved in attachment and entry of the toxin into the target cell. The holotoxin exhibits relatively little ADP-ribosyl transferase activity unless it is treated with trypsin and thiol which liberate fragment A (234,235,237,238).

The breakthrough experiment which eventually led to an understanding of the mode of action of diphtheria toxin was performed by Strauss and Hendee (239) who showed that the incorporation of amino acids into protein by growing HeLa cells stopped within 2 or 3 hrs in the presence of a low concentration of toxin. The effect on protein synthesis appeared to result from a primary action of the toxin, since other metabolic activities remained normal for several hours after amino acid incorporation had ceased (239). We now know that diphtheria toxin inactivates the eucaryotic translocating enzyme Elongation Factor-2 (240,241). All of the enzyme activity is located on fragment A which has been shown to catalyze at least three group transfer reactions involving NAD^+ (134):

1. NAD^+ + EF-2 $\rightleftarrows$ ADP-ribosyl-EF-2 + nicotinamide + H^+

2. NAD^+ + HOH $\rightleftarrows$ ADP-Ribose + nicotinamide + H^+

3. NAD^+ + TOXIN $\rightleftarrows$ ADP-Ribosyl-Toxin + nicotinamide + H^+

Only the first of these reactions is important in vivo (133,242,243). At physiological pH and at concentrations of EF-2 and NAD^+ comparable to those in living cells, reaction 1 goes almost to completion with $K=10^4$ (133,233). However, this reaction can be reversed by dialyzing out NAD^+, lowering the pH and adding in excess nicotinamide in the presence of fragment A (242,244). Active EF-2 is regenerated from ADP-ribosylated EF-2. Similarly, protein synthesizing systems derived from intoxicated HeLa cells or from heart muscle of severely intoxicated guinea pigs may be reactivated by adding excess nicotinamide and fragment A (242,245).

Under normal reaction conditions, EF-2 from all eucaryotic organisms so far tested serves as the ADP-ribose acceptor. No protein acceptor, other than EF-2, has been found in mammalian tissue extracts (233,244). Because of this extreme specificity, the reaction can be used to assay for EF-2 in extracts from cultured cells (237,246) and crude tissue extracts from normal and intoxicated animals (243). However, neither EF-G, the bacterial elongation factor corresponding in function to EF-2 nor elongation factor G from mitochondria is ADP-ribosylated in the presence of NAD^+ and fragment A (247,248). The NAD^+ requirement is also highly specific. NAD^+ cannot be replaced by α NAD^+, $NADP^+$ or NADH. Of a large series of analogues tested, only thionicotinamide AD^+ had activity comparable to NAD itself (249).

Under physiological conditions the equilibrium position of the reaction lies far to the right but can be shifted toward the left by adding high concentrations of nicotinamide (233,244,250). Honjo and coworkers demonstrated removal of the attached ADP-ribose and reactivation of EF-2 by incubation of ADP-ribose-EF-2 with nicotinamide and toxin. In addition, they have recovered an equivalent amount of authentic NAD^+ generated by the reversal (233).

The value for hydrolysis of the ADP-ribosyl-EF-2 linkage is about -4.0 kcal per mole at pH 7.0 and 25°C. The major implication of these findings with respect to the reaction in vivo is that the inactivation of EF-2 at equilibrium should be virtually complete under physiological conditions (135).

ii) Pseudomonas Exotoxin A

Pseudomonas exotoxin A, produced by Pseudomonas aeruginosa, is secreted as a single peptide chain (251,252). Molecular weight determination based on nucleotide sequence information has assigned an Mr of 66,583 to exotoxin A (253). Like diphtheria toxin, this toxin inhibits protein synthesis in susceptible cells (226,254). In cell free systems, the inhibition of protein synthesis by exotoxin A is dependent on NAD^+ and results from the ADP-ribosylation of EF-2 (226,255). ADP-ribosylation by Pseudomonas aeruginosa exotoxin A occurs at the same site on EF-2 as by diphtheria toxin. This is indicated by the fact that reversal of the ADP-ribosylation of EF-2 may be catalyzed by either toxin, regardless of the specific toxin used for the forward reaction (226,255). Like diphtheria toxin, the native exotoxin A exhibits virtually no enzymatic activity (252). Denaturation of the toxin with

urea plus thiol results in a large increase in ADP-ribosyltransferase activity (256). A form of exotoxin A (MW 25,000-27,000) that is catalytically active without denaturation has been isolated (252,257). Thus, exotoxin A appears to be similar to diphtheria toxin in molecular structure as well as in substrate specificity (226,258).

Some strains of P. aeruginosa produce, in addition to exotoxin A, another ADP-ribosyltransferase known as exoenzyme S (259). This protein is immunologically distinct from exotoxin A (259,260). Exoenzyme S catalyzes the ADP-ribosylation of a number of cellular proteins but not EF-2 (259). In contrast to Pseudomonas exotoxin A and diphtheria toxin, exoenzyme S is catalytically active without denaturation, reduction, or proteolytic digestion (259). Extracts of polyoma transformed baby hamster kidney cells (PyBHKR) can be used to distinguish the transferase activity of exoenzyme S from that of diphtheria toxin or Pseudomonas exotoxin A because these extracts do not contain any ADP-ribose acceptors for either of the latter two enzymes but do contain ADP-ribose acceptors for exoenzyme S (261).

References

1. Schimke, R.T. (1982). In R.T. Schimke (Ed.). Gene Amplification. Cold Spring Harbor Laboratory. pp. 317-333.

2. Tartof, K.D. (1975). Redundant genes. Ann. Rev. Gen. 9:355-385.

3. Brown, D.D. and Dawid, Igor B. (1968). Specific gene amplification in oocytes. Science. 160:272-280.

4. Wahl, G.M., Padgett, R.A., and Stark, G.R. (1979). Gene amplification causes overproduction of the first three enzymes of UMP synthesis in N-(phosphonacetyl)-L-aspartate-resistant hamster cells. J. Biol. Chem. 254:8679-8689.

5. Lewin, B. (1980). Somatic cell mutants. In Gene Expression 2. Vol. 2. Eucaryotic chromosomes. John Wiley and Sons, New York, pp. 142-188.

6. Walker, R. (1983). Structural organization of DNA in the nucleus of the eucaryotic cell. The Molecular Biology of Enzyme Synthesis. John Wiley and Sons, New York, pp. 38-61.

7. Samal, B., Worcel, A., Louis, C., and Schedl, P. (1981). Chromatin structure of the histone genes of D. melanogaster. Cell 23:401-409.

8. Wu, C. (1980). The 5' ends of Drosophila Heat Shock Genes in chromatin are hypersensitive to DNase I. Nature 286:854-860.

9. Gottesfeld, J.M. and Bloomer, L.S. (1980). Nonrandom alignment of nucleosomes on 5S RNA genes of X. Laevis. Cell 21:751-760.

10. Garel, A. and Axel, R. (1976). Selective digestion of transcriptionally active ovalbumin genes from oviduct nuclei. Proc. Natl. Acad. Sci. USA 73:3966-3970.

11. Weintraub, H. and Groudine, M. (1976). Chromosomal subunits in active genes have an altered conformation. Science 193:848-856.

12. Stalder, J., Groudine, M., Dodgson, J.B., Engel, J.D., Weintraub, H. (1980). Hb switching in chickens. Cell 19:973-980.

13. Stalder, J., Larsen, A., Engel, J.D., Dolan, M., Groudine, M., and Weintraub, H. (1980). Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNase I. Cell 20:451-460.

14. Wu, C. and Gilbert, W. (1981). Tissue-specific exposure of chromatin structure at the 5' terminus of the rat Preproinsulin II Gene. Proc. Natl. Acad. Sci. USA. 78:1577-1580.

15. Davie, J.R. and Candido, E.P.M. (1980). DNase I sensitive chromatin is enriched in the acetylated species of histone H4. FEBS. Lett. 110:164-168.

16. Kuo, M.T., Sahasrabuddhe, C.G., and Saunders, G.F. (1976). Presence of messenger specifying sequences in the DNA of chromatin subunits. Proc. Natl. Acad. Sci. USA. 73:1572-1575.

17. Lacy, E. and Axel, R. (1975). Analysis of DNA of isolated chromatin subunits. Proc. Natl. Acad. Sci. USA. 71:3978-3982.

18. Davie, J.R. and Candido, E.P.M. (1978). Acetylated histone H4 is preferentially associated with template-active chromatin. Proc. Natl. Acad. Sci. USA. 75:3574-3577.

19. ViDali, G., Boffa, L.C., Bradbury, E.M., and Allfrey, V.G. (1978). Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. Proc. Natl. Acad. Sci. USA. 75:2239-2243.

20. Hodo III, H.G., Sahasrabuddhe, C.G., Plishker, M.F., and Saunders, G.F. (1980). Use of RNA polymerase as an enzymatic probe of nucleosomal structure. Nuc. Acids Res. 8:3851-3864.

21. Wasylyk, B., Thevenin, G., Oudet, P., and Chambon, P. (1979). Transcription of in vitro assembled chromatin by Escherichia coli RNA polymerase. J. Mol. Biol. 128:411-440.

22. Wasylyk, B. and Chambon, P. (1980). Studies on the mechanism of transcription of nucleosomal complexes. Eur. J. Biochem. 103:219-226.

23. Chao, M.V., Gralla, J.D., and Martinson, H.G. (1980). Lac Operator Nucleosomes. 1. Repressor binds specifically to operator within the nucleosome core. Biochemistry 19:3254-3260.

24. Chambon, P. Eucaryotic nuclear RNA polymerases. (1975). Ann. Rev. Biochem. 44:613-638.

25. Breathnach, R. and Chambon, P. (1981). Organization and expression of eucaryotic split genes coding for proteins. Ann. Rev. Biochem. 50:349-383.

26. Benoist, C. and Chambon, P. (1981). In vivo sequence requirements of the SV40 early promoter region. Nature 290:304-310.

27. Mathis, D.J. and Chambon, P. (1981). The SV40 early region TATA box is required for accurate in vitro initiation of transcription. Nature 290:310-315.

28. Tsai, S.Y., Tsai, M-J., and O'Malley, B.W. (1981). Specific 5' flanking sequences are required for faithful initiation of in vitro transcription of the ovalbumin gene. Proc. Natl. Acad. Sci. USA. 78:879-883.

29. Grosschedl, R. and Birnstiel, M.L. (1980). Spacer DNA sequences upstream of the TATAAATA sequence are essential for promotion of H2A histone gene transcription in vivo. Proc. Natl. Acad. Sci. USA. 77:7102-7106.

30. Walker, R. (1983). Structural organization of DNA in the nucleus of the eucaryotic cell. The Molecular Biology of Enzyme Synthesis. John Wiley and Sons, New York, pp. 62-90.

31. Sakonju, S., Bogenhagen, D.F., and Brown, D.D. (1980). A control region in the center of the 5S RNA gene directs specific initiation of transcription: 1. The 5' border of the region. Cell 9:13-25.

32. Engelke, D.R., Ng, S-Y., Shastry, B.S., and Roeder, R.G. (1980). Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. Cell 19:717-728.

33. Bogenhagen, D.F., Sakonju, S., and Brown, D.D. (1980). A control region in the center of the 5S RNA gene directs specific initiation of transcription: II. The 3' border of the region. Cell 19:27-35.

34. Pelham, H.R.B. and Brown, D.D. (1980). A specific transcription factor that can bind either the 5S RNA gene or 5S RNA. Proc. Natl. Acad. Sci. USA 77:4170-4174.

35. Segall, J., Matsui, T., and Roeder, R.G. (1980). Multiple factors are required for the accurate transcription of purified genes by RNA polymerase III. J. Biol. Chem. 255:11986-11991.

36. Matsui, T., Segall, J., Weil, P.A., and Roeder, R.G. (1980). Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. J. Biol. Chem. 255:11992-11996.

37. Elgin, S.C.R. and Weintraub. H. (1975). Chromosomal proteins and chromatin structure. Ann. Rev. Biochem. 44:725-774.

38. Ajiro, K., Borun, T.W., and Cohen, L.H. (1981). Phosphorylation states of different histone 1 subtypes and their relationship to chromatin functions during the HeLa S-3 cell cycle. Biochemistry 20:1445-1454.

39. Ajiro, K., Borun, T.W., Shulman, S.D., McFadden, G.M., and Cohen, L.H. (1981). Comparison of the structures of human histones 1A and 1B and their intramolecular phosphorylation sites during the HeLa S-3 cell cycle. Biochemistry 20: 1454-1464.

40. Halleck, M.S. and Gurley, L.R. (1980). Histone H2A subfractions and their phosphorylation in cultured Peromyscus Cells. Exp. Cell Res. 125:377-388.

41. Sikorska, M., MacManus, J.P., Walker, R.P., and Whitfield, J.F. (1980). The protien kinases of rat liver nuclei. Biochem. Biophys. Res. Comm. 93:1196-1203.

42. Wolff, D.J., Ross, J.M., Thompson, P.N., Brostrom, M.A., and Brostrom, C.O. (1981). Interaction of calmodulin with histones. Alteration of histone dephosphorylation. J. Biol. Chem. 256:1846-1860.

43. Allfrey, V.G. (1980). Molecular aspects of the regulation of eukaryotic transcription: nucleosomal proteins and their postsynthetic modifications in the contol of DNA conformation and template function. In Goldstein, L. and Prescott, D.M. (Eds.) Cell Biology: A Comprehensive Treatise Vol. 3. Academic Press, New York, pp. 347-437.

44. Bode, J., Henco, K., and Wingender, E. (1980). Modulation of the nucleosome structure by histone acetylation. Eur. J. Biochem. 110:143-152.

45. Nishizuka, Y., Ueda, K., Honjo, T., and Hayaishi, O. (1968). Enzymic adenosine diphosphate ribosylation of histone and polyadenosine diphosphate ribose synthesis in rat liver nuclei. J. Biol. Chem. 243:3765-3767.

46. Janakidei, K. and States, J.C. (1980). Stabilization of chromatin by poly ADP-ribosylation. In M.E. Smulson and T. Sugimura (Eds.). Novel ADP-ribosylations of Regulatory Enzymes and Proteins. Elsevier, North Holland, pp. 111-120.

47. Hayaishi, O. and Ueda, K. (1982). Poly-and mono(ADP-ribosyl)ation reactions: their significance in molecular biology. In O. Hayaishi and K. Ueda (Eds.). ADP-Ribosylation Reactions. Academic Press, New York, pp. 3-16.

48. Yoshihara, K. (1972). Complete dependency of poly(ADP-Ribose) synthesis on DNA and its inhibition by actinomycin D. Biochem. Biophys. Res. Comm. 47:119-125.

49. Okayama, H., Edson, C.M., Fukushima, M., Ueda, K., and Hayaishi, O. (1977). Purification and properties of poly(adenosine diphosphate ribose) synthetase. Role of histone in poly(ADP-ribose) synthesis. J. Biol. Chem. 252:7000-7005.

50. Ohgushi, H., Yoshihara, K., and Kamiya, T. (1980). Bovine thymus poly(adenosine diphosphate ribose) polymerase. Physical properties and binding to DNA. J. Biol. Chem. 255:6205-6211.

51. Ueda, K., Kawaichi, M., and Hayaishi, O. (1982). Poly(ADP-ribose) synthetase. In O. Hayaishi and K. Ueda (Eds.). ADP-ribosylation Reactions. Academic Press, New York, pp. 117-155.

52. Roberts, J.H., Stark, P., Giri, C.P. and Smulson, M. (1975). Cytoplasmic poly(ADP-ribose) polymerase during the HeLa cell cycle. Arch. Biochem. Biophys. 171:305-315.

53. Yoshihara, K., Hashida, T., Tanaka, Y., Matsunami, N., Yamaguchi, A., and Kamiya, T. (1981). Mode of enzyme-bound poly(ADP-ribose) synthesis and histone modification by reconstituted poly(ADP-ribose) polymerase-DNA-cellulose complex. J. Biol. Chem. 256:3471-3478.

54. Kawaichi, M., Ueda, K., and Hayaishi, O. (1980). Initiation of poly(ADP-ribosyl) histone synthesis by poly(ADP-ribose) synthetase. J. Biol. Chem. 255:816-819.

55. Stone, P.R., Lorimer III, W.S., and Kidwell, W.R. (1977). Properties of the complex between histone H1 and Poly(ADP-ribose) synthesized in HeLa cell nuclei. Eur. J. Biochem. 81:9-18.

56. Smith, J.A. and Stocken, L.A. (1973). Identification of poly(ADP-ribose) covalently bound to histone F1 in vivo. Biochem. Biophys. Res. Comm. 54:297-300.

57. Ueda, K., Omachi, A., Kawaichi, M., and Hayaishi, O. (1975). Natural occurrence of poly(ADP-ribosyl) histones in rat liver. Proc. Natl. Acad. Sci. USA 72:205-209.

58. Ueda, K., Kawaichi, M., Okayama, H., and Hayaishi, O. (1979). Poly(ADP-ribosyl)ation of nuclear proteins. Enzymatic elongation of chemically synthesized ADP-ribose-histone adducts. J. Biol. Chem. 254:679-687.

59. Riquelme, P.T., Burzio, L.O., and Koide, S.S. (1979). ADP-ribosylation of rat liver lysine-rich histone in vitro. J. Biol. Chem. 254:3018-3028.

60. Burzio, L.O., Riquelme, P.T., and Koide, S.S. (1979). ADP-ribosylation of rat liver nucleosomal core histones. J. Biol. Chem. 254:3029-3037.

61. Ogata, N., Ueda, K., and Hayaishi, O. (1980). ADP-ribosylation of histone H2B. Identification of glutamic acid residue 2 as the modification site. J. Biol. Chem. 255:7610-7615.

62. Smulson, M., Butt, T., Nolan, N., Jump, D., and Decoste, B. (1980). Poly ADP-ribosylation leads to condensation of polynucleosomes in chromatin. In M.E. Smulson and T. Sugimura (Eds.). Novel ADP-ribosylations of Regulatory Enzymes and Proteins. Elsevier, North Holland, pp. 59-71.

63. Butt, T. and Smulson, M. (1982). Chromatin structure and poly(ADP-ribosyl)ation. In O. Hayaishi and K. Ueda (Eds.). ADP-Ribosylation Reactions. Academic Press, New York, pp. 173-191.

64. Butt, T.R. and Smulson, M. (1980). Relationship between nicotinamide adenine dinucleotide concentration and in vitro synthesis of poly (adenosine diphosphate ribose) on purified nucleosomes. Biochemistry. 19:5235-5242.

65. Halldorsson, H., Gray, D.A., and Shall, S. (1978). Poly (ADP-ribose) polymerase activity in nucleotide permeable cells. FEBS. Lett. 85:349-352.

66. Juarez-Salinas, H., Sims, J.L., and Jacobson, M. (1979). Poly(ADP-ribose) levels in carcinogen-treated cells. Nature. 282:740-741.

67. Wielckens, K., Schmidt, A., George, E., Bredehorst, R., and Hilz, H. (1982). DNA fragmentation and NAD depletion. Their relation to the turnover of endogenous mono(ADP-ribosyl) and poly(ADP-ribosyl) proteins. J. Biol. Chem. 257:12872-12877.

68. Wielckens, K., George, E., Pless, T., and Hilz, H. (1983). Stimulation of poly(ADP-ribosyl)ation during Erlich Ascites Tumor Cell "Starvation" and suppression of concomitant DNA fragmentation by benzamide. J. Biol. Chem. 258:4098-4104.

69. Durkacz, B.W., Omidiji, O., Gray, D.A., and Shall, S. (1980). $(ADP\text{-}ribose)_n$ participates in DNA excision repair. Nature. 283:593-596.

70. Creissen, D. and Shall, S. (1982). Regulation of DNA ligase activity by poly (ADP-ribose). Nature. 296:271-272.

71. Farzaneh, F., Shall, S., and Zalin, R. (1980). DNA strand breaks and poly(ADP-ribose) polymerase activity during chick muscle differentiation. In M.E. Smulson and T. Sugimura (Eds.). Novel ADP-ribosylations of Regulatory Enzymes and Proteins. Elsevier, North Holland, pp. 217-225.

72. Johnstone, A.P and Williams, G.T. (1982). Role of DNA breaks and ADP-ribosyl transferase activity in eucaryotic differentiation demonstrated in human lymphocytes. Nature. 300:368-370.

73. Rastl, E. and Swetly, P. (1978). Expression of poly(adenosine diphosphate-ribose) polymerase activity in erythroleukemic mouse cells during cell cycle and erythropoietic differentiation. J. Biol. Chem. 253:4333-4340.

74. Claycomb, W.C. (1976). Poly(adenosine diphosphate ribose) polymerase activity and nicotinamide adenine dinucleotide in differentiating cardiac muscle. Biochem. J. 154:387-393.

75. Pekala, P.H., Lane, M.D., Watkins, P.A., and Moss, J. (1981). On the mechanism of preadipocyte differentiation. Masking of poly(ADP-ribose) synthetase activity during differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. 256:4871-4876.

76. Kanai, M., Miwa, M., Kondo, T., Tanaka, Y., Nakayasu, M., and Sugimura, T. (1982). Involvement of poly(ADP-ribose) metabolism in induction of differentiation of HL-60 promyelocytic leukemia cells. Biochem. Biophys. Res. Comm. 105:404-411.

77. Terada, M., Fujiki, H., Marks, P.A., and Sugimura, T. (1979). Induction of erythroid differentiation of murine erythroleukemia cells by nicotinamide and related compounds. Proc. Natl. Acad. Sci. USA 76:6411-6414.

78. Lewin, B. (1980). Interrupted genes and RNA splicing. In Gene Expression 2. Vol. 2. Eucaryotic chromosomes. John Wiley and Sons, New York. pp. 761-864.

79. Williamson, R. (1980). The processing of hnRNA and its relation to mRNA. In Goldstein, L. and Prescott, D.M. (Eds.). Cell Biology: A Comprehensive Treatise Vol. 3. Academic Press, New York, pp. 547-562.

80. Crick, F. (1979). Split genes and RNA splicing. Science. 204:264-271.

81. Trapnell, B.C., Tolstoshev, P., and Crystal, R.G. (1980). Secondary structures for splice junctions in eucaryotic and viral messenger RNA precursors. Nuc. Acids Res. 8:3659-3672.

82. Murray, V. and Holliday, R. (1979). Mechanism for RNA splicing of gene transcripts. FEBS Lett. 106:5-7.

83. Rogers, J. and Wall, R. (1980). A mechanism for RNA splicing. Proc. Natl. Acad. Sci. USA 77:1877-1879.

84. Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L., and Steitz, J.A. (1980). Are snRNPs involved in splicing? Nature 283:220-224.

85. Hamer, D.H., Smith, K.D., Boyer, S.H., and Leder, P. (1979). SV40 recombinants carrying rabbit β-globin gene coding sequences. Cell 17:725-735.

86. Lai, C.-J. and Khoury, G. (1979). Deletion mutants of Simian Virus 40 defective in biosynthesis of late viral mRNA. Proc. Natl. Acad. Sci. USA 76:71-75.

87. Khoury, G., Gruss, P., Dhar, R., and Lai, C.-J. (1979). Processing and expression of early SV40 mRNA: a role for RNA conformation in splicing. Cell 18:85-92.

88. Gruss, P., Lai, C.-J., Dhar, R., and Khoury, G. (1979). Splicing as a requirement for biogenesis of functional 16S mRNA of Simian Virus 40. Proc. Natl. Acad. Sci. USA. 76:4317-4321.

89. Erikson, R.L. (1980). The expression of animal virus genes. In L. Goldstein and D.M. Prescott. (Eds.). Cell Biology: A Comprehensive Treatise Vol. 3. Academic Press, New York, pp. 265-300.

90. Berk, A.J. and Sharp, P.A. (1978). Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. USA. 75:1274-1278.

91. Ziff, E.B. (1980). Transcription and RNA processing by the DNA tumour viruses. Nature. 287:491-499.

92. Walker, R. (1983). Regulation by post-transcriptional modification of RNA transcripts. The Molecular Biology of Enzyme Synthesis. John Wiley & Sons, New York, pp. 93-121.

93. Hagenbuchle, O., Tosi, M., Schibler, U., Bovey, R., Wellauer, P.K., and Young, R.A. (1981). Mouse liver and salivary gland α-amylase mRNAs differ only in 5' non-translated sequences. Nature. 289:643-646.

94. Shatkin, A.J. (1976). Capping of eucaryotic mRNAs. Cell. 9:645-653.

95. Perry, R.P. and Kelley, D.E. (1976). Kinetics of formation of 5' terminal caps in mRNA. Cell. 8:433-442.

96. Darnell, Jr., J.E. (1983). The processing of RNA. Scientific American. 249:90-100.

97. Brawerman, G. and Diez, J. (1975). Metabolism of the polyadenylate sequence of nuclear RNA and messenger RNA in mammalian cells. Cell. 5:271-280.

98. Brawerman, G. (1974). Eukaryotic messenger RNA. Ann. Rev. Biochem. 43:621-642.

99. Revel, M. and Groner, Y. (1978). Post-transcriptional and translational controls of gene expression in eukaryotes. Ann. Rev. Biochem. 47:1079-1126.

100. Brawerman, G. (1981). The role of the poly(A) sequence in mammalian messenger RNA. CRC Crit. Rev. in Biochem. 10:1-38.

101. Proudfoot, N.J. and Brownlee, G.G. (1974). Sequence at the 3' end of globin mRNA shows homology with immunoglobulin light chain mRNA. Nature. 252:359-362.

102. Fitzgerald, M. and Shenk, T. (1981). The sequence 5'-AAUAAA-3' forms part of the recognition site for polyadenylation of late SV40 mRNAS. Cell. 24:251-260.

103. Aho, S., Tate, V., and Boedtker, H. (1983). Multiple 3' ends of the chicken pro α 2(I) collagen gene. Nuc. Acids Res. 11:5443-5450.

104. Gall, J.G. and Callan, H.G. (1962). H^3 uridine incorporation in lampbrush chromosomes. Proc. Natl. Acad. Sci. USA. 48:562-570.

105. Martin, T.E., Pullman, J.M., and McMullen, M.D. (1980). Structure and function of nuclear and cytoplasmic ribonucleoprotein complexes. In D.M. Prescott and L. Goldstein (Eds.). Cell Biology: A Comprehensive Treatise. 4:137-174.

106. Beyer, A.L., Miller, Jr., O.L., and McKnight, S.L. (1980). Ribonucleoprotein structure in nascent hnRNA is nonrandom and sequence-dependent. Cell. 20:75-84.

107. Schweiger, A. and Kostka, G. (1980). Identification of a 110,000 molecular weight protein associated with heterogenous nuclear RNA and messenger RNA in rat liver cells. Exp. Cell Res. 125:211-219.

108. McMullen, M.D., Shaw, P.H., and Martin, T.E. (1979). Characterization of poly(A^+) RNA in free messenger ribonucleoprotein and polysomes of mouse Taper ascites cells. J. Mol. Biol. 132:679-694.

109. Kinniburgh, A.J., McMullen, M.D. and Martin, T.E. (1979). Distribution of cytoplasmic poly (A^+) RNA sequences in free messenger ribonucleoprotein and polysomes of mouse ascites cells. J. Mol. Biol. 132:695-708.

110. Ochoa, S. (1983). Regulation of protein synthesis initiation in eucaryotes. Arch. Biochem. and Biophys. 223:325-349.

111. Siekierka, J., Manne, V., Mauser, L., and Ochoa, S. (1983). Polypeptide chain initiation in eucaryotes: reversibility of the ternary complex-forming reaction. Proc. Natl. Acad. Sci. USA. 80:1232-1235.

112. Siekierka, J., Mauser, L., and Ochoa, S. (1982). Mechanism of polypeptide chain initiation in eukaryotes and its control by phosphorylation of the α subunit of initiation factor 2. Proc. Natl. Acad. Sci. USA. 79:2537-2540.

113. Bermek, E. (1978). Mechanisms in polypeptide chain elongation on ribosomes. Prog. Nuc. Acid Res. Molec. Biol. 21:64-100.

114. Arlinghaus, R., Favelukes, G., and Schweet, R. (1963). A ribosome-bound intermediate in polypeptide synthesis. Biochem. Biophys. Res. Comm. 11:92-96.

115. Kemper, W.M., Merrick, W.C., Redfield, B., Liu, C.-K., and Weissbach, H. (1976). Purification and properties of rabbit reticulocyte elongation factor 1. Arch. Biochem. Biophys. 174:603-612.

116. Golinska, B. and Legocki, A.B. (1973). Purification and some properties of elongation factor 1 from wheat germ. Biochim. et Biophys. Acta 324:156-170.

117. Moon, H.-M., Redfield, B., Millard, S., Vane, F., and Weissbach, H. (1973). Multiple forms of elongation factor 1 from calf brain. Proc. Natl. Acad. Sci. USA. 70:3282-3286.

118. Iwasaki, K., Nagata, S., Mizumoto, K., and Kaziro, Y. (1974). The purification of low molecular weight form of polypeptide elongation factor 1 from pig liver. J. Biol. Chem. 249:5008-5010.

119. Iwasaki, K., Motoyoshi, K., Nagata, S., and Kaziro, Y. (1976). Purification and properties of a new polypeptide chain elongation factor, EF-1β, from pig liver. J. Biol. Chem. 251:1843-1845.

120. Prather, N., Ravel, J.M., Hardesty, B., and Shive, W. (1974). Evidence for activities of rabbit reticulocyte elongation factor 1 analogous to bacterial factors EF-Ts and EF-Tu. Biochem. Biophys. Res. Comm. 57:578-583.

121. Galasinski, W. and Moldave, K. (1969). Purification of aminoacyltransferase II (translocation factor) from rat liver. J. Biol. Chem. 244:6527-6532.

122. Raeburn, S., Collins, J.F., Moon, H.M., and Maxwell, E.S. (1971). Aminoacyltransferase II from rat liver. 1. Purification and enzymatic properties. J. Biol. Chem. 246:1041-1048.

123. Mizumoto, K., Iwasaki, K., Tanaka, M., and Kaziro, Y. (1974). Studies on polypeptide elongation factor-2 from pig liver. I. Purification and properties. J. Biochem. 75:1047-1056.

124. Twardowski, T. and Legocki, A.B. (1973). Purification and some properties of elongation factor 2 from wheat germ. Biochim. et Biophys. Acta. 324:171-183.

125. Merrick, W.C., Kemper, W.M., Kantor, J.A., and Anderson, W.F. (1975). Purification and properties of rabbit reticulocyte protein synthesis elongation factor 2. J. Biol. Chem. 250:2620-2625.

126. Arlinghaus, R., Shaeffer, J., and Schweet, R. (1964). Mechanism of peptide bond formation in polypeptide synthesis. Proc. Natl. Acad. Sci. USA. 51:1291-1299.

127. Chuang, D.-M. and Weissbach, H. (1972). Studies on elongation factor II from calf brain. Arch. Biochem. Biophys. 152:114-124.

128. Comstock, J.P. and Van, N.T. (1977). Purification and characterization of homogeneous protein synthesis elongation factor 2 from hen oviduct. Biochim. et Biophys. Acta. 477:199-220.

129. Legocki, A.B. (1979). Elongation factor EF-2 from wheat germ: purification and properties. Meth. Enzymol. 60:703-712.

130. Iglewski, W.J. and Lee, H. (1983). Purification and properties of an altered form of elongation factor 2 from mutant cells resistant to intoxication by diphtheria toxin. Eur. J. Biochem. 134:237-240.

131. Mizumoto, K., Iwasaki, K., Kaziro, Y., Nojiri, C., and Yamada, Y. (1974). Studies on polypeptide elongation factor 2 from pig liver. II. Molecular weight and amino acid composition. J. Biochem. 75:1057-1062.

132. Robinson, E.A. and Maxwell, E.S. (1972). Chemical properties of elongation factor 2. J. Biol. Chem. 247:7023-7028.

133. Honjo, T., Nishizuka, Y., Hayaishi, O., and Kato, I. (1968). Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyltransferase II and inhibition of protein synthesis. J. Biol. Chem. 243:3553-3555.

134. Pappenheimer, Jr., A.M. (1977). Diphtheria toxin. Ann. Rev. Biochem. 46:69-94.

135. Collier, R.J. (1975). Diphtheria toxin: mode of action and structure. Bacteriol. Rev. 39:54-85.

136. Brown, B.A. and Bodley, J.W. (1979). Primary structure at the site in beef and wheat elongation factor 2 of ADP-ribosylation by diphtheria toxin. FEBS. Lett. 103:253-255.

137. Tiboni, O. and Ciferri, O. (1971). Selective inhibition of the reactions catalyzed by ribosome-specific transfer factors G. FEBS. Lett. 19:174-179.

138. Robinson, E.A., Henriksen, O., and Maxwell, E.S. (1974). Elongation factor 2. Amino acid sequence at the site of adenosine diphosphate ribosylation. J. Biol. Chem. 249:5088-5093.

139. Van Ness, B.G., Howard, J.B., and Bodley, J.W. (1980). ADP-ribosylation of elongation factor 2 by diphtheria toxin. Isolation and properties of the novel ribosyl-amino acid and its hydrolysis products. J. Biol. Chem. 255:10717-10720.

140. Van Ness, B.G., Howard, J.B., and Bodley, J.W. (1980). ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed strucures of ribosyl-diphthamide and its hydrolysis products. J. Biol. Chem. 255:10710-10716.

141. Skogerson, L. and Moldave, K. (1967). The binding of aminoacyl transferase II to ribosomes. Biochem. Biophys. Res. Comm. 27:568-572.

142. Skogerson, L. and Moldave, K. (1968). Characterization of the interaction of aminoacyltransferase II with ribosomes. Binding of transferase II and translocation of peptidyl transfer ribonucleic acid. J. Biol. Chem. 243:5354-5360.

143. Skogerson, L. and Moldave, K. (1968). Evidence for the role of aminoacyltransferase II in peptidyl transfer ribonucleic acid translocation. J. Biol. Chem. 243:5361-5367.

144. Baliga, B.S. and Munro, H.N. (1972). Evidence of formation of a complex between GTP and elongation factor two and the binding of the complex to a specific site on mammalian ribosomes. Biochim. et Biophys. Acta. 277:368-383.

145. Traugh, J.A. and Collier, R.J. (1971). Interaction of transferase II with the 60S ribosomal subunit. FEBS. Lett. 14:285-288.

146. Henriksen, O., Robinson, E.A., and Maxwell, E.S. (1975). Interaction of guanosine nucleotides with elongation factor 2. I. Equilibrium Dialysis Studies. J. Biol. Chem. 250:720-724.

147. Bodley, J.W., Lin, L., Salas, M.L., and Tao, M. (1970). Studies on translocation V: fusidic acid stabilization of a eukaryotic ribosome-translocation factor-GDP complex. FEBS. Lett. 11:153-156.

148. Weissbach, H. and Ochoa, S. (1976). Soluble factors required for eukaryotic protein synthesis. Ann. Rev. Biochem. 45:191-216.

149. Montanaro, L., Sperti, S., and Mattioli, A. (1971). Interaction of ADP-ribosylated aminoacyl-transferase II with GTP and with ribosomes. Biochim. et Biophys. Acta. 238:493-497.

150. Bermek, E. (1972). Formation of a complex involving ADP-ribosylated human translocation factor, guanosine nucleotide and ribosomes. FEBS. Lett. 23:95-99.

151. McKeehan, W. (1972). The ribosomal subunit requirements for GTP hydrolysis by reticulocyte polypeptide elongation factors EF-1 and EF-2. Biochem. Biophys. Res. Comm. 48:1117-1122.

152. Baliga, B.S., Schechtman, M.G., and Munro, H.N. (1973). Competitive binding of EF-1 and EF-2 by mammalian ribosomes. Role of GTP hydrolysis in overcoming inhibition by EF-2 of aminoacyl-tRNA binding. Biochem. Biophys. Res. Comm. 51:406-413.

153. Palmiter, R.D., Mulvihill, E.R., Shepherd, J.H., and McKnight, G.S. (1981). Steroid hormone regulation of ovalbumin and conalbumin gene transcription. A model based upon multiple regulatory sites and intermediary proteins. J. Biol. Chem. 256:7910-7916.

154. Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M., and Darnell, Jr., J.E. (1981). Transcriptional control in the production of liver-specific mRNAs. Cell. 23:731-739.

155. Fan, H. and Penman, S. (1970). Regulation of protein synthesis in mammalian cells. II. Inhibition of protein synthesis at the level of initiation during mitosis. J. Mol. Biol. 50:655-670.

156. Kay, J.E., Ahern, T., and Atkins, M. (1971). Control of protein synthesis during the activation of lymphocytes by phytohaemagglutinin. Biochim. et Biophys. Acta. 247:322-334.

157. Jagus, R. and Kay. J.E. (1979). Distribution of lymphocyte messenger RNA during stimulation by phytohaemagglutinin. Eur. J. Biochem. 100:503-510.

158. Rudland, P.S., Weil, S., and Hunter, A.R. (1975). Changes in RNA metabolism and accumulation of presumptive messenger RNA during transition from the growing to the quiescent state of cultured mouse fibroblasts. J. Mol. Biol. 96:745-766.

159. Davies, E., Dumont, J.E., and Vassart, G. (1978). Thyrotropin-stimulated recruitment of free monoribosomes on to membrane-bound thyroglobulin-synthesizing polyribosomes. Biochem. J. 172:227-231.

160. Kay, J.E., Ahern, T., Lindsay, V.J., and Sampson, J. (1975). The control of protein synthesis during the stimulation of lymphocytes by phytohaemagglutinin. III. Poly(U) translation and the rate of polypeptide chain elongation. Biochim. et Biophys. Acta. 378:241-250.

161. Hassell, J.A. and Engelhardt, D.L. (1976). The regulation of protein synthesis in animal cells by serum factors. Biochemistry 15:1375-1381.

162. Fischer, I., Arfin, S.M., and Moldave, K. (1980). Regulation of translation. Analysis of intermediary reactions in protein synthesis in exponentially growing and stationary phase Chinese Hamster Ovary cells in culture. Biochemistry. 19:1417-1425.

163. Zucker, W.V. and Schulman, H.M. (1968). Stimulation of globin-chain initiation by hemin in the reticulocyte cell-free system. Proc. Natl. Acad. Sci. USA. 59:582-589.

164. Adamson, S.D., Herbert, E., and Godchaux III, W. (1968). Factors affecting the rate of protein synthesis in lysate systems from reticulocytes. Arch. Biochem. Biophys. 125:671-683.

165. Legon, S., Jackson, R.J., and Hunt, T. (1973). Control of protein synthesis in reticulocyte lysates by haemin. Nature New Biology. 241:150-152.

166. Maxwell, C.R., Kamper, C.S., and Rabinovitz, M. (1971). Hemin control of globin synthesis: an assay for the inhibitor formed in the absence of hemin and some characteristics of its formation. J. Mol. Biol. 58:317-327.

167. Gross, M. and Rabinovitz, M. (1972). Control of globin synthesis in cell-free preparations of reticulocytes by formation of a translational repressor that is inactivated by hemin. Proc. Natl. Acad. Sci. USA. 69:1565-1568.

168. Gross, M. and Rabinovitz, M. (1972). Control of globin synthesis by hemin: factors influencing formation of an inhibitor of globin chain initiation in reticulocyte lysates. Biochim. et Biophys. Acta. 287:340-352.

169. Clemens, M.J., Henshaw, E.C., Rahamimoff, H., and London, I.M. (1974). $tRNA_f^{met}$ binding to 40S ribosomal subunits: a site for the regulation of initiation of protein synthesis by hemin. Proc. Natl. Acad. Sci. USA. 71:2946-2950.

170. Farrell, P.J., Balkow, K., Hunt, T., Jackson R.J. and Trachsel, H. (1977). Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. Cell. 11:187-200.

171. Levin, D. and London, I.M. (1978). Regulation of protein synthesis: activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2. Proc. Natl. Acad. Sci. USA. 75:1121-1125.

172. Levin, D.H., Ranu, R.S., Ernst, V., London, I.M. (1976). Regulation of protein synthesis in reticulocyte lysates: phosphorylation of methionyl-tRNA$_f$ binding factor by protein kinase activity of translational inhibitor isolated from heme-deficient lysates. Proc. Natl. Acad. Sci. USA. 73:3112-3116.

173. Kramer, G., Cimadevilla, J.M., and Hardesty, B. (1976). Specificity of the protein kinase activity associated with the hemin-controlled repressor of rabbit reticulocyte. Proc. Natl. Acad. Sci. USA. 73:3078-3082.

174. Ernst, V., Levin, D.H., Leroux, A., and London, I.M. (1980). Site-specific phosphorylation of the α subunit of eukaryotic initiation factor eIF-2 by the heme-regulated and doubled-stranded RNA-activated eIF-2α kinases from rabbit reticulocyte lysates. Proc. Natl. Acad. Sci. USA. 77:1286-1290.

175. Revel, M. and Groner, Y. (1978). Post-transcriptional and translational controls of gene expression in eukaryotes. Ann. Rev. Biochem. 47:1079-1126.

176. Ochoa, S. and De Haro, C. (1979). Regulation of protein synthesis in eukaryotes. Ann. Rev. Biochem. 48:549-580.

177. Ochoa, S. (1979). Regulation of protein synthesis. CRC Crit. Rev. Biochem. 7:7-22.

178. Austin, S.A. and Clemens, M.J. (1980). Control of the initiation of protein synthesis in mammalian cells. FEBS. Lett. 110:1-7.

179. Amesz, H., Goumans, H., Haubrich-Morree, T., Voorma, H.O., and Benne, R. (1979). Purification and characterization of a protein factor that reverses the inhibition of protein synthesis by the heme-regulated translational inhibitor in rabbit reticulocyte lysates. Eur. J. Biochem. 98:513-520.

180. Leroux, A. and London, I.M. (1982). Regulation of protein synthesis by phosphorylation of eukaryotic initiation factor 2α in intact reticulocytes and reticulocyte lysates. Proc. Natl. Acad. Sci. USA. 79:2147-2151.

181. DeHaro, C., Datta, A., and Ochoa, S. (1978). Mode of action of the hemin-controlled inhibitor of protein synthesis. Proc. Natl. Acad. Sci. USA. 75:243-247.

182. DeHaro, C. and Ochoa, S. (1978). Mode of action of the hemin-controlled inhibitor of protein synthesis: studies with factors from rabbit reticulocytes. Proc. Natl. Acad. Sci. USA. 75:2713-2716.

183. Ranu, R.S. (1982). Regulation of eukaryotic protein synthesis by protein kinases that phosphorylate initiation factor eIF-2: further evidence for a common mechanism of inhibition of protein synthesis. Biochem. Biophys. Res. Comm. 107:828-833.

184. Siekierka, J., Mitsui, K.-I., and Ochoa, S. (1981). Mode of action of the heme-controlled translational inhibitor: relationship of eukaryotic initiation factor 2-stimulating protein to translation restoring factor. Proc. Natl. Acad. Sci. USA. 78:220-223.

185. Clemens, M.J., Pain, V.M., Wong, S.-T., and Henshaw. (1982). Phosphorylation inhibits guanine nucleotide exchange on eukaryotic initiation factor 2. Nature. 296:93-95.

186. Austin, S.A. and Kay, J.E. (1982). Translational regulation of protein synthesis in eukaryotes. Essays in Biochem. 18:79-120.

187. Kay, J.E., Benzie, C.R., Dicker, P., and Lindahl-Kiessling, K. (1978). Inhibition of initiation of protein synthesis in rabbit reticulocyte lysates by a factor present in lymphocyte cytoplasm. FEBS. Lett. 91:40-44.

188. Austin, S.A. and Kay, J.E. (1975). Defective initiation on natural messenger RNA by cell free systems from Krebs Ascites cells incubated at elevated temperatures. Biochim. et Biophys. Acta. 395:468-477.

189. Osterman, L.A. (1979). Participation of tRNA in regulation of protein biosynthesis at the translational level in eukaryotes. Biochimie. 61:323-342.

190. Pavlakis, G.N., Lockard, R.E., Vamvakopoulos, N., Rieser, L., Rajbhandary, U.L., and Vournakis, J.N. (1980). Secondary structure of mouse and rabbit α-and β-globin mRNAs:differential accessibility of α and β initiator AUG condons towards nucleases. Cell. 19:91-102.

191. Ben-Bassat, I., Mozel, M., and Ramot, B. (1974). Globin synthesis in iron-deficiency anemia. Blood. 44:551-555.

192. Lodish, H.F. (1974). Model for the regulation of mRNA translation applied to haemoglobin synthesis. Nature. 251:385-388.

193. Kabat, D. and Chappell, M.R. (1977). Competition between globin messenger ribonucleic acids for a discriminating initiation factor. J. Biol. Chem. 252:2684-2690.

194. DiSegni, G., Rosen, H., and Kaempfer, R. (1979). Competition between α - and β- globin messenger ribonucleic acids for eucaryotic initiation factor 2. Biochemistry. 18:2847-2854.

195. Nuss, D.L. and Koch, G. (1976). Variation in the relative synthesis of immunoglobulin G and non-immunoglobulin G proteins in cultured MPC-11 cells with changes in the overall rate of polypeptide chain initiation and elongation. J. Mol. Biol. 102:601-612.

196. Zähringer, J., Baliga, B.S., and Munro, H.N. (1976) Subcellular distribution of total poly(A)-containing RNA and ferritin-mRNA in the cytoplasm of rat liver. Biochem. Biophys. Res. Comm. 68:1088-1093.

197. Permutt, M.A. and Kipnis, D.M. (1972). Insulin biosynthesis. I. on the mechanism of glucose stimulation. J. Biol. Chem. 247:1194-1199.

198. Payvar, F. and Schimke, R.T. (1979). Methylmercury hydroxide enhancement of translation and transcription of ovalbumin and conalbumin mRNAs. J. Biol. Chem. 254:7636-7642.

199. Trachsel, H., Sonenberg, N., Shatkin, A.J., Rose, J.K., Leong, K., Bergmann, J.E., Gordon, J., and Baltimore, D. (1980). Purification of a factor that restores translation of vesicular stomatitis virus mRNA in extracts from poliovirus-infected HeLa cells. Proc. Natl. Acad. Sci. USA. 77:770-774.

200. Sonenberg, N., Skup, D., Trachsel, H., and Millward, S. (1981). In vitro translation in reovirus-and poliovirus-infected cell extracts. Effects of anti-cap binding protein monoclonal antibody. J. Biol. Chem. 256:4138-4141.

201. Storti, R.V., Scott, M.P., Rich, A., and Pardue, M.L. (1980). Translational control of protein synthesis in response to heat shock in D. Melanogaster cells. Cell 22:825-834.

202. Krüger, C. and Benecke, B.- J. (1981). In vitro translation of Drosophila heat-shock and non-heat-shock mRNAs in heterologous and homologous cell-free systems. Cell 23:596-603.

203. Scott, M.P. and Pardue, M.L. (1981). Translational control in lysates of Drosophila melanogaster cells. Proc. Natl. Acad. Sci. USA. 78:3353-3357.

204. Mukherjee, A.K., Guha, C., and Sarkar, S. (1981). The translational inhibitory 10S cytoplasmic ribonucleoprotein of chicken embryonic muscle is distinct from messenger ribonucleoproteins. FEBS. Lett. 127:133-138.

205. Wold, F. (1982). Posttranslational protein modifications: Perspectives and Prospectives. In B.C. Johnson (Ed.). Posttranslational Covalent Modifications of Proteins. Academic Press, New York, pp. 1-17.

206. Hayaishi, O. and Ueda, K. (1977). Poly(ADP-ribose) and ADP-ribosylation of proteins. Ann. Rev. Biochem. 46:95-116.

207. Vaughn, M. and Moss, J. (1981). Mono(ADP-ribosyl)transferases and their effects on cellular metabolism. Curr. Top. Cell. Reg. 20:205-246.

208. Mandel, P., Okazaki, H., and Niedergang, C. (1982). Poly(adenosine-diphosphate-ribose). Prog. Nuc. Acid Res. Molec. Biol. 27:1-51.

209. Moss, J., Stanley, S.J., and Watkins, P.A. (1980). Isolation and properties of an NAD-and Guanidine-dependent ADP-ribosyltransferase from turkey erythrocytes. J. Biol. Chem. 255:5838-5840.

210. Moss, J. and Stanley, S.J. (1981). Amino Acid-specific ADP-ribosylation. Identification of an arginine-dependent ADP-ribosyltransferase in rat liver. J. Biol. Chem. 256:7830-7833.

211. Moss, J and Stanley, S.J. (1981). Histone-dependent and histone-independent forms of an ADP-ribosyltransferase from human and turkey erythrocytes. Proc. Natl. Acad. Sci. USA. 78:4809-4812.

212. Moss, J. and Vaughan, M. (1980). Specificity of ADP-ribosylation by choleragen (cholera toxin), E. coli heat-labile enterotoxin and an arginine-and NAD-dependent avian erythrocyte transferase. In M.E. Smulson and T. Sugimura (Eds.). Novel ADP-Ribosylations of Regulatory Enzymes and Proteins. Elsevier, North Holland, pp. 391-399.

213. Moss, J. and Vaughan, M. (1982). Purification and properties of NAD:arginine ADP-ribosyltransferases from animal cells. In O. Hayaishi and K. Ueda (Eds.). ADP-ribosylation Reactions. Academic Press, New York, pp. 637-645.

214. Yost, D.A. and Moss, J. (1983). Amino acid-specific ADP-ribosylation. Evidence for two distinct NAD:arginine ADP-ribosyltransferases in turkey erythrocytes. J. Biol. Chem. 258:4926-4929.

215. Adamietz, P., Wielckens, K., Bredehorst, R., Lengyel, H., and Hilz, H. (1981). Subcellular distribution of mono(ADP-ribose) protein conjugates in rat liver. Biochem. Biophys. Res. Comm. 101:96-103.

216. Skala, H., Vibert, M., Weber, A., Kahn, A., and Dreyfus, J.-C. (1983). Protein ADP-ribosylation in rat liver cytosol. Biochem. Internat. 7:747-754.

217. Beckner, S.K. and Blecher, M. (1981). Endogenous and cholera toxin-catalyzed ADP-ribosylation of a plasma membrane protein of RL-PR-C cloned rat hepatocytes. Biochim. et Biophys. Acta. 673:477-486.

218. Reilly, T.M., Beckner, S., McHugh, E.M. and Blecher, M. (1981). Isoproterenol-induced ADP-ribosylation of a single plasma membrane protein of cultured differentiated RL-PR-C hepatocytes. Biochem. Biophys. Res. Comm. 98:1115-1120.

219. Hofstetter, W., Muhlebach, T., Lotscher, H.-R., Winterhalter, K.H., and Richter, C. (1981). ATP prevents both hydroperoxide-induced hydrolysis of pyridine nucleotides and release of calcium in rat liver mitochondria. Eur. J. Biochem. 117:361-367.

220. Moss, J. and Vaughn, M. (1979). Activation of adenylate cyclase by choleragen. Ann. Rev. Biochem. 48:581-600.

221. Katada, T. and Ui, M. (1981). Islet-activating protein. A modifier of receptor-mediated regulation of rat islet adenylate cyclase. J. Biol. Chem. 256:8310-8317.

222. Katada, T., and Ui, M. (1982). Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc. Natl. Acad. Sci. USA. 79:3129-3133.

223. Rodbell, M. (1980). The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature. 284:17-22.

224. Ross, E.M. and Gilman, A.G. (1980). Biochemical properties of hormone-sensitive adenylate cyclase. Ann. Rev. Biochem. 49:533-564.

225. Moss, J. and Vaughan, M. (1981). Mechanism of action of choleragen and E. coli heat-labile enterotoxin: activation of adenylate cyclase by ADP-ribosylation. Mol. Cell. Biochem. 37:75-90.

226. Iglewski, B.H. and Kabat, D. (1975). NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. USA. 72:2284-2288.

227. Ogata, N., Ueda, K., Kagamiyama, H., and Hayaishi, O. (1980). ADP-ribosylation of histone H1. Identification of glutamic acid residues 2, 14, and the COOH-terminal lysine residue as modification sites. J. Biol. Chem. 255:7616-7620.

228. Moss, J. and Vaughan, M. (1977). Mechanism of action of choleragen. Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. J. Biol. Chem. 252:2455-2457.

229. Moss, J. and Vaughan, M. (1978). Isolation of an avian erythrocyte protein possessing ADP-ribosyltransferase activity and capable of activating adenylate cyclase. Proc. Natl. Acad. Sci. USA. 75:3621-3624.

230. Manning, D.R., Fraser, B.A., Kahn, R.A., Gilman, A.G. (1984). ADP-ribosylation of transducin by islet-activating protein. Identification of asparagine as the site of ADP-ribosylation. J. Biol. Chem. 259:749-756.

231. Oppenheimer, N.J. and Bodley, J.W. (1981). Diphtheria toxin. Site and configuration of ADP-ribosylation of diphthamide in elongation factor 2. J. Biol Chem. 256:8579-8581.

232. Collier, R.J. and Cole, H.A. (1969). Diphtheria toxin subunit active in vitro. Science. 164:1179-1182.

233. Honjo, T., Nishizuka, Y., Kato, I., and Hayaishi, O. (1971). Adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis by diphtheria toxin. J. Biol. Chem. 246:4251-4260.

234. Gill, D.M. and Dinius, L.L. (1971). Observations on the structure of diphtheria toxin. J. Biol. Chem. 246:1485-1491.

235. Collier, R.J. and Kandel, J. (1971). Structure and activity of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymatically active and inactive fragments. J. Biol. Chem. 246:1496-1503.

236. Uchida, T., Gill, D.M. and Pappenheimer, Jr., A.M. (1971). Mutation in the structural gene for diphtheria toxin carried by temperate phage β. Nature New Biology. 233:8-11.

237. Gill, D.M. and Pappenheimer, Jr., A.M. (1971). Structure-activity relationships in diphtheria toxin. J. Biol. Chem. 246:1492-1495.

238. Drazin, R., Kandel, J., and Collier, R.J. (1971). Structure and activity of diphtheria toxin. II. Attack by trypsin at a specific site within the intact toxin molecule. J. Biol. Chem. 246:1504-1510.

239. Strauss, N. and Hendee, E.D. (1959). The effect of diphtheria toxin on the metabolism of HeLa cells. J. Exp. Med. 109:145-163.

240. Collier, R.J. (1967). Effect of diphtheria toxin on protein synthesis: Inactivation of one of the transfer factors. J. Mol. Biol. 25:83-98.

241. Goor, R.S. and Pappenheimer, Jr., A.M. (1967). Studies on the mode of action of diphtheria toxin. III. Site of toxin action in cell-free extracts. J. Exp. Med. 126:899-912.

242. Gill, D.M., Pappenheimer, Jr., A.M., Brown, R., and Kurnick, J.T. (1969). Studies on the mode of action of diphtheria toxin. VII. Toxin-stimulated hydrolysis of nicotinamide adenine dinucleotide in mammalian cell extracts. J. Exp. Med. 129:1-21.

243. Baseman, J.B., Pappenheimer, Jr., A.M., Gill, D.M., and Harper, A.A. (1970). Action of diphtheria toxin in the guinea pig. J. Exp. Med. 132:1138-1152.

244. Goor, R.S., Pappenheimer, Jr., A.M., and Ames, E. (1967). Studies on the mode of action of diphtheria toxin. V. Inhibition of peptide bond formation by toxin and NAD in cell-free systems and its reversal by nicotinamide. J. Exp. Med. 126:923-939.

245. Moehring, T.J. and Moehring, J.M. (1968). Response of cultured mammalian cells to diphtheria toxin. III. Inhibition of protein synthesis studied at the subcellular level. J. Bact. 96:61-69.

246. Gill, D.M. and Dinius, L.L. (1973). The elongation factor 2 content of mammalian cells. Assay method and relation to ribosome number. J. Biol. Chem. 248:654-658.

247. Johnson, W., Kuchler, R.J., and Solotorovsky, M. (1968). Site in cell-free protein synthesis sensitive to diphtheria toxin. J. Bacteriol. 96:1089-1098.

248. Richter, D. and Lipmann, F. (1970). Separation of mitochondrial and cytoplasmic peptide chain elongation factors from yeast. Biochemistry. 9:5065-5070.

249. Goor, R.S. and Pappenheimer, Jr., A.M. (1967). Studies on the mode of action of diphtheria toxin. IV. Specificity of the cofactor (NAD) requirement for toxin action in cell-free systems. J. Exp. Med. 126:913-921.

250. Honjo, T. and Hayaishi, O. (1973). Enzymatic ADP-ribosylation of proteins and regulation of cellular activity. Curr. Top. Cell. Reg. 7:87-127.

251. Leppla, S.H. (1976). Large-scale purification and characterization of the exotoxin of Pseudomonas aeruginosa. Infect. Immun. 14:1077-1086.

252. Vasil, M.L., Kabat, D., and Iglewski, B.H. (1977). Structure-activity relationships of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16:353-361.

253. Gray, G.L., Smith, D.H., Baldridge, J.S., Harkins, R.N., Vasil, M.L., Chen, E.Y., and Heyneker, H.L. (1984). Cloning, nucleotide sequence, and expression in Escherichia coli of the exotoxin A structural gene of Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA. 81:2645-2649.

254. Pavlovskis, O.R. and Shackelford, A.H. (1974). Pseudomonas aeruginosa exotoxin in mice: localization and effect on protein synthesis. Infect. Immun. 9:540-546.

255. Iglewski, B.H., Liu, P.V., and Kabat, D. (1977). Mechanism of action of Pseudomonas aeruginosa exotoxin A: adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.

256. Leppla, S.H., Martin, O.C., and Muehl, L.A. (1978). The exotoxin of P. aeruginosa: A proenzyme having an unusual mode of activation. Biochem. Biophys. Res. Comm. 81:532-538.

257. Chung, D.W. and Collier, R.J. (1977) Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of Pseudomonas aeruginosa. Infect. Immun. 16:832-841.

258. Pavlovskis, O.R., Iglewski, B.H., and Pollack, M. (1978). Mechanism of action of Pseudomonas aeruginosa exotoxin A in experimental mouse infections: adenosine diphosphate ribosylation of elongation factor 2. Infect. Immun. 19:29-33.

259. Iglewski, B.H., Sadoff, J., Bjorn, M.J., and Maxwell, E.S. (1978). Pseudomonas aeruginosa exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc. Natl. Acad. Sci. USA. 75:3211-3215.

260. Bjorn, M.J., Pavlovskis, O.R., Thompson, M.R., and Iglewski, B.H. (1979). Production of exoenzyme S during Pseudomonas aeruginosa infections of burned mice. Infect. Immun. 24:837-842.

261. Sokol, P.A., Iglewski, B.H., Hager, T.A., Sadoff, J.C., Cross, A.S., McManus, A., Farber, B.F., and Iglewski, W.J. (1981). Production of exoenzyme S by clinical isolates of Pseudomonas aeruginosa. Infect. Immun. 34:147-153.

MANUSCRIPTS

Paper 1.

Purification and Properties of an Altered Form of Elongation Factor-2 from Mutant Cells Resistant to Intoxication by Diphtheria Toxin

Abstract

Elongation factor-2 (EF-2) was isolated from wild-type and mutant-polyoma-virus transformed baby hamster kidney cells resistant to intoxication by diphtheria toxin. Cells were grown as tumors in hamsters and EF-2 was purified from tissue homogenates by column chromatography. Both forms of EF-2 chromatograph identically in Whatman DE-52 DEAE-cellulose, Sephadex DEAE-A50 and Sephracryl S-200 resins. However, wild-type and the mutant form of EF-2 elute from phosphocellulose at 0.16 and 0.24 M KCl, respectively. Both forms of EF-2 migrate in sodium dodecyl sulfate-polyacrylamide gels as a single band with an Mr of 93,000 and produce identical ^{125}I labeled tryptic peptide maps. However, additional labeled tryptic peptides are seen when wild-type EF-2 is ADP-ribosylated by fragment A of diphtheria toxin. The purified mutant protein is totally resistant to ADP-ribosylation and cannot be transformed into an ADP-ribosylatable form in a posttranslational modification system *in vitro*, indicating that resistance to ADP-ribosylation results from a mutation in the structural gene for EF-2.

Introduction

Diphtheria toxin fragment A specifically inactivates eukaryotic elongation factor 2 (EF-2) by catalyzing its ADP-ribosylation (1,2). The only known substrate for the enzyme under the usual reaction conditions is EF-2. Bodley and coworkers have isolated the ADP-ribosylated peptide and find that ADP-ribose is attached to EF-2 via an unusual amino acid, diphthamide, which is not known to occur in other proteins (3). The proposed structure of diphthamide is 2-[3-carboxyamide-3-(trimethylammonio)propyl]histidine.

Diphtheria-toxin-resistant cell lines have been developed in cell culture from toxin-sensitive parental lines by cloning survivors of an exposure to toxin (4-6). These mutants fall into two classes: (a) resistant to intoxication at the level of the plasma membrane (7) and (b) resistant to ADP-ribosylation at the level of EF-2 (4). Resistance at the level of EF-2 can reside in the structural gene for EF-2 (4) or in a component of a posttranslational modification system that directs the synthesis of the diphthamide side chain (8). To date, the mutant forms of EF-2 have yet to be purified and characterized.

In this paper we describe the purification of a mutant form of EF-2 from polyoma virus transformed baby hamster kidney cells (PyBHKR). EF-2 in lysates of these cells can not be ADP-ribosylated by fragment A of diphtheria toxin (6). Some properties of the mutant form of EF-2 are compared to those of wild-type EF-2 purified from the parental polyoma virus transformed baby hamster kidney cells (PyBHK).

Materials and Methods

Cell culture conditions and tumor induction. Polyoma-virus-transformed baby hamster kidney cells (PyBHK) and a mutant strain which is resistant to intoxication by diphtheria toxin (PyBHKR) were grown in cell culture as previously described (6). Extracts of these toxin-resistant cells are not ADP-ribosylated by fragment A of diphtheria toxin (6). Tumors were induced by subcutaneous inoculation of golden hamsters with 10^6 PyBHK or PyBHKR cells. Distinct tumors were found in all animals by five to six weeks and were surgically removed. Tumors averaging 4 g were stored at -70°C.

Purification of elongation factor-2 from PyBHK and PyBHKR cells. Approximately 100 g of tumor tissue was rapidly thawed in a 37°C water bath. All following steps were carried out between 0-5°C. Thirty milliliters of fresh Littlefield's medium (9) plus 25 g of tissue were homogenized in a Waring blender with 4 to 8 bursts of homogenization of 3s each. After each burst the homogenate was examined with a microscope for free nuclei. The lysate was centrifuged for 1 h at 10,400 X g and the postmitochondrial supernatant was filtered through glass wool. Ammonium sulfate was added to the sample to 80% saturation at pH 7.5, then centrifuged after 1 h at 10,400 X g for 1 h. The precipitate was dissolved and dialyzed extensively against Buffer A (9).

The sample was clarified by centrifugation and applied to a 5 X 40 cm column of Whatman DE-52 cellulose equilibrated with Buffer A, non-adsorbed material flushed from the column with Buffer A and the sample eluted with an 800 ml linear gradient of 0 to 0.2 M KCl in Buffer A at 600 ml per h. Fractions were assayed for EF-2 and dialyzed against Buffer A. After dialysis, the sample was clarified by centrifugation and applied to a 2.5 X 20 cm column of Sephadex DEAE-A50 and eluted with a 400 ml linear gradient of 0 to 0.2 M KCl in Buffer A at 120 ml per h. The enzyme was located by assay and precipitated by adding $(NH_4)_2\ SO_4$ to 80% saturation. The precipitate was collected by centrifugation, dissolved and dialyzed against Buffer A. The sample in 5 ml of Buffer A was applied to a 2.5 X 80 cm column of superfine Sephacryl S-200 and developed with Buffer A at a flow rate of 60 ml per h. The fractions rich in enzyme were located by assay and pooled. The sample was applied to a 2.5 X 20 cm Whatman P11 phosphocellulose column and eluted with a 400 ml linear gradient of 0 to 0.4 M KCl in Buffer A at a rate of 60 ml

per h. Fractions rich in enzyme were located by assay, pooled, dialyzed against Buffer A and frozen at -70°C.

Assay for elongation factor-2. Wild-type EF-2 from PyBHK cell extracts and from discrete chromatographic fractions was measured by the method of Gill and Dinius (10). The specific transfer of [^{14}C]ADP-ribose from NAD to EF-2 was measured in a reaction containing 25 µl of 2 times concentrated histamine buffer (10), 25 µl of the EF-2 containing sample, 2 µg of purified fragment A of diphtheria toxin (11) in 10 µl and 0.125 µCi of [^{14}C]NAD in 5 µl. The reaction mixtures were incubated at 37°C for 15 min and the reaction was terminated by the addition of 5% trichloroacetic acid. The precipitates were collected by filtration on 0.45 µm filters and counted (12).

Since the mutant form of EF-2 from PyBHKR cell extracts cannot be ADP-ribosylated, it was assayed by its ability to reconstitute a reticulocyte cell-free protein synthesizing system deficient in endogenous functional EF-2. A cell-free protein synthesis system from rabbit reticulocytes was prepared as described by Pelham and Jackson (13). The reticulocyte lysate was treated with 37 µg per ml of diphtheria toxin fragment A and 50 µg per ml of NAD. The mixture was incubated 15 min at 20°C to ADP-ribosylate the endogenous EF-2 and to eliminate the ability of the lysate to incorporate [^{3}H]leucine into acid precipitable protein. The protein synthesizing activity of the lysate could be restored by adding lysate from PyBHKR cells but could not be restored by adding a lysate from PyBHK cells. The presence of the mutant form of EF-2 in chromatographic fractions was assayed by its ability to restore the protein-synthesizing activity of the fragment A-treated rabbit reticulocyte lysate. A 2-µl sample of each chromatographic fraction was

added to 10 µl reticulocyte lysate containing [^{3}H]leucine and incubated at 30°C for 30 min. The reaction was terminated by chilling the sample on ice and adding 0.1 ml leucine (10 mg/ml) and 0.5 ml 1M NaOH containing 0.5 M H_2O_2. The labeled protein was precipitated by adding 0.5 ml 50% trichloroacetic acid. The precipitates were collected on 0.45 µm filters and counted (13).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out in the Laemmli buffer system (16) containing 0.1% sodium dodecyl sulfate (SDS). Proteins were separated in 14.5 cm X 12.0 cm X 1.5 mm slab gels containing 7.5% or 10% acrylamide in the separating gel and 4.5 % acrylamide in the stacking gel. The ratio of acrylamide to bis-acrylamide was 30:1.2. Samples were boiled in sample buffer containing 10% SDS, 10% glycerol and 1% 2-mercaptoethanol prior to being applied to the gel. The samples were electrophoresed with a constant current of 20 mA for 6 hr.

Radioiodination of proteins with Iodogen. Purified protein was iodinated using the method of Fraker and Speck (17). The reaction contained 20 µg of Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril), 90 µl of protein to be labeled at a concentration of 1 mg/ml in Buffer A without dithiothreitol and 10 µl of KI at 1 mg/100 ml containing 15 µCi of ^{125}I. After 5 min the iodinated sample was dialyzed extensively against Buffer A.

Tryptic peptide analysis. Peptide maps were performed following a modified procedure described by Elder et al. (15). Proteins were labeled with ^{125}I, dialyzed and electrophoresed on SDS-polyacrylamide gels. The selected gel slice was digested in 50 µg/ml of tolysolfonyl phenylalanyl chloromethyl ketone trypsin for 24 hr at 37°C. Samples were oxidized

with performic acid for 4 hr at 0°C and subsequently diluted in 2 ml of water and lyophilized. The peptide digest was washed twice with distilled water, resuspended in electrophoresis solvent (28% formic acid), and spotted on cellulose thin layer chromatography plates (Brinkman CEL 300, 20 X 20 cm, 0.25 mm thick). Electrophoresis was conducted for 2.5 h at 300 V in the first dimension and ascending chromatography was performed in the second dimension in N-butanol:pyridine:glacial acetic acid:H_2O (35:25:5:20). Labeled peptides were visualized by autoradiography using Kodak X-Omat film.

Posttranslational modification system. A posttranslational modification system was prepared as described by Moehring et al. (8). Briefly, a cell extract of PyBHK cells was incubated at 30°C in the presence of an energy-generating system and the wild-type or mutant form of EF-2. Samples were tested prior to incubation and after 2 h incubation in the ADP-ribosylation assay.

Protein determination. Protein was determined by the method of Bradford (14) modified by using a commerical reagent, Bio Rad Protein Assay Dye Reagent Concentrate (Bio Rad). Lysozyme was used as the standard.

Radioactive reagents. [^{14}C]NAD labeled in the adenine moiety (25 μCi/ml, specific activity 265 mCi/mmol) and [4,5-^{3}H]L-leucine (56.8 Ci/mmole) were obtained from ICN (Irvine, CA).

Results and Discussion

Previous studies on the mutant forms of EF-2 have used mutant cells or crude cell extracts. Although these studies have been highly informative, a more detailed characterization of the structure of the mutant forms of EF-2 will require purification of the proteins. In the present study we use a virus transformed cell line which facilitates the selection of mutants in cell culture and the production of large quantities of cells as tumors in animals. Our purification method is in some respects similar to that which Raeburn et al. (9) used to purify EF-2 from rat liver. To monitor the steps in purification of the mutant form of EF-2, an assay method was developed which utilizes a cell-free protein synthesis system deficient in endogenous functional EF-2. This assay, together with the ADP-ribosylation assay, provide convenient and highly sensitive means of assaying for the two forms of EF-2.

Table 1 shows the total protein recovered after each step in the purification and the amount of elongation factor-2 recovered based on the ^{14}C-ADP-ribosylation assay. Approximately 11 mg of EF-2 is recovered from 100 g of tumor tissue. The yield of EF-2 from an equivalent amount of PyBHK cells grown in cell culture is similar but much more laborious and costly to prepare (data not shown). Cell extracts from tumors induced by the mutant PyBHKR yield similar amounts of the modified form of EF-2. There is a small amount of ^{14}C-ADP-ribosylated protein in the tumor extracts of the mutant cells which is eliminated following chromatography on phosphocellulose. This ADP-ribosylated protein presumably represents infiltration of the tumor tissue by wild-type cells from the host rather than reversion to wild-type PyBHK phenotype since tumor cells remain highly resistant to intoxication by diphtheria toxin

after many passages in cell culture or in hamsters. Purified preparation of wild-type and mutant EF-2 produce a single band on SDS-polyacrylamide gels with an apparent Mr of 93,000 (Figure 1). Wild-type and mutant forms of EF-2 have identical chromatographic behavior on all resins except phosphocellulose. Both proteins elute from Sephadex DEAE-A50 at a salt concentration of 0.12 M KCl and elute with the second of the two main bands of protein from Sephacryl S-200. However, wild-type and the mutant form of EF-2 elute from phosphocellulose at a salt concentration of 0.16 and 0.24 M KCl, respectively (Figure 2). The assays used clearly distinguish between the mutant and wild-type forms of the enzyme and the behavior on the phosphocellulose resin allows elimination of contaminating wild-type EF-2 from preparations of the mutant form of EF-2. The purified mutant form of EF-2 is completely resistant to ADP-ribosylation demonstrating that resistance of PyBHKR cells to intoxication by diphtheria toxin (6) resides at the level of the EF-2 molecule (Table 1 and Figure 2). The mutation resulting in resistance to ADP-ribosylation by diphtheria toxin appears to alter the charge on the molecule as demonstrated by its binding to phosphocellulose and may reflect a configurational change in the molecule or the alteration of the diphthamide residue. Results presented by Van Ness et al. (18) show that besides diphthamide other regions of the EF-2 molecule are required for the ADP-ribosylation to occur. The mutation in the EF-2 of PyBHKR cells does not affect protein synthesis since both cell lines have an identical rate of protein synthesis in cell culture (data not shown). Similar findings have been reported by Moehring et al. (19) for structural EF-2 mutants of Chinese hamster ovary cells.

The inability to ADP-ribosylate the mutant form of EF-2 could result from a structural mutation in the molecule or a defect in the posttranslational modification system in PyBHKR cells. To examine the latter possibility, the purified mutant form of EF-2 was incubated in a posttranslational modification system, previously described by Moehring et al. (8), derived from wild-type PyBHK cells (Table 2). An ADP-ribosylated form of the mutant EF-2 could not be generated in the in vitro system implying that the alteration in the EF-2 resides in the primary structure of the molecule.

It is not feasible to resolve the numerous peptides in a tryptic peptide map of a protein the size of EF-2. To simplify the peptide map analysis, only [^{125}I] labeled peptides are resolved. The preparations of wild-type and the mutant form of EF-2 produce identical maps of the [^{125}I] labeled peptides (Figures 3a and 3b). These findings further support our conclusion that the 93,000 dalton protein isolated from PyBHKR tumors is EF-2. Since the [^{125}I] labeled peptides of the wild-type and the mutant form of EF-2 are identical, these regions of the molecule do not appear to be involved in the enhanced binding of the mutant form of EF-2 to phosphocellulose.

In contrast, ADP-ribosylation of wild-type EF-2 prior to iodination with [^{125}I] results in a significant change in the number of sites available for iodination (Figure 3c). Several additional labeled peptides are detected in digests of ADP-ribosylated EF-2. Also one peptide of the ADP-ribosylated EF-2 does not seem to contain as much [^{125}I] as the comparable peptide in the unmodified form of EF-2. As a control, the [^{125}I] labeled peptides were analyzed from wild-type EF-2 which had undergone a mock ADP-ribosylation in the absence of fragment A.

Tryptic peptide maps are generated which are identical to those shown in Fig. 3A and B, indicating that the changes observed in Fig. 3C are due to ADP-ribosylation of EF-2. It appears that ADP-ribosylation results in an alteration in the availability of sites for iodination and may cause a configurational change in the molecule. ADP-ribosylated EF-2 is incapable of catalyzing GTP hydrolysis and translocation (20). Why this occurs is not understood. ADP-ribosylated EF-2 can bind GTP almost as effectively as unmodified EF-2 (21) and can bind to ribosomes in the presence of GTP (22). It is possible that a configurational change in EF-2 resulting from ADP-ribosylation may affect its ability to catalyze the translocation event.

References

1. Pappenheimer, A.M., Jr. (1977) Ann. Rev. Biochem. 46: 69-94.

2. Collier, R.J. (1975) Bacteriol. Rev. 39:54-85.

3. Van Ness, B.G., Howard, J.B. and Bodley, J.W. (1980) J. Biol. Chem. 255:10710-10716.

4. Moehring, T.J., Donley, D.E. and Moehring, J.M. (1979) Somat. Cell. Genet. 5:469-480.

5. Gupta, R.S. and Siminovitch, L. (1978) Somat. Cell Genet. 4:554-571.

6. Sokol, P.A., Iglewski, B.H., Hager, T.A., Sadoff, J.C., Cross, A.S., McManus, A., Farber, B.F. and Iglewski, W.J. (1981) Infect. Immun. 34:147-153.

7. Moehring, J.M. and Moehring, T.J. (1979) Somat. Cell Genet. 5:453-468.

8. Moehring, J.M., Moehring, T.J. and Donley, D.E. (1980) Proc. Natl. Acad. Sci. USA 77:1010-1014.

9. Raeburn, S., Collins, J.F., Moon, H.M. and Maxwell, E.S. (1971) J. Biol. Chem. 246:1041-1048.

10. Gill, D.M. and Dinius, L.L. (1973) J. Biol. Chem. 248:654-658.

11. Pappenheimer, A.M., Jr., Uchida, T. and Avery-Harpter, A. (1972) Immunochemistry 9:891-906.

12. Vasil, M.L., Kabat, D. and Iglewski, B.H. (1977) Infect. Immun. 16:353-361.

13. Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67:247-256.

14. Bradford, M.M. (1976) Anal. Biochem. 72:248-254.

15. Elder, J.H., Gantsche, J.W., Jansen, F.C., Lerner, R.A., Hartley, J.W. and Rowe, W.P. (1977) Proc. Natl. Acad. Sci. USA 74:4676-4680.

16. Laemmli, U.K. (1970) Nature 227:680-685.

17. Fraker, P.J. and Speck, J.C. (1978) Biochem. Biophys. Res. Comm. 80:849-857.

18. Van Ness, B.G., Barrowclough, B. and Bodley, J.W. (1980) FEBS Lett. 120:4-6.

19. Moehring, J.M. and Moehring, T.J. (1979) Somat. Cell. Genet. 5:153-168.

20. Weissbach, H. and Ochoa, S. (1976) Ann. Rev. Biochem. 45:191-216.

21. Montanaro, L., Sperti, S. and Mattioli, A. (1971) Biochim. Biophys. Acta 238:493-497.

22. Bermek, E. (1976) J. Biol. Chem. 251:6544-6549.

Table 1

Purification of Elongation Factor-2

	PyBHK Cells		PyBHKR Cells	
Step	Total protein (mg)	EF-2[a] (mg)	Total protein (mg)	EF-2[a] (mg)
$(NH_4)_2 SO_4$ precipitate	3,088	49.6	3,000	0.130
DEAE cellulose	597	42.4	520	N.D.[b]
Sephadex DEAE-A50	105	20.2	150	0.087
Sephacryl S-200	44	19.2	36	0.064
Phosphocellulose	12	11.0	10	0.0

[a] Wild-type EF-2 content was calculated from the amount of [^{14}C]ADP-ribose specifically transferred from NAD to the EF-2 in the sample by diphtheria toxin fragment A.

[b] N.D. - not done.

Table 2

Effect of a Posttranslational Modification System on ADP-ribosylation of Wild-type and Mutant Form of Elongation Factor-2

[^{14}C]ADP-Ribose Incorporation by Elongation Factor-2 (p moles)

Time (min)	Wild type EF-2	Wild type EF-2 + modification extract[a]	Mutant EF-2 + modification extract[a]
0	9.7	20.2	8.2
120	8.8	20.3	8.2

[a] The background ADP-ribosylation of the endogenous EF-2 in the extract is 10.5 pmol. Extracts received 9 pmol of exogenous EF-2.

Fig. 1 Polyacrylamide gel electrophoresis of purified wild-type and mutant forms of elongation factor-2. Gels containing 7.5% acrylamide and 0.1% sodium dodecyl sulfate were prepared and run as described. From left to right: (a) 5 μg of the mutant form of EF-2 from PyBHKR cells; (b) 5 μg of EF-2 from PyBHK cells; (c) molecular weight standards including phosphorylase B with an Mr of 93,000.

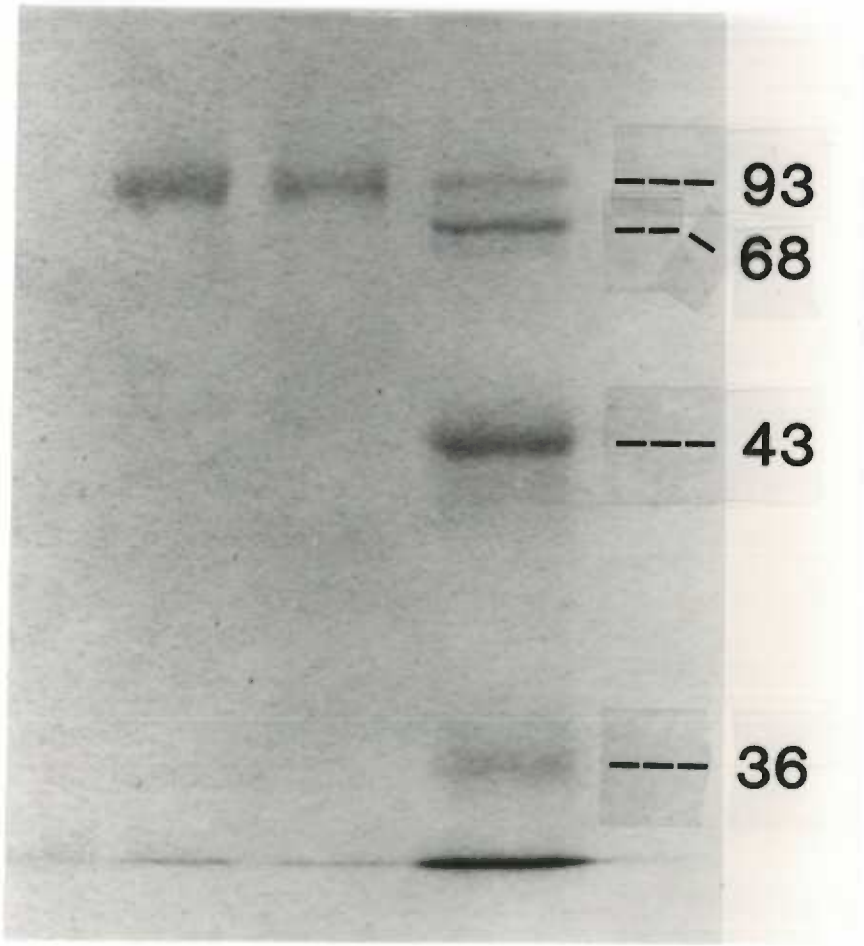

93
68
43
36

Fig. 2 Separation of wild-type from the mutant form of elongation factor-2 by chromatography on phosphocellulose. Both forms of EF-2 were purified as described under "Materials and Methods" and 30 μg of each protein were mixed together and chromatographed on a 1 x 4 cm phosphocellulose column. The proteins were eluted with a linear gradient 0 to 0.4 M KCl in Buffer A and assayed for [^{14}C]ADP-ribosylation of wild-type EF-2 and stimulation of *in vitro* protein synthesis by the mutant form of EF-2 as described under "Materials and Methods."

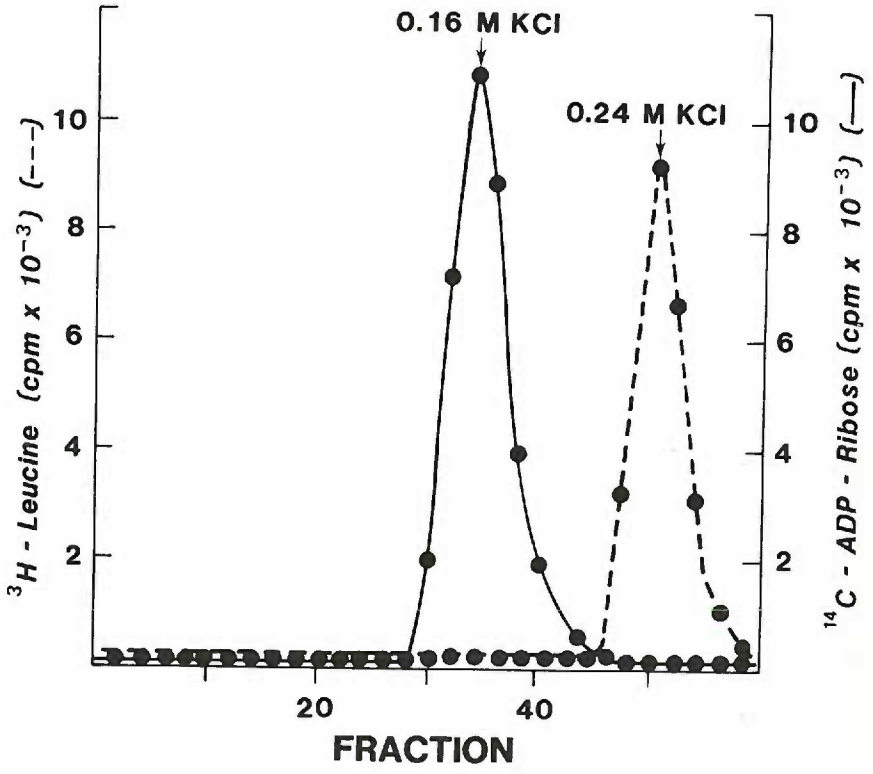
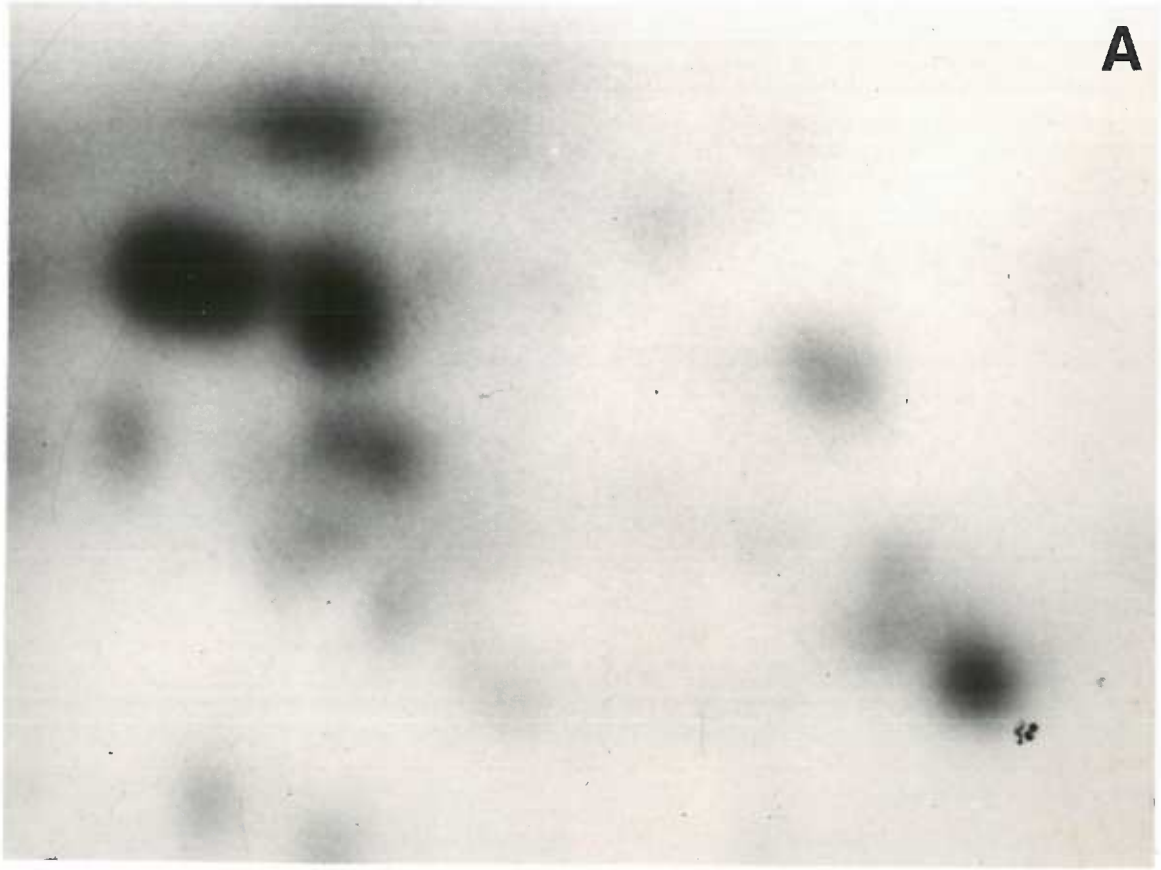

0.16 M KCl
0.24 M KCl
^3H - Leucine (cpm x 10^{-3}) (---)
^{14}C - ADP - Ribose (cpm x 10^{-3}) (—)
2
4
6
8
10
20
40
FRACTION

Fig. 3 Tryptic peptide analysis of [^{125}I] labeled elongation factor-2. The purified mutant form of EF-2, wild-type EF-2 and ADP-ribosylated EF-2 were iodinated with ^{125}I in the presence of Iodogen as described. The proteins were electrophoresed in 10% polyacrylamide gels and the labeled EF-2 was sectioned from the gel, digested with TPCK-trypsin and spotted on TLC plates as described. Electrophoresis was conducted in the vertical dimension from top to bottom and chromatography in the horizontal dimension from right to left. Proteins analyzed include: (a) EF-2 from mutant PyBHKR cells; (b) EF-2 from wild-type PyBHK cells; and (c) ADP-ribosylated EF-2 from PyBHK cells.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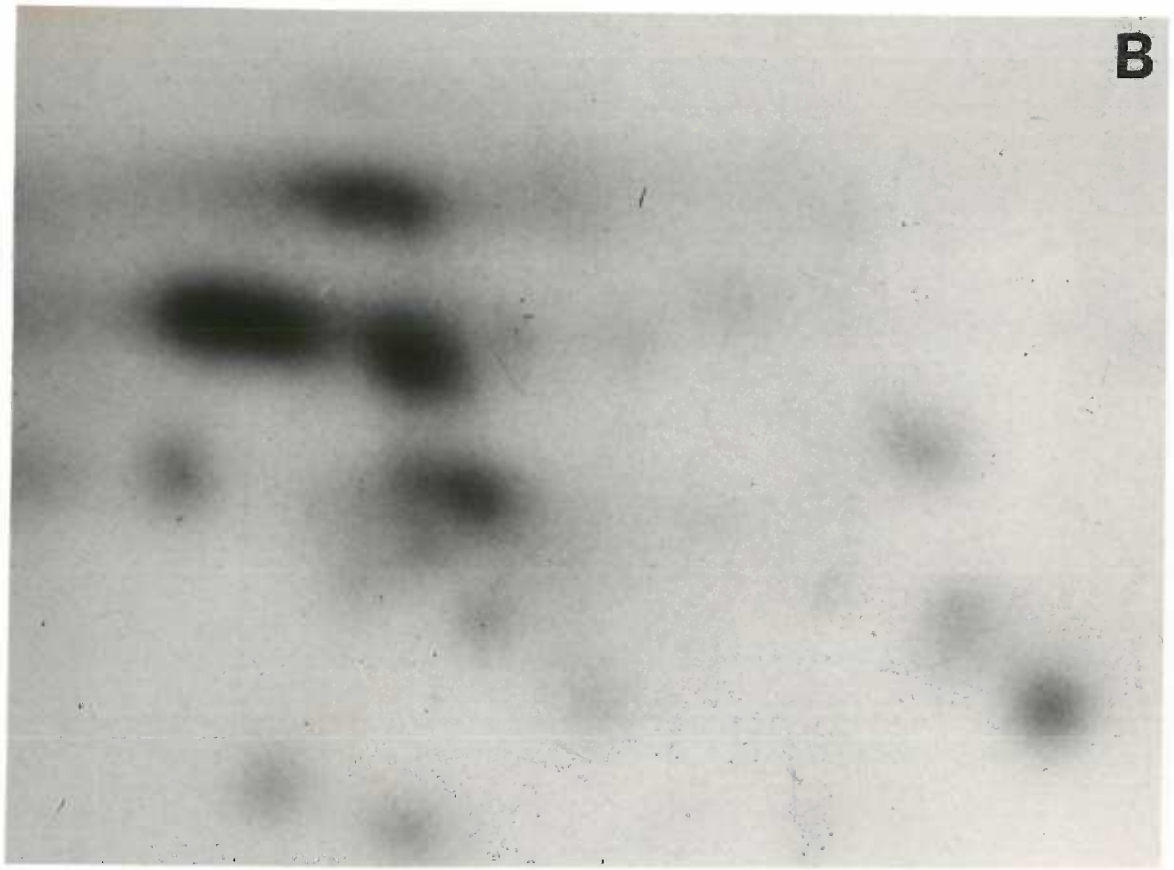
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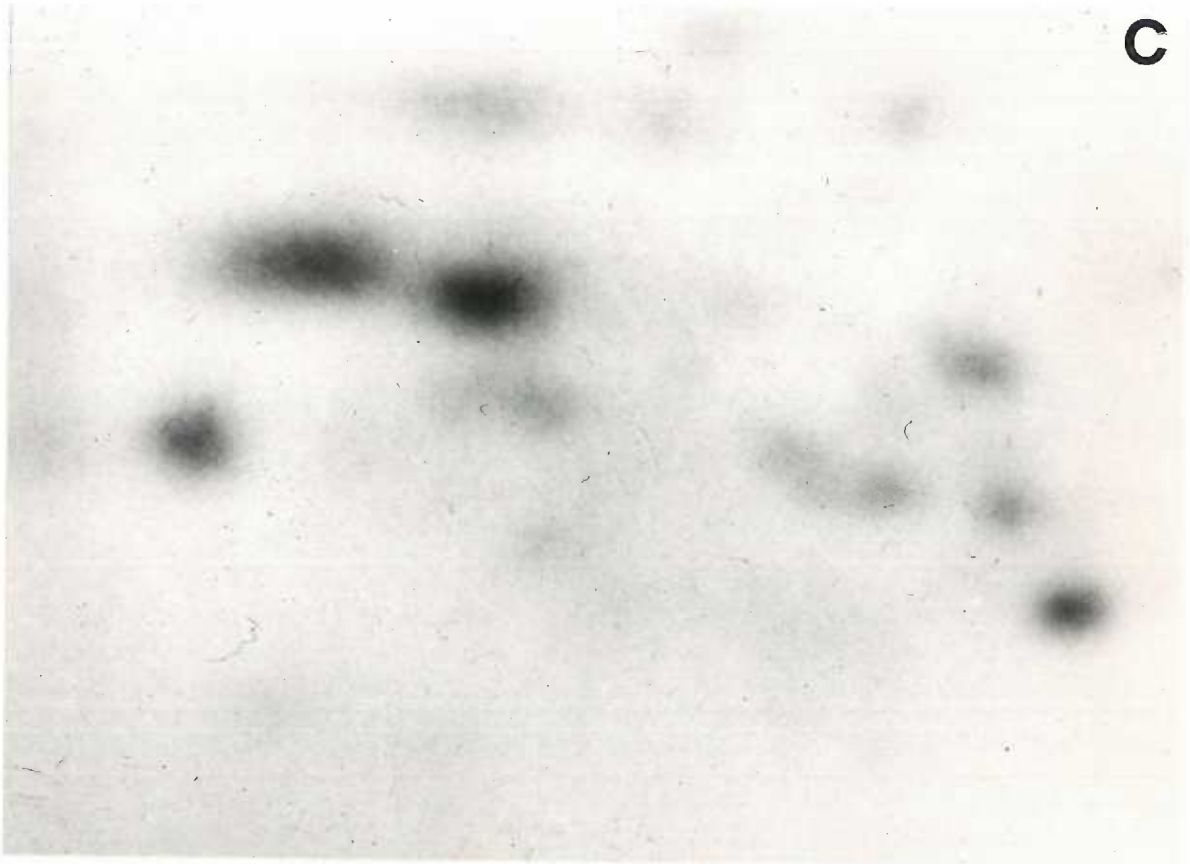

C

Paper 2.

Cellular ADP-ribosyltransferase with the Same Mechanism of Action as Diphtheria Toxin and *Pseudomonas* toxin A

ABSTRACT

An ADP-ribosyltransferase was found in elongation factor 2 (EF-2) preparations from polyoma virus-transformed baby hamster kidney (pyBHK) cells. Like fragment A of diphtheria toxin and Pseudomonas toxin A, this eukaryotic cellular enzyme transfers [^{14}C]adenosine from NAD^+ to EF-2. However, the cellular transferase is immunologically distinct from fragment A. The transferase also can be distinguished from fragment A and Pseudomonas toxin A by the inhibition of the activity of the former by cytoplasmic extracts and by histamine. Snake venom phosphodiesterase digestion of the [^{14}C]adenosine-labeled EF-2 product of the cellular transferase reaction yielded [^{14}C]AMP, indicating that the cellular enzyme is a mono(ADP-ribosyl)transferase. The forward ADP-ribosylation reaction catalyzed by the cellular enzyme is reversed by fragment A, yielding [^{14}C]NAD^+. The results strongly suggest that the cellular transferase is a mono(ADP-ribosyl)transferase, which ADP-ribosylates the same diphthamide residue of EF-2 as does fragment A and Pseudomonas toxin A.

Introduction

Mono(ADP-ribosyl)ated proteins have been found in a variety of eukaryotic tissues (1-3). These ADP-ribosylated proteins are present in practically every major compartment of the cell (4), suggesting a diversity of biological functions. However, little is known about the identity of these mono(ADP-ribosyl)ated acceptor proteins and their physiological functions. Moss and Vaughn (5) have described a cytosolic

ADP-ribosyltransferase from turkey erythrocytes that catalyzes the mono(ADP-ribosyl)ation of several endogenous proteins and the activation of brain adenylate cyclase. They were the first to suggest that the ADP-ribosyltransferase mechanisms of bacterial toxins, such as cholera toxin and heat-labile enterotoxin of Escherichia coli, are not entirely foreign to vertebrate cells.

The fragment A portion of diphtheria toxin and toxin A of Pseudomonas aeruginosa transfer ADP-ribose from NAD^+ to the diphthamide acceptor site of elongation factor 2 (EF-2) (6-10). ADP-ribosylated EF-2 no longer functions in protein synthesis, resulting in intoxication of the cells. In this paper we describe an endogenous mono(ADP-ribosyl)-transferase from polyoma virus-transformed baby hamster kidney (pyBHK) cells that also transfers ADP-ribose from NAD^+ to the diphthamide residue of EF-2.

Materials and Methods

Enzymatic Activity. EF-2 was purified from pyBHK cells as described (11). These EF-2 preparations also served as the source of the pyBHK transferase activity described in this report. Fragment A of diphtheria toxin was purified as described (12). ADP-ribosyltransferase activity was measured by the incorporation of radioactivity from [adenosine-U-^{14}C]NAD^+ into trichloroacetic acid-precipitable material in the presence of EF-2 by a modification of a previously described procedure (11,13). The modification was the absence of histamine and the use of purified EF-2 in the reaction. Unless otherwise noted, the reaction was performed at 22°C in 0.1 ml of 25 mM Tris HCl, pH 8.0/1 mM

dithiothreitol/6.33 μM[adenosine-U^{14}C]NAD$^+$ (534 mCi/mmol, New England Nuclear; 1 Ci = 37 GBq) containing EF-2 at 50 μg/ml and with or without various amounts of Pseudomonas toxin A, fragment A, or a mixture of fragment A and anti-fragment A antiserum. After various intervals of incubation, 10-μl samples were withdrawn and coprecipitated with 0.1 ml of bovine serum albumin at 0.1 mg/ml in 1 ml of 10% trichloroacetic acid. Precipitates were collected and washed, and the radioactivity was measured as described (11).

Analysis of Reaction Product. Radiolabeled products formed in the ADP-ribosyltransferase assays were electrophoresed in NaDodSO$_4$/polyacrylamide gels as described (11). Gels were dried and developed by autoradiography (11).

To determine if ADP-ribose was present in the acceptor protein as monomeric units or as poly(ADP-ribose), EF-2 was labeled by incubation for 160 min with [adenosine-U-^{14}C]NAD$^+$ and the endogenous pyBHK transferase or fragment A as described for our standard reaction mixture. The reaction mixture was dialyzed extensively to remove NAD$^+$, evaporated to dryness under nitrogen, and resuspended in 25 μl of 20 mM ammonium bicarbonate buffer (pH 9.0). Then 25 μl of snake venom phosphodiesterase (Worthington) at 1 mg/ml in 40 mM $MgCl_2$ was added (10), and the mixture was incubated at 37°C for 15 min, 30 min, and 6 hr. The digested products were then chromatographed on thin-layer polyethyleneimine (PEI)-cellulose plates (J.T. Baker) with 0.3 M lithium chloride as the solvent (14). AMP, adenosine, ADP-ribose, and NAD$^+$ (Sigma) were cochromatographed as markers. The chromatogram was exposed to x-ray film (Kodak XRP-5) to locate the ^{14}C-labeled products relative to the UV

light-absorbing markers. Radioactive material was scraped into vials and analyzed for radioactivity in a liquid scintillation spectrophotometer.

Reversal of the ADP-Ribosylation Reaction. Reverse reactions were done by incubating [U-^{14}C]adenosine-labeled EF-2 at pH 6.6 with an excess of fragment A (10 μg/ml) and 2 mM nicotinamide at 22°C as described (7). The radioactive products were analyzed by polyethyleneimine-cellulose thin-layer chromatography as described by Randerath and Randerath (15). The ^{14}C-labeled samples were cochromatographed with standards of NAD^+, AMP, adenosine, and ADP-ribose in 0.3 M LiCl. Radioactive compounds were located by autoradiography on x-ray film. Radioactive material was then scraped into vials and analyzed for radioactivity in a liquid scintillation spectrophotometer.

Results

Transfer of [^{14}C]Adenosine from NAD^+ to EF-2. Fragment A of diphtheria toxin rapidly ADP-ribosylated EF-2 in the presence of NAD^+ (Fig. 1). It was also possible to transfer [^{14}C]adenosine from NAD^+ to an acid-precipitable form in the absence of fragment A. Maximum transfer of label in the absence of fragment A occurred between 80 and 160 min, depending on the purified EF-2 preparation used, and always approached the maximum level of ADP-ribosylation catalyzed by fragment A. The transfer of [^{14}C]adenosine from NAD^+ to an acid-precipitable form suggested that our EF-2 preparations contained an unusual enzyme activity, which seemed to mimic the activity of fragment A. An autoradiogram of the [^{14}C]adenosine-labeled protein analyzed by

$NaDodSO_4$/polyacrylamide gel electrophoresis indicated that both fragment A and the endogenous enzyme in our EF-2 preparations tranferred most of the label to a protein with an apparent Mr of 93,000 (Fig. 2). We previously had characterized this protein in our preparations as EF-2 (11). The preparations of EF-2 also contained traces of products from the partial proteolysis of the EF-2 incurred during final steps in EF-2 purification and not during the ADP-ribosylation reaction. These products of partial proteolysis were readily detected in autoradiograms and were labeled identically with radioactivity by fragment A or the endogenous transferase in the EF-2 preparations. Similar labeling of ADP-ribosylated EF-2 degradation products by fragment A was observed by Van Ness et al. (16).

A comparison of tryptic peptides of EF-2 labeled in the presence of [adenosine-^{14}C]NAD^+ and fragment A or the endogenous transferase and then analyzed by thin-layer chromatography produced identical autoradiograms (data not shown). These data indicate that a similar peptide of the EF-2 molecule is labeled by both fragment A and the pyBHK endogenous transferase.

Properties of the Endogenous Transferase. Since large amounts of purified fragment A were used routinely in our laboratory, it was essential to eliminate the possibility that our EF-2 preparations were accidentally contaminated with traces of fragment A. Fragment A antibody greatly inhibited the transfer of label from NAD^+ to EF-2 in a fragment A-catalyzed reaction (Fig. 3A). In fact, increasing the antibody concentration 5-fold provided similar results (data not shown). The residual activity seen in the presence of anti-fragment A antiserum

is presumably due to the endogenous transferase activity present in the EF-2 preparation. In contrast, the fragment A antibody had essentially no effect on the endogenous transferase of the EF-2 preparations (Figure 3B), indicating that this ADP-ribosyltransferase associated with EF-2 is immunologically distinct from fragment A.

Two other properties clearly distinguish the pyBHK endogenous transferase from the known ADP-ribosyltransferases that specifically modify EF-2. First, addition of a charcoal-adsorbed cytoplasmic extract from pyBHK cells to purified EF-2 inhibited the endogenous transferase activity from the EF-2 preparations by 90% but had no effect on the ADP-ribosyltransferase activities of fragment A (Table 1). However, boiling the cytoplasmic extract prior to use did destroy the inhibitory effect on the endogenous transferase activity from the EF-2 preparation. Second, 0.25 M histamine almost totally inhibited the activity of the endogenous transferase but had no appreciable effect on the activity of either of the bacterial toxins (Table 1). Similar results were obtained at a histamine concentration of 0.15 M.

Endogenous Transferase is a Mono(ADP-Ribosyl)Transferase That Modifies Diphthamide of EF-2. Previously described bacterial ADP-ribosyltransferases have been shown to transfer mono(ADP-ribose) from NAD^+ directly to proteins (17). On the other hand, many eukaryotic ADP-ribosyltransferases yield poly(ADP-ribosyl)ated proteins (3). To determine if the EF-2 modified by the endogenous transferase contained monomers or polymers of ADP-ribose, we synthesized the product formed in the presence of the endogenous transferase, [adenosine-^{14}C]NAD^+, and EF-2 and then treated the labeled product with snake venom phosphodiesterase.

EF-2 modified by fragment A was used in an accompanying reaction mixture as a positive control. The digestion products were chromatographed and exposed to film. In both reactions the low molecular weight ^{14}C-labeled material chromatographed with AMP (Fig. 4). No radioactivity migrated with the marker ADP-ribose [which comigrates in the system with iso-ADP-ribose, the product of venom phosphodiesterase action on poly(ADP-ribose) (17)]. Therefore, the ADP-ribose is present on the acceptor in EF-2 as monomeric units, rather than as poly(ADP-ribose).

In addition, an extremely prolonged time of digestion (6 hr) resulted in the apparent conversion of [^{14}C]AMP to a ^{14}C-labeled material that comigrated with the adenosine marker (Fig. 4). This apparent conversion of AMP to adenosine represents a trace of contaminant enzyme activity in the snake venom phosphodiesterase preparation.

The ADP-ribosylation of EF-2 catalyzed by fragment A or Pseudomonas toxin A is reversible (7,18,19). This reverse reaction requires excess toxin (i.e., fragment A) and nicotinamide and has a lower pH optimum than that of the forward reaction. The reversibility of the forward reactions catalyzed by fragment A or the pyBHK endogenous mono(ADP-ribosyl)transferase are shown in Fig. 5. A preparation of [^{14}C]adenosine-labeled EF-2 containing a small amount of endogenous transferase (used in the forward reaction) was incubated in the absence or presence of excess fragment A and nicotinamide. Although little or no radioactivity was released from EF-2 in the absence of excess fragment A, over 60% of the radioactivity was released when both fragment A and nicotinamide were present in large excess. Similarly, radioactivity was released from EF-2 labeled in the forward reaction with fragment A and then reversed in the presence of excess fragment A and nicotinamide. The radioactive products of the

reverse reactions were analyzed by thin-layer chromatography. The low molecular weight soluble products found in the reverse reaction mixtures had the chromatographic behavior of NAD^+ (Fig. 6). In addition, the reaction mixtures contained some labeled EF-2, which remained at the origin.

Discussion

The mono(ADP-ribosyl)transferase from pyBHK cells can be distinguished from fragment A and Pseudomonas toxin A by the sensitivity of its activity to cytoplasmic extracts and to histamine. These properties may explain why similar cellular ADP-ribosyltransferases were not previously found by standard techniques. Crude preparations of EF-2 are commonly used to assay for fragment A and Pseudomonas toxin A, and histamine is commonly used to inhibit poly(ADP-ribosyl)transferase activity in reactions containing preparations of EF-2 (13). We observed that either condition inhibits the activity of the pyBHK ADP-ribosyltransferase.

Figure 1 presents the cpm of [^{14}C]adenosine incorporated per μg of EF-2. More than 95% of the protein in this preparation migrates as a single band with an Mr of 93,000 during $NaDodSO_4$/polyacrylamide gel electrophoresis and has been characterized as EF-2 (11). However, we calculated that the EF-2 preparation shown in Fig. 1 is 51% ADP-ribosylated by fragment A. The amount of EF-2 in six of our purified preparations able to accept the transfer of ADP-ribose catalyzed by fragment A ranges from 43% to 90% (11), with a typical preparation having about 50% ADP-ribosylatable EF-2. This variation in ADP-ribosylatable

EF-2 may represent an inactivation of the acceptor activity during purification . We have shown that ADP-ribose acceptor activity of EF-2 is inactivated by both freezing and thawing samples and by prolonged storage at -20 or -70°C (unpublished observations). Alternative explanations for the reduced acceptor activity of our EF-2 preparations are the presence of ADP-ribosylated EF-2 in EF-2 preparations extracted from cells or protein contaminants in our EF-2 preparations. The former explanation assumes that EF-2 is normally ADP-ribosylated in pyBHK cells and copurifies with EF-2.

Fragment A and Pseudomonas toxin A transfer ADP-ribose from NAD^+ specifically to the diphthamide acceptor site of EF-2 (6,9,10). Diphthamide is a unique, modified histidine residue only found in EF-2 (9,10). This residue apparently has been conserved throughout eukaryotic evolution (18,20,21), suggesting that it is involved in an important function. It seems unreasonable the diphthamide has been conserved for the convenience of microbial intoxication of cells. A more likely event is that diphthamide serves as the acceptor for an ADP-ribosylation reaction catalyzed by a cellular enzyme such as the one we have found in pyBHK cells. The microbial toxins simply take advantage of this existing system.

At the intracellular NAD^+ concentration in HeLa cells (about 0.5 mM), a steady-state concentration of a single molecule of fragment A is sufficient to inactivate all of the EF-2 within 24 hr (22). Yet the cellular enzyme activity described here has the same mechanism of action as that of fragment A. Such an endogenous enzyme must be under stringent control or it would cause cell death. This control may be exerted by a component (or components) in cytoplasmic extracts of pyBHK cells that we

found to inhibit the activity of the pyBHK ADP-ribosyltransferase. We propose that the interaction of the cellular ADP-ribosyltransferase and its inhibitor is a mechanism of controlling protein synthesis at the level of functional EF-2. Since cells with mutant EF-2, resistant to ADP-ribosylation by fragment A and presumably by the pyBHK transferase, synthesize protein and grow as well as wild-type cells do (11,23), the proposed mechanism for control of protein synthesis is probably not necessary for survival of all types of cells. It may only be operative under certain conditions in differentiated tissues. Nevertheless, the proposed control mechanism should result in cell death when out of balance and might explain pathological conditions characterized by cell death from unknown causes, such as cell senescence or diseases characterized by cell degeneration.

Using identical methods to those reported here, we have isolated a cellular ADP-ribosyltransferase from beef liver, which is also a mono(ADP-ribosyl)transferase that transfers ADP-ribose from NAD^+ to diphthamide of liver EF-2 (unpublished data). Thus, this enzyme activity can also be found in normal tissues from a different species and, therefore, may be ubiquitous.

To fully understand the reaction catalyzed by the pyBHK ADP-ribosyltransferase, a defined system containing purified enzyme, substrate and inhibitor must be established. In addition, this enzyme system should be shown to be operative *in vivo* in several cell types.

References

1. Bredehorst, R., Wielckens, K., Garteman. A., Lengyel, H., Klapproth, K. and Hilz, H. (1978) Eur. J. Biochem. 92:129-135.

2. Bredehorst, R., Wielckens, K., Adamietz, P., Steinhagen-Thiessen, E. and Hilz, H. (1981) Eur. J. Biochem. 120:267-274.

3. Hilz, H., Wielckens, K. and Bredehorst, R. (1982) in ADP-Ribosylation Reactions, eds. Hayaishi, O. and Ueda, K. (Academic, New York), pp. 207-219.

4. Adamietz, P., Wielckens, K., Bredehorst, R., Lengyel, H. and Hilz, H. (1981) Biochem. Biophys. Res. Commun. 101:96-103.

5. Moss, J. and Vaughan, M. (1978) Proc. Natl. Acad. Sci. USA 75: 3621-3624.

6. Honjo, T., Nishizuka, Y., Hayaishi, O. and Kato, I. (1968) J. Biol. Chem. 243:3553-3555.

7. Iglewski, B.H., Liu, P.V. and Kabat, D. (1977) Infect. Immun. 15: 138-144.

8. Iglewski, B.H., Sadoff, J.B., Bjorn, M.J. and Maxwell, E.S. (1978) Proc. Natl. Acad. Sci. USA 75: 3211-3215.

9. Van Ness, B.G., Howard, J.B. and Bodley, J.L. (1980) J. Biol. Chem. 255:10710-10716.

10. Oppenheimer, N.J. and Bodley, J.W. (1981) J. Biol. Chem. 256:8579-8581.

11. Iglewski, W.J. and Lee, H. (1983) Eur. J. Biochem. 134: 237-240.

12. Pappenheimer, A.J., Jr., Uchida, T. and Avery-Harpter, A. (1972) Immunochemistry 9:891-906.

13. Gill, D.M. and Dinius, L.L. (1973) J. Biol. Chem 248:654-658.

14. Roberts, J.H., Stark, P., Giri, C.P. and Smulson, M. (1975) Arch. Biochem. Biophys. 171:305-315.

15. Randerath, K. and Randerath, E. (1967) Methods Enzymol. 12:323-347.

16. Van Ness, B.G., Barrowclough, B. and Bodley, J.W. (1980) FEBS. Lett. 120:4-6.

17. Hayaishi, O. and Ueda, K. (1977) Annu. Rev. Biochem. 46:95-116.

18. Collier, R.J. (1975) Bacteriol. Rev. 39:54-85.

19. Honjo, T., Nishizuka, Y., Kato, I. and Hayaishi, D. (1971) J. Biol. Chem. 246:4251-4260.

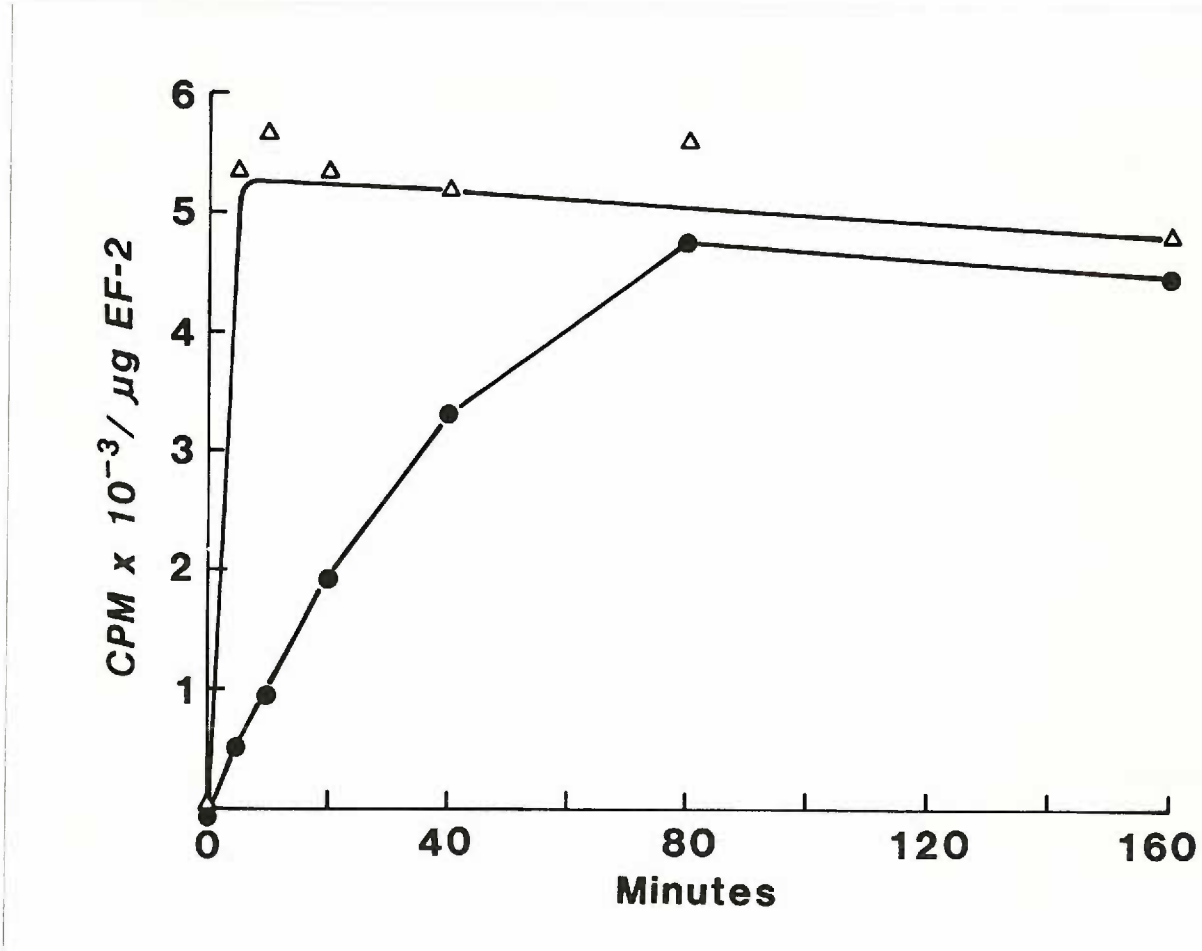
20. Pappenheimer, A.M., Jr. (1977) Annu. Rev. Biochem. 46:69-94.

21. Brown, B.A. and Bodley, J.W. (1979) FEBS. Lett. 103:253-255.

22. Pappenheimer, A.M., Jr., and Gill, D.M. (1973) Science 182:353-355.

23. Moehring, J.H. and Moehring, T.J. (1979) Somatic Cell Genet. 5:153-168.

Figure 1. Incorporation of label from NAD^+ into EF-2 catalyzed by fragment A or the pyBHK endogenous transferase found in EF-2 preparations. Five micrograms of an EF-2 preparation containing endogenous transferase plus 0.05 μg of fragment A (Δ) or 5 μg of an EF-2 preparation containing the endogenous transferase (•) were added to 0.1 ml of reaction buffer containing 6.33 μM [adenosine-^{14}C]NAD^+ and incubated at 22°C. At intervals, aliquots (10 μl) were taken, and trichloroacetic acid-precipitable material was assayed for radioactivity.


6
5
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2
1
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40
80
120
160
Minutes
CPM x 10⁻³/ µg EF-2

Figure 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of the proteins in the EF-2 preparations labeled with [^{14}C]adenosine from NAD^+ by fragment A or by the pyBHK endogenous transferase in the EF-2 preparations. EF-2 preparations were labeled as described in Figure 1, and the radioactive proteins were analyzed by electrophoresis on 12% $NaDodSO_4$/polyacrylamide gels along with marker proteins. Lanes: right, fragment A-labeled EF-2; left, endogenous transferase-labeled EF-2. The radioactive proteins were visualized by autoradiography. The major band coelectrophoresed with the phosphorylase b marker with an Mr of 93,000, as does the major band of stained protein (11).

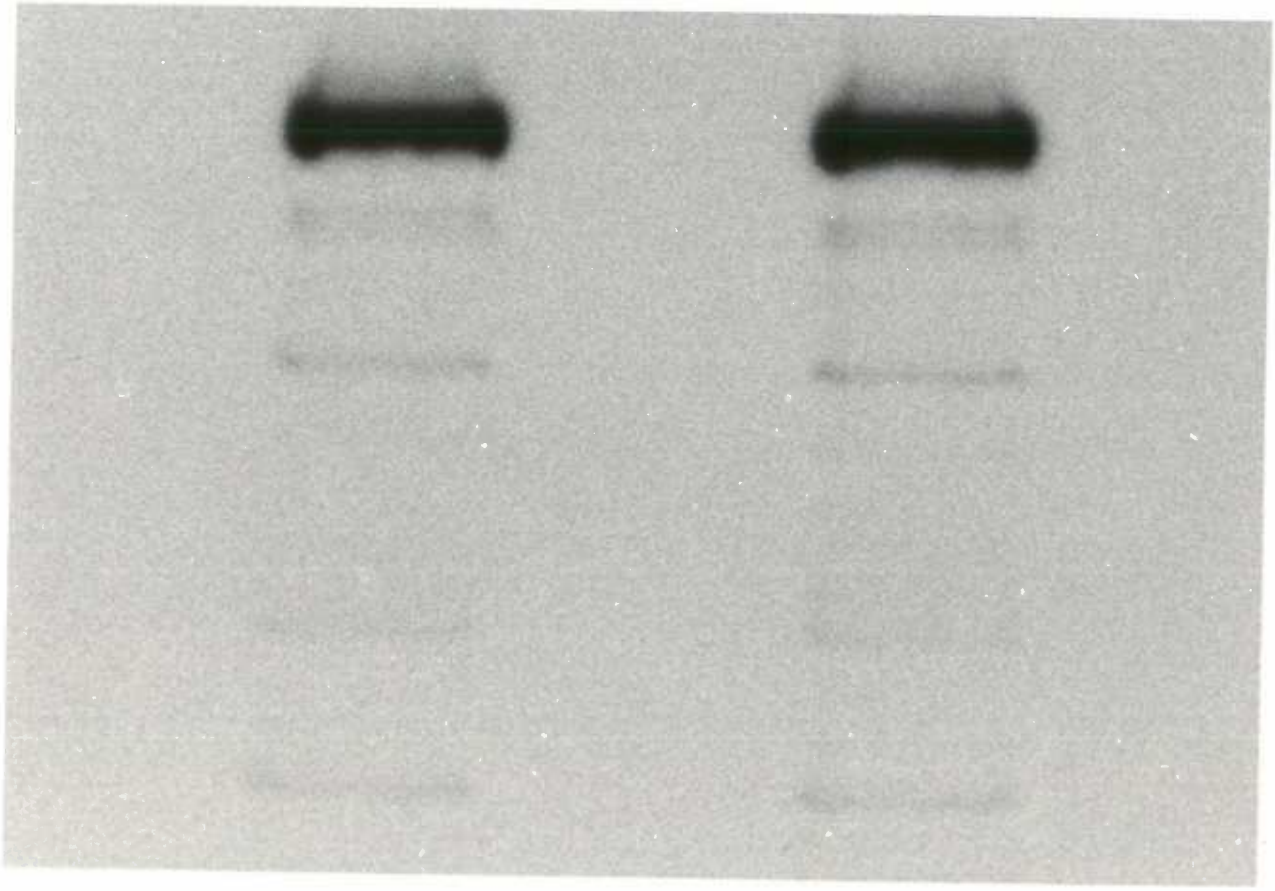
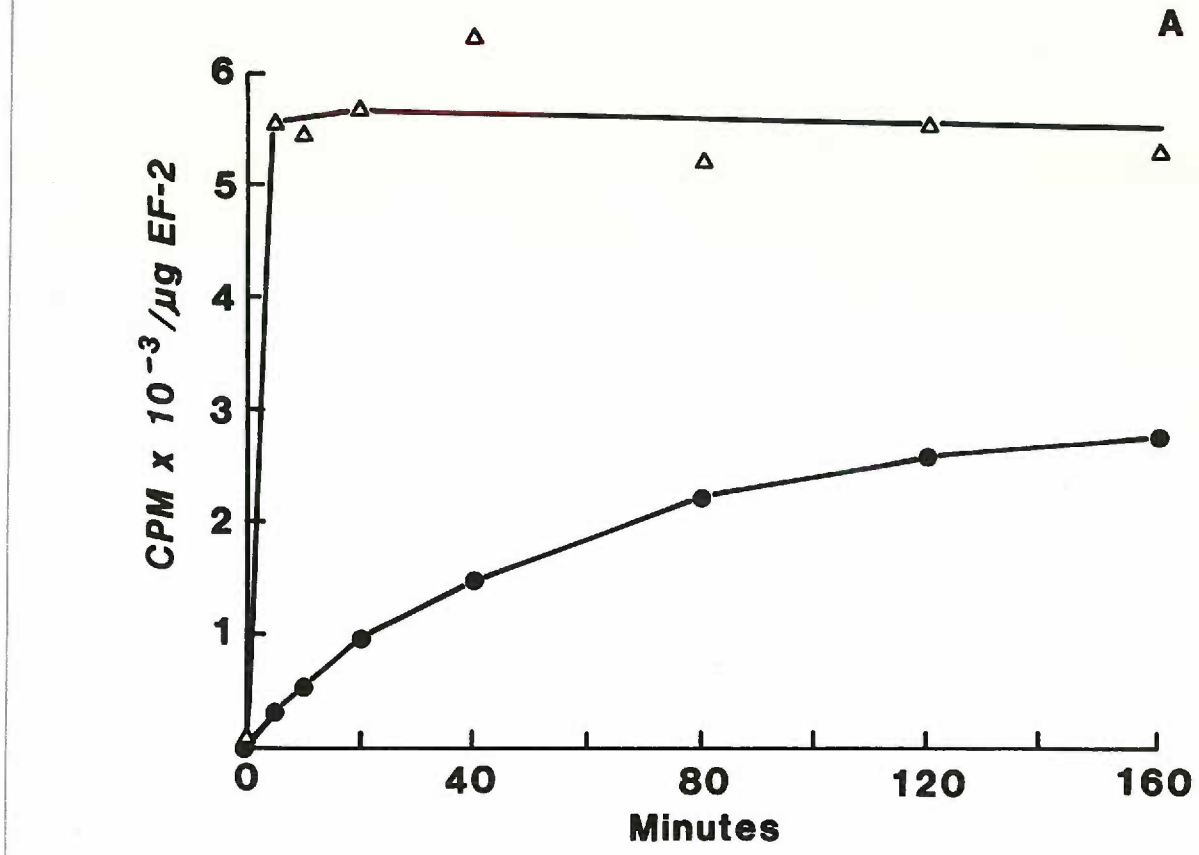


Figure 3. Effect of fragment A antibody on incorporation of [^{14}C]adenosine from NAD^+ into EF-2 catalyzed by fragment A or the pyBHK endogenous transferase. A 1:5 dilution of rabbit anti-fragment A antiserum or control serum was incubated with 0.05 μg of fragment A or an EF-2 preparation containing the pyBHK endogenous transferase activity for 5 min at 37°C. The enzyme and serum mixtures were then added to our standard transferase assay as described in Figure 1 and Materials and Methods. The transfer of [^{14}C]adenosine from NAD^+ to EF-2 is shown after various times of incubation. (A) Fragment A (Δ)-or fragment A/anti-fragment A antiserum (•)-catalyzed reaction. (B) Endogenous transferase (Δ)-or endogenous transferase/anti-fragment A antiserum (•)-catalyzed reaction.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CPM x 10⁻³/µg EF-2
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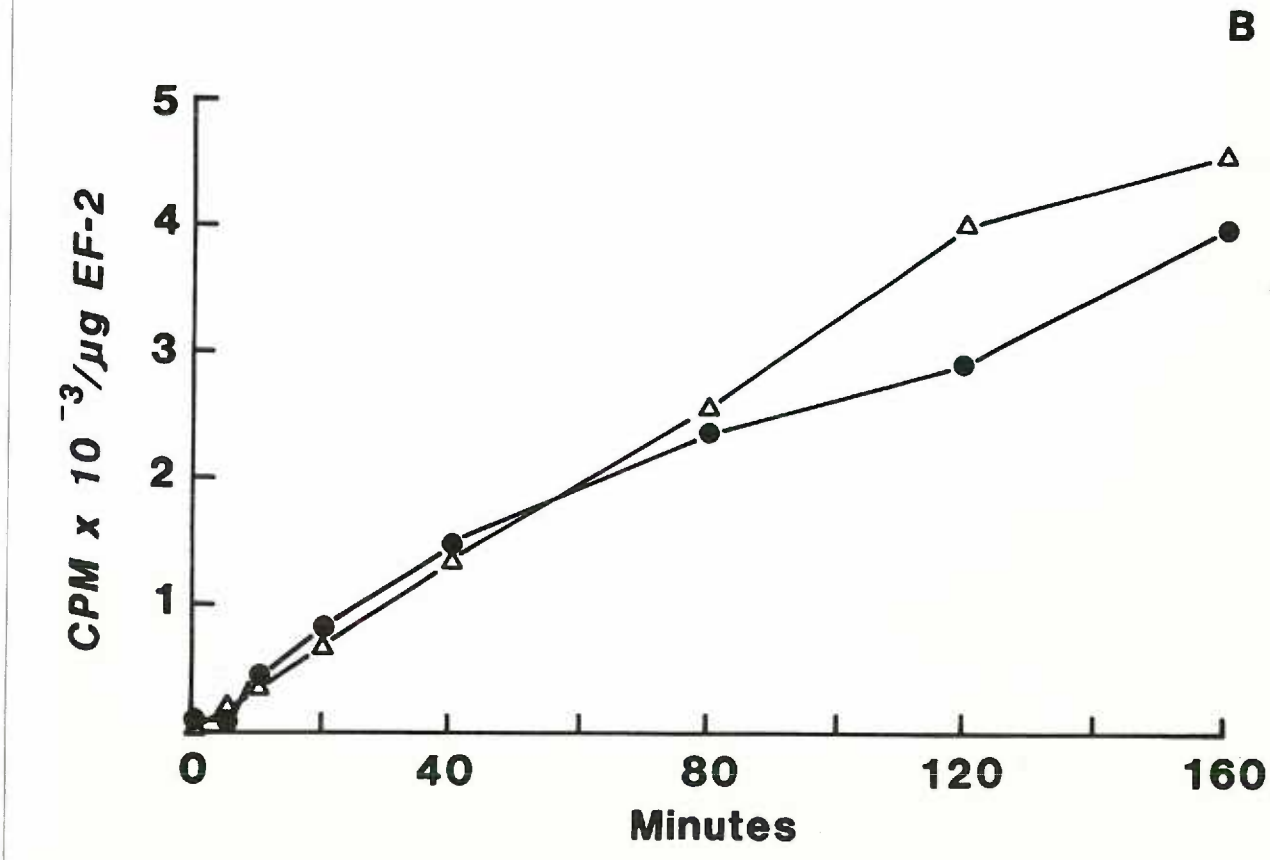
B
CPM x 10⁻³/µg EF-2
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4
3
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40
80
120
160
Minutes

Table 1. The effect of cytoplasmic extracts from pyBHK cells and histamine on transferase activity in EF-2 preparations.

	[^{14}C]Adenosine incorporated per μg of EF-2, cpm				
	Cytoplasmic extract (5 mg of protein per ml)			Histamine at 0.25 M	
Transferase	Absent	Present	Boiled and Centrifuged	Absent	Present
Fragment A	4700	4600	4600	4200	3900
Pseudomonas toxin A	NT	NT	NT	4500	4000
Endogenous transferase	4500	550	4000	4300	100

The standard reaction mixture described in Materials and Methods and Figure 1 was supplemented by the addition of 10 μl of a cytoplasmic extract from pyBHK cells having a protein concentration of 5 mg/ml or by adding histamine to a final concentration of 0.25 M. In some experiments cytoplasmic extract was boiled for 5 min and centrifuged to remove denatured protein before use. Cytoplasmic extracts were incubated with EF-2 for 5 min at 37°C before addition to the reaction mixture. The reaction mixtures were incubated until there was no further transfer of label to EF-2. The inhibitory effect of the cytoplasmic extract on the pyBHK endogenous transferase occurred at 5 min of incubation and was maximal by 10 min. The inhibitory effect of histamine on the endogenous transferase was maximal by 5 min of incubation, which was the first sampling time. Similar results were obtained when histamine was used at a concentration of 0.15 M. NT, not tested.

Figure 4. Thin-layer chromatography of the snake venom (SV) phosphodiesterase digestion products from EF-2 labeled by fragment A or the pyBHK endogenous transferase with [^{14}C]adenosine from NAD^+. The radioactive products are visualized by autoradiography. EF-2 was labeled by fragment A or by the endogenous transferase in our standard reaction mixture, and the products were digested with SV phosphodiesterase and chromatographed on thin-layer plates as described. Lanes show the soluble products from EF-2 labeled in the presence of [adenosine-^{14}C]NAD^+ by: fragment A (lane a), endogenous transferase (lane b), fragment A followed by a 15-min digestion with SV phosphodiesterase (lane c), endogenous transferase followed by a 15-min digestion with SV phosphodiesterase (lane d), fragment A followed by a 30-min digestion with SV phosphodiesterase (lane e), endogenous transferase followed by a 30-min digestion with SV phosphodiesterase (lane f), fragment A followed by a 6-hr digestion with SV phosphodiesterase (lane g), and endogenous transferase followed by a 6-hr digestion with SV phosphodiesterase (lane h). The major products of the 15- and 30-min digestions comigrate with AMP. Increasing the time of digestion to 6 hr results in the further conversion of AMP to the major product, which comigrates with adenosine. No soluble product cochromatographed with ADP-ribose. The R_f values of NAD^+, adenosine, AMP, and ADP-ribose are 0.65, 0.53, 0.40, and 0.25, respectively.

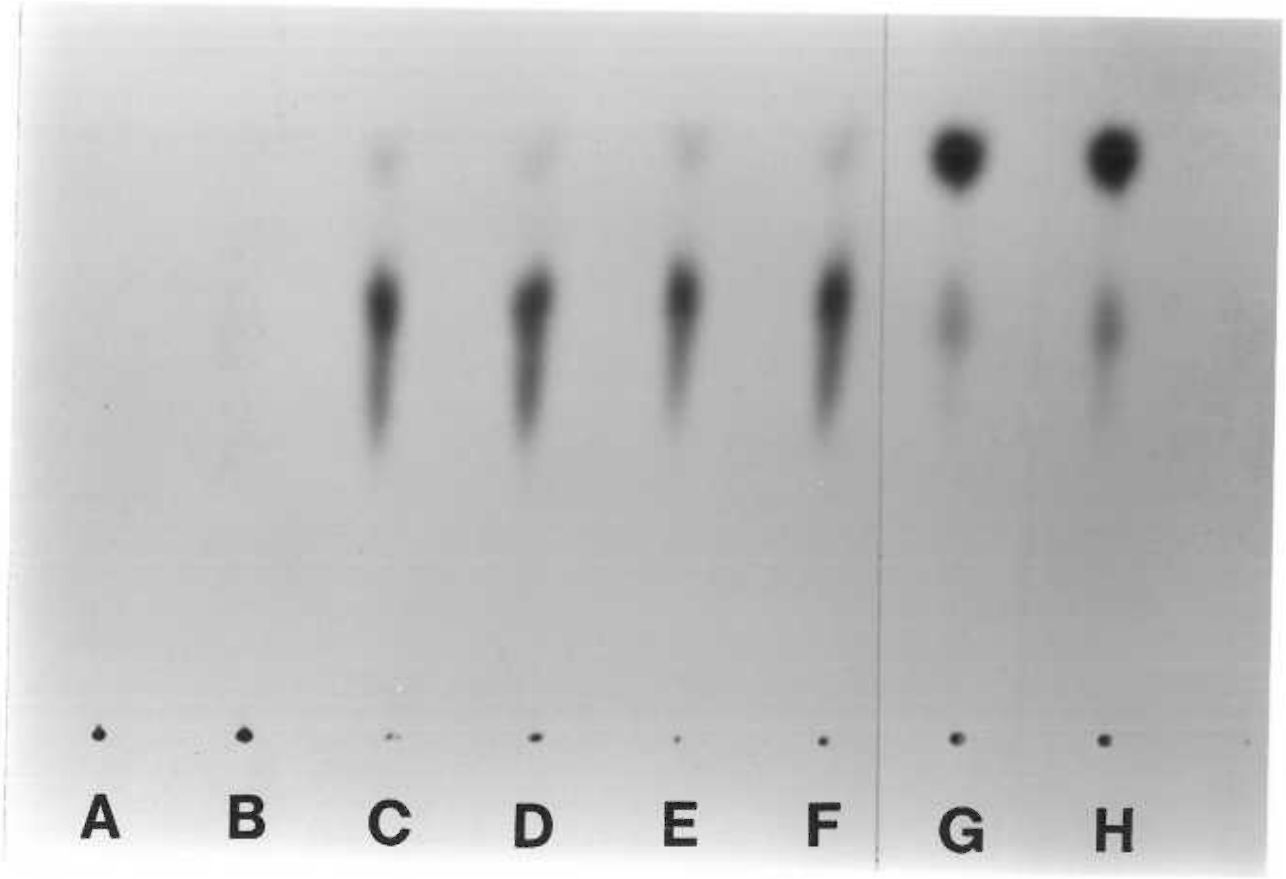
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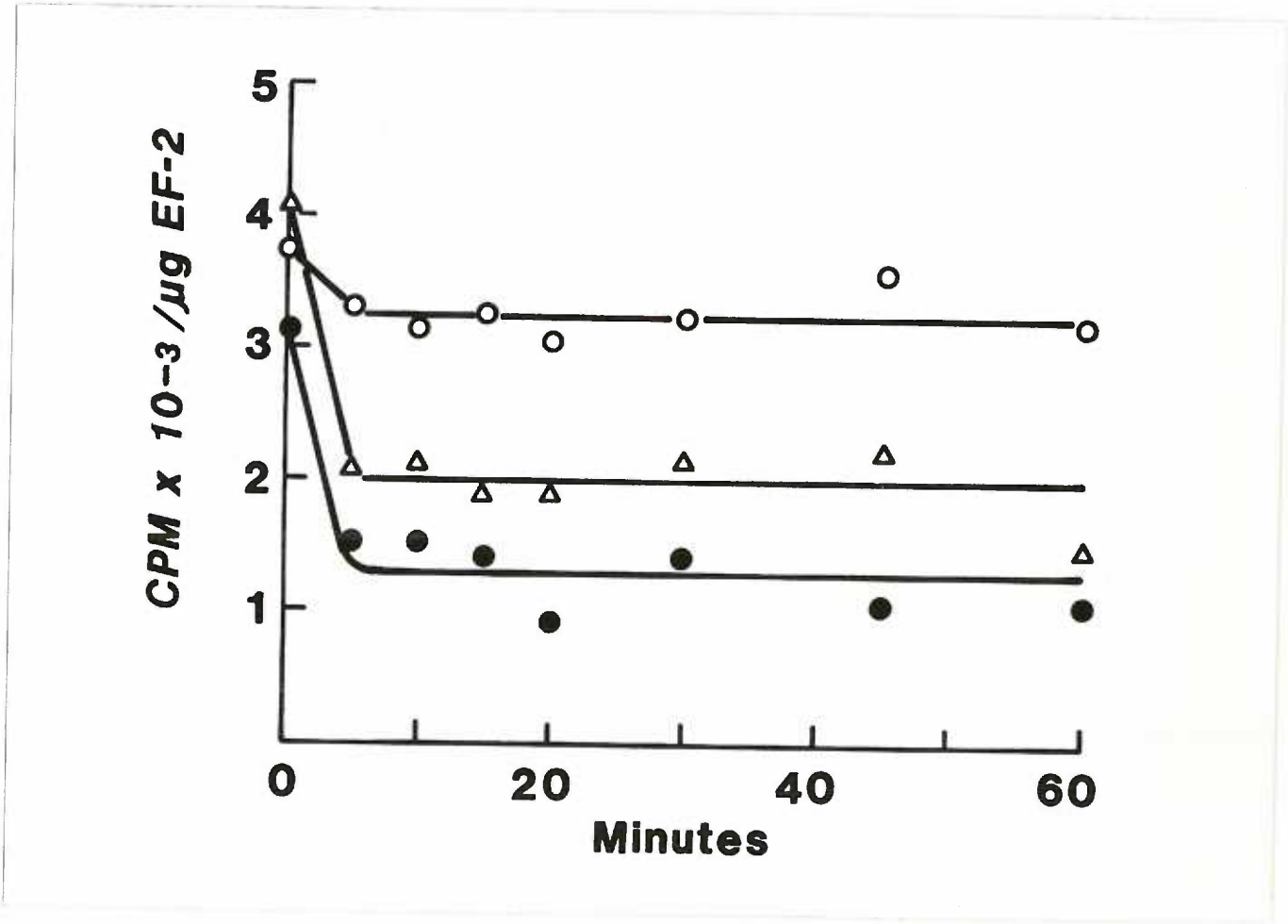
Figure 5. Reversal of ADP-ribosylation of EF-2 catalyzed by fragment A or the endogenous ADP-ribosyltransferase from pyBHK cells. EF-2 preparations were labeled by incubation with [adenosine-^{14}C]NAD^+ and a low concentration of fragment A (0.5 μg/ml) or the endogenous transferase in our standard reaction mixture. After exhaustive dialysis, which removes all unreacted NAD^+, the labeled proteins were incubated in Tris-HCl buffer (pH 6.6). For the reverse reaction, the EF-2 labeled by the endogenous transferase was incubated with fragment A (10 μg/ml) and 2 mM nicotinamide (•) or with 2 mM nicotinamide in the absence of fragment A (o). The fragment A-catalyzed forward reaction was also reversed with fragment A (10 μg/ml) and 2 mM nicotinamide (Δ).


CPM x 10-3 /µg EF-2
5
4
3
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1
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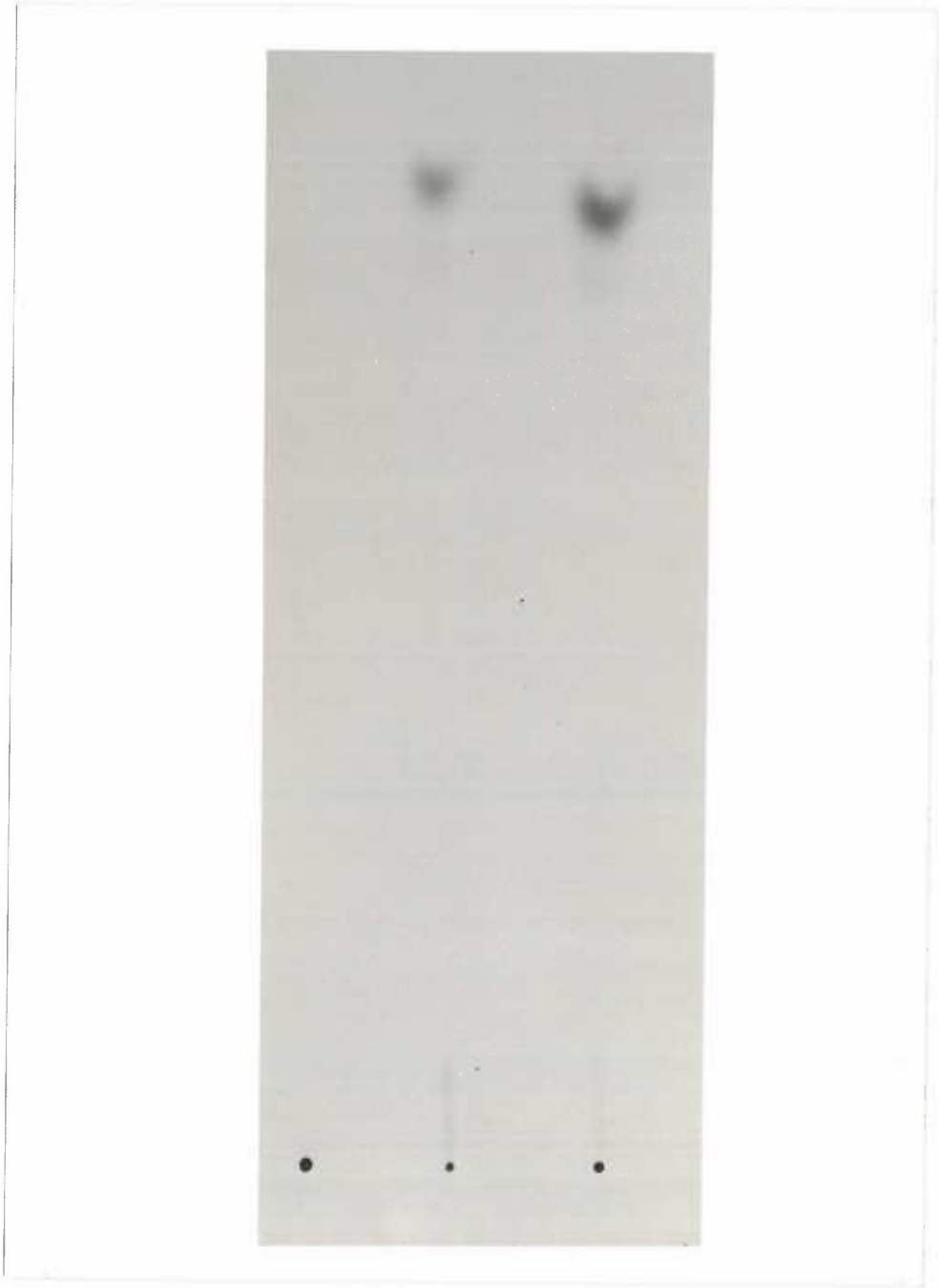
20
40
60
Minutes

Figure 6. Thin-layer chromatography of the products formed by the reversal reaction. Radioactive compounds were visualized by autoradiography. EF-2 was radioactively labeled by fragment A or the endogenous ADP-ribosyltransferase from pyBHK cells as described in Figure 1. After extensive dialysis, the reaction was reversed as described in Figure 5. The products of the reversal were analyzed by chromatography on CEL-300 thin-layer plates with 0.3 M LiCl as eluent. Lanes: right, reversal products from EF-2 labeled in the presence of [adenosine-^{14}C]NAD^+ by fragment A; left, reversal products of EF-2 labeled by the endogenous transferase. Only the major radioactive product of the reversal had the same chromatographic mobility as NAD^+. Radioactive EF-2 remains at the origin. No soluble radioactive labeled products were observed when labeled EF-2 from only the forward reaction was chromatographed. The R_f values of NAD^+, AMP, and ADP-ribose are 0.79, 0.74, and 0.84, respectively.



Paper 3.

ADP-Ribosyltransferase from Beef Liver Which ADP-Ribosylates Elongation Factor-2

ABSTRACT

Fragment A of diphtheria toxin and Pseudomonas toxin A intoxicate cells by ADP-ribosylating the diphthamide residue of elongation factor-2 (EF-2) resulting in an inhibition of protein synthesis [1-3]. A cellular enzyme from polyoma virus transformed baby hamster kidney (pyBHK) cells ADP-ribosylates EF-2 in an identical manner [4]. Here we describe a similar cellular enzyme from beef liver which transfers [^{14}C-adenosine]-ADP-ribose from NAD to EF-2. The ^{14}C-label can be removed from the EF-2 by snake venom phosphodiesterase as a soluble product which comigrates with AMP on TLC plates, indicating the ^{14}C-label is present on EF-2 as monomeric units of ADP-ribose. Furthermore, the forward transferase reaction catalyzed by the beef liver ADP-ribosyltransferase is reversible by excess diphtheria toxin fragment A, with the formation of ^{14}C-labeled NAD, indicating that both transferases ADP-ribosylate the same site on the diphthamide residue of EF-2. Thus, beef liver and pyBHK mono (ADP-ribosyl)transferases both modify the diphthamide residue of EF-2, in a manner identical to diphtheria toxin fragment A and Pseudomonas toxin A. These results suggest the cellular enzyme is probably ubiquitous among eukaryotic cells.

1. Introduction

We have recently described the first cellular mono(ADP-ribosyl) transferase from pyBHK cells which ADP-ribosylates eukaryotic EF-2 in a manner identical to fragment A of diphtheria toxin and Pseudomonas toxin

A [4]. We suggested that the cellular enzyme was part of an endogenous enzyme system of eukaryotic cells which is taken over by these two microbial toxins during the process of intoxication. Since these bacterial toxins affect a number of different types of cells [5-8], the endogenous cellular ADP-ribosyltransferase system should also have a broad distribution. The initial isolation of the cellular ADP-ribosyltransferase was from pyBHK cells which are both virus infected and transformed and consequently might contain a unique enzyme not found in normal tissue. In this report, however, we describe an ADP-ribosyltransferase which has now been isolated from normal beef liver. The finding of this enzyme in two different tissues from two different species of animals suggests it is probably ubiquitious among eukaryotic cells.

2. Materials and Methods

2.1 Partial purification of beef liver elongation factor-2

A fresh 21 lb beef liver was obtained from a local slaughterhouse, divided into 100 gm portions and frozen at -70°C. Elongation factor-2 (EF-2) from beef liver was partially purified according to a procedure for purification of EF-2 from hamster tumors [9]. Briefly, rapidly thawed liver was homogenized in a sucrose containing buffer at 5° C in a Waring blender. A postmitochondrial fraction was selected by centrifugation and precipitated with ammonium sulfate added to 80% saturation. The precipitate was dissolved in buffer A [4] and sequentially chromatographed on DEAE-cellulose, Sephadex DEAE A50, Sephacryl S-200 and phosphocellulose. Chromatographic fractions rich in

EF-2 were assayed by the specific transfer of [^{14}C-adenosine]-ADP-ribose from NAD (534 mCi/mM) to EF-2 by diphtheria toxin fragment A as described by Gill and Dinius [10]. The preparations of partially purified EF-2 were analyzed by SDS-polyacrylamide gel electrophoresis [9].

2.2 ADP-ribosylation of preparations of beef liver EF-2.

The partially purified liver EF-2 preparations also served as the source of the cellular ADP-ribosyltransferase. Diphtheria toxin fragment A was purified as described [11]. ADP-ribosyltransferase activity was measured by the incorporation of radioactivity from [U-^{14}C-adenosine]-NAD into trichloroacetic acid (TCA) precipitable material in the presence of EF-2 as described [4,9]. The reaction mixture contained 25 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 6.33 μM [U-^{14}C-adenosine]-NAD (534 mCi/mM) (New England Nuclear Co.), and EF-2 at 50μg/ml with or without added fragment A at 0.5μg/ml. After intervals of incubation at 22°C, 10 μl samples were TCA precipitated and the radioactivity was measured [4,9].

2.3 Snake venom phosphodiesterase digestion of ADP-ribosylated EF-2.

Digestion with snake venom phosphodiesterase (Worthington Biochemical) was used to distinguish between mono or poly-ADP-ribosylation of EF-2 by the endogenous transferase as described [4]. EF-2 was ADP-ribosylated by the cellular ADP-ribosyltransferase in the presence of ^{14}C-labeled NAD and then dialyzed to remove NAD. Then 25μl of snake venom phosphodiesterase was added and incubated 15 min at 37°C. The digestion products were chromatographed on thin-layer polyethyleneimine cellulose plates (J.T. Baker) with 0.3 M lithium chloride, dried and exposed to X-ray film (Kodak XRP-5) to locate the ^{14}C-labeled products

relative to the UV light absorbing AMP, adenosine, ADP-ribose and NAD markers (Sigma).

2.4 Reversal of the ADP-ribosylation reaction. Reversal reactions were done by incubating the product of the forward reaction (^{14}C-adenosine labeled EF-2) at pH 6.6 with an excess of fragment A (10μg/ml) and 2 mM nicotinamide at 22°C as previously described [4]. The radioactive products were analyzed by thin-layer polyethyleneimine cellulose chromatography as described above.

3. Results and Discussion

3.1 Transfer of ^{14}C-adenosine from NAD to EF-2 by the endogenous transferase.

Beef liver EF-2 was partially purified by sequential column chromatography on DEAE-cellulose, Sephadex DEAE A50, Sephacryl S-200 and phosphocellulose as previously described [9]. The partially purified beef liver EF-2 preparation consisted of a major band of protein having an Mr of 93,000 and trace bands of lower molecular mass proteins when reduced and analyzed by SDS-polyacrylamide gel electrophoresis (Figure 1A). The major band represented greater than 95% of the stainable protein on the gels. Incubation of the partially purified EF-2 preparation which contained the endogenous transferase with [^{14}C-adenosine]-NAD resulted in the transfer of ^{14}C-label to an acid precipitable form. EF-2 could also be labeled by addition of diphtheria toxin fragment A. Since the fragment A catalyzed reaction reaches saturation in < 5 min and the

endogenous transferase catalyzed reaction requires > 160 min of incubation, the ^{14}C-labeled acceptor could be preferentially labeled by either enzyme [4]. Moreover, the transfer of label to the acceptor by sequential addition of the two enzymes is not additive (unpublished data). SDS-polyacrylamide gel analysis of the products labeled by the cellular transferase or fragment A indicated that the label was transferred to a 93,000 dalton protein which comigrates with EF-2 [4,9], as shown in Figure 1B.

3.2 Snake venom phosphodiesterase digestion of ^{14}C-labeled EF-2.

To determine if the EF-2 modified by the beef liver ADP-ribosyltransferase contains monomers or polymers of ADP-ribose, we synthesized the labeled product formed in the presence of the endogenous cellular transferase, [^{14}C-adenosine]-NAD and EF-2 and then treated the labeled product with snake venom phosphodiesterase (SVP). EF-2 modified by fragment A was used in an accompanying reaction mixture as a positive control. In both reactions the soluble products resulting from digestion with snake venom phosphodiesterase cochromatographed with each other and with the UV light absorbing AMP marker (Fig 2). Some tailing of ^{14}C-labeled AMP in the chromatogram was observed due to the salt effect of the $MgCl_2$ component in the SVP catalyzed reaction. The same tailing effect of the AMP standard was observed when the standards were treated and chromatographed under the same conditions as the unknowns. The effect was identical in all reactions and does not obscure the region of the chromatogram where the ADP-ribose standard migrates. Since AMP is the product of snake venom phosphodiesterase action on mono ADP-ribosylated proteins, we conclude that ADP-ribose is present on the acceptor in EF-2 as monomeric units.

3.3 Reversal of the ADP-ribosylation reaction

The ADP-ribosylation of EF-2 by mono(ADP-ribosyl)transferases is reversible [4,12,13]. Since these ADP-ribosyltransferases synthesize identical products, it is possible to drive the forward reaction with one type of ADP-ribosyltransferase and then catalyze the reversal reaction with a second type of transferase [4,12,13]. A preparation of ^{14}C-adenosine labeled beef liver EF-2 containing a small amount of beef liver ADP-ribosyltransferase (used in the forward reaction) was incubated in the absence or presence of excess fragment A and nicotinamide to reverse the reaction. The ^{14}C-labeled product generated in this reversal reaction was analyzed by thin layer chromatography. The ^{14}C-labeled soluble product found in the reverse reaction mixtures cochromatographed with the marker NAD (Fig. 3), indicating that the beef liver enzyme ADP-ribosylates the diphthamide residue of EF-2 in a manner identical to that of fragment A of diphtheria toxin, Pseudomonas toxin A and pyBHK cellular mono(ADP-ribosyl)transferase.

The mono(ADP-ribosyl)transferases from beef liver and from pyBHK cells are found in EF-2 preparations when the EF-2 is partially purified as in [4,9]. Although the enzyme is found in our EF-2 preparations the activity can be separated from pyBHK EF-2 using an immunoadsorbant column containing polyvalent antibody prepared by immunizing Balb/c mice with an EF-2 preparation having the cellular transferase activity (unpublished data). The cellular ADP-ribosyltransferase binds to the immunoadsorbant column and can be eluted in an enzymatically active form, while pyBHK EF-2 does not bind to the resin. Thus, the transferase activity is distinct from EF-2 and either co-purifies with EF-2 or is a minor contaminant in our EF-2 preparations.

Beef liver serves as a convenient source for the cellular ADP-ribosyltransferase but we have found that the yield of beef liver EF-2 and its ADP-ribosyltransferase to be much lower (approximately 1/20) than the yield of pyBHK EF-2 and its copurifying ADP-ribosyltransferase enzyme. We have also found that the activity of the beef liver ADP-ribosyltransferase is much less stable than the enzyme isolated from pyBHK cells. Enzyme activity is rapidly lost when frozen at -70°C, suggesting the beef liver ADP-ribosyltransferase may be inactivated by the process of freezing. We normally store the beef liver EF-2 preparations in ice and use them as rapidly as possible. In contrast, the ADP-ribosyltransferase from pyBHK cells is stable when stored at -70°C and is more suitable for long term studies.

Similar mono(ADP-ribosyl)transferases have now been isolated from two different tissues of different species of animals. Since the unique diphthamide residue of EF-2 has been stringently conserved in eukaryotic cells from yeast to man [5,6,14] it seems likely that the mono(ADP-ribosyl)transferase which ADP-ribosylates this acceptor site will also be common to these eukaryotic cells. The stringent conservation of the diphthamide residue and, apparently, the cellular mono(ADP-ribosyl) transferase suggests an essential physiological function, perhaps "fine tuning" of protein synthesis at the level of functional EF-2. A model for the potential relationship of microbial toxins and cellular ADP-ribosyltransferases has recently been presented together with a potential explanation for the selective toxicity of the microbial ADP-ribosyltransferase over its cellular counterpart [15]. It will now be important to demonstrate the active form of this enzyme <u>in vivo</u> and to determine under what conditions that proposed "fine tuning" mechanism is operational.

References

1. Honjo, T., Nishizuka, Y., Hayaishi, O. & Kato, I. (1968) J. Biol. Chem. 243, 3553-3555.

2. Iglewski, B. H. & Kabat, D. (1975) Proc. Natl. Acad. Sci. USA 72, 2284-2288.

3. Van Ness, B. G., Howard, J. B. & Bodley, J. W. (1980) J. Biol. Chem. 255, 710-716.

4. Lee, H. & Iglewski, W. J. (1984) Proc. Natl. Acad. Sci. USA in press.

5. Collier, R. J. (1975) Bact. Rev. 39, 58-85.

6. Pappenheimer, A. M., Jr. (1977) Ann. Rev. Biochem. 46, 69-94.

7. Vasil, M. L. & Iglewski, B. H. (1978) J. Gen. Microbiol. 108, 333-337.

8. Taylor, A. & Iglewski, W. J. (1979) Virology 96, 177-185.

9. Iglewski, W. J. & Lee, H. (1983) Eur. J. Biochem. 134, 237-240.

10. Gill, D.M. and Dinius, L.L. (1973) J. Biol. Chem. 248, 654-658.

11. Pappenheimer, A.M., Jr., Uchida, T. and Avery-Harpter, A. (1972) Immunochemistry 9, 891-906.

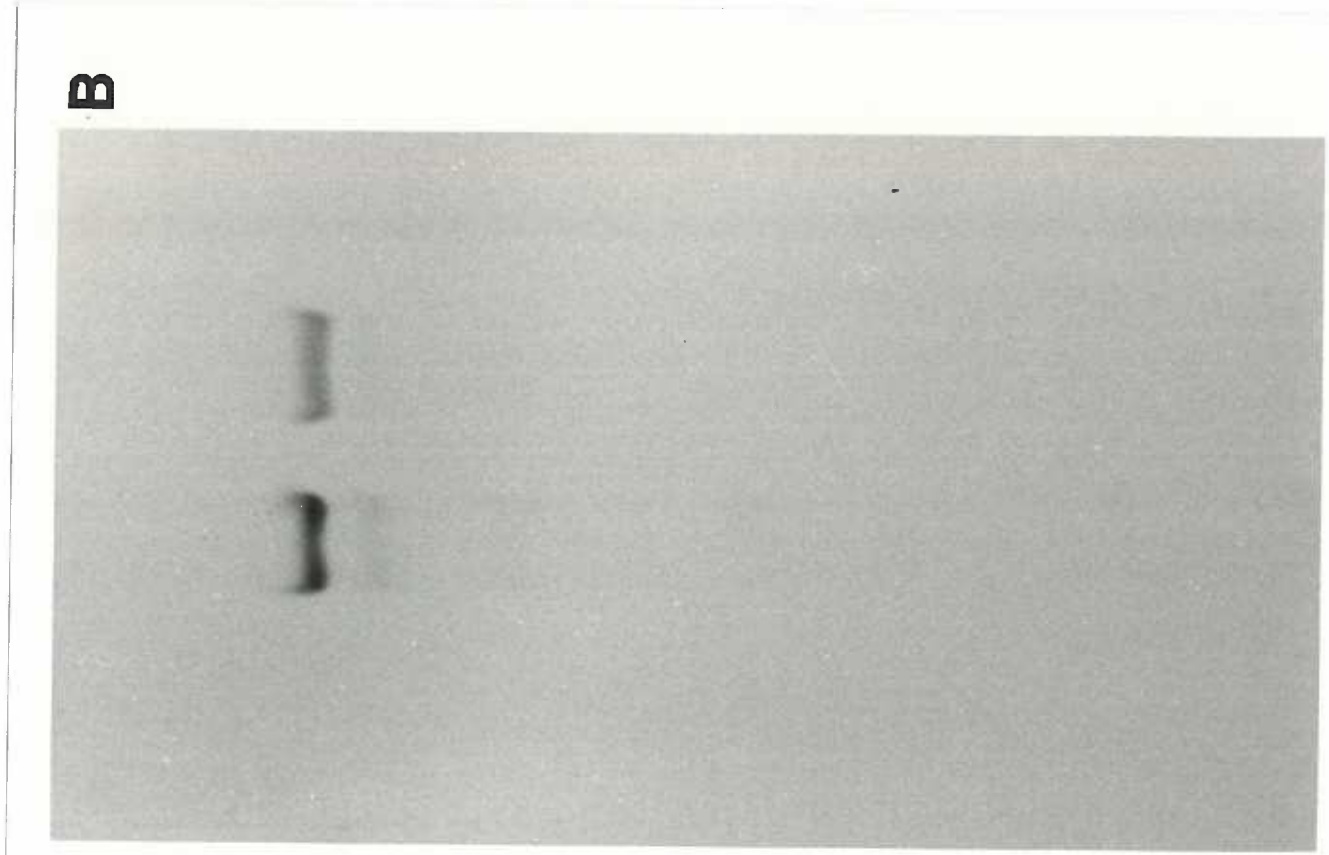
12. Iglewski, B. H., Liu, P. V. & Kabat, D. (1977) Infect. Immun. 15, 138-144.

13. Honjo, T., Nishizuka, Y., Kato, I. & Hayaishi, D. (1971) J. Biol. Chem. 246, 4251-4260.

14. Brown, B. A. & Bodley, J. W. (1979) FEBS. Lett. 103, 253-255.

15. Iglewski, W. J. (1984) ASM News 50, 195-198.

Figure 1. SDS-polyacrylamide gel analysis of partially purified beef liver EF-2 and of the EF-2 preparation labeled with ^{14}C-adenosine by diphtheria toxin fragment A and the endogenous transferase. EF-2 was partially purified from homogenates of beef liver as described in Materials and Methods. Gels containing 8.5% acrylamide and 0.1% sodium dodecyl sulfate (SDS) were prepared as previously described (9). Following electrophoresis the protein bands were stained with Coomassie Brilliant Blue stain as shown in (A). Lanes from left to right: molecular weight standards; beef liver EF-2 preparation incubated with [^{14}C-adenosine] NAD for 160 min; and beef liver EF-2 incubated with [^{14}C-adenosine] NAD and 0.05μg of diphtheria toxin fragment A for 160 min. The numbers represent Mr x 10^{-3} of the protein standards. Following electrophoresis, the dried gel was exposed to x-ray film to locate proteins labeled with ^{14}C-adenosine by the two transferases as shown in (B). Lanes from left to right: proteins labeled with ^{14}C-adenosine by fragment A; and proteins labeled with ^{14}C-adenosine by the endogenous transferase in beef liver EF-2 preparations. The major stained and ^{14}C-labeled protein comigrates with the 93,000 dalton phosphorylase b molecular mass marker protein.


B

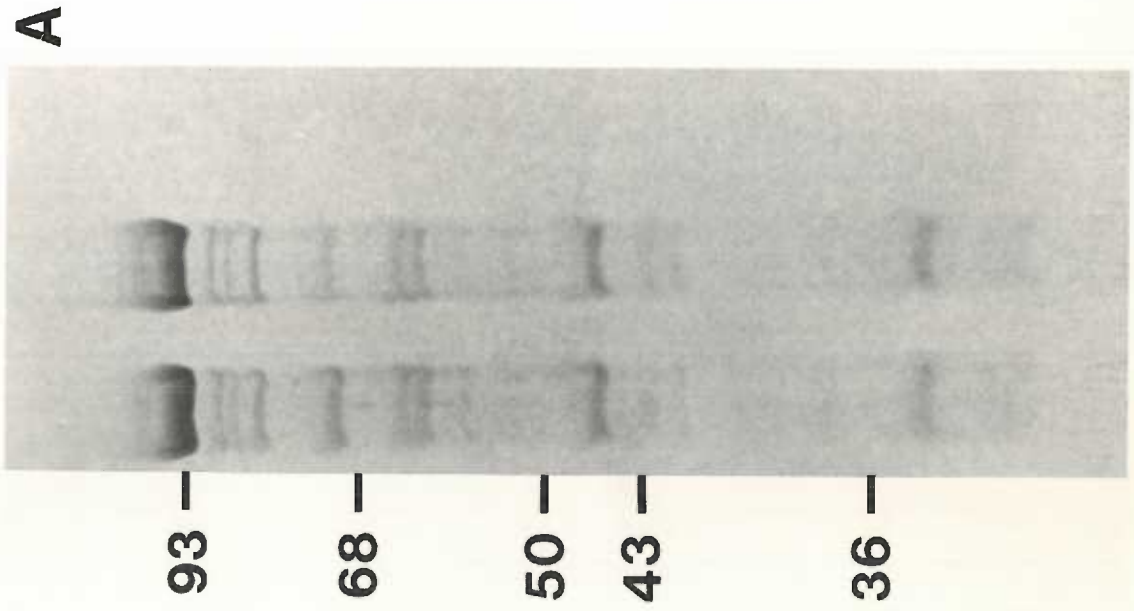

A
93
68
50
43
36

Figure 2. Thin-layer chromatography of the snake venom phosphodiesterase digestion products from beef liver EF-2 labeled by fragment A or the beef liver endogenous transferase with [^{14}C-adenosine] NAD. The beef liver EF-2 was labeled by fragment A or by the endogenous transferase in our standard reaction mixture, the products were digested with snake venom phosphodiesterase (SVP) and chromatographed on thin-layer plates as described in Materials and Methods. The radioactive products are visualized by autoradiography. The lanes from left to right represent the soluble products from EF-2 labeled in the presence of [^{14}C-adenosine] NAD by: a) fragment A; b) endogenous transferase; c) fragment A followed by 5 min digestion with SVP; d) fragment A followed by 15 min digestion with SVP; e) endogenous transferase followed by 5 min digestion with SVP; and f) endogenous transferase followed by 15 min digestion with SVP. ADPR, AMP, Aden. and NAD refer to the chromatographic positions of the UV-light absorbing standards of ADP-ribose, AMP, adenosine and NAD which have the R_f values of 0.65, 0.53, 0.40 and 0.25, respectively.

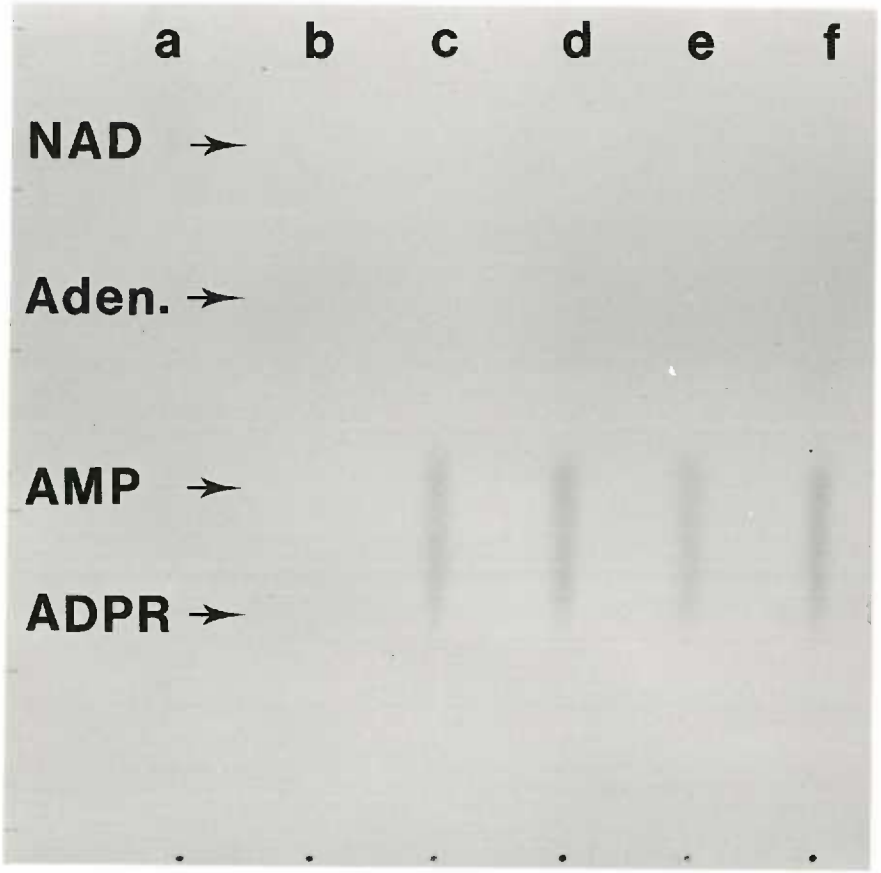
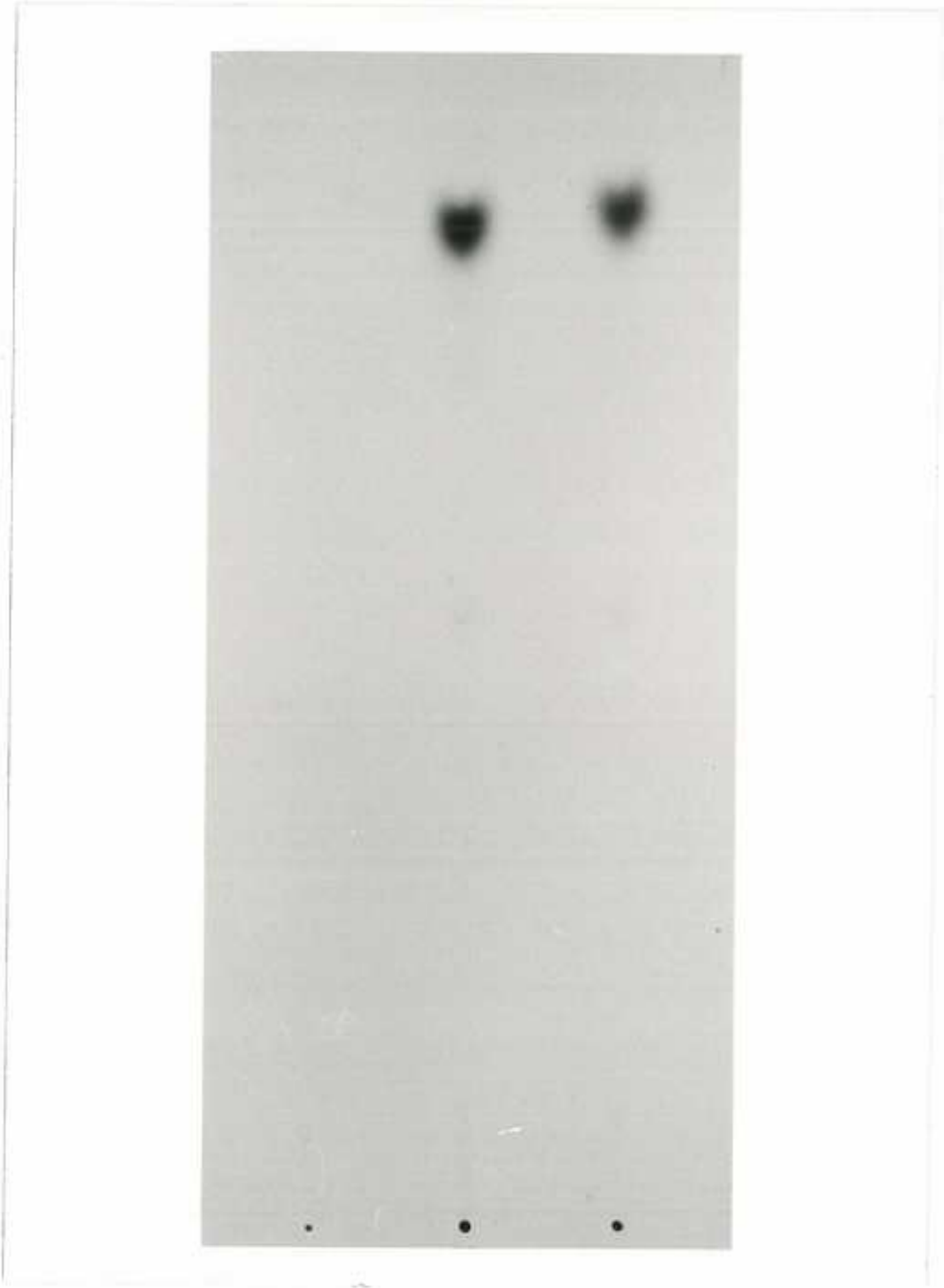

a
b
c
d
e
f
NAD
Aden.
AMP
ADPR

Figure 3. Thin-layer chromatography of the products formed by the reversal reaction. Beef liver EF-2 was radioactively labeled by diphtheria toxin fragment A (0.5μg/ml) or the endogenous ADP-ribosyl-transferase from the beef liver EF-2 preparation as shown in Figure 1B. After exhaustive dialysis to remove NAD, the labeled proteins were incubated in Tris-HCl buffer (pH 6.6). For the reverse reaction the ^{14}C-labeled EF-2 preparations were incubated with an excess of fragment A (10μg/ml) and 2 mM nicotinamide. The products of the reversal were analyzed by thin-layer chromatography using the same chromatographic system shown in Figure 2. Untreated ADP-ribosylated EF-2 was spotted at the origin of one lane as a control. Radioactive compounds were visualized by autoradiography. Lanes from left to right: untreated ADP-ribosylated EF-2; reversal product from EF-2 labeled in the presence of [adenosine-^{14}C] NAD by fragment A; and reversal product of EF-2 labeled by the beef liver endogenous transferase. The ^{14}C-labeled soluble product of the reversal cochromatographed with the NAD standard.



Paper 4.

Cellular Mono(ADP-ribosyl)transferase Inhibits Protein Synthesis

ABSTRACT

A rabbit reticulocyte cell-free protein synthesizing system deficient in functional endogenous EF-2 was used in reconstitution experiments with preparations of pyBHK EF-2, pyBHK mutant EF-2 which can not be ADP-ribosylated by diphtheria toxin fragment A and pyBHK EF-2 which was ADP-ribosylated by diphtheria toxin fragment A or pyBHK ADP-ribosyltransferase. Functional EF-2 was depleted in reticulocyte lysates by treatment with diphtheria toxin fragment A and NAD. The reticulocyte cell-free system was then reconstituted with pyBHK EF-2 following dialysis to remove NAD. Similar rates of protein synthesis were achieved with the cell-free system reconstituted with pyBHK EF-2 as with the unmodified reticulocyte EF-2 containing system. Adding NAD back to the cell-free system containing fragment A eliminates protein synthesis in systems reconstituted with pyBHK EF-2 but has no effect on systems reconstituted with mutant pyBHK EF-2. Cell-free systems reconstituted with pyBHK EF-2 which is ADP-ribosylated by diphtheria toxin fragment A or by pyBHK ADP-ribosyltransferase do not function in protein synthesis.

Introduction

Fragment A of diphtheria toxin and Pseudomonas toxin A are lethal toxins which kill cells by inhibiting protein synthesis (1,2,3). Cellular mono(ADP-ribosyl)transferases have recently been isolated from polyoma virus transformed baby hamster kidney (pyBHK) cells (4) and from beef liver which have the same molecular mechanism of action as fragment

A of diphtheria toxin and Pseudomonas toxin A (4). All four of these ADP-ribosyltransferases transfer ADP-ribose from NAD to the diphthamide residue of eukaryotic elongation factor-2 (EF-2). The effect of the ADP-ribosylation of EF-2 by the bacterial toxins is the inhibition of protein synthesis resulting in intoxication of cells (1,2,3). In this report we show that preparations of EF-2 from pyBHK cells which are ADP-ribosylated by the endogenous cellular ADP-ribosyltransferase or diphtheria toxin fragment A are no longer functional in the synthesis of proteins in a rabbit reticulocyte cell-free protein synthesizing system. These results further support an identical modification of the diphthamide residue of EF-2 by the cellular ADP-ribosyltransferase and by fragment A and also suggest a potential in vivo function for this cellular enzyme.

Experimental Procedures

Preparations of pyBHK EF-2 containing an endogenous cellular ADP-ribosyltransferase were prepared as previously described (5). The fractionation procedure removes a cytoplasmic inhibitor from the EF-2 and the ADP-ribosyltransferase which normally prevents the transfer of ADP-ribose from NAD to EF-2 by the endogenous enzyme in in vitro reactions (4).

A cell-free protein synthesizing system from rabbit reticulocytes was prepared as described by Pelham and Jackson (6). A portion of the reticulocyte lysate was treated with 37 μg/ml diphtheria toxin fragment A and 50 μg/ml NAD at 20°C for 15 min to ADP-ribosylate endogenous EF-2 in

the lysate and thus inactivate the EF-2 (5). The diphtheria toxin fragment A used in the reaction was purified as previously described (7). A portion of the fragment A treated reticulocyte lysate was then dialyzed extensively against deionized water at 0°C to remove NAD from the lysate. Dialyzed reticulocyte lysates could be effectively reconstituted by addition of preparations of pyBHK EF-2 since the fragment A in the lysate can no longer function in the absence of NAD. The concentration of EF-2 used to reconstitute reaction mixtures was calculated from the transfer of [^{14}C-adenosine]-ADP-ribose from NAD to EF-2 in the presence of fragment A as described by Gill and Dinius (8) and subsequently modified by the elimination of histamine (4). The reaction was performed at 22°C for 20 min in 0.1 ml of 25 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 6.33 μM [U-^{14}C-adenosine]-NAD (554 mCi/mM) (New England Nuclear Co.), 0.05 μg of fragment A and 10 μl of the EF-2 preparation. The trichloroacetic acid (TCA) precipitable radioactivity was collected on Millipore filters and counted in a low background counter (4). The EF-2 content of reticulocyte lysates was determined in a similar reaction mixture except a histamine containing buffer was used (8). Also, the lysate was pretreated with 0.5 M NaCl to free bound EF-2 from ribosomes and adsorbed with charcoal (Norit SG) to remove endogenous NAD from the lysate as previously described (8).

Preparations of pyBHK EF-2 used to reconstitute the in vitro protein synthesizing system were ADP-ribosylated by the endogenous ADP-ribosyltransferase or by addition of 0.05 μg of fragment A to 0.1 ml reaction mixtures containing 25 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol and 100 μg EF-2 with or without NAD or [^{14}C-adenosine]-NAD and incubated at 22°C. Aliquots (15 μl) were removed from the [^{14}C-adenosine]-NAD

containing reaction at time intervals and the TCA precipitable radioactivity transferred to EF-2 was determined in a low background counter (4). When the maximum transfer of label to an acid precipitable form had occurred, the reaction mixtures were transferred to dialysis tubing which had been presoaked in 100 µg/ml of bovine serium albumin and dialyzed extensively against buffer A (10) at 0°C. Following dialysis, the amount of acid precipitable ^{14}C-labeled EF-2 in solution and the amount adsorbed to the used dialysis tubing was determined by liquid scintillation.

The reticulocyte cell-free protein synthesis system was composed of 800 µl of reticulocyte cell lysate, 10 µl of 10 mg/ml creatine phosphokinase, 50 µl of 0.3 M creatine phosphate in 10 mM Tris-HCl (pH 7.5), 50 µl of 2 M KCl, 100 µl of the 19 essential amino acids at 0.5 mM each, 6 µl of 4 mM hemin, 10 µl of 400 mM $MgCl_2$ and, 6 µl of 200 mM dithiothreitol. The reaction system was mixed at 0°C and divided into 100 µl portions. The EF-2, ADP-ribosylated EF-2 or NAD was added to the reaction mixture and incubated at 30°C for 3 min, chilled to 0°C and then 5 µl of ^{3}H-leucine (4,5-^{3}H) L-leucine, 56.5 Ci/mM, New England Nuclear was added to complete the reaction mixture. A 15 µl sample at time 0 was taken and acid precipitated (6) and the remaining reaction mixture was incubated at 30°C. At time intervals, 15 µl samples were withdrawn from the reaction mixture, decolorized, acid precipitated, the precipitate was collected on Millipore filters and the amount of radioactivity was determined by liquid scintillation (6).

Results and Discussion

The incorporation of ^{3}H-leucine into trichloroacetic acid precipitable protein is linear during the initial 40 min. of incubation of the cell-free protein synthesizing system at 30°C. The reticulocyte lysate of this system has an endogenous EF-2 concentration of 90 μg/ml as determined by the specific transfer of [^{14}C-adenosine]-ADP-ribose from NAD to EF-2 by fragment A. Treatment of the lysate with fragment A and cold NAD results in a decreased amount of endogenous EF-2 still capable of accepting the transfer of ^{14}C-label from NAD by fragment A. The residual ADP-ribosylatable EF-2 in the fragment A treated reticulocyte lysate is 2.7 μg/ml. Following dialysis to remove cold NAD from the lysate, the cell-free protein synthesizing system was reconstituted by adding pyBHK EF-2 (Figure 1). Residual EF-2 in the fragment A treated system results in an extremely low level of protein synthesis as compared to the synthesis by the system containing untreated reticulocyte lysate. Increasing concentrations of pyBHK EF-2 added to the fragment A treated cell-free system result in progressively increasing rates of protein synthesis. At a pyBHK EF-2 concentration of 68 μg/ml the rate of protein synthesis of the reconstituted system is similar to that of the system containing untreated reticulocyte lysate (data not shown), indicating the translocation activity of the rabbit reticulocyte EF-2 is similar to the pyBHK EF-2 following the purification procedure.

The low level of protein synthesis of the fragment A treated reticulocyte lysate could be eliminated by the addition of NAD to 5mM (Figure 2). PyBHK EF-2 and mutant pyBHK EF-2 (mEF-2) which is totally resistant to ADP-ribosylation by diphtheria toxin fragment A (5,10) produce similar

rates of protein synthesis in the fragment A treated system indicating that removal of NAD from fragment A treated lysates by dialysis is sufficient to eliminate the ADP-ribosylation activity of the fragment A remaining in the lysate. Subsequent addition of NAD to the fragment A containing system totally eliminates protein synthesis of the system reconstituted with pyBHK EF-2 but has no effect on protein synthesis in the fragment A containing protein synthesizing system reconstituted with mEF-2.

PyBHK EF-2 was ADP-ribosylated by the endogenous pyBHK ADP-ribosyltransferase or by addition of diphtheria toxin fragment A in the presence of [^{14}C-adenosine]-NAD or cold NAD (Figure 3A). The fragment A catalyzed reaction is much more rapid than the cellular ADP-ribosyltransferase catalyzed reaction and is complete within 5 min. There is no further transfer of ^{14}C-adenosine from NAD to EF-2 by further incubation of the fragment A ADP-ribosylated EF-2 in the presence of its endogenous ADP-ribosyltransferase (Figure 3A). Similarly, EF-2 which accepts maximum transfer of ^{14}C-adenosine from NAD by the endogenous transferase can not be ADP-ribosylated further by the subsequent addition of fragment A (Figure 3B).

As shown in Figure 3A, reactions containing [^{14}C-adenosine]-NAD were used to indicate the time when maximum ADP-ribosylation had occurred in the parallel reactions containing cold NAD, pyBHK ADP-ribosyltransferase and fragment A. In addition, one ADP-ribosylation reaction mixture was identical except for the absence of NAD to determine the effect of the experimental manipulations on the translocation activity of the EF-2 in the reaction mixtures. The concentration of acid precipitable ^{14}C-labeled ADP-ribosylated EF-2 product of the reaction was monitored

during processing to determine loss of product. The only significant loss of ADP-ribosylated EF-2 occurred during dialysis to remove NAD from the reaction mixture. 40% of the labeled EF-2 adsorbed to the dialysis tubing even though it had been presoaked in 0.1 mg BSA/ml to presaturate protein adsorption sites on the tubing. ^{14}C-labeled NAD did not bind to the tubing.

As shown in Figure 4, the ability of the ADP-ribosylated EF-2 to reconstitute the fragment A treated protein synthesizing system was examined. ADP-ribosylation of pyBHK EF-2 by diphtheria toxin fragment A or by pyBHK ADP-ribosyltransferase eliminated the ability of the EF-2 to stimulate protein synthesis above the background levels of incorporation of the system. The mock treated EF-2 (treated identical to ADP-ribosylated EF-2 preparations, except NAD was absent) produced a rate of protein synthesis similar to untreated EF-2 in the reconstituted system. Therefore, ADP-ribosylation of the EF-2 by fragment A or the cellular ADP-ribosyltransferase inactivates the translocation activity of the EF-2 rather than an inactivation of the EF-2 by experimental manipulations.

In a previous report (4), we showed that pyBHK ADP-ribosyltransferase ADP-ribosylated the diphthamide residue of EF-2 in a manner identical to fragment A of diphtheria toxin. The identical modification of EF-2 by the two ADP-ribosyltransferases is supported by the absence of an additive transfer of ADP-ribose to EF-2 by the two enzymes and by their identical effect on the function of EF-2 in protein synthesis. Furthermore, these <u>in vitro</u> studies indicate the potentially lethal effect of the endogenous ADP-ribosyltransferase and predict a stringent control over this enzyme activity. A specific inhibitor of the pyBHK

ADP-ribosyltransferase has been reported (4), but its identity and mechanism of action is unknown.

It seems likely that the cellular ADP-ribosyltransferase might be used to "fine tune" protein synthesis at the level of functional EF-2. However, this could not be an essential control mechanism for cell survival in culture since mutants which contain non-ADP-ribosylatable EF-2 grow as well as wild-type cells (5). Control of protein synthesis by ADP-ribosylation of EF-2 may only function under certain conditions and may not be essential in cells adapted to growth in cell culture. To examine the potential role of cellular ADP-ribosyltransferase in regulating protein synthesis, conditions must be found in which the active form of the enzyme is demonstrated in vivo. Current studies are focused on the nature of the pyBHK ADP-ribosyltransferase and the mechanism of the specific inhibition of its activity by cytoplasmic extracts of pyBHK cells.

References

1. Collier, R.J. (1975), Bacteriol. Reviews 39, 54-85.

2. Iglewski, B.H. and Kabat, D. (1975), Proc. Natl. Acad. Sci. USA 72, 2284-2288.

3. Iglewski, B.H., Liu, P.V. and Kabat, D. (1977), Infect. Immun. 15, 138-144.

4. Lee, H. and Iglewski, W.J. (1984), Proc. Natl. Acad. Sci. USA, in press.

5. Iglewski, W.J. and Lee, H. (1983), Eur. J. Biochem. 134, 237-240.

6. Pelham, H.R.B. and Jackson, R.J. (1976), Eur. J. Biochem. 67, 247-256.

7. Pappenheimer, A.M. Jr., Uchida, T. and Avery-Harpter, A. (1972), Immunochemistry 9, 891-906.

8. Gill, D.M. and Dinius, L.L. (1973), J. Biol. Chem. 248, 654-658.

9. Raeburn, S., Collins, J.F., Moon, H.M. and Maxwell, E.S. (1971), J. Biol. Chem. 246, 1041-1048.

10. Sokol, P.A., Iglewski, B.H., Hager, T.A., Sadoff, J.C., Cross, A.S., McManus, A., Farber, B.F. and Iglewski, W.J. (1981), Infect. Immun. 34, 147-153.

FIGURE 1.

Reconstitution of the reticulocyte cell-free protein synthesizing system deficient in endogenous EF-2. A reticulocyte lysate containing 90 μg/ml ADP-ribosylatable EF-2 was treated with 37 μg/ml diphtheria toxin fragment A and 50 μg/ml NAD at 20°C for 15 min to inactivate endogenous EF-2. Following dialysis of the lysate to remove NAD, the protein synthesizing system was reconstituted by the addition of various concentrations of the pyBHK EF-2 preparations and the incorporation of ^{3}H-leucine into acid precipitable protein was determined in 0.1 ml reaction mixtures. (o---o) 6.6 μg EF-2 contributed by the reticulocyte lysate; (•---•) reaction mixture containing 0.2 μg of unADP-ribosylated EF-2 remaining following treatment of the lysate with fragment A; reconstituted with (▲——▲) 1.4 μg EF-2; (Δ——Δ) 4.1 μg EF-2; and (□——□) 5.5 μg EF-2.

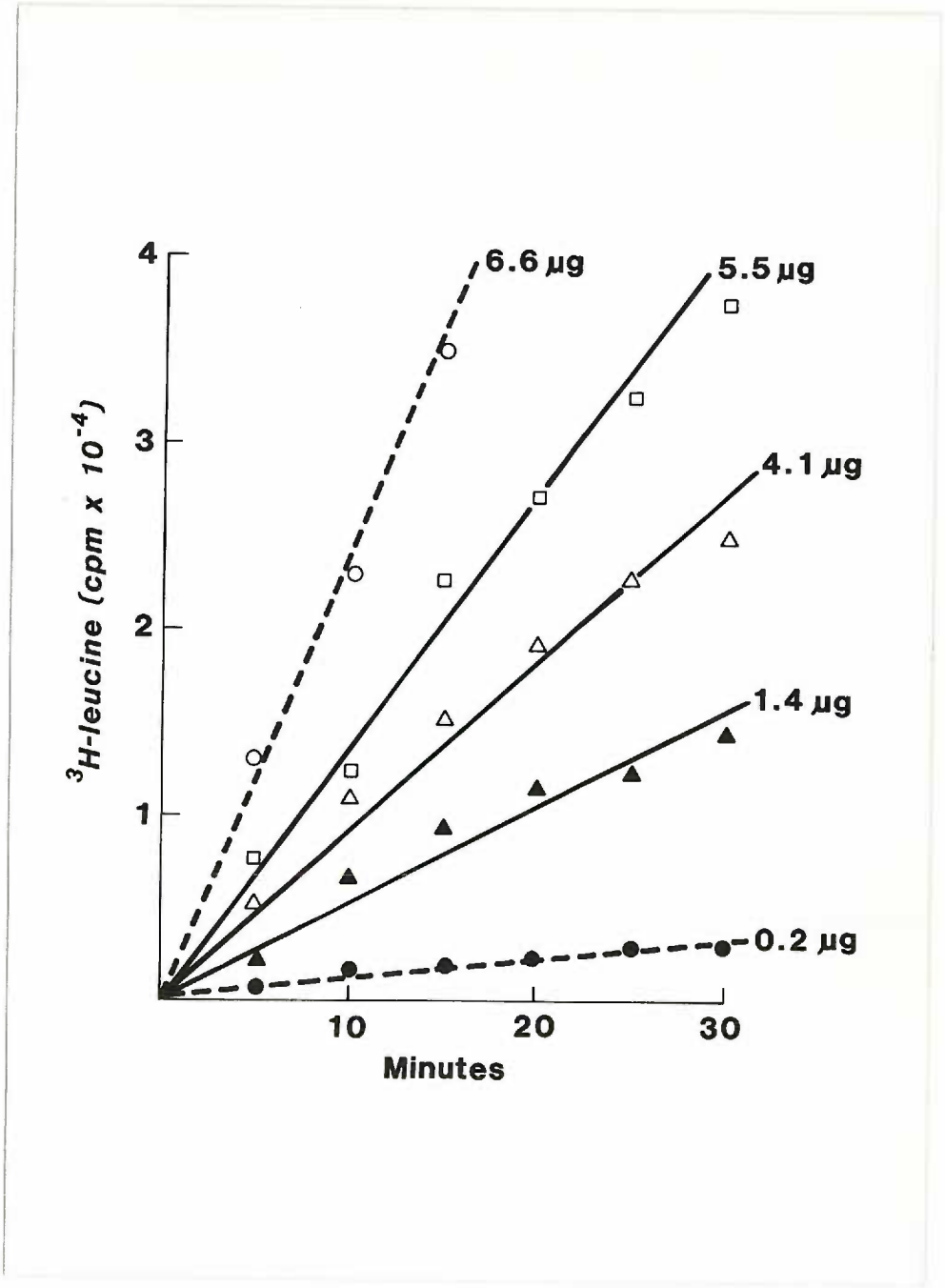

6.6 µg
5.5 µg
4.1 µg
1.4 µg
0.2 µg
^{3}H-leucine (cpm x 10^{-4})
4
3
2
1
10
20
30
Minutes

FIGURE 2.

The effect of the addition of mutant EF-2 and NAD on protein synthesis of the reticulocyte cell-free protein synthesizing system pretreated with diphtheria toxin fragment A. The EF-2 in a reticulocyte lysate was inactivated by treatment with fragment A and NAD as described in Figure 1. The lysate was dialyzed to remove NAD and was then reconstituted by the addition of pyBHK EF-2 or a mutant form of pyBHK EF-2 (mEF-2) which can not be ADP-ribosylated by fragment A (5,10). The incorporation of ^{3}H-leucine into acid precipitable protein was determined for 0.1 ml reactions containing: (▲---▲) 0.2 μg residual ADP-ribosylatable reticulocyte EF-2; reconstituted with (Δ——Δ) 5.5 μg pyBHK EF-2; and (■---■) 5.5 μg pyBHKR mEF-2. Incorporation of ^{3}H-leucine into protein was also determined in reactions containing 5 mM NAD plus: (●---●) 0.2 μg residual reticulocyte EF-2; (o---o) 5.5 μg pyBHK EF-2; and (□---□) 5.5 μg pyBHKR mEF-2.

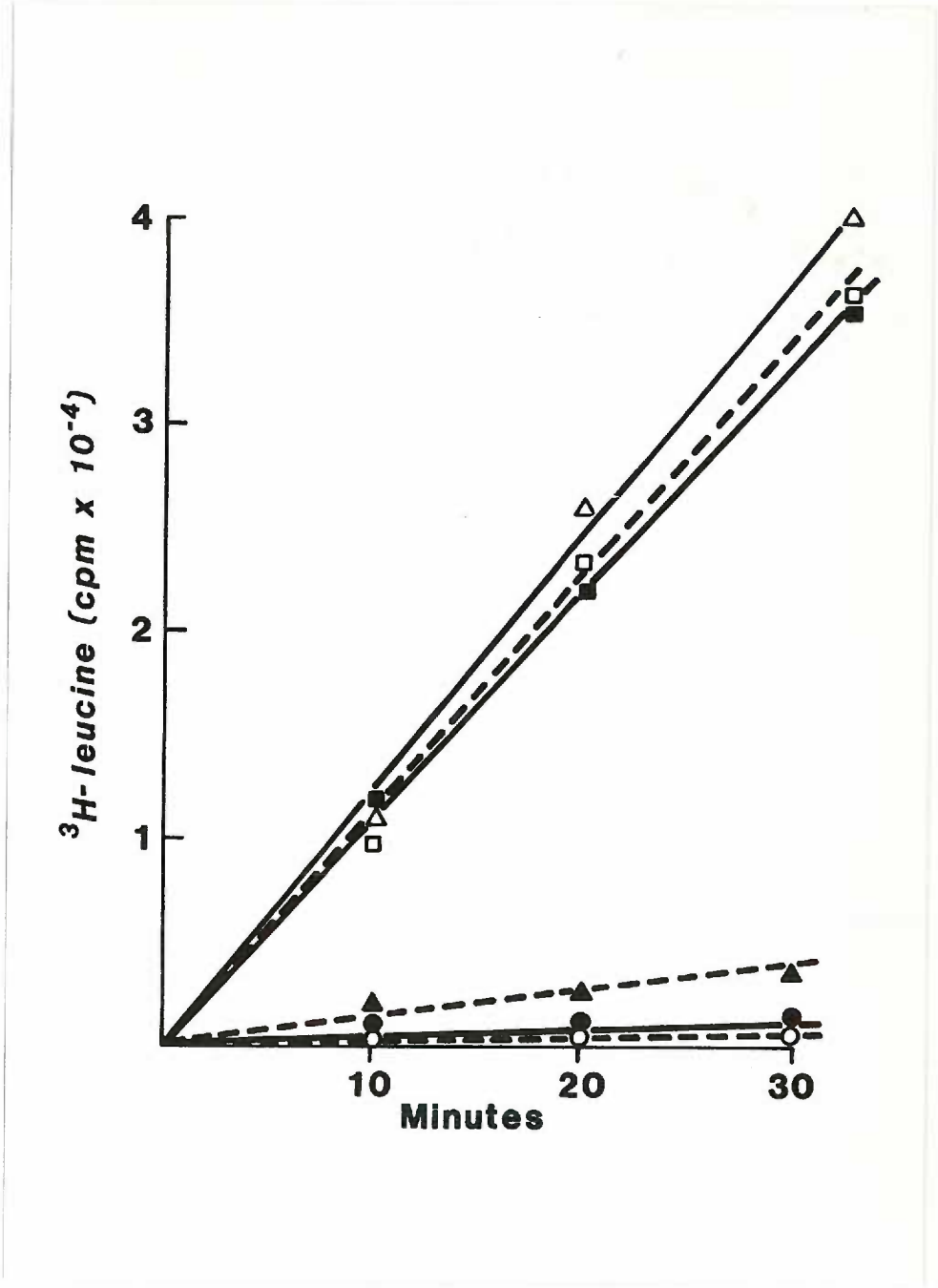
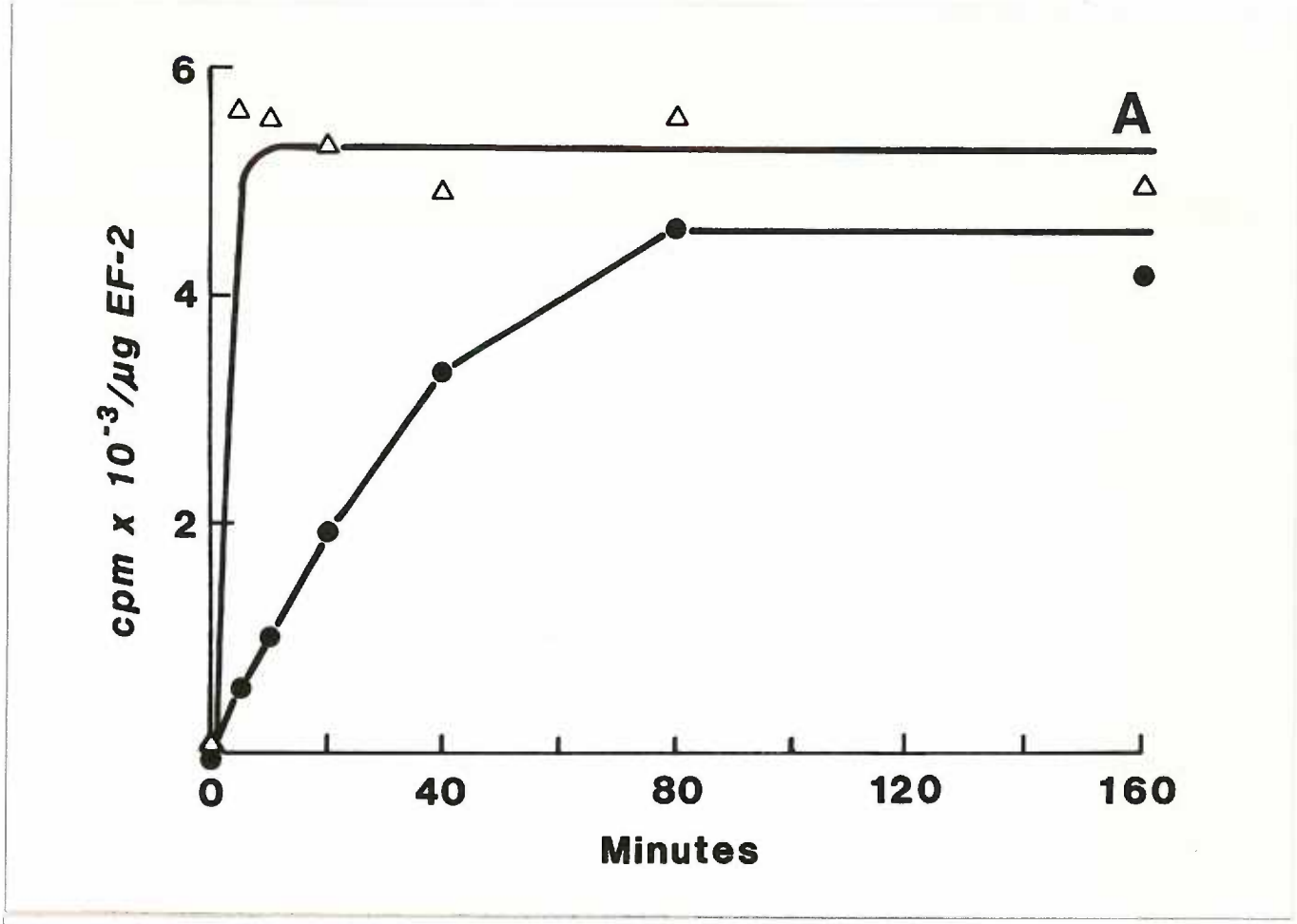

4
3
2
1
^3H-leucine (cpm x 10^{-4})
10
20
30
Minutes

FIGURE 3.

ADP-ribosylation of pyBHK EF-2 by diphtheria toxin fragment A and by pyBHK mono(ADP-ribosyl)transferase. The extent of ADP-ribosylation of pyBHK EF-2 preparations was monitored by the transfer of [^{14}C-adenosine]-ADP-ribose from NAD to EF-2 in the presence of fragment A and the endogenous pyBHK ADP-ribosyltransferase. The transfer of [^{14}C-adenosine]-ADP-ribose from NAD to EF-2 is shown after various times of incubation for (A) the (•——•) pyBHK ADP-ribosyltransferase catalyzed reaction; the (Δ——Δ) 0.05 μg of fragment A and pyBHK ADP-ribosyltransferase catalyzed reaction and (B) the (•——•) pyBHK ADP-ribosyltransferase catalyzed reaction to which 0.05 μg of fragment A was added after maximum transfer of ^{14}C-label to EF-2 had occurred. Additional EF-2 was subsequently added to demonstrate the rapid transfer of ^{14}C-label to EF-2 characteristic of the fragment A catalyzed reaction.


A
6
4
2
0
40
80
120
160
Minutes
cpm x 10-3/µg EF-2

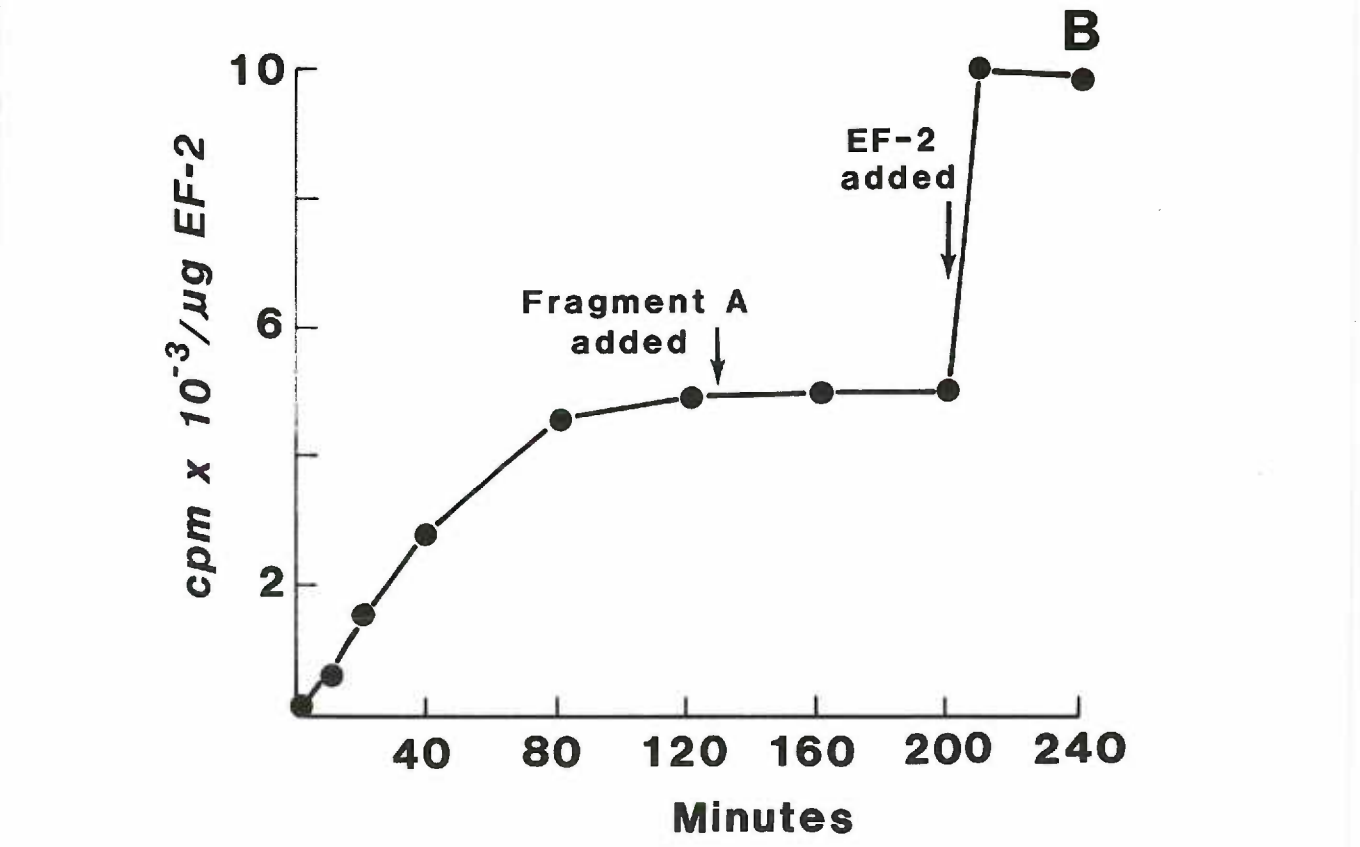
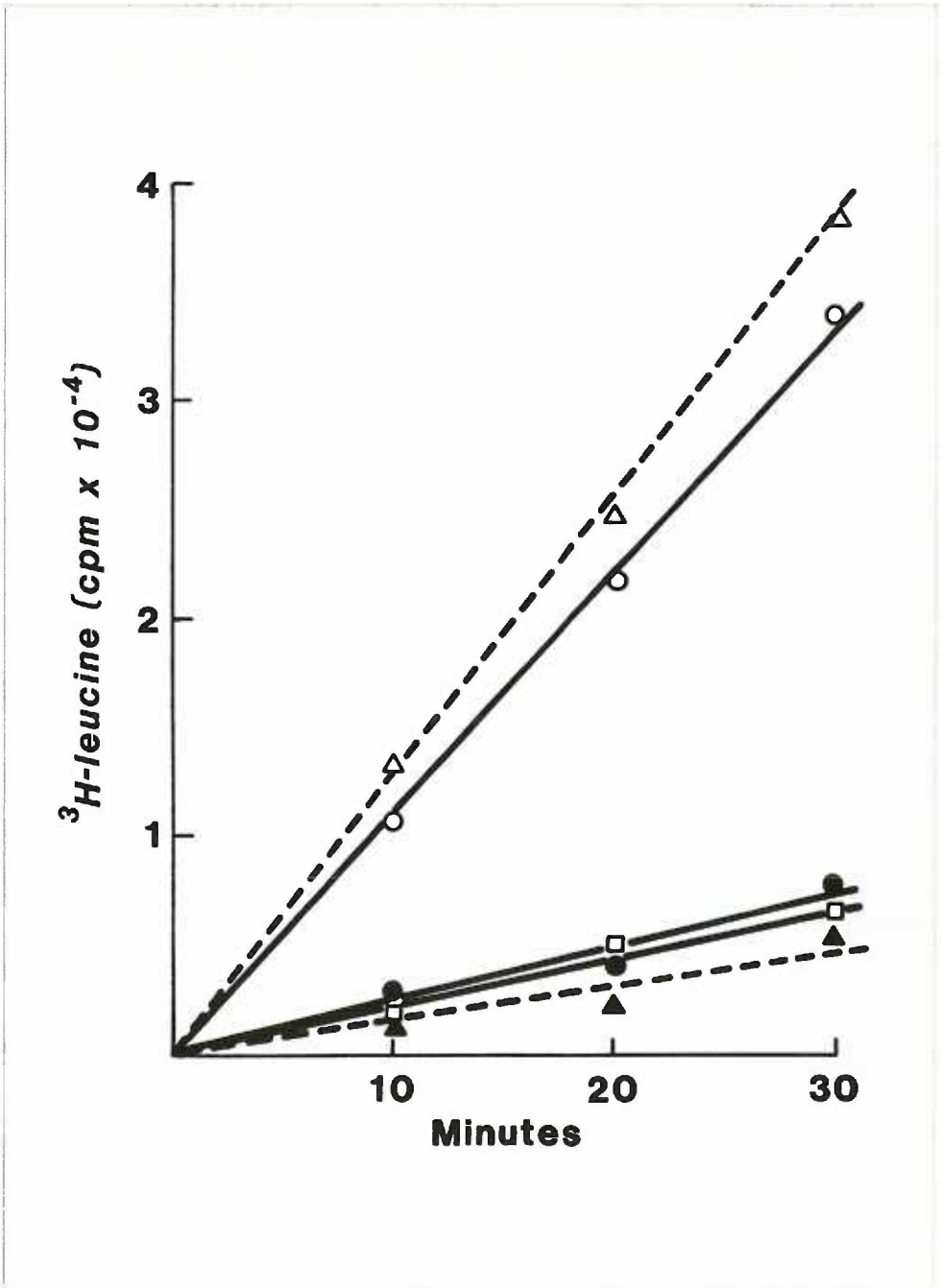

B
10
6
2
EF-2
added
Fragment A
added
40
80
120
160
200
240
Minutes
cpm x 10-3/µg EF-2

FIGURE 4.

Reconstitution of the reticulocyte cell-free protein synthesizing system deficient in endogenous EF-2 by addition of pyBHK EF-2 and pyBHK ADP-ribosylated EF-2. pyBHK EF-2 was ADP-ribosylated by either diphtheria toxin fragment A or pyBHK ADP-ribosyltransferase as described in Figure 3, except unlabeled NAD was used in this parallel reaction instead of ^{14}C-labeled NAD. When transfer of ADP-ribose to EF-2 had reached the maximum level, the ADP-ribosylated EF-2 was dialyzed and used to reconstitute the cell-free protein synthesizing system. One preparation of EF-2 (mock treated) was processed in an identical manner in the absence of NAD. The incorporation of ^{3}H-leucine into acid precipitable protein was determined in 0.1 ml reactions containing: (▲---▲) 0.2 µg residual ADP-ribosylatable reticulocyte EF-2; (Δ---Δ) 5.5 µg of untreated pyBHK EF-2; (o——o) 5.5 µg mock treated pyBHK EF-2; (●——●) 5.5 µg of pyBHK EF-2 ADP-ribosylated by fragment A; and (□——□) 5.5 µg of pyBHK EF-2 ADP-ribosylated by pyBHK ADP-ribosyltransferase.


4
3
2
1
^{3}H-leucine (cpm x 10^{-4})
10
20
30
Minutes

DISCUSSION

ADP-ribosylation reactions are a ubiquitous feature of biological entities. Enzymes that are able to catalyze these reactions have been found among viruses (1,2) procaryotes (3,4) and eucaryotes (5). Since ADP-ribosylated proteins are found in all eucaryotic cells examined to date (5) and in multiple compartments of the eucaryotic cell (6), it is assumed that these reactions are important in the metabolism of the cell. The most dramatic example of the effect of this kind of modification on eucaryotic cellular metabolism is seen during the intoxication of cells by diphtheria toxin or Pseudomonas exotoxin A, resulting in the inhibition of protein synthesis (7,8). Other effects, due to the modification of another ADP-ribose acceptor, include the choleragen or E. coli heat labile enterotoxin catalyzed ADP-ribosylation of the GTP binding protein of adenylate cyclase, resulting in the prolonged activation of this enzyme (8,9). Since it is evident that some ADP-ribosylation reactions have important consequences for the ultimate fate of the cell, the function of ADP-ribosylation reactions in general, insofar as these reactions all share the same mechanism of action, may be very significant in the regulation of cellular metabolism. Thus, the eucaryotic cellular counterparts of bacterial ADP-ribosyltransferases have been investigated as to their involvement in the regulation of various cellular activities.

ADP-ribosyltransferases can be divided into 2 groups: 1) those which catalyze the modification of proteins by mono(ADP-ribosyl)ation, the covalent transfer of single ADP-ribose units, and 2) those which catalyze poly(ADP-ribosyl)ation, the covalent transfer of a number of

ADP-ribose units, resulting in a homopolymer. All known bacterial ADP-ribosyltransferases, including those isolated from bacteriophages, transfer single ADP-ribose units. Mono(ADP-ribosyl)ation reactions, as catalyzed by endogenous or bacterial enzymes which transfer only single ADP-ribose units to proteins, produce a modified product which does not become further modified by subsequent ADP-ribosylations. Because there is only one modified product, functional assignments can be based on an all or none modification status. This is in contrast to poly(ADP-ribosyl)ation reactions, where an initial modification can be complicated by subsequent modifications. Even though cellular poly(ADP-ribosyl)ation reactions have been studied for many years and have been assigned putative roles in the regulation of eucaryotic DNA replication, DNA repair, cell division and cell differentiation (5,10), the study of mono(ADP-ribosyl)ation reactions offer the advantage that the physiological effects of a reaction can be attributed to a single reaction product.

The first endogenous mono(ADP-ribosyl)transferase to be discovered is one which uses arginine as an ADP-ribose acceptor (12). This enzyme has been isolated from turkey erythrocytes and catalyzes the transfer of ADP-ribose to a number of purified proteins. Furthermore, this transferase can catalyze the NAD^+-dependent activation of rat brain adenylate cyclase. However, it does not activate the cyclase from turkey erythrocytes and thus its true function remains unknown (8). A more recent finding concerning the adenylate cyclase system revealed an endogenous ADP-ribosylating activity in RL-PR-C rat hepatocyte plasma membranes that transfers ADP-ribose to the same membrane protein as that by cholera toxin. However, adenylate cyclase activity has not been

reported as a consequence of the endogenous reaction, and the acceptor residue is different for each process (13). Nonetheless, the activity of the endogenous hepatocyte plasma membrane ADP-ribosyltransferase can be stimulated by isoproterenol in a concentration dependent fashion, suggesting that transferase activity may be linked to catecholamine levels in vivo (14). In a separate report, Hofstetter proposed that the endogenous ADP-ribosylation of a mitochondrial membrane protein is involved in the release of calcium from rat liver mitochondria (15).

These examples have set the precedent for seriously considering the physiological significance of endogenous mono(ADP-ribosyl)ation reactions. What remains to be done to show an actual functional relationship between ADP-ribosylation and its role in the cell is, first, to characterize the enzyme-substrate interaction in vitro using purified components and, secondly, to show that the proposed ADP-ribosylation reaction proceeds in vivo.

With our EF-2 ADP-ribosylating enzyme system, we have initiated an approach that will permit further work to fulfill the two conditions as listed above. Using an EF-2 purification procedure that results in the attainment of a homogenous preparation of the protein from hamster tumors (16), we detected a copurifying NAD:EF-2 ADP-ribosylating enzyme activity (17). However, not all EF-2 preparations exibited the transferase activity to equal extents, even among preparations that originated from the same animal species, suggesting that this enzyme is not identical to EF-2 (unpublished observations). In order to obtain highly purified EF-2 ADP-ribosyl transferase, further processing of the hamster tumor extract beyond the EF-2 stage is required. Since we know that the endogenous enzyme activity can be completely neutralized by pseudomonas toxin A

antiserum, an antibody conjugated affinity column can be used to obtain the enzyme at the purity sufficient for biochemical analysis. Also, these same antibodies could be used as intracellular probes for the localization and quantitation of the transferase.

Another area that can be approached for study is the regulation of transferase activity. We know that PyBHK cell extracts contain a component(s) which inactivates or inhibits transferase activity (17) in a specific manner. The inhibition appears not to be the result of nonspecific degradation because fragment A can still catalyze the modification under identical conditions. Adding cellular extract to EF-2 that had already been fully modified by the cellular transferase does not lead to a decrease in TCA precipitable counts (Appendix). This inhibition is not limited to cells from which the transferase activity had been isolated but can also be similarly inhibited by extracts of rat liver cells (Appendix). This suggests that identical control mechanisms might exist to control a similar enzymatic activity in different organisms. Since an NAD:EF-2 ADP-ribosyltransferase has now been isolated from normal beef liver, (18) this suggests that this enzyme inhibitor system is probably ubiquitous among eucaryotic cells. Because the endogenous NAD:EF-2 ADP-ribosyltransferase shares a close resemblance to the diphtheria toxin fragment A catalyzed reaction, we investigated what effect the ADP-ribosylation of EF-2 by the endogenous cellular ADP-ribosyltransferase had on the ability of EF-2 to catalyze in vitro protein synthesis. Comparison of EF-2 which is ADP-ribosylated by diphtheria toxin fragment A with that modified by the endogenous transferase showed that PyBHK EF-2 is equally inactivated by the modification (19) regardless of which enzyme catalyzed the ADP-ribosylation.

The ADP-ribose acceptor site in EF-2 has been stringently conserved in eucaryotic cells (20,21,22). This, coupled with the apparent ubiquity of the cellular ADP-ribosyltransferase, suggests an essential physiological function for the modification. One possible role may be to fine tune the rate of protein synthesis at the level of functional EF-2 (18). Another possible involvement could be a mechanism which slows down the rate of translation sufficiently so that the accuracy of translation can be enhanced. Because cells containing a mutant form of EF-2 which is resistant to ADP-ribosylation by fragment A, and presumably by the PyBHK cellular transferase, can grow as well as wild type cells (16,23), control of protein synthesis at the level of functional EF-2 may not be necessary for the survival of all types of cells. However, an ADP-ribosyltransferase associated with the free mRNP which utilizes some proteins of the free mRNP as acceptors for mono and poly(ADP-ribosyl)-ation has been reported (24). Thus, it is possible that regulation of protein synthesis by ADP-ribosylation within the mRNP complex as a whole may be a generalized function. The modification, apart from any effect on the translocase activity of EF-2, may be connected with the repressed state of protein synthetic activity of free mRNP by affecting its tertiary structure.

On the other hand, in the cells which have the ADP-ribosylatable form of EF-2, it would be essential to maintain the endogenous transferase under stringent control, as we would otherwise expect a diphtheria toxin-like inhibition of protein synthesis to result. The kinetics of the inhibition reaction suggest that only a low level of transferase activity is permitted in the presence of cellular extracts (Appendix). It can be imagined that the inhibitor functions only upon

appropriate stimulation to limit transferase activity to an acceptable level within the cell. Since it appears that EF-2 undergoes a configurational change upon ADP-ribosylation (16), the stimulation may arise from a unique structure presented by ADP-ribosylated EF-2.

The possibility of an enzyme which can reverse the ADP-ribosylation reaction by removing the ADP-ribose moiety from its acceptor has been discussed (8,12). Because a single molecule of fragment A is thought to be sufficient for producing a lethal effect on a cell (25), it seems unlikely that intracellular removal of ADP-ribose occurs to any significant extent. Efforts to detect such a pathway by others have not yielded positive results (12). These findings are consistent with our working hypothesis. Control of the ADP-ribosylation reaction occurs at the point where the cell decides whether or not to let the reaction proceed. Once initiated, the reaction proceeds to the point where the concentration of the reaction product, ADP-ribosylated EF-2, triggers a response from the cell to inhibit the reaction. The interaction of the endogenous transferase with its specific inhibitor is a mechanism for down regulating the rate of translation at the translocation step. Taken together, our findings point to another level of regulation that can operate prior to the final expression of a particular gene product. From the extensive work carried out in recent years by investigators in different specialities, it is apparent that the expression of information that is stored in a gene is regulated at many different levels. Some of the control mechanisms may be a characteristic of a particular cell line, or may reflect a developmentally regulated step in a particular organism; however, most of the mechanisms for the control of gene expression are shared among cells.

Since the diphthamide residue is a conserved amino acid that is common to eucaryotic EF-2 (26,27) this suggests that it is involved in an important cellular function. The control of the enzymatic activity of EF-2 by modification of this residue may be a phenomenon that is related to the conserved presence of diphthamide in EF-2. Thus, the control of gene expression through the modification of EF-2 could represent an additional level of regulation that is shared among eucaryotic cells. Further work will be able to reveal if the diphthamide residue is concerned with any other functional roles.

SUMMARY AND CONCLUSIONS

A cellular ADP-ribosylating activity has been detected in EF-2 preparations purified from polyoma transformed baby hamster kidney (PyBHK) cells. A monomer of ADP-ribose from NAD^+ is covalently transferred to EF-2 in the cellular transferase catalyzed reaction that can be reversed using excess fragment A under suitable conditions. Furthermore, fragment A does not catalyze additional transfer of ADP-ribose to EF-2 once the EF-2 had been fully modified in the presence of the endogenous transferase. These results further support the conclusion that modification of the diphthamide residue of EF-2 by the endogenous transferase and by fragment A is identical. Thus, the reaction catalyzed by the cellular ADP-ribosyl transferase has the same mechanism of action as that of fragment A of diphtheria toxin. The endogenous transferase can be distinguished from fragment A and Pseudomonas exotoxin A by its behavior in the presence of cytoplasmic extracts and in the presence of histamine. Cellular extracts contain at least one non-dialyzable component which can specifically inhibit the cellular enzyme catalyzed reaction but has no effect on the reaction catalyzed by fragment A, representing a possible control mechanism exerted by these cells to restrict endogenous transferase activity to physiological levels. The cellular ADP-ribosylating activity is not unique to PyBHK cells, which might imply that this activity is a property of only virus infected and transformed cells. A similar ADP-ribosylating activity has been isolated from normal beef liver, thereby suggesting the ubiquitous presence of this enzyme among eucaryotic cells.

The ability of PyBHK EF-2 after ADP-ribosylation by diphtheria toxin fragment A or the endogenous transferase to catalyze the translocation step of protein synthesis were compared using a cell free protein synthesizing system derived from rabbit reticulocytes. The results showed that EF-2 which is ADP-ribosylated by either transferase no longer functions in protein synthesis. From these results, it is proposed that the cellular ADP-ribosyltransferase: ADP-ribosyltransferase inhibitor system interacts with EF-2 *in vivo* to control protein synthesis at the level of functional EF-2.

Discussion References

1. Rohrer, H., Zillig, W., and Mailhammer, R. (1975). ADP-ribosylation of DNA-dependent RNA polymerase of Escherichia coli by an NAD^+:protein ADP-ribosyltransferase. Eur. J. Biochem. 60:227-238.

2. Goff, C.G. (1974). Chemical structure of a modification of the Escherichia coli ribonucleic acid polymerase α polypeptides induced by bacteriophage T_4 infection. J. Biol. Chem. 249:6181-6190.

3. Skorko, R. and Kur, J. (1981). ADP-ribosylation of proteins in non-infected Escherichia coli cells. Eur. J. Biochem. 116:317-322.

4. Hayaishi, O. and Ueda, K. (1982). Poly-and mono(ADP-ribosyl)ation reactions: their significance in molecular biology. In O. Hayaishi and K. Ueda (Eds.) ADP-Ribosylation Reactions. pp. 3-16.

5. Hayaishi, O. and Ueda, K. (1977). Poly(ADP-ribose) and ADP-ribosylation of proteins. Ann. Rev. Biochem. 46:95-116.

6. Adamietz, P., Wielckens, K., Bredehorst, R., Lengyel, H., and Hilz, H. (1981). Subcellular distribution of mono(ADP-ribose) protein conjugates in rat liver. Biochem. Biophys. Res. Comm. 101:96-103.

7. Collier, R.J. (1975). Diphtheria toxin: mode of action and structure. Bacteriol. Rev. 39:54-85.

8. Vaughan, M. and Moss, J. (1981). Mono(ADP-ribosyl)transferases and their effects on cellular metabolism. Curr. Top. Cell. Reg. 20:205-246.

9. Moss, J. and Vaughan, M. (1979). Activation of adenylate cyclase by choleragen. Ann. Rev. Biochem. 48:581-600.

10. Hilz, H. and Stone, P. (1976). Poly(ADP-ribose) and ADP-ribosylation of proteins. Rev. Physiol. Biochem. Pharmacol. 76:1-58.

11. Pekala, P.H. and Moss. J. (1983). Poly ADP-ribosylation of protein. Curr. Top. Cell. Reg. 22:1-49.

12. Moss, J. and Vaughan, M. (1978). Isolation of an avian erythrocyte protein possessing ADP-ribosyltransferase activity and capable of activating adenylate cyclase. Proc. Natl. Acad. Sci. USA. 75:3621-3624.

13. Beckner, S.K. and Blecher, M. (1981). Endogenous and cholera toxin-catalyzed ADP-ribosylation of a plasma membrane protein of RL-PR-C cloned rat hepatocytes. Biochim. et Biophys. Acta 673:477-486.

14. Reilly, T.M., Beckner, S., McHugh, E.M. and Blecher, M. (1981). Isoproterenol-induced ADP-ribosylation of a single plasma membrane protein of cultured differentiated RL-PR-C hepatocytes. Biochem. Biophys. Res. Comm. 98:1115-1120.

15. Hofstetter, W., Muhlebach, T., Lotscher, H.-R., Winterhalter, K.H., and Richter, C. (1981). ATP prevents both hydroperoxide-induced hydrolysis of pyridine nucleotides and release of calcium in rat liver mitochondria. Eur. J. Biochem. 117:361-367.

16. Iglewski, W.J. and Lee, H. (1983). Purification and properties of an altered form of elongation factor 2 from mutant cells resistant to intoxication by diphtheria toxin. Eur. J. Biochem. 134:237-240.

17. Lee, H. and Iglewski, W.J. (1984). Cellular ADP-ribosyltransferase with the same mechanism of action as diphtheria toxin and pseudomonas toxin A. Proc. Natl. Acad. Sci. USA. 81:2703-2707.

18. Iglewski, W.J., Lee, H., and Muller, P. (1984). ADP-ribosyl-transferase from beef liver which ADP-ribosylates elongation factor-2. FEBS. Lett. 173:113-118.

19. Iglewski, W.J. and Lee, H. (1984). Cellular Mono(ADP-ribosyl)-transferase inhibits protein synthesis. Submitted.

20. Collier, R.J. (1975). Diphtheria toxin: mode of action and structure. Bacteriol. Rev. 39:54-85.

21. Pappenheimer, Jr., A.M. (1977). Diphtheria toxin. Ann. Rev. Biochem. 46:69-94.

22. Brown, B.A. and Bodley, J.W. (1979). Primary structure at the site in beef and wheat elongation factor 2 of ADP-ribosylation by diphtheria toxin. FEBS. Lett. 103:253-255.

23. Moehring, J.M. and Moehring, T.J. (1979). Characterization of the diphtheria toxin-resistance system in Chinese hamster ovary cells. Som. Cell Gen. 5:453-468.

24. Elkaim, R., Thomassin, H., Niedergang, C., Egly, J.M., Kempf, J. and Mandel, P. (1983). Adenosine diphosphate ribosyltransferase and protein acceptors associated with cytoplasmic free messenger ribonucleoprotein particles. Biochimie. 65:653-659.

25. Pappenheimer, Jr., A.M. and Gill, D.M. (1973). Diphtheria. Science. 182:353-358.

26. Van Ness, B.G., Howard, J.B., and Bodley, J.W. (1980). ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products. J. Biol. Chem. 255:10710-10716.

27. Oppenheimer, N.J. and Bodley, J.W. (1981). Diphtheria toxin. Site and configuration of ADP-ribosylation of diphthamide in elongation factor 2. J. Biol. Chem. 256:8579-8581.

Appendix A. Inhibition of PyBHK Cellular ADP-ribosyltransferase Activity by Rat Liver Cellular Extract

We have reported that crude cytoplasmic extracts of PyBHK cells inhibit the endogenous transferase activity in the PyBHK EF-2 preparations but not the enzyme activity of diphtheria toxin fragment A (1). When purified EF-2 is incubated with an equal volume of freshly thawed PyBHK cell extract for 10 min at 37°C prior to assay of transferase activity, the EF-2:extract mixture, in the absence of added fragment A, incorporates a low level of TCA precipitable [^{14}C]adenosine label. This is in contrast to the corresponding fragment A catalyzed reaction, which incorporates the same high level of TCA precipitable [^{14}C]adenosine label in the presence of cytoplasmic extracts as in the presence of extraction buffer only.

Rat liver extract was prepared in the same fashion as the PyBHK cell extract, described in (2). PyBHK EF-2 is incubated with an equal volume of rat liver extract or EF-2 purification buffer for 10 min at 37°C prior to use in the assay of transferase activity as described in (1). The control reactions contained 0.5 μg of purified fragment A in addition to the test reaction mixture. The values generated from this experiment (Table 1) show the level of TCA precipitable [^{14}C]adenosine label taken from each reaction at the last time point (160 min). Rat liver extract is seen to have a similar effect on endogenous transferase activity as PyBHK cell extracts. The activity of the endogenous transferase is approximately 4% of the corresponding fragment A catalyzed reaction or the level of incorporation seen in the presence of buffer only. However, fragment A activity is the same under either condition. The maximum

amount of TCA precipitable label incorporated by the endogenous transferase in the presence of buffer only is not significantly different from that of fragment A under either condition.

To further characterize the inhibitor present in PyBHK cell extracts, the same extract preparation as reported in (1) will be dialyzed against the extraction buffer as described in (2). After dialysis, the cellular extract is incubated with an equal volume of purified EF-2 for 10 min at 37°C. This mixture is later used in the modification assay. Undialyzed, boiled and centrifuged or unboiled and centrifuged PyBHK cell extract incubated with an equal volume of purified EF-2 served as positive and negative controls, respectively. This assay is performed as described (1) but modified by using EF-2 mixed with PyBHK cellular extracts prepared as described. The PyBHK cellular extract was dialyzed against 500 ml of extraction buffer for 8 hrs with 1 buffer change after 4 hrs. Before incubation with EF-2, all extracts were microfuged for 1 min on a Beckman microfuge B. Only the top portion of the microfuged solution is used for incubation with EF-2. The mixtures were incubated at room temperature (22°C). After 160 min of incubation, the amount of label incorporated in the presence of unboiled extract, dialyzed and undialyzed is 16% and 15%, respectively, of the amount of label incorporated in the presence of the undialyzed and boiled cellular extract.

To rule out any possible inhibitory effects due to the extraction buffer alone, a modification assay comparing the influence of extraction buffer on the reaction with that of deionized H_2O is performed. For this experiment, EF-2 is preincubated with an equal volume of either extraction buffer or deionized H_2O (control) at 37°C for 10 min.

Afterwards, the mixtures were cooled on ice and kept there until they were used for the experiment. At the end of this assay, incorporation of label in the presence of extraction buffer is higher (119%) compared with that in the presence of deionized H_2O (100%). Thus, the extraction buffer does not have any adverse effect on the incorporation of label as catalyzed by the endogenous transferase.

Taken together, these results show that both rat liver and PyBHK cellular extracts contain at least one component which specifically inhibits the endogenous transferase activity, preventing it from ADP-ribosylating EF-2. The ADP-ribose acceptor ability of EF-2 is not affected by the cellular inhibitor because fragment A can still catalyze the ADP-ribosylation reaction. In the case of PyBHK cell extract, the inhibitor is not dialyzable.

Table 1. Incorporation of Label Catalyzed by Fragment A and the Endogenous Transferase in the Presence of Rat Liver Cell Extract.

	Rat Liver Cell Extract[a]	EF-2 Purification Buffer[a]
Endogenous Transferase	162	3577
Fragment A	3734	3648

[a] Incorporation is recorded as counts per min per μg of purified hamster tumor EF-2.

Appendix B. Effect of Various Pseudomonas aeruginosa toxin A Antisera on the Activity of the Endogenous Transferase

To determine whether Pseudomonas aeruginosa toxin A (toxin A) antisera neutralize the activity of the endogenous enzyme, EF-2 is preincubated with an equal volume of either toxin A antiserum or normal rabbit serum for 5 min at 37°C. After preincubation, the mixtures were cooled on ice before use in the assay. The assay of transferase activity is performed as described (1) and modified by the inclusion of toxin A antiserum or normal rabbit serum in the reaction mixtures. The reaction mixtures were incubated at room temperature (22°C). 10 µl samples were periodically withdrawn and coprecipitated with 100 µl BSA (0.1 mg/ml) using 1 ml 10% TCA.

Both of the toxin A antisera used are inhibitory towards the endogenous transferase activity (Figures 1 and 2). Most of the incorporation of acid precipitable [^{14}C]adenosine label is inhibited in the presence of rabbit toxin A antiserum, whereas no incorporation of label occurred in the presence of sheep toxin A antiserum. These experiments show that the endogenous transferase can be neutralized by toxin A antisera.

To show that toxin A and endogenous transferase activities can be neutralized by toxin A antiserum, purified toxin A at 0.15 µg/10 µl is incubated with an equal volume of either toxin A antiserum or normal rabbit serum for 5 min at 37°C prior to assay. After incubation, the mixtures were cooled on ice. The assay is performed as described (1) and modified by the addition of the toxin A-antiserum or toxin A-serum mixture to the reaction. The mixtures were incubated at room temperature

(22°C). 10 µl samples were periodically withdrawn and coprecipitated with 100 µl BSA (0.1 mg/ml) using 1 ml 10% TCA. The TCA precipitable label was counted on a low background counter.

In the presence of toxin A antiserum (Figure 3), there was only a trace level of incorporated label with time. However, in the presence of control rabbit serum, there was a large increase in the amount of TCA precipitable label. The label incorporated in the presence of control rabbit serum is in 60 fold excess of that in the presence of toxin A antiserum. Thus, toxin A antiserum does neutralize toxin A activity; previous evidence that endogenous transferase activity can be neutralized by toxin A antiserum is reconfirmed. The level of TCA precipitable label in the presence of toxin A is similar to that attained in the presence of fragment A using the same EF-2 preparation.

Figure 1. Endogenous cellular transferase activity after pretreatment with affinity purified rabbit toxin A antiserum or normal rabbit serum. Purified PyBHK EF-2 preparations containing endogenous transferase activity is incubated for 5 min at 37°C with an equal volume of either rabbit toxin A antiserum or normal rabbit serum prior to the assay for ADP-ribosyltransferase activity. This assay is described as in (1) but modified by the additional presence of toxin A antiserum or control serum in the reaction. cpm x 10^{-3} is the acid-insoluble radioactive counts per min per μg EF-2.

Symbols: ADP-ribosyltransferase activity in the presence of rabbit toxin A antiserum (●) or control rabbit serum (Δ).

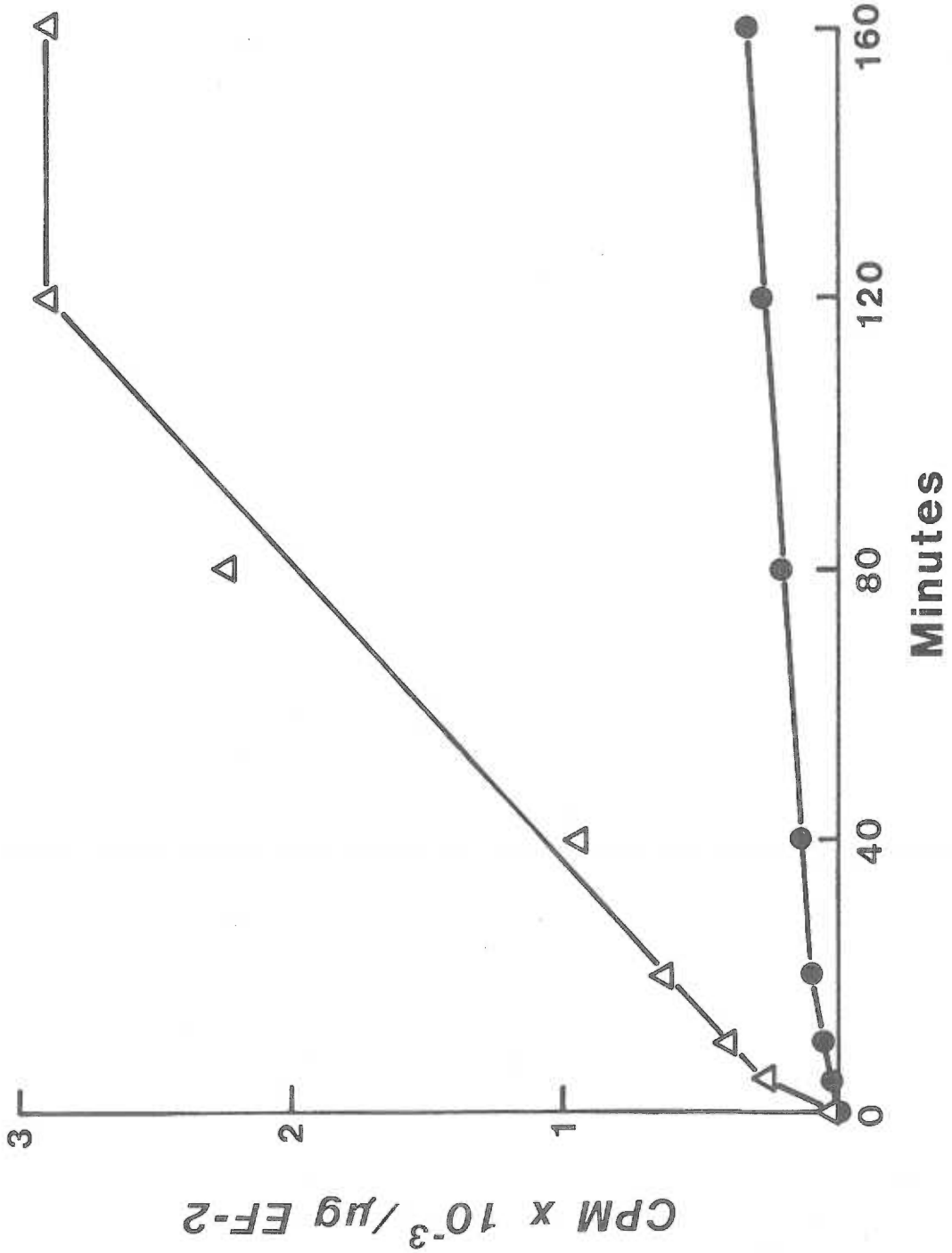

CPM x 10⁻³/μg EF-2
3
2
1
0
40
80
120
160
Minutes

Figure 2. Endogenous cellular transferase activity after pretreatment with a 50% ammonium sulfate cut of sheep toxin A antiserum or normal rabbit serum. Purified PyBHK EF-2 preparations containing endogenous transferase activity are each incubated for 5 min at 37°C with equal volumes of either sheep toxin A antiserum or normal rabbit serum prior to the assay for ADP-ribosyltransferase activity. This assay is described as in (1) but modified by the additional presence of toxin A antiserum or control serum in the reaction. cpm x 10^{-3} is the acid-insoluble radioactive counts per min per μg EF-2.

Symbols: ADP-ribosyltransferase activity in the presence of sheep toxin A antiserum (▲) or control rabbit serum (o).

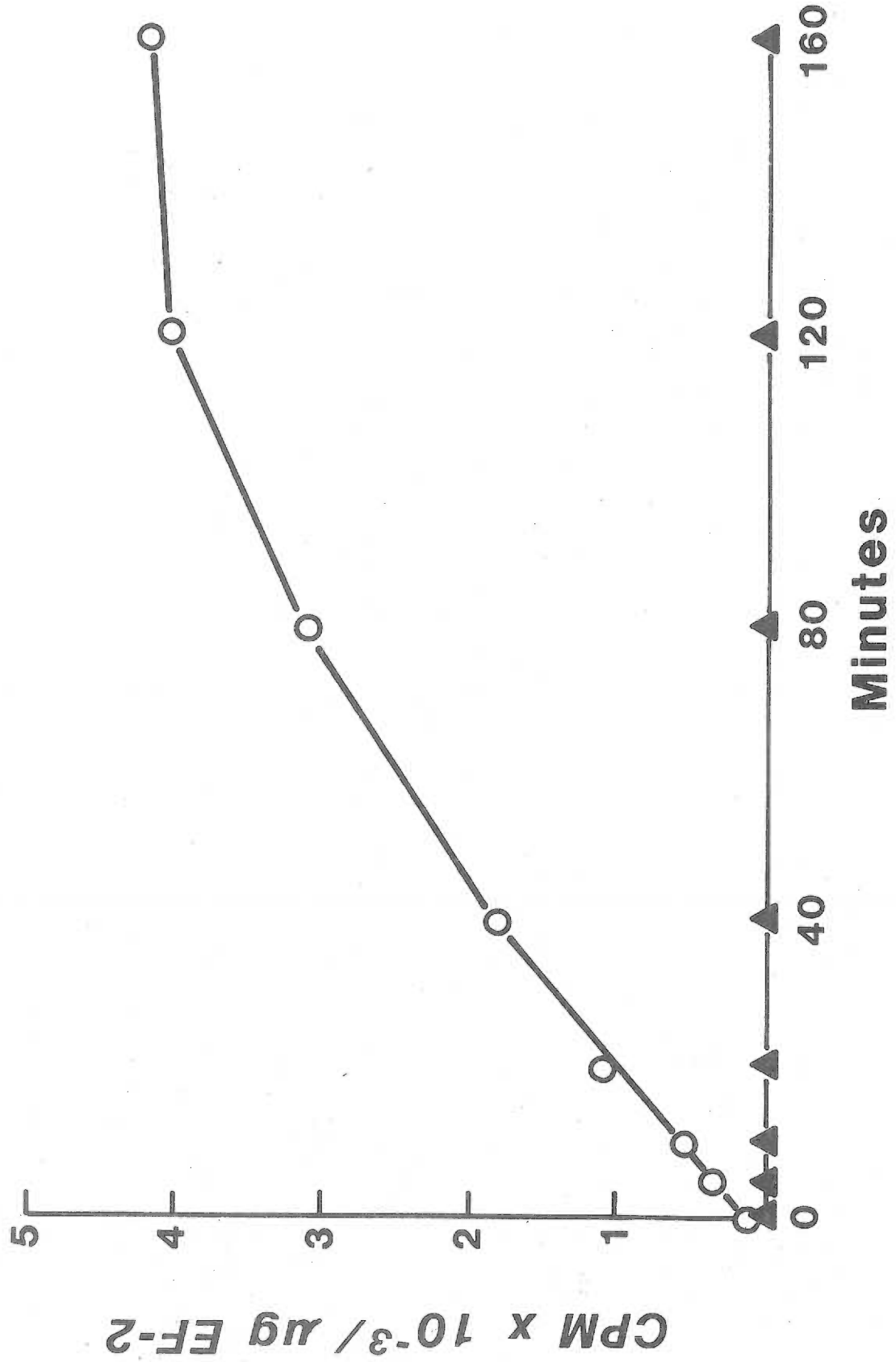
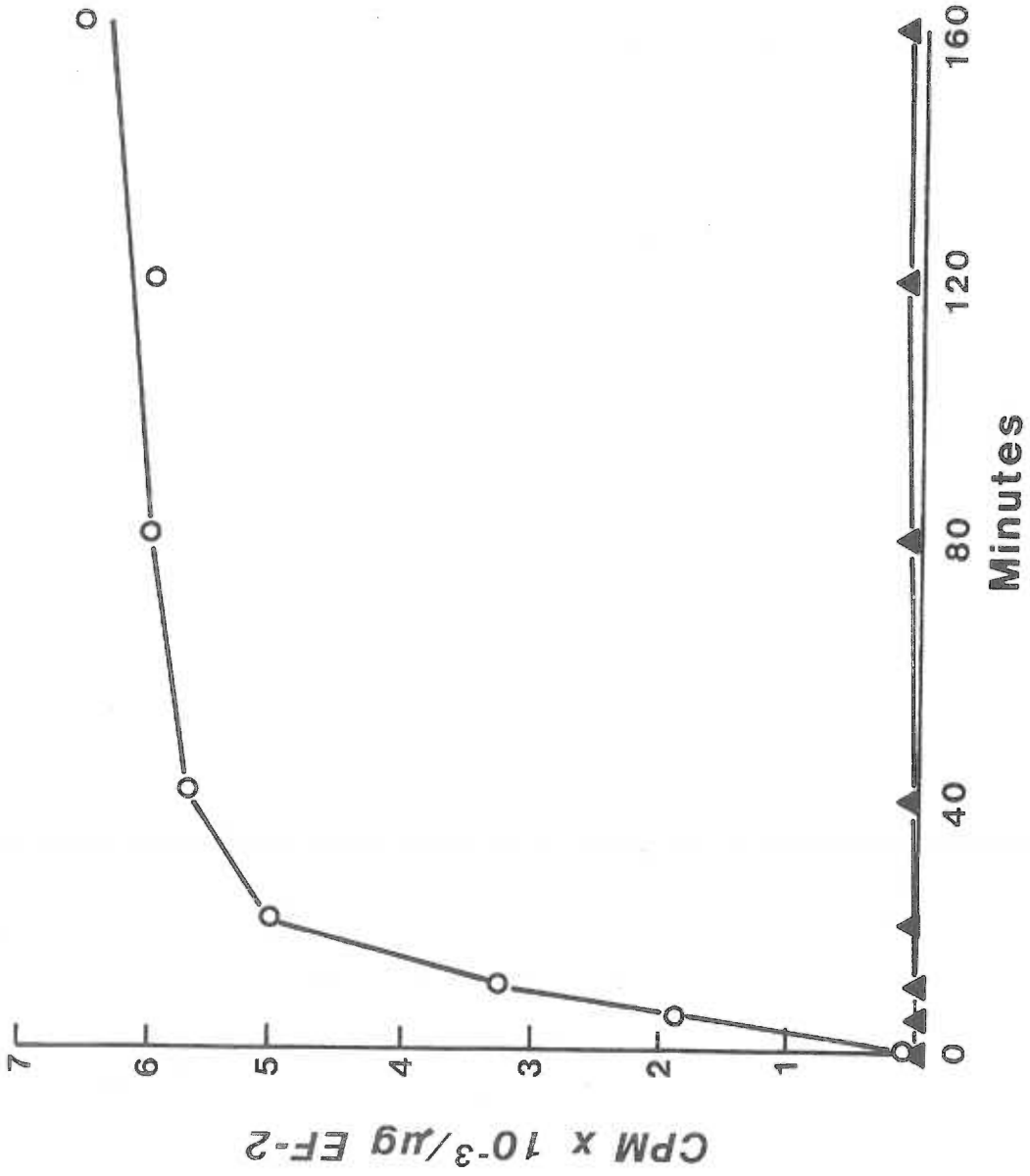

CPM x 10-3/ µg EF-2
5
4
3
2
1
0
40
80
120
160
Minutes

Figure 3. Effect of sheep toxin A antiserum or control rabbit serum on both toxin A ADP-ribosyltransferase and endogenous cellular transferase activities. Purified toxin A is incubated for 5 min at 37°C with an equal volume of either sheep toxin A antiserum or control rabbit serum prior to the assay for ADP-ribosyltransferase activity. This assay is described as in (1) but modified by the additional presence of toxin A mixed with toxin A antiserum or control rabbit serum. EF-2 is subsequently added to each reaction. cpm x 10^{-3} is the acid insoluble radioactive counts per min per μg EF-2.

Symbols: ADP-ribosyltransferase activities in the presence of sheep toxin A antiserum (▲) or control rabbit serum (o).

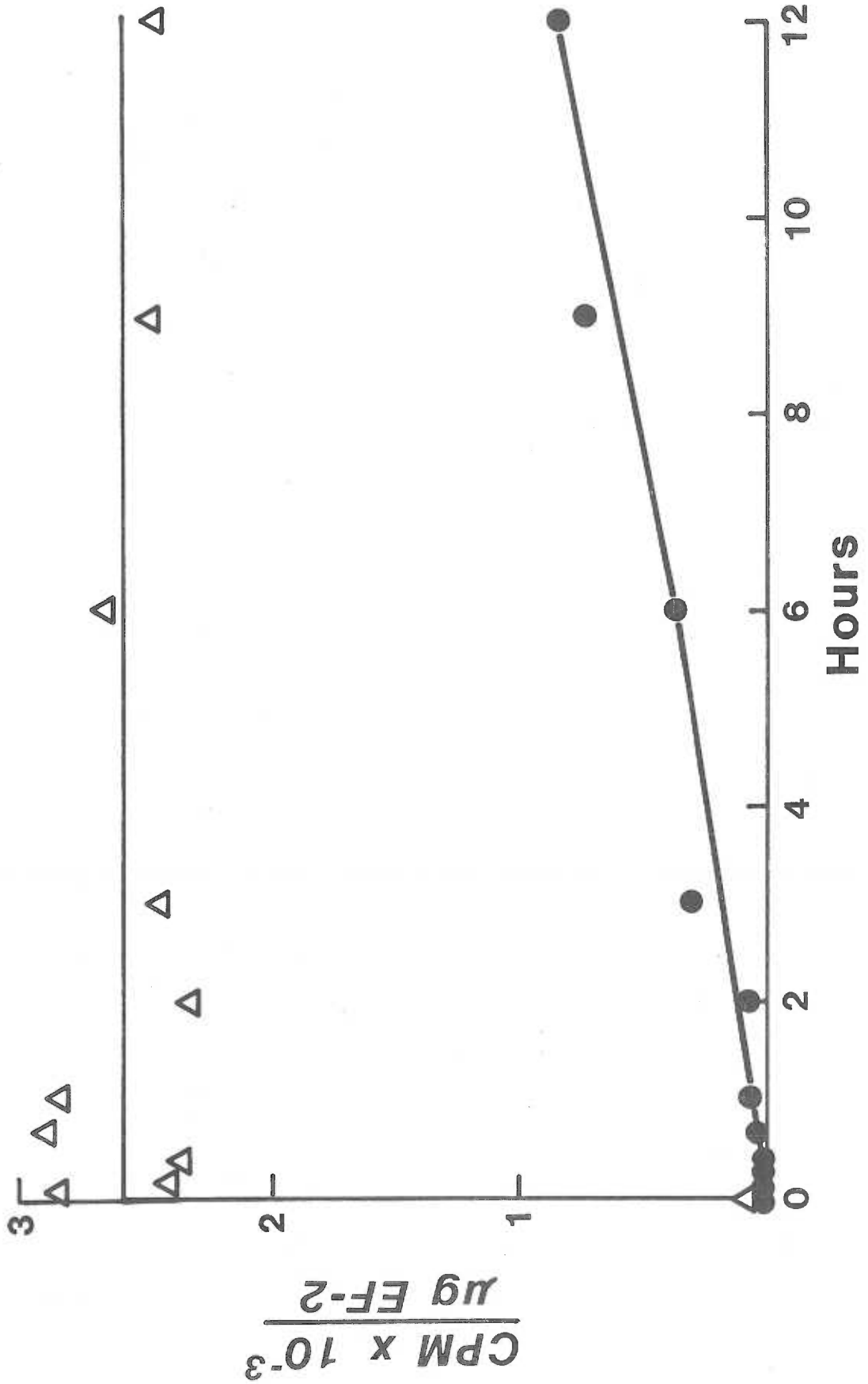

CPM x 10-3/µg EF-2
7
6
5
4
3
2
1
0
40
80
120
160
Minutes

Appendix C. Kinetics of EF-2 Modification by the Endogenous Transferase from Beef Liver.

The endogenous transferase of beef liver appears to modify EF-2 at a very slow rate (3) as determined by using the assay of transferase activity described in (1). To determine whether a larger than normal sampling time is required to observe the maximum transfer of TCA precipitable [^{14}C]adenosine label, the time allowed for endogenous ADP-ribosylation is extended to 12 hrs. The modification reaction is performed according to described methods (1) but modified by doubling the volume of each component of the standard reaction. Purified fragment A (0.1 μg) added to an identical reaction serves as a positive control for the maximum incorporation of label. With fragment A present, saturating levels of ADP-ribosylation were reached by 5 min of incubation (Figure 1). However, the endogenous transferase of beef liver does not completely modify EF-2 even after 12 hrs of incubation at room temperature (25°C). It is not known how long it takes for complete modification of EF-2 by the beef liver endogenous transferase. Perhaps the low level of activity is a reflection of a low enzyme yield, of the physical properties of the enzyme itself, or of modulating factor(s) that have copurified with EF-2 and the endogenous transferase.

Figure 1. EF-2 modification as catalyzed by the endogenous transferase of beef liver cells or diphtheria toxin fragment A over a 12 hr interval. Enzyme activity is assayed as described in (1). However, the volume of each component of the modification reactions is twice that used in the standard reaction. Reaction samples are taken periodically and TCA precipitated. cpm x 10^{-3} is the acid-insoluble radioactive counts per min per μg EF-2.

Symbols: (•) endogenous transferase activity; (Δ) transferase activity in the presence of fragment A.


CPM x 10-3
µg EF-2
3
2
1
0
0
2
4
6
8
10
12
Hours

Appendix D. Not All EF-2 Purification Protocols Result in Recovery of Endogenous Transferase Activity.

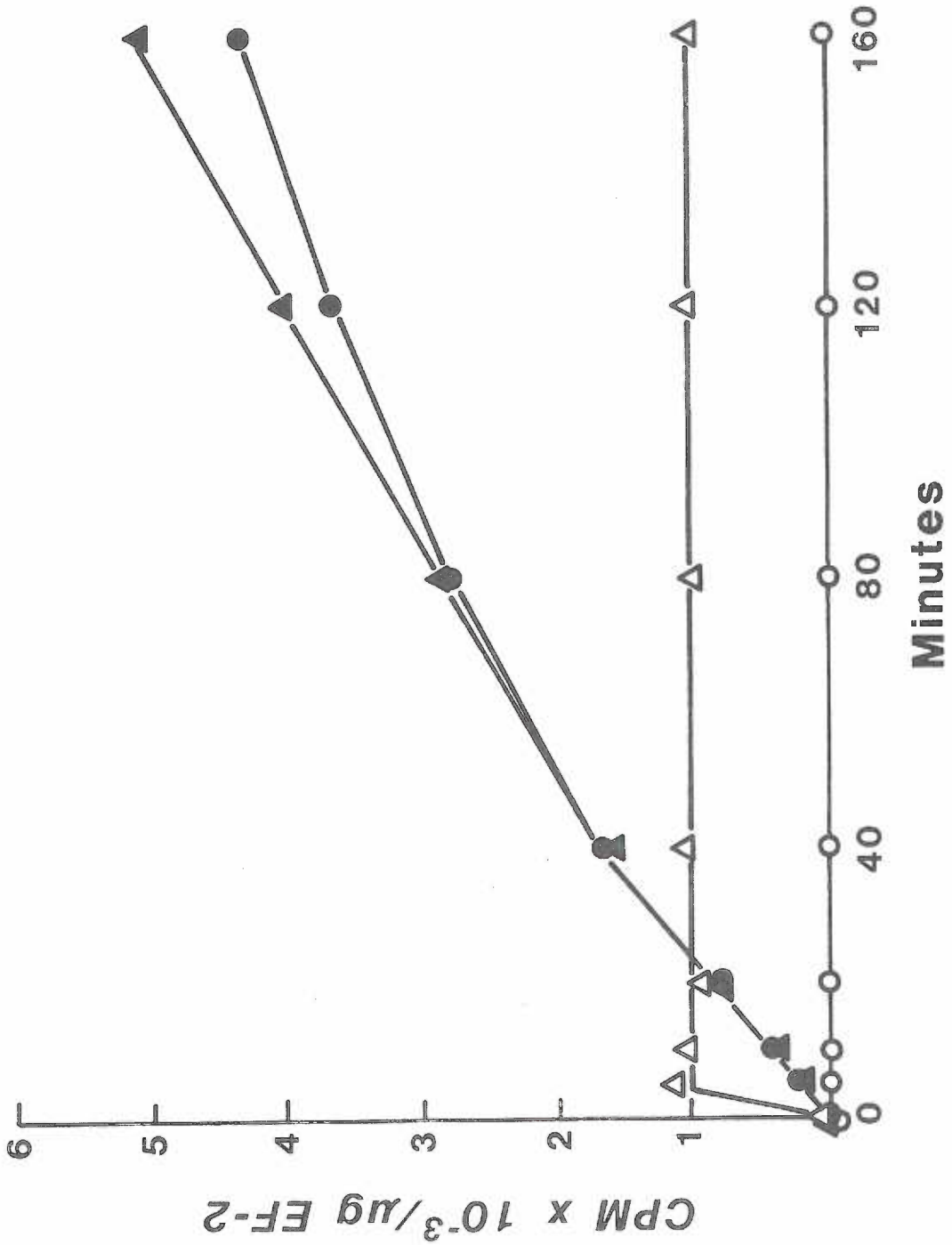
Purification of EF-2 according to the procedures described in (4) gave rise to endogenous transferase activity which appears to have copurified with PyBHK (1) and beef liver EF-2 (3). The enzyme activity appears to be recoverable using our EF-2 purification protocol independent of cell type. To determine whether EF-2 purified from rat liver by an independent technique (5) can also give rise to endogenous transferase activity, we took this purified EF-2 preparation and used it in our modification assay as described in (1). As a positive control for the ADP-ribose acceptor ability of this EF-2 preparation, fragment A is included in the control reaction. To determine whether or not there is an inhibitor present in the rat liver EF-2 preparation, this EF-2 is included with PyBHK EF-2 in the modification assay. As a control for the possible presence of an inhibitor in the rat liver EF-2 preparation, a modification reaction is carried out using only PyBHK EF-2 as the ADP-ribose acceptor. The assays were performed according to the method described in (1) and modified by the indicated additions to the basic reaction. These reactions were carried out simultaneously at room temperature (23.5°C). 10 µl samples were periodically withdrawn and coprecipitated with 100 µl BSA (0.1 mg/ml) using 1 ml 10% TCA. It is apparent that the rat liver EF-2 preparation does not contain any endogenous transferase activity (Figure 1). This is not due to the impairment of the ability of this EF-2 preparation to be ADP-ribosylated because saturating levels of radioactivity were incorporated by 5 min of incubation in the presence of fragment A. The lack of endogenous

transferase activity does not appear to be due to the presence of an inhibitor present in the rat liver EF-2 preparation because it had no effect on the endogenous transferase activity of PyBHK cells.[a] As a matter of fact, rat liver EF-2 and PyBHK EF-2 together gave a reaction profile identical to that obtained with PyBHK EF-2 alone during the first 80 min of incubation. Thereafter, there was even a slight increase in the incorporation of label using the combined EF-2 mixture as would be expected if rat liver EF-2 was being modified in addition to PyBHK EF-2. This experiment supports the conclusion that endogenous transferase activity is not due to EF-2 alone because the reaction requires more than just EF-2 as purified by Raeburn et al. (5); it requires something which we were able to recover using our purification protocol.

[a] It has been shown previously that crude rat liver cellular extract is capable of inhibiting the endogenous transferase activity of PyBHK cells (Appendix A).

Figure 1. Comparison of purified rat liver and PyBHK EF-2 preparations for their content of endogenous transferase activities. Rat liver and PyBHK EF-2 preparations are assayed separately for transferase activity as described in (1). Fragment A is included in a separate rat liver EF-2 containing reaction to serve as a positive control for the ability of this EF-2 preparation to be ADP-ribosylated. The combined effect of the two EF-2 preparations in the fourth reaction serves to determine whether or not there is a cellular transferase inhibitor present in the rat liver EF-2 preparation. Samples from each reaction were periodically withdrawn and TCA precipitated. cpm x 10^{-3} is the acid-insoluble radioactive counts per min per μg EF-2.

Symbols: Modification reaction containing purified rat liver and PyBHK EF-2 (▲); purified PyBHK EF-2 only (●); purified rat liver EF-2 only, in the presence of fragment A (Δ); and purified rat liver EF-2 only, in the absence of fragment A (o).

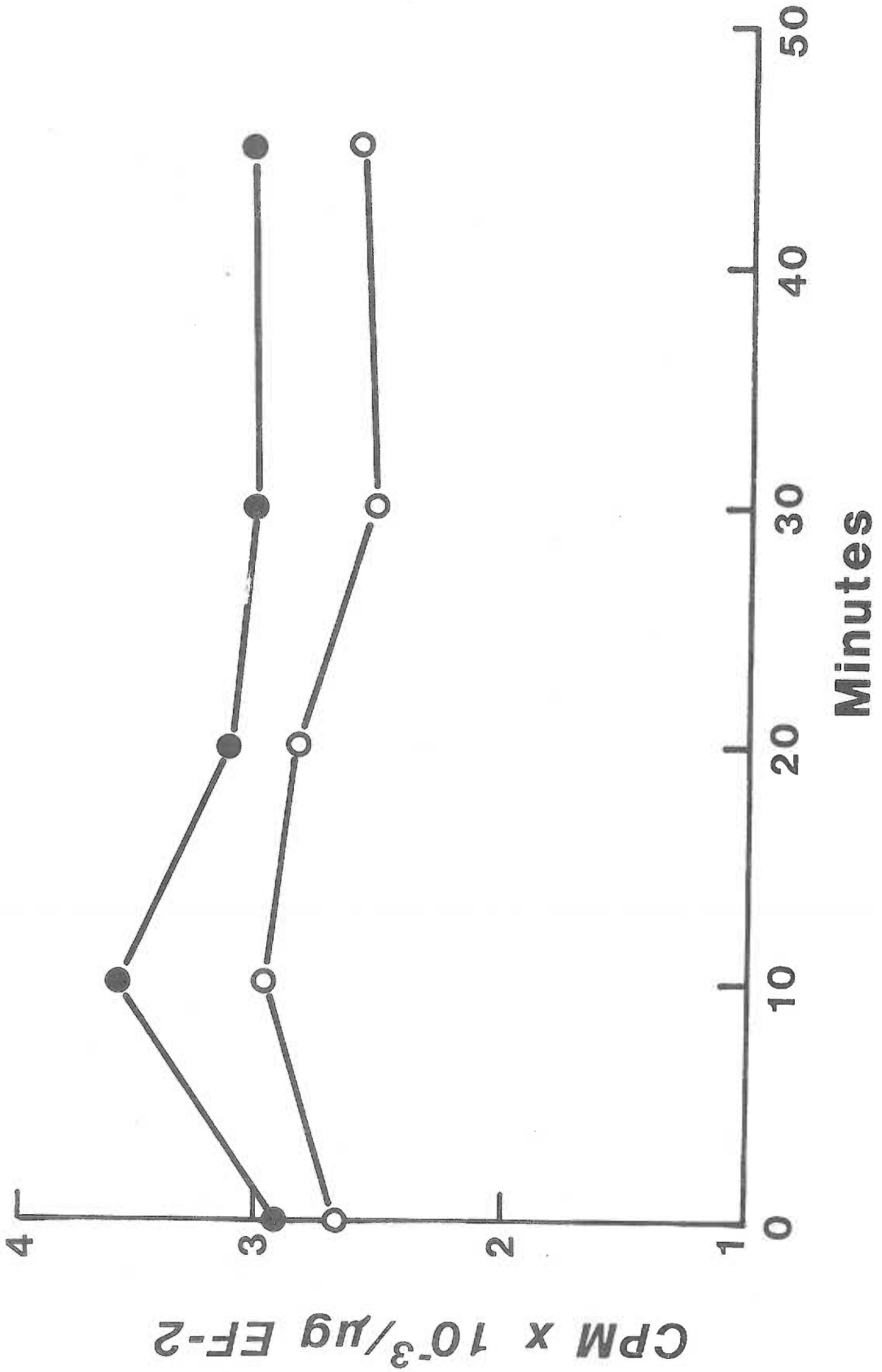

CPM x 10⁻³/μg EF-2
6
5
4
3
2
1
0
40
80
120
160
Minutes

Appendix E. Crude PyBHK Cell Extracts do not Reverse the ADP-ribosylation Reaction.

Vaughn and Moss (6) have stated that there is no evidence for the existence of enzymes that can remove ADP-ribose from products synthesized by any bacterial toxin. Because only a very few molecules of diphtheria toxin are required to produce a lethal effect on cells, it seems unlikely that removal of ADP-ribose from ADP-ribosylated proteins occurs at all. To test these statements on the PyBHK cell system, PyBHK EF-2 is first modified according to an established procedure (1). If there is an enzyme in the PyBHK cellular extract that can remove ADP-ribose from EF-2, we would expect a reduction of TCA precipitable counts after combining the modified EF-2 containing reaction mixture with the extract.

Two identical EF-2 modification reactions were carried out simultaneously for 200 min at room temperature (21.5°C). After this incubation, 10 µl samples were withdrawn from each reaction (0 time samples) and TCA precipitated. Thereafter, 10 µl of either PyBHK cellular extract or deionized H_2O (control) is added to the reaction mixture. The reactions were incubated at room temperature (21.5°C) and 10 µl samples were periodically withdrawn. All samples were co-precipitated with 100 µl BSA (0.1 mg/ml) using 1 ml 10% TCA. The results (Figure 1) show that there is no significant decrease in TCA precipitable label in either reaction by the end of the experiment. Since the presence of the cellular inhibitor in PyBHK cell extracts (1) can stop the activity of the endogenous transferase in 5 min under our assay conditions, these findings suggest that the PyBHK cell extract does not contain an enzyme which de-ADP-ribosylates ADP-ribosylated EF-2.

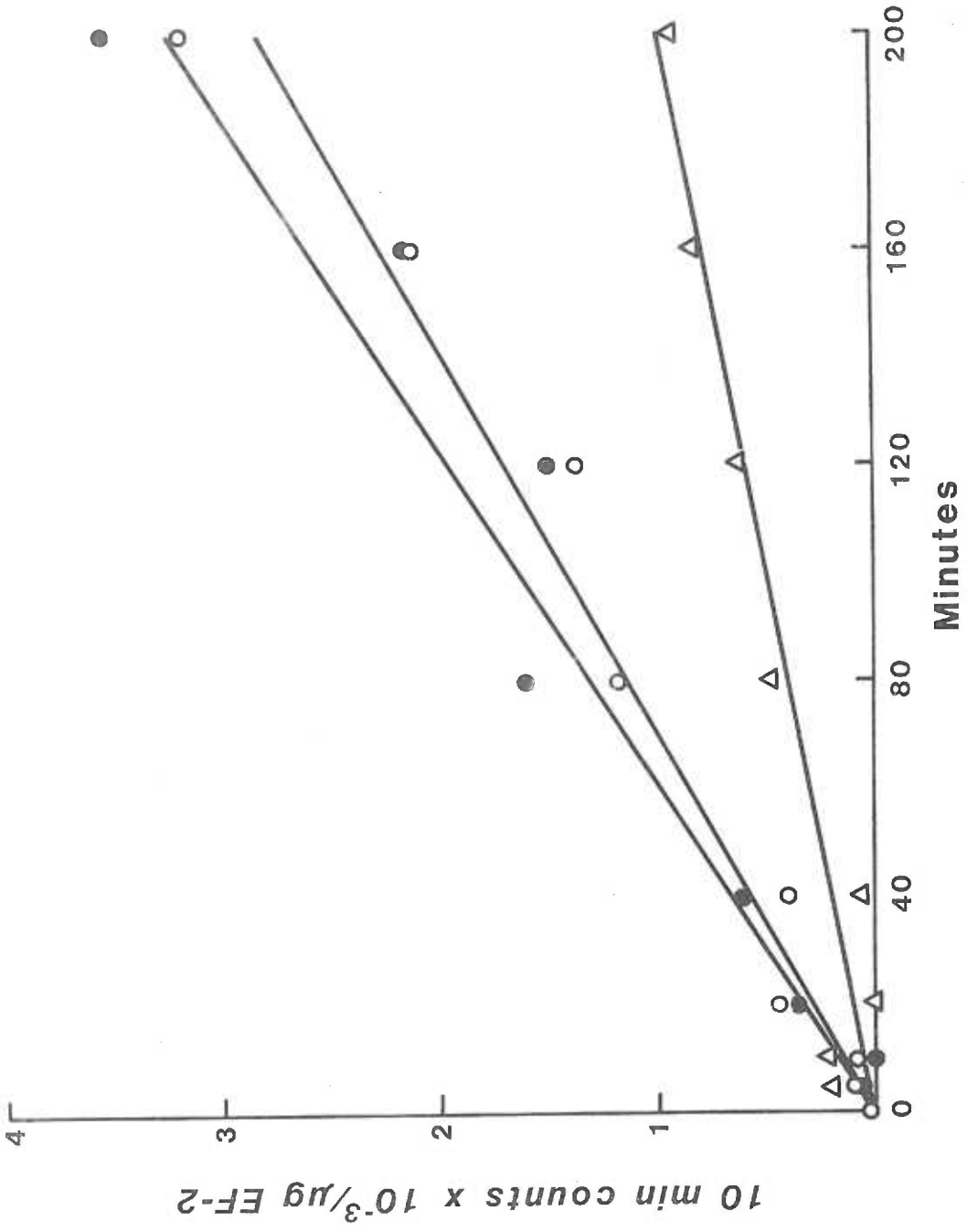
Figure 1. Fate of ADP-ribosylated EF-2 incubated in the presence of PyBHK cellular extract (•) or deionized H_2O (o). After PyBHK EF-2 has been ADP-ribosylated by the cellular transferase in the presence of [^{14}C-adenosine]NAD, PyBHK cellular extract or deionized H_2O is added to the reaction mixtures. The resulting mixtures were incubated at room temperature. Samples from these reactions were periodically withdrawn and TCA precipitated. cpm x 10^{-3} is the acid-insoluble radioactive counts per min per μg EF-2.


CPM x 10⁻³/μg EF-2
4
3
2
1
0
10
20
30
40
50
Minutes

Appendix F. ADP-ribose Inhibits Endogenous Transferase Activity.

Several reactions were performed to determine whether or not changing some reaction parameters can cause an increase in the activity of the endogenous transferase of beef liver to modify EF-2. Figure 1 shows the results of three modification reactions, two of which contain the standard final concentration of DTT (1mM) either with or without 10 mM ADP-ribose, and the remaining one has neither ADP-ribose nor DTT added to it. The modification reactions are performed as described in (1). The reactions were incubated at room temperature (23°C). 10 µl samples were periodically withdrawn and coprecipitated with 100 µl BSA (0.1 mg/ml) using 1 ml 10% TCA. A gradual increase in TCA precipitable [^{14}C]adenosine label is seen in all reactions; the highest increase occurred with the standard reaction (no ADPR, w/DTT), although the reaction gave only slightly lowered counts in the absence of any added ADPR or DTT. The presence of a final concentration of 10 mM ADPR significantly slows down the modification reaction. It has been reported (7) that 5 mM ADP-ribose inhibits 80-90% of the enzyme activity that catalyzes the splitting of the linkage between ADP-ribose and histone H2B. If such an enzyme exists for the ADP-ribose-EF-2 linkage, the ADP-ribose present in our assay medium may lead to a net increase in ADP-ribosylated EF-2 and thus to an increase in TCA precipitable counts. The absence of this increase and furthermore, a positive decrease (Figure 1) suggests that hydrolysis of the ADPR-EF-2 bond by an ADPR-histone 2B-like splitting enzyme is not a factor that is causing the low counts in the transferase catalyzed reactions with beef liver EF-2.

Figure 1. Effect of ADP-ribose and DTT on the EF-2 modification reaction catalyzed by the endogenous transferase of beef liver. Three ADP-ribosylation reactions are carried out using beef liver EF-2 as the ADP-ribose acceptor. One reaction has neither ADP-ribose nor DTT added to it. The remaining two reactions have a final concentration of 1 mM DTT, either with or without a final concentration of 10 mM ADP-ribose. The reaction mixtures were incubated at room temperature. Samples from these reactions were periodically withdrawn and TCA precipitated. The cumulative acid-insoluble radioactive counts for 10 min per μg EF-2 in the assay for ADP-ribosyltransferase activity is shown.
Symbols: Modification reaction containing a final concentration of 1 mM DTT (•); 10 mM ADP-ribose and 1 mM DTT (Δ); and neither ADP-ribose nor DTT (o).


10 min counts x $10^{-3}/\mu g$ EF-2
0
1
2
3
4
0
40
80
120
160
200
Minutes

Appendix References

1. Lee, H. and Iglewski, W.J. (1984). Cellular ADP-ribosyltransferase with the same mechanism of action as diphtheria toxin and Pseudomonas toxin A. Proc. Natl. Acad. Sci. USA 81:2703-2707.

2. Hager, T.A. (1978). The isolation and characterization of BHK and pyBHK cells highly resistant to diphtheria toxin: EF-2 mutants. Master's Thesis, Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon.

3. Iglewski, W.J., Lee, H., and Muller, P. (1984). ADP-ribosyltransferase from beef liver which ADP-ribosylates elongation factor-2. FEBS. Lett. 173:113-118.

4. Iglewski, W.J. and Lee, H. (1983). Purification and properties of an altered form of elongation factor 2 from mutant cells resistant to intoxication by diphtheria toxin. Eur. J. Biochem. 134:237-240.

5. Raeburn, S., Collins, J.F., Moon, H.M. and Maxwell, E.S. (1971). Aminoacyltransferase II from rat liver. I. Purification and enzymatic properties. J. Biol. Chem. 246:1041-1048.

6. Vaughan, M. and Moss, J. (1981). Mono(ADP-ribosyl)transferase and their effects on cellular metabolism. Curr. Top. Cell. Reg. 20:205-246.

7. Okayama, H., Honda, M. and Hayaishi, O. (1978). Novel enzyme from rat liver that cleaves an ADP-ribosyl histone linkage. Proc. Natl. Acad. Sci. USA 75:2254-2257.

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