Expression of the herpes simplex virus type-1 thymidine kinase gene in *Neurospora crassa*

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Dedication

To Karen

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Abstract

Reporter genes are indispensible tools in the study of gene regulation. The Herpes Simplex Virus type-1 thymidine kinase gene (*tk*) has been developed as a reporter gene for investigating *Neurospora crassa arg-2* gene regulation. The *arg-2* gene encodes the small subunit of mitochondrially-localized, Arg-specific carbamoyl phosphate synthetase (CPS-A). The *arg-2* transcript contains an upstream open reading frame (uORF) implicated in Arg-specific negative regulation of CPS-A. We constructed *arg-2-tk* fusion genes; in some constructs, a section of the 5′– gene region including the uORF was deleted. Using a plasmid to cotransform recipient cells to benomyl resistance, *arg-2-tk* constructs were introduced at ectopic sites in the genome of the recipient *arg-12^{s} pyr-3* strain, which is deficient in pyrimidine synthesis and overexpresses *arg-2*. Strains containing *arg-2-tk* fusion genes expressed thymidine kinase (TK) activity, as assayed by measurement of enzyme activities that phosphorylate (methyl-^{3}H)-thymidine. Strains transformed with uORF-containing *arg-2-tk* fusion genes exhibited negative regulation of TK activity when grown in Arg. TK activity was not present in strains lacking *arg-2-tk*. Expression of *tk* resulted in novel sensitivity of the cells to trifluorothymidine (TFT), a compound known to become toxic following phosphorylation by TK. Arg-specific negative regulation of *arg-2-tk* was also observed in strains containing constructs targeted to the *his-3* locus. In addition, the growth of these strains was more sensitive to TFT. Finally, due to *tk*'s apparent usefulness as a reporter gene in *Neurospora*, we designed and constructed the generalized reporter plasmid pDV8.

Chapter 1

Introduction

Neurospora crassa Shear & Dodge and *Saccharomyces cerevisiae* Hansen are useful model systems for elucidating mechanisms involved in the regulation and control of arginine (Arg) biosynthetic enzymes (reviewed by Davis, 1986). We are investigating the regulation of *N. crassa arg-2*, which encodes the small subunit of mitochondrially localized carbamoyl phosphate synthetase (CPS-A). The level of CPS-A activity controls flux through the Arg pathway by limiting the amount of carbamoyl phosphate available for citrulline synthesis in the mitochondrion (Davis, 1987).

Neurospora crassa and *S. cerevisiae* CPS-A enzymes consist of one small subunit and one large subunit encoded by unlinked nuclear genes. These enzyme complexes utilize NH_3, HCO_3^- and ATP for the production of carbamoyl phosphate for Arg biosynthesis. The small subunit functions as a glutamine aminotransferase to provide the required NH_3 from glutamine for carbamoyl phoshate synthesis (Davis, 1986). In *N. crassa*, the small and large subunits are encoded by *arg-2* and *arg-3*, respectively; in *S. cerevisiae*, they are specified by *CPA1* and *CPA2*. *S. cerevisiae* CPS-A is cytoplasmic. In *N. crassa*, CPS-A is mitochondrial, as is the related mammalian CPS I (Davis, 1986). This commonality suggests that Arg biosynthesis in *N. crassa* may more closely resemble mammalian Arg biosynthesis than *S. cerevisiae* does.

The *arg-2* gene is subject to unique, negative regulation by Arg and the expression of *arg-2* limits CPS-A activity under many circumstances (Davis, 1986). A 24-residue upstream open reading frame (uORF) in the 5′– region of the *arg-2* transcript appears important for negative regulation by Arg (Luo and Sachs, unpublished). A similar uORF is found in the 5′– region of the *CPA1* transcript and is implicated in Arg-specific regulation of that gene (Delbecq, *et al.*, 1994; Werner, *et al.*, 1987). One component of *arg-2* regulation appears to be translational (Davis, 1986; Luo, Freitag, and Sachs, unpublished; Orbach, *et al.*, 1990; Sachs and Yanofsky, 1991). In addition, the *arg-2* gene and the *arg-3* gene are positively regulated by the cross-pathway control

response to amino acid starvation (Davis, 1986; Sachs, in press); *arg-2* is developmentally controlled as well (Sachs and Yanofsky, 1991).

We chose to explore the use of the Herpes Simplex Virus type-1 thymidine kinase gene (*tk*) as a reporter gene in order to examine mechanisms involved in Arg-specific negative regulation of *N. crassa arg-2.* In contrast to mammals and certain viruses, *N. crassa*, like other fungi, lacks detectable thymidine kinase activities (Grivell and Jackson, 1968). Mammalian cells contain distinct cytosolic and mitochondrial thymidine kinase activities which are primarily involved in salvage pathways (Arnér, *et al.*, 1992). Fungi do not require these alternative pathways: dTMP is produced by thymidylate synthetase from dUMP. HSV-1 TK (TK) converts thymidine directly to dTMP. In addition, TK phosphorylates certain thymidine analogs which converts them into toxic forms capable of inhibiting cell growth (Crumpacker, *et al.*, 1980; Summers and Summers, 1977).

The *tk* gene has been used extensively as a genetic marker in mammalian cells (McNeil and Friesen, 1981). It has also been useful in yeast studies (Sclafini and Fangman, 1984). Here we describe the expression of *tk* in *N. crassa.* Due to the simplicity of the TK enzyme assay, the absence of a native TK in *N. crassa* (unlike *lacZ*, the *tk* gene provides quantifiable activity with no background rather than a relative unit scale with background activity), and TK-dependent drug sensitivity, the potential benefit of adapting this small (1.2 kb) intronless gene as a reporter in *N. crassa* appears substantial.

Chapter 2

Materials and Methods

2.1 *Neurospora crassa* strains

Wild-type (w.t.) strain 74-OR23-1*A* (74*A*) was obtained from D. Perkins at Stanford University. Mutant strains 6103 (*his-3* allele 1-234-723) and 6524 (*his-3* allele 1-234-1438) were obtained from the Fungal Genetics Stock Center. Strain 13-6 (*his-3* revertant from strain 6103) was isolated by Z. Lou at Oregon Graduate Institute. Strain *arg-12^s pyr-3* (*a*) was obtained from E. Selker at the University of Oregon.

2.2 Growth media, supplements, and storage

Medium contained Vogel's Minimal Salts (VM) and 2% sucrose or VM and FGS (FGS is 0.5% fructose/0.5% glucose/20% sorbose autoclaved at 10x concentration). *arg-12^s pyr-3* strains, when grown in 0.5 mg/ml Arg, were supplemented with 0.2 mg/ml uridine (Uri). Histidine (His, 0.5 mg/ml) was included in the media when growing strains 6103 and 6524 prior to transformation. Trifluorothymidine (TFT) from Sigma was added to final concentrations between 25 and 100 μM after cooling molten media to 50°C. Cultures were grown at room temperature or at 34°C on 1x VM/2% sucrose/2% agar slants for storage at -20°C.

2.3 Plasmids

Plasmids pNK2 and pNA3 were constructed by M. Sachs. The *tk* gene in plasmid pHSVΔ was obtained from G. Merrill at Oregon State University. Plasmid pM063 contains the benomyl resistance-conferring beta-tubulin gene (Orbach, *et al.*, 1986). Plasmid pZL505 was constructed by Z. Luo at Oregon Graduate Institute; it contains a *Hind*III–*Sma*I subfragment of the *N. crassa his-3* gene (Legerton and Yanofsky, 1985). *arg-2* cDNA fragments were obtained from M. Sachs' pAR1 (Orbach, *et al.*, 1990).

Plasmids pSP65 and pSP72 were obtained from Promega. Plasmid pAN52-1 contains the *gpd* promoter and *trpC* terminater from *A. nidulans* (Punt, *et al.*, 1990; Punt, *et al.*, 1987).

2.4 *Neurospora* transformation procedures

Competent *N. crassa* cells were prepared and transformed as described previously (Selitrennikoff and Sachs, 1991). For transformation, 2-5 μg of plasmid DNA was suspended in ice-cooled polystyrene Falcon tubes (#2058) with 25 μl of heparin-solution (5 mg heparin/ml in 1M STC (1 M sorbitol, 50 mM Tris-HCl pH 8.0, and 50 mM $CaCl_2$ (adjusted to pH 8.0)) and 100 μl of slowly-thawed cells (ca. 10^7 spheroplasts). This mixture was incubated on ice for 30 min before lipofectin (3.5 units, BRL) was added. The tubes were incubated at room temperature for 15 min before adding 875 μl of PTC (40% PEG 4000 (Sigma), 50 mM Tris-HCl (pH 8.0) and 1 M $CaCl_2$ (adjusted to pH 8.0)), and mixing by pipetting gently up and down. The mixture was incubated at room temperature for 20 min. Transformed cells (250 μl) were transferred into 10 ml of top (regeneration) agar (1x Vogel's/2% agar/1 M sorbitol/FGS), and inverted gently before being poured onto plates containing 15-20 ml of solidified bottom agar (1x Vogel's/1.5 % agar/FGS). These plates were incubated for 2-5 days at 34°C.

2.5 *Neurospora* DNA preparation

Conidia were collected from stock tubes; 10 μl of conidial suspension were inoculated into 25 ml of 1x VM/1.5% sucrose in 125 ml Erlenmeyer flasks, and cultures were grown at room temperature for 2 to 4 days (the formation of aerial hyphae and the onset of conidiation were avoided). Cells were then harvested onto #1 Whatman filter paper in a Büchner funnel. Samples were frozen at -80°C in 15 ml Falcon tubes (#2059 or equivalent) for at least 20 min.

DNA was extracted by a modification of a previously described procedure (Oakley, *et al.*, 1987) . Frozen tissue was lyophilized overnight and then pulverized by vortexing with a spatula for 30 sec. Samples were resuspended (by vortexing at high speed for 20 sec) in 1 ml of salt-detergent solution (4 mg/ml sodium deoxycholate, 10 mg/ml Brij 58 (polyoxyethylene20 cetyl ether), and 2 M sodium chloride (dissolved in order and stored at 4°C)), incubated at room temperature for 20 min while mixing on an end-over-end rotator, and centrifuged at 8000 rpm for 10 min in a Sorvall SS-34. The supernatant (300 μl) from each sample was transferred to Eppendorf tubes and gently

mixed with 1.2 ml of sodium trichloroacetic acid(TCA)/ethanol (1:4 vol:vol). The TCA/ethanol solution was prepared by dissolving 41.7 g NaTCA salt (Aldrich #19,078-0) in sterile water and adjusting to 50 ml (density is 1.43 g/ml) to make 4.5 M TCA. Fifty ml of ethanol was added, and the nucleic acids were precipitated at -20°C for at least 30 min. Precipitated nucleic acids were collected by centrifugation in a Speedfuge for 15 sec, washed with 300 μl of 70% ethanol, pelleted by another 15 sec spin, and dried briefly in a Speed Vac. Pellets were resuspended in 100 μl of 10 mM NH_4OAc, gently mixed with 100 μl of 10 mM NH_4OAc (containing 0.3 mg/ml RNaseA), and incubated at 50°C for 1 hr with gentle vortexing every 15 min to resuspend the pellet. Chloroform (200 μl) was added to each tube. The tubes were vortexed and then centrifuged in a Speedfuge for 5 min. The upper, aqueous phase was transferred to a new tube to which 107 μl of 7.5 M NH_4OAc and 0.8 ml isopropanol were added. The samples were mixed well by inversion, immediately centrifuged in a Speedfuge for 25 sec, washed with 300 μl of 70% ethanol, and dried briefly in a Speed Vac. Pellets were resuspended in 100 μl of TE buffer (10 mM Tris (pH7), 20 mM EDTA (pH8)) overnight at 4°C. The amount of DNA was quantified using a Hoefer fluorometer. Aliquots were stored at -80°C.

2.6 *Neurospora* RNA preparation

Small scale RNA preparations were obtained essentially as described previously (Sachs and Yanofsky, 1991). *Neurospora crassa* mycelium (50 mg frozen in liquid nitrogen and stored at -80°C) was added to 2 ml screw-cap Eppendorf tubes containing 1 g acid-washed glass beads (0.5 mm), 580 μl of extraction buffer (100 mM Tris HCl pH 7.5, 100 mM LiCl and 20 mM dithiothreitol), 420 μl phenol, 420 μl chloroform and 84 μl of 10% SDS. The tubes were capped and nucleic acids were immediately extracted by homogenizing with a mini-bead beater for 50 sec; the homogenate was mixed for at least an additional 5 min in an end-over-end rotator, and tubes were centrifuged in a Speedfuge for 30 sec. The aqueous phase was removed and extracted once with 0.8 ml of phenol:chloroform (1:1) using a mini-bead beater for 30 sec. The phases were separated by centrifuging 5 min in a Speedfuge. The aqueous phase was removed and extracted once with 0.8 ml chloroform by vortexing. Nucleic acids were precipitated from the aqueous phase with sodium acetate and ethanol. Following an additional precipitation step and an 80% ethanol wash, pellets were dried in a Speed Vac and redissolved in 100

μl sterile water. The amount of RNA was quantified using a Hoefer fluorometer. Aliquots were stored at -80°C.

2.7 Southern and northern analyses

For Southern analysis, DNA from *N. crassa* transformants was digested with the appropriate restriction endonucleases, and 1 μg samples were loaded into the wells of 0.8% agarose gels and electrophoresed at 60–80 V for 1.5 hr in TAE buffer (40 mM Tris base (pH 7), 20 mM NaOAc, 2 mM EDTA) containing 0.25 mg/ml ethidium bromide (EtBr).

For northern analyses (Sachs and Yanofsky, 1991), 6 μg of total RNA from transformed *N. crassa* strains was denatured, loaded into the wells of 1.5% denaturing agarose/formaldehyde gels, and electrophoresed at 80V for 1.5 hr. The RNA gels were stained with EtBr for 5 min and subsequently destained in diH_2O for several hours.

Gels were photographed and standards were marked by making plug-holes next to the bands with a Pasteur pipette. For Southern analysis, the gels were soaked in 0.25 M HCl for 15 min, rinsed, and soaked for 1 hr in 0.5 M NaOH/1.5 M NaCl/0.004% thymol blue; then 0.5% Tris-HCl (pH 7)/1.5 M NaCl was added for 1 hr (until the blue color turned yellow).

Nucleic acid transfer was accomplished by capillary action. Three large sponges were placed in a glass baking dish containing 10x SSPE (20x SSPE = 174 g NaCl, 27.6g NaH_2PO_4, 7.4 g Na_2EDTA adjusted to pH 7.4 in 800 ml H_2O). Filter paper (Fisher) was soaked in 10x SSPE and placed on top of the sponges (2 sheets/ gel). Plastic wrap was placed across the entire dish in a way that left only the minimum area of the filter paper uncovered for placing the gels upside-down. Appropriately marked Nytran membranes (MSI) were placed on top of the gels. Two sheets of filter paper were placed on the membranes followed by stacks of paper towels. This stack was compressed by placing a textbook on top. Transfers were accomplished overnight. The assembly was dismantled carefully, keeping the gel in contact with the membrane. Standards were marked with a dissecting needle. The membranes were separated from the gel, rinsed in 5x SSPE, and nucleic acids were cross-linked to the membranes using a Stratagene UV cross-linker.

Membranes were placed in plastic bags containing fresh prehybridization solution (50 ml prepared in a 50 ml screwcap tube: 25 ml formamide, 10 ml of 5x P (1% BSA, 1% polyvinylpyrollidone, 1% Ficoll 400,000, 250 mM Tris HCl (pH 7.5), 0.5% sodium

pyrophosphate, 5% SDS), 2.92 g NaCl, 0.5 ml of 10 mg/ml denatured and sheared salmon sperm DNA) and prehybridized with gentle shaking for a minimum of 5 hr.

Probes for Southern and northern analyses were labeled by the random primer method (Feinberg and Vogelstein, 1983). Appropriately digested and purified plasmid DNA (100 ng in 6 μl) was boiled for 3 min and put on ice for 1 min. This single-stranded DNA was diluted with 13 μl of LS (25 parts 1 M HEPES (pH 6.6), 25 parts DTM (100 μM dATP, 100 μM dGTP, 100 μM dTTP), 7 parts OL (1 mM Tris-HCl (pH 7.5), 1 mM EDTA, 90 O.D. units/ml hexamers), 5 parts 10 mg/ml BSA) before adding 5 μl of α-^{32}P-dCTP (50 μCi) followed by 1 μl of Klenow enzyme. Reactions were allowed to proceed at room temperature for 3 hr. Probes were recovered by centrifugation through Sephadex G-25 spin columns (Boehringer) into 150 μl of TE; 2 μl samples were counted in a scintillation counter (Beckman).

Radioactive probe (10^7 cpm ^{32}P/bag) was injected into plastic bags, using a needle and syringe, containing prehybridized membranes. Following 12-24 hr of incubation, filters were washed twice for 10 min and then twice for 15-30 min with 0.1x SSPE/1% SDS at 67°C. Film was exposed to moist filters wrapped in plastic wrap.

2.8 Thymidine kinase activity assay

Neurospora crassa conidia were collected and inoculated into 50 ml of medium (in 125 ml flasks) at 10^7 cells/ml. Cells were grown at 34°C for 5-6 hr on an orbital shaker. Mycelia were collected on Whatman #1 filter paper with a Millipore apparatus and washed once with sterile water. The fresh mycelia were immediately frozen in liquid nitrogen and stored at -80°C. This tissue was then assayed for TK enzyme activity by the modification of a previously described procedure (McNeil and Friesen, 1981) .

Crude cell extracts were prepared by adding approximately 200 mg frozen mycelia to 2 ml screw-top tubes (on ice) containing 1 g acid-washed glass beads (0.5 mm) and extraction buffer (10 mM Tris-HCl pH 8.0, 0.2 mM ATP, 1.4 mM fresh ß-mercapto-ethanol, 20% glycerol). Cells were beaten twice for 30 sec in a mini-bead beater at 4°C; tubes were cooled on ice between beatings. Extracts were clarified by centrifugation in a Speedfuge at 9,000 rpm for 10 min at 4°C. The supernatants were collected, aliquoted, frozen on dry ice and stored at -80°C. Bradford assays were performed to determine crude protein concentrations using bovine serum albumin as a

standard (Bradford, 1976). Samples were diluted with extraction buffer to 1 mg crude protein/ml prior to adding to the reaction mix.

Thymidine kinase reactions contained 0.25 mg crude protein/ml, 50 mM Tris-HCl pH 8.0, 10 mM ATP, 5 mM sodium fluoride, 5 mM calcium chloride, 10 mM ß-mercaptoethanol (freshly-diluted), and 0.02 mCi (methyl-^{3}H)-thymidine (ICN)/mg crude protein. Typical reaction volumes were 200 μl. Reactions were incubated at 37°C and then boiled for 2 min in a heatblock to stop the reaction. Reaction mixtures (50 μl) were spotted onto 2 cm DEAE-cellulose anion-exchange disks (Whatmann DE81) and allowed to dry. The disks were washed twice in 4 mM ammonium formate and then twice in 95% ethanol (at least 10 ml/disk, 5 min/wash). An apparatus for efficient washing was constructed by drilling multiple small holes (approximately 5 mm) in a 250 ml plastic beaker (disk beaker), which could be placed into a 500 ml pyrex beaker (washing beaker) containing a stir bar. Gentle stirring, combined with a slow up and down movement of the disk beaker by hand, gave the lowest background and the least amount of disk deterioration (data not shown). The disk beaker was lifted from and held over the wash beaker for pouring off solutions between washes. After the final wash, the disk beaker was removed from the wash beaker, allowed to drain briefly in a plastic weigh boat, and inverted so the clump of disks could be dropped into another plastic weigh boat. Individual disks were separated and gently transferred (with forceps) to 7 ml scintillation vials to which 5 ml of ScintVerse scintillation fluid (Fisher) was added. Levels of radioactivity in the samples were measured in a scintillation counter (Beckman). The counts reported represented the averages of reaction products spotted onto duplicate disks.

2.9 Trifluorothymidine (TFT) sensitivity assay

Conidia (500) from transformed *N. crassa* strains were collected and suspended in molten agar media (1.5% agar, VM, FGS) containing varying levels of TFT (25 μM–100 μM) and the appropriate nutritional supplements. Plates were incubated at 34°C for 3-5 days.

2.10 Molecular cloning techniques

Amplification and screening of plasmids were carried out in two *Escherichia coli* strains. Competent XL1-Blue cells were prepared and transformed by standard

techniques using $CaCl_2$ (Sambrook, *et al.*, 1989). Competent DH5-α cells were prepared and transformed by electroporation with an Electro Cell Maniplator®600 according to the manufacturer's instructions (BTX Inc.).

Restriction endonucleases from New England Biolabs (NEB) were used for DNA digestions. Nucleotides (Life Technologies) and Klenow enzyme (NEB) were used for 5'-overhang fill-in reactions. When necessary, calf-intestinal alkaline phosphatase (CIAP) (Pharmacia) was used to dephosphorylate DNA fragment ends. In some instances, DNA fragments were resolved on agarose gels and purified by either β-agarase (NEB) digestion of SeaPlaque™GTG (FMC) low-melting point agarose, or by DEAE-membrane elution (Schleicher & Schuell NA45) from SeaKem™GTG (FMC) agarose, following protocols supplied by the manufacturers.

Ligations using NEB T4 DNA ligase were performed as described by Promega (Titus, 1991) with one exception: pDV8 was constructed by adapting a rapid in-gel cloning technique described by Maniatis (Sambrook, *et al.*, 1989). In this case, pDVtrpC (0.6 μg) and pDVGPDTK (5 μl miniprep) were each digested with *Xho*I and *Bam*HI and separated in a 2% SeaPlaque low-melt agarose gel. The appropriate bands were excised with glass cover slips, placed in eppendorf tubes, and heated in a water bath at 65°C for 15 min. This molten agarose (pDVtrpC (2 μl) and pDVGPDTK (8 μl)) was combined in a 37°C water bath. The tubes were removed from the water bath and immediately mixed with 10 μl of ice-cold 2x ligase buffer, and 3 units of ligase (0.5 μl) was added. This reaction was incubated at 15°C overnight before introducing 5 μl of ligation products into 100 μl of competent *E. coli* by and transforming by electroporation.

2.11 Small scale plasmid DNA preparations

Plasmid DNA was prepared following a previously described procedure (Chung and Miller, 1988). Small scale bacterial cultures (1-3 ml of LB) from single ampicillin-resistant *E. coli* colonies were grown overnight at 37°C rotating at 200 rpm. Cultures (1.4 ml in microcentrifuge tubes) were pelleted in a Speedfuge for 25 sec; supernatants were removed by aspiration. Cells were resuspended in 0.5 ml of STET (8% sucrose, 50 mM EDTA, 5% Triton, 50 mM Tris-HCL (pH 8)) by vortexing; 0.02 ml of 10 mg/ml lysozyme was added and mixed by vortexing. Samples were incubated at room temperature for 5 min and then boiled for 2 min (tube caps open). Cell debris was removed by a 10 min spin in a Speedfuge at room temperature. The pellet of debris was

removed from the microcentrifuge tubes with sterile toothpicks, and 0.4 ml isopropyl alcohol was added to precipitate the plasmid DNA. Following a 10 min spin in a Speedfuge at room temperature, the supernatants were aspirated, and the nucleic-acid containing pellets were redissolved in 0.04 ml of TE.

2.12 Large scale plasmid DNA preparations

Large scale preparations of plasmid DNA were purified by equilibrium centrifugation in gradients of CsCl (Sambrook, *et al.*, 1989). Single ampicillin-resistant *E. coli* colonies (or 10 ml of LB starter cultures from single colonies) were inoculated into 200 ml of Superbroth (Sambrook, *et al.*, 1989) and grown overnight at 37°C with vigorous agitation. Cultures were transferred into 250 ml GSA bottles and pelleted at 2,000 rpm for 5 min in a Sorvall RC-5B centrifuge. Supernatants were poured off the pellets which were resuspended in 6 ml of 20 mM Tris (pH8)/10 mM EDTA. Cell suspensions were diluted with 13 ml of fresh lysis buffer (0.2 N NaOH/1% SDS), swirled, and incubated at room temperature for 10 min. Then 10 ml of NH_4Ac was added. The samples were mixed by inversion, placed on ice for 15 min, and centrifuged at 8,000 rpm for 20 min. The resulting plasmid-containing supernatants were filtered through cheesecloth into GSA bottles containing 34 ml of isopropanol; nucleic acids were precipitated at -20°C. Precipitated nucleic acids were pelleted by 8,000 rpm centrifugation for 15 min, supernatants were discarded, and the bottles were inverted and allowed to air dry. The pellets were resuspended in 4.0 ml of TE.

Plasmid-containing solutions for CsCl equilibrium centrifugation were prepared by adding 4.0 ml of plasmid solution and 0.3 ml of 5 mg/ml EtBr to 15 ml Beckman ultracentrafuge tubes containing exactly 4.46 g of CsCl. Tubes (balanced if necessary using a CsCl solution in which TE was substituted for TE-plasmid solution) were centrifuged at 45,000 rpm for 15–18 hr at 20°C using a VTi65.2 rotor in a Beckman Superspeed Centrifuge.

The plasmid DNA bands were removed from tubes by inserting #18 gauge needles attached to 3 ml syringes through the tube walls. The EtBr was removed by serial extraction using isopropyl alcohol equilibrated with a saturated solution of NaCl in sterile water. Nucleic acids were precipitated by adding 2 volumes of sterile water followed by 6 volumes of ethanol. Pellets were washed twice with 70% ethanol, vacuum dried, and redissolved in 400–800 μl of TE.

Chapter 3

Results and Discussion

3.1 TK activity in transformed *Neurospora* strains

Plasmids pNK2, and pNA3, containing *arg-2-tk* fusion genes (Fig. 1) were each integrated into the genome of *N. crassa* strain *arg-12^s pyr-3* by cotransformation with plasmid pM063 containing the benomyl-resistance gene: pNK2 contains *arg-2* 5′ regulatory sequences (uORF) upstream from *tk* and pNA3 does not. These strains would be expected to exhibit varying levels of TK activity due to the ectopic nature of the integration events. Expression of genes integrated in this manner can be affected by several factors including the location of the integration site and the number of integration events that occur. In addition, strain *arg-12^s pyr-3* is starved for arginine and overproduces carbamoyl phosphate due to the *arg-12* mutation that blocks citrulline production in the mitochondrion creating an accumulation of carbamoyl phosphate and a reduction in Arg levels. When this strain is grown in the presence of Arg, the regulatory effect on *arg-2* is significantly greater than in wild-type strains (Davis, 1987).

Under normal circumstances, mitochondrially produced carbamoyl phosphate reacts only with ornithine in the production of citrulline (Davis, 1986). However, a mutation at *arg-12* (98% reduced ornithine carbamoyl transferase activity) results in leakage of carbamoyl phosphate from the mitochondrion to the nucleus. This mutation is defined as a suppressor due to its ability to relieve the pyrimidine requirement of a *pyr-3* mutant strain which is deficient in nuclear carbamoyl phosphate synthesis. When the double mutant *arg-12^s pyr-3* is grown in Arg, CPS-A activity is reduced and there is insufficient leakage of carbamoyl phosphate from the mitochondrion to suppress the *pyr-3* mutation resulting in a uridine deficiency in the nucleus (Perkins, *et al.*, 1982).

The resulting benomyl resistant strains were screened by Southern analysis for integration of the intact *arg-2-tk* gene (M. Sachs, data not shown). Of the strains in which the *arg-2-tk* gene was integrated, four transformants containing each plasmid were selected for further analysis. Whole-cell extracts from the strains containing *arg-2-tk*

fusion genes exhibited TK activity in that they phosphorylated (methyl-^{3}H)-thymidine (Fig. 2.). In contrast, no apparent extract-dependent phosphorylation of (methyl-^{3}H)-thymidine was observed in untransformed strains. Three of four pNK2-containing strains exhibited significant Arg-specific negative regulation of TK; pNA3-containing strains did not.

Strain 19-56-208, transformed with pNK2, was chosen for further analyses of TK activity. We examined the linearity of enzyme activity in whole-cell extracts with time (Fig. 3). The TK+ strain (19-56-208) yielded a linear increase in product over the initial 15 min period. Neither endogenous TK activity, nor inhibitors of TK, appeared to be present in strains lacking TK, as demonstrated by measuring TK activity in mixtures of whole-cell extract from TK+ strain 19-56-203 and untransformed strain *arg-12^s pyr-3* (Fig. 4).

To specifically integrate a single copy of an *arg-2-tk* fusion gene at a known site in the *N. crassa* genome, two independent His$^-$ strains were transformed with plasmid pZL505 containing a truncated *N. crassa his-3* gene (Fig. 1). The complementaion of the histidine requirement in these strains would be expected to yield strains containing single copies of *arg-2-tk* fusion genes integrated specifically at the *his-3* locus. This type of integration provides more reliable and consistent results across a population of independent transformants than does cotransformation. In addition, the data obtained from targeted integrants should reflect wild-type levels of expression with respect to the *arg-2* gene and the regulatory effect that growth in Arg produces.

The resulting histidine prototrophs were isolated and integration at *his-3* was confirmed by Southern analysis (data not shown). Northern analysis (data not shown) indicated that the fusion gene produced a transcript of the predicted size. DNA and RNA blots were probed with a randomly labeled 1.2 kb *tk* gene (Orbach, *et al.*, 1990; Sachs and Yanofsky, 1991). Two strains containing single copies of pZL505 were assayed for TK activity in the presence and absence of Arg (Table 1). These strains differ in that they originated from independent strains with mutations in different alleles. Therefore, they would not be expected to give identical results.

The addition of Arg resulted in an approximate 3-fold decrease in TK activity, similar to Arg's regulatory effect on CPS-A activity in wild type *N. crassa* (Davis, 1986). Thus, these data suggest that strains that integrate single copies of *arg-2-tk* fusion genes containing the *arg-2* uORF respond to Arg by reducing TK activity in a manner similar to

the Arg-response of CPS-A in a wild-type strain. Strains containing pZL505 produced lower levels of TK than an *arg-12^s pyr-3* strain. Since strains containing the *arg-12^s* mutation overexpress *arg-2*, they might be expected to overexpress the *arg-2-tk* fusion genes, which is consistent with these results (Davis, 1986) .

3.2 TFT sensitivity in *Neurospora* strains

We examined the effect of TFT on *N. crassa* strains expressing *tk* (Table 2). Transformants were grown in minimal, Arg, Uridine, and Arg plus uridine media. The growth of the pNK2-containing cotransformants was slow in minimal media and completely inhibited by the addition of 25 μM TFT. In contrast, targeted integration produced strains that grew well in minimal media but were only partially inhibited by TFT. The *arg-12^s pyr-3* strains did not grow in the presence of Arg due to reduced leakage of carbamoyl phosphate from the mitochondria as a result of decreased CPS-A activity levels in response to Arg. Targeted integrants, however, responded to Arg with decreased TFT sensitivity, presumably by reducing TK activity through the *arg-2* uORF.

The addition of uridine to the media of *arg-12^s pyr-3* strains resulted in increased growth levels similar to those of targeted integrants. Surprisingly, uridine alone, which is required by strain *arg-12^s pyr-3* when Arg is present, relieves TFT sensitivity in that strain. The opposite is true for targeted integrants: uridine made those transformants more sensitive to TFT. The mechanisms responsible for these results are unclear but suggest that differing responses to uridine could possibly reflect alterations in metabolism arising as a consequence of the *arg-12^s pyr-3* mutations.

3.3 HSV-1 *tk* reporter plasmid construction

Our initial experiments demonstrated that active TK protein could be expressed in *N. crassa* under the control of *arg-2* uORF sequences. Therefore, we designed and constructed an expression vector, pDV8 that does not contain *arg-2* sequences so that specific *arg-2* sequences could subsequently be cloned into pDV8 to better analyze their role in *arg-2* gene expression. Figures 5 and 6 detail the steps and components involved in constructing pDV8. It is interesting to note that TK may be toxic to transformed *E. coli*. Some *tk*-containing plasmids produced transformed *E. coli* cultures that grew in abnormal cell aggregates and yielded low levels of plasmid DNA. pDV8 itself exhibited this phenomenon to a high degree.

To allow for proper transcriptional initiation and termination of *HSV-1 tk* (*Bgl*II–*Bam*HI), the *gpd* promoter (*Xho*I–*Pvu*II) and *trpC* terminater (*Bam*HI–*Hind*III) regions from *A. nidulans* were chosen to flank *tk* in pDV8 (Fig. 5). Both of these elements have been cloned and sequenced. Previously reported activity data are consistent with their retaining function in *N. crassa* (Staben, *et al.*, 1989). Plasmid pDV8 contains unique *Pvu*II and *Bgl*II sites juxtaposed between the *gpd* promoter and *tk* to allow for insertion of *5′– arg-2* sequences of choice.

For integration of pDV8 at the *his-3* locus, a unique *Not*I site was incorporated into pDV8 to accept the *Not*I fragment of *N. crassa his-3* from pH303-DN (Fig. 6). Studies on the expression of pDV8 remain to be accomplished.

Figure 1: Construction of *tk* expression vectors

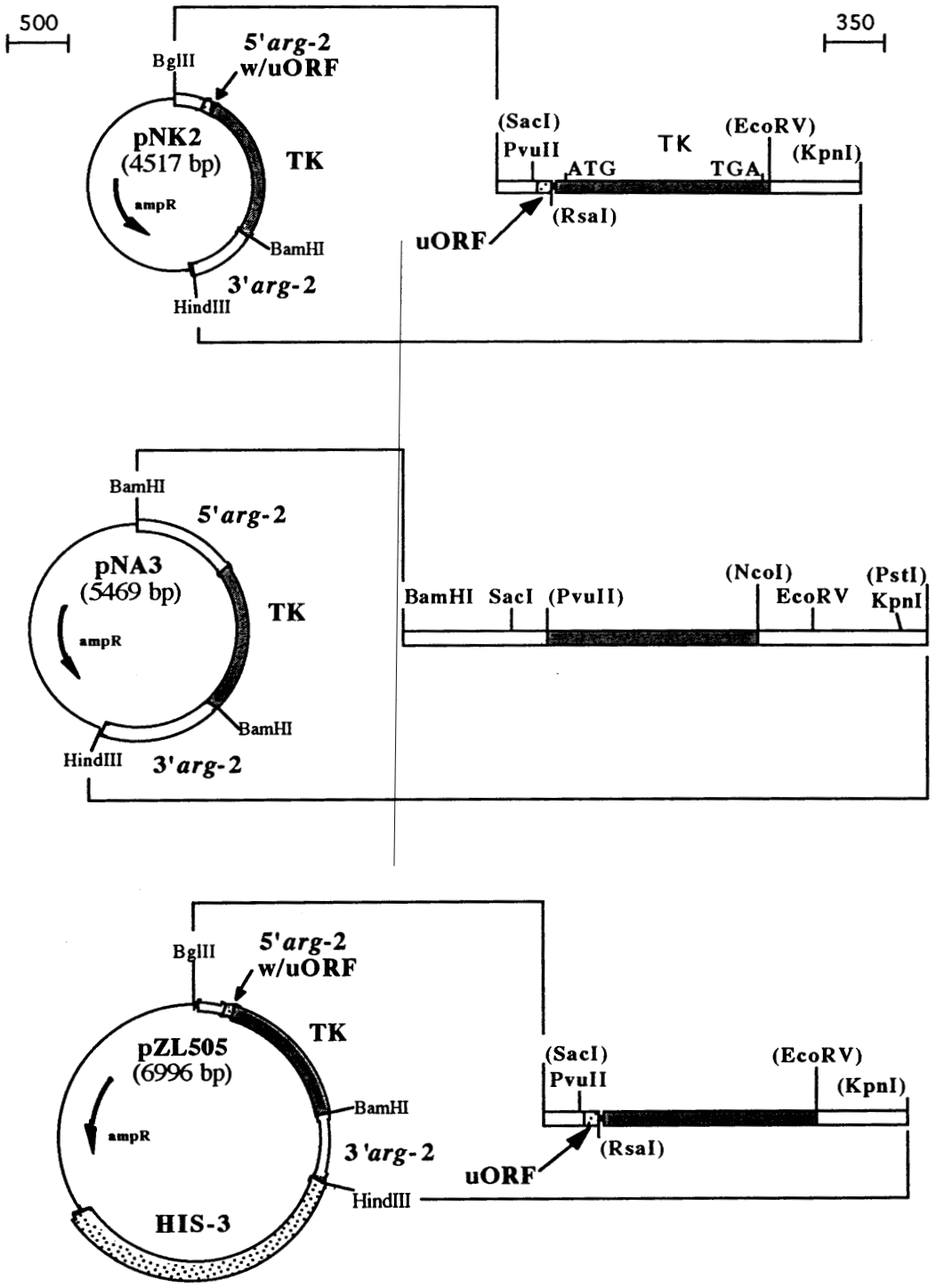

Figure 1: Construction of *tk* expression vectors— The *tk* fragment (*Bgl*II–*Bam*HI) in pNK2 contains the *tk* fragment from pMK13 inserted between *N. crassa arg-2* 5' (with the uORF) and *arg-2* 3' sequences. pNA3 contains a filled *tk* fragment (*Bgl*II–*Bam*HI) from pMK13 inserted between *N. crassa arg-2* 5' (without the uORF) and *arg-2* 3' sequences. pZL505 contains the *Bgl*II–*Hind*III fragment of pNK2 cloned into the same sites of pDE2. pDE2 contains a truncated *his-3* fragment from *N. crassa* genomic DNA. Parentheses indicate native *N. crassa arg-2* locus restriction sites.

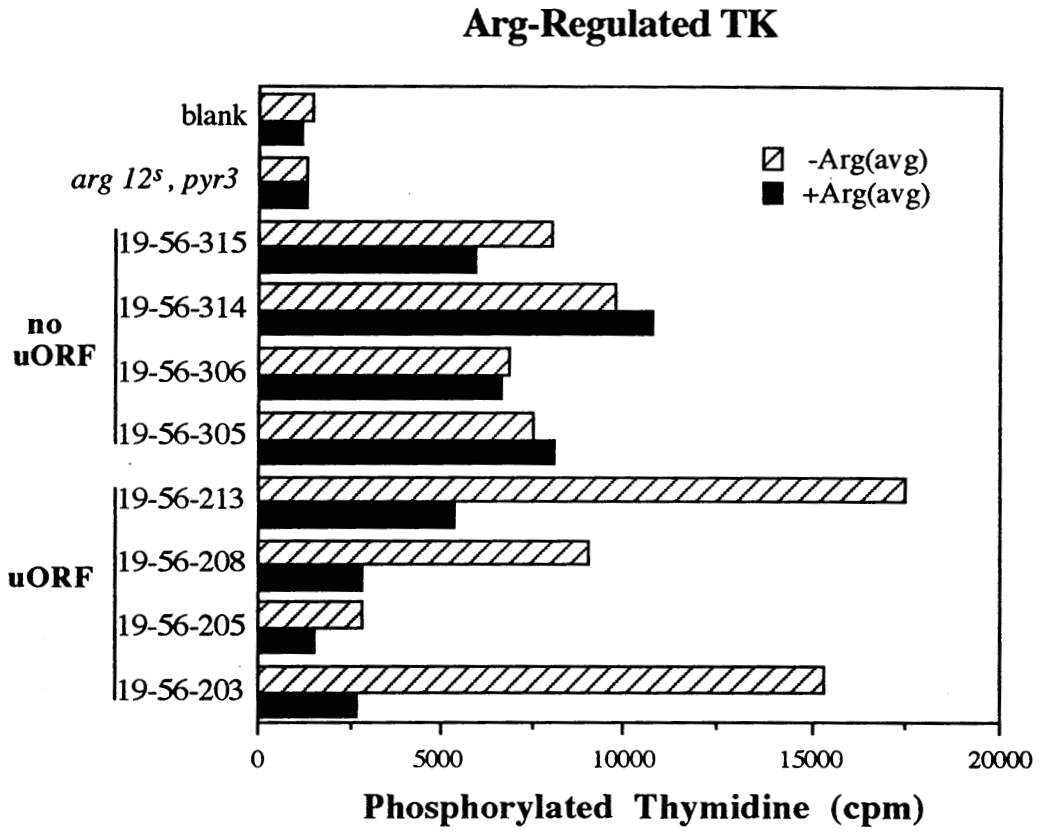

Figure 2: Arg-regulated thymidine kinase activity—*Neurospora crassa* strains were grown in VM or VM supplemented with Arg and Uridine. Extracts were prepared and assayed for TK activity (40 min reactions as described in the text).

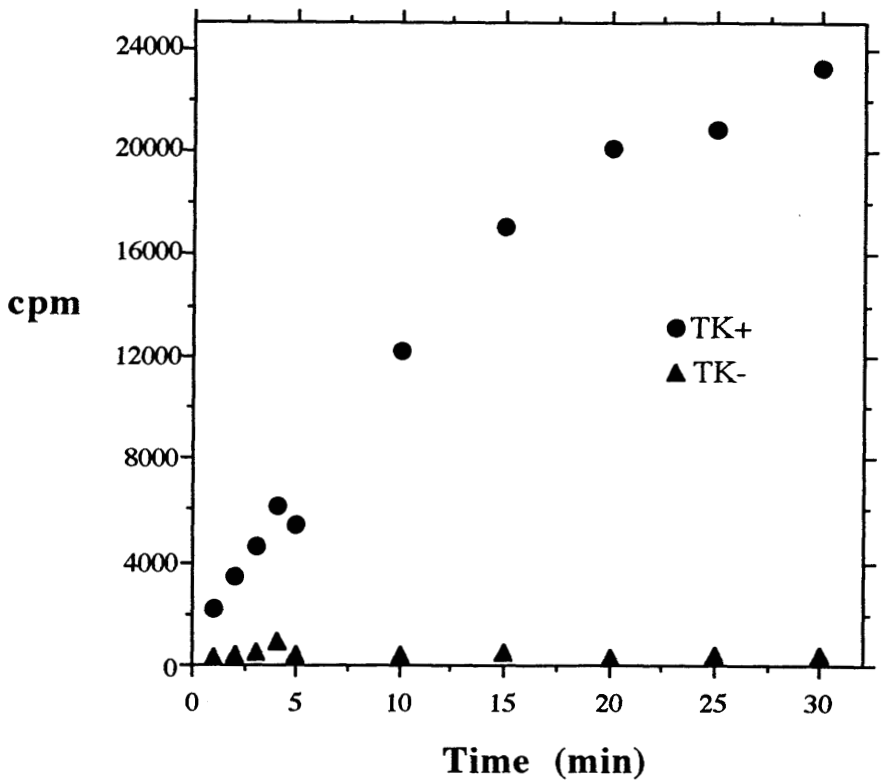

Figure 3: Time course analysis of thymidine kinase assay—*Neurospora crassa* extracts were prepared and assayed for TK activity (phosphorylated (methyl-^{3}H)-thymidine). Each 200 μl reaction contained 50 μg total *N. crassa* protein extract and 200 μCi (methyl-^{3}H)-thymidine. Reactions were incubated at 37°C for the time periods indicated. Filled circles represent *N. crassa* strain *arg-12^{s} pyr-3* transformed with pNK2. Filled triangles represent untransformed *N. crassa* strain *arg-12^{s} pyr-3*.

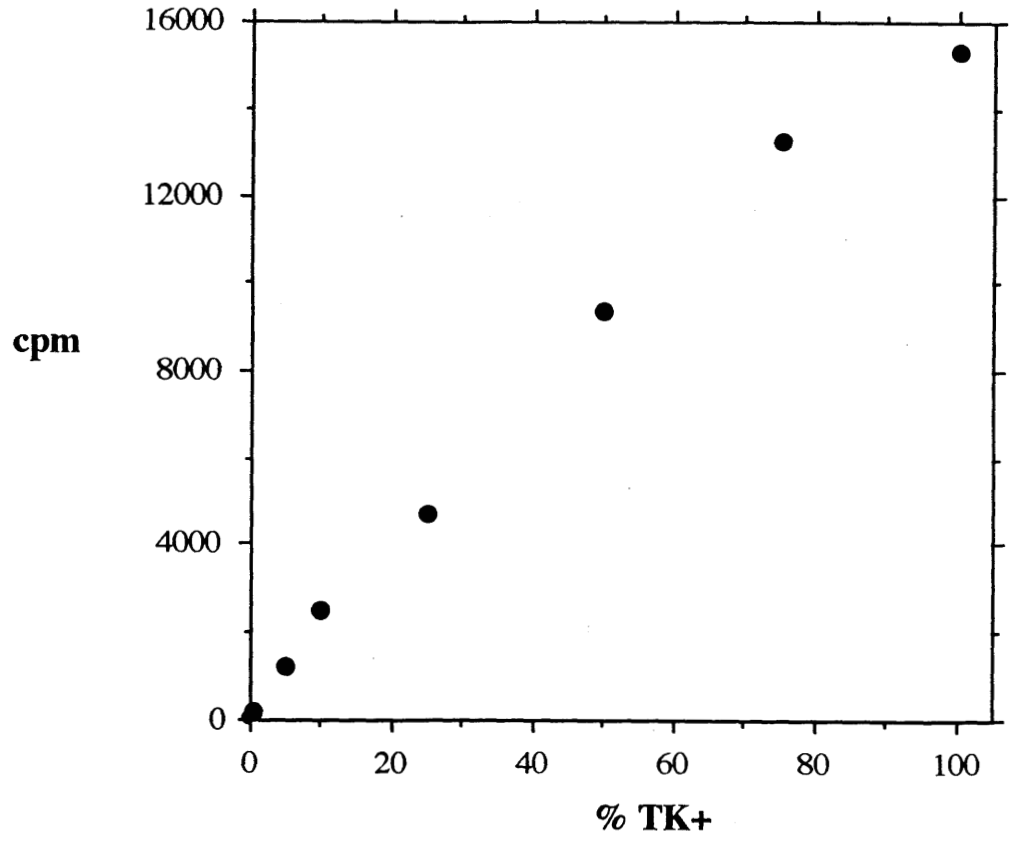

Figure 4: Thymidine kinase reconstruction experiment—Extracts from *N. crassa* strain *arg-12^s, pyr-3* transformed with pNK2 and untransformed *N. crassa* strain *arg-12^s pyr-3* were mixed to vary the proportion of TK+ extract from 0% and 100% as indicated. Each 200 μl reaction contained 50 μg of crude cell extract protein and 200 μCi (methyl-^{3}H)-thymidine. Reactions were incubated at 37°C for 10 minutes.

Table 1: Arg-regulated TK activity—Strains were grown in VM, VM plus Arg, or VM plus Arg and Uridine. Extracts were prepared and assayed for TK activity (40 min reactions as described in text).

STRAIN		**MEDIA**		
		Phosphorylated Thymidine (cpm)		
arg12^{s}, pyr-3	plasmid	minimal	Arg + Uri	fold regulation
(19-56-203)	**pNK2**	8777	1053	8.3
(19-56-314)	**pNA3**	6427	5497	1.2
his-3		minimal	Arg	
(6103)	**pZL505**	2443	520	4.7
(6524)	**pZL505**	1729	634	2.7

Table 2: Arg-regulated TFT sensitivity—Strains were grown in VM, VM plus Arg, or VM plus Arg and Uri. Conidia were collected and assayed for sensitivity to 25 μM TFT (2–3 day incubation). ++++ represents wild type growth.

STRAIN		**MEDIA**							
		minimal		**Arg**		**Uri**		**Arg + Uri**	
arg12^{s}, pyr-3	plasmid	-TFT	+TFT	-TFT	+TFT	-TFT	+TFT	-TFT	+TFT
(19-56-203)	**pNK2**	+	-	-	-	+++	+++	+++	+++
	none	+	+	-	-	+++	+++	+++	+++
his-3									
(6103)	**pZL505**	+++	++	+++	+++	+++	+	+++	+++
(6524)	**pZL505**	+++	++	+++	+++	+++	+	+++	+++
(13-6)	none	++	++	++	++	++	++	++	++
74A (w.t.)		++++	++++	++++	++++	++++	++++	++++	++++

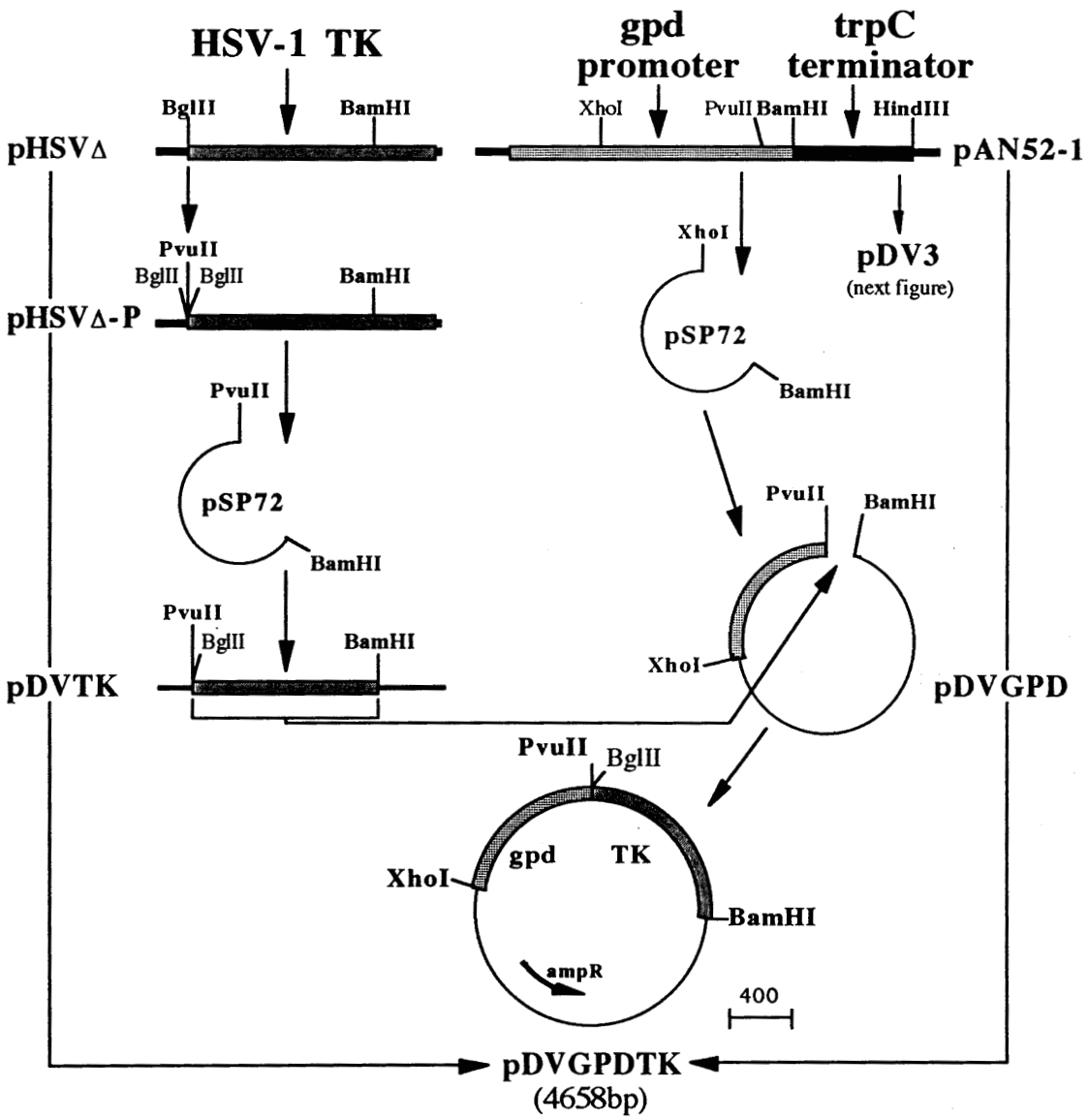

Figure 5: Subcloning HSV-1 *tk* and the *Aspergillus nidulans gpd* promoter— pHSVΔ was digested with *Bgl*II, Klenow filled, and then religated with a *Pvu*II linker (TACAGCTGTA) designed to regenerate flanking *Bgl*II sites when cloned into a filled *Bgl*II site to produce pHSVΔ-P. The resulting *tk* fragment (*Pvu*II-*Bam*HI) was then inserted into the multiple cloning site of pSP72 to produce pDVTK. The *tk* fragment (*Pvu*II-*Bam*HI) from pDVTK was then inserted downstream of the *gpd* promoter region of pDVGPD to produce pDVGPDTK. pDVGPD was constructed by cloning the gpd promoter region (*Xho*I-*Bam*HI) from pAN52-1 into the multiple cloning site of pSP72. **Unique sites** appear in bold type.

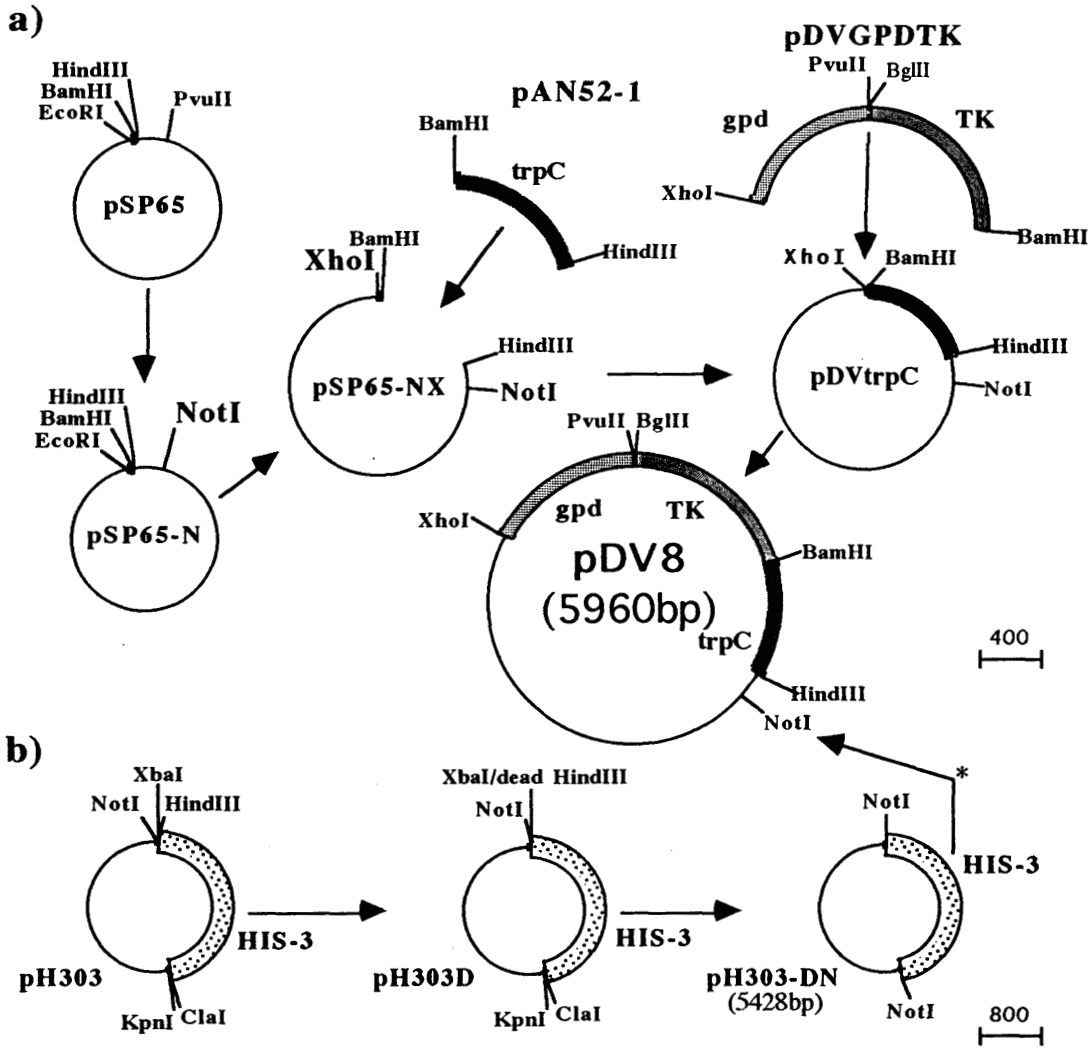

Figure 6: Construction of pDV8 and a *Not*I his-3 fragment— **a)** pSP65 was digested with *Pvu*II and religated with a *Not*I linker (AGCGGCCGCT) to produce pSP65-N. pSP65-N was then digested with *Eco*RI, Klenow filled, and religated with an *Xho*I linker (CCTCGAGG) to make pSP65-NX. The *Bam*HI–*Hind*III *trpC* fragment from pAN52-1 was cloned into pSP65-NX to make pDVtrpC. The *Xho*I–*Bam*HI fragment from pDVGPDTK (previous figure) was cloned into the same sites of pDVtrpC to make pDV8. **b)** pH303 was digested with *Xba*I and *Hind*III, Klenow filled, and religated. This plasmid (pH303D) was digested with *Cla*I, Klenow filled and religated with a *Not*I linker to produce pH303DN. *pH303DN's *Not*I fragment (*his-3*) inserted into the *Not*I site of pDV8. **Unique sites** appear in bold type.

Chapter 4

Conclusions and Future Perspectives

The Herpes Simplex Virus type-1 thymidine kinase gene (HSV-1 *tk*) can be expressed in *N. crassa*. Strains containing *arg-2-tk* fusion genes possess thymidine kinase (TK) activity as assayed by the phosphorylation of [methyl-^{3}H]-thymidine. Fusion genes containing *arg-2* uORF sequences produce strains with TK activity that is negatively regulated by Arg. In addition, the growth of *N. crassa* strains expressing *tk* is sensitive to trifluorothy-midine (TFT). This sensitivity is relieved when transformed his$^-$ strains containing *arg-2* uORF sequences are grown in the presence of Arg, possibly because Arg negatively regulates *tk* expression.

It is expected that future studies of the *N. crassa arg-2* locus, particularly of the uORF, will benefit from the *tk* reporter gene. Plasmid pDV8 makes it possible to construct *tk* fusion genes containing selected *arg-2* sequences. Unique *Pvu*II and *Bgl*II sites between *gpd* and *tk* provide an appropriately located insertion point for *arg-2* sequences based on the uORF. The unique *Not*I site in pDV8 allows the insertion of sequences for targeting these constructs to defined chromosomal locations (e.g. the *his-3* locus of *N. crassa*).

Future studies should include transformation of *N. crassa* strains with pDV8, and confirmation of TK activity in those strains. In addition, *arg-2* uORF sequences should be inserted between *gpd* and *tk* to examine whether these sequences are sufficient to reproduce Arg-specific regulation of TK activity. DNA and RNA should be analyzed to confirm that the gene is intact and properly transcribed. Novel *arg-2* uORF sequences, containing mutations or frame shifts, could be synthesized and cloned into pDV8. When transformed into the *N. crassa* genome, the effect of such changes in the uORF could be examined. In addition, CPS-A enzyme activity should be measured in order to compare it with levels of *arg-2*.

TK has possibilities beyond the study of Arg-specific regulation of CPS-A. The mechanism by which phosphorylated TFT inhibits the growth of cells could be examined using TK$^+$ and TK$^-$ *N. crassa* strains. For example, some thymidine analog drugs, i.e.

AZT (3′-azido-2′-deoxythymidine) and FIAU (1-2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl-5-iodouracil), have been used to treat human patients afflicted with Aquired Immune Deficiency syndrome (AIDS) . It is believed that drug toxicity, which has developed in some patients who were treated with these thymidine analogs, is in part due to the disruption of mitochondrial DNA synthesis as a result of phosphorylation of thymidine analogs by TK (Touchette, 1993). *Neurospora crassa*, in which mitochondrial defects are already known to cause senescence (Seidel-Rogel, *et al.*, 1989), would provide an excellent model system for this type of study.

Previously, it has been difficult to study DNA replication in *N. crassa* because the organism lacked TK and therefore could not readily incorporate tracers such as (methyl-^{3}H)-thymidine and bromodeoxyuridine (BrdU) into DNA. It has been demonstrated that TK+ *N. crassa* strains incorporate BrdU into DNA (E. Selker, M. Sachs, data not shown). Therefore, it should be possible to study the process of DNA replication using *tk* in *N. crassa.*

Clearly, HSV-1 *tk* has potential as a reporter gene in *N. crassa.* The HSV-1 *tk* gene will benefit the study of gene regulation in fungi, as well as provide a tool to assist in the effort to understand the mechanism of TK-induced cell damage in humans treated with thymidine analog drugs.

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