Nucleic Acids Research, 1997, Vol. 25, No. 12 **2389–2395**

Expression of herpes virus thymidine kinase in *Neurospora crassa*

Matthew S. Sachs*, Eric U. Selker[1], Baoyu Lin, Christopher J. Roberts[1,+], Zongli Luo[§], David Vaught-Alexander and Brian S. Margolin[1]

Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, PO Box 91000, Portland, OR 97291-1000, USA and [1]Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229, USA

Received March 6, 1997; Revised and Accepted April 22, 1997

ABSTRACT

The expression of thymidine kinase in fungi, which normally lack this enzyme, will greatly aid the study of DNA metabolism and provide useful drug-sensitive phenotypes. The herpes simplex virus type-1 thymidine kinase gene (*tk*) was expressed in *Neurospora crassa*. *tk* was expressed as a fusion to *N.crassa arg-2* regulatory sequences and as a hygromycin phosphotransferase–thymidine kinase fusion gene under the control of cytomegalovirus and SV40 sequences. Only strains containing *tk* showed thymidine kinase enzyme activity. In strains containing the *arg-2–tk* gene, both the level of enzyme activity and the level of mRNA were reduced by growth in arginine medium, consistent with control through *arg-2* regulatory sequences. Expression of thymidine kinase in *N.crassa* facilitated radioactive labeling of replicating DNA following addition of [^{3}H]thymidine or [^{14}C]thymidine to the growth medium. Thymidine labeling of DNA enabled demonstration that hydroxyurea can be used to block replication and synchronize the *N.crassa* mitotic cycle. Strains expressing thymidine kinase were also more sensitive than strains lacking thymidine kinase to anticancer and antiviral nucleoside drugs that are activated by thymidine kinase, including 5-fluoro-2′-deoxyuridine, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine and trifluorothymidine. Finally, expression of thymidine kinase in *N.crassa* enabled incorporation of bromodeoxyuridine into DNA at levels sufficient to separate newly replicated DNA from old DNA using equilibrium centrifugation.

INTRODUCTION

In contrast to all other growing cells, *Neurospora crassa*, like other fungi, lacks detectable thymidine kinase (TK) activity (1,2). Mammalian cells contain distinct cytosolic and mitochondrial TKs that are primarily involved in salvage pathways (3). Fungi do not appear to use the salvage pathway and TMP is produced solely by the *de novo* pathway from dUMP by thymidylate synthetase.

Here we describe properties conferred to the fungus *N.crassa* by expression of the herpes virus thymidine kinase gene (*tk*). We fused *tk* to regulatory sequences of the *N.crassa arg-2* gene (4,5), introduced the *arg-2–tk* fusion gene into the genome by transformation and obtained strains expressing TK. We also introduced a hygromycin phosphotransferase–*tk* fusion gene whose expression can be selected both positively and negatively (6). The simplicity of the TK enzyme assay and the absence of endogenous TK activity in *N.crassa* make this an excellent reporter gene in this fungus.

The expression of TK in mammalian systems has been exploited in many ways. The presence of endogenous TKs provides the basis for some important anticancer and antiviral drug therapies. Thymidine kinases phosphorylate nucleoside analogs such as fluorodeoxyuridine (FdU), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine (FIAU) and trifluorothymidine (TFT), which results in their conversion into toxic forms capable of inhibiting cell growth or viral infection (7–9). The different specificities of viral and host TKs have enabled the development of specific antiviral nucleoside analogs such as the anti-herpes drugs ganciclovir and acyclovir (10,11). The directed expression of recombinant TK has also enabled advances in genetic engineering and gene therapy. Thus, selection against integration and expression of the *tk* gene can increase the efficiency of gene targeting (12). Selection against the expression of recombinant *tk* genes integrated into the genome also enables the selective killing of specific cells in transgenic animals (13) and the killing of other organisms (14). Finally, ‘suicide’ gene therapy strategies using recombinant *tk* genes may ultimately prove clinically useful, as indicated by model studies (15,16). While the growth of *N.crassa* is normally relatively resistant to many of the nucleoside analogs that become toxic only after phosphorylation by TK, we found that expression of TK made the growth of *N.crassa* highly sensitive to such compounds.

* To whom correspondence should be addressed. Tel: +1 503 690 1487; Fax: +1 503 690 1464; Email: msachs@admin.ogi.edu

Present addresses: +The Seattle Project, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, M477, Seattle, WA 98104, USA and §Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-4318, USA

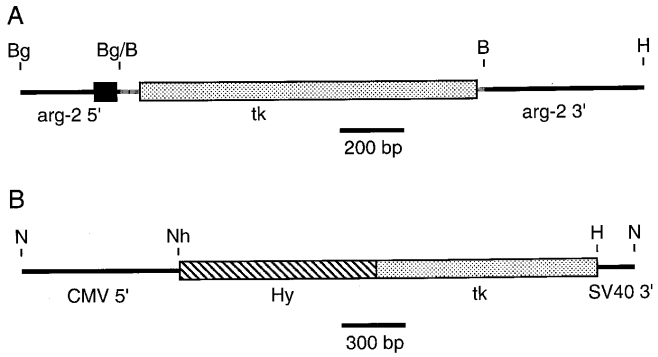

Figure 1. Diagrams of *arg-2–tk* and *Hy–Tk* constructs. (**A**) The *arg-2–tk* hybrid gene in plasmids pNK2 and pZL505 contains an intronless *arg-2* 5′-leader with the *arg-2* upstream open reading frame (black box) preceding the herpes virus TK coding region (stippled box) and the *arg-2* 3′-region distal to TK. (**B**) The *Hy–Tk* hybrid gene (6) contains the cytomegalovirus major early promoter 5′-proximal of a fusion gene with a hygromycin phosphotransferase domain (hatched box) and a TK domain (stippled box). The SV40 polyadenylation site is distal to the *Hy–Tk* fusion gene. Relevant restriction sites include: Bg, *Bgl*II; B, *Bam*HI; H, *Hin*dIII; N, *Not*I; Nh, *Nhe*I.

The presence of endogenous TK in mammalian cells facilitates investigations of DNA replication and repair. Compounds such as [^{3}H]thymidine and bromodeoxyuridine (BrdU) are converted by TK to the monophosphate forms, which are subsequently converted to the triphosphate form, enabling these compounds to be incorporated into DNA. The DNA of fungi cannot normally be labeled in this way (17). However, expression of TK in *N.crassa* enabled efficient incorporation of these compounds into DNA, providing new methods for studying DNA replication and repair in this organism.

MATERIALS AND METHODS

Plasmids

Techniques for plasmid construction and propagation were described previously (5,18). Plasmid pNK2 contained an *arg-2–tk* fusion gene that lacks *arg-2* intron sequences (Fig. 1). Plasmid pZL505 was constructed to target *arg-2–tk* genes to the *his-3* locus. The *Bgl*II–*Hin*dIII fragment of pNK2 containing *arg-2–tk* was ligated to the *Bgl*II–*Hin*dIII fragment of pDE2 (19), containing the truncated *his-3* locus and bacterial replication and selection sequences.

Plasmid pHyTk (6) contains a hygromycin phosphotransferase–*tk* gene fusion (*Hy–Tk*) flanked by the human cytomegalovirus IE94 promoter and the SV40 virus early region polyadenylation sequence (Fig. 1B). To create plasmid pBL105, for targeted integration of the *Hy–Tk* gene at the *his-3* locus, the *Not*I fragment of pHyTk was inserted into plasmid pH303 (K.Hager, Yale University), which contained the truncated *his-3* locus and bacterial replication and selection sequences.

Plasmid pM063 contains an allele of the β-tubulin gene that confers benomyl resistance and was modified to remove several restriction enzyme cleavage sites (a gift from M.J.Orbach, University of Arizona).

Construction and growth of *N.crassa* strains containing integrated copies of *tk* genes

Standard procedures were used to grow *N.crassa* (5,18). Strains were grown in minimal medium (Min; 1× Vogel's N, 2% sucrose; 20) supplemented as noted with 0.5 mg/ml uridine, 0.5 mg/ml arginine, both uridine and arginine, 0.3 mg/ml hygromycin B (Hyg; Calbiochem, La Jolla, CA), 1 μg/ml benomyl (DuPont). The *his-3* auxotroph was grown on Min supplemented with 0.5 mg/ml histidine. Tests of growth sensitivity to nucleoside analogs are described below.

The following *N.crassa* strains were from The Fungal Genetics Stock Center (FGSC): wild-type 74-OR23-1*A* (FGSC 987), *his-3* mutants [alleles 1-234-723 (FGSC 6103) and 1-234-1438 (FGSC 6524)]. The *arg-12^s pyr-3* strain was obtained from Robert Metzenberg. Competent *N.crassa* cells were prepared and transformed with DNA and homokaryotic cultures obtained as described (21,22). Pure cultures were maintained by isolating single colonies from plates streaked with conidia (23). *Neurospora* DNA and RNA were prepared and examined by Southern and northern blotting (5).

Thymidine kinase activity assays

Conidia were inoculated into 25 ml medium (in 125 ml flasks) at 10^7 conidia/ml. Cultures were grown at 34°C with orbital shaking (200 r.p.m.) for 13 h and harvested by vacuum filtration onto Whatman no. 541 filter paper. The collected mycelial mat was divided and quickly frozen in liquid nitrogen and stored at –80°C for subsequent preparation of crude whole-cell extracts or of total RNA.

Cell-free extracts were prepared and assayed for TK enzyme activity by a modification of a previously described procedure (24). Approximately 200 mg frozen mycelia were added to a 2-ml screw-top tube (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 β-mercaptoethanol, 20% glycerol). Cells were disrupted twice for 60 s in a mini-bead beater (BioSpec; Bartlesville, OK) at 4°C; tubes were cooled on ice between disruptions. Extracts were clarified by centrifugation in a microcentrifuge at 6600 *g* 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 (25).

Reaction mixtures for the assay of TK (125 μl) contained 30 μg protein, 50 mM Tris–HCl, pH 8.0, 10 mM ATP, 5 mM sodium fluoride, 5 mM calcium chloride, 10 mM β-mercaptoethanol (freshly diluted) and 30 μM [methyl-^{3}H]thymidine (7 Ci/mmol; Moravek Biochemicals, Brea, CA). Reaction mixtures were incubated at 37°C for the indicated periods, boiled for 2 min in a heat block to stop the reactions and centrifuged at 6600 *g* for 2 min. Supernatants (50 μl) were spotted onto 2 cm DEAE–cellulose anion exchange disks (Whatman DE81) and allowed to dry. The disks were washed four times in 4 mM ammonium formate and then twice in 95% ethanol (at least 10 ml/disk, 5 min/wash). TMP, but not thymidine, remains bound to the disks under these conditions. Individual disks were transferred to scintillation vials to which ScintiVerse scintillation fluid (Fisher) was added. Levels of radioactivity in the samples were measured

by scintillation counting. The counts reported represent the averages of duplicate or triplicate measurements.

Synchronization of DNA synthesis

Rapidly growing vegetative cultures (100 ml shaken in 500 ml flasks) of a *Neurospora* TK$^+$ transformant, typically grown for 12 h at 30°C from conidia (4–5 × 10^6/ml), were treated for 3–5 h with 0.1 M hydroxyurea (HU; Sigma) in Vogel's medium containing 2% sucrose. To simplify quantitation, [^{14}C]thymidine (~0.1 μCi/ml, 58 mCi/mmol; Amersham) was included in the culture prior to addition of HU in some experiments. The cultures were harvested by filtration, rinsed extensively with fresh minimal medium lacking HU and then resuspended in fresh, prewarmed minimal medium. [^{3}H]Thymidine (0.5–3 μCi/ml, 5 Ci/mmol; Amersham) was included in the fresh medium for some experiments or added to samples (5–20 ml) removed periodically from the primary culture for pulse labeling. Samples were harvested by filtration, rinsed, lyophilized and weighed; DNA was isolated as previously described (26). Radioactivity of samples was determined by scintillation counting in Ecoscint A scintillation fluid (National Diagnostics).

Nucleoside sensitivity assays

Conidia from wild-type, *arg-12^s pyr-3* and transformed *N.crassa* strains were collected, suspended in 3 ml molten 50°C agar medium (Min; 0.05% fructose, 0.05% glucose, 2% sorbose) containing specified levels of nucleoside analogs and plated on 35 ×10 mm disposable Petri dishes (Falcon). Analogs were prepared as stock solutions in water [10 mM FdU and 10 mM TFT (each from Sigma Chemicals); 2.5 mM FIAU (Moravek Biochemicals); 2 mM BrdU (Sigma); 180 mM gancyclovir (Hoffman-LaRoche)], sterilized by filtration and stored at –20°C. Analogs and conidia were added to medium immediately prior to plating. Cultures were incubated at 34°C for 3–5 days.

Separation of newly replicated DNA containing BrdU by equilibrium centrifugation

Neurospora strain Tk6103, which contained a single copy of the *arg-2–tk* gene integrated at *his-3*, was inoculated at 5 × 10^6 conidia/ml in 400 ml Vogel's minimal medium and shaken at 200 r.p.m. for 13 h at 30°C. At this time point (time 0), >90% of the conidia had germinated. At time 0, one quarter of the tissue (100 ml of culture) was harvested by filtration and frozen at –80°C. Aliquots of 20 μM BrdU and 2 μM FdU were immediately added to the remaining culture and the culture was incubated further at 30°C with shaking. All manipulations with BrdU were conducted in the dark to avoid possible DNA crosslinking. To replace the BrdU and FdU that was metabolized over the course of the experiment, additional aliquots of BrdU and FdU were added at the 60, 120 and 180 min time points (BrdU was added to increase its concentration by 4 μM and FdU was added to increase its concentration by 0.4 μM at these times). At the 45, 120 and 240 min time points, 100 ml aliquots of the culture were removed and the tissue harvested and frozen. The genomic DNAs were extracted (27) and 50 μg aliquots were analyzed by CsCl/ethidium bromide (0.083 g/ml CsCl; 28) equilibrium centrifugation. Samples were centrifuged for 13 h at 288 000 *g* in the TLN100 rotor of a Beckman Optima TL ultracentrifuge at 20°C and then photographed using short wave UV illumination.

RESULTS

Characterization of *N.crassa* strains expressing thymidine kinase

Two different *tk* constructs were introduced into *N.crassa*. The *arg-2–tk* construct has *N.crassa arg-2* sequences upstream and downstream of the herpes virus *tk* coding region (Fig. 1A). *arg-2* sequences were chosen because their regulatory effects on reporter gene expression are well established (18,29). An upstream open reading frame in the *arg-2* transcript that reduces gene expression when excess arginine is provided (5,18,29) is present in this construct. The second construct, *Hy–Tk*, was used to gain both positive and negative selection capabilities. The *Hy–Tk* construct contains the major early cytomegalovirus promoter and the SV40 early region polyadenylation site placed 5′ and 3′ respectively of a hygromycin phosphotransferase–TK fusion protein coding region (6). This gene was chosen because it was previously shown to confer positive selection for hygromycin resistance when introduced into *N.crassa* (18). In addition to allowing growth in medium containing hygromycin at concentrations up to 0.5 mg/ml (data not shown), integration of this construct at *his-3* also allowed for negative selection through TK expression, as shown below.

The *arg-2–tk* construct of plasmid pNK2 was introduced into the *arg-12^s pyr-3* strain by cotransformation with plasmid pMO63, which confers resistance to benomyl. The *arg-12^s pyr-3* strain was chosen because *arg-2* is more highly expressed in this strain in medium lacking arginine than in a wild-type strain (5,30). Benomyl-resistant transformants were screened by Southern analysis (data not shown) and a multicopy *arg-2–tk* strain, named Tk213, that showed arginine-regulated *tk* activity was selected for further studies. The *arg-2–tk* and *Hy–Tk* genes were also integrated as single-copy genes at the *his-3* locus by selecting transformants for histidine prototrophy using a truncated *his-3* gene in the vectors (5,31). Integration of single copies of *tk* reporter genes at *his-3* was confirmed by Southern analysis (data not shown). A representative strain containing the *arg-2–tk* gene, named Tk6103, and a strain containing the *Hy–Tk* gene, named HyTk1, were used for the studies described below. Similar results were obtained with other strains containing *arg-2–tk* or *Hy–Tk* genes integrated at *his-3* (data not shown).

Strains with or without the *tk* gene were assayed for TK activity by measuring the phosphorylation of [methyl-^{3}H]thymidine in whole-cell extracts. The wild-type *N.crassa* strain, which should lack TK, and strain Tk213 were chosen for initial analyses of TK activity. A time–course experiment to measure production of TK (Fig. 2A) confirmed that the wild-type strain lacked detectable TK, while strain Tk213 showed significant TK activity resulting in a linear increase in thymidylate over a period of 50 min and continued production of thymidylate for at least 120 min under these assay conditions. The *arg-12^s pyr-3* mutant, which was the transformation host used to generate strain Tk213, also lacked detectable TK activity (data not shown). Measurements of TK activity in mixtures of whole-cell extracts obtained from the wild-type strain and from strain Tk213 showed that the wild-type strain lacked inhibitors of TK activity (Fig. 2B).

All of the strains examined that contained *tk* genes showed TK enzyme activity and had a TK$^+$ phenotype (Table 1 and data not shown). Expression of TK did not appear to have negative effects on these strains' growth rates or on the viability of their conidial

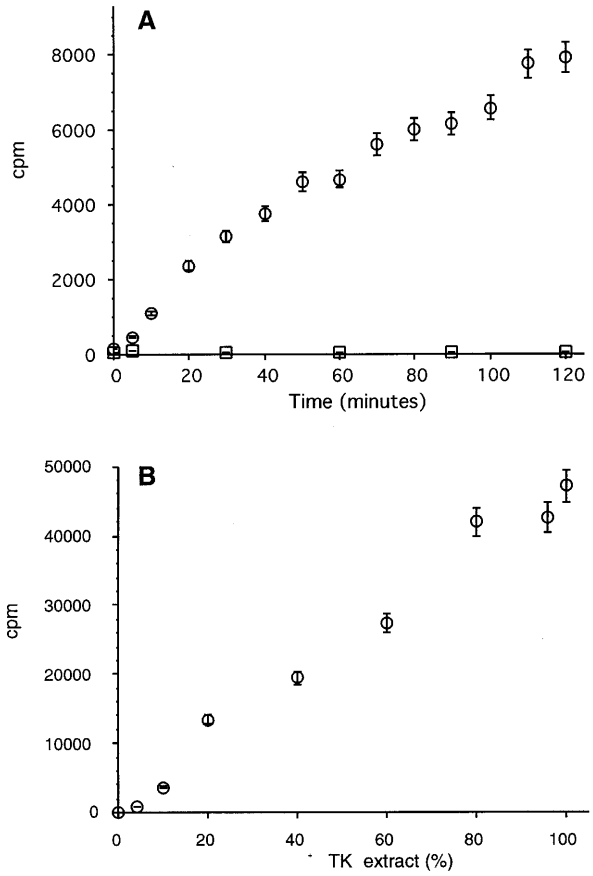

Figure 2. Assay of TK enzyme activity in *N.crassa*. (**A**) Whole-cell extracts prepared from strain Tk213 (circles) or wild-type (squares) were incubated for the indicated periods with [^{3}H]thymidine [diluted with unlabeled thymidine (Fluka) to 1 Ci/mmol] in TK assay buffer and the relative amount of phosphorylated [^{3}H]thymidine (thymidylate) produced was determined by retention on DEAE–cellulose filters as described in Materials and Methods. Yields of retained [^{3}H]thymidine are expressed in c.p.m. (**B**) Whole-cell extracts prepared from strain Tk213 and from wild-type were mixed in the proportions indicated, incubated with [^{3}H]thymidine (7 Ci/mmol) in TK assay buffer for 60 min and the relative amount of phosphorylated [^{3}H]thymidine produced determined. The mean value and standard deviations obtained from measurements of triplicate samples at each time point are indicated. In all cases, the c.p.m. of [^{3}H]thymidine retained on blank filters processed in parallel with experimental samples were subtracted.

spores (data not shown). The *arg-12^s pyr-3* strain Tk213 produced the highest level of TK activity; presumably, high expression was achieved because this strain contained the *arg-12^s* mutation, which boosts *arg-2* expression, and because it contained multiple copies of *arg-2–tk*. Strains containing *Hy–Tk* genes integrated at *his-3* in single-copy produced the lowest level of enzyme activity (Table 1 and data not shown). This lower level of expression possibly occurred because the cytomegalovirus promoter and SV40 polyadenlyation site did not function as efficiently in *N.crassa* as the corresponding *N.crassa arg-2* sequences and/or because the hygromycin phosphotransferase–TK fusion polypeptide was less stable or less active than the TK polypeptide.

The *arg-2* gene is negatively regulated by arginine (30) and transformants containing *arg-2–tk* gene fusions produced TK that was negatively regulated by arginine (Table 1 and data not shown). Strains containing *arg-2–tk* integrated at *his-3* showed a reduction in TK activity of ~3-fold when grown in arginine, similar to the effects of arginine on the expression of *arg-2* and other reporter genes containing the *arg-2* uORF in similar strains (5,18,29,30). The greater regulation of the *arg-2–tk* gene by arginine observed in the *arg-12^s pyr-3* host was expected, since *arg-12^s* strains overexpress *arg-2* in minimal medium (32). The *Hy–Tk* gene fusion, which lacks *arg-2* sequences, was not regulated by arginine, either at the level of TK enzyme activity (Table 1) or hygromycin phosphotransferase enzyme activity (18).

Table 1. Expression and regulation of TK in *N.crassa*[a]

Strain	–Arg	+Arg
74A	n.d.	n.d.
Tk213	53 160 ± 4030	4340 ± 320
Tk6103	4720 ± 640	1200 ± 270
HyTk1	1970 ± 60	2440 ± 190

[a]The TK$^-$ wild-type strain and TK$^+$ Tk213, Tk6103 and HyTk1 strains were grown in minimal medium (–Arg) or minimal medium supplemented with arginine (+Arg). For strain Tk213, uridine was added with arginine because the combination of the *arg-12^s pyr-3* mutant alleles present in this strain do not permit growth on arginine without uridine (30). Whole-cell extracts were prepared and assayed for TK as described in Materials and Methods (60 min reactions). The mean values and standard deviations obtained from measurements of duplicate samples are indicated. In all cases, the c.p.m. of [^{3}H]thymidine retained on blank filters (2450 c.p.m.) that were processed in parallel with experimental samples were subtracted; the wild-type *N.crassa* strain has no detectable TK (average value of 2440 c.p.m.).

Northern analyses indicated that fusion genes produced transcripts of the predicted sizes. Strains containing *arg-2–tk* constructs showed reduced levels of *arg-2–tk* mRNA when grown in arginine medium, as is also observed for the endogenous *arg-2* mRNA (data not shown; 32).

Synchronization of DNA synthesis

Hydroxyurea (HU), an inhibitor of ribonucleotide reductase, can be used to block DNA synthesis in *Neurospora*, as in other organisms (33,34). The availability of TK$^+$ *N.crassa* transformants allowed us to test whether HU could be used to synchronize DNA synthesis in this organism. Preliminary experiments with strain Tk213 showed that DNA synthesis was reversibly inhibited by 3–5 h treatments with 0.1 M HU, although the cultures continued to grow (data not shown). To test HU as a synchronizing agent, we treated a rapidly growing culture with HU for 4 h, washed out the drug and pulse labeled samples with [^{3}H]thymidine at various times up to 210 min after the HU treatment. DNA was isolated and the amount of [^{3}H]thymidine incorporated was determined. To avoid technical difficulties associated with accurately measuring DNA concentrations, we included [^{14}C]thymidine in the initial growth medium to label ‘old’ DNA. Newly synthesized DNA was labeled with ^{3}H and standardized to the ^{14}C-labeled DNA (Fig. 3). Similar results were also obtained by measuring DNA concentrations using a Hoefer DNA fluorometer (data not shown).

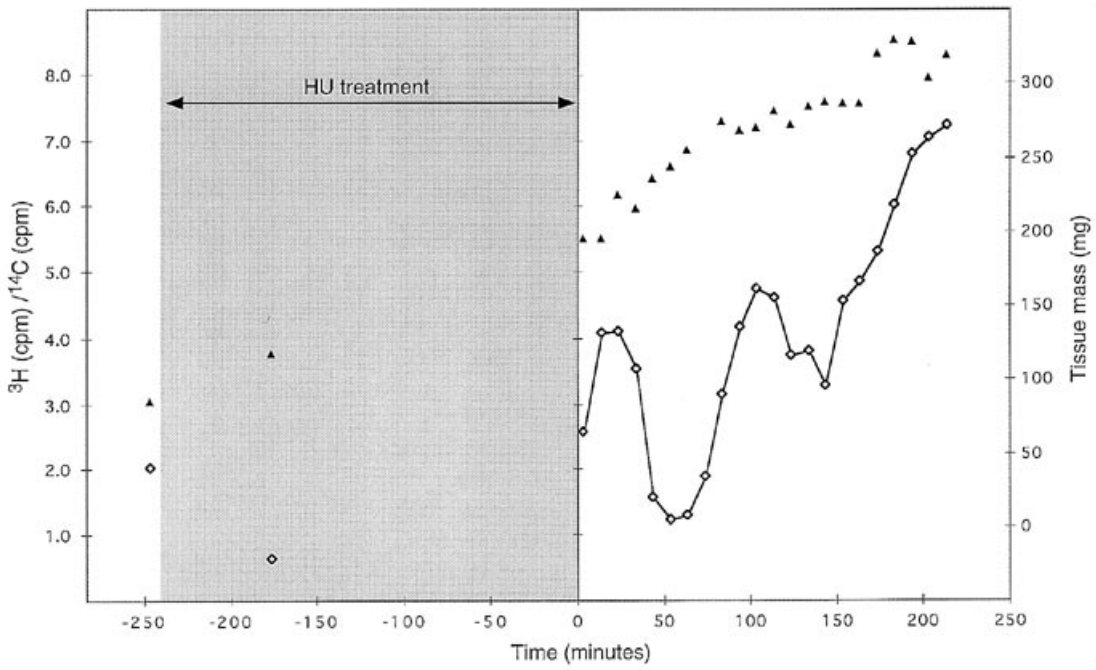

Figure 3. Synchronization of DNA synthesis by HU. An 11 h, 260 ml culture of germinating conidia (5×10^6/ml) of strain Tk213, grown at 30°C in medium spiked with 22 µCi [^{14}C]thymidine, was treated for 4 h with 0.1 M HU (shaded region), rinsed and resuspended in fresh medium without supplements. Ten milliliter aliquots were pulse labeled with [^{3}H]thymidine for 7 min before harvesting. Dried tissue samples were weighed to assess growth (filled triangles), DNA was isolated and 20% of it was used to measure [^{14}C]thymidine and [^{3}H]thymidine incorporation. ^{3}H/^{14}C ratios (open diamonds) are plotted using reference time points halfway through each pulse labeling period.

A sample taken after 1 h HU treatment demonstrated substantial, but incomplete, blockage of DNA synthesis relative to that immediately before the treatment (Fig. 3). DNA synthesis immediately increased after removal of the HU and continued to increase for ~20 min. Synthesis then sharply decreased, reaching a minimum at ~60 min following the release from HU block. These data suggest that one cycle of DNA synthesis was complete within 1 h and that DNA synthesis had, in large part, become synchronized.

A second round of DNA synthesis occurred without a long gap period and reached a peak at ~110 min. The second round of DNA synthesis occurred over a longer period than the first. Nevertheless, the time separating the two peaks (~90 min) is substantially shorter than the doubling time of *Neurospora* under these growth conditions. Indeed, tissue mass increased <50% in this period (Fig. 3). This is not unexpected, considering that a substantial increase in mass, approximately one doubling, occurred during the HU treatment. Thus, DNA synthesis likely 'caught up' after the block.

Effects of nucleoside drugs on TK$^+$ and TK$^-$ *N.crassa*

In mammalian cells, FdU, when phosphorylated by TK, covalently binds to thymidylate synthetase and inhibits enzymatic activity (35). Thus, we expected that the expression of TK in *N.crassa* would confer sensitivity to nucleoside analogs activated by TK, such as FdU. Comparison of the effects of FdU on TK$^+$ and TK$^-$ *N.crassa* strains revealed that TK$^+$ strains were much more sensitive to FdU than the TK$^-$ strains, as assayed by colony-forming ability on medium containing FdU (Fig. 4 and Table 2).

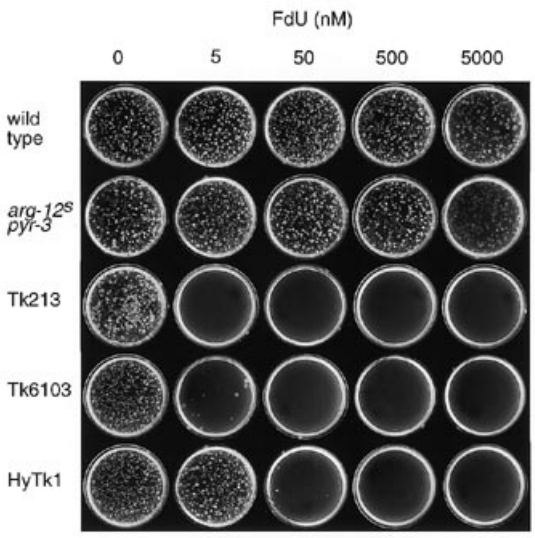

Figure 4. Effects of FdU on colony formation. Conidia from the indicated strains were suspended in molten agar containing the indicated concentrations of FdU, plated as described in Materials and Methods and photographed.

Strain Tk213 was >1000-fold more sensitive to FdU than strains lacking TK. The level of sensitivity of TK$^+$ strains to FdU (Fig. 4) by the colony-forming assay was qualitatively but not quantitatively correlated with the level of expression of TK as determined by enzyme assay (Table 1).

Table 2. Concentrations of FdU, FIAU, TFT and BrdU that inhibit colony formation by wild-type and TK$^+$ *N.crassa* strains[a]

Strain	FdU	FIAU	TFT	BrdU	Gancyclovir
74A	40 μM[b]	60 μM[b]	No inhibition[c]	No inhibition[d]	No inhibition[e]
Tk213	0.005 μM	0.3 μM	0.01 mM	0.02 μM	0.4 mM[b]
Tk6103	0.05 μM	3 μM	1 mM	No inhibition[d]	No inhibition[e]
HyTk1	0.5 μM	60 μM	No inhibition[c]	No inhibition[d]	No inhibition[e]

[a]Colony formation was tested as described in Materials and Methods.
[b]Severe but not total inhibition.
[c]Tested up to 1 mM.
[d]Tested up to 10 mM.
[e]Colonies were smaller at 0.4 mM; tested up to 4 mM.

At high concentrations, FdU also inhibits the growth of wild-type *N.crassa* (Table 2; 36). Both temperature and nitrogen source affect the inhibition of growth of the wild-type by fluoropyrimidines (36). While changing temperature and nitrogen source also affected the relative growth rates of TK$^+$ strains in medium containing FdU, these changes did not substantially affect colony-forming ability in FdU medium (data not shown).

Neurospora crassa strains expressing *arg-2–tk* were also sensitive to other anticancer and antiviral drugs (Table 2). Growth of all TK$^+$ strains was more sensitive than the wild-type strain to FIAU, a nucleoside analog which initially showed promise in treating viral infections in the laboratory but which had unexpected, lethal effects in human clinical trials (9). The *arg-2–tk* strains were sensitive to TFT; strains expressing *Hy–Tk* were not. Whether this difference in TFT sensitivity reflected the different level of expression of TK or differences in substrate specificities between TK and hygromycin phosphotransferase–TK was not determined. Strain Tk213, which expressed the highest level of TK, was also sensitive to BrdU and gancyclovir (Table 2). The addition of 2 mM uridine to the medium in addition to nucleoside analogs abrogated the inhibitory effects of these analogs (data not shown), consistent with the idea that these analogs act through pyrimidine utilization pathways and not by other means.

Separation of newly replicated DNA containing BrdU by equilibrium centrifugation

Incorporation of BrdU into replicating DNA increases its density and, like incorporation of heavy isotopes, should allow resolution of unreplicated and replicated DNA by equilibrium centrifugation in CsCl gradients (37). Specifically, unreplicated parental DNA (low density) should resolve from DNA that undergoes one round of semiconservative replication and BrdU incorporation (intermediate density) and this DNA should resolve from DNA that undergoes two rounds of replication and BrdU incorporation (high density). In TK$^+$ strains, BrdU partially offsets the toxicity of FdU (which presumably inhibits production of endogenous thymidine nucleotides; 35) over short growth periods and addition of both together enabled efficient incorporation of BrdU into DNA (data not shown). Equilibrium centrifugation analyses of DNA prepared from TK$^+$ *N.crassa* strains Tk213 and Tk6103 grown in the presence of these compounds showed time-dependent increases in density consistent with incorporation of BrdU into newly replicating DNA (strain Tk6103, Fig. 5; strain Tk213, data not shown). As expected, no change in the density of DNA of wild-type *N.crassa* was observed when cells were grown under these conditions (data not shown).

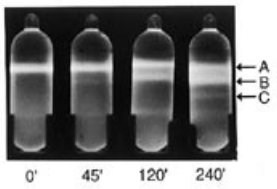

Figure 5. Incorporation of BrdU into replicating DNA in TK$^+$ *N.crassa*. A culture of strain Tk6103 was grown in BrdU/FdU-containing medium for the indicated periods and then DNA was isolated from the cells and examined by equilibrium centrifugation as described in Materials and Methods. Bands A, B and C correspond to DNAs of low, intermediate and high density respectively.

DISCUSSION

The expression of herpes virus TK in *Neurospora* confers novel phenotypes. Thymidine kinase expression is readily detectable in genetically modified strains containing *arg-2–tk* and *Hy–Tk* genes but not in wild-type *N.crassa* lacking either of these genes. Thus, like *Escherichia coli lacZ* (29,38–40) and *E.coli hph* (18), this gene is a useful reporter for quantitative measurements of gene expression in *Neurospora*. Furthermore, TK expression in *N.crassa* renders the organism sensitive to toxic nucleoside analogs that are activated by TK. Thus, expression of TK can be used for negative selection in this organism. Finally, expression of TK facilitates the labeling of newly replicated *N.crassa* DNA with radioactively labeled thymidine and BrdU.

Neurospora crassa expressing TK should prove useful for testing the effects of drugs that are toxic following activation through TK. There are few eukaryotic non-animal systems for testing the action of these drugs. *Neurospora crassa* is a convenient model system in which genetic and biochemical approaches can be combined for understanding the molecular bases of cellular phenomena (41). For example, mutations affecting enzymes such as uridine kinase and phosphoribosyl transferase affect the sensitivity of TK$^-$ *N.crassa* strains to high concentrations of fluorodeoxypyrimidines (42,43). The isolation and analyses of mutations in TK$^+$ cells that confer resistance to nucleoside analogs should yield insight into the pathways that metabolize these compounds.

The demonstration in TK$^+$ strains that *N.crassa* DNA replication appears to be synchronized for at least two cycles by

release from an HU block is consistent with *N.crassa* possessing an 'S/M checkpoint' that ensures that DNA replication is complete before the onset of mitosis, as do other organisms, including yeasts and mammals (44). Mutants in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* exist that disrupt this checkpoint, allowing the cell cycle to continue in HU, resulting in loss of viability. While several mutagen-sensitive mutants in *N.crassa* are known to be sensitive to HU (45), whether they are checkpoint-defective was not determined, presumably because of the technical difficulties of demonstrating synchronized DNA synthesis in TK^- strains. There is evidence, however, that DNA synthesis in wild-type *N.crassa* stops in response to DNA damage by ceasing initiation of new replicons, while two γ-ray-sensitive mutants, *uvs-6* and *mus-9*, show no replicon initiation checkpoint control (46,47). Understanding how these mutations and other mutations affect DNA metabolism in *N.crassa* should be facilitated by expression of TK in this organism.

ACKNOWLEDGEMENTS

This work was supported by US Public Health Service grants GM47498 (M.S.S.) and GM35690 (E.U.S.) from the National Institutes of Health, a grant from the Medical Research Foundation of Oregon (M.S.S.) and a Senior International Fellowship from the Fogarty International Center of the National Institutes of Health (E.U.S.). E.U.S. gratefully acknowledges the hospitality and comments of Adrian Bird (University of Edinburgh) for the development of labeling and synchronization procedures. We also thank Michael Freitag for helpful discussions.

REFERENCES

1 Grivell,A.R. and Jackson,J.F. (1968) *J. Gen. Microbiol.*, **54**, 307–317.
2 Kornberg,A. and Baker,T.A. (1992) *DNA Replication*, 2nd Edn. W.H.Freeman, New York, NY.
3 Arnér,E.S.J., Spasokoukotskaja,T. and Eriksson,S. (1992) *Biochem. Biophys. Res. Commun.*, **188**, 712–718.
4 Orbach,M.J., Sachs,M.S. and Yanofsky,C. (1990) *J. Biol. Chem.*, **265**, 10981–10987.
5 Luo,Z., Freitag,M. and Sachs,M.S. (1995) *Mol. Cell. Biol.*, **15**, 5235–5245.
6 Lupton,S.D., Brunton,L.L., Kalberg,V.A. and Overell,R.W. (1991) *Mol. Cell. Biol.*, **11**, 3374–3378.
7 Machover,D. (1991) *J. Surg. Oncol.*, **2** (suppl.), 42–50.
8 Bean,B. (1992) *Clin. Microbiol. Rev.*, **5**, 146–182.
9 McKenzie,R. *et al*. (1995) *N. Engl. J. Med.*, **333**, 1099–1105.
10 St Claire,M.H., Lambe,C.U. and Furman,P.A. (1987) *Anitmicrob. Agents Chemother.*, **31**, 844–849.
11 De Clercq,E. (1993) *Adv. Virus Res.*, **42**, 1–55.
12 Mansour,S.L., Thomas,K.R. and Capecchi,M.R. (1988) *Nature*, **336**, 348–352.
13 Allen,R.G., Carey,C., Parker,J.D., Mortrud,M.T., Mellon,S.H. and Low,M.J. (1995) *Mol. Endocrinol.*, **9**, 1005–1016.
14 Vald'es,J., Taylor,M.C., Cross,M.A., Ligtenberg,M.J., Rudenko,G. and Borst,P. (1996) *Nucleic Acids Res.*, **24**, 1809–1815.
15 Dilber,M.S., Abedi,M.R., Bjorkstrand,B., Christensson,B., Gahrton,G., Xanthopoulos,K.G. and Smith,C.I. (1996) *Blood*, **88**, 2192–2200.
16 Beck,C., Cayeux,S., Lupton,S.D., Dorken,B. and Blankenstein,T. (1995) *Hum. Gene Ther.*, **6**, 1525–1530.
17 Dien,B.S. and Srienc,F. (1991) *Biotechnol. Prog.*, **7**, 291–298.
18 Freitag,M., Dighde,N. and Sachs,M.S. (1996) *Genetics*, **142**, 117–127.
19 Ebbole,D. (1990) *Fungal Genet. Newsl.*, **37**, 15–16.
20 Vogel,H.J. (1956) *Microbiol. Genet. Bull.*, **13**, 42–43.
21 Selitrennikoff,C.P. and Sachs,M.S. (1991) *Fungal Genet. Newsl.*, **38**, 90–91.
22 Ebbole,D. and Sachs,M.S. (1990) *Fungal Genet. Newsl.*, **37**, 17–18.
23 Davis,R.H. and de Serres,F.J. (1970) *Methods Enzymol.*, **27A**, 79–143.
24 McNeil,J.B. and Friesen,J.D. (1981) *Mol. Gen. Genet.*, **184**, 386–393.
25 Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
26 Foss,H.M., Roberts,C.J., Claeys,K.M. and Selker,E.U. (1993) *Science*, **262**, 1737–1741.
27 Selker,E.U., Cambareri,E.B., Jensen,B.C. and Haack,K.R. (1987) *Cell*, **51**, 741–752.
28 Sambrook,J., Fritsch,E.F. and Maniatis,T.M. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29 Luo,Z. and Sachs,M.S. (1996) *J. Bacteriol.*, **178**, 2172–2177.
30 Davis,R.H. (1986) *Microbiol. Rev.*, **50**, 280–313.
31 Sachs,M.S. and Ebbole,D. (1990) *Fungal Genet. Newsl.*, **37**, 35–36.
32 Sachs,M.S. and Yanofsky,C. (1991) *Dev. Biol.*, **148**, 117–128.
33 Serna,L. and Stadler,D. (1978) *J. Bacteriol.*, **136**, 341–351.
34 Srivastava,V.K., Pall,M.L. and Schroeder,A.L. (1988) *Mutat. Res.*, **200**, 45–53.
35 Daher,G.C., Harris,B.E. and Diasio,R.B. (1990) *Pharmac. Ther.*, **48**, 189–222.
36 Buxton,F.P. and Radford,A. (1982) *Mol. Gen. Genet.*, **186**, 259–262.
37 Meselson,M. and Stahl,F.W. (1958) *Proc. Natl. Acad. Sci. USA*, **44**, 671–682.
38 Bell-Pedersen,D., Dunlap,J.C. and Loros,J.J. (1996) *Mol. Cell. Biol.*, **16**, 513–521.
39 Corrochano,L.M., Lauter,F.R., Ebbole,D.J. and Yanofsky,C. (1995) *Dev. Biol.*, **167**, 190–200.
40 White,B.T. and Yanofsky,C. (1993) *Dev. Biol.*, **160**, 254–264.
41 Perkins,D.D. (1992) *Genetics*, **130**, 687–701.
42 Buxton,F.P. and Radford,A. (1982) *Mol. Gen. Genet.*, **185**, 129–131.
43 Buxton,F.P. and Radford,A. (1982) *Mol. Gen. Genet.*, **185**, 132–135.
44 Elledge,S.J. (1996) *Science*, **274**, 1664–1672.
45 Schroeder,A.L. (1988) In Friedberg,E.C. and Hanawalt,P.C. (eds), *DNA Repair: A Laboratory Manual of Research Procedures*. Marcel Dekker, New York, NY, Vol. 3, pp. 77–98.
46 Koga,S.J. and Schroeder,A.L. (1987) *Mutat. Res.*, **183**, 139–148.
47 Schroeder,A.L., Inoue,H. and Sachs,M.S. (In press) In Hoekstra,M.F. and Nickoloff,J.A. (eds), *DNA Damage and Repair*. Humana Press, Totowa, New Jersey.