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Thymidine-Requiring Mutants of *Dictyostelium discoideum*

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Two thymidine auxotrophs of *Dictyostelium discoideum* were isolated which improve the efficiency of in vivo DNA-specific radiolabeling. Mutant HPS400 lacked detectable thymidylate synthetase activity, required 50 μg of thymidine per ml, and incorporated sixfold more [^{3}H]thymidine into nuclear DNA than did a wild-type strain. Either dTMP or exogenously provided DNA also permitted growth of this strain. The second mutant, HPS401, was isolated from HPS400 and also lacked thymidylate synthetase activity, but required only 4 μg of thymidine per ml for normal growth and incorporated 55 times more thymidine label than did a control strain. Incorporation of the thymidine analog 5′-bromodeoxyuridine was also markedly increased in the mutants. Catalytic properties of the thymidylate synthetase of *D. discoideum* investigated in cell extracts were consistent with those observed for this enzyme in other organisms. These strains should facilitate studies of DNA replication and repair in *D. discoideum* which require short-term labeling, DNA of high specific activity, or elevated levels of substitution in DNA by thymidine analogs.

The cellular slime mold *Dictyostelium discoideum* has served as an experimental model for development and differentiation and for the repair of DNA damage in eucaryotic cells. Although genetic studies of DNA repair in this organism have been useful (31, 32), many complementary molecular studies have been hampered by the lack of a procedure for efficient DNA-specific radiolabeling. Previous methods for radiolabeling the DNA of *D. discoideum* have relied either on the direct provision of DNA precursors in the growth medium (14) or on supplying bacteria which contain radiolabeled DNA (8). In the latter procedure, the degradation products of DNA derived from ingested bacteria are utilized by *D. discoideum* cells for the synthesis of DNA. Direct labeling is inefficient due to limited uptake or to competition between pools of exogenously provided and de novo-synthesized DNA precursors. Although the incorporation of label by means of an isotopically labeled bacterial food source achieves a higher specific activity of DNA, this circuitous method is cumbersome and unsuitable for short-term labeling studies.

The isolation of mutants which increase the efficiency of DNA precursor incorporation has been an important element in the development of isotopic labeling procedures for a number of commonly studied organisms, including *Escherichia coli* (21) and *Saccharomyces cerevisiae* (5). Similar mutants in *D. discoideum* would facilitate molecular studies of DNA replication and repair. Unlike some other simple eucaryotes (11), *D. discoideum* possesses a thymidine kinase (19). This allowed us to consider the isolation of thymidine-requiring mutants suitable for DNA radiolabeling. Here we report the isolation and characteristics of two thymidine auxotrophic strains of *D. discoideum* which permit convenient and specific radiolabeling of DNA in vivo.

MATERIALS AND METHODS

Strains, media, and cell growth. The origin of haploid strain HPS83 (*cyc*A1, *axe*A1, *tsg*A1, *axe*B1, *acr*D369, *ebr*B364, *spr*H351, *man*A1), used to derive the auxotrophic mutants, has been described previously (31). Cells were grown in complex medium HL5 modified to contain, per liter, 14.3 g of Difco Proteose Peptone (Difco Laboratories), 7.15 g of yeast extract (Difco), 15.4 g of glucose, 0.507 g of Na_2HPO_4, 0.486 g of KH_2PO_4, 0.1 g of streptomycin, and 0.2 MU of penicillin, adjusted to a final pH of 6.7, or in the defined medium of Franke and Kessin (9), modified to contain twice the normal concentration of vitamins and minerals. Cultures of 5 to 50 ml per 250-ml flask were incubated at 23°C with swirling as described previously (14). Cell number was determined with a Coulter counter.

The concentration of compounds used in nutritional studies (all obtained from Sigma Chemical Co.) was determined spectrophotometrically. Calf thymus DNA was purified by phenol-chloroform extraction, precipitated in ethanol, and suspended in defined medium before use. The average molecular weight of this DNA, determined electrophoretically, was $>10^8$.

Isolation of HPS400. Cell suspensions (10^7/ml) shaken in phosphate-buffered saline (22) were treated for 45 min with 115 μg of nitrosoguanidine per ml, washed extensively, and then suspended in HL5 (2×10^6/ml) supplemented with 100 μg of thymidine per ml. Cell viability was reduced to 35% after this treatment. Cultures were incubated for 40 h, during which time the cell density increased twofold, to allow for recovery of growth and expression of induced mutations. These cells were washed free of thymidine and then held for 24 h in HL5 containing 10 μg of netropsin (a gift from Lederle Laboratories) per ml. This treatment reduced the viability of cells surviving the nitrosoguanidine treatment to 13%. These cultures were diluted appropriately and spread in association with *E. coli* B/r (22) to produce approximately 10 to 40 colonies per plate (100 by 15 mm). Cells from individual colonies were transferred into 0.2 ml of HL5 contained in wells of 96-well, multitest plates (Flow Laboratories). One-half (0.1 ml) of the cell suspension from each well was transferred into wells of corresponding plates which contained enough thymidine to bring the final solution concentration to 100 μg/ml. Plates were incubated at 23°C in a humidified chamber for up to 4 weeks. Growth in wells with and without added thymidine was compared, and colonies judged to have reached higher cell densities in the presence of thymidine were rescreened for thymidine-en-

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hanced growth before final determination of growth kinetics in the presence or absence of 100 μg of thymidine per ml.

Analysis of thymidine and bromodeoxyuridine (BUdR) incorporation. Incorporation of thymidine into acid-insoluble cellular material was measured by sampling aliquots of culture at intervals during incubation in HL5 containing 10 μCi of [^{3}H]thymidine (ICN Pharmaceuticals) per ml, followed by collection of cells on cellulose-nitrate filters. The cells were washed on the filters and then treated with 10% ice-cold trichloroacetic acid. After 20-min exposure to trichloroacetic acid, acid-insoluble material was collected on filters, washed extensively, and then dried and prepared for scintillation counting. In this and all other thymidine incorporation studies involving HPS400, the medium was supplemented with 20 μg of thymidine per ml to allow some cell growth.

The procedures used for preparing cell lysates and CsCl density gradients used in the analysis of thymidine incorporation into nuclear and mitochondrial DNA were essentially those of Clark and Deering (8). The CsCl solutions, prepared with an average density of 1.620 g/cm^3, always contained netropsin (50 μg added to lysates prepared from 1×10^7 to 2×10^7 cells) to increase the density difference between nuclear and mitochrondrial DNA (14).

Fractions containing nuclear or mitochondrial DNA were pooled separately from CsCl gradients, omitting one or two fractions that divided these DNA species to avoid cross-contamination. The DNA concentration of pooled fractions was determined after dialysis against 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid)-10 mM NaCl (pH 7.0) by using a sensitive assay based on the enhanced fluorescence of DNA-bound Hoechst 33258 dye (Calbiochem) (7). *D. discoideum* DNA was used as the calibration standard. Additional aliquots of the dialyzed DNA samples were precipitated with trichloroacetic acid and prepared for counting (14).

For the analysis of BUdR incorporation, cells were incubated for 3 h in HL5 containing [^{3}H]thymidine (10 μCi/ml) and various amounts of BUdR. The medium used for HPS400 was also supplemented with sufficient thymidine to produce a total nucleoside concentration of 206 μM (equivalent to 50 μg of thymidine per ml) to provide for the nutritional requirement of this auxotrophic strain. Lysates and CsCl gradients used in BUdR density shift experiments were prepared in a manner similar to those described for the thymidine incorporation studies, with the exceptions that the average gradient density was 1.630 g/cm^3 and gradients included a normal density marker of ^{14}C-labeled DNA extracted from cultures not exposed to BUdR. Gradient profiles were analyzed after correction for overlap, using standards containing only ^{3}H or ^{14}C. In agreement with work demonstrating no difference in the binding of netropsin to A-T or A-BUdR base pairs (30), the number of fractions separating peaks of BUdR-substituted and normal DNA was found to be identical in gradients prepared with or without netropsin. Because addition of this antibiotic enhanced nuclear and mitochondrial DNA density differences without interfering with the DNA density shift analysis, netropsin was included in all CsCl gradients. Measurement of the CsCl density gradient revealed a density difference of 0.0025 g/cm^3 between fractions over the bulk of the gradient. This value and published values for the thymine content of the *D. discoideum* genome (15) were used in calculating the extent of BUdR substitution for thymidine by applying the equation of Luk and Bick (17). In cases in which the density shift was great enough to merge peaks of nuclear and mitochondrial DNA, the density obtained from the position of the broad hybrid peak was used in the calculation.

Determination of thymidylate synthetase activity. The thymidylate synthetase activity of cell extracts was determined by the method of Ayusawa and co-workers (1) with minor modifications. Tetrahydrofolate (THF) was synthesized in this laboratory by dimethylamine-borane reduction of folic acid (Sigma) (18). Solutions of 150 μM 5,10-methylene-THF (5,10-CH_2THF) were prepared shortly before use by adding THF to a deoxygenated solution of 50 mM Tris, 170 mM NaF, 25 mM 2-mercaptoethanol, and 0.15% formaldehyde (pH 7.5). The active cofactor 5,10-CH_2THF is formed rapidly and quantitatively in this solution by formaldehyde oxidation of THF (3). The specific activity of the [^{3}H]dUMP (ICN) solution, purified by passage over a cellulose-Norit A (Fisher Chemical Co.) column (12), was 6×10^3 cpm/nmol. Samples containing at least 10^8 cells were harvested from HL5 during log-phase growth (4×10^6 to 8×10^6/ml), washed in Tris (10 mM; pH 7.5)-sucrose (250 mM) buffer, and then suspended on ice with 2 to 3 volumes of Tris-sucrose buffer containing 250 ng of leupeptin (Sigma) and 50 ng of pepstatin (Sigma) per ml. After lysis by sonication, clarified cell extracts were prepared for assay by centrifugation (100,000 × *g*, 30 min, 4°C) to remove subcellular debris. Assays, always performed within 2 h of lysis, were initiated by adding 0 to 100 μl of extract to tubes containing 40 μl of 1.25% bovine serum albumin, 20 μl of 5,10-CH_2THF (150 μM), 18 μl of [^{3}H]dUMP (900 μM), and sufficient Tris-sucrose buffer to yield a final reaction volume of 178 μl. Reactions, which normally proceeded for 30 min at 23°C, were terminated by addition of a suspension (0.5 ml) of 12% Norit A in 0.1 N HCl. After 10 min of incubation, Norit A was removed by centrifugation, and an aliquot of the supernatant containing ^{3}H liberated from the 5′ position of dUMP during synthesis of dTMP was prepared for scintillation counting. Experimental values were corrected by subtracting blank values from assay tubes incubated without enzyme. Protein concentrations were determined with a commercially available assay kit (Bio-Rad Laboratories), with ovalbumin as the protein standard.

RESULTS

Isolation of thymidine-requiring mutants. The rationale for the isolation of thymidine-requiring mutants of *D. discoideum* was to enrich by applying negative selection against cells capable of growth in thymidine-poor medium and then to screen colonies individually for auxotrophy. Based on the ability to support the growth of the thymine- or thymidine-requiring *E. coli* strain 15T$^-$, the HL5 growth medium used in these experiments was estimated to contain a maximum of 6 μg of thymidine per ml (W. Ford, personal communication). Cells requiring thymidine in excess of this amount were not expected to grow or be affected by agents reducing the viability of growing cells only.

The antibiotic netropsin has been reported to selectively inactivate replicating eucaryotic cells (34). Experiments with axenically grown cultures of *D. discoideum* indicated that treatment with netropsin was effective in reducing the viability of growing cells as much as 1,000-fold below that of cells reversibly blocked in growth by exposure to 5′-fluorodeoxyuridine (C. A. Michrina and R. A. Deering, unpublished data). A protocol making use of such netropsin-mediated, preferential killing of replicating cells was devised for the isolation of thymidine-requiring mutants. The genetically marked strain HPS83 was chosen as a parent because the potential genetic analysis of any derived mutants would

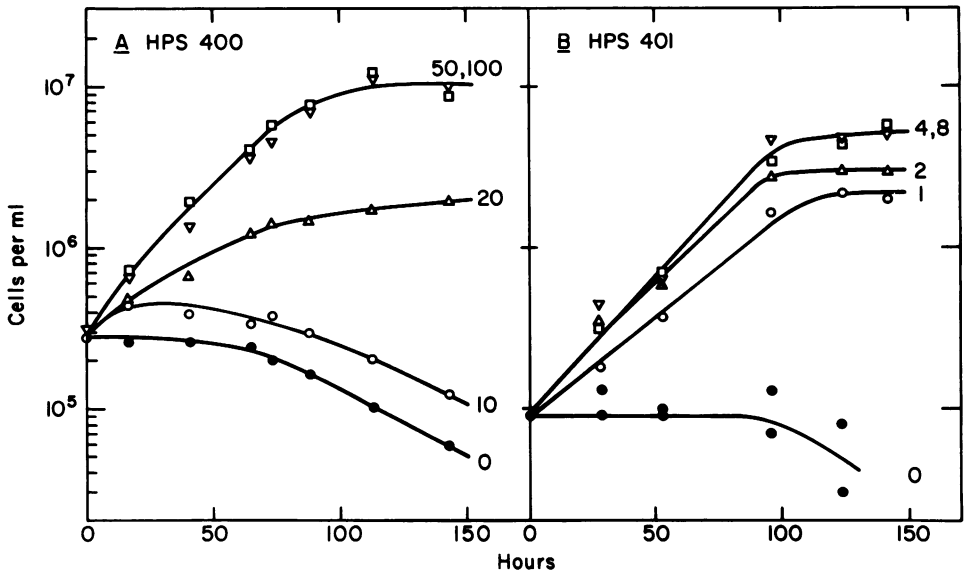

FIG. 1. Effect of thymidine concentration (micrograms per milliliter, as indicated) on growth of thymidine-requiring mutants in defined medium. (A) HPS400: ●, 0 μg/ml; ○, 10 μg/ml; △, 20 μg/ml; □, 50 μg/ml; ▽, 100 μg/ml. (B) HPS401: ●, 0 μg/ml; ○, 1 μg/ml; △, 2 μg/ml; □, 4 μg/ml; ▽, 8 μg/ml. The non-thymidine-requiring parental strain HPS83 grown in defined medium with 0 or 100 μg of thymidine per ml gave growth curves indistinguishable from HPS400 grown with 50 μg of thymidine per ml.

be simplified by using a strain carrying mutations mapped to each linkage group. Cells were treated with nitrosoguanidine and allowed to recover in thymidine-supplemented HL5 to express induced mutations and to allow resumption of growth before exposure to netropsin. These cultures were washed and transferred to medium without added thymidine to inhibit growth of auxotrophs before netropsin treatment. Cells were exposed to the drug and then grown clonally for later scoring for thymidine auxotrophy. A single thymidine-requiring mutant, designated HPS400, was isolated from 8,300 colonies screened in this manner. The mutation conferring thymidine auxotrophy was designated *tmp*A600.

When an alternate procedure for obtaining thymidine auxotrophs was followed in which netropsin treatment of the mutagenized culture was eliminated, no thymidine-requiring strains were recovered, even after screening more than 11,000 colonies.

In initial experiments designed to determine the spontaneous frequency of reversion of HPS400 to prototrophy, a single clone capable of growth in unsupplemented HL5 was isolated from 1.7×10^8 cells. This strain, HPS401, was initially thought to be a revertant to thymidine independence, but later experiments (to be described below) showed that it remained auxotrophic for thymidine and carried an additional mutation, termed *tdr*A600, which lowered the level of thymidine required for growth. Spontaneously arising prototrophic revertants of HPS400 were never observed in these experiments, indicating a reversion frequency of $<7 \times 10^{-9}$. Later attempts, using nitrosoguanidine mutagenesis, to derive additional mutants from HPS400 with low thymidine requirements proved unsuccessful, although prototrophic revertants could be isolated with this treatment at frequencies of about 10^{-5}.

Nutritional characteristics of HPS400 and HPS401. Both HPS400 and HPS401 demonstrated an absolute requirement for thymidine (or thymidine-containing precursors; see below), although the levels necessary to support growth in these strains differed widely (Fig. 1). Thymidine concentrations ranging from 40 to 100 μg/ml (165 to 400 μM) were optimal for growth of HPS400, allowing a 16-h doubling time and a maximum culture density of approximately 10^7 cells per ml. As little as 4 μg of thymidine per ml was sufficient to provide for maximal growth of HPS401, although the final cell densities observed in this strain grown in defined medium were consistently lower than for HPS400 or the parental strain HPS83. For HPS400, supplementation of the medium with 10 μg of thymidine per ml or less did not permit cell division and in fact led to cell loss, whereas concentrations of 500 and 1,000 μg/ml allowed somewhat less growth than did 40 to 100 μg/ml. The doubling times observed in the parental strain HPS83 and the two derived auxotrophs were consistently 4 to 6 h longer than in other strains carrying fewer mutations.

Incubation of HPS400 in growth medium lacking adequate thymidine led to cell death. Small but measurable decreases in viability were observed after 6 to 10 h in unsupplemented defined medium, whereas times of 24 and 48 h yielded 30 and 10% viability, respectively. This phenomenon, commonly referred to as thymidineless death, is widely observed in other thymidine or dTMP auxotrophs (2). It is likely to have reduced the effectiveness of the initial netropsin-induced enrichment step used in the mutant isolation protocol by causing cell lethality among thymidine auxotrophs.

Endogenous dTMP is provided for DNA synthesis by the enzyme thymidylate synthetase in a reaction which transfers the methylene group of 5,10-CH_2THF to dUMP, producing dTMP and dihydrofolate (3). Therefore, mutations affecting either thymidylate synthetase or enzymes in pathways leading to 5,10-CH_2THF production will produce thymidine auxotrophy. The nutritional consequences of blocks in these two areas will differ; inactivation of thymidylate synthetase produces a simple requirement for thymidine or dTMP, whereas blockage of enzymes involved in the folic acid cycle leads to complex requirements for amino acids and purines as well as thymidine or dTMP because of the requirement for folates in the synthesis of these compounds (16).

To better understand the nature of the metabolic defect in HPS400, a series of compounds other than thymidine were

TABLE 1. Growth supplementation of HPS400 by DNA or DNA precursors

Compound	Growth supplementation	Concn (μm)[a]
Deoxythymidine	+	200
dTMP	+	310
DNA[b]	+	310[b]
Thymine	−	2,000
UdR	−	1,700
dUMP	−	1,500

[a] Minimum concentration required to permit growth to the maximum cell density or maximum concentration tested in the case of substances incapable of supporting growth.

[b] Calf thymus DNA concentration expressed as the concentration of dTMP available from the DNA.

tested for the capacity to satisfy the nutritional requirements of this strain (Table 1). Neither thymine, deoxyuridine (UdR), nor dUMP supplementation allowed growth, whereas dTMP or exogenous calf thymus DNA did support growth. The defined medium used for growth of *D. discoideum* already contains histidine and methionine, amino acids dependent on reduced folates for synthesis. Addition of the purines guanine and adenine (each at 50 μg/ml) and serine (300 μg/ml), other compounds requiring folates for synthesis, did not improve growth (data not shown).

The inability of dUMP and UdR to provide for the growth of HPS400, and the lack of a purine or serine requirement, is consistent with a role for the *tmp*A600 mutation in reducing or eliminating thymidylate synthetase activity.

Thymidylate synthetase activity. Because the nutritional experiments suggested that the *tmp*A600 mutation might be affecting thymidylate synthetase, this enyzmatic activity was assayed in extracts of the parental and mutant strains. Some initial characterization of the optimum assay condition for the parental activity was necessary, since prior studies of *D. discoideum* thymidylate synthetase have not been reported.

Under our standard conditions, the parental enzyme gave linear reactions with time and amount of protein for times up to 30 min and for amounts of protein as high as 3.5 mg per reaction (Fig. 2, HPS83). A concentration of 20 μM 5,10-CH_2THF was found to be sufficient for maximal enzyme activity. The specific activity of extracts ranged from 17 to 22 pmol of ^3H released/min per mg of protein. This thymidylate synthetase activity was highly unstable, decaying exponentially with a half-life of approximately 2 h at 0 to 4°C, even with the inclusion of the protease inhibitors leupeptin and pepstatin before cell lysis. The activity decayed three- to fivefold more rapidly in the absence of these inhibitors.

Characteristics of the parental wild-type thymidylate synthetase activity and the assay system are shown in Table 2. The activity was destroyed by boiling. Omitting THF from the assay mixture did not completely eliminate activity, but did cause a reduction to 10 to 20% of control levels, suggesting that the cell lysates contained or were capable of generating significant amounts of 5,10-CH_2THF. The enzyme activity was not stimulated by addition of Mg^{2+} and was weakly inhibited by dTMP, the end product of the reaction. Kinetic measurements made in the cell lysates indicated an apparent K_m for 5,10-CH_2THF of 2 to 3 μM.

Thymidylate synthetase activity was not detected in either of the strains carrying *tmp*A600 (Fig. 2, HPS400 and HPS401), even after reactions for up to 60 min with 4 mg of protein. The absence of activity in HPS400 was not due to the presence of an inhibitor, since mixtures of parental and mutant cell extracts yielded the activity anticipated for the parental strain alone (Table 2). Thymidylate synthetase activity of HPS83 cultures grown with or without added thymidine was identical. The possibility that the apparent lack of activity was due to an enzyme with altered optimum reaction requirements was tested by varying the concentration of 5,10-CH_2THF used in the assay from 0 to 160 μM and assaying with dUMP concentrations of 20 and 90 μM. Thymidylate synthetase activities were never observed for HPS400 or HPS401 under any of our assay conditions.

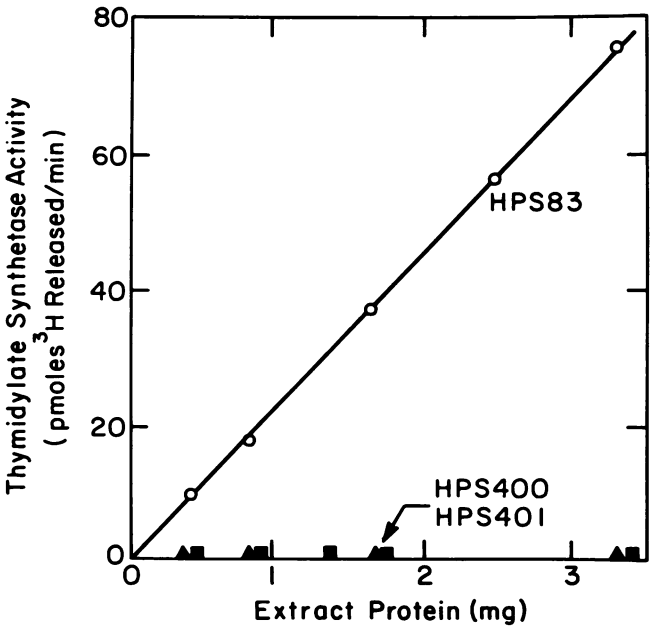

FIG. 2. Thymidylate synthetase activity for strains HPS83 (○), HPS400 (■), and HPS401 (▲) as a function of cell extract protein added to the assay system.

Incorporation of [^3H]thymidine by auxotrophic mutants. Initial experiments on the incorporation of [^3H]thymidine into acid-insoluble cellular material revealed increased thymidine incorporation for both auxotrophic mutants, with HPS400 and HPS401 incorporating an average of 3- and 11-fold more total label, respectively, than did HPS83. Alkaline hydrolysis of lysates before acid precipitation did not decrease the recovered counts, indicating that exoge-

TABLE 2. Factors affecting assay of *D. discoideum* thymidylate synthetase activity

Condition	Relative activity
Complete system	
With fresh extract	1.0
With boiled extract	0.0
With fresh extract plus equal amt of HPS400 extract protein	1.0
With thymidylate at	
0.01 mM	0.96
0.25 mM	0.73
1.00 mM	0.47
With 10 mM Mg^{2+}	0.75
With fresh extract HPS83 grown in presence of 50 μg of thymidine per ml	1.0
Minus THF	0.14

nously provided thymidine was not being incorporated into RNA. These results must be interpreted cautiously with regard to DNA-specific labeling, since some trichloroacetic acid-precipitable label may be present in material other than nucleic acids, as observed in *Neurospora crassa* (33) and *Chlamydomonas reinharti* (29), or label may be taken up selectively by either mitochondrial or nuclear DNA. Because there have been indications of such problems during the labeling of *D. discoideum* with thymidine (14), it was important to study incorporation specifically into DNA.

In studies utilizing HPS400, 20 μg of thymidine per ml was added to the HL5 medium to permit cell growth. Experiments comparing the specific activity of nuclear DNA obtained from cells incubated with 10 μCi of [^{3}H]thymidine and 0, 20, or 50 μg of cold thymidine per ml indicated that label incorporation in HPS400 was most efficient when the intermediate level of thymidine supplementation was used. This is due to opposing effects of label dilution at high thymidine concentrations and the requirement of HPS400 for sufficient thymidine to support growth.

Typical CsCl density gradient profiles of thymidine-labeled *D. discoideum* DNA obtained from the parental auxotrophic strains are shown in Fig. 3. An unusual feature of the *D. discoideum* genome is the relatively high abundance of mitochondrial DNA (15). This mitochondrial DNA, comprising ~30% of the genome, can be separated from the bulk of nuclear DNA in CsCl gradients because of its slightly greater buoyant density, and it accounts for the bimodal distribution of label seen in the gradient profiles. The results of this experiment reveal two aspects of thymidine incorporation by the mutants. In previously available axenic strains, label incorporation was skewed heavily in favor of the mitochondrial DNA (14). This is seen in the gradient profile of HPS83 where the bulk of the counts were observed in the mitochondrial DNA, even though ~70% of the total DNA is of nuclear origin. The preferential mitochondrial incorporation is especially apparent during labeling periods of shorter than one generation time and seriously limits experiments intended to study nuclear DNA synthesis or repair. A comparison of label distribution between strains reveals that the preferential uptake of [^{3}H]thymidine into mitochondrial DNA is largely eliminated in HPS400 and HPS401. The increased incorporation of thymidine observed in HPS400 is primarily due to enhanced nuclear incorporation alone, whereas significantly more label is incorporated into both forms of HPS401 DNA.

The specific activity of nuclear and mitochondrial DNA from cells labeled with [^{3}H]thymidine for up to 24 h was determined by separate collection of the mitochondrial and nuclear DNA from gradients of the type shown in Fig. 3, followed by independent quantitation of the amounts of DNA and radioactivity. The results of this study (Fig. 4) demonstrated large interstrain differences in DNA specific activity. Relative to the prototroph HPS83, HPS400 and HPS401 incorporate 6 and 55 times more thymidine, respectively, into nuclear DNA during 6 h of labeling. Differences between strains in the specific activity of mitochondrial DNA were less pronounced. Equal amounts of label were incorporated into the mitochondrial DNA of HPS83 and HPS400, whereas HPS401 mitochondrial DNA contained approximately eightfold more radioactivity for all labeling periods tested.

Incorporation of BUdR. The incorporation of thymidine analogs into DNA has been an important part of many studies of DNA repair synthesis (10) and has served as a selective agent for mutant isolation (13). A study of the incorporation of BUdR, a commonly used analog, showed an enhancement of uptake by the auxotrophic mutants similar to that observed for thymidine.

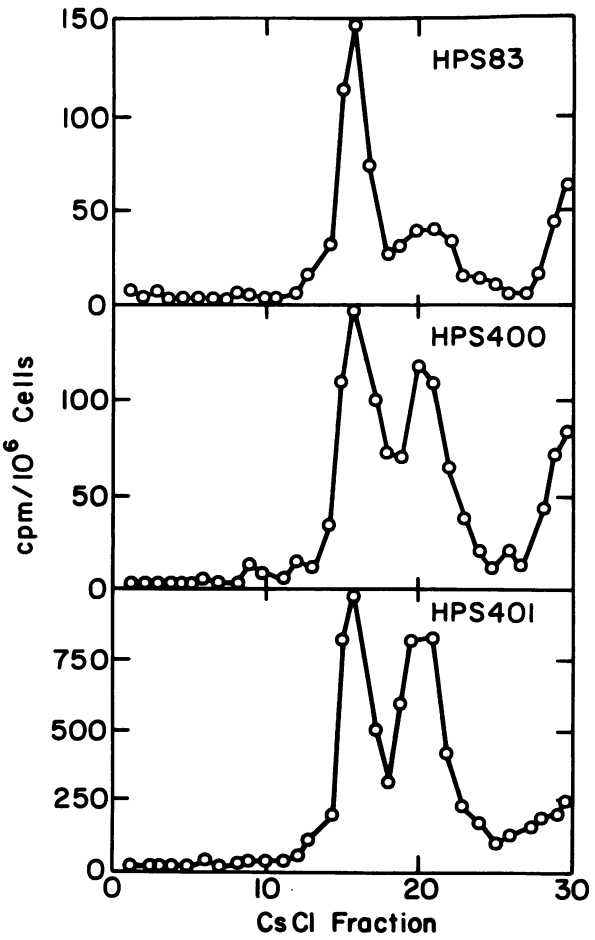

FIG. 3. CsCl density gradient profiles for HPS83, HPS400, and HPS401 labeled for 6 h in HL5 with 10 μCi of [^{3}H]thymidine per ml. The medium for HPS400 was supplemented with 20 μg of unlabeled thymidine per ml. The density of the CsCl solution increases from right to left. The mitochondrial DNA peak is to the left (higher density) and the nuclear DNA peak is to the right (lower density). Note the change in the cpm scale in the panel representing HPS401 DNA. Labeling levels were normalized to uptake by 10^6 cells. Netropsin was added to cell lysates to enhance resolution of nuclear and mitochondrial DNA.

Growth inhibition by BUdR was more pronounced in the thymidine-requiring strains, suggesting more efficient incorporation of this analog. The concentrations of BUdR required to inhibit growth to 50% of control values were 75, 12, and 3 μg/ml for HPS83, HPS400, and HPS401, respectively.

The uptake of BUdR into DNA was followed by measuring the increase in buoyant density which results from incorporation of this analog. Typical CsCl density gradient profiles of DNA from cells grown in the presence of 12 μg of BUdR per ml are shown in Fig. 5. The density of DNA from parental strain HPS83 was not detectably altered, whereas a shift of nuclear and mitochondrial components from their normal positions was clearly seen in HPS400 DNA. The BUdR-induced density shift was most pronounced in HPS401 and resulted in complete separation of BUdR-substituted and normal DNA. Experiments comparing density shifts of whole-cell DNA and DNA from isolated nuclei of the

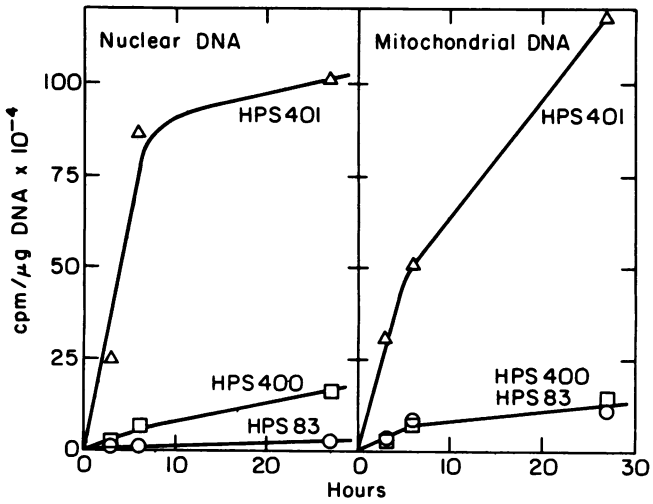

FIG. 4. Specific activity of nuclear and mitochondrial DNA of strains HPS83 (○), HPS400 (□), and HPS401 (△). Cells were grown in HL5 containing 10 μCi of [^{3}H]thymidine per ml for the times indicated. The growth medium for HPS400 was also supplemented with 20 μg of unlabeled thymidine per ml.

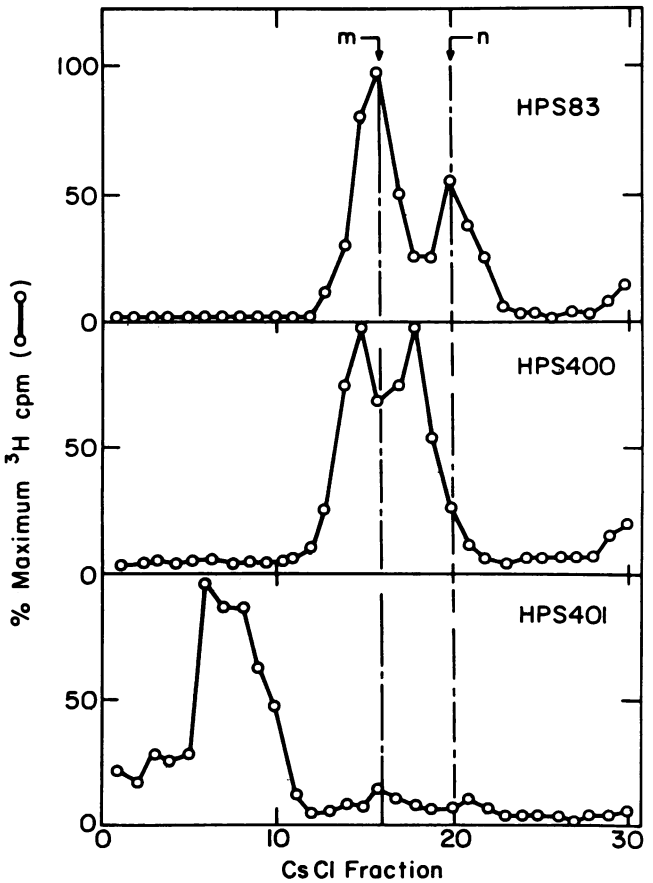

FIG. 5. CsCl density gradient profiles of HPS83, HPS400, and HPS401 DNA from cells incubated for 3 h in HL5 containing 12 μg of BUdR per ml. The density of the CsCl increases from right to left. The positions of normal nuclear (n) and mitochondrial (m) marker DNAs are indicated by the broken vertical lines.

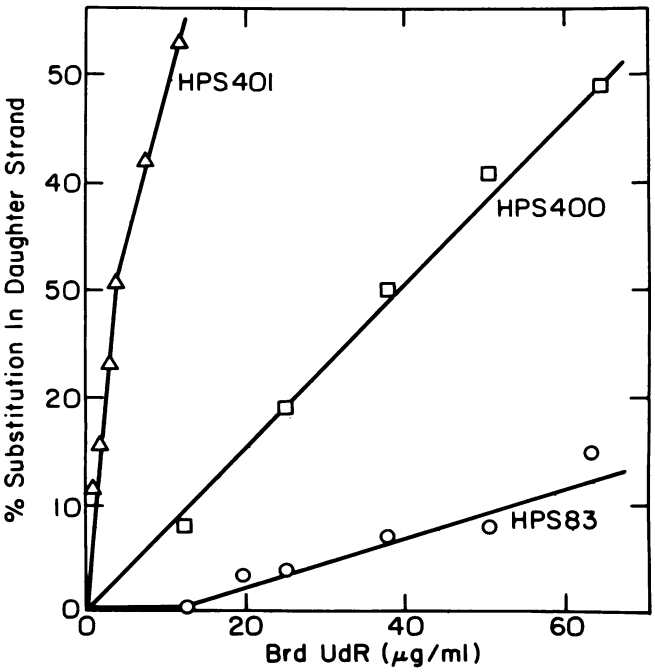

FIG. 6. Percent BUdR substitution for thymidine in the newly synthesized strand of nuclear DNA from strains HPS83 (○), HPS400 (□), and HPS401 (△) incubated for 3 h in HL5 containing BUdR.

auxotrophic mutants indicated that nuclear and mitochondrial components banded together whenever the peaks were shifted more than four to six fractions (0.010 to 0.015 g/cm^3), indicating some preferential uptake into the normally less dense nuclear DNA. This is most evident in the gradient profile for HPS401 where only a broad single peak of BUdR-containing DNA is visible. The density shifts evident in these experiments are due only to substitution in a single strand because the 3-h period used for labeling comprises <25% of the normal generation time.

The extent of BUdR substitution for thymidine in newly synthesized daughter strands of nuclear DNA was determined from DNA density shift data for cells grown in medium supplemented with various concentrations of BUdR (Fig. 6). These results indicate markedly enhanced incorporation of BUdR by both auxotrophic strains.

DISCUSSION

The thymidine auxotrophs HPS400 and HPS401 significantly increase the efficiency of DNA-specific radiolabeling in *D. discoideum*. For example, after 6 h of labeling with [^{3}H]thymidine, the specific activity of nuclear DNA of HPS400 was sixfold greater than that of the parental control strain, whereas nuclear DNA of HPS401 contained 55 times the control activity.

The biased incorporation of thymidine label into mitochondrial DNA, which has hindered studies of nuclear DNA replication and repair with this organism (14), is not apparent in either HPS400 or HPS401. The large proportion of label incorporated into the mitochondrial DNA of wild-type cells suggests the existence of a small mitochondrial precursor pool contributed to only by enzymes localized elsewhere in the cell. Exogenously provided DNA precursors may compete more effectively with this limited mitochondrial pool than with the nuclear precursor pool which may be provided by de novo synthesis at or near the site of nuclear replication. The mutational inactivation of thymidylate synthetase

would eliminate competition between the salvage and de novo pathways, leading to more equal specific activities of nuclear and mitochondrial DNA. This explanation is consistent with observations in mammalian cells that thymidylate synthetase and other enzymes involved in DNA replication are primarily located within the nucleus (25) and that differences exist in the size of cytoplasmic and nuclear DNA precursor pools (28).

Nutritional studies demonstrated that dTMP and DNA, compounds transported poorly if at all into most cells, were nearly as effective as thymidine in supporting the growth of HPS400. Although these results might be interpreted by postulating the occurrence of efficient degradation of exogenous DNA by secreted nucleases, coupled with conversion of dTMP to thymidine by a nucleotide phosphatase before entry into the cell, we favor the interpretation that effective growth supplementation by dTMP and DNA is possible because transport into *D. discoideum* proceeds through nonspecific endocytosis of the medium (20). Therefore, these compounds, which are excluded from entry into most cells, may be readily accessible to cells of *D. discoideum*.

The inability of thymine to allow growth of HPS400 is consistent with results demonstrating that this compound cannot be used to label *D. discoideum* DNA (Deering, unpublished data). Although enzymes of de novo synthesis are ubiquitous, those of the salvage pathways show wide variation in distribution and in the types of reactions catalyzed (16). The enzyme thymidine phosphorylase, which reversibly catalyzes the conversion of thymine to thymidine (27), is present in most organisms capable of utilizing thymine. *D. discoideum* may be low in or lack this activity.

The inability of UdR or dUMP to support growth of HPS400, and the lack of an adenine, guanine, or serine requirement, suggested that the *tmp*A600 mutation of HPS400 affected thymidylate synthetase activity. A direct assay of extracts of HPS400 and HPS401 failed to detect this activity in either mutant. It is not known whether the TMPA locus encodes the structural gene for this enzyme or whether this gene secondarily affects the synthesis or activity of thymidylate synthetase.

The *tmp*A600 mutation is unusually stable, reverting at a frequency of $<7 \times 10^{-9}$. Only the *bsg*A mutation associated with an inability to utilize the bacterium *Bacillus subtilis* as a food source has been reported to revert with a similarly low frequency of 4.5×10^{-9} (24). Although spontaneous revertants were not observed, the frequency of prototrophs could be increased in a dose-dependent manner by treatment with nitrosoguanidine. The low spontaneous reversion frequency, coupled with the ability to induce prototrophic revertants with mutagens, may make the *tmp*A600 marker useful for quantitative mutation studies.

The metabolic alteration associated with the *tdr*A600 mutation in HPS401 is unknown. This mutation lowers the amount of thymidine required for growth to 4 μg/ml. In bacteria, the labeling efficiency of thymidylate synthetase mutants is enhanced by secondary mutations which eliminate activities responsible for thymidine degradation (21). The effect of *tdr*A600 may be to inactivate or reduce analogous enzymatic activities in *D. discoideum*, thereby increasing the availability of thymidine within the cell.

Partial characterization of thymidylate synthetase activity in cell extracts allows comparison of the *D. discoideum* enzyme with thymidylate synthetases of other organisms. The average specific activity of 20 pmol of ^{3}H/min per mg of protein in HPS83 extracts is similar to values of 25 to 60 pmol of ^{3}H/min per mg reported for *S. cerevisiae* (3), but is 10- to 20-fold less than that of *E. coli* (26). The estimated K_m for 5,10-CH_2THF of 3 to 4 μM is 2- to 10-fold below that observed in bacteria, yeast, or mammalian systems (3, 23). Inhibition of thymidylate synthetase by dTMP occurs with thymidylate synthetase isolated from a variety of procaryotic and eucaryotic sources (6), as well as from *D. discoideum*. The *D. discoideum* thymidylate synthetase is similar to enzymes of higher eucaryotes in not being stimulated by Mg^{2+} (23).

The residual activity observed in assays which omitted 5,10-CH_2THF suggests that *D. discoideum* contains, or is capable of generating, appreciable quantities of this compound. Estimates based on the amount of 5,10-CH_2THF necessary to provide for the observed levels of activity indicate an intracellular concentration of this compound of 13 μM. This closely matches values reported for *S. cerevisiae* (3).

A serious problem encountered in this initial study of *D. discoideum* thymidylate synthetase was the lability of enzyme activity in cell extracts. Effective means of improving enzyme stability must be developed before more refined work can be initiated.

The thymidine auxotrophs HSP400 and HPS401 should facilitate experiments with *D. discoideum* which require DNA-specific radiolabeling or incorporation of thymidine analogs into DNA. These include studies of DNA replication and repair involving pulse-labeling or BUdR-induced DNA density shifts. These strains may also find use in "suicide" techniques for the enrichment of a population for auxotrophs or other classes of conditional growth mutants. Construction of repair-deficient thymidine auxotrophs, useful for the molecular characterization of DNA repair in this organism, should now be possible, using conventional techniques of *D. discoideum* genetics.

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LITERATURE CITED

1. **Ayusawa, D., H. Koyama, K. Iwata, and T. Seno.** 1981. Selection of mammalian thymidine auxotrophic mutants defective in thymidylate synthetase by their reduced sensitivity to methotrexate. Somat. Cell Genet. **7**:523–534.
2. **Barclay, B. J., and B. A. Kunz, J. G. Little, and R. H. Haynes.** 1982. Genetic and biochemical consequences of thymidine stress. Can. J. Biochem. **60**:172–194.
3. **Bisson, L., and J. Thorner.** 1977. Thymidine 5'-monophosphate-requiring mutants of *Saccharomyces cerevisiae* are deficient in thymidylate synthetase. J. Bacteriol. **132**:44–50.
4. **Bisson, L. F., and J. Thorner.** 1981. Thymidylate synthetase from *Saccharomyces cerevisiae*. J. Biol. Chem. **256**: 12456–12462.
5. **Bisson, L. F., and J. Thorner.** 1982. Exogenous dTMP utilization by a novel *tup* mutant of *Saccharomyces cerevisiae*. J. Bacteriol. **152**:111–119.
6. **Blakley, R. L.** 1963. The biosynthesis of thymidylic acid. IV. Further studies in thymidylate synthetase. J. Biol. Chem. **238**:2113–2118.
7. **Brunk, C. F., K. C. Jones, and T. W. James.** 1979. Assay for nanogram quantities of DNA in cellular homogenates. Anal. Biochem. **92**:497–500.
8. **Clark, J. M., and R. A. Deering.** 1981. Excision of pyrimidine dimers from nuclear deoxyribonucleic acid in ultraviolet-irradi-

ated *Dictyostelium discoideum*. Mol. Cell. Biol. **1**:121–127.
9. **Franke, J., and R. Kessin.** 1977. A defined medium for axenic strains of *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. U.S.A. **74**:2157–2161.
10. **Friedberg, E. C., and P. C. Hanawalt (ed.).** 1981. DNA repair: a laboratory manual of research procedures. Marcel Dekker, New York.
11. **Grivell, A. R., and J. F. Jackson.** 1968. Thymidine kinase: evidence for its absence from *Neurospora crassa* and some other microorganisms. J. Gen. Microbiol. **54**:307–317.
12. **Kammen, H. O.** 1966. A rapid assay for thymidylate synthetase. Anal. Biochem. **17**:553–556.
13. **Kessin, R. H., K. L. Williams, and P. C. Newell.** 1974. Linkage analysis in *Dictyostelium discoideum* using temperature-sensitive growth mutants selected with bromodeoxyuridine. J. Bacteriol. **119**:776–783.
14. **Kielman, J. K., and R. A. Deering.** 1980. Ultraviolet light-induced inhibition of cell division and DNA synthesis in axenically grown repair mutants of *Dictyostelium discoideum*. Photochem. Photobiol. **32**:149–156.
15. **Kimmel, A. R., and R. A. Firtel.** 1982. The organization and expression of the *Dictyostelium discoideum* genome, p. 234–324. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
16. **Kornberg, A.** 1980. DNA replication, p. 39–86. W. H. Freeman Co., San Francisco.
17. **Luk, D. C., and M. D. Bick.** 1977. Determination of 5′-bromodeoxyuridine in DNA by buoyant density. Anal. Biochem. **77**:346–349.
18. **Martinelli, J. E., and M. Chaykovsky.** 1980. A convenient preparation of tetrahydrofolate analogs. Synthesis of tetrahydro-3′,5′-dichloromethotrexate. Prep. Biochem. **10**:161–166.
19. **Michrina, C. A., and R. A. Deering.** 1980. Thymidine kinase activity in *Dictyostelium discoideum*. J. Gen. Microbiol. **119**:263–266.
20. **Murray, E. A.** 1982. Membranes, p. 71–116. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
21. **O'Donovan, C. A.** 1978. Thymidine metabolism in bacteria (and "how, or how not, to label DNA"), p. 219–253. *In* I. Molineux and M. Kohiyama (ed.), DNA synthesis: past and future. Plenum Press, New York.
22. **Podgorski, G., and R. A. Deering.** 1980. Quantitation of induced mutation in *Dictyostelium discoideum*: characterization and use of a methanol-resistance mutation assay. Mutat. Res. **74**: 459–468.
23. **Rader, J. I., and F. M. Huennekens.** 1973. Folate coenzyme-mediated transfer of one-carbon groups, p. 197–225. *In* P. D. Boyer, H. Landy, and K. Myrbäck (ed.), The enzymes, vol. 9B. Academic Press, Inc., New York.
24. **Ratner, D. I., T. E. Ward, and A. Jacobson.** 1981. Evidence for the transformation of *Dictyostelium discoideum* with homologous DNA, p. 595–605. *In* D. D. Brown and C. F. Fox (ed.), Developmental biology using purified genes: ICN-UCLA Symposia on Molecular and Cellular Biology, vol. 23. Academic Press, Inc., New York.
25. **Reddy, G. P. U., and A. B. Pardee.** 1980. Multienzyme complex for metabolic channeling in mammalian DNA replication. Proc. Natl. Acad. Sci. U.S.A. **77**:3312–3316.
26. **Roodman, S. T., and G. R. Greenberg.** 1971. A temperature-sensitive *thy* mutant blocked in the synthesis of thymidylate synthetase. J. Biol. Chem. **246**:2609–2617.
27. **Schwartz, M.** 1978. Thymidine phosphorylase from *Escherichia coli*. Methods Enzymol. **51**:442–445.
28. **Skoog, L., and G. Bjursell.** 1974. Nuclear and cytoplasmic pools of deoxyribonucleoside triphosphates in Chinese hamster ovary cells. J. Biol. Chem. **249**:6434–6438.
29. **Swinton, D. C., and P. C. Hanawalt.** 1972. In vivo specific labeling of *Chlamydomonas* chloroplast DNA. J. Cell Biol. **54**:592–597.
30. **Wartell, R. M., J. E. Larson, and R. D. Wells.** 1974. Netropsin: a specific probe for A-T regions of duplex deoxyribonucleic acid. J. Biol. Chem. **149**:6719–6731.
31. **Welker, D. L., and R. A. Deering.** 1979. Interactions between radiation-sensitive mutations in double-mutant haploids of *Dictyostelium discoideum*. Mol. Gen. Genet. **167**:265–270.
32. **Welker, D. L., and K. L. Williams.** 1981. Temperature-sensitive DNA repair mutations in the cellular slime mold *Dictyostelium discoideum*. Curr. Genet. **3**:167–172.
33. **Williams, L. G., and J. K. Mitchell.** 1969. Mutants affecting thymidine metabolism in *Neurospora crassa*. J. Bacteriol. **100**:383–389.
34. **Young, J. D., J. W. Gorman, J. A. Gorman, and R. M. Bock.** 1976. Indirect selection for auxotrophic mutants of *Saccharomyces cerevisiae* using the antibiotic netropsin. Mutat. Res. **35**:423–428.