

## Temperature-Sensitive DNA Mutant of Chinese Hamster Ovary Cells with a Thermolabile Ribonucleotide Reductase Activity

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**JB3-B is a Chinese hamster ovary cell mutant previously shown to be temperature sensitive for DNA replication (J. J. Dermody, B. E. Wojcik, H. Du, and H. L. Ozer, *Mol. Cell. Biol.* 6:4594-4601, 1986). It was chosen for detailed study because of its novel property of inhibiting both polyomavirus and adenovirus DNA synthesis in a temperature-dependent manner. Pulse-labeling studies demonstrated a defect in the rate of adenovirus DNA synthesis. Measurement of deoxyribonucleoside triphosphate (dNTP) pools as a function of time after shift of uninfected cultures from 33 to 39°C revealed that all four dNTP pools declined at similar rates in extracts prepared either from whole cells or from rapidly isolated nuclei. Ribonucleoside triphosphate pools were unaffected by a temperature shift, ruling out the possibility that the mutation affects nucleoside diphosphokinase. However, ribonucleotide reductase activity, as measured in extracts, declined after cell cultures underwent a temperature shift, in parallel with the decline in dNTP pool sizes. Moreover, the activity of cell extracts was thermolabile in vitro, consistent with the model that the JB3-B mutation affects the structural gene for one of the ribonucleotide reductase subunits. The kinetics of dNTP pool size changes after temperature shift are quite distinct from those reported after inhibition of ribonucleotide reductase with hydroxyurea. An indirect effect on ribonucleotide reductase activity in JB3-B has not been excluded since human sequences other than those encoding the enzyme subunits can correct the temperature-sensitive growth defect in the mutant.**

In procaryotic organisms, conditional lethal mutations affecting DNA replication have been useful in identifying proteins essential to the synthesis of DNA and its precursors (21). By contrast, few conditional DNA-defective mutants are available in mammalian cells, a fact that limits the use of genetic approaches to an understanding of their DNA metabolism (28). Dermody et al. (9) have previously reported the isolation, utilizing a variety of selective and nonselective conditions, of a large collection of Chinese hamster cell mutants that are temperature sensitive for growth. Biochemical screening has identified a subset of nine mutants that were designated temperature-sensitive DNA (tsDNA) mutants, as they exhibited rapid inhibition of DNA synthesis but not protein synthesis upon shift of growing cultures from the permissive temperature (33°C) to the nonpermissive or restrictive temperature (39.5°C). In an effort to further identify the possible bases for the temperature-sensitive defects, these nine mutants were tested for their ability to support the DNA replication of two different classes of DNA viruses: mouse polyomavirus and human type C adenovirus types 2 and 5 (Ad2 and Ad5). Four mutants restricted polyomavirus but not Ad, and only one mutant (JB3-B) restricted replication of both viral DNAs in a temperature-dependent fashion. Examination of functions known to be shared for DNA synthesis by cells and the two viruses suggested a defect in deoxyribonucleotide metabolism in JB3-B (and conversely excluded it in the other four mutants).

Mutations that specifically affect the synthesis of deoxyribonucleoside triphosphates (dNTPs) have helped to clarify metabolic and genetic relationships between deoxyribonucleotide synthesis and DNA replication (41, 42). For example, mutations in the genomes of both *Escherichia coli* and bacteriophage T4 have been used to generate evidence supporting a direct physical connection between the replication apparatus and the enzymatic machinery for dNTP synthesis (29). No such temperature-sensitive mutants have been described, however, for mammalian cells. Consequently, we investigated the nature of the biochemical defect in JB3-B. We report here evidence demonstrating that a defect in dNTP synthesis is responsible for the temperature-sensitive phenotype in JB3-B. Infected mutant cells exhibited a temperature-dependent defect in Ad DNA synthesis. There was also a depletion of dNTP but not ribonucleoside triphosphate (rNTP) pools when uninfected cells were shifted from 33 to 39°C. Furthermore, the mutant cells were found to contain a thermolabile form of ribonucleotide reductase activity. Unexpectedly, the changes in dNTP pool size that resulted from shifting a culture of JB3-B to a nonpermissive temperature (NPT) was quite distinct from those seen in various laboratories after inhibition of ribonucleotide reductase in mammalian cells with hydroxyurea (4, 13). This finding, together with preliminary studies on correction of the temperature-sensitive growth defect in JB3-B by DNA-mediated gene transfer, raise the possibility that the primary defect is in a gene product other than that encoding either the M1 or M2 subunit of ribonucleotide reductase.

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### MATERIALS AND METHODS

**Cells and culture methods.** The temperature-sensitive mutant JB3-B was obtained from CHO-S cells after mutagenesis

TABLE 1. Growth properties of JB3-B and its sublines

Cell line <sup>a</sup>	Doubling time (h) <sup>b</sup> at:		Efficiency of colony formation (%) at <sup>c</sup> :	
	33°C	39.5°C	33°C	39.5°C
CHO-S	16	11	100	100
Temperature sensitive				
JB3-B/2	28	>60 <sup>d</sup>	58	0.00017
JB3B.OUA <sup>r</sup>	ND <sup>e</sup>	ND	45	ND
JB3B.THO	42	>60	53	ND
Temperature independent				
Revertants				
3B2R1	29	26	ND	ND
JB3B.OUA <sup>r</sup> (Ts <sup>+</sup> )	29	22	ND	ND
JB3B.THO(Ts <sup>+</sup> )	42	34	ND	ND
Transfectants				
JB3B.B51 (primary)	55	48	22	2.3
JB3B.206 (secondary)	42	36	ND	ND

<sup>a</sup> Origins of cells lines are described in Materials and Methods.

<sup>b</sup> Dishes (60-mm diameter) were seeded with  $2 \times 10^4$  to  $5 \times 10^4$  cells at 33°C. After 24 to 40 h, replicate sets of dishes were shifted to 39.5°C. Cell number was determined as previously described (35).

<sup>c</sup> Cells were seeded at various densities, and colonies were counted after 2 to 3 weeks.

<sup>d</sup> No doubling in cell number in 60 h.

<sup>e</sup> ND, Not determined.

with ethyl methanesulfonate, utilizing the stratagem of repeated brief exposures to selective conditions (9). It was isolated from cultures which had been shifted from 33 to 37.5°C for 2 h plus an additional 4 h with bromodeoxyuridine followed by Hoechst 33258 and UV light. The mutant was recloned (JB3-B/2) at 33°C before its use in these studies. A ouabain-resistant derivative (designated JB3B.OUA<sup>r</sup>) was obtained from JB3-B/2 after mutagenesis with ethyl methanesulfonate and selection in  $3 \times 10^{-3}$  M ouabain at 33°C. A doubly drug-resistant derivative (designated JB3B.THO) was isolated from temperature-dependent JB3B.OUA<sup>r</sup> in  $5 \times 10^{-5}$  M thioguanine without further mutagenesis. Spontaneous temperature-resistant revertants [3B2R1 and JB3B.OUA<sup>r</sup>(Ts<sup>+</sup>)] were isolated at 39.5°C from JB3-B and JB3B.OUA<sup>r</sup>, respectively. No spontaneous temperature-resistant revertants were obtained in multiple attempts with more than  $10^8$  JB3B.THO cells in toto; JB3B.THO(Ts<sup>+</sup>) was isolated after ethyl methanesulfonate mutagenesis. All CHO cell lines were cultured in a 1:1 mixture of Dulbecco modified Eagle medium and F12 Ham's medium (DF medium) supplemented with 10% newborn or fetal bovine serum and proline as previously described (9, 22). All mutant cell lines were used within a few passages from frozen storage. The cell lines used are identified in Table 1.

**Materials and reagents.** Radioactively labeled deoxyribonucleoside 5'-triphosphates obtained from ICN Pharmaceuticals were [8-<sup>3</sup>H]dATP (22 Ci/mmol), [methyl-<sup>3</sup>H]dTTP (22 Ci/mmol), [5-<sup>3</sup>H]dCTP (25 Ci/mmol), and [8-<sup>3</sup>H]dGTP (11 Ci/mmol). [5-<sup>3</sup>H]cytidine 5'-diphosphate (25.3 Ci/mmol), [<sup>3</sup>H]thymidine ([<sup>3</sup>H]TdR) (74 Ci/mmol), and [<sup>35</sup>S]methionine (800 Ci/mmol) were purchased from Dupont, NEN Research Products (Boston, Mass.). Unlabeled nucleotides and adenylylimidodiphosphate ( $\beta'$ - $\gamma$ -imidoadenosine 5'-triphosphate) were obtained from Sigma Chemical Co. The alternating copolymers poly(dA-dT) and poly(dI-dC) were from Pharmacia. Purified *E. coli* DNA polymerase I (6,600 U/mg of protein) and phenylmethylsulfonyl fluoride were purchased from Boehringer Mannheim Biochemicals. Tri-*N*-

octylamine was obtained from ICN Pharmaceuticals. Freon-113 (1,1,2-trichlorotrifluoroethane) was from Aldrich Chemical Co. Nonidet P-40 was obtained from Particle Delta Laboratories.

**Macromolecular synthesis.** Cells were incubated with [<sup>3</sup>H]TdR or [<sup>35</sup>S]methionine for estimation of DNA and protein synthesis, respectively, as previously described (9).

**Cell cycle analysis.** Cells were harvested by trypsinization, centrifuged at low speed, and suspended in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M Na<sub>2</sub>HPO<sub>4</sub> · NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4) at a concentration of  $10^6$  cells per ml. One milliliter was added to 9 ml of cold 90% ethanol, and the mixture was stored at 4°C until analysis. The fixed cells were then sedimented, washed once, suspended in PBS (at  $10^6$  cells per ml), and stained with propidium iodide (50  $\mu$ g/ml) in the presence of Triton X-100 (1% wt/vol) and RNase for 20 min at room temperature as described elsewhere (20). Cells were analyzed for DNA content by using a system 50H flow cytometer (Ortho Diagnostics Institutes, Westwood, Mass.) interfaced to an Ortho 2150 data analysis system.

**Nuclear isolation.** We used the rapid technique developed by Leeds et al. (25) for isolation of nuclei within 20 s from monolayer cultures. Briefly, cultures on 100-mm dishes were placed on ice, and the medium was removed by aspiration. The cells were lysed by the addition of 2.5 ml of 1% Nonidet P-40 detergent in nuclear isolation buffer. After the plate was rocked for 10 s in the presence of the buffer, the buffer was aspirated and the cells were washed for 5 s with 5.0 ml of ice-cold PBS. After aspiration of the PBS, the nuclei remaining on the plate were immediately extracted for analysis of dNTP content. Whole-cell monolayers under the same growth conditions were treated identically, except for omission of the Nonidet P-40 treatment.

**dNTP analysis.** Both whole cells and nuclei were extracted for nucleotide analysis by the two-step extraction procedure of North et al. (36) with the following modifications: 5% trichloroacetic acid was used in place of 5% perchloric acid for the second step of the extraction, and acid extracts were neutralized with tri-*N*-octylamine as described by Garret and Santi (15). The contents of three 100-mm dishes were pooled for whole-cell assays, and the contents of four dishes were pooled for nuclear assays. dNTP pool sizes were determined by the DNA polymerase-based enzymatic assay (36), which measures incorporation of a limiting dNTP into an alternating copolymer template [poly(dA-dT) or poly(dI-dC)] by DNA polymerase I in the presence of an excess of the labeled complementary dNTP. dAMP (100 mM) was added to each reaction mixture to limit product breakdown. Samples from each assay reaction mixture were taken for measurement at three different times of incubation to ensure that plateau incorporation values were always reached. In addition, two different amounts of extract were assayed to ensure that counts per minute incorporated were directly proportional to volume of extract assayed. The values were corrected for dilution of the specific activity of added radioactive dNTPs by the sample as well as for background incorporation. Results are expressed as picomoles of dNTP per  $10^6$  whole cells or  $10^6$  nuclei.

**rNTP analysis.** The whole-cell extracts used for dNTP assays were also analyzed for rNTP content by high-performance liquid chromatography by utilizing a Varian high-pressure liquid chromatograph (model 5000) coupled with a Vista data analysis system (model 401). Samples (50  $\mu$ l) of each extract were analyzed on a strong anion-exchange column (Partisil 10-SAX, 4.6 mm by 25 cm; Whatman) plus

a guard column of the same material. The chromatography was performed in an isocratic mode at 30°C with a flow rate of 1.5 ml/min. The eluant was 0.35 M potassium phosphate buffer, pH 3.85. The UV detector operated at 254 nm. The rNTPs in the cell extracts were identified by comparing retention times of the unknown peaks with those of standard rNTPs chromatographed under the same conditions just before the sample under observation. The amount of each rNTP was determined by calculating the peak area in comparison with a standard curve. Results are expressed as nanomoles of rNTP per  $10^6$  cells.

**Ribonucleotide reductase assay.** Preparation of the enzyme extracts and assays of ribonucleotide reductase were performed as previously described (43) with minor modifications. Exponentially growing cells were seeded at a density of  $2 \times 10^6$  cells per 100-mm dish, incubated at 33°C for 40 h, and shifted to 39°C. At the indicated time points, the cells were collected and the enzyme extracts were prepared as described by Slabaugh et al. (43), except that 2 mM  $MgCl_2$ , 20  $\mu M$   $FeCl_3$ , and 20  $\mu M$  phenylmethylsulfonyl fluoride were added to the lysis buffer. Samples representing  $5 \times 10^6$  cells were assayed for intracellular CDP reductase activities. Reactions were performed at both 33 and 39°C for 30 min. Samples from each assay reaction mixture were taken for measurement at three different times of incubation to ensure that the activity of the enzyme was linear with respect to the incubation time. All assays were done in duplicate. The duplicate assays agreed within 10% of each other. Enzyme activity is expressed as picomoles of CDP reduced per milligram of protein.

To determine the thermolability of the enzyme, 20  $\mu l$  of enzyme preparation was mixed with 20 mM dithiothreitol, 40 mM  $FeCl_3$ , 4 mM magnesium acetate, and 40  $\mu M$  CDP and then brought to the desired temperature by placing the tubes in a water bath at either 39 or 42°C. At the indicated times, the samples were removed from the water bath and immediately cooled on ice. The remaining enzyme activity was then measured by incubation for 30 min (0, 15, 30 min) at 33°C after the addition of 110  $\mu M$  CDP and [ $^3H$ ]CDP (100 cpm/pmol), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and adenylylimidodiphosphate at the final concentration of the reaction mixture for CDP reduction (43).

**Analysis of Ad DNA synthesis.** Ad2 stocks were prepared in HeLa cells by conventional methodology, and titers for infectious units were determined by indirect immunofluorescence on CV-1 cells as described previously (38). Chinese hamster cell lines were infected at a multiplicity of 400 infectious units per cell. Viral DNA synthesis was determined by radiolabeling and alkaline sucrose gradient analysis as described by Longiaru and Horwitz (26). Briefly, at various times of interest postinfection (p.i.), cells were pulse-labeled for 1 h with [ $^3H$ ]TdR (5  $\mu Ci/ml$ ; Dupont, NEN Research Products) in thymidine-free medium followed by the addition of nonradioactive TdR for 30 min to chase intermediates into full genomic length, washed with PBS, scraped, and lysed directly on a 5 to 20% alkaline sucrose gradient with deoxycholate. A cesium chloride cushion was used to collect cellular DNA. Centrifugation was performed in a SW27 rotor for 16 h at 22,000 rpm at 4°C. Fractions of 0.5 ml were collected, and radioactivity was determined after trichloroacetic acid precipitation. Each gradient represented triplicate pooled infected cultures.

**Cell fusion.** For the complementation analysis, cells were fused at 33°C with 50% polyethylene glycol as previously described (19). On the day after fusion, the cells were split

into 100-mm dishes and refed with complete medium containing hypoxanthine ( $6.5 \times 10^{-5}$  M), aminopterin ( $5 \times 10^{-6}$  M), thymidine ( $1.15 \times 10^{-5}$  M) (designated HAT medium), and ouabain ( $2 \times 10^{-3}$  M) at 33°C. For hybrids involving human cells,  $10^{-5}$  M ouabain was used.

**Genetic analysis of correcting sequences.** DNA-mediated gene transfer was performed by the calcium phosphate coprecipitation technique (16) with DNA from a human cosmid library constructed in the cosmid vector pCV103 (SV2-*gpt*) provided by Y.-F. Lau (23). Twenty 100-mm dishes of JB3B.THO cells ( $5 \times 10^6$  cells per dish) were transfected with 25  $\mu g$  of cosmid DNA apiece. After 24 h, each culture was split into five 150-mm dishes and selected for *gpt* expression in HAT medium at 33°C. After 4 weeks, *gpt*<sup>+</sup> colonies (approximately 50,000 in toto) were shifted to 39.5°C for determination of temperature correction. Three temperature-independent primary transfectants were obtained; one (JB3B.B51) was chosen for further study. High-molecular-weight DNA was isolated from B51 and used to generate a secondary transfectant by the same procedure. Approximately 800 *gpt*<sup>+</sup> colonies were obtained at 33°C. Two temperature-independent secondary transfectants were obtained in a single dish; one (JB3B.206) was studied further. Plasmid DNAs pBLUR8, pSV2*gpt*, pER-WT, and pRRM2 were obtained from W. Jelinek (18), P. Berg (33), I. Caras (6), and L. Thelander (45), respectively. The cosmid and plasmid DNAs were propagated in appropriate strains of *E. coli* and harvested by standard methodology (27). High-molecular-weight cellular DNA was isolated as previously described (44). Southern analysis for exogenously introduced sequences was performed as described elsewhere (34) or with modification as indicated in the text. High-molecular-weight DNA and plasmid DNA controls were digested with different restriction endonucleases overnight at concentrations and under conditions specified by the supplier. Fragments of digested DNA were separated by electrophoresis in 0.8% agarose gels. Hybridizations were performed with the gel-purified inserts of the 270-base-pair *Alu* fragment from pBLUR8, the 1.8-kilobase *gpt* containing the *Bgl*III to *Bam*HI fragment of pSV2*gpt*, the 2.9-kilobase M1 *Bam*HI fragment of pER-WT, or the 1.5-kilobase *Pst*I fragment of M2 cDNA cloned in pUC18, respectively. Probes were  $^{32}P$  labeled by the random primer method (14).

## RESULTS

**Growth properties of JB3-B.** JB3-B was isolated from wild-type (wt) CHO cells by utilizing an enrichment scheme involving brief exposure to an intermediate temperature of 37.5°C. The mutant cells grew readily at 33°C, the permissive temperature, although the doubling time (28 h) was prolonged compared with that of the CHO parent, which had a doubling time of 16 h under comparable conditions. JB3-B cells formed colonies at high efficiency (50 to 100%) when plated at low cell density at 33°C but at a reduced efficiency (13%) at the initial selection temperature of 37°C. However, only rare colonies were observed even when  $10^6$  cells were plated at 39.5°C (Table 1). Consequently, we elected to use 39 to 39.5°C as the NPT for the further characterization of the mutant phenotype. CHO-S cells grow well throughout this temperature range. A series of spontaneous revertants were isolated on the basis of colony formation at the NPT, and one (3B2R1) was studied in some detail. Although it grew well at both 33 and 39.5°C, its doubling time in both cases was prolonged compared with that of the wt and similar to that of JB3-B. We have, consequently, empha-

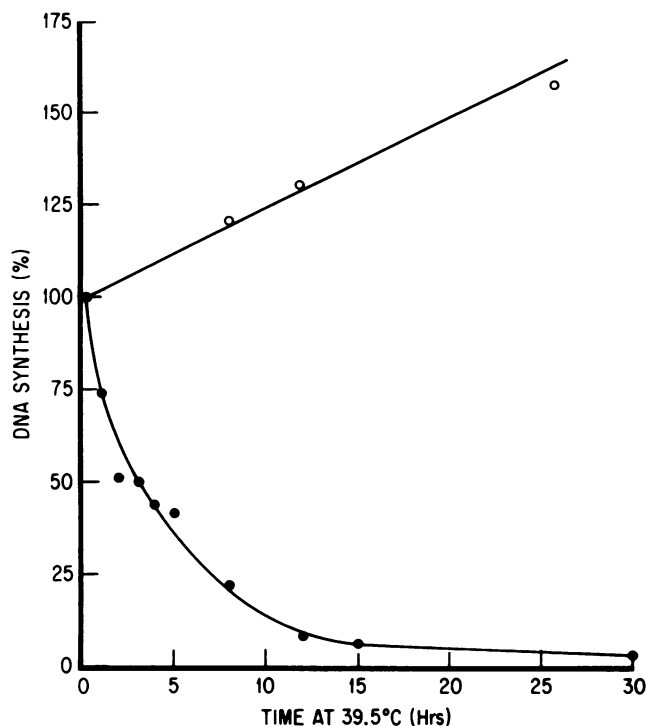


FIG. 1. DNA synthesis in mutant (JB3-B) and revertant (3B2R1) cells. Cells ( $5 \times 10^4$ ) were seeded into flat-base culture tubes at  $33^\circ\text{C}$  for 40 h and shifted to  $39.5^\circ\text{C}$  at time zero. Cultures were pulse-labeled with [ $^3\text{H}$ ]TdR for 60 min at appropriate intervals and analyzed for radioactivity as described in Materials and Methods and elsewhere (5). All values were normalized to the radioactivity incorporated shortly (30 to 60 min) after temperature shift, designated as 100% for each cell line. Symbols: ●, JB3-B; ○, 3B2R1.

sized comparisons between JB3-B and its revertant rather than solely with wt to ensure that any biochemical difference is due to the mutant gene responsible for the temperature-sensitive defect. The growth properties of drug-resistant mutants of JB3-B (which retain the temperature-sensitive phenotype) and their respective temperature-independent revertants are also included in Table 1 for comparison.

When JB3-B cells were shifted from  $33^\circ\text{C}$  to the NPT, the rate of incorporation of [ $^3\text{H}$ ]TdR fell, as previously reported, such that there was an 80% inhibition by 8 h at the NPT (Fig. 1). There was little or no increase in cell number (Fig. 2). Although there was a gradual loss of viability, detachment of cells, and fall in protein synthesis at the NPT as commonly observed for temperature-sensitive DNA mutants, these effects occurred at later times (e.g., after 16 to 24 h at the NPT) than the inhibition of DNA synthesis (Table 2). Cell cycle analysis by flow cytometry indicated a gradual accumulation of cells with an S-phase content of DNA compared with cultures maintained at  $33^\circ\text{C}$ , which showed a more typical log-phase profile (Fig. 3). These results are consistent with a primary defect in DNA synthesis, although the effects were most dramatic at later times (e.g., 24 h in Fig. 3F), i.e., after the fall in the rate of DNA synthesis.

**Ad DNA replication in JB3-B.** We previously reported that the synthesis of polyomavirus and Ad DNA is restricted when infected JB3-B cells are incubated at the NPT (7). Moreover, this effect was observed even when the cells had been previously infected at  $33^\circ\text{C}$ , indicating that ongoing viral DNA synthesis was also affected. Since viral DNA synthesis would not be expected to be cell cycle dependent

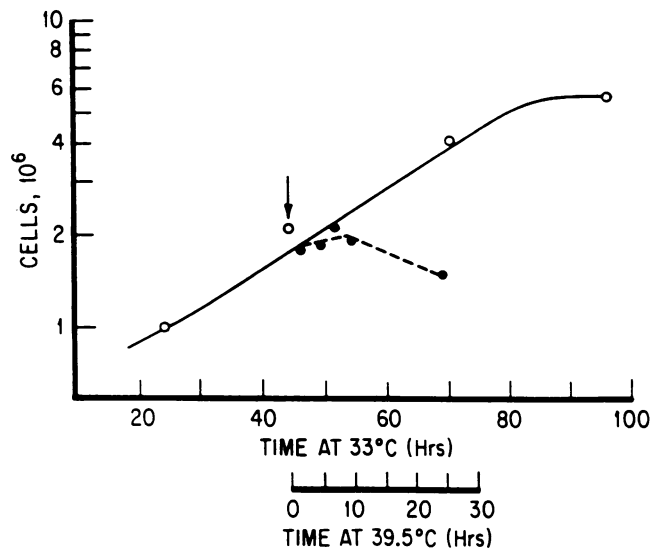


FIG. 2. Growth curve of JB3-B. Dishes (100-mm diameter) were seeded with  $5 \times 10^5$  cells at  $33^\circ\text{C}$ . At 44 h, cultures were refed with fresh medium and replicate cultures were shifted to  $39.5^\circ\text{C}$ . Cell number per culture was determined as described in Table 1, footnote b. Symbols: ○,  $33^\circ\text{C}$ ; ●,  $39.5^\circ\text{C}$ .

under such experimental conditions, these results indicate that the effect on viral DNA directly reflects the defect in cell DNA synthesis.

The previous initial synthesis of Ad DNA replication in JB3-B by dot blotting allowed only the measurement of the accumulation of progeny DNA molecules. To permit a more direct examination of the rate of viral DNA synthesis, we initiated studies using pulse-labeling of nascent viral DNA. The synthesis of viral intermediates was analyzed on alkaline sucrose gradients in which a discrete peak of genome-length viral DNA can be obtained free from cellular DNA, which pellets at the bottom of the gradient. Longiaru and Horwitz (26) reported for Ad-infected wt CHO cells that the

TABLE 2. Viability of JB3-B at the NPT

Time (h) at 39–39.5°C	Cell no. ( $10^4$ ) <sup>a</sup>	Colony formation (%) <sup>b</sup>	Dye exclusion (%) <sup>c</sup>	Amino acid incorporation (%) <sup>d</sup>
0	7.1	100	100	100
9	ND <sup>e</sup>	85	ND	83
15	7.5	ND	100	100
27	ND	6.2	ND	100
37	5.7	ND	10	3.3
48	5.1	<1	<1	ND

<sup>a</sup> Cell number was determined as described in Table 1, footnote b. Cultures maintained at  $33^\circ\text{C}$  showed a doubling time of 30 h.

<sup>b</sup> Dishes (60-mm diameter) were seeded with 100 cells at  $33^\circ\text{C}$  and shifted to  $39.5^\circ\text{C}$  for 9 to 48 h before being returned to  $33^\circ\text{C}$  to permit colonies to appear. The culture maintained at  $33^\circ\text{C}$  continuously had 56 colonies and was normalized to 100%.

<sup>c</sup> Dishes (60-mm diameter) were seeded with  $4 \times 10^4$  cells at  $33^\circ\text{C}$  and shifted to  $39.5^\circ\text{C}$  after 32 h. At the times indicated, trypsinized cultures were assayed for dye exclusion with trypan blue. The same cultures were used for determination of cell number. All cultures maintained at  $33^\circ\text{C}$  showed 100% dye exclusion throughout the experiment.

<sup>d</sup> Cells ( $5 \times 10^4$ ) were seeded into flat-base culture tubes at  $33^\circ\text{C}$  h before the shift to  $39.5^\circ\text{C}$ . Cultures were incubated with [ $^{35}\text{S}$ ]methionine in methionine-free medium for 2 h, and incorporation into macromolecules was determined as previously described (9).

<sup>e</sup> ND, Not determined.

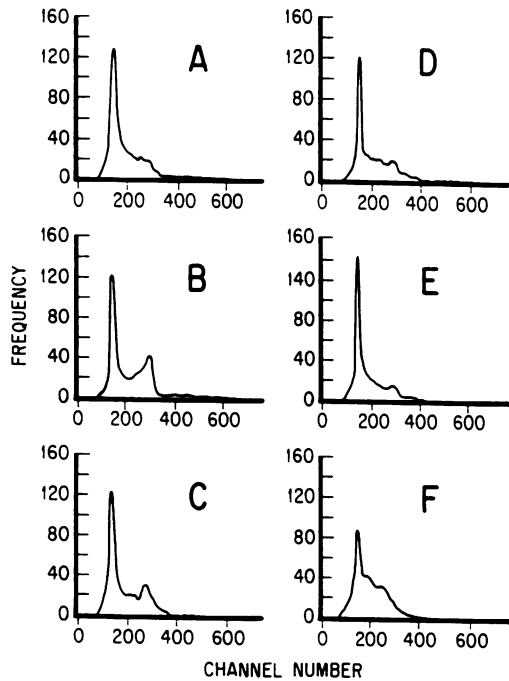


FIG. 3. Cell cycle distribution of JB3-B. Cultures used to generate the growth curve in Fig. 2 were analyzed by flow cytometry as described in Materials and Methods. The time at which cultures were shifted is designated 0 h. (A) 33°C, 0 h; (B) 33°C, 10 h; (C) 33°C, 24 h; (D) 39.5°C, 5 h; (E) 39.5°C, 10 h; (F) 39.5°C, 24 h.

maximum rate of viral DNA synthesis occurs between 36 and 42 h p.i. at 37°C. The course of infection in JB3-B cells was similar, although delayed. The maximum rate of Ad2 DNA synthesis occurred between 48 and 50 h p.i. at 33°C. To facilitate the interpretation of the data, cultures of JB3-B infected at 33°C were shifted to 39.5°C 36 and 42 h p.i., when the rate of viral DNA synthesis is increasing. In this manner, observation of the effect of the NPT on viral DNA synthesis should be maximized. The results are shown in Fig. 4. The rate of viral DNA synthesis in control cultures maintained at 33°C continued to increase. In parallel cultures that were shifted to the NPT, however, no increase in the rate of viral DNA synthesis could be observed. In cultures shifted at 42 h p.i., there was an immediate fall in viral DNA synthesis, whereas in cultures shifted at 36 h p.i., the marked increase failed to occur. Thus, incubation of Ad-infected JB3-B cells at the NPT leads to a prompt inhibition of viral DNA synthesis. These results confirm and extend the conclusion of the previous viral studies that the temperature-dependent inhibition of DNA synthesis in JB3-B is a direct result of the cellular mutation. Additional experiments have demonstrated that this inhibition is not attributable to artifacts such as a degradation of viral DNA or a selective loss of the infected cells (data not shown).

**Effects of JB3-B mutation on nucleotide pool sizes.** The preceding findings involving cell cycle kinetics and inhibition of viral DNA synthesis suggested that a defect existed in dNTP metabolism. Preliminary experiments indicated that after shift of a JB3-B culture from 33 to 39°C, one or more dNTP pools declined, but that appreciable quantities remained after DNA replication had virtually halted (data not shown). It was, however, possible that the residual pools at the NPT were located far from replication sites and, hence, no longer able to support replication; for example, there

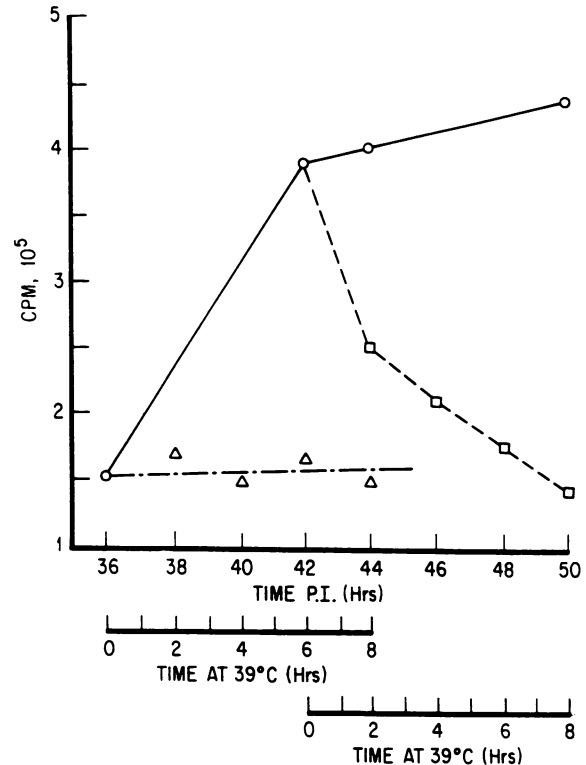


FIG. 4. Ad DNA synthesis in JB3-B. Cultures of JB3-B cells were infected at 33°C and analyzed for viral DNA by alkaline sucrose gradients after pulse-labeling with [<sup>3</sup>H]TdR for 1 h as described in Materials and Methods. The figure is a composite of several overlapping experiments in which cultures were maintained throughout at 33°C (○), infected at 33°C for 36 h before the shift to 39.5°C (△), or infected at 33°C for 42 h before the shift to 39.5°C (□).

could be a more extensive specific depletion of dNTP pools within the nucleus. Quantitative studies can be performed on dNTP pools in rapidly isolated (ca. 20 s) mammalian cell nuclei as shown by Mathews and co-workers (24, 25) by using a method, described originally by Rapaport et al. (39), which involves brief treatment of a cell monolayer with 1% Nonidet P-40 detergent, followed by rapid washing and nucleotide extraction. The dNTP pool sizes in such isolated nuclei remain constant over at least 30 s of extraction and 30 s of washing.

When we shifted cultures of JB3-B from 33 to 39°C, we observed an immediate decline in the dNTP pools measured in whole-cell extracts (Fig. 5). All four pools declined at similar rates, reaching minimum values by 12 h after the temperature shift. The decline roughly paralleled the loss in DNA-synthesizing capacity, as measured by [<sup>3</sup>H]TdR incorporation (Fig. 1). However, substantial residual pools remained at 12 h, ranging from 20 to 50% of the respective pool sizes in cultures at 33°C. On the other hand, the intranuclear pools declined by 4- to 15-fold. The most dramatic effect was observed with dCTP, for which the level dropped to 1 pmol/10<sup>6</sup> cells, close to the lower limit of detection for this assay. Since we extract for 10 s and wash for 5 s, the nuclear pool sizes that we determine probably represent intranuclear pools as they exist in the intact cell. It seems likely, therefore, that the inhibition of DNA synthesis at the NPT in JB3-B cells is related to the depletion of DNA precursors within the nucleus. Similar experiments were performed with the revertant 3B2R1 to assess whether the defect was

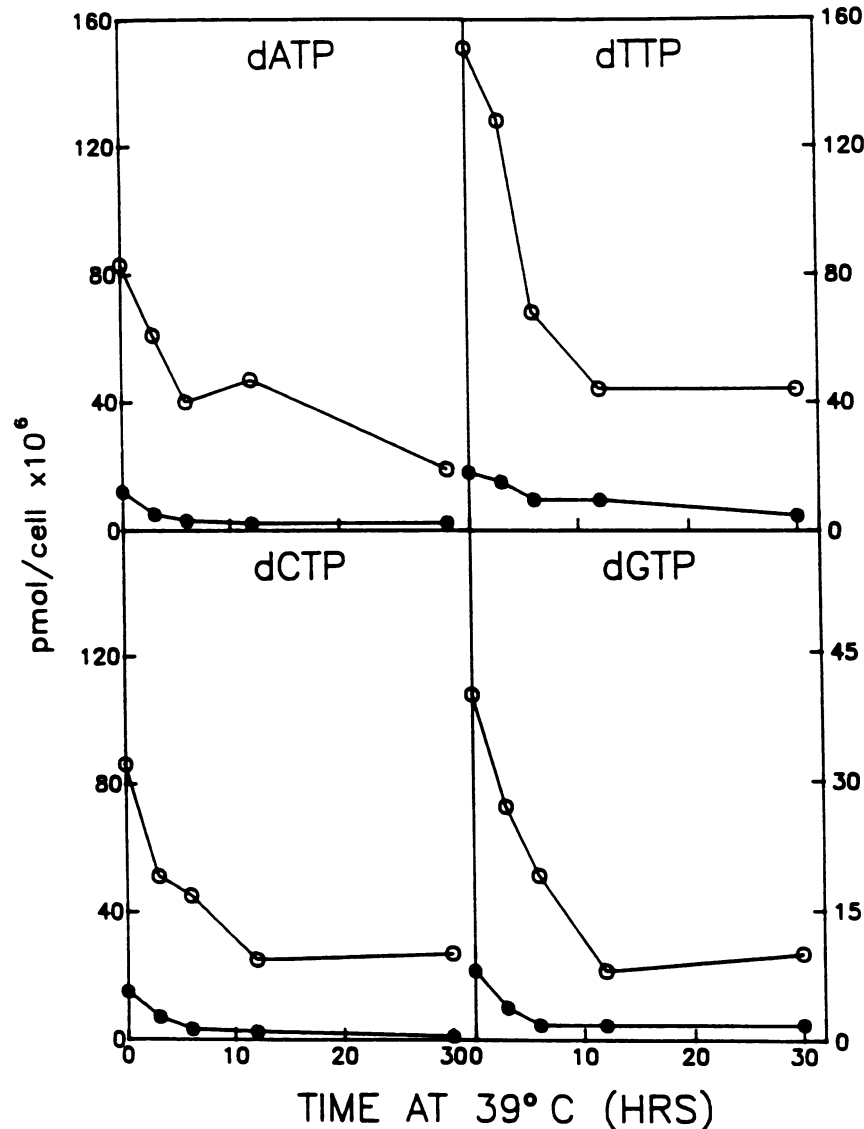


FIG. 5. Mutant (JB3-B) dNTP pools after temperature shift from 33 to 39°C. Cells were seeded at a density of  $5 \times 10^5$  cells per 100-mm dish, incubated at 33°C for 40 h, and shifted to 39°C at time zero. At the indicated times, whole cells and nuclei were extracted and the dNTP pools were quantitated as described in the text. Symbols: ○, whole cell; ●, nucleus.

associated with the temperature-sensitive mutation. These cells showed no depletion after a temperature shift to 39°C in either whole-cell or nuclear extracts (Fig. 6). In fact, most of the pools increased over the 12 h of the experiment, consistent with the finding that the cells continue to synthesize DNA (Fig. 1) and grow at 39°C. Therefore, the dNTP pool depletions seen in the JB3-B cells represent a direct consequence of the temperature-sensitive mutation in these cells.

The only enzymes that participate in the synthesis of all four DNA precursors are ribonucleotide reductase and nucleoside diphosphokinase. Since nucleoside diphosphokinase is involved in the synthesis of rNTPs as well as dNTPs, the most direct way to evaluate nucleoside diphosphokinase as the defective gene product was to analyze rNTP pools as a function of time after shift of JB3-B cells from 33 to 39°C. We observed no significant rNTP pool changes in whole-cell extracts under these conditions (Fig. 7). Therefore, nucleoside diphosphokinase appears to be ruled out as the mutant protein in JB3-B cells.

**Temperature sensitivity of ribonucleotide reductase in JB3-B cells.** Results of the above experiments suggested a critical role for ribonucleotide reductase activity in mutant and revertant cells, and this was tested by assaying directly for activity of this enzyme. Figure 8 shows the results when enzyme activity was assayed (with CDP as the substrate) as a function of time after temperature shift. Activities in the mutant declined when JB3-B cells were incubated at 39°C before the preparation of extracts; the kinetics were similar to those described for the dNTP pool depletions. No such loss in activity was seen with the revertant cells, and in fact, there was a significant increase in activity during the period analyzed. Thus, the JB3-B mutation affects ribonucleotide reductase activity, either directly or indirectly.

Note, however, that in mutant cell extracts there were no differences in specific enzyme activity whether the extracts were assayed at 33 or 39°C. Indeed, the revertant extracts consistently showed increased activity at 39°C compared with corresponding assays performed at 33°C, as one might

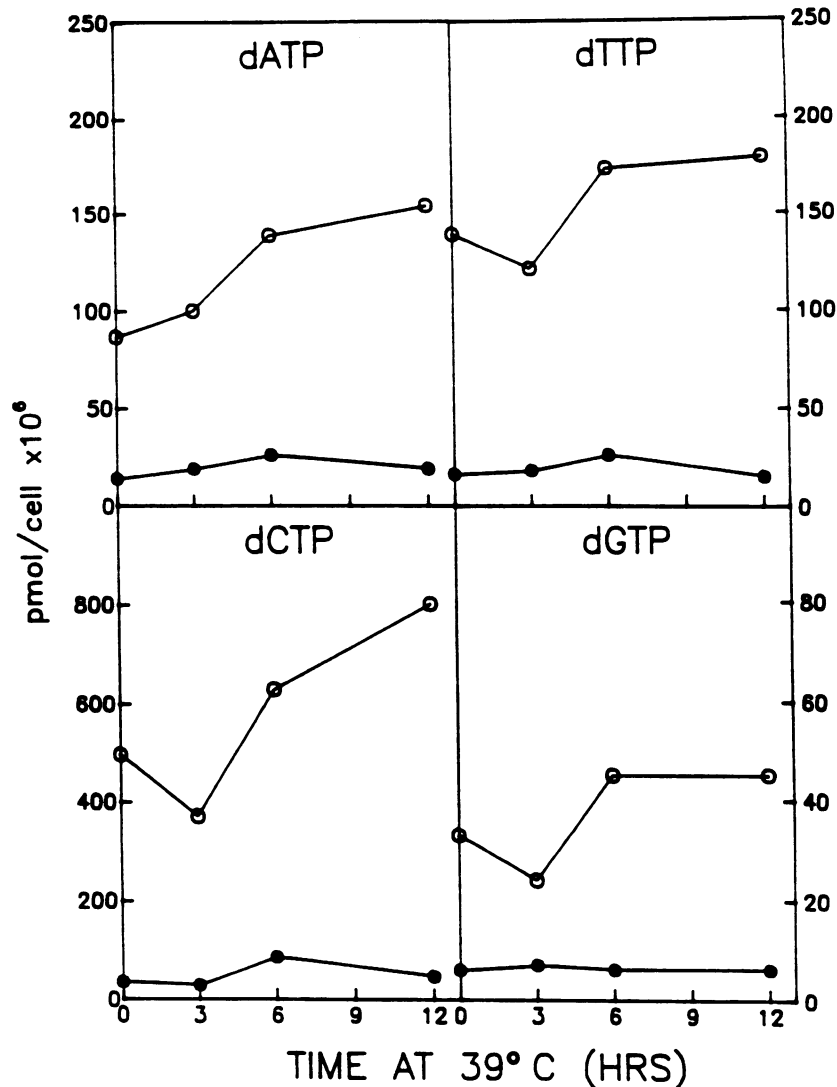


FIG. 6. Revertant (3B2R1) dNTP pools after temperature shift from 33 to 39°C. Revertant cells were analyzed as described in the legend to Fig. 5. Symbols: ○, whole cell; ●, nucleus.

expect for most wt mammalian enzymes. However, the fact that the JB3-B ribonucleotide reductase showed no loss of activity at 39°C suggests that this enzyme is not the mutant gene product. Another explanation for the data is that the JB3-B ribonucleotide reductase protein is thermolabile but that the kinetics of enzyme inactivation are slow with respect to the incubation period of the enzyme assay. We tested this latter possibility by assaying reductase activity at 33°C in mutant and revertant extracts as a function of time of preincubation of the extracts at either 39 or 42°C. The JB3-B extracts did show declines in activity after preincubation, while activity in the revertant extracts was unaffected (Fig. 9). Note that there was a 30-min lag with the JB3-B extract at 39°C before any enzyme inactivation was detectable. Since the incubation period for the ribonucleotide reductase assay is also 30 min, it is apparent why the 39°C assays depicted in Fig. 8 showed no evidence for thermolability of the JB3-B ribonucleotide reductase. More important, the data of Fig. 9 provide evidence that the JB3-B ribonucleotide reductase protein is thermolabile. In addition, the figure shows that a temperature-independent cell line (JB3B.206)

which was obtained by transfecting JB3-B with human DNA (described in the next section) also contains a thermostable ribonucleotide reductase.

**Genetic studies with JB3-B.** Cell hybrid analyses were performed with JB3-B and its temperature-sensitive, thioguanine-resistant, ouabain-resistant subclone (JB3B.THO) to determine whether the temperature-sensitive phenotype is recessive to that of wt cells, as a prelude to attempted isolation of the gene which corrects the temperature-sensitive defect. When JB3B.THO and CHO cells were fused with polyethylene glycol, hybrids could be isolated at both 33°C and the NPT in medium supplemented with HAT and ouabain. Furthermore, colonies isolated at 33°C were also able to proliferate at 39.5°C (data not shown). Hybrids isolated between JB3B.THO and other temperature-sensitive DNA mutants isolated from CHO cells (which do not restrict Ad DNA synthesis) were similarly temperature independent (data not shown). Finally, hybrids isolated between JB3B.THO and JB3-B at 33°C were temperature sensitive for growth, ruling out the possibility that a gene dosage effect is responsible for the correction of the temper-

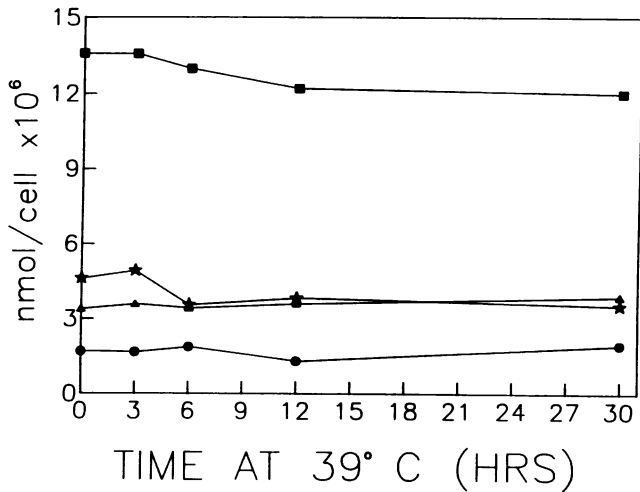


FIG. 7. Mutant (JB3-B) whole-cell rNTP pools after temperature shift from 33 to 39°C. The whole-cell extracts used for dNTP pool determination (Fig. 5) were quantitated for rNTP content by high-performance liquid chromatography as described in the text. The pool size is presented as nanomoles of rNTP per 10<sup>6</sup> whole cells. Symbols: ■, ATP; ★, UTP; ▲, CTP; ●, GTP.

ature-sensitive growth defect. We therefore concluded that the temperature-sensitive defect in JB3-B is recessive.

The temperature-sensitive defect in JB3-B can also be corrected by DNA from human cells as demonstrated by cell

fusion of JB3B.THO with human fibroblastic cell lines (B. E. Wojcik and H. L. Ozer, unpublished data). An attempt to isolate the correcting human DNA sequences was initiated. A cosmid library containing genomic human DNA linked to the bacterial guanine phosphoribosyltransferase gene (*gpt*) (23) was used to transfect JB3B.THO cells. Temperature-independent primary transfectants (e.g., B51 in Table 1) were isolated after transfected colonies were identified by initial selection at 33°C in HAT medium followed by shift of cultures to 39°C. High-molecular-weight DNA was prepared from B51 and used to transfect additional JB3B.THO cells, resulting in selection of temperature-independent secondary transfectants as above. Such a secondary transfectant (e.g., 206 in Table 1) is only enriched for the correcting sequence since Southern analysis reveals multiple copies of the vector sequences. We were able to confirm the authenticity of the primary and the secondary transfectants by back-selection experiments. The transfectants, in contrast to their parental JB3B.THO cells, were sensitive to thioguanine owing to expression of the *gpt* sequences in the vector. When the B51 or 206 cells were plated in medium containing 5 × 10<sup>-5</sup> M thioguanine at 33°C, clones were isolated which had lost the *gpt* sequences. They also had lost the linked human repetitive sequences detectable with BLUR8 (data not shown) and now demonstrated a temperature-sensitive growth phenotype.

Since the biochemical data indicate that a temperature-dependent ribonucleotide reductase activity could be responsible for the properties of JB3-B, we tested for the presence of human ribonucleotide reductase DNA se-

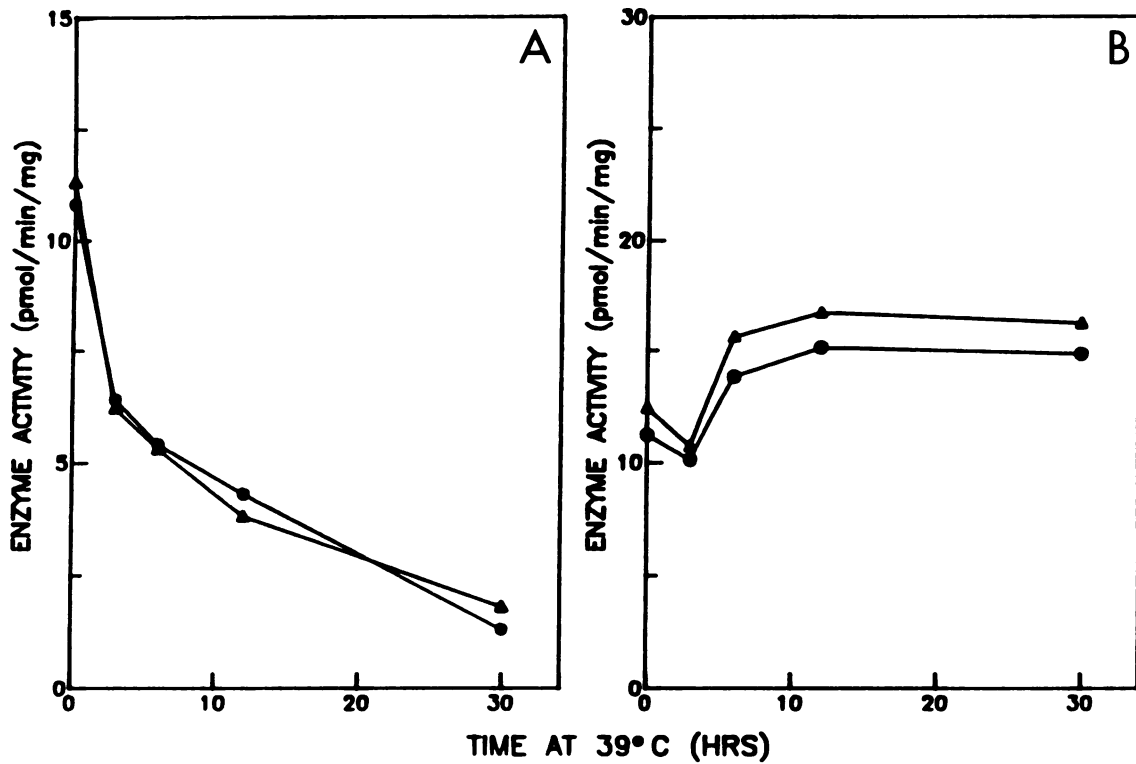


FIG. 8. Ribonucleotide reductase activity in extracts of mutant (JB3-B) and revertant (3B2R1) cells after temperature shift from 33 to 39°C. Mutant and revertant cells were seeded at a density of 2 × 10<sup>6</sup> cells per 100-mm dish, incubated at 33°C for 40 h, and shifted at 39°C at time zero. At the indicated times, enzyme extracts were prepared and samples representing 5 × 10<sup>6</sup> cells were assayed for CDP reductase activity at 33 and 39°C as described in the text. Enzyme activity is expressed as picomoles of dCDP formed per minute per milligram of protein. Each data point represents the average value of duplicate reductase assays. The mutant (A) was assayed at 33°C (●) and at 39°C (▲); the revertant (B) was assayed at 33°C (●) and 39°C (▲).



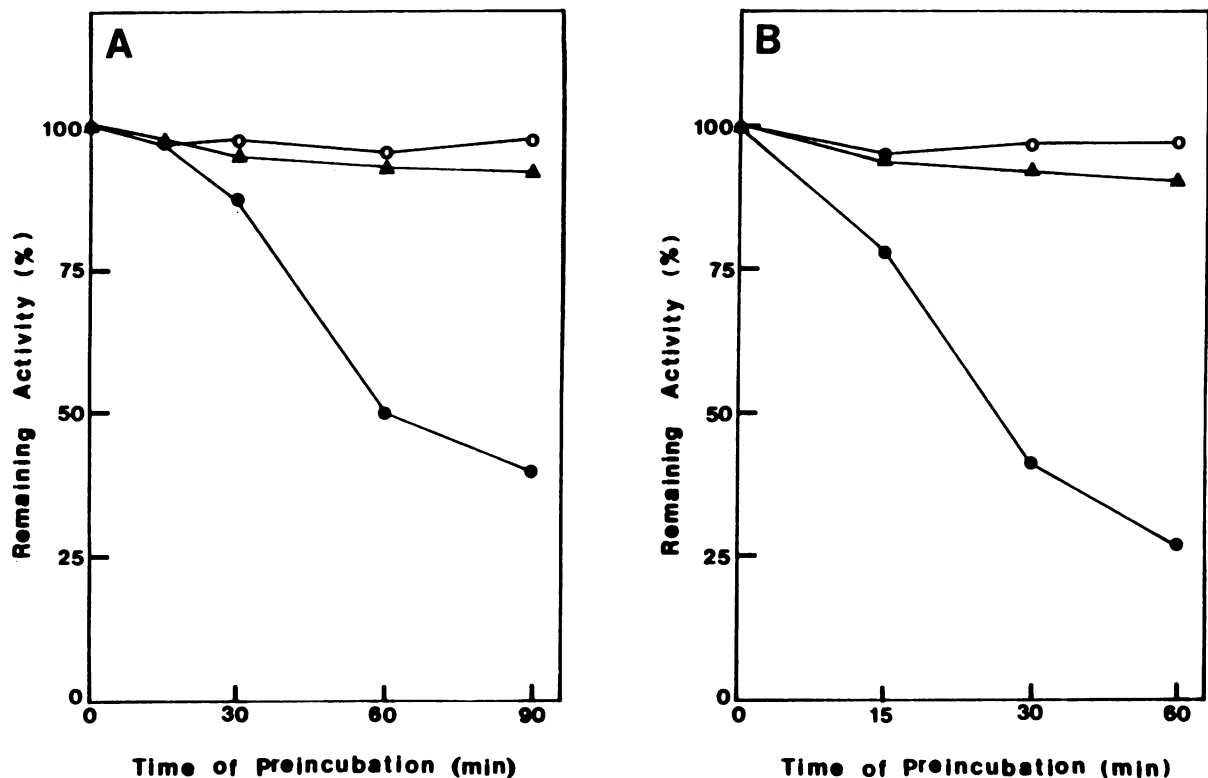


FIG. 9. Thermolability of ribonucleotide reductase activity from mutant (JB3-B), revertant (3B2R1), and transfectant (JB3B.206) cells. Enzyme extracts like those depicted in Fig. 8 were preincubated at 39°C (A) and 42°C (B) for the times indicated in the presence of 40  $\mu$ M CDP. The enzyme activities were then assayed at 33°C in duplicate (average value plotted) as described in the text. The activities without preincubation at 39 or 42°C are designated 100%. These values were all between 10 and 12 pmol/min/mg of protein. Symbols: ●, mutant; ○, revertant; and △, transfectant.

quences in this secondary transfectant, which had also regained a thermostable enzyme activity. Previous studies have shown that ribonucleotide reductase consists of two subunits, designated M1 and M2 (30). The cDNAs which code for these subunit proteins from the mouse have been cloned (6, 45). We obtained DNA corresponding to both M1 and M2 from other laboratories and used these DNAs to probe filters containing restriction enzyme-digested DNA isolated from the secondary transfectant 206 (Fig. 10). Southern analysis of high-molecular-weight DNA digested with appropriate restriction enzymes demonstrated the previously reported pattern for human (lane 1) and Chinese hamster (lane 2) DNA for M1 (5) in Fig. 10A and M2 (47) in Fig. 10B. Analysis of two independent DNA preparations from the secondary transfectant showed a pattern consistent with the endogenous M1 gene and failed to reveal any additional human-specific bands (lanes 4 and 5 in Fig. 10A). DNA from a back-selected primary transfectant is included (in lane 3) for comparison. Analysis of DNA from these cell lines after digestion with *Eco*RI similarly failed to reveal any human-specific bands in 206 (data not shown). Analysis for M2 sequences also failed to demonstrate human-specific sequences (see lanes 3 and 4 in Fig. 10B), although alteration in the pattern of at least one allele of the endogenous gene appears to have occurred in the secondary transfectant upon repeated passage; the DNA in lane 4 was prepared subsequent to that in lane 3. Note that the hybridization conditions employed were of reduced stringency in an effort to facilitate the detection of the donor sequences.

## DISCUSSION

As discussed elsewhere (28, 37), relatively few mammalian cell mutants have been described with temperature-sensitive lesions affecting DNA synthesis (9, 12). We believe that JB3-B is the first one in which defective DNA replication is known to result from aberrant DNA precursor biosynthesis. Several experimental findings support our conclusion that defective dNTP metabolism results from the mutation in JB3-B. (i) The observed decrease in dNTP pools correlates closely with the temperature-sensitive phenotype. (ii) The magnitude and time course of the inhibition of DNA synthesis observed by [<sup>3</sup>H]TdR incorporation for both cellular and viral DNA are similar to each other and to the pool changes. (iii) There is a defect in Ad DNA synthesis which is consistent with the observed dNTP levels. (iv) The levels of activity of ribonucleotide reductase, an enzyme responsible for dNTP synthesis, are reduced in extracts prepared from the mutant at the NPT. Finally, all these phenotypic defects are corrected in a revertant selected for growth at the NPT.

Other mammalian cell mutations affecting DNA precursor biosynthesis have been described, but they do not have a conditional lethal phenotype. Mutations affecting ribonucleotide reductase have been described based on resistance to hydroxyurea or to deoxyribonucleosides (30, 46). Other mutations include thymidylate synthase deficiencies that yield a thymidine auxotrophy (1), dCMP deaminase deficiencies that cause dNTP pool imbalances (46), and loss of feedback control of CTP synthetase, which unbalances both

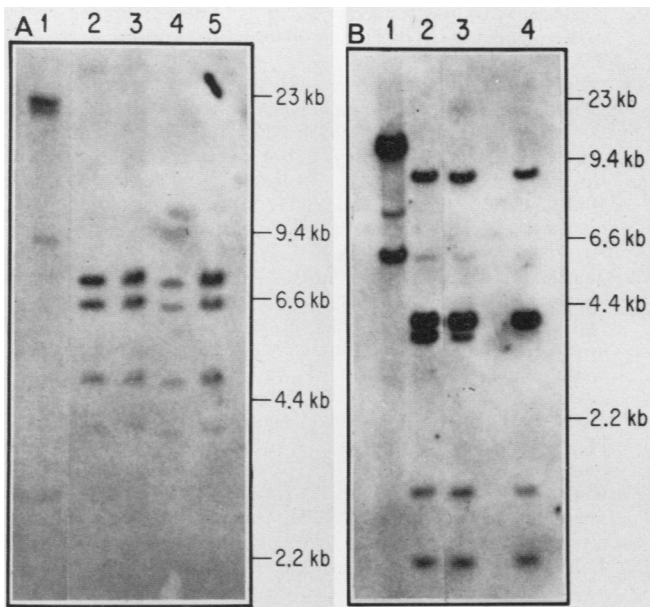


FIG. 10. Southern blot analysis of temperature-independent transfectants. High-molecular-weight DNA was prepared and analyzed for ribonucleotide reductase gene sequences. (A) DNA was digested with *Bam*HI and probed for M1 sequences. Lanes: 1, human fibroblast; 2, JB3-B; 3, back-selected (temperature-sensitive) primary transfectant B51; 4, secondary transfectant 206, preparation 1; 5, secondary transfectant 206, preparation 2. (B) DNA was digested with *Eco*RI and probed for M2 sequences. Lanes: 1, human fibroblast; 2, JB3-B; 3, secondary transfectant 206, preparation 1; 4, secondary transfectant, preparation 2. Probes were prepared and used for hybridization as described in Materials and Methods except that hybridization was performed at 37°C and filters were washed at 42°C. kb, Kilobases.

rNTP and dNTP pools (31). These cell lines have generated useful insights into biological processes such as mutagenesis (32, 46), thymineless death (3), and the likely existence of multienzyme complexes for dNTP synthesis (2). Similarly, we expect the JB3-B mutation to be useful in several ways for further exploration of the relationships between DNA replication and DNA precursor biosynthesis in mammalian cells. For example, the estimated DNA synthesis rates decline in parallel with the depletion of the four dNTP pools as a function of time after shift to the NPT. Observations from several laboratories have suggested that dNTP levels control the rate of DNA replication through interaction with allosteric sites on replicative DNA polymerases (10, 17, 35, 40). Such models, we believe, would lead to prediction of more complex relationships between dNTP pool sizes and DNA replication rates than observed in our experiments. These data must be considered preliminary, however, because we have not yet measured true DNA synthesis rates. Nevertheless, the data at this stage suggest a simple relationship between intracellular dNTP concentrations and replication rates, as though polymerase activity was limited after temperature shift simply by substrate-level control. Such relationships are suggested also by recent kinetic analyses of DNA polymerase  $\alpha$  and of replication in permeabilized mammalian cells (11).

Further studies are needed to precisely identify the basis for the mutation in JB3-B. The mutation affects a component of ribonucleotide reductase, because extracts show thermolability of the enzyme in the mutant and thermostability of the enzyme in the revertant and secondary transfectant. The

relative ease with which temperature-independent revertants can be isolated from JB3-B and JB3B.OUR<sup>r</sup> suggests that a single mutational event is involved. The simplest interpretation is that the primary mutation is in the sequence encoding for one of the two subunits (M1 or M2) of ribonucleotide reductase. However, there are no direct genetic data to support this model, and one must consider the possibility of an indirect effect on ribonucleotide reductase activity. In fact, preliminary studies with DNA-mediated gene transfer failed to demonstrate human sequences encoding M1 or M2 in JB3B.THO temperature corrected after transfection with human DNA (i.e., JB3B.206). Although unexpected, this should not be viewed as definitive evidence against the primary defect involving such a mutation. For example, temperature correction might occur through a compensatory mechanism (8). Supporting this possibility is the suggestion that several doses of the responsible gene may be required in JB3B.206 since the secondary transfectants appear to contain multiple copies of human cosmids and repeated efforts to obtain tertiary transfectants have been unsuccessful so far (Wojcik and Ozer, unpublished data). As an alternative approach, we are currently attempting to correct the defect in JB3-B by introduction of functional copies of the respective subunits. Identification of the nature of the mutation takes on added importance because the metabolic consequences of inhibiting the ribonucleotide reductase activity in JB3-B cells are distinctly different from the consequences of inhibition of DNA replication in wt cells by addition of hydroxyurea. Inactivating the enzyme in JB3-B cells by a temperature shift causes all four dNTP pools to decline in parallel. By contrast, a hydroxyurea block leads to a particularly steep decline in dATP and dGTP pools, while dTTP actually accumulates (4). These latter results have been interpreted in terms of rapid turnover of purine dNTP pools, substrate cycles that synthesize pyrimidine dNTPs from nucleosides (4), and the ready conversion of intracellular deoxycytidine nucleotides to thymidine nucleotides (41). One might expect similar consequences to ensue from a temperature inactivation of the enzyme in JB3-B cells. However, hydroxyurea inhibition is targeted to the small subunit (M2) of the tetrameric ribonucleotide reductase, which contains a tyrosine free radical essential to catalytic activity (30, 41). If the JB3-B mutation is found to affect the large subunit, which contains the allosteric control sites for the enzyme, it might account for the differences observed. Alternatively, destabilization of the entire tetramer through its increased heat lability could be responsible. An additional possibility is that the mutation affects one of the other components of the ribonucleotide reductase system, such as glutaredoxin or glutathione reductase.

Finally, we note that even at 30 h after shift to the NPT, substantial dNTP levels were seen in whole-cell extracts, even though the levels seen in nuclei were nearly undetectable. We do not conclude that this implies the existence of cytosolic pools of dNTP which do not equilibrate with pools in the nucleus. Our earlier experiments with synchronized CHO cells showed that G1-phase cells contain substantial dNTP pools, which are obviously not available for use in DNA replication (24). By contrast, under most conditions, dNTP pools in S-phase cells behave as though the cytosol and nucleus constitute a single metabolic compartment (24, 25). Since the experiments in the present study were all done with nonsynchronized cultures, we suggest that the residual pools seen in whole-cell extracts are confined primarily to cells that are not undergoing DNA replication. These observations are consistent with our earlier suggestion that studies

of the relationship between dNTP pools and DNA replication should be done with synchronized cell cultures. Moreover, the nuclear dNTP measurements more closely reflect dNTP pools available for replication than do those measured in whole-cell extracts.

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#### LITERATURE CITED

- Ayusawa, D., H. Koyama, K. Iwata, and T. Seno. 1980. Single-step selection of mouse FM3A cell mutants defective in thymidylate synthetase. *Somatic Cell Genet.* **6**:261-270.
- Ayusawa, D., K. Shimizu, H. Koyama, K. Takeishi, and T. Seno. 1983. Unusual aspects of thymidylate synthase in mouse cells introduced by DNA-mediated gene transfer. *J. Biol. Chem.* **258**:48-54.
- Ayusawa, D., K. Shimizu, H. Koyama, K. Takeishi, and T. Seno. 1983. Accumulation of DNA strand breaks during thymineless death in thymidylate synthase-negative mutants of mouse FM3A cells. *J. Biol. Chem.* **258**:12448-12454.
- Bianchi, V., E. Pontis, and P. Reichard. 1986. Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. *J. Biol. Chem.* **261**:16037-16042.
- Brissenden, J. E., I. Caras, L. Thelander, and U. Francke. 1988. The structural gene for the M1 subunit of ribonucleotide reductase maps to chromosome 11, band 15, in human and to chromosome 7 in mouse. *Exp. Cell Res.* **174**:302-308.
- Caras, I. W., and D. W. Martin, Jr. 1988. Molecular cloning of the cDNA for a mutant mouse ribonucleotide reductase M1 that produces a dominant mutator phenotype in mammalian cells. *Mol. Cell. Biol.* **8**:2698-2704.
- Challberg, M. D., and T. J. Kelly. 1989. Animal virus DNA replication. *Annu. Rev. Biochem.* **58**:671-718.
- Choi, W.-J., J. L. Campbell, C. Kuo, and A. Y. Jong. 1989. The *Saccharomyces cerevisiae* SOC 8-1 gene and its relationship to a nucleotide kinase. *J. Biol. Chem.* **264**:15593-15599.
- Dermody, J. J., B. E. Wojcik, H. Du, and H. L. Ozer. 1986. Identification of temperature-sensitive DNA<sup>-</sup> mutants of Chinese hamster cells affected in cellular and viral DNA synthesis. *Mol. Cell. Biol.* **6**:4594-4601.
- Diffley, J. F. X. 1988. Affinity labeling the DNA polymerase complex. *J. Biol. Chem.* **263**:19126-19131.
- Dresler, S. L., M. G. Frattini, and R. M. Robinson-Hill. 1988. In situ enzymology of DNA replication and ultraviolet-induced DNA repair synthesis in permeable human cells. *Biochemistry* **27**:7247-7254.
- Eki, T., T. Enomoto, A. Miyajima, H. Miyazawa, Y. Murakami, F. Hanaoka, M. Yamada, and M. Ui. 1990. Isolation of temperature-sensitive cell cycle mutants from mouse FM3A cells. *J. Biol. Chem.* **265**:26-33.
- Eriksson, S., S. Skog, B. Tribukait, and B. Wallstrom. 1987. Deoxyribonucleoside triphosphate metabolism and the mammalian cell cycle. *Exp. Cell Res.* **168**:79-88.
- Feinberg, A. P., and R. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13.
- Garret, C., and D. V. Santi. 1979. A rapid and sensitive high pressure liquid chromatography assay for deoxyribonucleoside triphosphate in cell extracts. *Anal. Biochem.* **99**:268-273.
- Graham, F., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5. *Virology* **52**:456-457.
- Grindey, G. B., M. Winkler, M. Otten, and J. A. Steinberg. 1980. Inhibition of calf thymus DNA polymerase- $\alpha$  by deoxyribonucleoside triphosphate. *Mol. Pharmacol.* **17**:256-261.
- Jelinek, W. R., T. P. Toomey, L. Leinward, C. H. Duncan, P. A. Biro, P. V. Chouday, S. M. Weissman, C. M. Rubin, C. M. Houck, P. I. Deininger, and C. W. Schmid. 1980. Ubiquitous, interspersed repeated sequences in the mammalian genome. *Proc. Natl. Acad. Sci. USA* **77**:1398-1402.
- Jha, K. K., M. Siniscalco, and H. L. Ozer. 1980. Temperature-sensitive mutants of Balb/3T3 cells. III. Hybrids between ts2 and other mouse mutant cells affected in DNA synthesis and correction of ts2 defect by human X chromosome. *Somatic Cell Genet.* **6**:603-614.
- Klein, C. E., H. L. Ozer, F. Traganos, J. Atzpodien, H. F. Oettgen, and L. J. Old. 1988. A transformation-associated 130 kD cell surface glycoprotein is growth regulated in normal human cells. *J. Exp. Med.* **167**:1684-1696.
- Kornberg, A. 1980 and 1982. DNA replication and 1982 supplement to DNA replication. W. H. Freeman & Co., San Francisco.
- LaBella, F., and H. L. Ozer. 1985. Differential replication of SV40 and polyoma DNA in Chinese hamster ovary cells. *Virus Res.* **2**:329-343.
- Lau, Y.-F., and Y. W. Kan. 1983. Versatile cosmid vectors for the isolation, expression, and rescue of gene sequences: studies with the human alpha-globin gene cluster. *Proc. Natl. Acad. Sci. USA* **80**:5225-5229.
- Leeds, J. M., and C. K. Mathews. 1987. Cell cycle-dependent effects on deoxyribonucleotide and DNA labeling by nucleoside precursors in mammalian cells. *Mol. Cell. Biol.* **7**:532-534.
- Leeds, J. M., M. B. Slabaugh, and C. K. Mathews. 1985. DNA precursor pools and ribonucleotide reductase activity: distribution between the nucleus and cytoplasm of mammalian cells. *Mol. Cell. Biol.* **5**:3443-3450.
- Longiaru, M., and M. S. Horwitz. 1981. Chinese hamster ovary cells replicate adenovirus DNA. *Mol. Cell. Biol.* **1**:208-215.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Marcus, M., A. Fainsod, and G. Diamond. 1985. The genetic analysis of mammalian cell-cycle mutants. *Annu. Rev. Genet.* **19**:389-421.
- Mathews, C. K., L. K. Moen, Y. Wang, and R. G. Sargent. 1988. Intracellular organization of deoxyribonucleotide-synthesizing enzymes. *Trends Biochem. Sci.* **13**:394-397.
- McClarty, G. A., A. K. Chan, Y. Engstrom, J. A. Wright, and L. Thelander. 1987. Elevated expression of M1 and M2 components and drug-induced posttranscriptional modulation of ribonucleotide reductase in a hydroxyurea-resistant mouse cell line. *Biochemistry* **26**:8004-8011.
- Meuth, M. 1981. Sensitivity of a mutator gene in Chinese hamster ovary cells to deoxynucleoside triphosphate pool alterations. *Mol. Cell. Biol.* **1**:652-660.
- Meuth, M. 1989. The molecular basis of mutations induced by deoxyribonucleoside triphosphate pool imbalances in mammalian cells. *Exp. Cell Res.* **181**:306-316.
- Mulligan, R. C., and P. Berg. 1981. Selection for animal cells that express the *E. coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78**:2072-2076.
- Neufeld, D. S., S. Ripley, A. Henderson, and H. L. Ozer. 1987. Immortalization of human fibroblasts transformed by origin-defective simian virus 40. *Mol. Cell. Biol.* **7**:2794-2802.
- Nicander, B., and P. Reichard. 1981. Aphidicolin sensitivity of variant 3T6 cells selected for changes in ribonucleotide reductase. *Biochem. Biophys. Res. Commun.* **103**:148-155.
- North, T. W., R. K. Bestwick, and C. K. Mathews. 1980. Detection of activities that interfere with the enzymatic assay of deoxyribonucleoside 5'-triphosphates. *J. Biol. Chem.* **255**:6640-6645.

37. Ozer, H. L., J. J. Dermody, and B. E. Wojcik. 1987. Mammalian *ts* DNA mutants, p. 475–483. *In* DNA replication and recombination. Alan R. Liss, Inc., New York.
38. Radna, R., B. Foellmer, L. A. Feldman, U. Francke, and H. L. Ozer. 1987. Restriction of human adenovirus replication in Chinese hamster cell lines and cell hybrids with human cells. *Virus Res.* **8**:277–299.
39. Rapaport, E., A. Garcia-Blanco, and P. C. Zamecnik. 1979. Regulation of DNA replication in S phase nuclei by ATP and ADP pools. *Proc. Natl. Acad. Sci. USA* **76**:1643–1647.
40. Reichard, P. 1978. From deoxynucleotides to DNA synthesis. *Fed. Proc.* **37**:9–14.
41. Reichard, P. 1988. Interactions between deoxyribonucleotide and DNA synthesis. *Annu. Rev. Biochem.* **57**:349–374.
42. Sargent, R. G., and C. K. Mathews. 1987. Imbalanced deoxyribonucleoside triphosphate pools and spontaneous mutation rates determined during dCMP deaminase-defective bacteriophage T4 infections. *J. Biol. Chem.* **262**:5546–5553.
43. Slabaugh, M. B., T. L. Johnson, and C. K. Mathews. 1984. Vaccinia virus induces ribonucleotide reductase in primate cells. *J. Virol.* **52**:507–514.
44. Small, M. B., E. Simmons, K. K. Jha, H. L. Ozer, L. A. Feldman, J. Pyati, S. Hann, and D. Dina. 1984. Genetic analysis of the transformed phenotype in mouse cells. *In* M. L. Pearson and N. L. Sternberg (ed.), *Gene transfer and cancer*. Raven Press, New York.
45. Thelander, L., and P. Berg. 1986. Isolation and characterization of expressible cDNA clones encoding the M1 and M2 subunits of mouse ribonucleotide reductase. *Mol. Cell. Biol.* **6**:3433–3442.
46. Weinberg, G., B. Ullman, and D. W. Martin, Jr. 1981. Mutator phenotypes in mammalian cell mutants with distinct biochemical defects and abnormal deoxyribonucleoside triphosphate pools. *Proc. Natl. Acad. Sci. USA* **78**:2447–2451.
47. Yang-Feng, T. L., D. E. Barton, L. Thelander, W. H. Lewis, P. R. Srinivasan, and U. Francke. 1987. Ribonucleotide reductase M2 subunit sequences mapped to four different chromosomal sites in humans and mice: functional locus identified by its amplification in hydroxyurea-resistant cell lines. *Genomics* **1**:77–86.