MOLECULAR AND CELLULAR BIOLOGY, Dec. 1986, p. 4594–4601
0270-7306/86/124594-08$02.00/0

Vol. 6, No. 12

Identification of Temperature-Sensitive DNA$^-$ Mutants of Chinese Hamster Cells Affected in Cellular and Viral DNA Synthesis

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Received 17 March 1986/Accepted 11 July 1986

We described a strategy which facilitates the identification of cell mutants which are restricted in DNA synthesis in a temperature-dependent manner. A collection of over 200 cell mutants temperature-sensitive for growth was isolated in established Chinese hamster cell lines (CHO and V79) by a variety of selective and nonselective techniques. Approximately 10% of these mutants were identified as ts DNA$^-$ based on differential inhibition of macromolecular synthesis at the restrictive temperature (39°C) as assessed by incorporation of [^{3}H]thymidine and [^{35}S]methionine. Nine such mutants, selected for further study, demonstrated rapid shutoff of DNA replication at 39°C. Infections with two classes of DNA viruses extensively dependent on host-cell functions for their replication were used to distinguish defects in DNA synthesis itself from those predominantly affecting other aspects of DNA replication. All cell mutants supported human adenovirus type 2 (Ad2) and mouse polyomavirus DNA synthesis at the permissive temperature. Five of the nine mutants (JB3-B, JB3-O, JB7-K, JB8-D, and JB11-J) restricted polyomavirus DNA replication upon transfection with viral sequences at 33°C and subsequent shift to 39°C either before or after the onset of viral DNA synthesis. Only one of these mutants (JB3-B) also restricted Ad2 DNA synthesis after virion infection under comparable conditions. No mutant was both restrictive for Ad2 and permissive for polyomavirus DNA synthesis at 39°C. The differential effect of these cell mutants on viral DNA synthesis is expected to assist subsequent definition of the biochemical defect responsible.

Temperature-sensitive mutants have been a powerful tool in understanding the molecular mechanism of many cellular processes. The paucity of such conditionally lethal variants has hampered severely the analysis of DNA replication in higher eucaryotes. For this discussion we wish to make a distinction between DNA replication which involves the totality of functions which are required for reproduction of a cellular (or viral) genome and the biochemistry of DNA synthesis itself. Whereas several temperature-sensitive mutants affected in progression through G1 of the cell cycle have been investigated within collections of mutants in Syrian hamster BHK cells (2, 29), rat 3Y1 fibroblasts (31), and other cell lines (23, 46), rarely have mutants been found to be defective in DNA synthesis. Temperature-sensitive mutants with biochemical defects in DNA synthesis have been identified in mouse cell lines such as L (5, 10, 43), BALB/3T3 (37, 48), and FM3A (27). Those in the former two mouse cell lines may, however, be interrelated since they do not complement each other in cell hybrids (14). Methods of selection commonly utilized to isolate temperature-sensitive mutants have involved prolonged exposure to restrictive temperature, and the mutants in DNA synthesis described above typically lose viability under such conditions. Taken together, these results indicate limitations in the prevailing strategies used to isolate and identify such mutants.

Although Chinese hamster ovary (CHO) cells have often been used for isolation of mutants, few mutants directly affecting DNA synthesis have been described (24, 38), and no studies have been reported on extensive isolation of temperature-sensitive mutants in CHO cells. Several considerations indicated that this cell line would be a good choice to serve as the parent of a mutant collection. First, it has favorable growth properties over a broad range of temperatures (33 to 41°C), and its efficiency of colony formation approaches 100%. Hirschberg and Marcus (11) have demonstrated the efficiency of direct screening by replica plating for identification of mutants temperature sensitive for growth in V79 cells. Replica plating has been successfully applied to CHO cells as well (6). Second, autosomal recessive mutants dispersed throughout the genome have been isolated in CHO cells at unusually high frequency (9, 36). If genes important to DNA synthesis reside in regions of functionally hemizygous chromosomes, mutants at these loci should be easier to obtain. Third, human adenovirus (type C) and the papovaviruses simian virus 40 and polyomavirus replicate in CHO cells. Such viruses have been well characterized genetically and biochemically, including the availability of in vitro assays for viral DNA synthesis (3, 20). Adenovirus type 2 (Ad2) synthesizes its DNA in CHO cells normally, although it is delayed and no virions are formed (21; Radna et al., manuscript in preparation). CHO cells are resistant to infection by papovavirus virions. However, polyomavirus DNA is replicated efficiently when introduced into CHO cells by DNA-mediated gene transfer; simian virus 40 DNA is replicated less well (19). Cell mutants producing a thermolabile protein necessary for DNA synthesis might be more readily identified if the protein was used in viral DNA synthesis as well. For example, a determination of the biochemical lesion would be much simplified for mutants that will not support viral DNA synthesis in vitro and could be complemented by wild-type extracts.

We isolated over 200 mutants of CHO and V79 cells which are temperature sensitive for growth by utilizing a variety of selective and nonselective strategies. A minority (approximately 10%) were identified which preferentially affect DNA replication. These were analyzed further as to whether the cellular defect affected polyomavirus and adenovirus DNA synthesis in an effort to identify those mutants likely to be defective in macromolecules involved in DNA synthesis.

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Five of nine mutants restrict polyomavirus DNA synthesis at 39°C, the restrictive temperature for cellular DNA synthesis; one of these restricts adenovirus as well.

MATERIAL AND METHODS

Cells and culture methods. Two sublines of Chinese hamster ovary fibroblasts were employed: CHO-S (Toronto) was obtained from P. Stanley (39), and CHO-K1 (Denver) was from L. Chasin (16). CHO thy⁻49 (26), a mutant of the Toronto subline with disturbed nucleotide pools resulting in a mutator phenotype, was obtained from M. Meuth. V79 Chinese hamster lung cells (4) (clone 380-6) were obtained from U. Francke. TsH1, a mutant of the Toronto subline with a temperature-sensitive leucyl-tRNA synthetase (42), was obtained from L. Thompson. TsH1 TG^r, resistant to 5×10^{-5} M thioguanine, was isolated in this laboratory. Cells were cultured in Dulbecco modified Eagle medium supplemented with proline or an equal mixture of Dulbecco modified Eagle medium-proline and F12 Hams medium (DF medium) with 10% newborn calf serum (M.A. Bioproducts) as previously described (19). To facilitate recovery of large numbers of mutants, individual colonies were picked with 6-in. (15-cm) wood applicators (Thomas) for transfer to other dishes.

Virus and viral DNA. Ad2 virus stocks were prepared in HeLa cells by conventional methodology, and titers for infectious units were determined by indirect immunofluorescence as described elsewhere (Radna et al., in preparation). Recombinant DNAs containing viral sequences were propagated and harvested from DH-1 (*dam*$^+$) bacteria by standard methodology (22). pLA1 contains the 0 to 9.4 *Bgl*II fragment of the Ad5 genome cloned into pAT (41). It was kindly supplied by B. Stillman. p53A6.6 DNA contains a complete polyomavirus genome cloned at the *Bam*HI site in pAT153 and was kindly provided by R. Kamen (45). All recombinant DNAs were used in accordance with National Institutes of Health guidelines.

Mutagenesis. Exponentially growing subconfluent cultures, incubated at 39°C for 3 days to minimize any preexisting temperature-sensitive cells in the population, were exposed to 250 μg of ethyl methanesulfonate (Eastman Kodak Co., Rochester, N.Y.) per ml for 16 h at 33°C, followed by 3 days of incubation to allow phenotypic expression and recovery from the mutagen. Approximately 20% of the cells survived this treatment. Cultures were suspended in trypsin-EDTA, diluted 10-fold, and subcultured overnight at 33°C immediately before the procedures for identification of mutants.

Single selection with prolonged exposure to selective conditions. Mutagenized cells were seeded at 2×10^5 cells per 100-mm dish in Dulbecco modified Eagle medium-proline, incubated at 33°C for 24 h, and then shifted to 39°C. Sixteen hours later, 2×10^{-5} M bromodeoxyuridine (BUdR) was added for 48 h, followed by Hoechst 33258 (1.0 μg/ml) for 2 h and exposure to long-wave UV light for 60 s. Cultures were refed with DF medium and incubated at 33°C to allow colonies to form (2 weeks). A total of 20 to 50 colonies were observed per dish. A representative number was picked, grown at 33°C, and subcultured at 33 and 39°C to identify putative temperature-sensitive mutants.

Multiple selections with brief exposures to selective conditions. Mutagenized cells growing at 33°C were refed with prewarmed medium at 37.5 to 40.5°C. After 2 to 4 h at the restrictive temperature, BUdR (2×10^{-5} M) was added for an additional 2 to 4 h, followed by Hoechst 33258 and UV light as above. Cultures were refed with DF medium and incubated for 16 h at 33°C, and the procedure was repeated. Each cycle resulted in the survival of 1 to 3% of the viable cells present before selection. After three cycles of selection, colonies were allowed to grow at 33°C, picked, and tested as above.

Nonselective replica plating in microwells. Mutagenized cells were diluted in DF medium to 20 cells per ml. Samples of 200 μl (four cells per well) were distributed into 96-well microtiter plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.), resulting in colonies in 45 to 70% of the wells. After 7 days at 33°C, medium was aspirated, 150 μl of trypsin-EDTA was added to each well, and plates were incubated at 33°C until the cells were dispersed. A small volume was transferred into each of two 96-well dishes via a 96-well replicator (noncommercial design), one incubated at 33°C, the other at 39°C. Four days later, individual wells in plates at 39°C were examined under low magnification (×40). Wells with aberrant or no cells were compared with the replica at 33°C. Putative mutants at 33°C were suspended in trypsin-EDTA and subcultured in 12-well plates at the two temperatures for confirmation of the temperature-sensitive phenotypes.

Determination of rates of DNA and protein synthesis. Cells (5×10^4) in 3 ml were seeded into flat-based culture tubes (Nunc 1409) in the prone position and incubated for 24 h at 33°C. Medium (2 ml) was added, and tubes were shifted to an upright position to mimic later labeling conditions (some mutants had altered morphology and might be less adherent) for an additional 16 h. Subsequently, one set of tubes was shifted to a 39°C water bath, the rest remaining at 33°C. At appropriate intervals thereafter, duplicate cultures were pulse-labeled at 33 and 39°C: the growth medium was aspirated and replaced with methionine-free Dulbecco modified Eagle medium-proline (which lacks thymidine as well) supplemented with 1 μCi of [^{35}S]methionine per ml (800 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and [^{3}H]thymidine (74 Ci/mmol; New England Nuclear Corp.). After 2 h of incubation, labeling medium was removed, the monolayer was washed with phosphate-buffered saline, and the cells were lysed by the addition of 1 ml of lysis solution (0.1% sodium dodecyl sulfate, 10 mM Tris [pH 7.5], 1 mM EDTA). Samples were precipitated with trichloroacetic acid, collected onto GF/A filters (Whatman, Inc., Clifton, N.J.), and dissolved with an NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.). Radioactivity was determined in Liquifluor (New England Nuclear Corp.) by liquid scintillation spectroscopy after double-label correction as previously described (47).

Determination of adenovirus DNA replication. Cell lines were seeded at 3×10^5 cells per 60-mm dish and infected the following day with Ad2 at a multiplicity of infection of between 400 and 700 for CHO or 50 and 75 for V79. Cultures in duplicate were incubated at 33 or 39.5°C. At various times postinfection (p.i.), viral DNA was selectively extracted by a modified Hirt procedure which included pronase in the lysis buffer (18). Hirt supernatants were extracted with organic solvents, and DNA was precipitated in 2.5 volumes of cold ethanol. Dried pellets were dissolved in 50 μl of 10 mM Tris–1 mM EDTA (pH 7.9) per culture. Dot-blot analysis was performed as described by Kafatos et al. (15). Briefly, 15 μl of each DNA sample was denatured in 0.3 M NaOH, serially diluted by one-third, and spotted onto a nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) filter with the Schleicher & Schuell manifold apparatus. The plasmid pLA1, nick translated with [^{32}P]dATP and

[^{32}P]dCTP [New England Nuclear Corp.) by the method of Rigby et al. (33), was used for hybridization as previously described (19). Known amounts of purified pLA1 DNA were used as a quantitation standard.

Determination of polyomavirus DNA replication. Cell lines were seeded at 5×10^5 cells per 60-mm dish and transfected with 1 μg of p53A6.6 DNA (44) plus 9 μg of carrier (calf thymus) DNA by the calcium phosphate coprecipitation technique (8) for 16 h as previously described (19). Cultures in duplicate were incubated and processed as in the preceding section except that conventional Hirt extractions were performed (12). Replicated recombinant DNA was identified after digestion with *Sal*I and *Dpn*I (Promega Biotech, Madison, Wis.) under conditions specified by the supplier and quantitated by Southern analysis with nick-translated p53A6.6 DNA (19).

RESULTS

Isolation of temperature-sensitive mutants by drug selection. Mutants have been isolated with a variety of selective agents (see references 1, 23, and 46 for reviews). The commonly utilized selection regimens depend on slowing (or arrest) of DNA synthesis of the rare temperature-sensitive mutants after shift of the mass population to an elevated (restrictive) temperature. The culture is then exposed to an agent which will kill the wild-type cells that vigorously incorporate it into their DNA. Upon removal of the agent and return of the culture to the lower (permissive) temperature, the surviving population becomes enriched for mutants. High-specific-activity [^{3}H]TdR, arabinofuranosylcytosine, and fluorodeoxyuridine have been successfully used for recovery of temperature-sensitive mutants (28, 43). BUdR in conjunction with Hoechst 33258 and visible light has also been shown to be highly toxic to rapidly growing cells (17, 40). We compared these agents for their kinetics of cell killing and found that BUdR (with dye and UV light) is the most efficient. For example, exposure to BUdR at 39°C for 40 h reduced wild-type survival by 10,000- to 100,000-fold, and exposure for 6 to 8 h reduced survival by 100-fold. A reconstruction experiment with TsH1 TG^r as a prototype mutant defective in DNA synthesis (albeit indirectly) confirmed that exposure of a mixed population of cells to BUdR at 39°C for 6 h results in an enrichment (14-fold) for the mutant. We therefore elected to use three cycles of such short-term exposure to BUdR or a single long-term exposure (48 h) for mutant isolation.

Ethyl methanesulfonate mutagenesis was performed in 25 separate dishes to maximize deriving mutants of independent origin. Each culture was then split, and the sister subcultures were subjected to different selections, using restrictive temperatures of 37.5, 39, and 40.5°C and different lengths of incubation in BUdR-containing medium as described in Materials and Methods. Mutants temperature sensitive for growth had either of two phenotypes: cells stopped growing at 39°C, most often followed by loss of attachment 1 to 3 days later (designated RD for rapid death), or cells continued growing very slowly at 39°C and remained attached, forming microcolonies (designated SG for slow growth). Most colonies picked from either selection showed wild-type growth at 39°C, as expected, and were discarded.

All temperature-sensitive cell lines were examined for their kinetics of macromolecular synthesis at the permissive and restrictive temperatures. Since the primary goal was to identify mutants with rapid onset of the temperature-sensitive defect, we determined the rate of DNA synthesis (incorporation of [^{3}H]TdR) only within the first 24 h after shift of growing cultures from 33 to 39°C. As a control, cells were simultaneously monitored for protein synthesis (incorporation of [^{35}S]methionine). This permitted us to distinguish at the outset those mutants which were secondarily affected in DNA synthesis. Mutants were divided into four categories based on these results (Table 1). Category 1, designated ts DNA$^-$, shows reduced incorporation of [^{3}H]TdR at 39°C relative to 33°C without concomitant decline in [^{35}S]methionine incorporation. Category 2, designated slow ts pro$^-$, shows a parallel decline in protein and DNA synthesis. The fall in DNA synthesis is presumed secondary to inhibition of protein synthesis. Category 3, designated rapid ts pro$^-$, mimics a mutant in leucyl-tRNA synthetase (TsH1) already described which shows a marked inhibition of protein and DNA synthesis almost immediately after shift to 39°C. Category 4, designated wild type, shows no obvious defect in protein or DNA synthesis. This would include mutants with a delayed onset of phenotype requiring more than one cell generation at the restrictive temperature or mutants in cellular processes that do not rapidly affect protein or DNA synthesis. As expected, the ts DNA$^-$ mutants represent a minority of the total mutants isolated; they display both RD and SG growth patterns.

TABLE 1. Phenotypes of temperature-sensitive mutants derived by selection with BUdR

Selection	Total no. of mutants	Phenotypic category			
		Wild-type-like	ts DNA$^-$	Slow ts pro$^-$	Rapid ts pro$^-$
Multiple short selections[a]	84	26	5	31	22
Single long selection[b]	43	27	4	12	0

[a] A total of 10^7 CHO cells (from 25 individual mutageneses) was subjected to one of several selective conditions involving 4 to 6 h at different restrictive temperatures in 2×10^{-5} M BUdR, Hoechst 33258, and 60 s of exposure to UV light as described in the text. Clones formed at 33°C were picked, and those exhibiting temperature-sensitive growth were tested for macromolecular synthesis. Assignment of phenotypes was based on [^{3}H]TdR and [^{35}S]methionine incorporation as described in the text.

[b] Mutagenized (5×10^5) cells were incubated for 16 h at the restrictive temperature and then for 48 h with 2×10^{-5} M BUdR followed by Hoechst dye and UV treatment as described in footnote *a*. Phenotypes of temperature-sensitive colonies were assigned as described in footnote *a*.

Isolation of temperature-sensitive mutants by replica plating. A difficulty associated with selective procedures as a method of mutant isolation is that the deleterious exposure

TABLE 2. Phenotypes of temperature-sensitive mutants derived by replica plating[a]

Parent strain	No. of colonies observed	No. of mutants found	Mutant frequency[b]	Phenotypic category			
				Wild type-like	ts DNA$^-$	Slow ts pro$^-$	Rapid ts pro$^-$
CHO-K1	1,645	18	1/92	7	4	7	0
CHO-S	2,170	22	1/97	9	4	9	0
CHO thy$^-$49	1,542	10	1/154	6	2	2	0
V79	1,120	10	1/112	5	2	3	0

[a] Mutagenized cells were seeded into 96-well plates and screened for temperature-sensitive mutants as described in the text. Phenotypes of putative mutants were assigned as described in Table 1, footnote *a*.

[b] Total, 1/108.

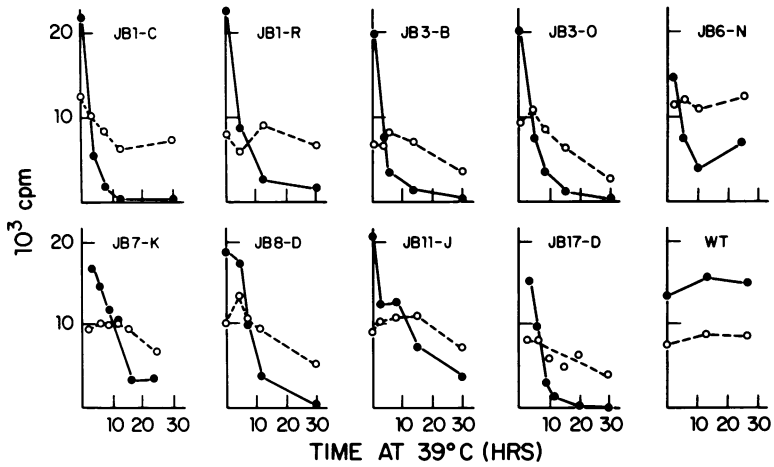

FIG. 1. Macromolecular synthesis in temperature-sensitive mutants. Cultures of each cell line were seeded and incubated at 33°C for 40 h; half were shifted to 39°C at time 0. Cultures were pulse-labeled with [^{3}H]TdR (●) or [^{35}S]methionine (○) at appropriate intervals and analyzed for radioactivity as described in the Materials and Methods. WT, wild type.

to restrictive temperature can be ameliorated but not eliminated. Our reconstructive experiments with TsH1 demonstrated that even our least restrictive selections killed temperature-sensitive mutants in the population (data not shown). Therefore, we isolated a series of mutants without selection by a replica plating technique similar to that described by Marcus et al. (23). Four cell lines were used in these experiments for comparative purposes: CHO-K1 and CHO-S, which were also the parents of the mutants derived by selection; CHO thy⁻49, a cell line with a mutator phenotype owing to disturbance of intracellular nucleotide pools; and V79, a Chinese hamster lung fibroblast cell line. The results are listed in Table 2. The CHO sublines and V79 yielded mutants at a rate of one mutant per 100 clones examined, consistent with the previous results with V79. Slightly fewer mutants arose in CHO thy-49. The distribution of mutants obtained was similar to that observed in selection experiments, except for the absence of the tsH1-like phenotype. ts DNA⁻ variants represent 20% of the mutants isolated by this technique, which compares favorably with selection experiments.

Phenotypic properties of ts DNA⁻ mutants. Twenty putative ts DNA⁻ mutants from the initial series of labeling experiments were retested at multiple times after shift to the restrictive temperature (0, 2, 5, 10, 15, and 25 h). Nine which showed the most pronounced phenotype were recloned and subjected to further study. Figure 1 depicts the data obtained on macromolecular synthesis. All the mutants showed a rapid inhibition of [^{3}H]TdR incorporation with delayed or no inhibition of protein synthesis upon shift to 39°C. Seven mutants showed progressive decreases from the initial time; two (JB11-J and JB8-D) maintained high incorporation for 5 to 10 h, followed by a progressive inhibition.

Table 3 summarizes their growth properties at 39°C. All the mutants showed normal frequencies of colony formation at 33°C. Some, however, exhibited moderate colony formation at 39°C (1 of 10^4 cells). This occurred particularly in the mutants that were derived from long selection and appears to be a phenotypic property since retesting the survivors after 2 weeks at 39°C led to comparable efficiencies of colony formation at the two temperatures (data not shown). Revertants, recognizable as large wild type-like colonies, occurred at frequencies of 10^{-5} to 10^{-6} for JB3-B, JB11-J, and JB17-D.

Viral DNA replication in ts DNA⁻ mutants. In an effort to facilitate the further characterization of these mutants, we examined the effect of the mutation on the ability of the cells to support viral DNA synthesis at the permissive and restrictive temperatures. This approach offers several potential advantages. First and foremost, it identifies mutants amenable to intensive biochemical investigations. However, the approach also permits an initial characterization of the primary defect as well, limiting greatly the number of possible biochemical bases for the ts DNA⁻ phenotype (44). A

TABLE 3. Growth properties of temperature-sensitive mutants of CHO cells[a]

Cell line	Parent strain	Selection[b]	Efficiency of colony formation (%) at:		Ratio (33°C/39°C)	Colony morphology at 39°C
			33°C	39°C		
JB1-C	CHO-K1	I	86	0.01	1×10^4	Smaller size than 33°C
JB1-R	CHO-S	I	48	0.005	$\geq 1 \times 10^4$	Normal appearance
JB3-B	CHO-S	II	58	0.0002	3×10^5	Revertants only
JB3-O	CHO-S	I	60	0.001	$\geq 6 \times 10^4$	Microcolonies only; no revertants
JB6-N	CHO-S	I	48	0.005	$\geq 1 \times 10^4$	Microcolonies only; no revertants
JB7-K	CHO-S	I	60	0.005	$\geq 1 \times 10^4$	Microcolonies only; no revertants
JB8-D	CHO-K1	II	100	0.005	$\geq 2 \times 10^4$	Microcolonies only; no revertants
JB11-J	CHO-S	III	86	0.0001	1×10^6	Revertants only
JB17-D	V79	III	90	0.0001	1×10^6	Revertants only

[a] Cells were seeded at various densities to determine the efficiency of colony formation at 33 and 39°C.
[b] I, Single long selection; II, multiple short selections; III, nonselective replica plating.

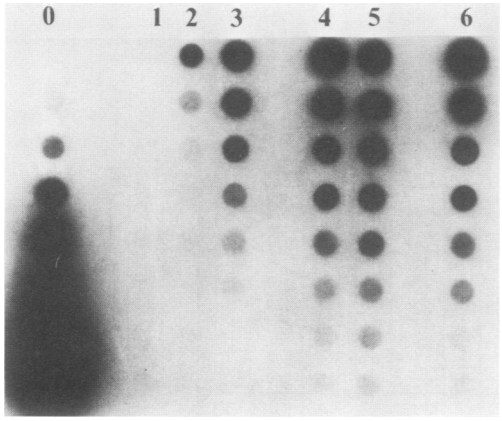

FIG. 2. Ad2 DNA replication in a temperature-sensitive mutant nonrestrictive for viral DNA synthesis. Cells were infected with Ad2 virions, and viral DNA was extracted and quantitated by dot-blot hybridization with nick-translated pLA1 DNA as described in Materials and Methods. Lanes: 0, pLA1 DNA, 10-fold-increasing concentrations from 0.1 pg to 1.0 mg; 1, uninfected cells (for lanes 1 to 6 DNA was serially diluted threefold); 2, cells infected for 24 h at 33°C; 3, cells infected for 24 h at 39°C; 4, cells infected for 48 h at 33°C; 5, cells infected for 48 h at 39°C; 6, cells infected for 24 h at 33°C followed by 24 h at 39°C.

differential effect on the two classes of viruses could be particularly informative.

Ad2 DNA replication in ts DNA$^-$ mutants. Mutant cell lines were infected with Ad2 virions at 33°C for 2 h and either shifted to 39°C or reincubated at 33°C. At 24 and 48 h p.i., the cells were lysed, and the level of viral DNA was quantitated by the dot-blot hybridization technique. All the mutants tested supported Ad2 DNA synthesis at 33°C. Based on the results of screening at 39°C, the mutants were placed into two groups. The first group included those mutants which exhibited no defect in Ad2 DNA synthesis. A representative of this group is shown in Fig. 2. The accumulation of progeny viral DNA occurred equally well at 39°C as at 33°C. In some mutant cell lines, as well as wild type, more Ad2 progeny DNA accumulated at 39°C than at 33°C. Viral DNA was detectable by 24 h p.i. at both temperatures (lanes 2 and 3), and it increased markedly between 24 and 48 h p.i. (lane 4 versus lane 2 at 33°C; lane 5 versus lane 3 at 39°C). Infected cells incubated for 24 h at 33°C followed by 24 h at 39°C showed levels equal to or greater than those seen after 48 h at 33°C (compare lane 6 with lane 4). This result is consistent with pulse-labeling experiments which found that maximal rates of viral DNA synthesis occur at 30 to 35 h p.i. in wild-type CHO cells (21; Radna et al., in preparation).

Five of the mutants fell into the second group. These mutants showed some inhibition of viral DNA synthesis at 24 or 48 h p.i. at 39°C. The reduced level of viral DNA could be explained by multiple mechanisms, some of which would be expected to be secondary to the arrest of cell growth. To distinguish further between such and a primary defect, cultures were infected at 33°C, incubated at 33°C for 24 h, and shifted to 39°C for an additional 24 h. Four of these showed no significant difference between cultures incubated for 24 h at 33°C plus 24 h at 39°C and those incubated at 33°C for 48 h, indicating that the observed defect is probably secondary. For example, there may be a loss of progeny viral DNA owing to cell death or loss after 2 days at the restrictive temperature or both.

One mutant (JB3-B), however, demonstrated a marked inhibition at 39°C under all conditions (Fig. 3). This result was most dramatic when cultures were maintained at 39°C throughout infection (lanes 2 and 4). However cultures which were incubated initially at 33°C before shift to 39°C (lane 5) showed a 10-fold reduction as compared with those incubated continuously at 33°C (lane 3). This result is indicative of a rapid, primary defect in Ad2 DNA synthesis.

Polyomavirus DNA replication in ts DNA$^-$ mutants. Since the papovaviruses require more host factors than the adenoviruses for their efficient replication, the mutants were also examined for their ability to support polyomavirus DNA synthesis at both the permissive and restrictive temperatures. Previous studies from this laboratory had shown that CHO cells are refractory to polyomavirus infection by virions; therefore, transfection with p53A6.6 was used to assess the nine ts DNA$^-$ mutants by Southern analysis. New viral DNA synthesis could be distinguished from persistent input DNA by resistance to the enzyme *Dpn*I, which digests only the methylated unreplicated DNA. For convenience of quantitation, all viral forms were converted to linear form III by digestion with *Sal*I. Cultures were incubated at either 33 or 39°C for 1 to 4 days. For reasons discussed above, cultures were also incubated initially at 33°C for 1 or 2 days followed by a subsequent shift to 39°C. Newly replicated p53A6.6 DNA was not detectable at 1 day posttransfection at either 33 or 39°C in any cell line tested (data not shown). However, all the mutants subsequently showed polyomavirus replication at 33°C. Most mutants also supported replication at 39°C as well as wild type (Fig. 4; adjacent lanes corresponded to duplicate transfected cultures). Viral DNA is evident at 48 h at 33°C and thereafter in the higher-molecular-weight region as a single band at 8.8 kilobases. The multiple smaller fragments represent *Dpn*I-digested (input) sequences. The level of viral DNA increased between 48 and 72 h at 33°C (compare lanes 1 and 2 with lanes 7 and 8). As expected, viral DNA synthesis was enhanced at 39°C; cells incubated at 39°C for 48 h (lanes 5 and 6) or 24 h at 33°C followed by 24 h at 39°C (lanes 3 and 4) had more viral synthesis than those incubated continuously at 33°C.

Five of the mutants, JB3-B, JB3-O, JB7-K, JB8-D, and

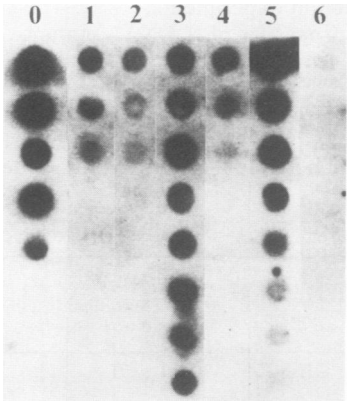

FIG. 3. Ad2 DNA replication in a temperature-sensitive mutant restrictive for viral DNA synthesis. JB3-B cells were infected with Ad2 virions, and viral DNA was extracted and quantitated as described in the legend to Fig. 2. Lanes: 0, pLA1 DNA, 10-fold-decreasing concentrations from 1.0 mg to 0.1 pg; 1, cells infected for 24 h at 33°C; 2, cells infected for 24 h at 39°C; 3, cells infected for 48 h at 33°C; 4, cells infected for 48 h at 39°C; 5, cells infected for 24 h at 33°C followed by 24 h at 39°C; 6, uninfected cells.

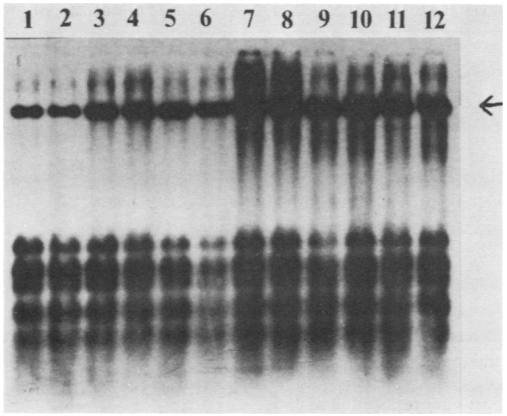

FIG. 4. Polyomavirus DNA replication in a temperature-sensitive mutant nonrestrictive for viral DNA synthesis. Cells were transfected with p53A6.6 and viral DNA was extracted and quantitated by Southern analysis with nick-translated p53A6.6 DNA as described in Materials and Methods. The arrow corresponds to linear unit-length p53A6.6. Lanes: 1 and 2, infected for 48 h at 33°C; 3 and 4, infected for 24 h at 33°C followed by 24 h at 39°C; 5 and 6, infected for 48 h at 39°C; 7 and 8, infected for 72 h at 33°C; 9 and 10, infected for 48 h at 33°C followed by 24 h at 39°C; 11 and 12, infected for 24 h at 33°C followed by 48 h at 39°C. Adjacent lanes correspond to duplicate transfected cultures.

JB11-J, demonstrated an inhibition in polyomavirus DNA replication at 39°C. An example of one of these (JB7-K) can be seen in Fig. 5. All five showed little or no detectable levels of replication when incubated for 48 h at 39°C (lanes 5 and 6). Moreover, they also showed diminished accumulation of viral DNA after shift of cultures from 33 to 39°C as can be seen by comparing lanes 1 and 2 (48 h at 33°C) with lanes 3 and 4 (24 h at 33°C plus 24 h at 39°C) and lanes 7 and 8 (72 h at 33°C) with lanes 9 and 10 (48 h at 33°C plus 24 h at 39°C). Some residual viral DNA synthesis at 39°C is evident since, in each case, the cultures incubated at 33°C plus 39°C had more DNA than those harvested before the shift to 39°C (e.g., compare lanes 9 and 10 with lanes 1 and 2). Thus, these five mutants exhibit a marked defect in polyomavirus DNA replication at the temperature restrictive for cellular DNA replication.

DISCUSSION

The isolation of many temperature-sensitive mutants and the use of simple (viral) model systems to characterize them, the primary approach which led to the fruitful study of procaryotic DNA replication, was applied in our study of eucaryotic DNA replication. Previous mutant isolations in diverse cell lines have resulted in several large collections of temperature-sensitive mutants (1, 23). However, few of these mutants have thus far proved to be defective in DNA synthesis. One possible reason for this phenomenon is suggested by genetic studies done with ts DNA⁻ mutants. In mouse cells, independent mutants such as ts2 (14), ts20 (J. J. Donegan and H. L. Ozer, unpublished data), and tsC1 (7) are clustered on the X chromosome and fail to complement each other by somatic cell hybridization, thus suggesting repeated isolation of mutations in the same gene or related genes. Nishimoto and colleagues (30) observed a similar phenomenon in Syrian hamster cells. They initially analyzed their collection of BHK temperature-sensitive mutants by somatic cell hybridization and found that approximately 50% were members of a single complementation group, which was corrected by the wild-type X chromosome. It is likely that these BHK mutants are ts DNA⁻ because of arrest in the G1 phase of the cell cycle since a prototype does not complement ts 13, which arrests in G1 (25). CHO cell lines were selected as parents of our temperature-sensitive mutant cell lines because of the previous demonstration of regions of functional hemizygosity (36). It was anticipated that these regions could be exploited to derive increased numbers of mutations in autosomal genes required for DNA synthesis. Multiple sublines were also examined.

Another possible explanation for the paucity of mutants in DNA synthesis is the long exposure of the cell population to the restrictive temperature during the selection process. Such a protocol would tend to select against any mutants which exhibit a rapid inhibition of DNA synthesis. Indeed, mutants previously reported from this laboratory and elsewhere often lose viability when exposed to the restrictive temperature for periods exceeding one cell cycle. In an effort to ameliorate this condition in our study, the selection methodology was changed. A regimen involving multiple short (4 to 6 h) exposures to the restrictive temperature was used. Such conditions would be expected to have minimal lethality as they resulted in only a 100-fold killing of wild-type cells per cycle of selection with BUdR. Additional experiments involved a single exposure of 48 h. Both approaches resulted in significant numbers of ts DNA⁻ mutants (6% for the multiple short selections and 9% for the single long selection) (Table 1). Since ameliorating the selective conditions might still not allow isolation of rapid-onset ts DNA⁻ mutants in great numbers, we also used nonselective conditions to isolate temperature-sensitive mutants. Although no enrichment for defects in DNA synthesis might be expected, we found that a relatively high percentage (20%) of the mutants temperature sensitive for growth were ts DNA⁻ mutants. Viability studies with ts DNA⁻ mutants isolated in this study confirm the expectation that there is cell lethality associated with prolonged exposures to the restrictive temperature (data not shown).

Nine of the ts DNA⁻ mutants isolated in this study, including representatives from all three isolation regimens, were chosen for further analysis. The kinetics of DNA synthesis at the restrictive temperature differed for some of the mutants (e.g., a delay in the onset of inhibition). No

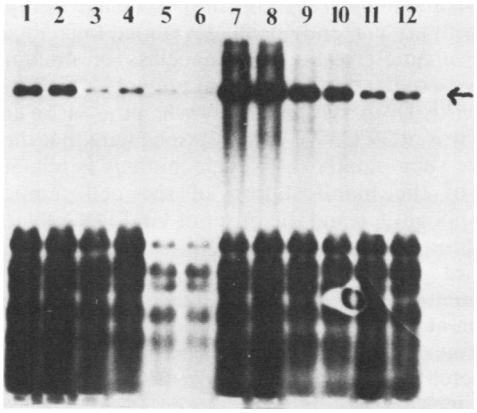

FIG. 5. Polyomavirus DNA replication in a temperature-sensitive mutant restrictive for viral DNA synthesis. JB7-K cells were transfected and analysis was performed as described in the legend to Fig. 4.

obvious correlation exists between these kinetics of DNA synthesis in an individual mutant and the regimen used to isolate it, although the single long selection tended to result in mutants which showed a greater persistence of limited cell growth as evidenced by the formation of microcolonies at the restrictive temperature.

Our overall goal was, however, the isolation of mutants temperature sensitive in DNA synthesis itself, which would be expected to be a subset among these ts DNA$^-$ mutants. Therefore, it would be most important to identify those mutants which involve macromolecules which are most relevant to cellular DNA synthesis in the S phase, i.e., replicon initiation and elongation. It would also be advantageous to distinguish those mutants which were unable to complete other stages of the cell cycle, precluding replication of DNA. Each of our ts DNA$^-$ mutants was, therefore, screened for the ability to support viral DNA synthesis at 39°C. Ad2 and polyomavirus were chosen because of the ability of CHO cells to support appreciable levels of viral replication for both (19, 21; Radna et al., in preparation) and the multifold but varied dependence of both viruses on host functions for viral replications. A summary chart of the analysis of the mutants for intracellular viral DNA synthesis is shown in Table 4. The mutants can be classified into three groups. The first includes those mutants which support the replication of both Ad2 and polyomavirus at the restrictive temperature. Four of the mutants fall into this category. The lesion in these mutants may be in a gene not directly involved with DNA synthesis (e.g., cell cycle mutants) or may be in a gene coding for a function required for cellular DNA synthesis but either supplied by the virus itself or not required for viral DNA replication (5, 34, 35).

The second group includes the mutants JB3-O, JB7-K, JB8-D, and JB11-J. These mutants failed to support polyomavirus DNA synthesis at 39°C but did support Ad2 DNA synthesis at wild-type levels at the same temperature. A defect in cell cycle progression is unlikely to be responsible for the temperature-sensitive phenotype. It had been previously demonstrated that only the onset of papovavirus DNA synthesis is cell cycle dependent (32, 37); however, polyomavirus DNA synthesis was inhibited even when infected cultures were shifted to 39°C after its onset. Furthermore, the differential effect on replication of the two viruses precludes that a defect in generating deoxyribonucleotide pools adequate for DNA synthesis is responsible since both viruses depend on cellular nucleotide pools for their synthesis. In vivo and in vitro systems for polyomavirus replication in CHO cells are currently being developed to facilitate more definitive characterization of this class of mutants. One might have expected to have found mutants which restricted polyomavirus DNA synthesis only when the entire course of infection was at 39°C. We can only speculate that the failure to observe such putative cell cycle mutants is related to the kinetics of the manifestation of the cell temperature-sensitive phenotype and the onset of viral DNA synthesis in an asynchronous cell population.

The third group of mutants has a single member, JB3-B, which failed to support either Ad2 or polyomavirus DNA replication at the restrictive temperature. The defect in Ad2 DNA synthesis has been confirmed by pulse-labeling studies with infected cells shifted to the restrictive temperature at various times p.i. (B. E. Wojcik and H. L. Ozer, unpublished data). As in the discussion of the mutants of the second group, it is unlikely that a defect in cell cycle progression is involved. Since both viruses are dependent on several cell functions, it is not possible to define the exact step at this time; however, it should be noted that a defect in a function relevant to deoxyribonucleotide synthesis is a prime candidate. Direct evidence in regard to that possibility could be obtained by assessment of adenovirus DNA synthesis in vitro and by determination of intracellular nucleotide pool levels. Such experiments are currently in progress.

TABLE 4. Summary of DNA virus replication studies

Cell line	Viral replication[a]	
	Ad 2	Polyomavirus
JB1-C	+	+
JB1-R	+	+
JB3-B	−	−
JB3-O	+	−
JB6-N	+	+
JB7-K	+	−
JB8-D	+	−
JB11-J	+	−
JB17-D	+	+

[a] +, Viral replication is supported at the temperature restrictive for cellular growth (39°C); −, the cell line fails to support viral replication at 39°C. All cell lines support viral replication at 33°C.

In summary, it can be demonstrated that both selective and nonselective regimens result in the isolation of ts DNA$^-$ mutants as defined by differential effects on macromolecular synthesis. In all cases, such mutants represent a minority of those obtained. The majority of the ts DNA$^-$ mutants isolated in this study display a rapid inhibition of DNA replication. It can also be concluded that the use of DNA viruses as simple model systems to facilitate the further characterization of ts DNA$^-$ mutants is a valid and feasible approach. Cell mutants which are also defective in supporting viral DNA synthesis represent a significant proportion of ts DNA$^-$ mutants (five of nine mutants) isolated in this manner. These well-characterized DNA viruses should facilitate the further definitive characterization of the cellular lesion.

ACKNOWLEDGMENTS

We thank Sara Viteri for excellent technical assistance in the isolation of many of the temperature-sensitive mutants.

This work was supported by Public Health Service grant CA 23002 from the National Cancer Institute and the auspices of the RCMI Center for Gene Structure and Function at Hunter College.

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