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Characterization of a *ts* Mutant of BALB/3T3 Cells and Correction of the Defect by In Vitro Addition of Extracts from Wild-Type Cells

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ts20 is a temperature-sensitive mutant cell line derived from BALB/3T3 cells. DNA synthesis in the mutant decreased progressively after an initial increase during the first 3 h at the restrictive temperature. RNA and protein synthesis increased for 20 h and remained at a high level for 40 h. Cells were arrested in S phase as determined by flow microfluorimetry, and DNA chain elongation was retarded as measured by fiber autoradiography. Infection with polyomavirus did not bypass the defect in cell DNA synthesis, and the mutant did not support virus DNA replication at the restrictive temperature. After shift down to the permissive temperature, cell DNA synthesis was restored whereas virus DNA synthesis was not. Analysis of virus DNA synthesized at the restrictive temperature showed that the synthesis of form I and replicative intermediate DNA decreased concurrently and that the rate of completion of virus DNA molecules remained constant with increasing time at the restrictive temperature. These studies indicated that the mutation inhibited ongoing DNA synthesis at a step early in elongation of nascent chains. The defect in virus and cell DNA synthesis was expressed in vitro. [^{3}H]dTTP incorporation was reduced, consistent with the in vivo data. The addition of a high-salt extract prepared from wild-type 3T3 cells preferentially stimulated the incorporation of [^{3}H]dTTP into the DNA of mutant cells at the restrictive temperature. A similar extract prepared from mutant cells was less effective and was more heat labile as incubation of it at the restrictive temperature for 1 h destroyed its ability to stimulate DNA synthesis in vitro, whereas wild-type extract was not inactivated until incubated at that temperature for 3 h.

DNA *ts* mutants have been isolated from cultured mammalian cells. These should eventually prove useful for studying the process of DNA replication in such cells. In procaryotic cell systems, conditional-lethal mutants of bacteria and their phages have enabled the identification of replication proteins, so that we now have detailed knowledge of the multitude of enzymes and cofactors involved in the complex processes of initiation and chain elongation (15, 16). Comparatively little is known about DNA replication in mammalian cells. Despite the availability of mammalian DNA *ts* mutants (12), they have not been used for in vitro studies with complementation assays of the type that have been so successful in procaryotic systems. The DNA *ts* mutants that have been isolated and partially characterized have been divided into two groups: those whose defect prevents the cells from entering S phase, and those whose defect directly affects the replication process (2). The study of the biochemical defects in this second group could be facilitated by the use of DNA viruses. Simian virus 40 and polyomavirus DNA represent simple models for mammalian replicons (4). The steps involved in DNA chain elongation are more easily analyzed with virus DNA than with cell DNA if the parental cell line is permissive for virus DNA synthesis and the infected mutant cell line expresses the DNA *ts* lesion.

Here we present the characterization of a mutant, *ts*20, which is derived from mouse BALB/3T3 cells (13). Our results indicate that its defect inhibited cell DNA replication and that the mutant cells were unable to support virus DNA replication. The defect preferentially blocked a step early in the elongation of nascent virus DNA chains.

Much of what we know about procaryotic DNA replication was obtained from in vitro systems in which the metabolic processing of nucleosides and nucleotides is bypassed and the replicative mechanism can therefore be studied more directly. We have used an in vitro eucaryotic system to study DNA synthesis in *ts*20 cells. In this system, cells are made permeable to nucleoside triphosphates by treatment with a dilute solution of the detergent Brij 58. The requirements for the system and the extent of synthesis have been established. Under appropriate conditions, the cells use exogenous triphosphates for semiconservative DNA synthesis. This synthesis is a continuation of the in vivo process. The initial rate of in vitro synthesis is 30% of the in vivo rate with up to 3% of the genome replicated within a 60-min incubation period. The partially lysed cells retain 70% of their protein content (21). There is evidence from centrifugation studies (8) and from fiber autoradiography experiments (9) that indicates that the in vitro system preserves some initiation as well as elongation, with elongation at up to 50% of the in vivo rate. We show here that the mutant *ts*20 cells expressed their phenotype in this system and that a high-salt protein extract from wild-type 3T3 cells partially restored inactivated DNA synthesis. The active factor in this crude extract from mutant cells was heat labile.

MATERIALS AND METHODS

Cells and virus. The mutant *ts*20 cells were isolated at 33°C from BALB/3T3 cells after mutagenesis with *N*-methyl-*N*^{1}-nitrosoguanidine (Aldrich Chemical Co., Inc.) and exposure at 39°C to medium containing [^{3}H]thymidine (dThd) of high specific activity for greater than one generation time (48 h) by procedures described previously (13, 24, 28). Non-temperature-sensitive revertants (*ts*20R) were isolated from *ts*20 on the basis of spontaneous colony formation at 39°C, using cells grown from low inocula. Cell culture techniques were as previously described (13, 14). The *ts* mutant was main-

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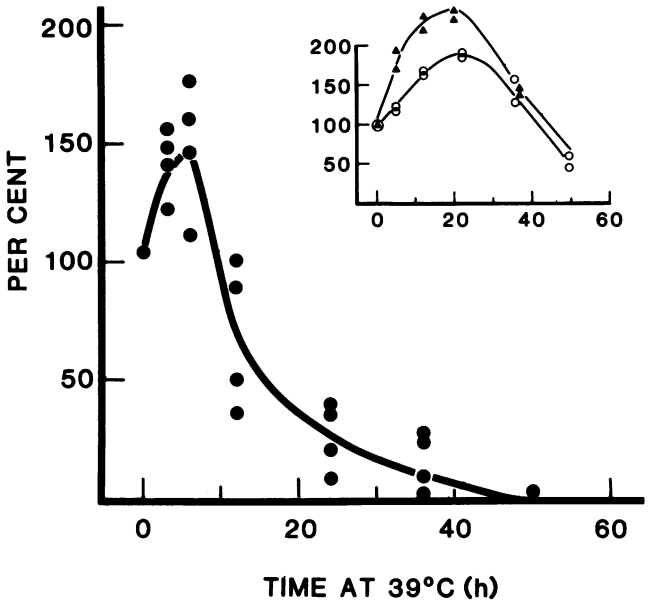

FIG. 1. Macromolecular synthesis in *ts*20 at 39°C. Culture dishes containing 5×10^4 cells were seeded at 33°C and shifted to 39°C after 24 h. At various times, cultures were pulsed for 30 min with [^{3}H]dThd (●), [^{3}H]uridine (○), or ^{3}H-amino acids (▲). Incorporation of radioactivity into TCA-precipitable material was determined as described in the text. The samples at 33°C immediately before temperature shift (0 h) were designated 100%. Points represent individual determinations in the same experiments.

tained at the permissive temperature of 33°C in the Dulbecco-Vogt modification of Eagle medium supplemented with 10% calf serum. The restrictive temperature for these studies was 39°C. The large-plaque strain of polyomavirus was propagated at a low multiplicity of infection on primary mouse kidney cells. Cells were infected with virus at a multiplicity of infection of 10 to 20 at 24 h after the dishes were seeded. All experiments were completed within 2 months after reactivation of the cells from liquid nitrogen. No mycoplasma contamination was detected in cultures of these cells when they were examined by fluorescent staining (1) or by uridine phosphorylase assay (19). Cell number was measured with a Coulter Counter.

Analysis of dATP and dTTP pools. Cellular deoxyribonucleotide triphosphate pools were extracted with 60% methanol as previously described (29). Enzymatic analysis of dATP and dTTP pools was performed with *Escherichia coli* DNA polymerase I (Boehringer Mannheim Biochemicals) and the template poly d(A-T) (Boehringer Mannheim), as described by Lindberg and Skoog (20).

Measurements of macromolecular synthesis. Measurements of macromolecular synthesis were performed as described by Talavera and Basilico (27). About 5×10^4 cells from a culture in exponential growth were plated in 2 ml of fresh medium in 35-mm culture dishes. For measurements of DNA synthesis, [^{3}H]dThd with a specific activity of 74 Ci/mmol was added to a final concentration of 2 μCi/ml. For measurements of RNA synthesis, [^{3}H]uridine with a specific activity of 40 Ci/mmol was added to a final concentration of 2 μCi/ml. For measurement of protein synthesis, a mixture of ^{3}H-amino acids was added to give a final concentration of 2 to 5 μCi/ml in medium from which unlabeled amino acids had been omitted. All radioisotopes were obtained from New England Nuclear Corp. After a 30-min incubation with radioisotopes, 1 ml of lysing solution (50 mM Tris-hydrochloride [pH 7.5], 5 mM EDTA, 150 mM NaCl, and 0.6% sodium dodecyl sulfate) was added to each dish, and the dishes were then left at room temperature for 5 to 10 min. The lysate was precipitated by the addition of 1 ml of 20% trichloroacetic acid (TCA) at 4°C for 20 min. The precipitates were collected on Whatman GF/C filters and washed repeatedly with cold 5% TCA, ethanol, and acetone. Radioactivity was determined in a Beckman LS7500 scintillation counter in 5 ml of toluene-based scintillation fluid. For measurement of cell and virus DNA synthesis in infected cells, mutant cells were infected with polyomavirus and maintained at 33°C or shifted back and forth from 33 to 39°C according to the experimental protocols described below. At different times, cells were pulse-labeled for 2 h with [^{3}H]dThd at 5 μCi/ml (74 Ci/mmol; New England Nuclear Corp.). At the conclusion of the labeling period, virus and cell DNAs were separated by the Hirt procedure (11). The samples were precipitated with trichloroacetic acid (TCA) and processed for liquid scintillation counting.

Flow microfluorimetry (FMF). Estimation of the DNA content per cell was made on cultures grown at 33°C and at various times after shift to the restrictive temperature. Cells were stained with propidium iodide (17) and analyzed with a flow cytometer (1CP 22A; Ortho Instruments).

DNA fiber autoradiography. DNA fiber autoradiography was performed as described previously (10) except when the technique was modified for use with temperature-sensitive cells (6). In brief, this modification involved warming the isotope-containing medium to 39°C before it was added to cell cultures held at that temperature.

Neutral sucrose gradients of virus DNA. Virus DNA extracted by the Hirt procedure (11) was sedimented through neutral sucrose gradients (5 to 20% [wt/vol]) prepared in 0.5 M NaCl–10 mM EDTA–10 mM sodium acetate, pH 6.0. Gradients were centrifuged at 4°C for 3 h at 46,000 rpm in a Beckman SW50.1 rotor. Fractions were collected from the bottom and analyzed for acid-insoluble radioactivity by TCA precipitation. Polyomavirus DNA was run on parallel but separate gradients as a marker.

Determination of rate of completion of virus replicative intermediates in vivo. Mutant and revertant (*ts*20R) cells in 25-ml flasks were infected at 33°C and analyzed for incorporation of [^{3}H]dThd into replicative intermediate molecules at 36 h after infection directly or after shift to 39°C for 1 to 8 h. For pulse-labeling, the medium was removed and replaced with 1 ml of prewarmed Dulbecco-modified Eagle medium containing 100 μCi of [^{3}H]dThd for 2.5 min at the appropriate temperature. For chase, the labeling solution was removed and 5 ml of prewarmed Dulbecco-modified Eagle medium containing 5×10^{-4} M dThd and 1×10^{-6} M deoxycytosine was added for 2.5 to 15 min at the same temperature. Incorporation of radioactivity was terminated by immersion in ice. Virus DNA was extracted by the Hirt procedure (11) and sedimented on a preparative neutral sucrose gradient (5 to 20% [wt/vol]) in 1.0 M NaCl–1 mM EDTA–10 mM Tris-hydrochloride, pH 7.8. Gradients were centrifuged at 4°C for 16 h at 24,000 rpm in a Beckman SW27 rotor. The region corresponding to replicative intermediates and completed form I (and II) DNA molecules was pooled and concentrated by precipitation with ethanol; fractions were identified based on admixed [^{14}C]dThd-labeled purified virus DNA. The DNA was then dissolved in TES buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-hydrochloride, pH 7.8) and fractionated by benzoylated naphtholated DEAE-cellulose chromatography. The benzoylated naphtholated

DEAE-cellulose was obtained from Boehringer Mannheim and utilized as described elsewhere (18, 23). Fractions were eluted sequentially with 0.25 M NaCl (fraction A), 1 M NaCl (fraction B), and 1 M NaCl in 2% caffeine (fraction C). In control experiments, replicative intermediates fractionated almost entirely (>90%) in fraction C. (The DNA in this fraction contained extensive single-stranded regions.) Purified form I and form II DNA distributed in fraction B (ca. 80% of recovered material) and fraction C (ca. 20%). The proportion of radioactivity in intracellular replicative intermediates was calculated after correction for cross-contamination of the respective fractions.

In vitro DNA synthesis. The preparation of the lysed cell system for in vitro DNA synthesis has been described previously (21). In brief, infected or uninfected cells were labeled in vivo overnight with [^{14}C]dThd (0.005 μCi/ml, 60 mCi/mmol). After appropriate temperature shifts, the cells were lysed with 0.01% Brij 58 for 20 min. Over 90% of the cells lost their defined plasma membrane boundaries as seen through a light microscope. The lysed cells were centrifuged through a buffer solution to wash them free of detergent. They were suspended in a complete in vitro reaction mixture that included four deoxynucleoside triphosphates, four ribonucleoside triphosphates, and an energy source. The template was endogenous cell or virus DNA and the radioactive tracer was [^{3}H]dTTP (200 μCi/ml, 800 Ci/mmol; New England Nuclear Corp.). Total concentration of dTTP was one-tenth that of the other three deoxynucleoside triphosphates. The average [^{14}C]dThd incorporation in a sample was used to correct for variation in the cell number in each reaction mixture (20 μl containing about 10^5 cells). When infected cells were used the virus DNA was extracted by the Hirt procedure (11). Radioactivity was measured after TCA precipitation.

Preparation of cell extracts. Whole-cell extracts were prepared by the method of Reinhard et al. (22). The starting material was more than 10^9 cells grown in roller bottle cultures at 33°C. All subsequent procedures were performed at 4°C. The cells were scraped from the plastic surfaces, washed with phosphate-buffered saline, and suspended in homogenization buffer (40 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.6], 80 mM KCl, 2 mM dithiothreitol, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid, 4 mM $MgCl_2$, 2 mM ATP). They were disrupted with 20 strokes of a Dounce homogenizer. The broken cells were then extracted in the same buffer, except that the concentration of KCl was raised to 0.38 M. The homogenate was centrifuged for 60 min in an SW56 rotor (Beckman Instruments) at 30,000 rpm. The supernatant was dialyzed against a dialysis buffer composed of the homogenization buffer to which 0.3 M sucrose had been added, and it was concentrated twofold by vacuum dialysis.

Nuclear and cytoplasmic extracts were prepared in the following manner. The cells were scraped from the roller bottles, washed, and suspended in hypotonic buffer (0.01 M sodium phosphate [pH 6.0], 0.01 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 150 μg of phenylmethylsulfonyl fluoride per ml). The cells were disrupted with 20 strokes of a Dounce homogenizer, and the nuclei were separated from the cytoplasm by centrifugation. The pellet (nuclei) was suspended in LiCl extraction buffer (0.01 M Tris-hydrochloride [pH 8.0], 1 mM EDTA, 0.5 mM dithiothreitol, 150 μg of phenylmethylsulfonyl fluoride per ml, 0.4 M LiCl) and extracted for 30 min. The supernatant (cytosol) was dialyzed against homogenization buffer and extracted with the same buffer containing 0.38 M KCl. Debris was removed from the extracts by centrifugation at low speed. The extracts were subjected to high-speed centrifugation in an SW56 rotor at 30,000 rpm for 1 h, dialyzed against the same buffer used in the dialysis of whole-cell extracts, and concentrated by vacuum dialysis. All the concentrated samples from extracts were diluted before use with dialysis buffer to the required concentration such that the extract, when added to the in vitro reaction mixture, contained in 20 μl the same amount of cell material as was present in 200 μl of the reaction mixture. Control samples without extract were diluted with an equivalent volume of dialysis buffer.

TABLE 1. Pools of dATP and dTTP[a]

Pool	Time (h) at:		pmol per 10^6 cells with the following cell line:		
	33°C	39°C	3T3	*ts*20R	*ts*20
dATP	36	0	55	56	50
	34.5	1.5	52	53	56
	33	3	53	60	52
	30	6	53	59	51
	27	9	56	57	55
	24	12	61	56	49
	21	15	67	63	50
	18	24	68	67	52
dTTP	36	0	42	40	43
	34.5	1.5	45	44	51
	33	3	49	51	50
	30	6	52	53	52
	27	9	52	53	42
	24	12	50	54	38
	21	15	53	52	31
	18	24	56	54	28

[a] Cells were cultured at a cell density of 4×10^5 cells per 50-mm dish and incubated at 33°C and then shifted to 39°C. At the time of harvest duplicate dishes were used to count the number of cells. The pool size is presented as pico moles per 10^6 cells.

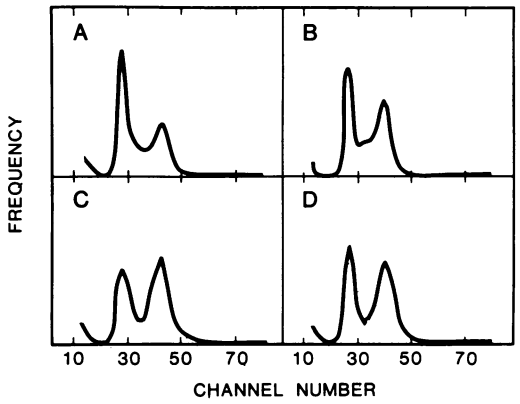

FIG. 2. FMF analysis of *ts*20 cells. Mutant cells, growing at 33°C, were shifted to 39°C and examined by FMF as described in the text at the following times at the restrictive temperature: 0 h (A), 9 h (B), 16 h (C), and 24 h (D). The profiles were traced from photographs of the display. Frequency on the ordinate represents number of cells, and channel number on the abscissa represents relative DNA content per cell.

RESULTS

At 33°C, *ts*20, 3T3, and revertant cells exhibited similar growth characteristics. At 39°C, the mutant cells began to lose their ability to form colonies within the first 16 h. The

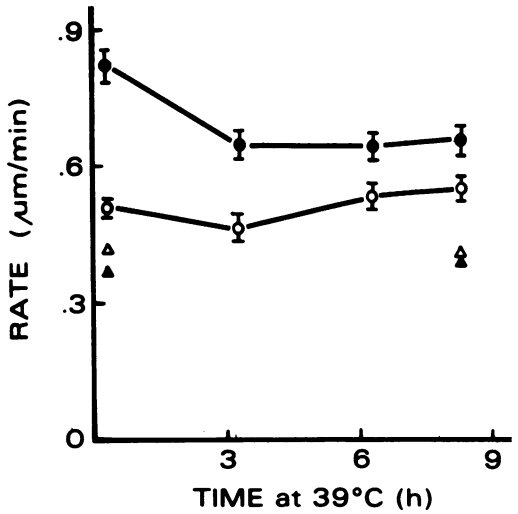

FIG. 3. Rate of DNA chain elongation in mutant and revertant (*ts*20R) cells. Mutant and revertant cell cultures in exponential cell growth were shifted to 39°C at zero time. At subsequent times, samples were labeled for consecutive 30-min periods with [^{3}H]dThd of high and low specific activity and processed for fiber autoradiography. Each point at 39°C represents 100 measurements; each point at 33°C represents 50 measurements. The error bars are the standard errors of the means. Symbols: ●, revertant cells, 39°C; ○, *ts*20 cells, 39°C; ▲, revertant cells, 33°C; △, *ts*20 cells, 33°C.

decrease in cell number resulting from the loss of attachment was slight at 16 h (less than 8%) but increased at later times. The growth of wild-type and revertant cells at 39°C was about 20% faster than that at 33°C (data not shown).

Analysis of macromolecular synthesis. The synthesis of DNA, RNA, and protein in *ts*20 was measured by incorporation of tritiated precursors (Fig. 1). When *ts*20 was shifted to 39°C, there was an initial 1.5-fold increase in [^{3}H]dThd incorporation. This began to decrease within 3 to 4 h and reached 50% inhibition by 12 h and 80% inhibition by 24 h. (The inhibition was 67% at 12 h and 85% at 24 h when compared with the maximum rate at 39°C.) We attributed the initial increase in dThd incorporation to increased metabolism at the higher temperature before the expression of the mutation. Incorporation of uridine and amino acids increased for 24 h before declining. The decreased levels of incorporation of these two precursors after 24 h at 39°C can be attributed to death of cells or their loss of attachment (15% by 24 h and 47% by 48 h) or both.

Deoxynucleotide triphosphate pools. Since a change in the size of the intracellular deoxyribonucleotide triphosphate pools could contribute to the decrease of exogenous dThd incorporation, the pools of dATP and dTTP were measured (Table 1). In exponentially growing cultures of 3T3 and *ts*20R cells, both pools increased 20 to 33% after shift to 39°C for 24 h. In mutant cells, the dATP pool remained the same and the dTTP pool decreased as DNA synthesis was inhibited. The decrease of the dTTP pool in *ts*20 cells (11% at 12 h and 35% at 24 h) was much less than that of dThd incorporation at the same time points (Fig. 1). The deoxyribonucleotide triphosphate pool levels correlate with DNA synthesis (3, 25, 26). Specifically, in 3T3 cells, when DNA synthesis is inhibited by increased cell density, the pools of dCTP, dGTP, and dTTP decrease while the pool of dATP remains the same (25). *ts*20 gave similar results in this respect. Certainly, pool dilution could not account for the decreased dThd incorporation in the mutant. Data presented below show that DNA synthesis was directly inhibited in *ts*20; we interpret the decrease in the dTTP pool as secondary to this.

Ongoing DNA synthesis. Two types of analyses were performed to determine whether DNA synthesis is directly inhibited in *ts*20: FMF and DNA fiber autoradiography. Growing cultures at 33°C were shifted to 39°C for various lengths of time and analyzed for DNA content per cell by FMF. The profiles obtained are shown in Fig. 2. A readily apparent shift from the typical pattern of exponentially growing cells at 33°C was observed. Cells did not accumulate with a DNA content expected for a G1 population. The pattern is consistent with arrest of cells throughout S phase since DNA levels in early and late S phase would be expected to overlap G1 and G2 levels.

We used DNA fiber autoradiography to measure the rate of DNA chain elongation in vivo. If it was retarded in *ts*20 at 39°C, this would be evidence in favor of *ts*20 being an S-phase mutant. The results (Fig. 3) show that immediately after shift to 39°C, the rate of chain elongation was less than in revertant cells at 39°C. It remained lower for the duration of the 8-h experiment, despite a slight decrease with time in the rate in revertant cells. At 33°C, the rates were lower and very similar in both lines, as observed in earlier experiments with *ts* mutants of hamster cells (6). The differences between cell lines at 39°C were statistically significant at all time points. The results provided evidence that the mutation in *ts*20 directly affected ongoing DNA replication and indicated that *ts*20 is an S-phase mutant.

Virus DNA synthesis. We next examined whether the mutation affected polyomavirus DNA synthesis. Mutants cells were infected with polyomavirus and maintained at 33°C or shifted from 33 to 39°C and back for various times. Samples were harvested at 38 h after infection after they had been labeled for 2 h with [^{3}H]dThd. Virus and cell DNAs were separated from the same sample by the Hirt procedure (11), and the incorporation into each was analyzed (Table 2). Cell and virus DNA synthesis in infected cells decreased 84 and 90%, respectively, after 16 h at 39°C. Thus, despite the stimulation of cell DNA synthesis normally caused by polyomavirus infection, the virus could not bypass the block to DNA synthesis in *ts*20. This also indicated that the mutant gene product was required for polyomavirus DNA synthesis and that the need for the gene product for virus DNA synthesis was at least as much as for cell DNA synthesis.

When infected cells were shifted back to 33°C, cell DNA synthesis began to increase. This was also true in uninfected

TABLE 2. Reversibility of block to virus and cell DNA synthesis in temperature-inactivated *ts*20 cells[a]

Hours			cpm/10^6 cells	
At 33°C	Shift up (39°C)	Shift down (33°C)	Cell DNA (10^5)	Virus DNA (10^4)
38	0	0	8.0	6.0
35	3	0	10.5	8.6
32	6	0	5.2	3.3
28	10	0	2.4	2.1
22	16	0	1.3	0.6
22	10	6	5.2	0.7
11	27	0	0.7	0.2
11	10	17	6.2	0.2

[a] Culture dishes containing 10^6 *ts*20 cells in exponential growth were infected with polyomavirus at 33°C. Samples were shifted up to 39°C and back down to 33°C for the times indicated in the table. They were labeled with [^{3}H]dThd for the last 2 h of the experiment. Virus and cell DNAs were separated from the same samples by the Hirt procedure (11), and the incorporation of radioactivity was measured.

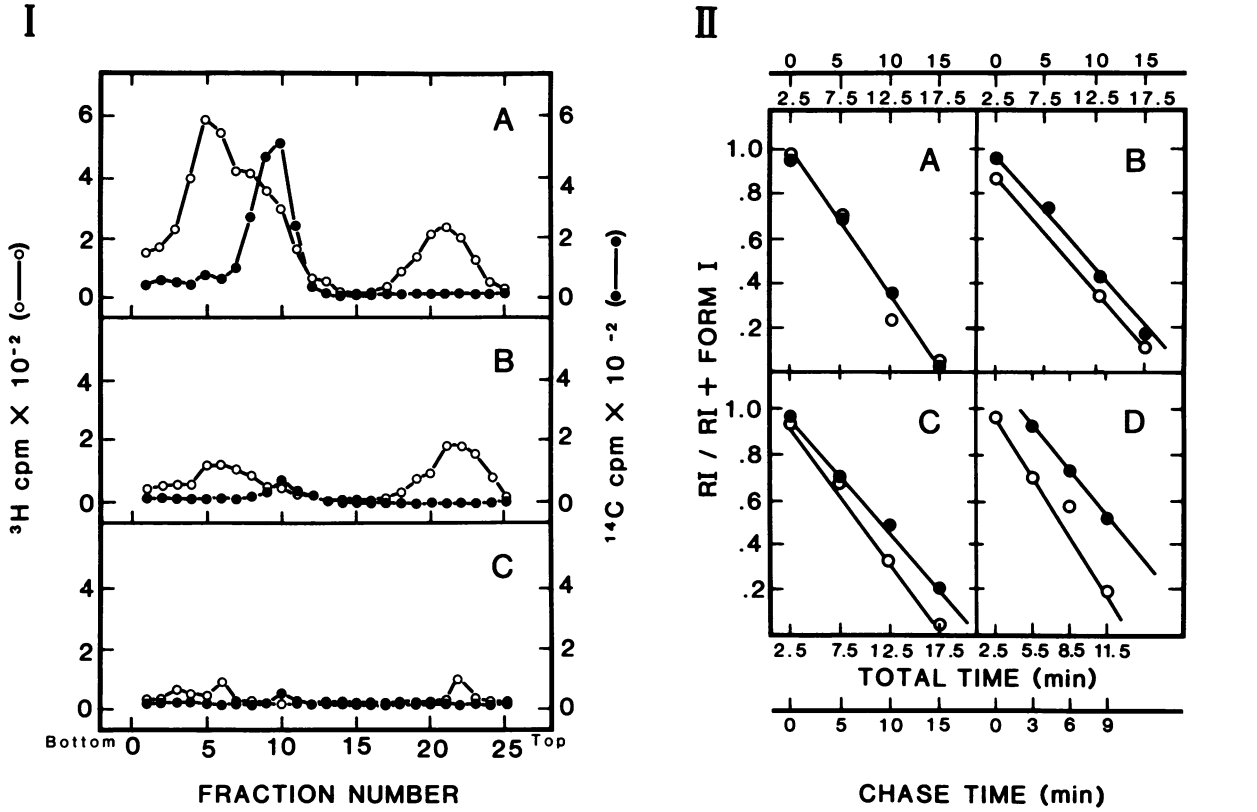

FIG. 4. (I) Velocity sedimentation analysis in neutral sucrose gradients of DNA synthesized in polyomavirus-infected *ts*20 cells at 39°C. Cells were infected with polyomavirus, incubated at 33°C for 24 h, and then shifted to 39°C. After 3 h (A), 5 h (B), and 8 h (C) at 39°C, the cells were pulse-labeled for 2 h with [^{14}C]dThd (2.5 μCi/ml). Immediately before termination of the experiment, the cells were labeled with [^{3}H]dThd (50 μCi/ml) for 5 min. Virus DNA was extracted by the Hirt procedure (11) and centrifuged in neutral sucrose gradients. Symbols: ●, ^{14}C counts per minute; ○, ^{3}H counts per minute. (II) Rate for completion of virus replicative intermediates in *ts*20 cells. Cells were infected for 36 h and pulse-labeled for 2.5 min with [^{3}H]dThd followed by chase in nonradioactive dThd. Virus DNA was quantitated after fractionation on benzoylated naphtholated DEAE-cellulose as described in the text. (A) *ts*20 shift to 39°C for 1 h (●) or 4 h (○) before pulse; (B) *ts*20 shift to 39°C for 1 h (●) or 8 h (○) before pulse; (C) *ts*20 pulse at 33°C (●) or shift to 39°C for 1 h (○) before pulse; (D) *ts*20R pulse at 33°C (●) or shift to 39°C for 1 hr (○) before pulse.

cells (data not shown). Virus DNA synthesis did not resume after the shift back to 33°C.

To study the defect of the gene product on the pattern of virus DNA synthesis, we examined polyomavirus DNA synthesized at the restrictive temperature by velocity centrifugation on neutral sucrose gradients. Mutant cells were infected with polyomavirus at 33°C. After 24 h, the cells were shifted to 39°C. They were labeled with [^{14}C]dThd for 2 h and then with [^{3}H]dThd for 5 min at several times after shift to the restrictive temperature. Virus DNA in the Hirt supernatant was subjected to centrifugation. The results are shown in Fig. 4I. The synthesis of form I DNA, labeled with ^{14}C, as well as the synthesis of replicative intermediate DNA, labeled with ^{3}H, were inhibited progressively with increasing time at 39°C. Since there was no preferential accumulation of replicative intermediates, this suggested that the defect blocked a step early in the synthesis of polyomavirus DNA molecules.

This interpretation was examined further in a series of experiments in which the distribution of radioactivity in virus DNA was determined by pulse-chase experiments at various times at the restrictive temperature. Infected cells shifted to 39°C were pulsed with [^{3}H]dThd for 2.5 min followed by medium change and addition of excess nonradioactive dThd (chase) to minimize further incorporation of radioactivity. After chase times of 2.5 to 15 min, virus DNA was extracted by the Hirt procedure (11) and sedimented on neutral sucrose gradients. The peak corresponding to replicative intermediates and form I DNA was pooled and analyzed on benzoylated naphtholated DEAE-cellulose to quantitate the amount of each of these forms. In Fig. 4II, the data are presented as the proportion of radioactivity that fractionated as replicative intermediates after the different chase times. The slope permitted comparison of the rate of completion (i.e., the rates of strand elongation and segregation) of virus replicative intermediates at the permissive and restrictive temperatures. Several points are evident. First, there was a gradual decrease of molecules with extensive single-stranded regions under all conditions; hence, there was no accumulation of replicative intermediates. Second, the slope did not change with longer times at 39°C, although the absolute level of incorporation decreased as in the experiment shown in Fig. 4I. The rates were 6.4% per min for 1 and 4 h at 39°C in Fig. 4IIA, and 5.0 and 5.1% per min for 1 and 8 h at 39°C in Fig. 4IIB. Differences in rates could be demonstrated by this method, as shown in Fig. 4IIC and 4IID in which *ts*20 and *ts*20R were compared at 33°C and within 1 h of shift to 39°C. In this case of *ts*20R (Fig. 4IID), there was a 40% increase in rate upon shift (5.9% per min at 33°C to 8.4% per min at 39°C). These values are consistent

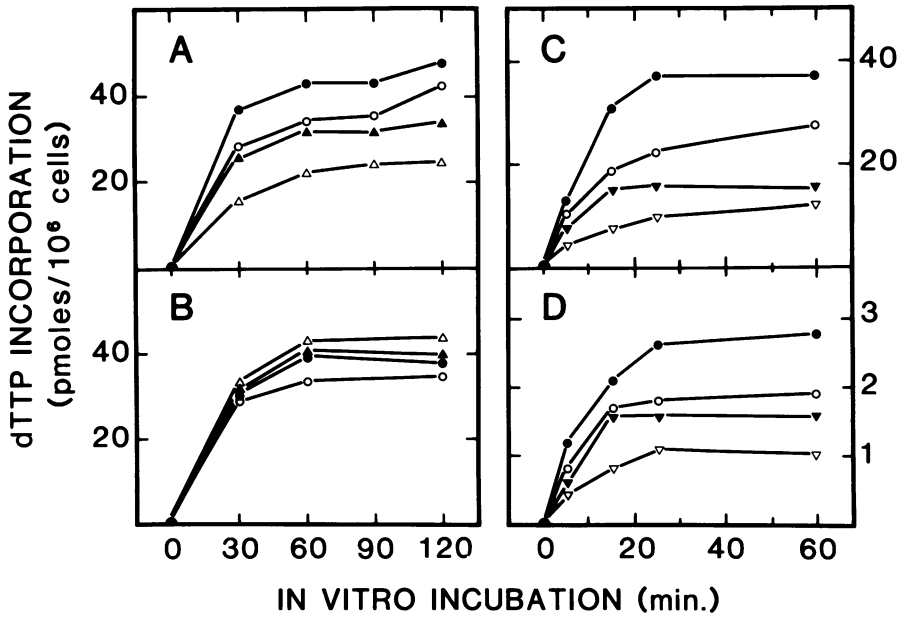

FIG. 5. Effects of in vivo incubation at 39°C on in vitro synthesis. Culture dishes containing 5×10^6 cells of uninfected *ts*20 (A), wild-type 3T3 (B), or infected *ts*20 (C and D) were labeled with [^{14}C]dThd at 33°C. After 16 h (for uninfected cells) or 24 h (for infected cells), the medium was changed and the cells shifted to 39°C for 0 h (●), 6 h (○), 9 h (▲) or 10 h (▼), or 12 h (△) or 24 h (▽). The cells were then lysed with 0.01% Brij 58, and in vitro DNA synthesis was carried out at 39°C. The DNA in infected *ts*20 cells was subjected to extraction by the Hirt procedure (11). Incorporation of [^{3}H]dTTP into uninfected *ts*20 cell DNA (A), wild-type 3T3 cell DNA (B), infected *ts*20 cell DNA (C), and virus DNA in infected *ts*20 cells (D) was measured. The ^{14}C counts were used to correct differences in dTTP incorporation caused by variation in the number of cells in each sample.

with those reported by Francke and Eckhart (7) for polyomavirus DNA maturation with a *ts*A mutant. An increase was also observed with *ts*20 before expression of the mutation (Fig. 4IIC), although to a lesser extent (4.9% per min at 33°C to 6.0% per min at 39°C). Our inability to detect a marked or progressive defect in rate of completion of DNA molecules at the restrictive temperature confirmed the interpretation that the defect preferentially blocked a step early in the synthesis of virus DNA.

DNA synthesis in vitro. On the basis of the findings above, we examined in vitro DNA synthesis in *ts*20 with cells lysed with Brij 58. First we determined whether the DNA *ts* phenotype was expressed in vitro. Wild-type 3T3 cells and infected and uninfected mutant *ts*20 cells that had been maintained at 33°C in the presence of [^{14}C]dThd to uniformly label their DNA were shifted to the restrictive temperature of 39°C for different times before lysis. Controls were maintained at 33°C throughout the experiment. The [^{14}C]dThd was removed at the time of temperature shift. Incorporation of [^{3}H]dTTP was measured in control and shifted cultures after Brij treatment. Virus and cell DNA synthesized in vitro was prepared from infected cells by the Hirt procedure (11). In the case of 3T3 cells (Fig. 5B), shift to 39°C had little effect on the rate of DNA synthesis. On the other hand, incubation of mutant cells at 39°C in vivo inhibited incorporation into both virus and cell DNA; the inhibition was more marked as the time at 39°C was prolonged. Incubation of uninfected *ts*20 cells at 39°C for 12 h reduced [^{3}H]dTTP incorporation 54% at 30 min and 40% at the plateau time of 1 to 2 h compared with mutant cells maintained at 33°C (Fig. 5A). The reduction of [^{3}H]dTTP incorporation into virus and cell DNA was more marked in infected mutant cells (Fig. 5C and D). The time course of inhibition was similar to that seen in vivo (Fig. 1 and Table 2). This showed that the mutant phenotype was expressed similarily in vivo and in vitro.

Stimulation of DNA synthesis by cell extracts has been demonstrated in Brij 58-lysed cell systems (22). We examined whether the depressed DNA synthesis in the mutant cells at 39°C could be restored by the addition of cell extracts from wild-type cells to the in vitro reaction mixtures. If the wild-type cell extracts contained the corresponding wild-type gene product, it should correct the depressed DNA synthesis in the mutant cells with the *ts* phenotype. Extracts from wild-type cells were able to stimulate [^{3}H]TTP incorporation in lysates of the mutant cells at the restrictive temperature (Table 3). The effect was dose dependent up to ×5 concentration of cell extract and then remained constant up to ×10 (data not shown); the ×10 concentration was used in all experiments. The mutant cells were maintained at 39°C for 12 h before lysis to establish the DNA *ts* phenotype. Controls for this experiment were mutant cells maintained at 33°C and wild-type cells maintained at 33 and 39°C for 12 h. In each case the samples supplemented with cell extracts were compared with replicate samples supplemented with dialysis buffer instead of extract. Since mutant cells would be expected to show different growth rates at 39 and 33°C, the cell numbers in parallel cultures at both temperatures were determined before lysis and used to correct the dTTP incorporation. The addition of extract increased dTTP incorporation by 55% in 3T3 cells, by 83% in *ts*20 cells kept at 33°C, and by 165% in *ts*20 cells kept at 39°C for 12 h. Therefore, the effect of the wild-type cell extract on *ts*20 cells with the DNA *ts* phenotype expressed was three times that on wild-type cells and twice that on mutant cells under permissive conditions.

If the extract from wild-type cells truly complemented the lysates of mutant cells, then the stimulatory effect of extract

from mutant cells should be more heat labile than that of extract from wild-type cells. Comparable extract from mutant cells maintained at 33°C was able to stimulate dTTP incorporation but was about half as effective as extract from wild-type cells (Table 3). There was no difference in the total protein content of these two extracts, and the *ts* extract showed the same dose dependence curve as wild-type extract (data not shown). We also tested the heat lability of the extracts. The stimulatory activity of the wild-type extract was maintained for 1 h at 39°C but was inactivated after 3 h at this termperature. In contrast, the activity of the extract from mutant cells was destroyed by 1 h of incubation at 39°C.

We also monitored the stimulatory activity during fractionation of the cell extract. Wild-type cells or mutant cells were separated into nuclei and cytosol, and extracts were prepared from both fractions. These were added to lysed mutant cells that has been incubated at 39°C for 12 h before lysis. The cytosol extracts gave almost identical results as the whole-cell extracts (Table 3), including the increased heat lability in the mutant extracts. The nuclear extracts showed no activity. Cell extracts were unable to stimulate virus DNA synthesis in vitro (data not shown), probably because the mutation irreversibly blocks such synthesis.

TABLE 3. Effect of incubation at 39°C on extracts from wild-type 3T3 or *ts*20 cytosol and nuclei[a]

Cell line	Time (h) for in vivo incubation of cells at 39°C	Extract	Time (h) for in vitro incubation of extracts at 39°C	Increase of DNA synthesis above control (%)
3T3	0	3T3 cell	0	55
	12	3T3 cell	0	64
*ts*20	0	3T3 cell	0	83
	12	3T3 cell	0	165
	12	*ts*20 cell	0	47
	12	3T3 cytosol	0	125
	12	3T3 cytosol	1	100
	12	3T3 cytosol	3	5
	12	3T3 nucleus	0	5
	12	*ts*20 cytosol	0	45
	12	*ts*20 cytosol	1	10
	12	*ts*20 cytosol	3	−5
	12	*ts*20 nucleus	0	0

[a] In vitro DNA synthesis was performed at 39°C for 60 min. The control was the same cell line incubated under identical condition at the same temperature but with buffer substituted for the cell extract. The dTTP incorporation was 25 pmol/10^6 cells in the control sample of *ts*20 cells which had been incubated at 39°C for 12 h, and about 42 pmol/10^6 cells in other control samples.

DISCUSSION

These results provide the initial characterization of *ts*20, a temperature-sensitive mutant of mouse cells. Cell growth was inhibited within one cell generation of shifting cells from the permissive to the restrictive temperature. The mutation in *ts*20 specifically inhibited DNA synthesis in vivo at the restrictive temperature. Incorporation of dThd into DNA decreased markedly, whereas synthesis of RNA and protein continued at normal rates for more than 24 h after shift to 39°C. FMF analysis showed that the cells were arrested in S phase, and fiber autoradiography experiments revealed that DNA chain elongation was retarded. These results ruled out the possibility that the mutation directly affected the uptake of exogenous dThd. Our experiments measuring the pool size of dTTP eliminated the possibility that the lesion primarily affected some biochemical process leading to deoxynucleotide triphosphate synthesis since the dATP pool was not changed by restrictive conditions and the dTTP pool decrease was later and less marked than the inhibition of dThd incorporation. In vitro experiments also showed that DNA synthesis was directly inhibited since the mutation was expressed under these conditions. By using polyomavirus DNA replication as a model system for cell DNA replication in this mutant, we provided evidence that the mutation is not a defect in initiation since such a defect should have been bypassed by virus infection. Data obtained in this system indicate an early block since the synthesis of polyomavirus replicative form as well as form I DNA was inhibited by restrictive conditions. Pulse-chase studies indicated that there was no block to the steps after the formation of replicative intermediates. Taken together, these experiments indicate that *ts*20 is defective at some point early in DNA chain elongation.

Temperature-shift experiments showed, on one hand, that the inhibition of cell DNA synthesis in vivo was at least partially reversible and that inhibition in vivo could be partially reversed in vitro by the addition of concentrated extracts from wild-type cells. On the other hand, there was no reversibility of arrested virus DNA synthesis in vivo and in vitro. Dinter-Gottlieb and Kaufmann (5) reported that aphidocolin, an inhibitor of polymerization, irreversibly inhibits SV40 DNA relication whereas its effect on cellular DNA synthesis is reversible. It could be that in mutant cells arrested virus DNA molecules are in a topological state under restrictive conditions that makes them unable to act as a template at a later time when the function of the mutant protein is restored by permissive conditions.

The stimulatory activity in cell extracts had properties consistent with a specific factor associated with the temperature sensitivity of the mutant. It preferentially stimulated lysates of *ts*20 at the restrictive temperature, and extracts from *ts*20 were less active and more readily inactivated by incubation at 39°C in vitro before assay. This activity fractionated with the cytosol in both wild type and mutant; however, we do not consider that this finding precludes a direct role in nuclear DNA synthesis for the factor, since many enzymes involved in DNA synthesis are readily extractible into the cytosol under comparable conditions.

The temperature sensitivity of this factor suggested that the lysed cell system with *ts*20 has the potential for use as a complementation assay to which crude or purified extracts might be added in attempts to identify the mutant gene product and its wild-type counterpart. However, there are difficulties with this approach. DNA synthesis is stimulated by factors that are normally contained in cell extracts (22), and these might interfere with the measurement of complementing activity when mutant cells are used. In the experiments presented here, we found that the relative increase in DNA synthesis in DNA *ts* mutant cells was greater than in wild-type cells and in mutant cells under permissive conditions. We conclude that this extra stimulation is a complementing activity: however, the high background from nonspecific stimulation in the extracts will hinder further purification of that activity. Separation of nonspecific stimulatory activity for complementing activity would likely result in extracts that only minimally stimulate DNA synthesis. Indeed, we have encountered such problems in our own

preliminary efforts to extend purification of the putative protein beyond salt fractionation and gel filtration (Zeng and Hand, unpublished data).

We should also note that the phenotype of this mutant is similar to another mutant, *ts*2, also isolated by one of us (24). Both have defective virus and cell DNA synthesis at the restrictive temperature. The two mutants were isolated in separate experiments. They do not complement each other, and the defect in both is corrected by the product of a human gene that is located on the X chromosome. We believe that the mutation in *ts*20 could be distinct from that in *ts*2 and as such will be valuable in studies aimed at elucidating the biochemical steps in mammalian DNA replication.

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