terpretation of the structural changes that occur during the structural transition. This transition may be a prelude to formation of M1 RNA dimers, needed for catalytic activity of the enzyme (Guerrier-Takada et al., 1986), or may involve a rearrangement of parts of M1 RNA directly involved in the active site.

Further work is required to clarify the details of the conformational changes undergone by M1 RNA to generate the catalytically active species. However, our results underline the importance of RNA conformation as a means of regulating function and also highlight the capability of RNA molecules to exhibit unexpected ionization properties that are due, presumably, to the details of the folding in solution of these molecules.

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Articles

DNA Synthesis in BalB/C-3T3 *ts* 2 Cells Is Restricted by a Temperature-Sensitive Function of Late G_1 Phase†

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ABSTRACT: *ts* 2 BalB/C-3T3 mouse fibroblasts are *cdc* mutants, which arrest late in G_1, at or near the G_1/S traverse, upon full expression of the heat-sensitive lesion. The kinetics of temperature inhibition of DNA synthesis in logarithmically growing cultures reveal three stages of heat inactivation. During the first generation time equivalent, normal semiconservative, semidiscontinuous replication proceeds but is reduced as cells exit and do not reenter S phase. During a second such period, a minimal rate of normal DNA synthesis is maintained. Thereafter, as the cells move into a third aborted cell division cycle, the rate of DNA synthesis increases. However, all semiconservative synthesis is then replaced by DNA repair replication. Temperature inactivation of the *ts* 2 protein results in shutdown of nuclear DNA synthesis. In contrast, normal replication of mitochondrial DNA proceeds at control rate throughout the first stage of temperature inactivation. Synthesis of this organellar genome is quantitatively reduced as the cells move into the second phase of heat inhibition. Titration of chromatin-bound DNA with ethidium bromide revealed that wild-type cells exhibit a changing DNA topology as the temperature is raised. Temperature-inactivated *ts* 2 cells behave as though their DNA has been topologically frozen in the configuration of control cells at or near entry into S phase.

Progression of mammalian cells into DNA synthesis is dependent upon one or more biochemical events that permit or induce traverse of the G_1/S interface of the cell duplication cycle [cf. Pardee et al. (1978) and Sheinin et al. (1978b)]. Little is yet known about the nature of these processes. As one approach to this problem, we have been studying a number of mutant *dna*^{ts} mammalian cells, i.e., cells that are temperature-sensitive (*ts*) in DNA synthesis [cf. Sheinin (1980, 1984)]. These cells are higher eukaryotic analogues of what have been designated as cell division cycle, or *cdc*, mutants of yeast cells [cf. Pringle & Hartwell (1981) and Dickinson (1984)].

The present study is concerned with BalB/C-3T3 *ts* 2 mouse fibroblasts, isolated by Slater and Ozer (1976). We have now demonstrated that temperature inactivation of the *ts* 2 protein brings cells to arrest very late in G_1, at or near the G_1/S traverse (Sheinin et al., 1985b). The cells are therefore *dna*^{ts}/G_1^{ts}. Expression of the *ts* 2 defect results in inactivation

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of cellular DNA formation, under conditions in which de novo synthesis of RNA and protein is apparently unaffected [see also Sheinin et al. (1986)].

Sheinin and Lewis (1980) found that the *ts* 2 cells could be distinguished from dna^{ts}/S^{ts} cells by an important property. The latter cells exhibit coupled shutdown of synthesis of histones, which begins 6–12 h after initial onset of inactivation of DNA synthesis. In contrast, continuing synthesis of histones is uncoupled from declining DNA replication in temperature-inactivated *ts* 2 cells. In addition (Sheinin et al., 1985b), the latter do not undergo the disaggregation of the facultative, condensed heterochromatin that characterizes temperature-inhibited dna^{ts}/S^{ts} mouse L-cells (Setterfield et al., 1978) which arrest early in S phase [cf. Sheinin (1980) and Guttman & Sheinin (1979)].

Temperature-inactivated *ts* 2 cells are unable to support replication of polyoma (Py) virus DNA (Slater & Ozer, 1976; Sheinin et al., 1985a). In contrast, they do carry out synthesis of mouse adenovirus DNA (Sheinin et al., 1985a) and of linear, double-stranded Moloney murine leukemia virus (MoMuLV) DNA (Richter et al., 1984). In the present study we have further examined the phenotypic expression of the BalB/C-3T3 *ts* 2 mutation, as it impacts on DNA replication.

Materials and Methods

Cells. Wild-type BalB/C-3T3 mouse fibroblasts (Todaro & Green, 1963) and the *ts* 2 derivative (Slater & Ozer, 1976) were cultivated on a glass or plastic surface according to routine methods (Shopsis & Sheinin, 1976). Unless otherwise noted, the medium was minimum Eagle's medium (type α) (α-MEM) supplemented with 7.5% (v/v) fetal calf serum. The permissive temperature (pt) was 34 °C, and the nonpermissive temperature (npt) was 38.5 °C. Cells were cultured in incubators continually flushed with a mixture of 95% air and 5% CO_2, humidified to 90%.

General Experimental Regimens. Unless otherwise indicated, BalB/C-3T3 and *ts* 2 cells were inoculated at a concentration of (1–3) × 10^4 cells/cm^2 of growing surface to allow for 4–5 doublings before cells approached the G_0 state at confluence. Cell concentrations were measured by using an electronic cell counter in suspensions of cells released from the growing surface with trypsin. Freshly plated cells were incubated at 34 °C for at least 24 h before any experimental manipulation, to permit regeneration of surface components released by protease treatment and effective reentry of cells into the cycle (Onodera & Sheinin, 1970; Sheinin & Onodera, 1970).

Where noted, cells were prelabeled in their DNA with [^{14}C]thymidine (dThd) by growth at 34 °C through 3–4 generations in medium lacking nucleosides, but supplemented with [2-^{14}C]dThd (0.01 μCi/mL; approximate concentration, 2 μM). When cells attained confluence, they were subcultured in nonradioactive medium and after 24 h were incubated at 34 °C or at 38.5 °C. To label DNA newly made under test conditions, [*methyl*-^{3}H]dThd was used at 10 or 100 μCi/mL (approximate specific activity, 20 Ci/mmol). After being labeled, the cells were washed in situ 3 times with ice-cold medium containing 41 μM dThd. They were lysed with 1% sodium dodecyl sulfate (SDS) in 0.15 M NaCl + 0.015 M sodium citrate [SSC (Marmur, 1961)] either directly or later, after release from the growth surface with trypsin.

Measurement of DNA Synthesis. (*a*) *Incorporation of dThd.* The rate of DNA synthesis was measured in triplicate samples of 10^6 cells exposed for 1 h to [^{3}H]dThd at 10 μCi/mL. After incubation, the cells were chilled, washed 3 times with ice-cold complete medium, frozen and thawed 3 times, and precipitated with 5% (w/v) trichloroacetic acid (TCA). The precipitated macromolecules were collected onto GF/C glass fiber filters, and their radioactivity was measured by liquid spectrometry (Sheinin, 1966).

(*b*) *Autoradiography.* BalB/C-3T3 or *ts* 2 cells, on coverslip cultures, were exposed for 1 h to [^{3}H]dThd at 10 μCi/mL and then processed for autoradiography with Kodak NT B2 emulsion as described elsewhere (Sheinin & Quinn, 1965).

(*c*) *Equilibrium Centrifugation Analysis in Neutral and Alkaline $CsCl_2$ Density Gradients.* Equilibrium centrifugation analysis was performed according to previously described methodology (Sheinin & Guttman, 1977). Cells ($\simeq 10^7$) suspended in 1 mL of SSC containing 2% (w/v) sarkosyl NL-30 (Geigy Industrial Chemicals) were incubated at room temperature to permit cell lysis and dissociation of nucleoprotein complexes. A 21-gauge stainless steel needle was used to shear the DNA to $M_r \simeq 10^8$. The sheared lysate was diluted to 5 mL with saturated $CsCl_2$ in 0.01 M tris(hydroxymethyl)aminomethane (Tris) + 0.01 M ethylenediaminetetraacetic acid (EDTA) (pH 7.4) to give a final density of 1.70 gm/cm^3. Equilibrium centrifugation was at 48 000 rpm for 48 h at 2 °C in a Beckman SW 50.1 rotor, with poly(dA·dT) and poly(dC·rG) as markers. Samples were prepared for analysis in alkaline $CsCl_2$ gradients by incubation for 30 min at 50 °C in 0.1 M NaOH. The starting density of $CsCl_2$ was 1.76 gm/cm^3. After centrifugation, gradient fractions were collected and monitored for OD_{260nm}, TCA-insoluble radioactivity, and refractive index, from which was calculated the buoyant density.

(*d*) *Velocity Sedimentation Analysis in Sucrose Density Gradients.* Velocity sedimentation analysis in neutral and alkaline sucrose density gradients of native and denatured DNA was performed as described elsewhere (Cheevers et al., 1970). Fractions collected were monitored as under (c). It should be noted that, after experimental manipulation, the cells were washed in situ with prewarmed phosphate-buffered saline [PBS (Dulbecco & Vogt, 1954)]. They were then scraped from the substratum with a rubber policeman, combined in SSC, and collected by centrifugation at 800*g*. This procedure took about 10–15 min in all. The cells were then subjected to analysis.

Analysis of DNA Conformation in Chromatin by Titration with Ethidium Bromide. The conformation of DNA in chromatin was examined by titration with the intercalating dye ethidium bromide [EtdBr (Brazell & Cook, 1971)]. Cells cultured at 34 or 38.5 °C for 24 h were collected by centrifugation, washed, and resuspended in PBS at a density of $\simeq 5 \times 10^6$/mL. Aliquots (100 μL) were layered onto six preformed 5.2-mL linear density gradients of 5–30% (w/v) sucrose in 50 mM Tris, pH 7.8, 10 mM EDTA, 1.0 NaCl, containing six different concentrations of EtdBr, ranging from 0 to 25 μg/mL. Lysis buffer [100 μL of 50 mM Tris, pH 7.8, 10 mM EDTA, 1.0 M NaCl, and 1% (w/v) Triton X-100] was added to the cell layer. Lysis was allowed to proceed for 20 min at 20 °C. The cell preparations were spun at 6000 rpm for 60 min at 20 °C in a SW 50.1 rotor. For each experiment six different concentrations of EtdBr were used, giving six gradients for analysis. Each experiment with matched cell type, temperature, and periods of incubation was analyzed in the same rotor head under exactly the same conditions. At least three sets of experiments were performed for each cell type and temperature condition. Although the absolute data varied from experiment to experiment, the relative results were the same.

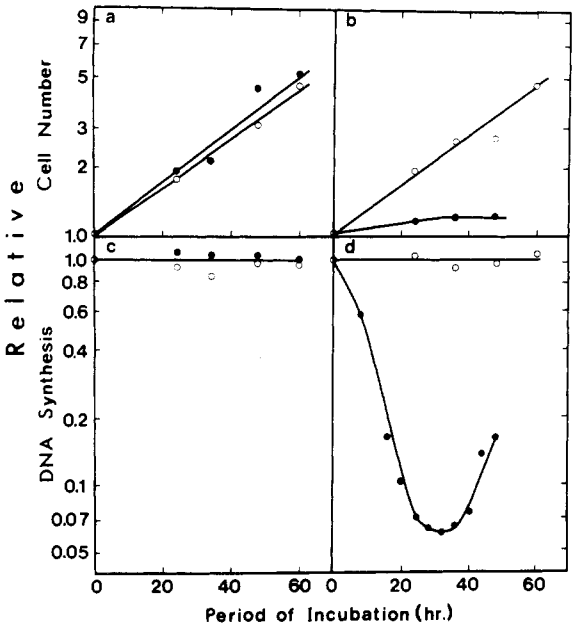

FIGURE 1: Growth and DNA synthesis in wild-type and *ts* 2 mouse fibroblasts. Two sets of cultures (in 2-oz Brockway bottles) of BalB/C-3T3 and *ts* 2 cells were grown to early logarithmic phase at 34 °C in medium containing [^{14}C]dThd to prelabel normal cellular DNA. One set was upshifted to 38.5 °C (●); the other was maintained at 34 °C (○). At the intervals noted triplicate cultures were pulse-labeled with [^{3}H]dThd (5 mL, 10 μCi/mL, 1 h). The cultures were washed 3 times with complete nonradioactive medium; the cells were released from the growth surface by brief trypsinization. An aliquot was used to measure cell number. The remainder was processed to determine radioisotope incorporation. Incorporation of [^{3}H]dThd was normalized on the basis of the recovery of [^{14}C]DNA.

The material so sedimented was processed on an ISCO Model 640 gradient fractionator and monitored for OD_{254nm}. For each sample the relative distance sedimented was calculated, as the ratio of the distance sedimented in EtdBr-containing gradients as compared with that in EtdBr-free gradients. From the data obtained for each cell type, an "equivalence concentration" was determined. It is that amount of EtdBr required to convert the chromatin-bound DNA to the fully relaxed state from its original superhelical conformation. This experiment was repeated 3 times with wild-type and *ts* 2 cells matched for cell number and for incubation at 34 and 38.5 °C. This procedure was validated in earlier studies [cf. Colwill & Sheinin (1982)].

Mitochondrial DNA Synthesis. Cells generally prelabeled in their DNA with [^{14}C]dThd, as noted above, were subcultured in nonradioactive medium for 24 h at the pt. Half of each culture set was upshifted to the npt. At various intervals thereafter about 10^8 cells were incubated with [^{3}H]dThd for 3 h at the appropriate temperature. Each cell batch was then processed for isolation of nuclei and mitochondria, which were extensively purified. The mitochondrial (mt) DNA was analyzed by equilibrium centrifugation in EtdBr-containing $CsCl_2$ density gradients, as described elsewhere (Sheinin et al., 1977). Recovery was monitored in terms of the total normal, marker [^{14}C]DNA.

Results

Growth and DNA Synthesis in ts 2 Cells. The patterns of division and rate of DNA synthesis in *ts* 2 cells incubated at 34 and 38.5 °C are shown in Figure 1. For comparative purposes, panels a and c show normal growth and DNA formation in wild-type BalB/C-3T3 mouse fibroblasts. That *ts* 2 cells exhibit wild-type parameters at the pt is seen in panels b and d. In contrast, when the *ts* 2 cells are upshifted to 38.5 °C, cell division ceases within 24 h post-temperature shift (pts), as noted in panel b. Panel d reveals that inhibition of [^{3}H]-dThd incorporation into the DNA of cultures of *ts* 2 cells incubated at the npt is readily detectable within 4–6 h pts. It proceeds through three phases designated I, II, and III, each equivalent to about one generation period. During phase I (0–20 h pts) ongoing DNA synthesis undergoes initial inactivation to a minimum level. This is maintained throughout phase II (20–34 h pts). During phase III (i.e., beyond 32–36 h pts) incorporation of [^{3}H]dThd rises once again.

Table I: Autoradiographic Analysis of DNA Synthesis in BalB/C-3T3 *ts* 2 Mouse Fibroblasts

interval (h)	cells labeled (%) at 34 °C	cells labeled (%) at 38.5 °C
0	39.2	61.9
2	44.7	80.1
4	49.8	63.6
8	73.2	40.0
12	57.2	57.4
16	48.2	22.2
20	39.2	16.4
24	29.8	12.3
28	28.0	13.1
36	19.2	5.24[a]
40	15.7	2.76[a]
44	12.1	3.79[a]
48	8.21	9.64[a]

[a] Nuclei with less than 20 grains.

The cellular basis of this phenomenon has been examined by autoradiography, in cultures brought to early S phase, in parasynchrony as described elsewhere (Sheinin et al., 1985b). These cultures were maintained at 34 °C or were upshifted to 38.5 °C. After various intervals the cells were incubated with [^{3}H]dThd and processed by autoradiography to assess the number of cells making DNA.

The results of one such study, presented in Table I, indicate the degree of synchrony obtained with respect to S phase cells at time zero. With continued incubation of the *ts* 2 cells at the pt, cells continued to flow into S, to reach a peak at ≃8 h. Thereafter, the number of cells making DNA declined, with kinetics that reflected exit from and continuing entry into S phase of cells as the culture moved through successive division cycles and approached confluence at ≃48 h. Similar results have been obtained with wild-type cells grown at low or high temperature (Sheinin & Onodera, 1970).

In the case of the *ts* 2 cultures upshifted to 38.5 °C, the proportion of cells in DNA synthesis remained high for ≃4 h, and then declined continuously, in a pattern suggesting outflow and no influx into S phase. It should be noted that after 28 h any cells that were labeled at the npt carried fewer than 20 grains over their nuclei. Such very lightly labeled cells were never seen in the control culture. Indeed, this labeling pattern was reminiscent of "unscheduled DNA synthesis" characteristic of repair replication [cf. Stich et al. (1977) and Sheinin et al. (1985b)].

Characteristics of DNA Synthesis in Temperature-Inactivated ts 2 Cells. The foregoing observations are compatible with the model that temperature inactivation has little effect on normal DNA replication already initiated in S phase cells. In contrast, heat denaturation of the *ts* 2 gene product leads to inhibition of entry into the DNA-synthetic period and, secondarily, to repair replication late after temperature inactivation. We therefore examined the mechanism of DNA synthesis in *ts* 2 and wild-type cells incubated at 34 °C or at 38.5 °C, in a number of ways.

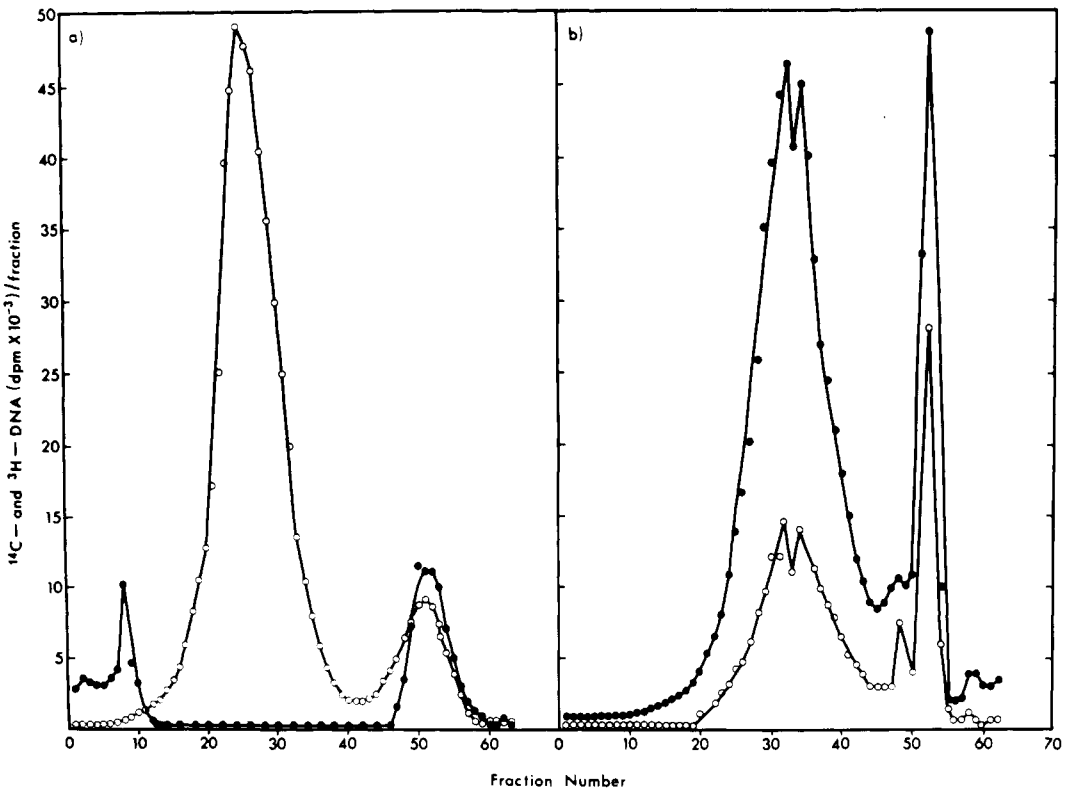

FIGURE 2: Velocity sedimentation analysis in alkaline sucrose density gradients of DNA synthesized by *ts* 2 cells. *ts* 2 cells prelabeled in their DNA with [^{14}C]dThd were subcultured at 34 °C in 32-oz Brockway bottles. After 24 h, half the cultures were shifted to 38.5 °C (●), and half were maintained at 34 °C (○). Sixteen hours later the cells were incubated for 2 min in 10 mL of medium containing 100 μCi/mL of [^{3}H]dThd. Half of each culture set was processed immediately. The medium of the other half was decanted and replaced with 40 mL of complete medium containing unlabeled dThd. This set was processed after 1 h of further incubation. The doubly labeled DNA was denatured and analyzed by velocity sedimentation in alkaline sucrose density gradients. Sedimentation was from left to right.

(*a*) *Buoyant Density Analysis.* We first looked at the average buoyant density of the DNA of cells incubated at the low and high temperatures, as an index of gross DNA composition. Cells were grown through 3–4 doublings at 34 °C in medium containing [^{14}C]dThd to label normal DNA. These were subcultured in dThd-free medium and incubated at the pt or npt, for periods of up to 44 h, at which time *ts* 2 cells begin to detach from the growth surface. Newly made DNA was labeled with [^{3}H]dThd for 6-h periods at the appropriate temperature. The doubly labeled cellular DNA was analyzed by equilibrium centrifugation as described under Materials and Methods.

The DNA of BalB/C-3T3 and *ts* 2 cells gave the same centrifugation profile in this test, irrespective of temperature of incubation of interval pts, of labeling with [^{3}H]dThd (data not shown). Thus [^{3}H]DNA cobanded at equilibrium with the marker, normal [^{14}C]DNA at a buoyant density of 1.707 gm/cm^3, as expected for mouse DNA [cf. Kit (1961) and Sheinin & Guttman (1977)]. These findings indicate that the DNA synthesized by *ts* 2 cells at the npt does not differ greatly from the total normal cellular genome, in terms of its primary structure.

(*b*) *Pulse–Chase Studies.* The pattern of DNA synthesis in *ts* 2 cells undergoing temperature inactivation was performed in a pulse–chase study, according to the legends of Figures 2 and 3. BalB/C-3T3 cells were included for comparison.

The sedimentation behavior of DNA from BalB/C-3T3 and *ts* 2 mouse fibroblasts, incubated at the high and low temperatures for intervals up to 44 h, was qualitatively the same, whether examined after a 2-min pulse or a 1-h chase interval (data not shown). In neutral sucrose density gradients, the undenatured [^{3}H]DNA newly made at the npt cosedimented along with the [^{14}C]DNA, preformed at the pt. These double-stranded molecules, of large molecular weight, collected onto a 70% sucrose cushion. There was no evidence for degradation of preformed DNA, nor for formation of small molecular weight, double-stranded DNA during short or longer term incubation of cells at the low or high temperature.

Figure 2 presents sedimentation profiles obtained with denatured DNA from *ts* 2 cells subjected to pulse labeling and chase at 23 h pts. As indicated in panel a, the [^{3}H]DNA synthesized during a 2-min pulse was recovered as three components, in fractions 1–11, 10–42, and 44–60, respectively.

Of special interest is the low molecular weight ($\simeq$10–12 S) [^{3}H]DNA in the first component, well separated from the bulk of the large molecular weight, ^{14}C-marker chromosomal DNA, running primarily as the second peak. Panel b reveals that this [^{3}H]DNA is fully recovered after the 1-h chase period as large molecular weight DNA, cosedimenting with this ^{14}C-labeled chromosomal DNA.

The third component at the very bottom of the gradient (fractions 45–62) is frequently observed when large numbers of cells are processed for alkaline degradation of DNA, by the experimental procedure used. It has been shown to be associated with undissolved cellular material (Cheevers et al., 1970) and is therefore unrevealing for the present purposes.

There was no evidence for degradation of the preformed DNA, or that made at the npt, under the test conditions. This was verified by quantitative analysis (data not shown).

Sedimentation behavior similar to that shown in Figure 2 was observed with DNA derived from pulse–chase experiments performed with wild-type BalB/C-3T3 cells, still growing at the low or high temperature. The same was seen with *ts* 2 cells multiplying at the pt, and also with *ts* 2 cells incubated at the npt up to 36 h pts.

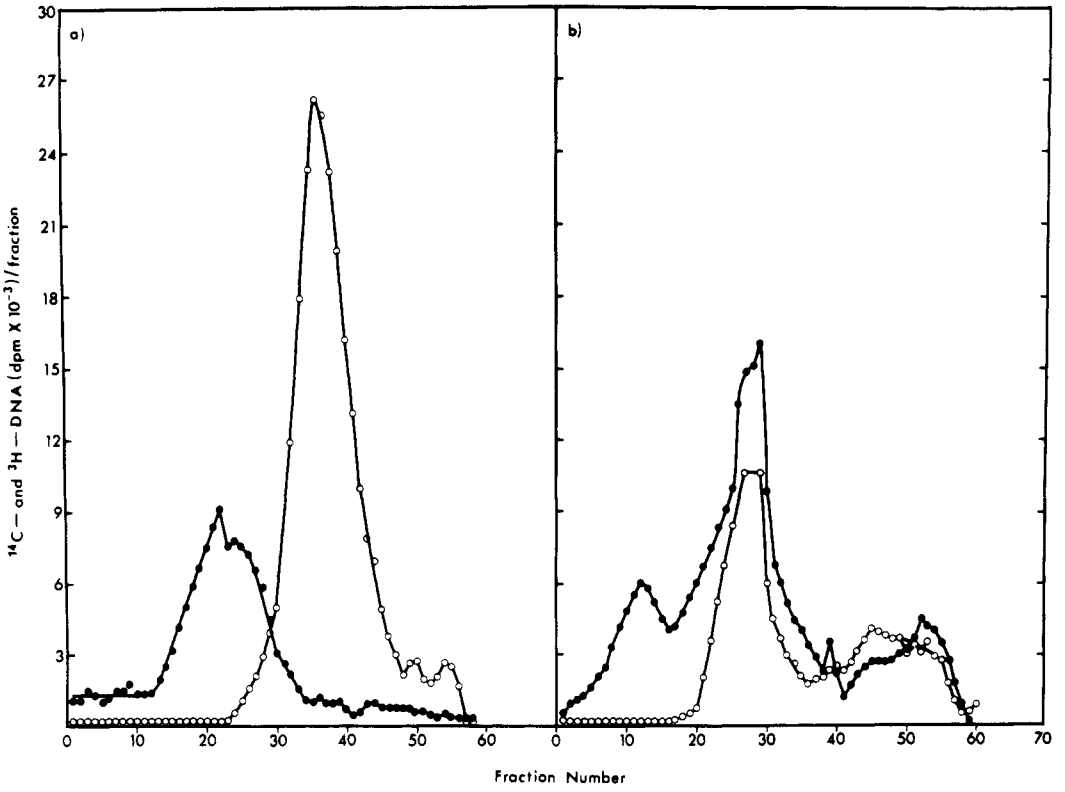

FIGURE 3: Velocity sedimentation analysis in alkaline sucrose density gradients of DNA synthesized by *ts* 2 cells at 47 h pts. *ts* 2 cells were processed as described in the legend to Figure 2, except that they were incubated at the npt for 47 h prior to pulse (panel a) and chase (panel b) labeling. Great care was taken to recover all the cells.

Figure 3 presents analogous sedimentation behavior for the alkaline-denatured DNA of *ts* 2 cells subjected to pulse–chase analysis at 47 h pts. Panel a reveals that the [^{3}H]DNA made during the 2-min pulse sedimented in the upper half of the gradient but overlapped somewhat with the marker [^{14}C]DNA. These data suggest that this material was of fairly large molecular weight, although still considerably smaller than the normal, linear, chromosomal DNA, as extracted. Panel b shows the sedimentation profile of the [^{3}H]DNA after the 1-h pulse interval. Clearly, the majority of this DNA cosedimented with the ^{14}C-marker DNA. However, a small proportion ran as DNA of smaller molecular weight. The reasons for this are not clear.

These various results suggest that DNA synthesis in control cells, and in *ts* 2 cells undergoing phase I, II, or III temperature inactivation, proceeds by the semidiscontinuous mode [cf. Sheinin et al. (1978a,b)].

(*c*) *Semiconservative or Repair Replication.* The next experiment was designed to test for semiconservative DNA replication in *ts* 2 cells incubated at the pt or npt. Cells were prelabeled in their DNA with [^{14}C]dThd, during growth at 34 °C, and then cultured for varying periods at 34 °C or 38.5 °C. They were further incubated for 6 h with BrdUrd to density-label the newly made DNA, which was then radioactively tagged with [^{3}H]dThd, as noted in the legend of Figure 4. The triply marked DNA was analyzed by equilibrium centrifugation in neutral and alkaline $CsCl_2$ density gradients.

Qualitatively similar results were obtained with wild-type cells growing at 34 °C or at 38.5 °C, with *ts* 2 cells multiplying at 34 °C, and with *ts* 2 cells incubated for up to 32 h at 38.5 °C, i.e., throughout phases I and II of temperature inactivation. Illustrative data are shown in Figure 4a, for *ts* 2 cells temperature-inhibited for a total of 32 h.

The native parental [^{14}C]DNA was recovered in two fractions. About 40% was of unreplicated buoyant density of $\simeq$1.706 gm/cm^3. The remainder was of increasingly higher density, suggesting semiconservative synthesis in the presence of BrdUrd. Of the newly made, ^{3}H-labeled progeny DNA, $\simeq$60% was recovered in hybrid density DNA, and the remainder cobanded with the ^{14}C-labeled template. The corresponding banding profile for alkaline-denatured DNA is shown in panel b. Of the total newly synthesized ^{3}H- and BrdUrd-labeled DNA, less than 15% was recovered in association with the single-stranded, ^{14}C-marked, parental DNA. The major fraction was distributed throughout the gradient as single-stranded material of increasingly higher density. These data suggest that at 32 h pts *ts* 2 cells still synthesize their DNA primarily by the semiconservative mode.

The banding profiles for DNA synthesized by *ts* 2 cells temperature-inactivated for a total of 47 h are shown in Figure 4c,d. Both native DNA and alkaline-denatured DNA give evidence of almost complete cobanding of ^{14}C-labeled template and ^{3}H-labeled DNA newly made at the npt. These observations point to non-semiconservative replication.

Analysis of Conformation of Chromatin DNA of BalB/C-3T3 and ts 2 Mouse Fibroblasts. In the following experiments we compared the conformation of nuclear DNA in wild-type cells and in the *ts* 2 derivative incubated at the low temperature or the npt. Cells brought to early S phase were incubated for 24 h at different temperatures and processed for velocity sedimentation analysis of their nucleoid DNA, using EtdBr as a probe of superhelicity, according to the procedure set out under Materials and Methods and in the legend of Figure 5.

In all cases the DNA of cells, released to interact with concentrations of EtdBr of 1–20 μg/mL, yielded the expected sedimentation pattern [cf. Mattern & Painter (1979) and Colwill & Sheinin (1982)]. This is seen in panel a of Figure 5 for the wild-type cells. At low concentration of EtdBr the DNA–dye complex sedimented rapidly at a rate approximating that for the free nuclear DNA. As the EtdBr concentration

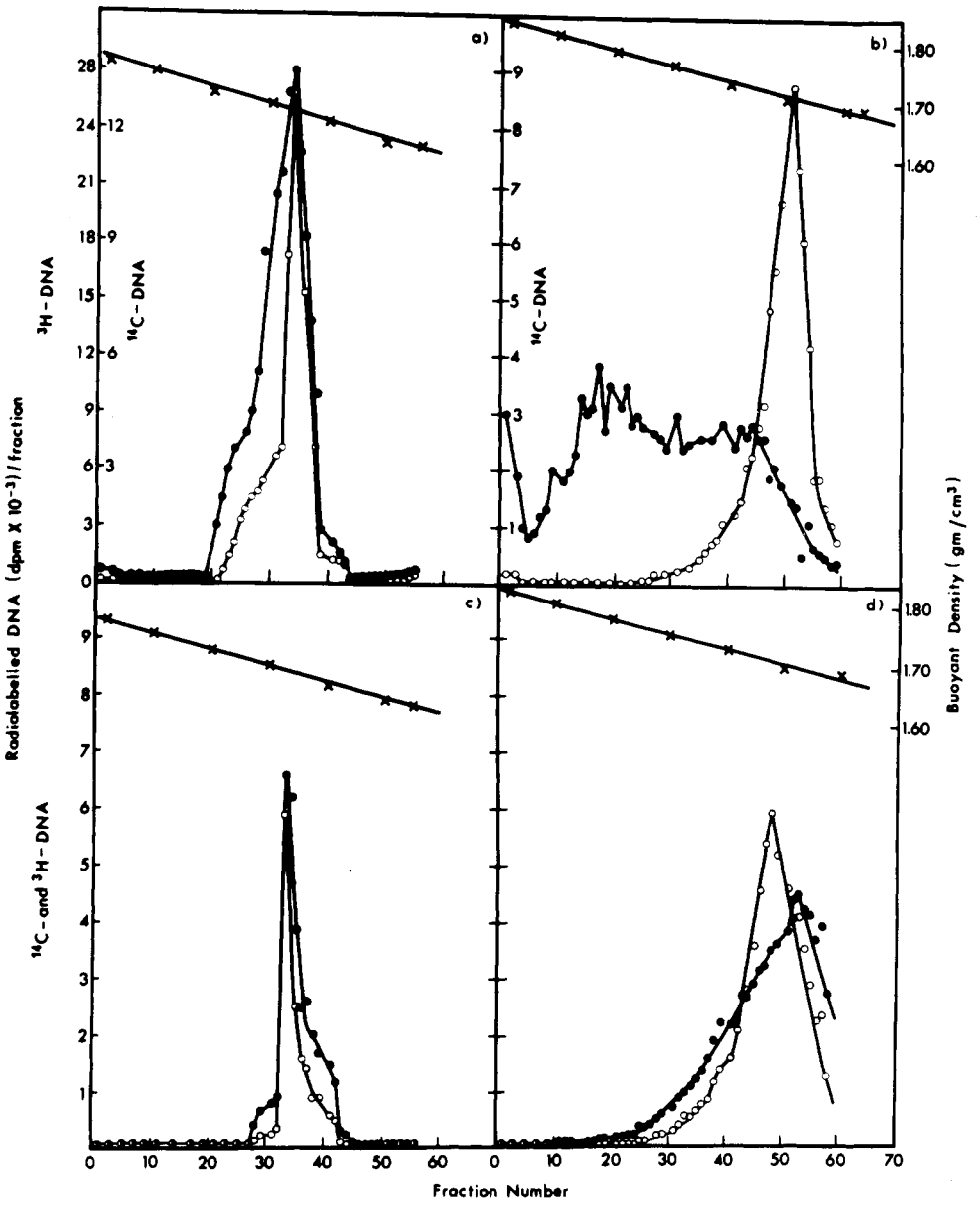

FIGURE 4: Isopycnic centrifugation analysis of DNA synthesized in *ts* 2 BalB/C-3T3 mouse fibroblasts incubated at 34 °C or at 38.5 °C in the presence of BrdUrd. *ts* 2 cells, prelabeled in their DNA with [^{14}C]dThd, were subcultured at 34 °C in 32-oz Brockway bottles. After 24 h, half the cultures were shifted to 38.5 °C (●), and half were maintained at 34 °C (○). At various intervals thereafter sets of cultures ($\simeq 10^8$ cells) were incubated for 6 h in medium containing 160 μM BrdUrd + 10 μM FdUrd, and then for 1 h further with [^{3}H]dThd (10 μCi/mL). The cells were then harvested and processed for isopycnic centrifugation analysis in $CsCl_2$ density gradients as set out under Materials and Methods. The density of fractions is indicated by ×. Panels a and b: Neutral and alkaline gradients of native and denatured DNA from cells incubated at the npt for 32 h. Panels c and d: The same for cells temperature-inactivated for 47 h.

was increased, the EtdBr-bound DNA moved more slowly down the gradient. Its effective size-density declined due to relaxation, by the dye, of the initially negatively supercoiled DNA. The sedimentation rate reached its lowest value at the equivalence concentration of EtdBr, i.e., that amount required to yield fully relaxed chromatin DNA. A further increase in EtdBr concentration gave a heavier DNA-dye complex undergoing positive resupercoiling, thereby acquiring an increased density.

Panel a of Figure 5 shows the sedimentation behavior of the chromatin–DNA of wild-type BalB/C-3T3 mouse fibroblasts incubated at 34, 37, and 38.5 °C. The minimal equivalence concentrations of EtdBr were approximately 3.5, 4.2, and 4.5 μg/mL, respectively. The chromatin–DNA of cells growing at the higher temperature was present in a less supercoiled configuration than that of cells growing at the lower temperatures. This was manifest in three parameters: the equivalence concentration, the extent of sedimentation relative to untreated DNA, and subsequent positive supercoiling at higher EtdBr concentrations.

Panel b presents the data obtained with the *ts* 2 cells incubated for 24 h at the same three temperatures. There is general similarity in the shapes of the EtdBr titration curves obtained, when compared both within this set and with the data given by wild-type cells. However, the details differ somewhat. Thus all *ts* 2 cell preparations exhibited a relatively sharp equivalence concentration of $\simeq 4$ μg/mL, and all gave almost superimposable titration patterns.

Mitochondrial DNA Synthesis. The aforementioned studies indicate that synthesis of DNA by *ts* 2 cells was inhibited by at least 90%, by the end of the first stage of temperature inactivation, implicating nuclear activity as the primary target of heat denaturation of the *ts* 2 protein. In the following experiments we therefore examined the impact on mitochondrial (mt) DNA replication.

As noted in the legend to Figure 6, cells were grown at 34

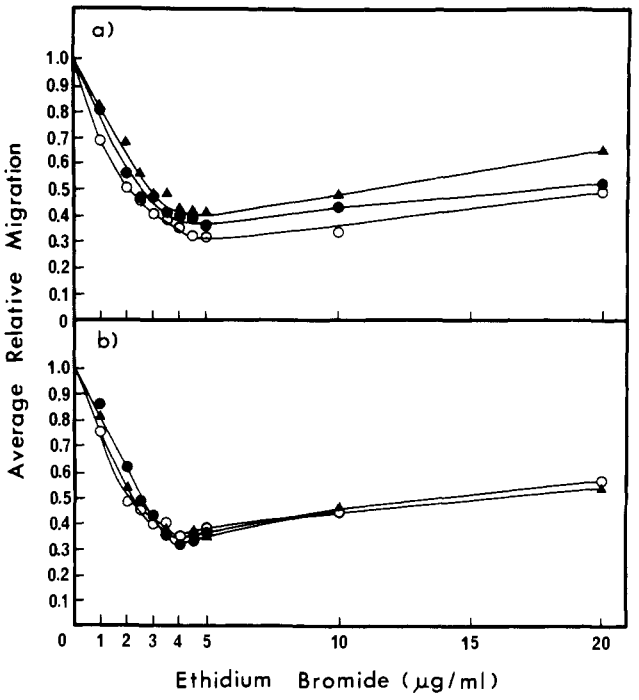

FIGURE 5: EtdBr titration of the nucleoid DNA of BalB/C-3T3 and *ts* 2 mouse fibroblasts. BalB/C-3T3 (panel a) and *ts* 2 cells (panel b) were subcultured from G_0 confluence to obtain $^1/_3$ coverage of the substratum and incubated for 24 h at 34 °C. They were then cultured further for 24 h at this temperature (○), or at 37 (▲) or 38.5 °C (●). The cells were then processed as described under Materials and Methods, for analysis of DNA topology by titration with EtdBr.

°C in the presence of [^{14}C]dThd to prelabel normal DNA. Upon subculture, they were further cultivated at this temperature for 24 h, and then half of the culture sets were upshifted to 38.5 °C. They were labeled for 3 h under test conditions with [^{3}H]dThd and processed for the isolation of nuclei and mitochondria. The latter were subjected to further analysis by equilibrium centrifugation in the presence of EtdBr.

Panels a and b of Figure 6 are characteristic of the banding profiles observed in all wild-type cell experiments, and for *ts* 2 cells incubated at the pt, i.e., under conditions permitting active cell growth. It is clear that the marker ^{14}C-labeled mtDNA and the ^{3}H-labeled mtDNA made under test conditions cobanded, with an apparent buoyant density of 1.46 gm/cm^3. This is characteristic of EtdBr-bound, superhelical, and covalently closed mtDNA [cf. Sheinin et al. (1977)]. In some instances a shoulder of EtdBr-associated mtDNA replicating forms was also detected at a somewhat lower bouyant density. The data for the [^{14}C]DNA reveals that no degradation of the preformed mtDNA had occurred.

The banding behavior of mtDNA produced by *ts* 2 cells similarly temperature-manipulated is shown in panels c and d. The former indicates that the *ts* 2 cells make control amounts of normal mtDNA after incubation at the npt for 16 h. In contrast, they make much less such organellar DNA after 40 h of temperature inactivation (panel d).

The quantitative aspects of three such experiments were analyzed. The mtDNA was found to comprise ≃5.6% of the total DNA of growing BalB/C-3T3 and *ts* 2 cells. The data in Table II show a comparison of the rates of nuclear DNA and mtDNA synthesis in wild-type and *ts* 2 cells, cultured at the pt and the npt.

In the BalB/C-3T3 cells, the ratio of synthetic rates for nuclear and mtDNA remained relatively constant over the experimental period. For *ts* 2 cells the ratio for nuclear activity began to increase shortly after temperature upshift, such that

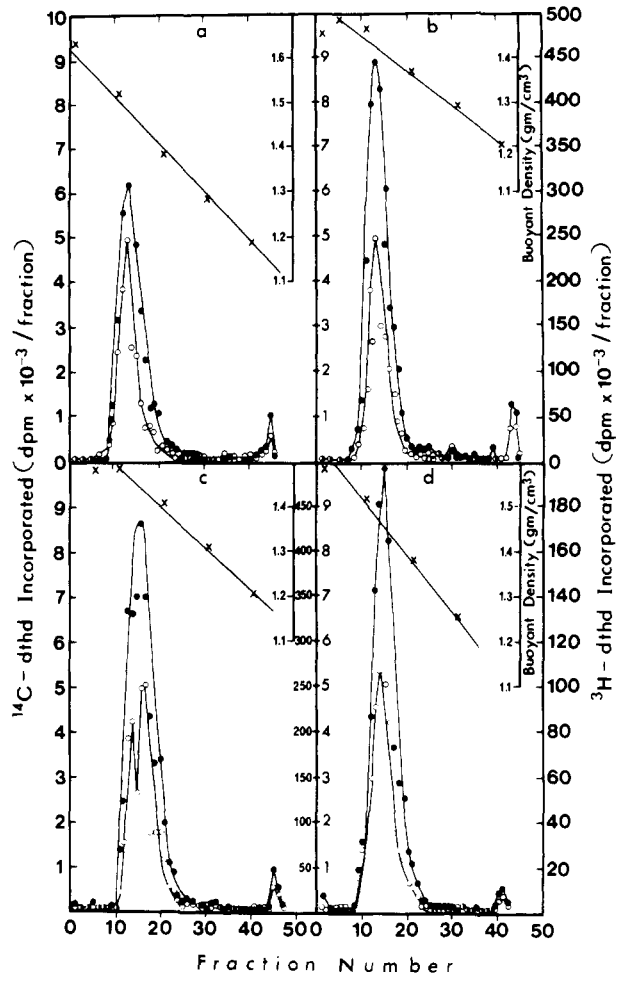

FIGURE 6: Isopycnic centrifugation of mitochondrial DNA synthesized in BalB/C-3T3 and *ts* 2 mouse fibroblasts incubated at 34 °C and at 38.5 °C. BalB/C-3T3 (panels a and b) and *ts* 2 cells (panels c and d), prelabeled in their normal DNA by growth in medium containing [^{14}C]dThd at 34 °C (○), were subcultured at this low temperature in radioisotope-free medium. After 24 h one culture set was upshifted to 38.5 °C, while the other was maintained at 34 °C. At various intervals thereafter, ≃10^8 cells were labeled for 3 h with [^{3}H]dThd (●). The cultures were then harvested and processed for extraction and analysis of the doubly labeled mtDNA by equilibrium centrifugation in $CsCl_2$ density gradients containing 100 μg/mL EtdBr, as described under Materials and Methods. Buoyant density is denoted as ×.

Table II: Ratio of DNA Synthesis in BalB/C-3T3 and *ts* 2 Cells Incubated at Different Temperatures

	ratio of [^{3}H]DNA[a] synthesis at 34 °C/38.5 °C in			
	BalB/C-3T3		*ts* 2	
interval (h)	nuclei	mt	nuclei	mt
0–3	1.76	1.10	1.23	1.38
8–11	1.42	1.13	2.93	1.21
12–15			3.40	1.06
24–27	1.05	0.93	73.2	3.72
36–39	0.92	1.01	90.0	7.63
40–43	1.13	1.74	86.2	17.2

[a] [^{3}H]dThd incorporated into the total DNA of BalB/C-3T3 and *ts* 2 cells was 1.53 × 10^8 and 1.03 × 10^8 dpm/10^8 cells, respectively, at 34 °C during 0–3 h.

in phase II the normal activity was ≃100 times that in the temperature-inactivated cells. The mtDNA synthetic capacity of the *ts* 2 cells incubated at pt and npt remained relatively unchanged until stage II of temperature inhibition. Thereafter, the ratio of activity for the mitochondria of control cells to

activity for mitochondria of heat-inhibited cells also rose, but not nearly as severely.

Discussion

The kinetics of heat inactivation of DNA synthesis in *ts* 2 cultures resemble, in a general way, those observed with dna^{ts}/S^{ts} mouse L-cells [cf. Sheinin (1976, 1980), Sheinin & Guttman (1977), Setterfield et al. (1978), Sheinin et al. (1977, 1978a), Guttman & Sheinin (1979), Sheinin & Lewis (1980), and Ganz & Sheinin (1983)]. Any DNA synthesis carried out by such cells incubated at the npt for an elapse time equivalent to two generation periods is effected by normal semiconservative, semidiscontinuous replication. Thereafter, as the cells move into a third aborted generation period, all semiconservative DNA synthesis is replaced by repair replication.

The kinetic and autoradiographic analyses reveal that *ts* 2 cells already in DNA synthesis when upshifted to the npt progress apparently normally through the current S phase. They indicate further that the mutant cells are unable to enter S phase, once temperature inactivation of the *ts* 2 gene product has taken place. These results are readily contrasted with those obtained in autoradiographic analyses of dna^{ts}/S^{ts} cells, which have been shown to block in S phase (Sheinin, 1980, 1984) because they are defective in a protein that functions in the process of DNA replication, e.g., the *ts* A1S9 and *ts* C1 mouse L-cells, which arrest early in S phase (Sheinin & Guttman, 1977; Sheinin et al., 1977, 1978b; Guttman & Sheinin, 1979; Sheinin & Lewis, 1980), as a result of the heat denaturation of a DNA topoisomerase II enzyme (Colwill & Sheinin, 1983) and a DNA chain elongation factor, respectively (Ganz & Sheinin, 1986).

It was found here, in confirmation of earlier work (Sheinin & Lewis, 1980), that DNA preformed at 34 °C by *ts* 2 and wild-type BalB/C-3T3 mouse fibroblasts remained intact for at least 96 h at this pt or at 38.5 °C. We obtained no indication of degradation of DNA newly made, under test conditions, in wild-type or *ts* cells upon subsequent incubation at either temperature. The pulse–chase experiments set out here show that all DNA synthesis observed was by the semidiscontinuous mode. It is therefore not surprising to discover that the DNA replicated by *ts* 2 cells under restrictive conditions exhibits the same buoyant density in $CsCl_2$ density gradients as does the normal DNA made by *ts* 2 cells at 34 °C and that produced by wild-type BalB/C-3T3 mouse fibroblasts growing at the low or high temperature.

Taken all together, the data indicate that *ts* 2 cells already in S phase move through it without difficulty, while undergoing primary inactivation of the mutant protein. Thereafter, these cells are prevented from initiating or reinitiating entry into DNA synthesis by traverse of the G_1/S interface.

ts 2 cells continued to synthesize mtDNA at the control high rate throughout the entire first stage of temperature inactivation. Thereafter, mtDNA replication underwent reduction but was relatively much higher than that occurring in the nuclei. The data suggest that when the cells have arrested at the G_1/S interface, the ratio of nuclear DNA to mtDNA synthesis is put into serious imbalance. As the cells attempt to reestablish the normal equilibrium between the two cellular DNA compartments, this ratio is reduced. Such regulation of nuclear DNA:mtDNA synthesis may be a general property of temperature-arrested dna^{ts} mammalian cells. Thus, it has been observed with the dna^{ts}/S^{ts} *ts* A1S9 (Sheinin et al., 1977) and *ts* C1 mouse L-cells (Guttman & Sheinin, 1979).

The EtdBr titration profiles for the chromatin-bound DNA of wild-type BalB/C-3T3 cells growing at 34 or 38.5 °C, and of *ts* 2 cells incubated at the pt or the npt, are reminiscent of those obtained with other wild-type (Brazell & Cook, 1975; Colwill & Sheinin, 1982; Mattern & Painter, 1979) and dna^{ts} mammalian cells (Colwill & Sheinin, 1982). They provide evidence for a supercoiled conformation for the nuclear DNA in BalB/C-3T3 and *ts* 2 mouse fibroblasts. They indicate that expression of the *ts* 2 defect prevents the chromosomal DNA of mutant cells from undergoing the conformational modulations that characterize wild-type cells progressing normally into the DNA-synthetic phase, and from one cycle to another.

The *ts* 2 gene product is clearly required for initiation of new rounds of normal cellular DNA replication. This is shown here and elsewhere in a separate study of cell cycle progression (Sheinin et al., 1985b). On the other hand, DNA repair replication proceeds handily even after the *ts* 2 protein has undergone inactivation.

Denaturation of the *ts* 2 protein does not directly prevent replication of the mouse adenovirus DNA (Sheinin et al., 1985a) or of the linear, double-stranded DNA form of the MoMuLV genome (Richter et al., 1984). On the other hand, synthesis of Py DNA and conversion of the first MoMuLV DNA product to its covalently closed, superhelical intermediate are dependent upon the functional integrity of the *ts* 2 protein.

It is of interest that both the adenovirus [cf. Lichy et al. (1983) and Stillman & Tamanoi (1983)] and the MuLV [cf. Bishop (1983)] carry genetic information for viral proteins that effect initiation and synthesis of the viral genome. There is, as yet, no direct evidence that the Py genome encodes a protein that participates directly in viral DNA synthesis, although it is assumed that the Py T-antigen serves functions in initiation [cf. Eckhart (1983)].

These several observations acquire added interest from recent preliminary findings which suggest that the *ts* 2 protein may participate in that process of initiation of DNA replication that is mediated by interaction of the DNA primase with the DNA polymerase α holoenzyme [cf. Philippe et al. (1984)]. We anticipate that further studies with the *ts* 2 gene product will provide unique insights into the molecular biology and enzymology of the several modes of initiation of DNA synthesis effected by mammalian cells as they progress into and through the S phase.

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The *ts* 2 cells were a generous gift of Dr. Harvey Ozer.

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Photosensitive DNA Cleavage and Phage Inactivation by Copper(II)–Camptothecin

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ABSTRACT: Upon irradiation with 365-nm light, copper(II)–camptothecin significantly produced single- and double-strand breaks of DNA and also induced a marked inactivation of bacteriophage. The nucleotide sequence analysis exhibited considerably random DNA cleavage. The DNA strand scission by the camptothecin–Cu(II)–UV light system, as well as the phage inactivation, was strongly suppressed by bathocuproine and catalase, indicating participation of cuprous species and hydrogen peroxide in the reaction. The present results suggest that (1) Cu(II) ion may play an important role as a cofactor in antitumor action of camptothecin and (2) the combination of copper–camptothecin plus long-wave ultraviolet light is useful against certain cancer treatment as a new photochemotherapy.

Camptothecin (CPT) isolated from *Camptotheca acuminate* (Nyssaceae), a tree widely distributed in the sourthern part of China, is a typical cytotoxic alkaloid and has some therapeutic effects on gastric, rectum, and bladder tumors (Cai & Hutchinson, 1983; Wall & Wani, 1980). Although CPT exhibits dose-dependent toxic side effects, the 10-hydroxy derivative of the compound has been clinically used in the cancer treatment in the People's Republic of China with considerable success against liver carcinoma and head–neck cancer. In Japan, the clinical evaluation of CPT derivatives as an antineoplastic agent has been actively continued.

The alkaloid inhibits nucleic acid synthesis rather than protein synthesis, and its rapid suppression of the former is probably responsible for the cytotoxic action of CPT (Cai & Hutchinson, 1983; Wall & Wani, 1980). In contrast with the DNA synthesis, the RNA synthesis is immediately restored

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