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Characterization of DNA Synthesis and DNA-Dependent ATPase Activity at a Restrictive Temperature in Temperature-Sensitive tsFT848 Cells with Thermolabile DNA Helicase B

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A temperature-sensitive mutant defective in DNA replication, tsFT848, was isolated from the mouse mammary carcinoma cell line FM3A. In mutant cells, the DNA-dependent ATPase activity of DNA helicase B, which is a major DNA-dependent ATPase in wild-type cells, decreased at the nonpermissive temperature of 39°C. DNA synthesis in tsFT848 cells at the nonpermissive temperature was analyzed in detail. DNA synthesis measured by incorporation of [^{3}H]thymidine decreased to about 50% and less than 10% of the initial level at 8 and 12 h, respectively. The decrease in the level of thymidine incorporation correlated with a decrease in the number of silver grains in individual nuclei but not with the number of cells with labeled nuclei. DNA fiber autoradiography revealed that the DNA chain elongation rate did not decrease even after an incubation for 10 h at 39°C, suggesting that initiation of DNA replication at the origin of replicons is impaired in the mutant cells. The decrease in DNA-synthesizing ability coincided with a decrease in the level of the DNA-dependent ATPase activity of DNA helicase B. Partially purified DNA helicase B from tsFT848 cells was more heat sensitive than that from wild-type cells. Inactivation of DNA-dependent ATPase activity of DNA helicase B from mutant cells was considerably reduced by adding DNA to the medium used for preincubation, indicating that the DNA helicase of mutant cells is stabilized by binding to DNA.

The unwinding of double-stranded DNA is crucial for the processes of DNA replication, repair, recombination, and transcription. A class of enzymes, designated DNA helicases, accomplish this task by consuming energy produced by hydrolysis of ATP (20–22, 47). Thus, these enzymes contain an intrinsic DNA-dependent ATPase activity.

A cell-free simian virus 40 (SV40) DNA replication system provided an effective functional approach to identifying and characterizing the cellular proteins required for DNA replication in mammalian cells, and many enzymes and proteins such as DNA polymerase, proliferating cell nuclear antigen, and single-stranded-DNA-binding protein have been identified by using this system (13, 15, 42). However, the DNA helicase and the origin-binding protein involved in DNA replication in mammalian cells have not been identified by using the SV40 DNA replication system because large T antigen, which is the only virally encoded protein required for the system, has both DNA helicase and origin-binding activities (2). Thus, identification of the DNA helicase involved in mammalian cell DNA replication is necessary for understanding the molecular mechanism of DNA replication in mammalian cells.

A large number of mammalian DNA helicases have been identified mainly by biochemical assays. A list of these helicases has been presented elsewhere (21, 47). However, only a few mammalian helicases have been characterized with respect to their roles in cellular metabolism. The excision repair cross-complementing *ERCC3* and *ERCC2* gene products have helicase activity (32, 43) and are subunits of transcription factor TFIIH (5, 31, 32). *ERCC3* and *ERCC2* are human genes isolated on the basis of complementation of excision repair defects in rodent cell lines (49, 51), and they are responsible for the defect of xeroderma pigmentosum complementing group B (52) and D (8) cells, respectively. It is apparent that the ERCC3 and ERCC2 helicases are involved in the processes of transcription and DNA excision repair.

Both genetic and biochemical approaches are necessary to identify DNA helicases involved in DNA replication in mammalian cells. In this study, we isolated temperature-sensitive mutants in DNA replication that had thermolabile DNA helicase activity to identify the DNA helicases involved in DNA replication. Mutants were screened by measuring the DNA-dependent ATPase activity in crude extracts developed by fast protein liquid chromatography (FPLC) Mono Q column chromatography, based on the fact that DNA helicases have DNA-dependent ATPase activity, and a mutant designated tsFT848 was found to have thermolabile DNA helicase activity. We characterized DNA synthesis in the mutant cells at the restrictive temperature in detail and studied the relationship between the defective DNA synthesis and the thermosensitivity of DNA-dependent ATPase activity of the DNA helicase.

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MATERIALS AND METHODS

Cells and cell culture. FM3A cells (wild type) were originally established from a spontaneous mammary carcinoma from a C3H/He mouse (27). ts1, ts2, ts232, ts240, ts244, and ts245 cells were isolated as described previously (48), and other mutant cells were isolated as described by Eki et al. (7). Cells were maintained

in suspension culture in RPMI 1640 medium supplemented with 10% calf serum, 100 μg of streptomycin sulfate per ml, and 100 U of penicillin G potassium per ml. Cells were cultured either at 33°C (permissive temperature) or at 39°C (nonpermissive temperature).

Measurements of macromolecular synthesis. Exponentially growing tsFT848 cells (2×10^5 cells) were inoculated with 1 ml of RPMI 1640 containing 10% dialyzed calf serum, 10^{-6} M thymidine, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5) and incubated at 33°C for 6 h. The cells were pulse-labeled with 37 kBq of [^{3}H]thymidine or [^{3}H]uridine for 30 min after various periods of incubation at 33 or 39°C. For labeling with [^{3}H]leucine, all procedures were the same as those described above except that leucine-free minimal essential medium containing 1/100 volume of RPMI 1640 and dialyzed calf serum at a final concentration of 10% was used for the culture medium to increase the specific activity of the [^{3}H]leucine. Pulse-labeling was terminated by adding 0.2 ml of 1-mg/ml NaN_3 to the cultures and chilling the culture tubes in an ice water bath. The cells were collected by centrifugation and lysed with 0.2 ml of 0.1 M NaOH. After neutralization with 0.1 M HCl, trichloroacetic acid was added at a final concentration of 5%. Acid-insoluble materials were collected on Whatman GF/C glass fiber filters, and the radioactivity was measured with a liquid scintillation counter.

Measurement of labeling index and grain number. Cells were labeled with [^{3}H]thymidine as described above and centrifuged at 1,500 rpm for 5 min. The pellet was washed once with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) by centrifugation. The pellet was resuspended in 1 ml of 0.5% sodium citrate and maintained at 37°C for 5 min, and then 5 ml of fixative (methanol-acetic acid, 3:1) was added. The suspension was left at room temperature for 5 min and then centrifuged at 1,500 rpm for 5 min. The pellet was washed once with the fixative by centrifugation, and the pellet was suspended in a small volume of the fixative. The suspension was dropped onto a glass slide and dried in air. The slide was washed four times with cold 2% perchloric acid and then with water. Twofold-diluted Sakura NR-M2 emulsion was mounted on a glass slide, which was exposed at 4°C for 10 days and then developed. The percentage of labeled nuclei (labeling index) and the number of silver grains in individual nuclei were measured.

DNA fiber autoradiography. Cells (1.5×10^5/ml) were inoculated into glass culture tubes (15 by 105 mm) containing 2 ml of growth medium and incubated at 33°C for 1 day and then at either 33 or 39°C for various periods. Thirty minutes before pulse-labeling, 5-fluoro-2′-deoxyuridine was added to the cultures at a final concentration of 3×10^{-8} M to exhaust the endogenous thymidylate. The cells were precipitated by centrifugation at 1,500 rpm for 5 min at room temperature and resuspended in 0.5 ml of prewarmed labeling medium which contained 9.25 MBq of [^{3}H]thymidine (4.26 TBq/mmol) and 3×10^{-8} M 5-fluoro-2′-deoxyuridine. The cells were pulse-labeled with this medium for 10, 20, and 30 min at 39°C. Pulse-labeling was terminated by adding cold thymidine and NaN_3 at final concentrations of 10^{-4} M and 200 μg/ml, respectively, followed by chilling in an ice water bath. The labeled cells were collected by centrifugation, washed with cold CMF-PBS containing 10^{-4} M thymidine, and resuspended in a fourfold dilution of CMF-PBS containing 10^{-4} M thymidine. About 5,000 cells were dropped onto bovine serum albumin-coated glass slides and mixed with lysis medium (2% sodium dodecyl sulfate, 0.05 M EDTA [pH 8.0]). DNA fibers released from the cells were spread with a glass rod over the surface of the slide. Air-dried glass slides were washed with 10% trichloroacetic acid once, 5% trichloroacetic acid three times, and ethanol once and then dipped in twofold-diluted Sakura NR-M2 emulsion. Exposure was performed at 4°C for about 3 months.

Preparation of cell extracts and Mono Q column chromatography to screen mutants. Cells were cultured at 39°C for 8 h, harvested, and stored at −80°C until use. The frozen stored cells (2×10^8 cells) were thawed, suspended in 5 ml of buffer 1 (20 mM potassium phosphate buffer [pH 7.5], 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.25 mM phenylmethylsulfonyl fluoride), and sonicated. The sonic extract was suspended in a final concentration of 0.3 M KCl by adding 1/10 volume of buffer 1 containing 3.3 M KCl. After being stirred for 30 min at 0°C, the extract was centrifuged at 105,000 × *g* for 45 min at 2°C. One milliliter of DEAE-Sephacel equilibrated with 0.3 M KCl in buffer 1 was added to the supernatant, which was rocked for 30 min at 4°C to absorb nucleic acids and centrifuged at 3,000 rpm for 20 min. The supernatant was dialyzed against buffer 2 (20 mM potassium phosphate buffer [pH 7.5], 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.25 mM phenylmethylsulfonyl fluoride, 0.01% Triton X-100, 20% ethylene glycol). The dialysate was filtrated through a Millipore filter (pore size, 0.22 μm) and loaded onto an FPLC Mono Q HR5/5 column equilibrated with 50 mM KCl in buffer 2. The column was washed with 5 ml of equilibration buffer. Proteins were eluted from the column with a 45-ml linear gradient of KCl from 50 to 400 mM in buffer 2. Aliquots (20 μl) of each fraction were assayed for ATPase activity in the presence or absence of 5 μg of heat-denatured DNA.

Preparation of cell extracts by the modified method. Freshly harvested cells (5×10^8) were suspended in 5 ml of buffer 1 and disrupted by 40 strokes in a Dounce homogenizer. Glycerol was added to the lysate at a final concentration of 20%, and proteins were extracted with 0.3 M KCl by adding a 1/10 volume of buffer 1 containing 3.3 M KCl. After centrifugation at 105,000 × *g* for 45 min, the supernatant was recovered, and the concentration of KCl in the supernatant was adjusted to 0.1 M by adding buffer 1 containing 20% glycerol.

Partial purification of DNA helicase B. Freshly harvested tsFT848 (3.0×10^9 cells) and wild-type (8.9×10^9) cells were suspended in buffer 1 at a concentration of 1.25×10^8 cells per ml and sonicated. The sonic extract was mixed with an 11/50 volume of glycerol and 0.1 volume of 3.3 M KCl in buffer 1 to bring the concentrations of glycerol and KCl to 20% and 0.3 M, respectively. After being stirred for 30 min at 0°C, the extract was centrifuged for 1 h at 100,000 × *g*. The supernatant was loaded onto a DEAE-cellulose column (20 ml) equilibrated with 0.3 M KCl–20% glycerol in buffer 1. The flowthrough fractions were pooled, and Triton X-100 was added to the fraction at a final concentration of 0.01% (vol/vol). The fraction was dialyzed against buffer 1 containing 0.01% Triton X-100 and 20% glycerol (buffer 3) with 0.1 M KCl. The dialysate was loaded onto a second DEAE-cellulose column (20 ml) equilibrated with 0.1 M KCl in buffer 3. The column was washed with 5 bed volumes of equilibration buffer, and proteins were eluted from the column with 0.5 M KCl in buffer 3. The eluted fractions were pooled and dialyzed against 0.1 M KCl in buffer 3. The dialysate was applied onto a phosphocellulose column (4 ml) equilibrated with 0.1 M KCl in buffer 3 and eluted with a linear gradient of KCl from 0.1 to 1 M in buffer 3. The active fractions of DNA-dependent ATPase were pooled and dialyzed against 0.1 M KCl in buffer 3. The dialysate was further purified by Mono Q FPLC. DNA-dependent ATPase bound to a Mono Q HR5/5 column was eluted with 40 ml of a linear gradient of KCl from 0.1 to 0.4 M in buffer 3.

ATPase assay. The standard reaction mixture (50 μl) contained 50 mM Tris-HCl, (pH 7.5), 20 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM ATP, and 25 μg of bovine serum albumin. DNA-dependent and -independent ATPase activities were assayed in the presence and absence of 5 μg of heat-denatured calf thymus DNA. The amount of produced ADP was determined by the method of Korn and Yanofsky (16).

DNA helicase assay. DNA helicase substrates were prepared and DNA helicase activity was assayed as described previously (36).

RESULTS

Screening for temperature-sensitive mutants with a defect in DNA-dependent ATPase activity. DNA helicases have DNA-dependent ATPase activity, and it is difficult to quantify DNA helicase activity in crude extracts. We therefore screened collections of mutants to identify those defective in DNA helicase activity by measuring DNA-dependent ATPase activities in crude extracts separated by FPLC Mono Q column chromatography. The screened mutants were isolated from mouse FM3A cells and included tsFT20 and tsFT5 cells, which are temperature-sensitive mutants for DNA polymerase α (26) and the ubiquitin-activating enzyme E1 (24), respectively. DNA synthesis in these mutant cells decreased rapidly after an increase to the nonpermissive temperature of 39°C. The cells were incubated at 39°C for 8 h, then harvested, and stored at −80°C until use. Crude extracts prepared from wild-type or mutant cells were applied onto a Mono Q column. Figure 1 shows the elution profiles of DNA-dependent ATPase activities in crude extracts. The extracts prepared from wild-type cells and all of the mutants except tsFT848 cells exhibited one major and several minor peaks. However, the major peak of DNA-dependent ATPase activity disappeared from tsFT848 cells.

Growth properties and macromolecular syntheses in tsFT848 cells at the nonpermissive temperature. Figure 2 shows the growth curves of tsFT848 and wild-type cells. Wild-type and tsFT848 cells grew at 33°C with doubling times of 14.4 and 21.7 h, respectively. Wild-type cells showed a higher growth rate at 39°C than at 33°C, while the number of tsFT848 cells did not increase at 39°C.

The levels of DNA, RNA, and protein synthesis were measured in tsFT848 cells cultured at the nonpermissive temperature. Figure 3 shows the rates of incorporation of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine into acid-insoluble materials after a temperature shift from 33 to 39°C. The incorporation of [^{3}H]thymidine increased slightly up to 4 h and decreased to about 50% and less than 10% of the initial level at 8 and 12 h, respectively. The incorporation of [^{3}H]uridine and [^{3}H]leucine did not decrease but rather increased considerably up to 8 h and then declined. The incorporation of the three precursors increased gradually when the cells were cultured at 33°C. Thus,

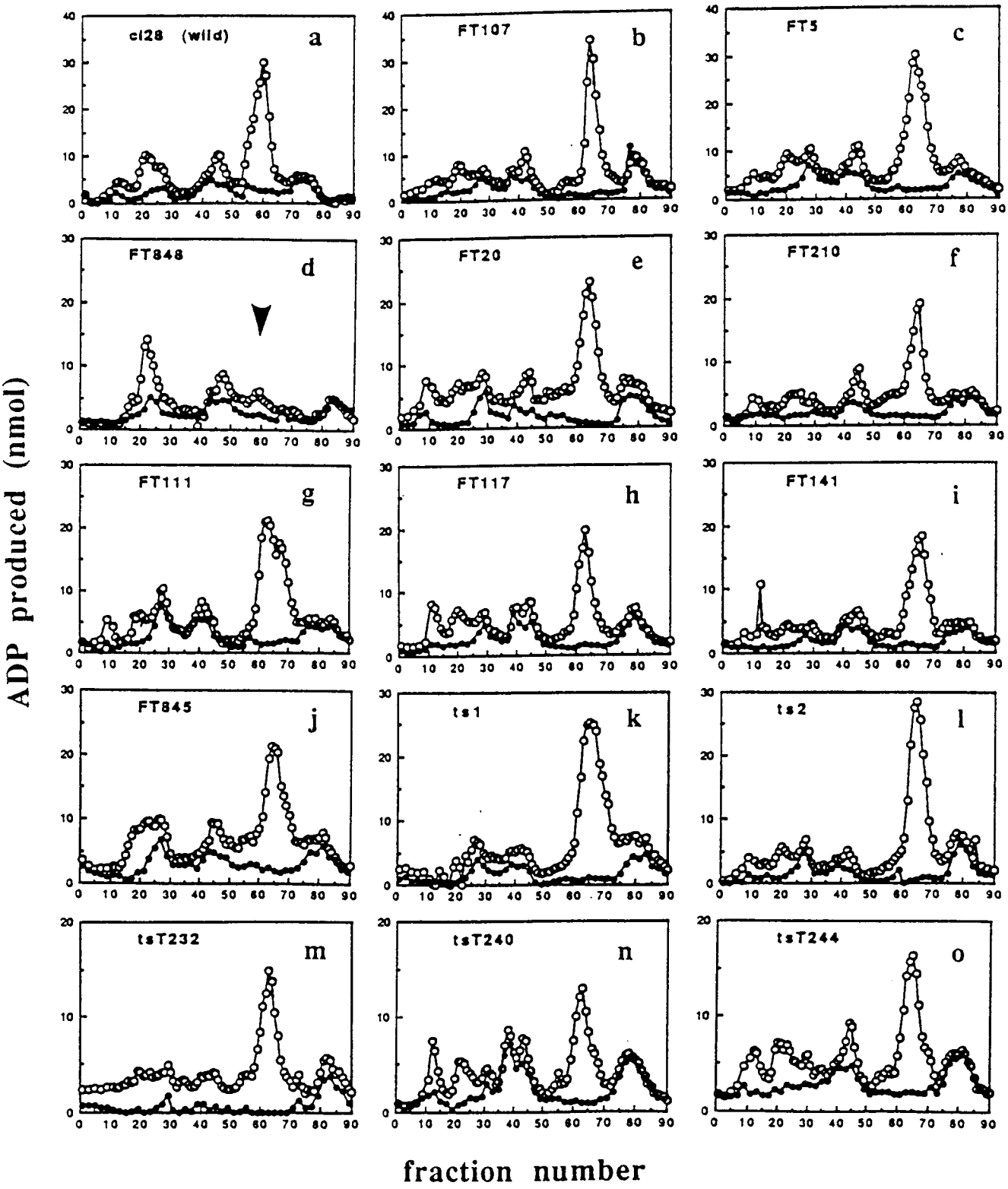

FIG. 1. Screening of mutants having a defect in DNA-dependent ATPase activity. Cells were cultured at 39°C for 8 h, harvested, and stored at −80°C until use. Cell extracts were prepared and subjected to Mono Q column chromatography as described in Materials and Methods. Aliquots (20 μl) of the eluted fraction were assayed for ATPase activity at 37°C for 90 min in the presence (○) or absence (●) of 5 μg of heat-denatured DNA. The arrow indicates the position where the DNA-dependent ATPase B eluted.

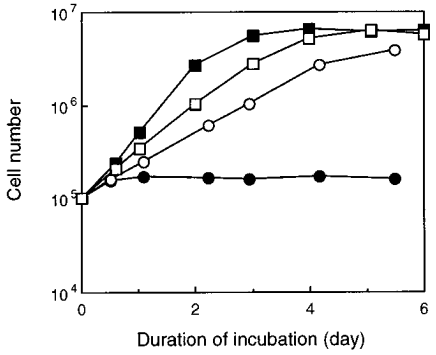

FIG. 2. Growth of tsFT848 and FM3A cells at permissive and nonpermissive temperatures. Exponentially growing cells (10^5) were inoculated and cultured at 33 or 39°C. ○, tsFT848 cells cultured at 33°C; ●, tsFT848 cells cultured at 39°C; □, FM3A cells cultured at 33°C; ■, FM3A cells cultured at 39°C.

tsFT848 cells have a defect in DNA synthesis at the nonpermissive temperature.

Analysis of DNA synthesis in tsFT848 cells by autoradiography and DNA fiber autoradiography. A change in the labeling index, which is defined as the percentage of labeled nuclei, was measured in tsFT848 cells during incubation at the nonpermissive temperature (Fig. 4A). The labeling index of tsFT848 cells and of wild-type cells was little changed during incubation periods of up to 12 h.

The number of silver grains per nucleus, which represents the amount of synthesized DNA in an individual nucleus, did not decrease for up to 4 h but was decreased at 8 h (Fig. 4B). At 16 h after the temperature shift to the nonpermissive temperature, a slight decrease in labeling index (Fig. 4A) and a marked decrease in the number of silver grains (Fig. 4B) were observed in the mutant cells. No decrease in the grain number was observed in wild-type cells during incubation at the nonpermissive temperature for up to 16 h (Fig. 4C).

We next performed DNA fiber autoradiography to determine whether the decrease in the grain number in an individual nucleus was due to a decrease in the rate of DNA chain elongation. tsFT848 cells were cultured at 39°C for 6 and 10 h and pulse-labeled with [^{3}H]thymidine for 10, 20, and 30 min. In tsFT848 cells cultured at 39°C for 6 and 10 h, a linear relationship was observed between the duration of labeling and the mean of the track lengths of silver grains as in wild-type cells cultured at 39°C for 10 h or in mutant cells cultured at 33°C (Fig. 5). From the slope of a plot of track

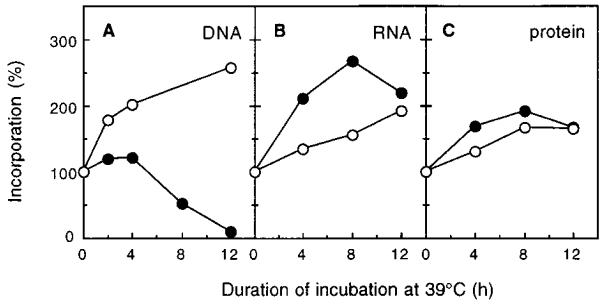

FIG. 3. DNA, RNA, and protein synthesis in tsFT848 cells at 39°C. Cells were pulse-labeled with [^{3}H]thymidine (A), [^{3}H]uridine (B), and [^{3}H]leucine (C) for 30 min at the indicated times after a temperature shift to 39°C (●). The radioactivity incorporated into acid-insoluble materials was measured. The incorporation of the precursors into the cells cultured at 33°C was also determined (○). Values are expressed as percentages of the initial level.

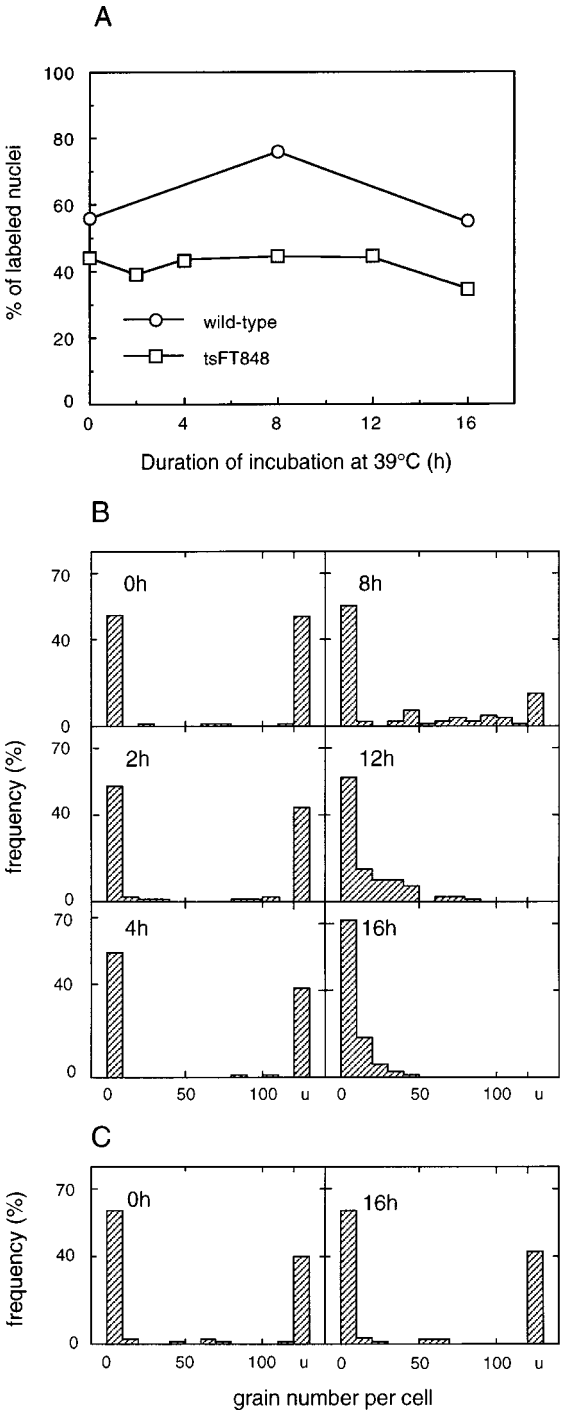

FIG. 4. Analysis of DNA synthesis by autoradiography. Cells were cultured at 33 or 39°C for the indicated periods, then labeled with [^{3}H]thymidine for 30 min, and assessed by autoradiography as described in Materials and Methods. (A) Labeling index, defined as the percentage of cells with labeled nuclei, for tsFT848 (□) and FM3A (○) cells. (B) Distribution histograms of the number of silver grains in the nuclei of tsFT848 cells. (C) Distribution histograms of the number of silver grains in the nuclei of FM3A cells. The nuclei containing more than 120 grains are indicated as uncountable (u).

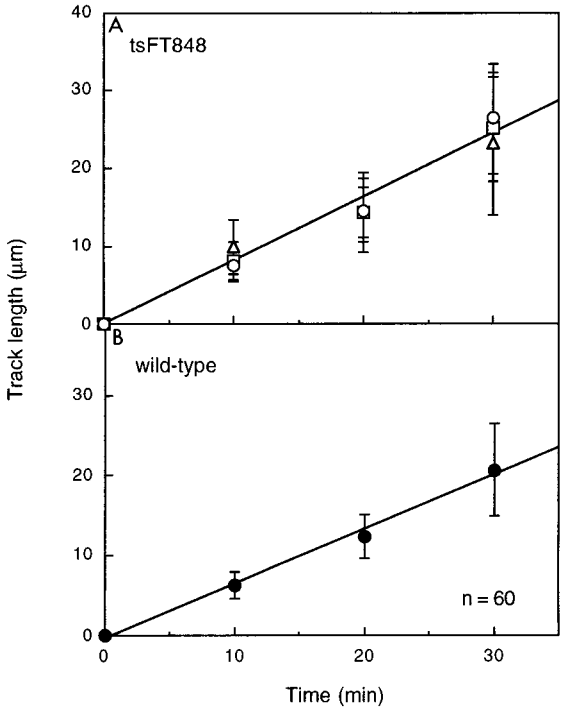

FIG. 5. DNA chain elongation rates in tsFT848 (A) and FM3A (B) cells. Wild-type and tsFT848 cells were cultured at 33°C (○) or 39°C for 6 h (□) or 10 h (△ and ●) and pulse-labeled with [^{3}H]thymidine for 10, 20, and 30 min. DNA fiber autoradiography was performed as described in Materials and Methods. The mean lengths of silver grain tracks were plotted against the labeling time. Vertical lines indicate standard deviations.

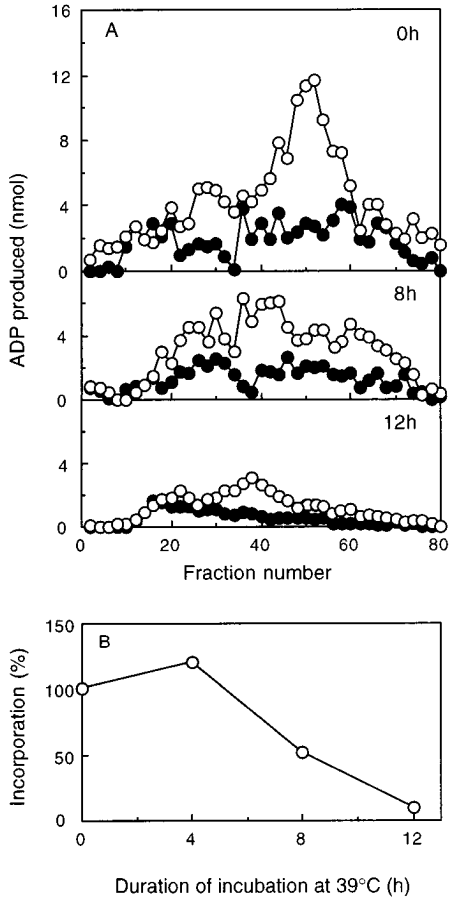

FIG. 6. Relationship between the levels of DNA-dependent ATPase activity and DNA-synthesizing ability in tsFT848 cells. (A) Cell extracts were prepared from tsFT848 cells cultured at 33 or 39°C for 8 and 12 h by the modified extraction method and loaded onto a Mono Q column as described in Materials and Methods. The Mono Q column was developed with a linear gradient of KCl from 0.1 to 0.4 M. Aliquots (20 μl) of the eluted fraction were assayed for ATPase activity at 37°C for 60 min in the presence (○) or absence (●) of 5 μg of heat-denatured DNA. (B) Cells were pulse-labeled with [^{3}H]thymidine for 30 min at the indicated times after a temperature shift to 39°C, and the radioactivity incorporated into acid-insoluble materials was determined. Values are expressed as percentages of the initial level.

length versus the pulse-labeling period, DNA chain elongation rates were estimated to be 0.74, 0.81, and 0.86 μm/min for tsFT848 cells cultured at 33 and 39°C for 6 and 10 h, respectively, and 0.68 μm/min for wild-type cells cultured at 39°C for 10 h.

Relationship between the decrease in DNA-dependent ATPase activity and the decrease in DNA-synthesizing ability of tsFT848 cells. To examine the relationship between the decrease in DNA-dependent ATPase activity and the decrease in DNA-synthesizing ability of the cells, extracts were prepared from the mutant cells cultured at the permissive temperature, 33°C, or at 39°C for 8 and 12 h.

When prepared from frozen cells, DNA-dependent ATPase activity of DNA helicase B was undetectable even in the extracts of tsFT848 cells cultured at 33°C (data not shown). Extracts were prepared from freshly harvested cells by the modified method described in Materials and Methods. As shown in Fig. 6A, a major peak of DNA-dependent ATPase activity was detected in the extracts of tsFT848 cells cultured at 33°C. The peak was considerably decreased in the extracts prepared from the mutant cells cultured at 39°C for 8 h (Fig. 6A, middle panel). Virtually no DNA-dependent ATPase activity was detected in the position of the main peak in the extracts prepared from tsFT848 cells cultured at 39°C for 12 h (Fig. 6A, lower panel). The decrease in DNA-dependent ATPase activity seemed to precede or coincide with the decrease in DNA synthesis (Fig. 6B).

Partial purification of DNA helicase B and examination of its thermosensitivity. We purified the major DNA-dependent ATPase from FM3A cells and designated it DNA-dependent ATPase B (35). We later renamed it DNA helicase B since it had DNA helicase activity (34). To confirm that DNA helicase B itself is temperature sensitive in tsFT848 cells, it was partially purified from tsFT848 and wild-type cells by serial column chromatography on DEAE-cellulose twice, phosphocellulose, and FPLC Mono Q. Figure 7 shows the elution profile of the DNA-dependent ATPase activity of DNA helicase B from wild-type and tsFT848 cells from the Mono Q column. Both DNA-dependent ATPase activities eluted at the same position. However, the specific activity of DNA-dependent ATPase activity of tsFT848 cells was considerably lower than that of wild-type cells. In addition, DNA helicase activity of tsFT848 cells was also lower than that of wild-type cells (data not shown). The heat sensitivity of the Mono Q fractions was then studied. As shown in Fig. 8, DNA helicase B from tsFT848 cells is more heat sensitive than that from wild-type cells.

Figure 9 shows the heat inactivation curves of DNA-dependent ATPase activity of DNA helicases B from tsFT848 and wild-type cells in the presence or absence of DNA. Under the latter condition, like DNA helicase activity, DNA-dependent ATPase activity of DNA helicase B from tsFT848 cells was inactivated more rapidly than that from wild-type cells. How-

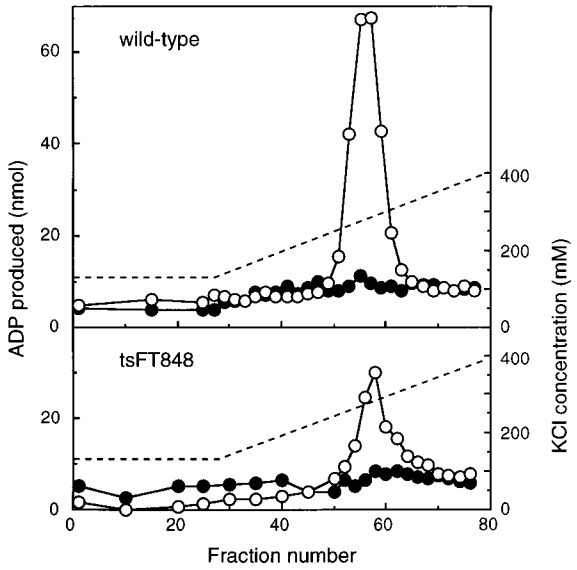

FIG. 7. Mono Q column chromatography of partially purified DNA-dependent ATPase B from tsFT848 and wild-type cells. Cell extracts were prepared from tsFT848 and wild-type cells and fractionated by serial column chromatography on DEAE-cellulose twice and phosphocellulose. The phosphocellulose column fraction containing DNA-dependent ATPase activity was loaded onto a Mono Q column. Aliquots (20 μl) of the eluted fraction were incubated at 33°C for 90 min in the presence (○) or absence (●) of 5 μg of heat-denatured DNA. Dashed lines indicate the concentration of KCl in the eluted fraction.

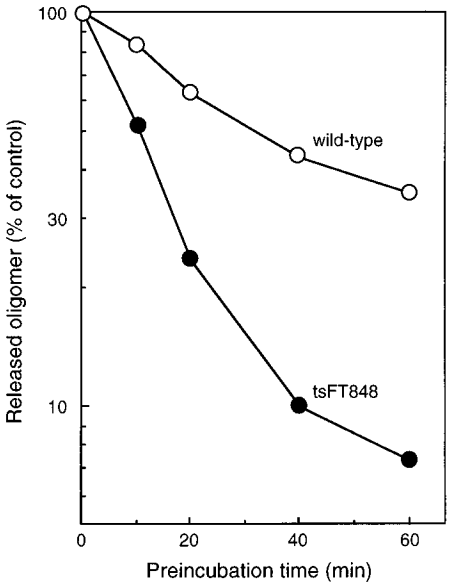

FIG. 8. Heat sensitivity of DNA helicase B from tsFT848 cells. The peak fractions of the Mono Q column (Fig. 7) were dialyzed against 0.1 M KCl in buffer 2 containing 50% glycerol instead of 20% ethylene glycol. The dialysate was used as partially purified DNA helicase B. The partially purified DNA helicase B from tsFT848 or wild-type cells was incubated at 40°C for the indicated periods in buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM 2-mercaptoethanol, 500 μg of bovine serum albumin per ml, and 2.5% glycerol prior to the DNA helicase assay. DNA helicase activity was assayed at 33°C for 10 min as described previously (36). The amount of released oligomers was quantified with a BAS2000 image analyzer (Fuji-Photofilm Co. Ltd.). The 100% values correspond to 12 and 6 fmol of oligonucleotides released for DNA helicase B from tsFT848 cells and from wild-type cells, respectively.

ever, in the presence of DNA, DNA-dependent ATPase activities from both mutant and wild-type cells were considerably stabilized, and there was little decrease in the enzyme activity even after incubation for 60 min.

DISCUSSION

A large number of DNA helicases have been isolated from eukaryotes (21, 47). Several DNA helicases that copurify with various DNA polymerases during purification have been described, indicating the direct interaction of these helicases with the DNA polymerase (1, 19, 30, 41, 46). The activities of some DNA helicases are stimulated by the single-stranded-DNA-binding protein RP-A (11, 38, 39, 45), which has been suggested to be involved in replication, repair, and recombination. These observations indicate but not prove roles for DNA helicases in some aspects of DNA transaction.

Several eukaryotic DNA helicases have been characterized biochemically and genetically, and their roles have been indicated. These include the SV40 and polyomavirus large tumor antigens (2, 33); the herpes simplex virus type 1 origin-binding helicase (3) and helicase-primase complex (4); the bovine papillomavirus E1 protein (40, 53); three helicases from the yeast *Saccharomyces cerevisiae*, the Rad3 (12, 28, 44), Pif1 (9, 17), and Srs2 (18, 29) proteins; and two human helicases, the ERCC2 and ERCC3 helicases (6, 10, 50).

In this study, we confirmed that a temperature-sensitive mouse cell mutant, tsFT848, contains thermolabile DNA helicase B activity (Fig. 8). The inability to detect DNA-dependent ATPase activity of DNA helicase B in extracts prepared from frozen tsFT848 cells cultured at 33°C indicates that DNA helicase B in the mutants is sensitive not only to temperature but also to freezing and thawing.

A cell-free system for polyomavirus DNA replication has been established (25). This system uses the polyomavirus large tumor antigen and mouse cell extracts as sources of DNA replication proteins. By analogy to the cell-free system for SV40 DNA replication, the polyomavirus replication system should require DNA polymerase α-primase, DNA polymerase δ, DNA topoisomerases I and II, RP-A, and other enzymes and proteins. The extracts prepared from tsFT20 cells, which have a temperature-sensitive DNA polymerase α, cultured at the nonpermissive temperature were unable to support polyomavirus DNA replication as described previously (7). On the other hand, extracts from tsFT848 cells cultured at the nonpermissive temperature efficiently supported DNA replication (37), indicating that the defect in tsFT848 cells is not in any of the replication proteins described above.

The decrease in DNA-synthesizing ability in tsFT848 cells coincided with a decrease in the level of DNA-dependent ATPase activity (Fig. 6). The decrease in DNA-dependent ATPase activity cannot be ascribed to the decrease in DNA synthesis because the extracts prepared from tsFT20 and tsFT5 cells cultured at 39°C for 8 h, in which the level of DNA synthesis decreased to less than 10% of the initial level as described previously (24, 26), exhibited DNA-dependent ATPase activity as shown in Fig. 1c and e. Considering this fact together with the observations presented above, it seems very likely that DNA helicase B is defective in tsFT848 cells and that this defect is responsible for the defect in DNA synthesis at the nonpermissive temperature in the mutant cells. Thus, DNA helicase B seems to be involved in DNA replication in mammalian cells.

DNA fiber autoradiography showed that the DNA chain elongation rate in the mutant cells did not change at the nonpermissive temperature (Fig. 5). This finding indicated that once integrated into the DNA replication machinery, the tem-

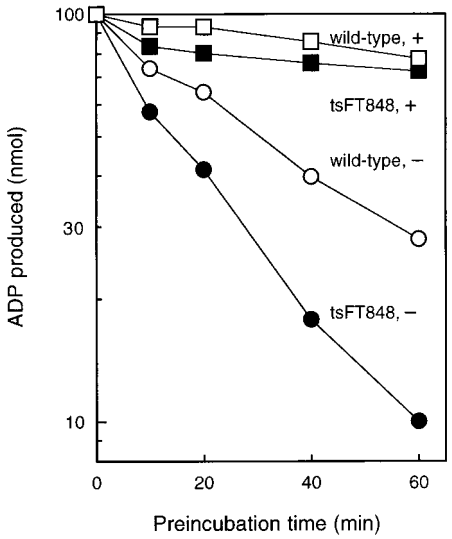

FIG. 9. Stabilization of DNA-dependent ATPase activity of DNA helicase B from tsFT848 cells by the presence of DNA. Partially purified DNA helicase B from tsFT848 (●, ■) or wild-type (○, □) cells was incubated at 40°C for the indicated periods in 25 μl of medium containing 50 mM Tris-HCl (pH 7.5), 20 mM 2-mercaptoethanol, 0.5 mg of bovine serum albumin per ml, and 6% glycerol with (■, □) or without (●, ○) 5 μg of heat-denatured DNA. Twenty five microliters of 50 mM Tris-HCl (pH 7.5) containing 20 mM 2-mercaptoethanol, 0.5 mg of bovine serum albumin per ml, 10 mM ATP, and 10 mM $MgCl_2$ was added to the samples, and 5 μg of heat-denatured DNA was added to the samples that were preincubated without DNA. The mixtures were incubated at 33°C for 90 min to estimate the level of DNA-dependent ATPase activity.

perature-sensitive protein retains its stability or is protected from temperature inactivation. The data in Fig. 9, showing the protection of DNA helicase B from heat inactivation by the presence of DNA, seem to support this notion.

The use of cell-free systems to study the biochemistry of DNA replication will facilitate understanding the roles of DNA helicase B in DNA replication. A model system that consists of plasmid DNA containing an autonomously replicating sequence from *S. cerevisiae*, SV40 T antigen, single-stranded-DNA-binding protein, DNA polymerase α-primase complex, and DNA gyrase has been constructed (14). In this system, T antigen acts as a DNA helicase and DNA synthesis starts from the autonomously replicating sequence region and proceeds bidirectionally. It is noteworthy that among mammalian helicases so far tested, only DNA helicase B replaced T antigen and functioned in the DNA replication system (23).

To further confirm the mutation in DNA helicase B in tsFT848 cells and its involvement in DNA replication, we are in the process of isolating a cDNA for DNA helicase B.

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