J. Biochem. **134**, 71–82 (2003)
DOI: 10.1093/jb/mvg126

A Temperature-Sensitive Mutant of the Mammalian RNA Helicase, DEAD-BOX X Isoform, DBX, Defective in the Transition from G1 to S Phase

Junko Fukumura, Eishi Noguchi*, Takeshi Sekiguchi and Takeharu Nishimoto†
Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582

Received March 28, 2003; accepted April 22, 2003

ts ET24 cells are a novel temperature-sensitive (ts) mutant for cell proliferation of hamster BHK21 cells. The human genomic DNA which rescued the temperature-sensitive lethality of ts ET24 cells was isolated and screened for an open reading frame in the deposited human genomic library. *X* chromosomal *DBX* gene encoding the RNA helicase, DEAD-BOX X isoform, which is homologous to yeast Ded1p, was found to be defective in this mutant. The single point mutation (P267S) was localized between the Motifs I and Ia of the hamster DBX of ts ET24 cells. At the nonpermissive temperature of 39.5°C, ts ET24 cells were arrested in the G1-phase and survived for more than 3 days. In ts ET24 cells, total protein synthesis was not reduced at 39.5°C for 24 h, while mRNA accumulated in the nucleus after incubation at 39.5°C for 17 h. The amount of *cyclin A* mRNA decreased in ts ET24 cells within 4 h after the temperature shift to 39.5°C, consistent with the fact that the entry into the S-phase was delayed by the temperature shift.

Key words: DBX, Ded1, protein synthesis, somatic ts mutant, ts ET24.

Temperature-sensitive (ts) mutation is a valuable tool for clarifying genes essential for cell proliferation (*1*). We have isolated a series of ts mutants from the hamster BHK21 cell line (*2*, *3*). These were classified according to the ability of hybrid cells created by cell-cell fusion of the mutants to grow at the nonpermissive temperature of 39.5°C. The ts ET24 cell line turned out to be a novel mutant of the hamster BHK21 cell line. The gene responsible for a temperature-sensitive phenotype of ts ET24 cells was identified, as reported (*2*). It was the hamster *X* chromosomal gene, *DBX*/*DDX3*, which encodes a RNA helicase, homologous to the human DEAD-BOX X isoform (*4*, *5*).

Helicases are enzymes that catalyze strand separation of double- stranded DNAs or RNAs, in a manner dependent on the hydrolysis of a nucleoside triphosphate, preferentially ATP (*6*). RNA helicases have common ATPase motifs, which are normally comprised of Motif I and a variant Motif II which contains either a DEAD or DexH amino acid sequence: (*7*). According to the genomic sequence, *S. cerevisiae* possesses 26 DEAD-box proteins and 13 DexH-box proteins of RNA helicases (*8*). Most of these yeast RNA helicases have homologues in mammalian cells, and the crystal structure of hepatitis C virus NS3 RNA helicase domain is similar to that of the bacterial DNA helicases (*9*). This suggests that the folding of helicase motifs is well conserved through evolution.

RNA helicases are required for various aspects of cellular RNA metabolism, such as transcription, pre-mRNA splicing, ribosome biogenesis, tRNA and snRNA processing, RNA export, translation and RNA degradation (*8*). *S. cerevisiae* RNA helicase, Ded1p, which is reported to be required for general translation together with the eIF4A DEAD–box RNA helicase (*8*, *10*, *11*), is well conserved through evolution. *Xenopus* Ded1p-homologue, An3, was identified as a protein tightly bound to Crm1p/exportin 1 (*12*). Its helicase activity is coupled with the CRM1-mediated nuclear export of An3 (*13*). A functional mammalian homologue of *S. cerevisiae* Ded1p is reported to be murine PL10 (*14*), which rescues the lethality of *S. cerevisiae ded1* mutants (*10*, *15*); however, PL10 is expressed only in the germ cells of mice (*14*), contrary to the general cellular functions assumed for *S. cerevisiae* Ded1p. Human genomic sequences reveal that humans have two genes encoding *S. cerevisiae* Ded1p-homologues, *DBX*/*DDX3* and *DBY* (*4*, *5*), which are expressed ubiquitously (*4*).

Our present results indicated that the *DBX* gene is essential for cell proliferation. Its mutation in ts ET24 cells caused the G1-arrest at 39.5°C. Total protein synthesis was not inhibited in ts ET24 cells, in contrast to *S. cerevisiae ded1* mutants (*10*). DBX was not detected on polyribosome RNA fractions of human 293 cells. Together, these results indicated that mammalian DBX was not directly involved in protein translation. We found that the amount of *cyclin A* mRNA was reduced in ts ET24 cells within 4 h after the temperature shift to 39.5°C. Thus, the stability of *cyclin A* mRNA was suggested to be affected in ts ET24 cells, resulting in the G1-arrest of ts ET12 cells at 39.5°C. Interestingly, the nuclear accumulation of mRNA was also observed in ts ET12 cells after incubation at 39.5°C for 17 h, which is reported to occur in the mutants defective in *S. cerevisiae* DEAD-box RNA helicase, Dbp5 (*16*) or Sub2p (*17*). These findings suggest that the mammalian DBX RNA helicase has several

*Present address: Department of Molecular Biology, The Scripps Research Institute, La Jolla, California, 92037, USA.
†To whom correspondence should be addressed. Tel: +81-92-642-6175, Fax: +81-92-642-6183, E-mail: tnishi@molbiol.med.kyushu-u.ac.jp

unknown functions, which should be clarified in the future by use of ts ET24 cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—All cell lines were cultured in Dulbecco's modified Eagle (DME) medium supplemented with 10% calf serum or 10% fetal calf serum in a humidified atmosphere containing 10% CO_2. ts ET24 cells were maintained at 33.5°C, the permissive temperature, and ts$^+$ cells were selected at 39.5°C, the nonpermissive temperature.

FACScan Analysis—Cells were washed with TD (Tris-buffered saline without Ca^{2+} and Mg^{2+}) and collected by trypsin treatment. Collected cells were again washed with TD, fixed with 50% methanol for 1 h at 4°C, then treated with RNase A (1 μg/ml), stained with propidium iodide (0.1% sodium citrate, 50 μg/ml propidium iodide) and subjected to FACScan analysis (Becton Dickinson, Franklin Lakes, NJ). The data were analyzed with CELLQuest and ModFit LT (Becton Dickinson) software.

Mutagenesis and Selection of ts Mutants—Exponentially growing cultures of hamster BHK21 cells (4×10^5 cells) were synchronized in the G0-phase by incubation in low-serum DME medium and then in isoleucine-free DME medium, each for 24 h as described (*18*). Every 4 h after release into fresh DME medium containing 20% fetal calf serum, cells were incubated in DME medium containing 1 μg/ml of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and 50 μg/ml of thymidine, as described (*2*, *3*). Mutagenized cells were incubated in DME medium, either with or without 0.1 μg/ml of benomyl, for 72 h. Cells were then synchronized in the G0-phase as described above, and ts mutants for cell-proliferation were selected at 37°C using *5*-fluoro-*2*′-deoxyuridine (FUdR) (*2*).

Stable ts$^+$ Transformation of ts ET24 Cells—The high-molecular-weight HeLa cell DNA (20 μg/dish) or the cloned genomic DNA or cDNA carried on the expression vector (2 μg/dish) was transfected into ts ET24 cells (seeded at 2×10^5 cells/100-mm dish) along with pSV2neo^r (2 μg/dish), using the calcium phosphate coprecipitation method, as described previously (*19*), or the lipofectamine method as described by the supplier (Invitrogen Inc. Carlsbad, CA). Transfected cells were incubated at 33.5°C for 18 h, then washed with TD buffer. After further incubation at 33.5°C for 48 h, transfected cells were cultured in the presence of G418 (800 μg/ml) at either 33.5°C or 39.5°C. The surviving colonies were fixed, stained with crystal violet and counted as described (*19*).

Construction and Screening of Genomic DNA Libraries—λ phage library: The genomic DNA of the ts$^+$ secondary transformant, 24H2-94, was partially digested with *Sau*3AI and fractionated with a sucrose gradient (10–40%). The resulting DNA fragments of 12 to 24 kb were ligated with λ DashII DNA (Stratagene, La Jolla, CA) which had been digested with *Bam*HI, then packed into λ phage using Giga pack III gold packaging extract (Stratagene). The resulting recombinant phages (about 1×10^4 pfu/ml) were screened for human sequences using a human-specific *Alu*-sequence as a probe.

Cosmid library: The genomic DNA of the ts$^+$ secondary transformant, 24H2-94, was partially digested with *Sau*3AI and dephosphorylated by calf intestine alkaline phosphatase (CIAP). The resulting DNA fragments were ligated with SuperCos1 vector (Stratagene) that had been digested with *Xba*I, then treated with CIAP and further digested with the *Bam*HI enzyme. The resulting ligated DNA fragments were packaged *in vitro* with the Giga pack III XL-packaging extracts and then infected into *E. coli* XL1-blue MR. Colonies were screened for human sequences by colony hybridization using a 0.6-kb human-specific *Alu*-free *Eco*RI fragment as a probe, as reported (*19*).

Amplification and cloning of DBX cDNA—HeLa poly A$^+$ RNA encoding the human DBX, was amplified by the RT-PCR method using four oligonucleotides as primers (primer 1, ATG AGT CAT GTG GCA GT; primer 2, GCA AGG ACG AAC TCT AGAT; primer 3, TTT TCA TAC CGA TCT AGAG; and primer 4, TCA GTT ACC CCA CCA GTC AA). The positions of these primers in the human *DBX* gene are shown in Fig. 5A. The resulting cDNA fragment was inserted into the *Bgl*II site of the pcDEBΔ vector. The hamster *DBX* cDNA clone was isolated from the cDNA library of the wild-type (wt) BHK21 cells using the human *DBX* cDNA fragment as the probe. According to the nucleotide sequence of obtained hamster wt *DBX* cDNA (deposited as Genbank Accession No. AB080116), the *DBX* cDNA of ts ET24 cells was amplified from the poly A$^+$ RNA of the hamster ts ET24 cells by the RT-PCR method using four synthetic oligonucleotides as the primers (primer 5, CGG TAC TCT TCA GGG ATGA; primer 6, CAT GGA CGA ACT CGA GATC; primer 7, AGA TCT ATG AGG AAG CCAG; and primer 8, CTA CTG CAA AGC AGG CTCA). The positions of these primers in the hamster *DBX* gene are shown in Fig. 5A. The hamster *DBX* cDNA fragments derived from ts ET24 and wt BHK21 cells were inserted into the *Sac*I–*Hin*dIII sites of pcDEBΔ.

Immunofluorescence Staining and Antibodies—Cells were washed with phosphate-buffered saline (PBS) containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.2, then fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton-X-100 in PBS for 5 min. After a wash with PBS, cells were incubated in a blocking solution of PBS containing 3% bovine serum albumin (Sigma Fraction V) for 1 h at room temperature, followed by the same solution containing the primary antibody for 1 h at room temperature. After three washes with PBS, cells were treated with the FITC-conjugated goat anti-mouse IgG (Bio Source International) in blocking solution for 45 min at room temperature. They were then stained with 1 μg/ml of Hoechst 33342. Cells on coverslips were mounted on Vectashield (Vector Laboratory, Burlingame, CA). Digital imaging of stained cells was obtained using the Olympus laser-scanning microscope LSM-GB200 system. The antibodies used were as follows: anti-eIF4E (Cat. no. 610269) and anti-cyclin D1 (Cat. no. 556470) derived from BD Transduction Laboratories, anti- ribosome S6 (Cat. no. sc-13007) and anti-ribosome L28 (Cat. no. sc-14151) derived from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-cyclin A and anti-cyclin B antibodies were described previously (*37*). The anti-α-tubulin derived

from Sigma (Cat. no. T9026). The DBX antibody was prepared as following. According to the sequence of hamster DBX cDNA, a peptide with the sequence YHEGYACTSIHGDSQ was synthesized. The peptide coupled to the carrier protein was used to immunize rabbits by MBL (Nagoya).

Fluorescence In Situ Hybridization (FISH)—Cells fixed and permeabilized as described above were washed with PBS twice at room temperature, then incubated in 2× SSC buffer containing 300 mM NaCl and 30 mM sodium citrate for 10 min at room temperature. The resulting cells were subjected to hybridization in buffer (2× SSC containing 1 mg/ml yeast tRNA, 10% dextran sulphate, 25% formamide and 4 ng/μl FITC-oligo-dT 50-mer) for 20 h at 42°C. After hybridization, cells were washed with 2× SSC for 15 min twice, 0.5× SSC for 15 min and PBS for 5 min at room temperature, then processed for fluorescence microscopy. An oligo-dT 50-mer, the 3′ end of which had been labeled with FITC, was obtained from Hokkaido System Science (Sapporo).

In Vivo Labeling of Proteins and Immunoprecipitation—Cells were labeled with 20 μCi/ml of [^{35}S]methionine (Promix, Amersham Bioscience) and washed with PBS three times. The radioactivity incorporated into acid-insoluble materials was determined as described (*3*). The incorporated radioactivity was counted with a scintillation counter.

Cells were lysed in 1 ml of lysis buffer [50 mM Tris pH 7.4, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM PMSF and10 μg/ml each of aprotinin, leupeptin, pepstatin and antipain] and clarified by centrifugation at 10,000 ×*g* for 10 min at 4°C. Cell-extracts (500 μg) were incubated with 1 μl of antibodies for 1 h at 4°C, then with 50 μl of protein G-Sepharose 4 Fast Flow (Amersham Pharmacia) (50% v/v) for an additional 1 h. Immunoprecipitates were collected by centrifugation at 10,000 ×*g* for 2 min at 4°C, washed five times with 1 ml of the lysis buffer at 4°C, then suspended in 50 μl of the sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10 mM 2-mercaptoethanol, 3% (wt/vol) SDS, and 20% glycerol, before being boiled. The resulting protein samples were electrophoresed in a 10% or 12.5% SDS–polyacrylamide slab gel and analyzed by immunoblotting or autoradiography as described previously (*20*). Immunoblotting was performed using an ECL kit (Amersham Bioscience) as recommended by the supplier.

RESULTS

Isolation of the ts ET24 Cell and Its Preliminary Characterization—Most mammalian ts mutants possess a mutation in the *X* chromosome (*2*), consistent with the fact that *X* chromosomal genes are expressed in a haploid dose. To isolate autosomal mutants, wt BHK21 cells were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, then incubated in the presence of 0.2 μg/ml of benomyl, an anti-microtubule drug, prior to FUdR selection. Irrespective of the benomyl addition, however, the majority of the isolated ts mutants belonged to the complementation group of ts BN462 cells which has a defect in the *X* chromosomal *CCG1/TAF$_{II}$250* gene (*21*). Even so, we obtained two novel ts mutants, ts ET12 and ts ET24. ts ET12 cells rapidly died and floated out of dishes following incubation at 39.5°C, the nonpermissive temperature. In contrast, ts ET24 cells remained attached to culture dishes after incubation for two weeks at 39.5°C, suggesting that this mutant may have a defect in a gene involved in the G1-phase progression. ts ET24 cells were therefore chosen for further analysis.

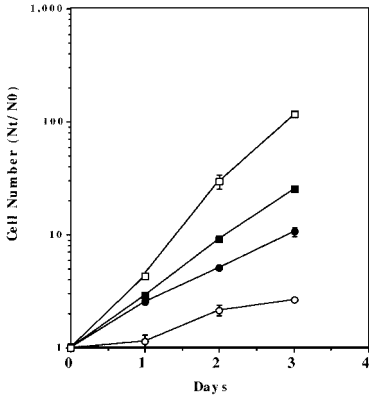

Fig. 1. **ts ET24 cells grew slowly at 39.5°C.** wt BHK21 (solid squares, open squares) and ts ET24 (solid circles, open circles) cells were seeded at densities of 5 × 10^4/50-mm dish, and then incubated at 33.5°C for one day. After feeding with fresh medium, half the cultures were shifted to 39.5°C. At the indicated time, cells were harvested and counted by a Coulter counter. The ratio of cell number (Nt) at each time point relative to the initial number (N0) at the temperature shift was plotted against the time of incubation. (solid squares, solid circles) 33.5°C; (open squares, open circles) 39.5°C. Error bars (standard deviation) from triplicate samples are shown.

When cultures of ts ET24 cells exponentially growing at 33.5°C were incubated at 39.5°C, their growth ratio was significantly reduced within just one day; however, ts ET24 cells did not cease to proliferate for at least three days (Fig. 1). To determine what phase of the cell cycle was affected in ts ET24 cells at 39.5°C, cell-cycle progression was analyzed by fluorescence-activated cell sorting (FACS). When exponentially growing cells were incubated at 39.5°C, ts ET24 cells in the S-phase were rapidly reduced by the temperature shift 33.5°C to 39.5°C (Fig. 2A), but wt BHK21 cells in the S-phase were not affected by the shift (Fig. 2A). A hypophosphorylated form of Rb (retinoblastoma protein, an inhibitor of cell-cycle progression) also accumulated in ts ET24 cells incubated at 39.5°C (data not shown). On prolonged incubation (17 h to 48 h) at 39.5°C, ts ET24 cells in the S-phase slowly increased (Fig. 2A, b), consistent with the increment of ts ET24 cells in the G2-M phase after incubation for 48 h at 39.5°C (Fig. 2A, a).

To confirm the defect of the S-phase entry, cultures of ts ET24 cells and wt BHK21 cells were synchronized at the G0/1 phase by serum starvation, then incubated at 33.5°C or 39.5°C in a normal medium (Fig. 2B). Compared to wt BHK21 cells, the entry of ts ET 24 cells into the S-phase was delayed even at 33.5°C and its frequency was lower. At 39.5°C, ts ET24 cells also entered into the S-phase, although their entry was delayed compared to that at 33.5°C. Thus, it was clear that the entry into the S-phase was not completely inhibited in ts ET24 cells at 39.5°C within 48 h, consistent with the fact that ts ET24 cells proliferated for at least 3 days at 39.5°C.

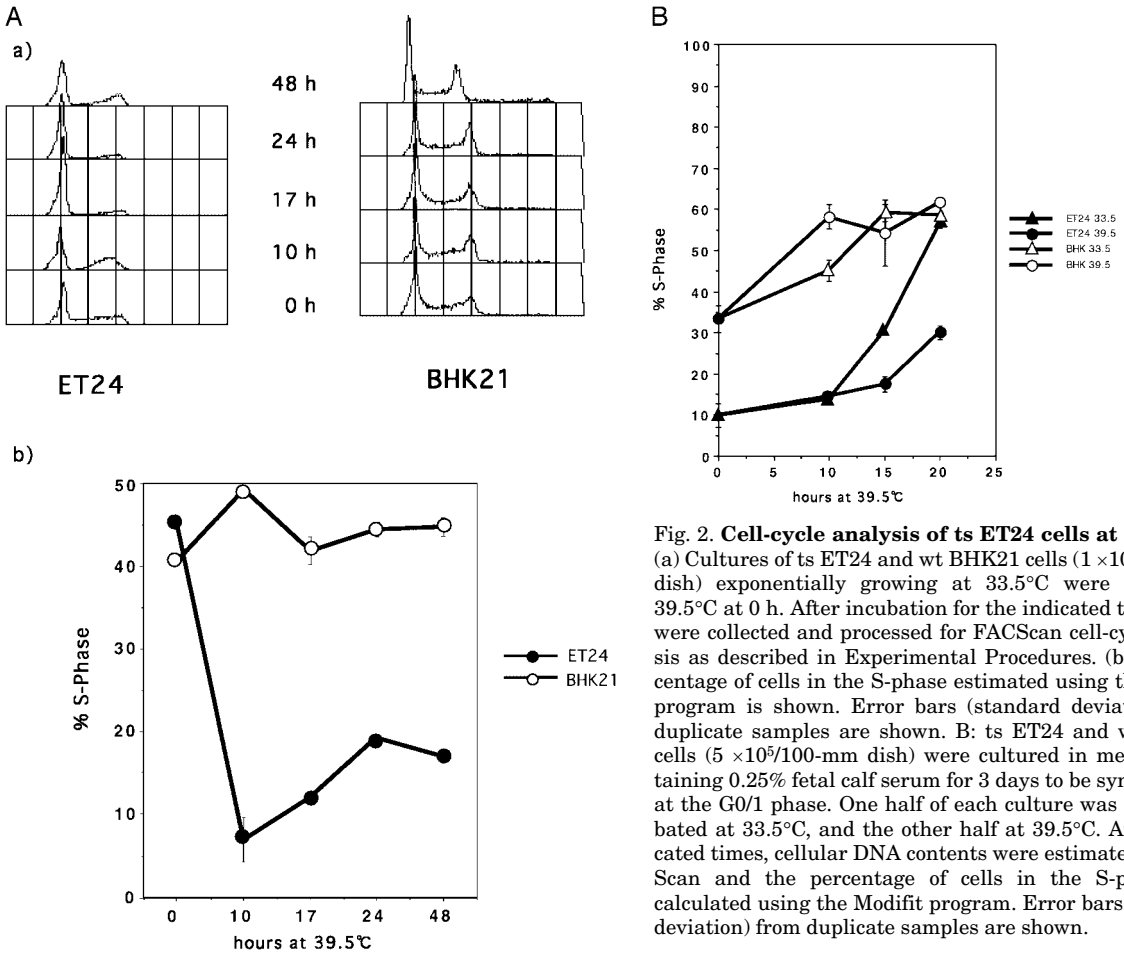

Fig. 2. **Cell-cycle analysis of ts ET24 cells at 39.5°C.** A: (a) Cultures of ts ET24 and wt BHK21 cells (1×10^6/100-mm dish) exponentially growing at 33.5°C were shifted to 39.5°C at 0 h. After incubation for the indicated times, cells were collected and processed for FACScan cell-cycle analysis as described in Experimental Procedures. (b) The percentage of cells in the S-phase estimated using the Modifit program is shown. Error bars (standard deviation) from duplicate samples are shown. B: ts ET24 and wt BHK21 cells (5×10^5/100-mm dish) were cultured in medium containing 0.25% fetal calf serum for 3 days to be synchronized at the G0/1 phase. One half of each culture was then incubated at 33.5°C, and the other half at 39.5°C. At the indicated times, cellular DNA contents were estimated by FACScan and the percentage of cells in the S-phase was calculated using the Modifit program. Error bars (standard deviation) from duplicate samples are shown.

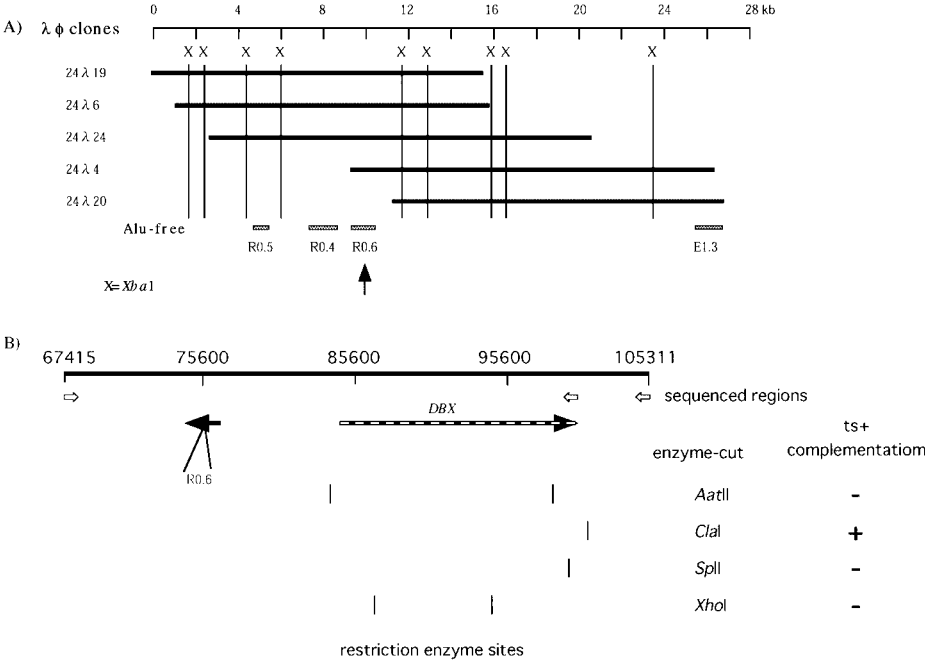

Fig. 3. **Isolated human genomic DNA fragments.** A: Alignment of human DNA fragments inserted into the λ phage vectors. The solid lines indicate the position of human DNA inserted into the indicated recombinant phage clones. The restriction sites for *Xba*1 are shown. The stippled boxes indicate the position of human genomic DNA without the human-specific *Alu*-sequence (*Alu*-free). B: Human genomic DNA inserted into the Cosmid J clone. The nucleotide number of the obtained human *X* chromosomal genomic DNA was estimated from Genbank Accession No. AL391647. The position of DKFZp566MO51 and the DEAD-Box X isoform (DBX) is shown by closed arrows. The open boxes of *DBX* (shown by the white sections in the arrow) indicate the position of exons. The position of the R0.6 *Alu*-free fragment in the cosmid J that was used as a probe, is shown. Open arrows indicate regions of which the nucleotide sequences were determined. Restriction enzymes that cut the *DBX* genome are shown along with their positions. (–) did not rescue the ts phenotype of ts ET24 cells. (+) rescued the ts phenotype of ts ET24 cells.

Table 1. **Rescue of mutant ts ET24 phenotype by DNA transfection.**

	Transfected DNA	Number of colonies per dish		
		ts$^+$	hygro^r	neo^r
a.	Cosmid J	126.0 ± 4.8	–	133.3 ± 26.6
	Human DBX cDNA	68.0 ± 34.4	29.5 ± 11.5	–
	pSV2 neo	0.0 ± 0.0	–	9.1 ± 4.6
b.	Human DBX cDNA	44.6 ± 22.9	75.5 ± 22.5	
	Hamster DBX cDNA (wt-mt*)	10.8 ± 2.6	90.5 ± 22.1	
	Hamster DBX cDNA (mt-mt**)	0.0 ± 0.0	54.0 ± 0	
	pcDEBD vector	0.3 ± 0.7	58.3 ± 15.4	

*The N-terminal 0.8-kb fragment derived from the wild-type DBX cDNA (wt) was fused in-frame with the C-terminal 1.2-kb fragment derived from the mutant-type DBX cDNA (mt). **Mutant-type DBX cDNA (mt-mt).

The DBX/DDX3 Gene Complements the ts ET24 Phenotype—To identify what gene was mutated in ts ET24 cells, high-molecular-weight DNA of HeLa cells was introduced into ts ET24 cells along with pSV2neo^r plasmid DNA, as a marker of transfection. Transfected cells were cultured at 39.5°C or 33.5°C in normal medium containing G418 (800 μg/ml) as a control. From the resulting primary ts$^+$ colonies which grew at 39.5°C, total cellular DNA was extracted to be analyzed for the presence of human DNA using the human-specific *Alu*-sequence. To exclude any extra human DNAs that were not required for rescuing the temperature-sensitive lethality of ts ET24 cells, total cellular DNAs of primary ts$^+$ transformants were transfected into ts ET24 cells. Transfected cells were incubated at 39.5°C. The resulting secondary ts$^+$ transformant, 24H2-94, was chosen to prepare the genomic DNA library using the λ phage vector λ-DASHII. The genomic library of the ts$^+$ transformant, 24H2-94 cells, was screened for the human-specific *Alu*-sequence. The human DNA fragments carried by the resulting recombinant λ phage-vectors were aligned based on the *Xba*I site (Fig. 3A). They covered about 27 kb of the human genomic DNA. The resulting human genomic DNA fragments were then digested with *Eco*RI to be screened for the DNA fragments without a human-specific *Alu*-sequence (*Alu*-free fragments). The resulting human *Alu*-free genomic DNA R0.6 (indicated by an arrow in Fig. 3A) was used as a probe for screening the cosmid genomic DNA library of the secondary ts$^+$ transformant, 24H2-94. The resulting 15 cosmid clones (A to P) were transfected into ts ET24 cells in order to determine their ability to rescue the temperature-sensitive lethality of ts ET24 cells. Two cosmid clones, E and J, were found to be able to rescue the lethality of ts ET24 cells. Representative results are shown in Fig. 4 and Table 1.

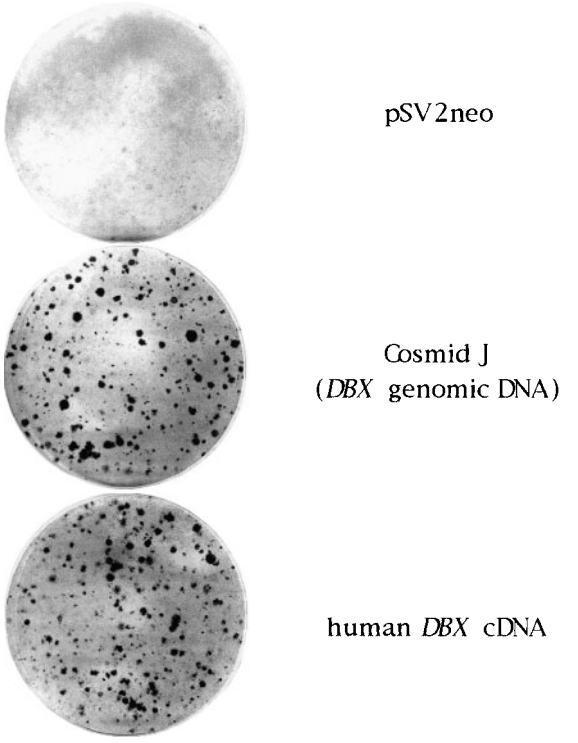

Fig. 4. **Complementation of ts ET24 mutation by human *DBX* genomic and cDNA clones.** ts ET24 cells (2×10^5 cells/100-mm dish) were transfected with the plasmid pSV2neo^r, super COS J (carrying the human *DBX* genomic DNA) and the pCDEBΔ-human *DBX* cDNA, using the calcium phosphate co-precipitation method. Transfected cells were incubated at 33.5°C for 18 h, washed with TD, and incubated for a further 48 h at 33.5°C, cultured at 39.5°C for about two weeks and stained with crystal violet, as described (*19*).

The nucleotide sequences of both ends of the cosmid J clone (Fig. 3B, indicated by open arrows) revealed that the obtained human genomic DNA covers a region from the nucleotide number 67415 to the nucleotide number 105311 of the human *X* chromosome sequence (Genbank Accession No. AL391647). This region encodes the DEAD-Box X isoform (DBX/DDX3) (*4*, *5*) (Fig. 3B, DBX) and the unknown mRNA (cDNA deposited as DKFZp566MO51) (Fig. 3B, indicated by a closed arrow). Analysis of the deposited human genomic sequence revealed that the *DBX* gene possesses restriction enzyme sites of *Cla*I, *Spl*I, *Aat*II and *Xho*I, as indicated in Fig. 3B.

Digestion of the human DNA inserted into the cosmid J clone with *Spl*I, *Aat*II and *Xho*I, but not *Cla*I, was found to destroy the ability of the cosmid J clone to rescue the lethality of ts ET24 cells (Fig. 3B, ts$^+$ complementation). Thus, the *DBX* gene was shown to be required for complementing the ts ET24 mutation. Consistent with this finding, human *DBX* cDNA amplified from the poly A$^+$ RNA of human HeLa cells, as shown in Fig. 5A, rescued the temperature-sensitive lethality of ts ET24 cells (Fig. 4, *DBX* cDNA, Table 1a).

The Hamster DBX Gene of ts ET24 Cells Contains a Single-Point Mutation Responsible for the ts Phenotype—To determine whether ts ET24 cells contain a mutation in the hamster *DBX* gene, we isolated a series of hamster

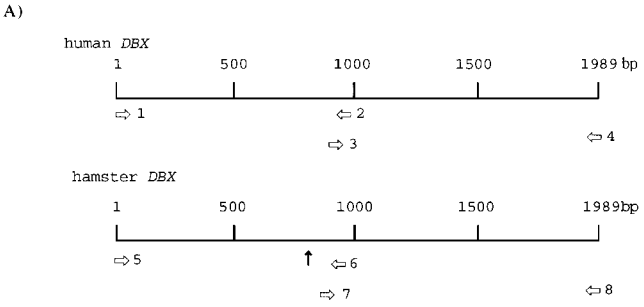

Fig. 5. **Single point mutation in hamster *DBX* cDNA.** A: Human and hamster *DBX* cDNA was amplified using the two sets of nucleotide primers indicated by open arrows. The resulting cDNA fragments were fused in-frame to construct the full-length cDNA. The closed arrow in the hamster *DBX* gene indicates the mutation site of the ts ET24 cell line. B: Alignment of the amino acid sequence of hamster DBX, *Xenopus* An3 and *S. cerevisiae* Ded1p. The position of the motif shown in blue letters is according to Tanner and Linder (*7*). The position of the mutated amino acids in ts ET24 cells is shown in red letters.

B)

```
hamster   MSHVAVENALGLDQQFAGLDLNSSDNQTGGSTASKGRYIPPHLRNREATK----------------------------------------------GFYDKD
xenopus   MSHVAVENVLNLDQQFAGLDLNSADAESGVA-GTKGRYIPPHLRNKEASRNDSNWDSGRGGNGYINGMQDDRDGRMNGYDRGGYGSRGTGRSDRGFYDRE
S.cere.   MAE--------LSEQVQNLSINDNNENG---------YVPPHLR-------------------------------------GKPRSARNNSSNYNNN
          *..        * .*   * .*  . ..         *.*****                                                . *. .

hamster   SSGWSSSKDKDAYSSFGSRGDSRGKSSFFGDRGSGSRGRFDDRGRGDYDGIGSRGDRSGFGKFERGGNSRWCD-KSDEDDWSKPLPPSERLEQELFSGGN
xenopus   NSGWNSGRDKDAYSSFGSRGD-RGKGSLFNERGSGSR-RTDDRRQDGFDGMGNRSDKSGFGRFDRG-NSRWSDDRNDEDDWSKPLAPNDRVEQELFSGSN
S.cere.   NGGYNGGRGGGSFFS-NNRRGGYGNGGFFGGNNGGSRS----------------NGRSG---------GRWIDGKHV------PAPRNEKAEIAIFGVPE
          ..* ..... ... *  .* .  *....*    .***                  ..**         .** * .         * . ... *  .*.  .
                                                                                                Motif I
hamster   ------TGINFEKYDDIPVEATGNNCPPHIESFSDVEMGEIIMGNIELTRYTRPTPVQKHAIPIIKEKRDLMACAQTGSGKTAAFLLPILSQIYADGPGE
xenopus   ------TGINFEKYDDIPVEATGSNCPPHIESFHDVTMGEIIMGNIQLTRYTRPTPVQKHAIPIIIEKRDLMACAQTGSGKTAAFLLPILSQIYADGPGD
S.cere.   DPNFQSSGINFDNYDDIPVDASGKDVPEPITEFTSPPLDGLLLENIKLARFTKPTPVQKYSVPIVANGRDLMACAQTGSGKTGGFLFPVLSESFKTGPS-
                .****..******.*.* . *  *  *     .. ... ** *.*.*.****** ..**. . **************..**.*.**. .  **.
                                  Motif Ia                                                Motif Ib                Motif
hamster   ALRAMKENGRYGRRKQYPISLVLAPTRELAVQIYEEARKFSYRSRVRPCVVYGGAEIGQQIRDLERGCHLLVATPGRLVDMMERGKIGLDFCKYLVLDEA
xenopus   AMKHLQENGRYGRRKQFPLSLVLAPTRELAVQIYEEARKFAYRSRVRPCVVYGGADIGQQIRDLERGCHLLVATPGRLVDMMERGKIGLDFCKYLVLDEA
S.cere.   --PQPESQGSFYQRKAYPTAVIMAPTRELATQIFDEAKKFTYRSWVKACVVYGGSPIGNQLREIERGCDLLVATPGRLNDLLERGKISLANVKYLVLDEA
                .* . .** .* ....******* **..**.**.***.*..**.****. **.*.*..****.********* *..******.*   ********
                II                          Motif III                                                        Motif
hamster   DRMLDMGFEPQIRRIVEQDTMPPKGVRHTMMFSATFPKEIQMLARDFLDEYIFLAVGRVGSTSENITQKVVWVEEMDKRSFLLDLLNATGKDSLTLVFVE
xenopus   DRMLDMGFEPQIRRIVEQDTMPPKGVRQTMMFSATFPKEIQILARDFLDEYIFLAVGRVGSTSENITQKVVWVEEMDKRSFLLDLLNATGKDSLTLVFVE
S.cere.   DRMLDMGFEPQIRHIVEDCDMTPVGERQTLMFSATFPADIQHLARDFLSDYIFLSVGRVGSTSENITQKVLYVENQDKKSALLDLLSAST-DGLTLIFVE
          ************.***.  * * * *.*.******* .** ****** .****.***************. **. **.* *****.*.  *.***.***
          IV                                        Motif V                                  Motif VI
hamster   TKKGADSLEDFLYHEGYACTSIHGDRSQRDREEALHQFRSGKSPILVATAVAARGLDISNVKHVINFDLPSDIEEYVHRIGRTGRVGNLGLATSFFNERN
xenopus   TKKGADALEDFLYHEGYACTSIHGDRSQRDREEALHQFRSGKSPILVATAVAARGLDISNVKHVINFDLPSDIEEYVHRIGRTGRVGNLGLATSFFNEKN
S.cere.   TKRMADQLTDFLIMQNFRATAIHGDRTQSERERALAAFRSGAATLLVATAVAARGLDIPNVTHVINYDLPSDVDDYVHRIGRTGRAGNTGLATAFFNSEN
          **. ** * ***  . .  *.*****.* .** **  **** . .************.** ****.*****...********** ** ****.***  *

hamster   INITKDLLDLLVEAKQEVPSWLENMAFEHHYKGSSRGRSKSSRFSGGFG---ARDYRQSSGASSSSFSSSRSSSSRSGGGGHGSSRGF-GGGGYGGFYNS
xenopus   INITKDLLDLLVEAKQEVPSWLENMAYEQHHKSSSRGRSKS-RFSGGFG---AKDYRQSSGAGSS-FGSSRGGRS----SGHGGSRGF-GGG-YGGFYNS
S.cere.   SNIVKGLHEILTEANQEVPSFLKDAM-----MSAPGSRSNSRR--GGFGRNNNRDYRKAGGASAGGWGSSRSRDN-----------SFRGGSGWGSDSKS
           ** *.* ..* **.***** * .        ... .**.* *  ****    .***...**...  .***.  .           .* **.  *.  .*

hamster   DGYGGNYNSQG-VDWWGN
xenopus   DGYGGNYGGSSQVDWWGN
S.cere.   SGWGNS-GGSNNSSWW--
           * * .  .      **
```

DBX cDNA fragments from the cDNA library of wt BHK21 cells using human *DBX* cDNA as a probe. The resulting hamster *DBX* cDNA revealed that the amino acid sequence of hamster *DBX* (deposited as Genbank Accession No. AB080116) is 98.3% identical to that of human *DBX* (Fig. 5B). According to the nucleotide sequence of hamster DBX cDNA, the poly A^+ RNAs of ts ET24 cells were amplified using two sets of primers, as shown in Fig. 5A. The resulting two cDNA fragments were sequenced. Of 1,989 base-pairs of the hamster *DBX* ORF, only one base substitution was identified: a C → T transition at the first position of codon 267, converting proline to serine (Fig. 5B, indicated by a red letter). The genomic DNA of ts ET24 cells, which had been amplified by PCR, was proven to contain the same nucleotide change as the cDNA (data not shown).

Finally, the N-terminal 0.8-kb fragment of the wt BHK21 *DBX* cDNA (wt), was replaced with that of the ts ET24 *DBX* cDNA (mt), which contains the ts ET24 mutation site, in order to confirm that the C-terminal half of the ts ET24 *DBX* cDNA has no mutation. The resulting recombinant hamster *DBX* cDNA clone (wt-mt) was inserted into the vector pcDEBΔ containing the hygromycine-resistance gene ($hygro^r$), then transfected into ts ET24 cells (Table 1b). As a control, human *DBX* cDNA and mutated hamster *DBX* cDNA (mt-mt) were transfected on the same vector into ts ET24 cells. While the number of $hygro^r$ colonies was comparable in all the transformation assays, the hamster *DBX* cDNA (wt-mt), but not the hamster mutated *DBX* (mt-mt) cDNA, rescued the temperature-sensitive lethality of ts ET24 cells, similar to human *DBX* cDNA.

Both mutated and wild-type hamster *DBX* genes rescued the cold-sensitive lethality of *S. cerevisiae ded1-21* (*22*) (data not shown). Thus. the hamster DBX is a functional homologue of *S. cerevisiae* Ded1p. This result also indicated that mutated hamster DBX of ts ET24 cells was functional at the lower temperature, 14°C.

DBX Is Not Involved in General Protein Synthesis—The *DBX* mutation of the ts ET24 cell line is localized between Motif 1 and Motif 1a of the DEAD-box RNA helicase family (Fig. 5B) (*7*). Such a mutation has not been

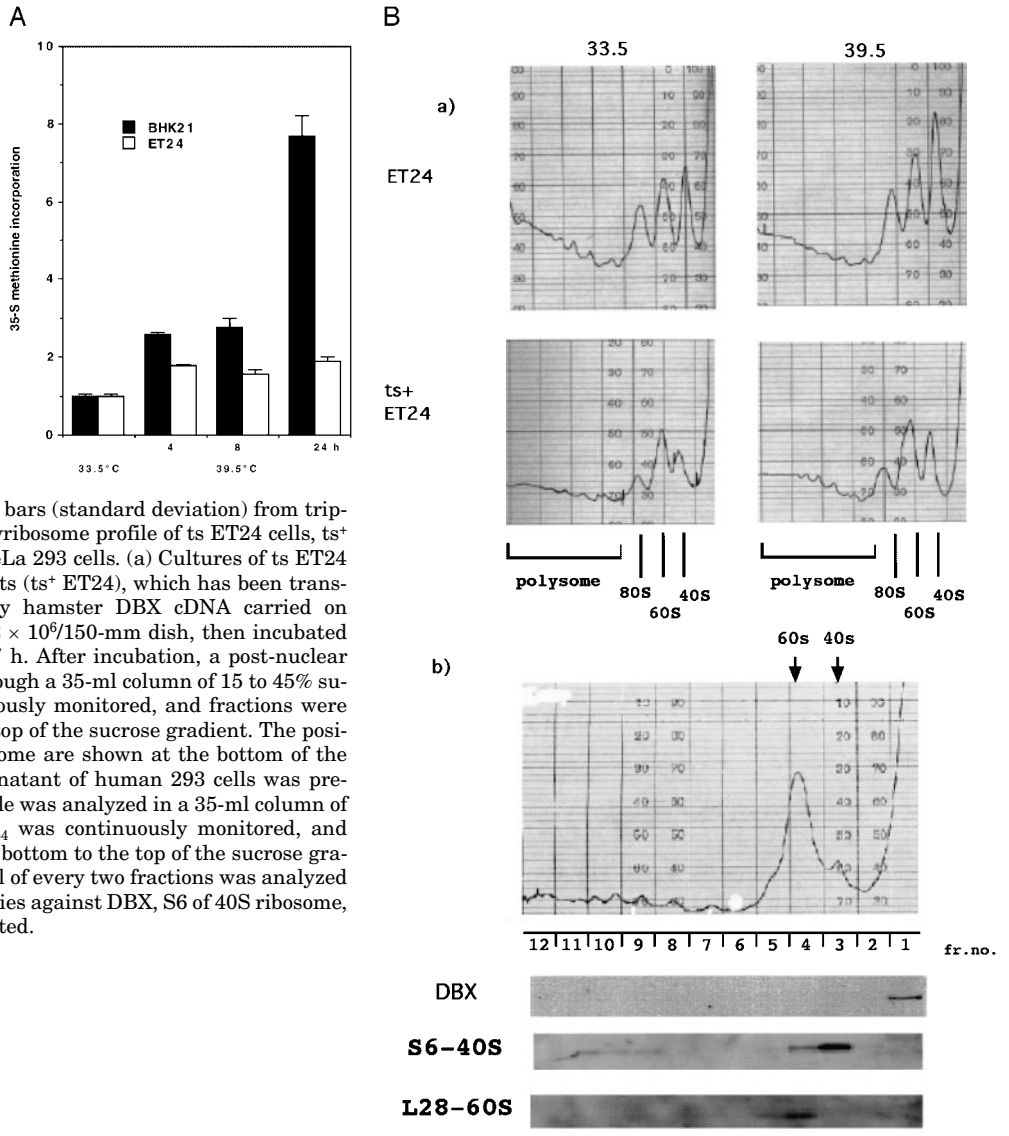

Fig. 6. **General protein synthesis in ts ET24 and wild-type BHK21 cells.** (A) Series of exponentially growing cultures of ts ET24 (open bars) and wild-type BHK21 cells (closed bars) (1×10^5 cells per 20-mm dish) were prepared at 33.5°C, then incubated at 39.5°C. At the indicated time (h), cells were washed with TD, fed with methionine-free DME medium containing 20 μCi/ml of [^{35}S]methionine, and then incubated for 1 h. After labeling, cells were washed with TD and the total radioactivity incorporated into the acid-insoluble material (on the ordinate) was measured, as described in "EXPERIMENTAL PROCEDURES." Error bars (standard deviation) from triplicate samples are shown. B: Polyribosome profile of ts ET24 cells, ts$^+$ transformants of ts ET24 and HeLa 293 cells. (a) Cultures of ts ET24 cells (ET24) and ts$^+$ transformants (ts$^+$ ET24), which has been transformed to the ts$^+$ phenotype by hamster DBX cDNA carried on pcDEBΔ vector, were seeded at 2×10^6/150-mm dish, then incubated at either 33.5°C or 39.5°C for 17 h. After incubation, a post-nuclear supernatant was centrifuged through a 35-ml column of 15 to 45% sucrose gradient. A_{254} was continuously monitored, and fractions were collected from the bottom to the top of the sucrose gradient. The positions of 40S, 60S, 80S and polysome are shown at the bottom of the panels. (b) A post-nuclear supernatant of human 293 cells was prepared and the polyribosome profile was analyzed in a 35-ml column of 15 to 45% sucrose gradient. A_{254} was continuously monitored, and fractions were collected from the bottom to the top of the sucrose gradient, as above. An aliquot of 3 ml of every two fractions was analyzed by immunoblotting using antibodies against DBX, S6 of 40S ribosome, or L28 of 60S ribosome, as indicated.

previously reported. *S. cerevisiae ded1* mutants, which have amino acid substitutions of either glycine^{108} → asparagine and glycine^{494} → asparagine (*ded1-120*) or glycine^{368} → asparagine (*ded1-199*), have been reported to be defective in general protein translation (*10*). In contrast, total protein synthesis is less strongly affected in *S. pombe ded1* mutants (*23*).

With this in mind, we addressed the question of whether protein synthesis in ts ET24 cells is affected by the mutation. Series of exponentially growing cultures of wt BHK21 and ts ET24 cells were prepared at 33.5°C and then incubated at 39.5°C. Cultures were pulse-labeled for 1 h with [^{35}S]methionine at the indicated time after the temperature shift to 39.5°C (Fig. 6A). Total protein synthesis of ts ET24 cells was not reduced at 39.5°C compared to that at 33.5°C (Fig. 6A, 33.5°C), although it was lower than that of wt BHK21 cells incubated at 39.5°C (Fig. 6A, 39.5°C). These results may reflect the fact that the growth rate of ts ET 24 cells was strongly reduced at 39.5°C, while ts ET24 cells themselves proliferated for at least 3 days (Fig. 1).

To confirm the effect of the ts ET24 mutation of DBX on protein translation, we analyzed the polyribosome profile on sucrose density gradients, since this profile has been reported to be affected in *S. cerevisiae ded1* mutants (*10*). Cultures of ts ET24 cells and ts$^+$ transformants of ts ET24 cells were incubated at 33.5°C or 39.5°C for 17 h, then the polyribosome profile was analyzed. After incubation at 39.5°C for 17 h, the peaks of 40S seemed to increase in ts ET24 cells, when compared to ts$^+$ cells, but no significant accumulation of 80S monosomes was observed (Fig. 6B, a). These results are consistent with the finding that the ability of protein synthesis was not reduced in ts ET24 cells by incubation at 39.5°C (Fig. 6A). In contrast to previous reports (*10*), the above findings indicated that DBX is not involved in protein translation. To confirm this, the distribution of DBX within polyribosomes was determined using human 293 cells. As expected, DBX proteins were not localized in polyribosome fractions, while components of 40S and 60S ribosome subunits were observed in polyribosome fractions under the same experimental conditions (Fig. 6B, b). DBX, thus,

may not be directly involved in protein translation in mammalian cells.

Amount of Cyclin A Decreased in ts ET24 Cells at 39.5°C—It is noticeable that *S. pombe ded1* mutants were isolated as cell-cycle mutants defective in cdc2 activity (*23*, *24*). In *S. pombe ded1-1D5*, translation of the B-type cyclins Cig2 and Cdc13 was reduced at the non-permissive temperature, while total protein synthesis was not affected (*23*). According to these previous reports, the cellular amounts of cyclins A and B were examined in ts ET24 cells at both 33.5°C and 39.5°C. Although the amount of cyclin B, cyclin D1 and α-tubulin were not reduced, that of cyclin A was reduced after incubation at 39.5°C for 4 h in ts ET24 cells, however, no reduction of cyclins was seen in wt BHK21 cells or in ts$^+$ transformants of ts ET24 cells (Fig. 7A). To examine whether the reduction in cyclin A was due to the defect in protein synthesis, series of cultures of ts ET24 cells and, as controls, wt BHK21 cells and ts$^+$ transformants, were incubated at 39.5°C. Every 4 h, cells were pulse-labeled for 1.5 h with [^{35}S]methionine after the temperature shift to 39.5°C. As shown in Fig. 7C, the production of both cyclin A and cyclin B was apparently reduced after incubation at 39.5°C for 4 h in ts ET24 cells, but not in wt BHK21 cells or in ts$^+$ transformants of ts ET24 cells (Fig. 7C, compare * band with cyclin A or cyclin B).

The reduction of cellular amount of cyclin A, but not cyclin B, may reflect their stability, since a cellular amount of cyclin A, but not cyclin B, was reduced within 4 h after the addition of a protein-synthesis inhibitor, cycloheximide (10 μg/ml), in wt BHK21 cells (Fig. 7B).

The amount of cyclin A mRNA decreased in ts ET24 cells at 39.5°C—Xenopus An3 which is homologus to mammalian DBX, has been identified as a protein binding to Crm1, which functions for nuclear protein export depending on Ran (*12*, *13*). In this context, we examined the localization of mRNA in ts ET24 cells by *in situ* hybridization using the oligo-dT probe (Fig. 8A, mRNA). As expected, mRNA accumulated in the nucleus of ts ET24 cells at 39.5°C (Fig. 8A, compare 0 h with others). Such nuclear accumulation of mRNA in ts ET24 cells became apparent after incubation for 17 h. In contrast, no nuclear accumulation of mRNA was observed after incubation at 39.5°C for 17 h in wt BHK21 cells (Fig. 8A, wild-type BHK21, 17 h). The poly A-tailed mRNA contains the cap-structure (*11*). In this regard, the endogenous eIF4E, a cap-binding protein (*11*), was stained in ts ET24 cells (Fig. 8B). The eIF4E proteins were accumulated in the nucleus of ts ET24 cells after incubation at 39.5°C for 17 h, but not at 33.5°C. There was no nuclear accumulation of eIF4E in wild-type BHK21 cells (data not shown).

Although the observed nuclear accumulation of mRNA was not rapid compared with the reduction of cyclin A and B production (Fig. 7C), it raised the question of whether a particular group of mRNAs encoding proteins

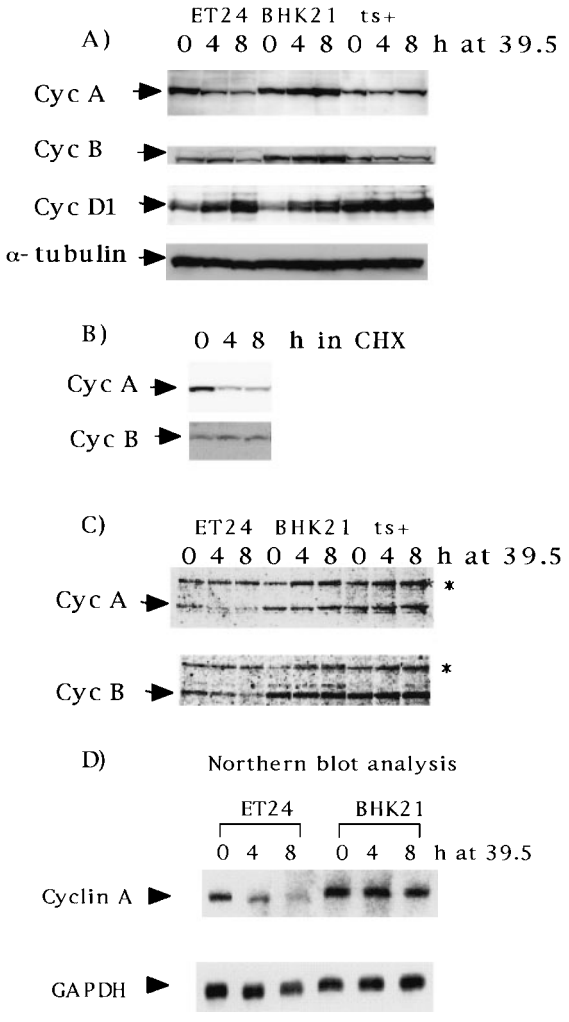

Fig. 7. **Decrease of cyclin A and cyclin B mRNAs in ts ET24 cells during incubation at 39.5°C.** A: Series of cultures of ts ET24, wt BHK21 and ts$^+$ transformed cells exponentially growing at 33.5°C were incubated at 39.5°C. Every 4 h, total cellular extracts were prepared for Western blotting analysis as described in Experimental Procedures. The extracts were analyzed by immunoblotting with the antibodies against cyclin A1, cyclin B1, cyclin D1 and α-tubulin. The positions of these proteins are indicated by arrows. B: Exponentially growing cultures of wt BHK21 cells were incubated in the presence of 10 μg/ml of cycloheximide at 33.5°C. At the indicated time, total cell-extracts were prepared and analyzed for the presence of cyclin A and B1 by immunoblotting with the antibodies against cyclin A and cyclin B1. C: Series of cultures of ts ET24, BHK21 and ts$^+$ transformed cells exponentially growing at 33.5°C were incubated at 39.5°C. Every 4 h, [^{35}S]methionine (50 μCi/ml) was added to cultures for 1.5 h. Cells were collected and processed for immunoprecipitation using anti-cyclin antibodies. Immunoprecipitates were run on SDS polyacrylamide gel, and the dried gel was exposed to an image film in a Fuji image analyzer. The positions of cyclin A and cyclin B are indicated by arrows. The asterisk shows an internal control protein which appeared in the background. D: Exponentially growing cultures of ts ET24 and wt BHK21 cells (5×10^6 cells) were prepared at 33.5°C, and half of each culture was incubated at 39.5°C. Every 4 h, cells were collected, frozen at –80°C, then completely lysed by use of a lysis buffer [50 mM NaCl, 0.3 M sucrose, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.1% NP40, and RNase inhibitor (80 U/ml)]. The extracts were kept on ice for 10 min, then centrifuged at 3000 ×*g* for 5 min. Nuclear RNA was isolated with triazol solution (Invitrogen, Carlsbad CA) as recommended by the supplier and ethanol precipitation. Twenty micrograms of nuclear RNA was electrophoresed and transferred onto a nylon membrane. Northern blotting analysis was carried out using human *cyclin A* and mouse *GAPDH cDNA* as probes.

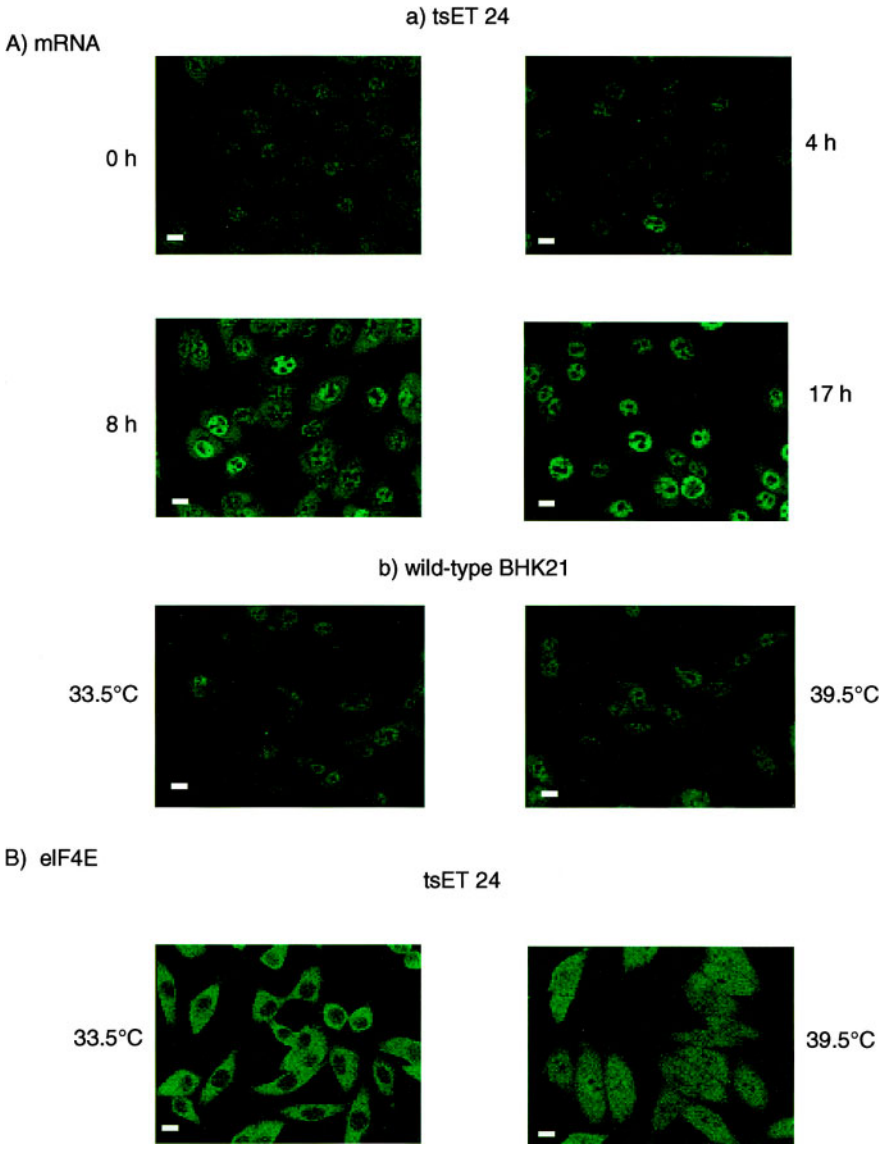

Fig. 8. **Nuclear accumulation of mRNA and eIF4E in ts ET24 cells at 39.5°C.** A: (a) A series of cultures of ts ET24 cells exponentially growing on coverslips at 33.5°C was incubated at 39.5°C. After incubation at 39.5°C for the indicated time (h), cells were processed for FISH as described in Experimental Procedures by using oligo-dT as a probe. As a control, ts ET24 cells growing at 33.5°C were also processed for FISH. Scale bar, 10 μm. (b) Half of the cultures of wt BHK21 cells exponentially growing on coverslips at 33.5°C were incubated at 39.5°C for 17 h. Cells incubated at 33.5°C or 39.5°C were then processed for FISH. Scale bar, 10 μm. B: Half of the cultures of ts ET24 cells exponentially growing on coverslips at 33.5°C were incubated at 39.5°C for 17 h. Cells incubated at 33.5°C or 39.5°C were fixed and stained with the anti-eIF4E antibody as described in Experimental Procedures. Scale bar, 10 μm.

such as cyclin A and B might be rapidly accumulated in the nucleus, resulting in the inhibition of mRNA translation. To address this question, series of exponentially growing cultures of ts ET24 cells, wt BHK21 cells and ts$^+$ transformants of ts ET24 cells were prepared at 33.5°C, and then half of cultures were incubated at 39.5°C. The total amount of *cyclin A* and *cyclin B* mRNAs was apparently reduced in ts ET24 cells, but not in the other cells (data not shown), after incubation for 17 h at 39.5°C. Taken with the nuclear accumulation of mRNA at 39.5°C, this result suggested that both *cyclin A* and *cyclin B* mRNAs might become unstable due to the nuclear accumulation. To confirm this, series of exponentially growing cultures of ts ET24 cells and, as a control, wt BHK21 cells, were prepared and then a half of cultures was incubated at 39.5°C. Every 4 h, cells were collected, and their cytoplasmic and nuclear fractions were separated. Total RNAs were extracted from each fraction and analyzed for the presence of mRNAs encoding cyclin A, and as a control, GAPDH, using as probes, *cyclin A* and *GAPDH* cDNA clones. The amount of mRNAs encoding cyclin A, but not GAPDH, was reduced in both cytoplasmic and nuclear fractions of ts ET24 cells after incubation at 39.5°C for 4 h. A representative result of nuclear *cyclin A* mRNA is shown in Fig. 7D (ET24). No reduction of *cyclin* A mRNA was seen in wt BHK21 cells at 39.5°C (Fig. 7D, BHK21).

DISCUSSION

Isolation of Somatic ts Mutants—Conditional mutations, particularly temperature-sensitive mutations, are the main avenue employed to clarify the cellular function of genes in yeast, as well as in mammalian cells. In contrast to yeast, the isolation of mammalian ts mutants and the identification of their mutated genes have been both laborious and time-consuming. Recently, the nucleotide sequences of the human genome and cDNAs have been almost completely determined and made available. Therefore, the partial sequence of the obtained human genomic DNA fragment that rescues the lethality of ts mutants was able to reveal the gene(s) carried on the iso-

lated human genomic DNA fragments, as described here. In light of these advantages, it will be possible to quickly identify what genes are defective in mammalian ts mutants in the future. The problem highlighted in the present study, is how to selectively isolate mammalian ts mutants defective in autosomal genes. We attempted to isolate autosomal ts mutants using benomyl, an anti-microtubule drug. However, this was not successful. We again obtained a lot of mutants defective in the *X* chromosomal genes.

Mammalian DBX and Nuclear mRNA Accumulation—The ts ET24 cell line is the first ts mutant of mammalian cell lines defective in the DEAD-box RNA helicase, *DBX*. Since the hamster *DBX* complemented a cold-sensitive *S. cerevisiae ded1-21*, the obtained hamster *DBX* should be a functional homologue of *S. cerevisiae DED1*. Similar to *S. pombe ded1* mutants (*23*), ts ET24 cells did not show any strong inhibition of protein synthesis. Instead, they showed the nuclear accumulation of mRNA. A cap-binding protein, eIF4E, which binds to mRNA, also accumulated in the nucleus of ts ET24 cells during incubation at 39.5°C. Since eIF4E is imported into the nucleus, and since the *Xenopus* DBX homologue, An3, is exported to the cytoplasm with the aid of the Ran cycle, the cellular localization of the other proteins involved in the Ran pathway was investigated. However, the localization of these proteins did not seem to be affected by the ts ET24 mutation (data not shown). The nuclear accumulation of elF4 with mRNA may suggest that eIF4E forms the eIF4F complex (the cap-binding protein complex) (*11*) in the nucleus. In this regard, it is noteworthy that *S. cerevisiae DED1* is a multicopy suppressor of *S. cerevisiae cdc33* (eIF4E) and *tif1* (eIF4A) (*25*). Even so, the nuclear accumulation of mRNA may not be a direct effect of the ts ET24 mutation of DBX, since it became obvious in ts ET24 cells after incubation at 39.5°C for 17 h. This assumtion is consistent with the previous reports. *S. cerevisiae* mutants showing nuclear accumulation have been systematically screened (*26*, *27*). There are no *ded1* mutants among them. So far, three DEAD-Box RNA helicases are reported to be involved in mRNA export. The nuclear Mtr4p was identified in the *mtr* mutant screen (*28*). It turned out to be one of the components of the exosome (*29*). *S. cerevisiae* SUB2 is another DEAD-Box RNA helicase (*8*). Sub2p directly interacts with the nuclear protein Yra1p, which is an essential export factor for mRNA (*17*). The defect of SUB2 as well as its overexpression causes the nuclear accumulation of mRNA. Finally, Dbp5 is reported to function for mRNA export by collaborating with CAN/Nup159p (*16*, *30*).

DBX in Cell Cycle Regulation—We do not yet know why the amount of *cyclin A* mRNA decreased at the non-permissive temperature. The promoter assay of cyclin A suggested that the transcription of *cyclin A* seemed to be unaffected by the mutation (data not shown). There are no reports indicating that DBX is involved in mRNA stability, although several *S. cerevisiae ded1* mutants have been isolated. Mutant *spp81-1* was isolated as an extragenic suppressor of *prp8-1* which is defective in mRNA splicing (*31*); and *ded1-21* was isolated as an extragenic suppressor of *srm1-1* and *mtr1,* mutant alleles of *PRP20*, which encodes the *S. cerevisiae RCC1* homologue, the GDP/GTP exchange factor of a small GTPase Ran (*32*). The *S. pombe ded1*$^+$ gene was identified as a multicopy suppressor of a checkpoint defect (*33*), or of sterility (*34*). Related to these reports, Ded1p is reported to interact with Chk1 and Cdc2 in *S. pombe* (*24*), and with Srm1p/Prp20p in *S. cerevisiae* (*32*). The interaction between Ded1p and Chk1 would suggest that Ded1p is involved in cell-cycle control. Consistent with this, Grallert *et al*. (*23*) have isolated *S. pombe ded1* mutants as a mutation defective in B-cyclin function or expression of *cyclin B* by two independent screens. Our present result is similar to their report, regarding to the cell cycle specific function of Ded1p.

The stability of *cyclin A* and *cyclin B* mRNAs is reported to be regulated by HuR, which binds the 3′-untranslated region of *cyclin A* and *B1* mRNAs, forming RNA-protein complexes in a cell-cycle dependent manner (*35*). It is possible that the formation of HuR-*cyclin A* and *cyclin B* mRNA complexes is inhibited by the ts ET24 mutation of DBX. The ts ET24 mutation of DBX thus makes *cyclin A* and *B* mRNAs unstable, causing a delay of entry into the S-phase. It is noticeable that the amount of cyclin D1 increased in ts ET24 cells at 39.5°C. This result suggests that DBX may not be involved in the stability of mRNAs encoding G1 cyclins.

The above findings together suggest that Ded1p/DBX is a multifunctional protein. The phenotypes of *ded1* mutants hitherto reported may depend on a mutated allele. The ts ET24 cell line has a single-point mutation, P267S, in DBX, which is localized between Motif I and Motif Ia. The P267 is conserved from yeast to mammals. A yeast mutant possessing the same mutation as the ts ET24 cell line has not been reported. Probably, the mutation site of *DBX* of ts ET24 cells is required for interaction between DBX and HuR in order to stabilize *cyclin A* and *cyclin B* mRNAs. It has been reported that *S. cerevisiae* cold-sensitive *ded1-199* and *ded1-120* have a defect in translation initiation (*10*). In these mutants, no nuclear accumulation of mRNA was observed, in contrast to our present finding. Instead, a general protein translation is affected in these *S. cereviae ded1* mutants (*10*). The discrepancy between hamster ts ET24 cells and *S. cerevisiae ded1-199* and *ded1-120* remains to be clarified.

Expression of Mammalian DBX—The *wt hamster DBX* gene carried on the CMV (Cytomegalo Virus) vector, which is used for overproduction, was not able to rescue the temperature-sensitive phenotype of ts ET24 cells (data not shown). Taken together with the report that the overexpression of *SUB2*, which is essential for mRNA export, causes the nuclear accumulation of mRNA (*17*), the inability of hamster *DBX* carried on the CMV vector to rescue the lethality of ts ET24 cells suggests that overexpression of DBX has a detrimental effect on cell proliferation. This assumption is consistent with the report that PL10 is only expressed in male germ cells (*14*). PL10 is probably lethal for somatic cells due to the higher activity of RNA helicase. In this regard, it should be noted that human DBX has 92% sequence identity at the protein level with human DBY (*36*). Human *DBY* has two alternative transcripts, which differ only in the length of 3′-UTR (*36*). The human DBY with a long 3′-UTR, alternative transcript 2, is expressed ubiquitously, while the human DBY with a short 3′-UTR, alternative transcript 1, is only expressed in the testis. The question of which

transcript of *DBY* can complement ts ET24 mutation is an interesting problem which awaits future clarification. We think that a variability of ts$^+$ transformation frequency with *DBX* cDNA may reflect such a 3′-UTR problem as described above.

What is clear, however, is that the unknown function of DBX will undoubtedly be clarified using ts ET24 cells.

This work was supported by Grants-in-Aid for Specially Promoted Research (TN), and a Grant-in-Aid for Scientific Research on Priority Areas (TS), from the Japan Ministry of Education, Science, Sport and Culture. The English used in this manuscript was revised by Miss K. Miller (Royal English Language Centre, Fukuoka, Japan) and Professor David Maruyama (Chapman University, California).

REFERENCES

1. Marcus, M., Fainsod, A., and Diamond, G. (1985) The genetic analysis of mammalian cell-cycle mutants. *Annu. Rev. Genet.* **19**, 389–421
2. Nishimoto, T. (1997) Isolation and characterization of temperature-sensitive mammalian cell cycle mutants. *Methods Enzymol.* **283**, 292–309
3. Nishimoto, T. and Basilico, C. (1978) Analysis of a method for selecting temperature sensitive mutants of BHK21 cells. *Somat. Cell. Genet.* **4**, 323–340
4. Lahn, B.T. and Page, D.C. (1997) Functional coherence of the human Y chromosome. *Science* **278**, 675–680
5. Park, S.H., Lee, S.-G., Kim, Y., and Song, K. (1998) Assignment of a human putative RNA helicase gene, DDX3, to human X chromosome bands p11.3→p11.23. *Cytogenet. Cell Genet.* **81**, 178–179
6. von Hippel, P.H. and Delagoutte, E. (2001) A general model for nucleic acid helicases and their "coupling" within macromolecular machines. *Cell* **104**, 177–190
7. Tanner, N.K. and Linder P. (2001) DExD/H box RNA helicases: from generic motors to specific dissociation functions. *Mol. Cell* **8**, 251–262
8. De La Cruz, J., Kressler, D., and Linder, P. (1999) Unwinding RNA in Saccharomyces cerevisiae: DEAD-box proteins and related families. *Trends Biochem. Sci.* **24**, 192–198
9. Bird, L.E., Subramanya, H.S., and Wigley, D.B. (1998) Helicases: a unifying structural theme? *Curr. Opin. Struct. Biol.* **8**, 14–18
10. Chung, R.-Y., Weaver, P.L., Liu, Z., and Chang, T.-H. (1997) Requirement of the DEAD-BOX protein Ded1p for messenger RNA translation. *Science* **275**, 1468–1471
11. Gingras, A.-C., Raught, B., and Sonenberg, N. (1999) elF4 initiation factors: effectors of mRNA recruitment to ribosome and regulators of translation. *Annu. Rev. Biochem.* **68**, 913–963
12. Askjaer, P., Bachi, A., Wilm, M., Bischoff, F.R., Weeks, D.L., Ogniewski, V., Ohno, M., Niehrs C., Kjems, J., Mattaj, I.W., and Fornerod, M. (1999) RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-BOX helicase. *Mol. Cell. Biol.* **19**, 6276–6285
13. Askjaer, P., Rosendahl, R., and Kjems, J. (2000) Nuclear export of the DEAD box An3 protein by CRM1 is coupled to An3 helicase activity. *J. Biol. Chem.* **275**, 11561–11568
14. Leroy, P., Alzari, P., Sassoon, D., Wolgemuth, D., and Fellous, M. (1989) The protein encoded by a murine male germ cell-specific transcript is a putative ATP-depdendent RNA helicase. *Cell* **57**, 549–559
15. Mamiya, N. and Worman, H.J., (1999) Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. *J. Biol. Chem.* **274**, 14751–15756
16. Tseng, S.S.-T., Weaver, P.L., Liu, Y., Hitomi, M., Tartakoff, A.M., and Chang, T.-H. (1998) Dbp5p, a cytosolic RNA helicase, is required for poly(A)$^+$ RNA export. *EMBO J.* **17**, 2651–2662
17. Straber, K. and Hurt, E. (2001) Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. *Nature* **413**, 648–652
18. Nishimoto, T., Ishida, R., Ajiro, K., Yamamoto, S., and Takahashi, T. (1981) The synthesis of protein(s) for chromosome condensation may be regulated by a post-transcriptional mechanism. *J. Cell Physiol.* **109**, 299–308
19. Watanabe, M., Furuno, N., Goebl, M., Go, M., Miyauchi, K., Sekiguchi, T., Basilico, C., and Nishimoto. T. (1991) Molecular cloning of the human gene, CCG2, that complements the BHK-derived temperature-sensitive cell cycle mutant tsBN63: identity of CCG2 with the human X chromosomal SCAR/RPS4X gene. *J. Cell Sci.* **100**, 35–43
20. Sekiguchi, T., Nohiro, Y., Nakamura, Y., Nisamoto, N., and Nishimoto, T. (1991) The human CCG1 gene, essential for progression of the G1 phase, encodes a 210-kilodalton nuclear DNA-binding protein. *Mol. Cell. Biol.* **11**, 3317–3325
21. Sekiguchi, T., Miyata, T., and Nishimoto, T. (1988). Molecular cloning of the cDNA of human X chromosomal gene(CCG1) which complements the temperature-sensitive G1 mutants, tsBN462 and ts13, of the BHK cell line. *EMBO J.* **7**, 1683–1687
22. Hayashi, N., Yokoyama, N., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1995) RanBP1, a Ras-like nuclear G protein binding to Ran/TC4, inhibits RCC1 via Ran/TC4. *Mol. Gen. Genet.* **247**, 661–669
23. Grallert, B., Kearsey, S.E., Lenhard, M., Carlson, C.R., Nurse, P., Boye, E., and Labib, K. (2000). A fission yeast general translation factor reveals links between protein synthesis and cell cycle controls. *J. Cell Sci.* **113**, 1447–1458
24. Liu, H.-Y., Nefsky, B.S., and Walworth, N.C. (2002) The Ded1 DEAD Box helicase interacts with Chk1 and Cdc2. *J. Biol. Chem.* **277**, 2637–2643
25. De La Cruz, J., Iost, I., Kressler, D., and Linder, P. (1997) The p20 and Ded1p proteins have antagonistic roles in elF4E-dependent translation in *Saccharomyces cerevisiae*. *Proc. Nat. Acad. Sci. USA* **94**, 5201–5206
26. Amberg, D.C., Goldstein, A.L., and Cole, C.N. (1992) Isolation and characterization of RAT1: an essential gene of Saccharomyces cerevisiae required for the efficient nucleocytoplasmic trafficking of mRNA. *Genes Dev.* **6**, 1173–1189
27. Kadowaki, T., Zhao, Y., and Tartakoff, A.M. (1992) A Conditional Yeast Mutant Deficient in mRNA Transport from Nucleus to Cytoplasm *Proc. Natl Acad. Sci. USA* **89**, 2312–2316
28. Liang, S., Hitomi, M., Hu, Y.-H., Liu, Y., and Tartakoff, A.M. (1996) A DEAD-Box-family protein is required for nucleocytoplasmic transport of yeast mRNA. *Mol. Cell. Biol.* **16**, 5139–5146
29. De La Cruz, J., Kressler, D., Tollervey, D., and Linder P. (1998) Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3′ end formation of 5.8S rRNA in *Saccharomyces cerevisiae*. *EMBO J.* **17**, 1128–1140
30. Schmitt, C., von Kobbe, C., Bachi, A., Pante, N., Rodrigues, J.P., Boscheron, C., Rigaut, G., Wilm, M., Seraphin, B., Carmo-Fonseca, M., and Izaurralde, E. (1999) Dbp5, a DEAD-box protein required for mRNA export, is recruited to the cytoplasmic fibrils of nuclear pore complex via a conserved interaction with CAN/Nup159p. *EMBO J.* **18**, 4332–4347
31. Jamieson, D.J., Rahe, B., Pringle, J., and Beggs, J.D. (1991) A suppressor of a yeast splicing mutation (prp8-1) encodes a putative ATP-dependent RNA helicase. *Nature* **349**, 715–717
32. Hayashi, N., Seino, H., Irie, K., Watanabe, M., Clarl, K.L., Matsumoto, K., and Nishimoto, T. (1996) Genetical interaction of *DED1* encoding a putative ATP-dependent RNA helicase with *SRM1* encoding a mammalian RCC1 homologue in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **253**, 149–156
33. Forbes, K.C., Humphrey, T., and Enoch, T. (1998) Suppressors of Cdc25p overexpression identify two pathways that influence the G2/M checkpoint in fission yeast. *Genetics* **150**, 1361–1375
34. Kawamukai, M. (1999). Isolation of a novel gene, moc2, encoding a putative RNA helicase as a suppressor of sterile strains

in *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **1446**, 93–101
35. Wang, W., Caldwell, C.M., Lin, S., Furneaux, H., and Gorospe, M. (2000) HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. *EMBO J.* **19**, 2340–2350
36. Foresta, C., Ferlin, A., and Moro, E. (2000). Deletion and expression analysis of *AZFa* genes on the human Y chromosome revealed a major role for *DBY* in male infertility. *Hum. Mol. Genet.* **9**, 1161–1169
37. Sekiguchi, T., Noguchi, E., Hayashida, T., Nakashima, T., Toyoshima, H., Nishimoto, T., and Hunter, T. (1996) D-type cyclin expression is decreased and p21 and p27 CDK inhibitor expression is increased when tsBN462 CCG1/TAF$_{II}$250 mutant cells arrest in G1 at the restrictive temperature. *Genes to Cells* **1**, 687–705