THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 33, Issue of August 15, pp. 31149–31158, 2003

Printed in U.S.A.

Functional Domains of Chicken Mitochondrial Transcription Factor A for the Maintenance of Mitochondrial DNA Copy Number in Lymphoma Cell Line DT40*

Received for publication, April 11, 2003, and in revised form, May 15, 2003
Published, JBC Papers in Press, May 20, 2003, DOI 10.1074/jbc.M303842200

Yuichi Matsushima‡, Kiyoshi Matsumura‡, Shoji Ishii‡, Hidetoshi Inagaki§, Tomohiro Suzuki¶, Yoichi Matsuda¶, Konrad Beck‖‡‡, and Yasuo Kitagawa‡§§**

From the ‡Graduate Courses for Regulation of Biological Signals, ¶Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, and ‖Bioscience Center, Nagoya University, Nagoya 464-8601 and the §Neuroscience Research Institute, National Institute of Advanced Industrial Science and Technology, Tukuba, Ibaragi, 305-8566, Japan

Nuclear and mitochondrial (mt) forms of chicken mt transcription factor A (c-TFAM) generated by alternative splicing of a gene (*c-tfam*) were cloned. *c-tfam* mapped at 6q1.1-q1.2 has similar exon/intron organization as mouse *tfam* except that the first exons encoding the nuclear and the mt form-specific sequences were positioned oppositely. When cDNA encoding the nuclear form was transiently expressed in chicken lymphoma DT40 cells after tagging at the C terminus with c-Myc, the product was localized into nucleus, whereas the only endogenous mt form of DT40 cells was immunostained exclusively within mitochondria. c-TFAM is most similar to *Xenopus* (*xl*-) TFAM in having extended C-terminal regions in addition to two high mobility group (HMG) boxes, a linker region between them, and a C-terminal tail, also found in human and mouse TFAM. Similarities between *c*- and *xl*-TFAM are higher in linker and C-terminal regions than in HMG boxes. Disruption of both *tfam* alleles in DT40 cells prevented proliferation. The *tfam*$^{+}$/*tfam*$^{-}$ cells showed a 50 and 40–60% reduction of mtDNA and its transcripts, respectively. Expression of exogenous wild type *c-tfam* cDNA in the *tfam*$^{+}$/*tfam*$^{-}$ cells increased mtDNA up to 4-fold in a dose-dependent manner, whereas its transcripts increased only marginally. A deletion mutant lacking the first HMG box lost this activity, whereas only marginal reduction of the activity was observed in a deletion mutant at the second HMG box. Despite the essential role of the C-terminal tail in mtDNA transcription demonstrated *in vitro*, deletion of c-TFAM at this region reduced the activity of maintenance of the mtDNA level only by 50%. A series of deletion mutant at the tail region suggested stimulatory and suppressive sequences in this region for the maintenance of mtDNA level.

Transcription of mitochondrial (mt)[1] DNA is best understood for *Saccharomyces cerevisiae* (1, 2), in which a core RNA polymerase of $M_r \sim 145$ with sequence similarity to viral RNA polymerases of T3, T7, and SP6 (3) initiates transcription together with a dissociable 39-kDa mt transcription factor B (*sc*-mtTFB) (4, 5). This protein functions like a bacterial σ factor (6–8), but amino acid sequence comparisons (9) and mutational analyses (10) do not strongly support the hypothesis that *sc*-mtTFB is homologous to bacterial σ factor. The *Xenopus* equivalent (*xl*-mtTFB) having this activity has been characterized (11–12), but direct evidence confirming the structural similarity of *xl*-mtTFB to *sc*-mtTFB has not been reported. Human mt-TFB has been cloned and shown to be structurally related to RNA adenine methyltransferase (13). These two classes of proteins bind cooperatively to a nonanucleotide promoter sequence (4, 14) present at multiple locations in the 75-kb circular yeast mt genome (15). Thus, mtTFBs acts as a specific factor enabling the RNA polymerase to locate the appropriate start sites.

Our knowledge about the transcription of human mtDNA, mainly based on the *in vitro* transcription system, is somewhat different and depends on a transcription factor (*h*-TFAM; formerly referred to as *h*-mtTFA) containing two HMG boxes (2, 16, 17). The yeast homologue *sc*-mtTFA is an abundant protein composed almost entirely of two HMG boxes separated by a rather short linker region (18). *sc*-mtTFA was originally described as ABF2, which could bind to the replication origin of yeast nuclear DNA but was later found to be localized in mitochondria (19). Disruption of the ABF2 gene led to a loss of respiratory competence and mtDNA when cells were grown in the presence of glucose. Because expression of *h*-TFAM in the yeast *abf2* strain improved the phenotype, a potential functional homology of *h*-TFAM to *sc*-mtTFA was confirmed (20). Nonetheless, the role of *sc*-mtTFA in mtDNA transcription remains unclear since *in vitro* it can hardly activate transcription. By replacing the various regions of h-TFAM with the corresponding parts of the yeast homologue, the linker region and the C-terminal tail abbreviated in *sc*-mtTFA were shown to be significant for the recognition and transcriptional activation of the human mtDNA (21). Mouse (22) and *Xenopus mtTFA* (25) (will be referred to as m- and *xl*-TFAM hereafter, respectively) have been cloned and shown to have the long linker region and the C-terminal tail as h-TFAM does. *xl*-TFAM stim-

* This work was supported by Research for the Future Program of the Japan Society for the Promotion of Science (to Y. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB021166 and AB059657.

** A Japan Society for the Promotion of Science Research fellow.

‡‡ A visiting researcher supported by the Ministry of Education, Science, Culture, and Sports of Japan.

§§ To whom correspondence should be addressed: Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya-shi 464-8601, Japan. Tel.: 81-52-789-5227; Fax: 81-52-789-4296; E-mail: yasuok@agr.nagoya-u.ac.jp.

[1] The abbreviations used are: mt, mitochondrial; kb, kilobase pair(s); HMG, high mobility group; *MPA*, mycophenolic acid-resistant gene; *neo*^{r}, neomycin-resistant gene; PBS, phosphate-buffered saline; SSC, standard saline citrate solution; TFAM, mt transcription factor A.

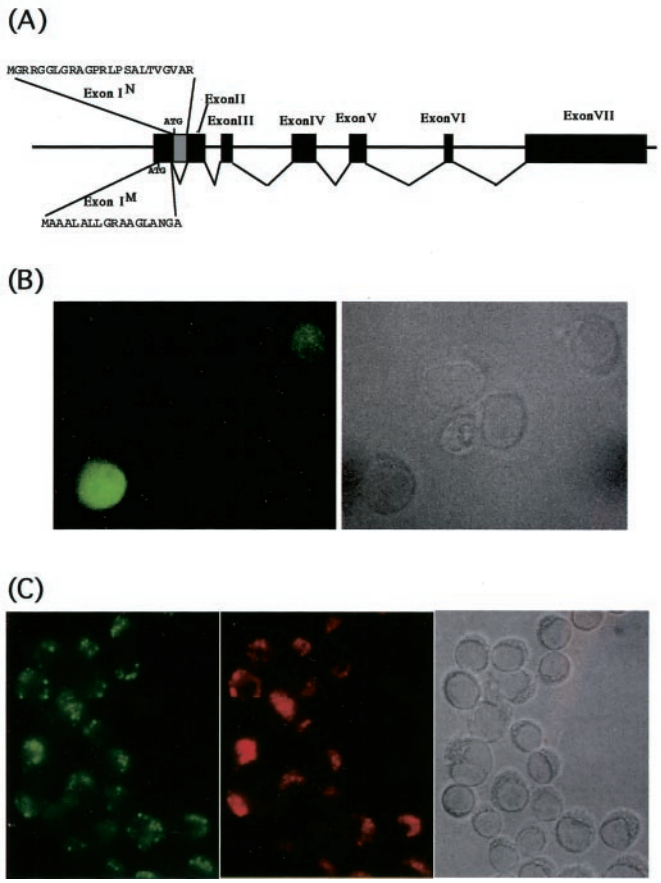

FIG. 1. **Nuclear and mitochondrial c-TFAMs generated by alternative splicing of duplicated first exons in *c-tfam*.** *c-tfam* has exon I^M and exon I^N encode independent N-terminal sequences starting their own ATG codons as indicated. They are alternatively spliced to be connected in-frame to the common downstream sequence encoded by exon II through VII arranged as depict in *A*. When DT40 cells were transfected with cDNA sequence encoding the nuclear form and tagged with c-Myc sequence, a population of the cells transiently expressing the transgene was indirectly immunostained by the combination of anti-c-Myc rabbit polyclonal IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG as in the *left panel* of *B*. Comparing the phase contrast micrograph in the *right panel* of *B*, it is clear that the staining is limited within the nucleus with the exclusion from several spots probably corresponding to nucleolus. When wild type DT40 cells were indirectly immunostained by a combination of anti-c-TFAM antiserum and fluorescein isothiocyanate-conjugated anti-rabbit IgG, all cells exhibited dotted staining within the cytoplasm excluded from nucleus as in the *left panel* of *C*. This staining well overlapped with the staining of living dye for mitochondria (MitoTracker Red) as in the *middle panel* of *C*. The *right panel* of *C* shows a phase contrast micrograph of the same field of view.

ulates transcription *in vitro* up to 10-fold for the H-strand, but only ~3-fold for the L-strand (23).

For better understanding of the function of TFAM, the evolutionary variation of the control region of mtDNA may be worthwhile to be considered. This is the region between the genes for $tRNA^{Pro}$ ($tRNA^{Glu}$ in birds) and $tRNA^{Phe}$ containing the H-strand replication origin, the promoters for transcription activation, and H- and L-strand start sites (24). In *Xenopus*, the distance of the transcription start sites of the L- and H-strands is rather short, with 50 bp (25) *versus* 150 bp in the case of human (26). Lower dependence of *Xenopus* mtDNA transcription on *xl*-TFAM compared with the human system can be due to this difference of the control region. From this aspect, chicken mtDNA provides an opportunity to expand our knowledge of mtDNA transcription in higher vertebrates. The chicken mitochondrial genome has a characteristic organization of the genes compared with other vertebrates (27), and its H- and L-strand sites have no separating base pair (28). We have cloned and determined the cDNA sequence encoding chicken (c-)TFAM, which is more similar to *xl*-TFAM than to the human and mouse proteins. Targeted disruption of both *c-tfam* alleles in a chicken cell line DT40 showed that TFAM is essential for proliferation of this cell line. In cells with heterozygous disrupted *c-tfam*, mtDNA and its transcripts were reduced. Expression of exogenous wild type *c-tfam* cDNA in those cells increased mtDNA up to 4-fold in a dosage-dependent manner, whereas its transcripts increased only marginally. We thus show that c-TFAM is important for maintenance of the mtDNA copy number.

MATERIALS AND METHODS

Cell Culture, Gene Transfection, and Screening—DT40 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO_2. Cells were split to 1×10^5/ml every third day. For DNA transfection, 10^7 cells were suspended in 0.5 ml of PBS containing 30 μg of linearized plasmid and electroporated with a Gene Pulser (Bio-Rad) at 550 V and 25 microfarads. The cells were transferred to 20 ml of fresh medium and incubated for 24 h. After resuspending in 100 ml of medium containing either 30 μg/ml mycophenolic acid or 2 mg/ml G418, cells were divided into five 96-well plates and cultured for 7–10 days. Drug-resistant colonies were transferred to 50 ml of T-flasks for preparation of genomic DNA to confirm heterozygous disruption of *tfam* alleles by Southern blotting. For screening of DT40 clones having both *tfam* alleles disrupted, the colonies with heterozygous disruption were subjected to another round of transfection/screening cycle using a medium supplemented with 50 μg/ml uridine and 110 μg/ml sodium pyruvate. To obtain the colony expressing exogenous *c-tfam* cDNA and its deletion mutants under the background of heterozygous *tfam* allele disruption, plasmids having either of these sequences and neomycin-resistance gene (neo^r) in tandem, both under the control of β-actin promoters, were transfected to the cells with either *tfam* allele targeted by *MPA*, and the G418-resistant colonies were selected. The colonies positive to exogenous c-TFAM were further selected by Western blotting of the cell lysate.

Screening and Sequencing of cDNA and Genomic DNA Clones—A chicken cDNA library in which mRNAs isolated from the day 5 embryos were reverse-transcribed using oligo-dT primers, and double-stranded cDNA were inserted into *Eco*RI-*Xho*I sites of λZAPII vector was a gift from Dr. Atsushi Kuroiwa. ~1×10^7 phages were screened using the *Sal*I fragment of *h-tfam* cDNA (17) as a probe in a buffer containing 50 mM Tris-HCl (pH 7.5), 5× SSC, 5× Denhardt's solution, 1% SDS, and 100 μg/ml salmon sperm DNA. After hybridization (16 h, 53 °C), filters were washed 3 times with 2× SSC containing 0.1% SDS at room temperature and once with 0.2× SSC containing 0.1% SDS at 53 °C for 30 min and exposed to x-ray film (Fuji). Because the cDNA clones isolated from this library were found to encode only nuclear c-TFAM (GenBank™ accession number AB21166), the 5′ sequence encoding the N terminus of mt c-TFAM (GenBank™ accession number AB059657) was obtained by reverse transcription of RNA extracted from DT40 cells with SMART rapid amplification of cDNA ends cDNA amplification kit (Clontech) using a primer of 5′-CACCAGCTCCAAGCTGTTCAG-3′. The amplified fragment was sequenced with a primer of 5′-GCTGGTTGTCCCTCAGGAAG-3′. For transient expression of the nuclear or mt *c-tfam* cDNA tagged with a c-Myc sequence at 3′ end, either cDNA clone was amplified with 3′ primer of 5′- GCGGGATCCTTACAAGTCTTCTTCAGAAATAAGCTTTTGTTCTTCTTCAGATTTTTTCAGCTTCAGTTTCGCCAG-3′ paired with 5′-primer of 5′-CCCggTCgACACCGCGGTGGCGGCCGCCATGGGGCGGAGGGGCGGGCTG-3′ or 5′- GGCGGTCGACTCCGTGGGGAGGGTGGGCTGCGGCATG-3′, respectively. The amplified fragments were inserted downstream of β-actin promoter after *Sal*I/*Bam*HI digestion.

A DT40 genomic DNA library with *Sau*3A partial digests (15–25 kb) inserted into the *Xho*I site of a λFIXII vector was a gift from Dr. Tatsuo Nakayama. ~1×10^6 phages were screened at a hybridization temperature of 65 °C using a *c-tfam* cDNA clone as a probe. Genomic DNA clones were analyzed by restriction endonuclease mapping and Southern blot analysis. Pertinent restriction fragments were subcloned into pBluescript. DNA was sequenced by the dideoxynucleotide method on an Applied Biosystems (model 377) DNA sequencer.

Preparation of Anti-chicken TFAM Antiserum—To express c-TFAM in *Escherichia coli*, the *Sac*I-*Hin*dIII fragment of *c-tfam* cDNA coding for amino acid residues Ala-42 to Leu-248 was cloned into the *Sac*I-

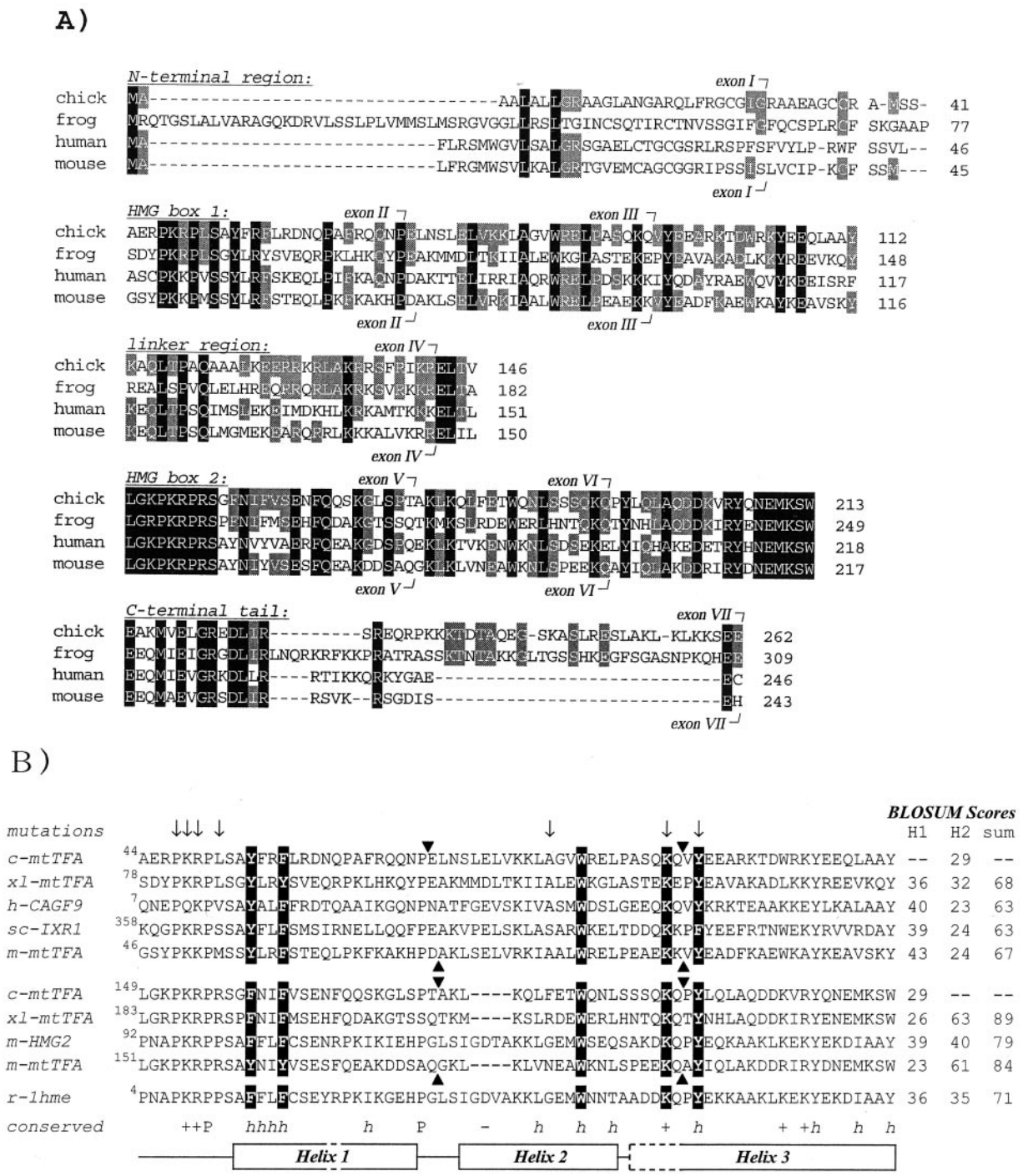

FIG. 2. **Alignment of chicken-TFAM amino acid sequence with *Xenopus*-, human-, and mouse-TFAM (*A*) and sequence comparison of HMG boxes (*B*).** In *panel A*, sequences are divided according to their putative domain organization as the N-terminal region, HMG box 1, linker region, HMG box 2, and C-terminal tail. Residues identical in all four proteins are printed in *reverse font*. Other residues identical with those of c-TFAM are *highlighted* with a *gray background*. *Dashes* indicate gaps introduced for optimum alignment. *Residue numbers* are given at the *right*. Exon boundaries for the chicken (*top*) and mouse (*bottom*) TFAM genes are indicated by *brackets*. In *panel B*, HMG box sequence regions of chicken (*c*), *Xenopus* (*xl*, Ref. 23), and mouse (*m*, Ref. 22) TFAMs are aligned with those of a human polyglutamine/CAG repeat-containing protein CAGF9 (40), the yeast intrastrand cross-link recognition protein *sc*-IXR1 (41), and mouse HMG2 (42). The secondary structure of the second HMG box of rat HMG1 as evaluated by NMR (Protein Data Bank code 1hme (43)) is depicted as a schematic *below* the alignment. In the structure of the corresponding domain of hamster HMG1, helix 1 is interrupted near the middle, and helix 3 is shortened by five residues as indicated by *dashed lines* (44). Motif boundaries were chosen based on deletion mutants in DNA binding assays (45–47) and extensive sequence alignments. *Arrows* denote residues for which point mutation studies performed on different HMG box-containing proteins have shown a loss of DNA binding capacity. Conserved residue types (*h*, hydrophobic; +/−, charged; *P*, proline) shown in the *bottom line* are assessed qualitatively from more complete alignments. *Triangles* mark the positions of exon boundaries within *c*- and *m-tfam*. *Columns* on the *right* give the BLOSUM62 scores resulting from pairwise comparison of the first (H1) and second (H2) c-TFAM HMG box regions with the adjacent sequences as well as the sum of the two values.

*Hin*dIII sites of *pQE31* (Qiagen, Chatsworth, CA). A bacterial culture harboring the plasmid was grown in LB medium containing 100 μg/ml ampicillin, and expression was induced by 0.1 mM isopropil-1-thio-β-D-galactoside. The N-terminal His_6 tag was utilized for purification of the product on a nickel nitrilotriacetic acid column according to the manufacture's instructions. New Zealand White rabbits were immunized by intramuscular injections of 0.3 mg of purified protein in complete Freund's adjuvant and boosted once with 0.3 mg of protein in incom-

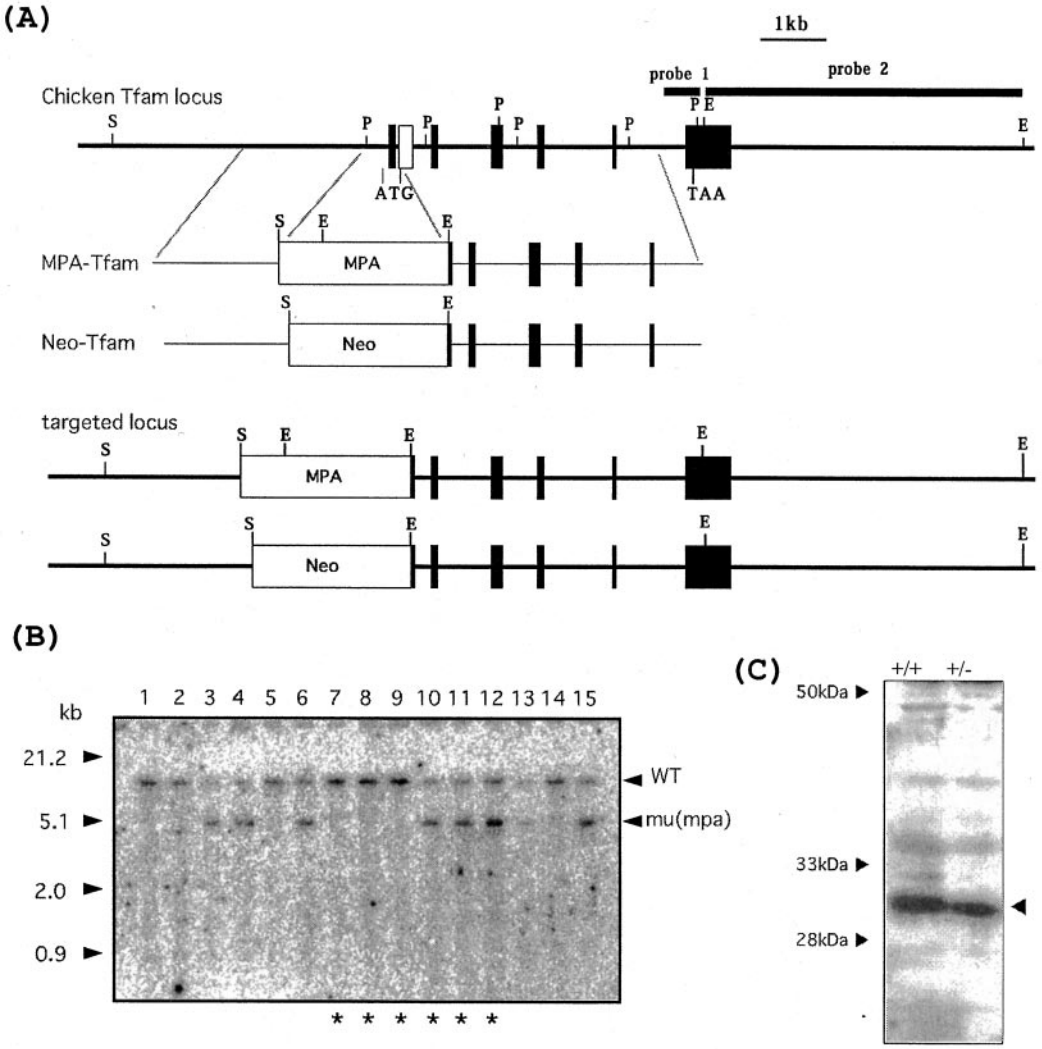

FIG. 3. **Strategy for the targeted disruption of *c-tfam* in a lymphoma cell line of DT40.** *A*, restriction map of *c-tfam* locus, design of the targeting vectors, and expected organization of the disrupted *Tfam* locus after homologous recombination are shown. The translation initiation ATG and termination TAA sites are indicated. *Shaded* and *open boxes* represent exons and drug resistance genes, respectively. Relevant restriction sites shown are: *E*, *Eco*RI; *P*, *Pst*I, *S*, *Sal*I. Positions and lengths of probes 1 and 2 are shown at the *top*. *B*, result of a typical Southern analysis of DNA from drug-resistant DT40 clones is shown. DNA isolated from untransfected DT40 cells (*lane 1*) and from 14 mycophenolic acid-resistant clones (*lane 2–15*) was digested with *Sal*I and *Eco*RI, electrophoresed, and hybridized with the probe 1. Eight clones (*lane 3*, *4*, *6*, *10–13*, *15*) gave a 5-kb band created by the homologous recombination. The clones having genotype of either $tfam^{+}/tfam^{+}$ (*lanes 7–9*) or $tfam^{+}/tfam^{-}$ (*lanes 10–12*) were selected as indicated by *asterisks* and analyzed for mtDNA levels in Fig. 4. The migration positions of *tfam* fragments digested from wild type (*WT*) and disrupted (*mu(mpa)*) alleles are indicated by *arrowheads* at the *right margin*. Migration positions of DNA size markers are indicated by *arrowheads* at the *left margin*. *C*, reduced levels of c-TFAM *in* $tfam^{+}/tfam^{-}$ cells was confirmed by Western blotting with antiserum raised against recombinant c-TFAM. Cellular protein (10 μg) extracted from $tfam^{+}/tfam^{+}$ (*lane* +/+) and $tfam^{+}/tfam^{-}$ (*lane* +/−) cells was separated by SDS-polyacrylamide gel electrophoresis, transferred to a filter, and Western-blotted.

plete Freund's adjuvant. Sera were collected 4 weeks after initial injection.

Indirect Immunofluorescence Microscopy—DT40 cells were grown on coverslips. After labeling with MitoTracker Red CMXRos (Molecular Probes) according to the manufacturer's instructions, cells were fixed with methanol/water/acetic acid (95:4:1, v/v) for 15 min, permeabilized with cold methanol for 10 min, incubated with anti-c-TFAM antiserum or anti-c-Myc rabbit polyclonal IgG (Santa Cruz Biotechnology) diluted 1:500 or 1:100, respectively, in buffer A (PBS containing 0.1% Tween 20) for 1 h, washed 4 times with PBS, incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Cappel; 1:100 dilution in buffer A) for 1 h, washed 4 times with PBS, and mounted in 90% (v/v) glycerol containing 2 mM Tris-HCl (pH 8.0), 0.2 M 1,4-diazabicyclo-(2,2,2)octane.

Western Blotting—Cell extracts corresponding to 10 μg of total protein were separated in 15% SDS-polyacrylamide gels and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose (Amersham Biosciences). Filters were preincubated with 5% skim milk in PBS, incubated with anti-c-TFAM (1:500 in buffer A) for 1 h, washed 4 times with buffer A, incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) for 1 h, and washed with buffer A. Protein bands were visualized using the ECL Western blotting reagents (Amersham Biosciences).

Chromosome Preparation and in Situ Hybridization—Chromosomal assignment of *c-tfam* was performed using the direct R-banding fluorescence *in situ* hybridization method as described (29, 30) with minor modifications. Mitogen-stimulated splenocytes were cultured at 39 °C and synchronized by a thymidine block, and 5-bromodeoxyuridine was incorporated during the late replication stage for differential replication staining after release of excessive thymidine. R-bands were stained by exposure of Hoechst 33258-stained chromosome slides to UV light. Chromosome slides were heated at 65 °C for 2 h, denatured at 70 °C in 70% formamide in 2× SSC, and dehydrated in a 70, 85, 100% ethanol series at 4 °C. The 15-kb chicken genomic DNA fragment inserted into the *Sal*I site of pBluescript was labeled by nick translation with biotin 16-dUTP (Roche Applied Science), ethanol-precipitated together with chicken whole genomic DNA, salmon sperm DNA, and *E. coli* tRNA, and denatured at 75 °C in 100% formamide for 10 min. The denatured probe was mixed with an equal volume of hybridization solution to a final concentration of 50% formamide, 2× SSC, 10% dextran sulfate, and 2 mg/ml bovine serum albumin. 20 μl of the solution containing 250 ng of labeled DNA were put on the denatured slide, covered with

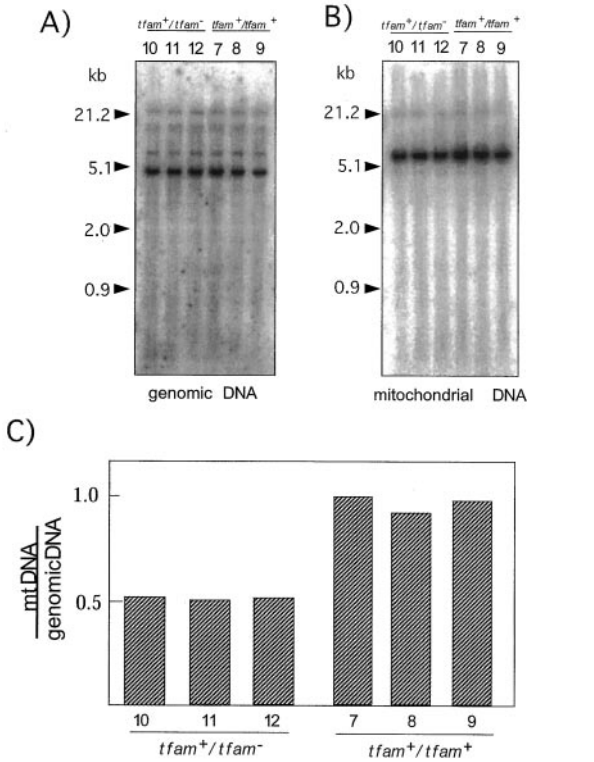

FIG. 4. **Reduced levels of mtDNA in *tfam*$^+$/*tfam*$^-$ cells.** DNA extracted individually from *tfam*$^+$/*tfam*$^+$ clones (*lanes 7–9* of Fig. 3*B*) and *tfam*$^+$/*tfam*$^-$ clones (*lanes 10–12*) was digested with *Sal*I and *Eco*RI. 10 μg were separated on a 1.0% agarose gel and transferred to a nylon membrane. As an internal control, the membrane was first hybridized with ^{32}P-labeled probe 2 (see Fig. 3*A*) (*A*) and then rehybridized with a ^{32}P-labeled mtDNA fragment spanning the ATPase 6/8 genes (*B*). *C*, mtDNA signals of *tfam*$^+$/*tfam*$^+$ and *tfam*$^+$/*tfam*$^-$ cells were quantified as described under "Materials and Methods" and normalized by the internal control.

Parafilm, and incubated overnight at 37 °C. Slides were washed in 50% formamide in 2× SSC at 37 °C for 20 min, and 2× and 1× SSC at room temperature for 20 min. After rinsing in 4× SSC, slides were incubated with Cy2-labeled streptavidin (Amersham Biosciences) at 1:500 dilution in 1% bovine serum albumin, 4× SSC at 37 °C for 1 h. After washing with 4× SSC, 0.1% Nonidet P-40 in 4× SSC, and 4× SSC for 10 min each on a shaker, slides were rinsed with 2× SSC and stained with 0.75 μg/ml propidium iodide. Hybridization was visualized by excitation at 450–490 nm (Nikon filter set B-2A) and ~365 nm (UV-2A), and recorded on Kodak Ektachrome 100 film.

Southern Blotting—Genomic DNA was purified from DT40 cells with DNAZOL (Invitrogen). 10 μg of DNA were separated by 1% agarose gel electrophoresis after digestion with *Eco*RI, *Sal*I, and/or *Pst*I and transferred to Hybond N+ (Amersham Biosciences). Hybridization was performed in a buffer containing 50 mM Tris-HCl (pH 7.5), 5× Denhardt's solution, 1% SDS, and 0.1 mg/ml salmon sperm DNA at 65 °C for 16 h. Filters were washed 3 times with 2× SSC containing 0.1% SDS at room temperature for 10 min and once with 0.2× SSC containing 0.1% SDS at 65 °C for 30 min and analyzed with a Fuji BAS 2000 image analyzer. For the screening of DT40 clones, of which *tfam* was disrupted, a 0.5-kb probe (*probe 1* in Fig. 3A) was prepared by amplifying the *Sal*I-*Eco*RI fragment inserted in pBluescript KS(+) with a paired primer of 5′-TAGTTCCCTTTCTGTCAAAG-3′ and T7 primer and digesting it with *Eco*RI. For semi-quantification of mtDNA, the *Eco*RI-*Stu*I fragment of chicken ATPase 6/8 cDNA was used. A 5-kb *Eco*RI fragment of *tfam* (*probe 2* in Fig. 3A) was used for the normalization of the quantification of mtDNA. Because this probe sequence is localized outside of the targeted region, its signal cannot be affected by the disruption of *tfam* in DT40 cells.

Northern Blotting—Total RNA was extracted by the acid guanidinium thiocyanate/phenol/chloroform method (31). 10 μg of RNA was separated by 1.5% formaldehyde agarose gel electrophoresis and transferred to Hybond N+. Filters were hybridized to ^{32}P-labeled probes in 5× standard saline phosphate -EDTA, 0.5% SDS, 5× Denhardt's solution, 50% formaldehyde, 0.1 mg/ml of denatured salmon sperm DNA at 42 °C for 16 h. Filters were washed 3 times with 2× SSC containing 0.1% SDS at room temperature and once with 0.1× SSC containing 0.1% SDS at 65 °C for 30 min and analyzed with a Fuji Film BAS 2000 Image Analyzer. Probes for *c-tfam*, ATPase α, ATPase γ, and ATPase 6/8 mRNAs were individual cDNA clones isolated in our laboratory. Probes for β-actin RNA (5′-ATGGATGATGATATTGCTGC-3′; 5′-TTCATCGTACTCCTGCTTGC-3′), 12 S/16 S rRNA (5′-AAGCTAGGACCCAAACTGG-3′; 5′-GTGAAGAGTTGTGGTCTGTG-3′), and ND6 (NADH dehydrogenase subunit VI) mRNA (5′-CAACCCACGCACAAGCTC-3′; 5′-GTAGCGTCTGTGATAGG-3′) were prepared by reverse transcription-PCR of chicken RNA with primers designed based on the reported sequences.

Targeting Vector Construction—As a source of targeting vectors, plasmids with *MPA* (32) and *neo*^{r} (33) under the control of the chicken β-actin promoter were inserted into pBluescript II. Plasmids were gifts of Dr. Shunichi Takeda. Selection marker genes were sandwiched by a 1.8-kb sequence upstream of exon I^M and a 4.6-kb sequence spanning the 3′ terminus of exon II through intron VI as left and right arms, respectively (*cf.* Fig. 3*A*). For the left arm insertion, a *Pst*I site of the 1.8-kb *Sal*I-*Pst*I fragment was modified to a *Sal*I site. For the right arm insertion, a *Bam*HI-*Bam*HI fragment was prepared by PCR using paired primers of 5′-CCCGGGATCCCTTCCTGAGGGACAACCAGC-3′ and 5′-CCCGGGATCCCACCTGTGCAGTACTCATCC-3′.

Expression Vectors for TFAM Deletion Mutants—Paired *c-tfam* cDNA fragments that sandwich the domain to be deleted were prepared by PCR so as to have restriction ends of 5′-*Sal*I/blunt-3′ or 5′-blunt/*Bam*HI and were inserted downstream of β-actin promoter by a three-piece ligation technique. The deleted sequences of c-TFAM were Lys-46–Try-112 for the first HMG box, Lys-z13–Val-146 for the linker, Leu-147–Trp-213 for the second HMG box, and Glu-214–Glu-262E for the tail. A series of deletion mutants at the tail was also prepared; Val-218 to Glu-262 for del M, Ser-228 to Glu-262 for del R, Ser-244 to Glu-262 for del G, and Lys-254 to Glu-262 for del A. The vectors for expression of the mutants had *neo*^{r} under the control of β-actin promoter for the selection of positive colonies.

Sequence Analysis—General sequence editing and analysis were performed using MacDNASIS, version 3.7 (Hitachi Software). Similarity searches against the non-redundant GenBank™ data base (June 1999) were done using BLAST 2.0 (34). HMG boxes were compared with those of the Pfam data base (version 4.0; Ref. 35). Multiple sequence alignments were performed with ClustalW (36). Pairwise comparisons were done using the BLOSUM62 matrix and default values for gap penalties.

Quantification of the Blot Signals—A Southern blot was analyzed by a Fuji Film BAS 2000 Image Analyzer, and the radioactivity was quantified by determining the photostimulated luminescence count with a Fuji Film Image Gauge, version 3.4. The ECL signal Western blotting was semiquantified with Image ID program (Amersham Biosciences).

RESULTS AND DISCUSSION

cDNA Cloning of Nuclear and mt c-TFAMs—Low stringency screening of 1 × 10^7 independent cDNA clones of mRNAs expressed in the day 5 chicken embryos with *h-tfam* cDNA probes yielded five selected clones. Partial sequencing and restriction fragment analysis of the inserts showed that they share identical 3′-sequences with different extensions in the 5′ direction. Three clones had the same sequence (AB021166) of 1324 bp, whereas the other two clones contained sequences of 1174 and 1008 bp, respectively. The longest sequence encodes an open reading frame of 792 bp starting with ATG at the fourth base and terminating at TAA at the 769th base to code for a 264-residue amino acid sequence. Neither poly(A)-adding signal nor poly(A) tail was found.

Attempts of rapid amplification of cDNA ends (37) using mRNA extracted from DT40 cells as the template yielded 5′-extended sequence (AB059657) encoding different N-terminal sequence (Fig. 1*A*), suggesting that chicken cells express two forms of c-TFAM. When these cDNA sequences obtained from the day 5 chicken embryos were transiently expressed in DT40 cells after tagging with the c-Myc sequence at the C terminus, indirect immunofluorescence staining of the cells with anti-c-Myc polyclonal IgG showed localization of the product into the nucleus, whereas the endogenous form in DT40 cells was local-

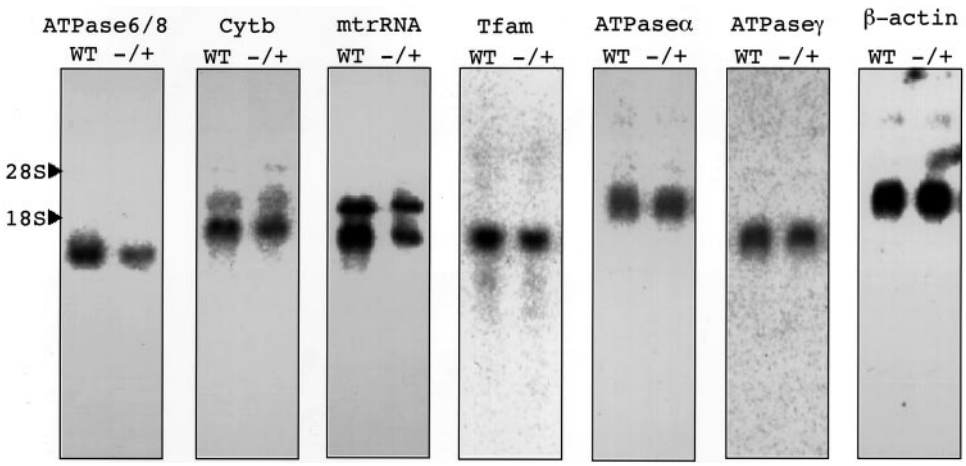

FIG. 5. **Northern blot analysis of *tfam*$^+$/*tfam*$^+$ and *tfam*$^+$/*tfam*$^-$ cells.** RNA (10 μg each) extracted from *tfam*$^+$/*tfam*$^+$ (*WT*) and *tfam*$^+$/*tfam*$^-$ (−/+) cells was separated on 1.5% agarose gels, transferred to filters, and hybridized with ^{32}P-labeled probes as indicated at the *top* of each *panel*. Migration positions of 28 S and 18 S rRNA are indicated at the *left*. *Cytb*, cytochrome *b*.

ized in mitochondria (Fig. 1*B*; see below). We thus cloned mt and nuclear forms of c-TFAM.

For mouse spermatocytes, a testis-specific TFAM isoform resulting from alternative splicing of a different exon I^T has been characterized in addition to mt mouse m-TFAM, and this isoform is targeted to the nucleus (22). Screening of a DT40 genomic DNA library with the mt c-TFAM cDNA sequence as a probe yielded three overlapping fragments. Analysis of restriction sites and partial sequencing resulted in the exon/intron organization of *c-tfam* as shown in Fig. 1*A*. The N-terminal sequence of nuclear c-TFAM is encoded by exon I^N, which is directly connected to exon II without insertion of an intron, whereas that of mt c-TFAM is encoded by exon I^M and continued to the sequence encoded by exon II by splicing out of the exon I^N. Thus, the mechanism of generating the nuclear and mt c-TFAMs is similar to *m-tfam*, but the exons encoding the mt and nuclear form-specific sequences (exon I^N and exon I^M) are positioned oppositely. The downstream cDNA sequence is encoded by additional six exons. All exon/intron boundaries had well conserved consensus sequences for splicing both at the 5′- and 3′-end of introns (not shown). Immunostaining of DT40 cells with antiserum prepared against c-TFAM showed staining of mitochondria well overlapping with that of living dye for mitochondria (MitoTracker Red), and no signal was indicated in the nuclear region (Fig. 1*C*).

Sequence Comparison of c-TFAM with xl-, m-, and h-TFAMs—Alignment of the deduced c-TFAM amino acid sequence with those of *xl*- (23), h- (17), and m- (22) TFAMs in Fig. 2*A* shows an identity of 42, 37, and 40%, respectively. In contrast to h- and m-TFAMs, c-TFAM shares with *xl*-TFAM the C terminus-extended regions. Its calculated molecular mass (M_r = 29,985) is larger than that of h- (M_r = 29,097) and m-TFAM (M_r = 27,988) but smaller than that of *xl*-TFAM (M_r = 35,501). Its theoretical isoelectric point of 10.7 is comparable with that of *Xenopus* (pI ~ 10.2), which is slightly more basic than that of h- and *m*-TFAMs (pI ~ 9.7). As do other TFAMs, the chicken sequence contains two HMG boxes separated by a ~35-amino acid residue linker. Whereas the HMG-box sequences show high correlations between the different species, the closest relationship between *c*- and *xl*-TFAMs in the whole sequences is mainly due to the similarities of the C-terminal extensions and the linker region.

By replacing the various regions of h-TFAM with the corresponding parts of the yeast homologue, the significance of the linker region and the C-terminal tail for the recognition and transcriptional activation of the human mtDNA promoter has been established (21). For c-TFAM, the isoelectric point of the linker region is predicted as pI ~ 11.9, which is similar to that estimated for *xl*- and m-TFAMs and higher than for h-TFAM (pI ~ 10.0). Assuming that the two HMG regions recognize the repeated half-sites in the control region of mtDNA, as suggested for human, mouse, and *Xenopus* (21, 38), it is interesting to note that the mouse and *Xenopus* half-sites are separated by three to six A/T-rich sequences, whereas those of human consist of G/C rich regions. Chicken mtDNA promoter sequences are not yet specified in such detail, but the highly basic character of the linker region shared with that of mouse and *Xenopus* might indicate a similarity in the binding sites. Besides, the chicken and *Xenopus* linker regions have a 60% sequence identity, and the C-terminal tail of both is similarly extended by ~20 (chick) and ~30 (*Xenopus*) residues over that of mammalian TFAMs (Fig. 2*A*). Such similarity could be of importance with respect to the considerably shorter distance of 50 (25) and 0 bp (28) between the transcription start sites of the L- and H-strands found for *Xenopus* and chicken, respectively, which in human extends over 150 bp (26).

HMG domains have the ability to bend DNA and to wrap distorted DNA with low nucleotide sequence specificity (39). The 367 HMG boxes collected in the Pfam data base (35) have an average length and sequence identity of 66 residues and 36%, respectively. Although in most cases they occur as single motifs, in 34 proteins they appear twice in close proximity, and four proteins contain three or four HMG domains. Fig. 2*B* shows an alignment of the two c-TFAM HMG regions with those of some other proteins, selected on the basis of high similarity. The combined similarity values as expressed as BLOSUM62 scores are highest when compared with *xl*-TFAM, which is due to the close similarities between the second HMG boxes, whereas the first HMG box has a lower similarity, which is generally found for other proteins having two HMG boxes. The structure of several HMG domains has been solved to high resolution by multidimensional NMR techniques (43, 44). As one example, the sequence of the second HMG box of rat HMG1 (r-1hme) of known structure is included at the bottom of Fig. 2*B*. It contains three α-helices of which the first (N terminus) runs antiparallel to the second (middle) helix, and the third (C terminus) helix bends at an angle of 70−80° relative to the middle helix, forming a V-shaped configuration. The aromatic and basic character of residues corresponding to Phe-14, Phe-17, Trp-45, Lys-53, and Tyr-56 of the r-1hme structure is also found in both c-TFAM HMG boxes (printed in *reverse font* in Fig. 2*B*), which in rat and hamster HMG1 cluster to a concave

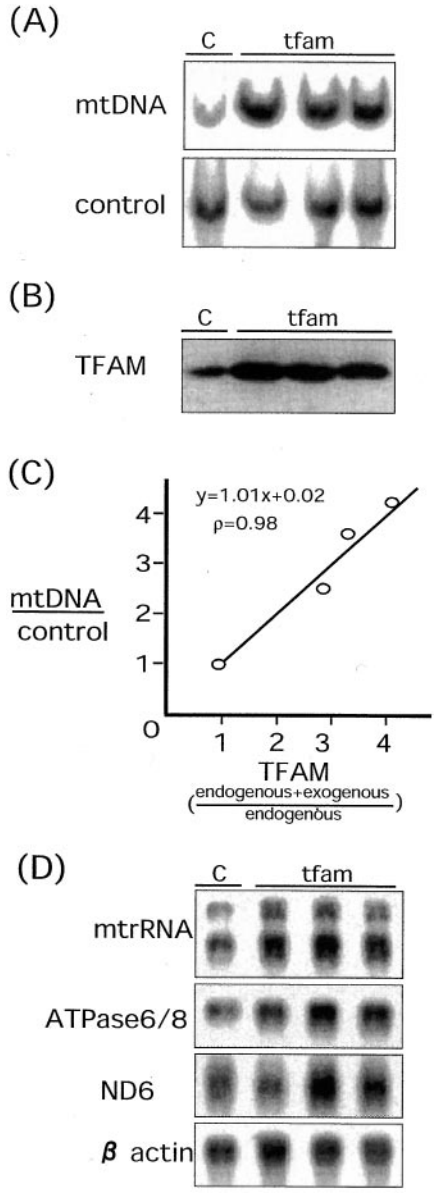

FIG. 6. **Dosage-dependent increase of mtDNA copy number in DT40 cells by exogenous c-TFAM.** Wild type *c-tfam* cDNA under the control of β-actin promoter and with *neo*^{r} as the selection marked was introduced into the *tfam*$^+$/*tfam*$^-$ DT40 cells, and the drug-resistant clones were selected. *A*, Southern blot analysis on DNA extracted from three independent clones (*tfam*) together with a control clone (*c*) with ^{32}P-labeled mtDNA fragment (*top panel*) or ^{32}P-labeled control probe (*lower panel*) was carried out as in Fig. 4. *B*, Western blot analysis on c-TFAM in the proteins extracted from individual clones was carried out as in Fig. 3*C*. In this experiment, the exogenous and the endogenous c-TFAM had the same size and could not be distinguished. *C*, normalized mtDNA signal of an individual clone quantified as in Fig. 4*C* is plotted against c-TFAM signal quantified as described under "Materials and Methods." A regression line with regression coefficient (slope) and cutting point of the vertical axis of 1.01 and 0.02, respectively, is drawn to connect the plots by the least squares method. The correlation coefficient (ρ) of the line was 0.98. *D*, Northern blot analysis on RNA extracted from individual clones was carried out as in Fig. 5.

surface formed between the three helices (43, 44). Point mutation studies on a number of HMG box-containing proteins indicate the importance of several residues for its DNA binding capacity (*arrows* in Fig. 2*B*; Refs. 45–47). Most of the corresponding residues within the c-TFAM HMG boxes appear consistent with those of active DNA binding sequences.

Exon/Intron Organization and Chromosomal Assignment of c-tfam—When compared with the cDNA-derived amino acid sequence, all boundaries are well aligned with those of m- and h-TFAM (Fig. 2*B*). Within the HMG domains, exon boundaries II/III and V/VI are localized within the turn region separating helices 1 and 2 (Fig. 2*B*). Boundaries III/IV and VI/VII correspond to the beginning of helix 3, with exons IV and VII including the linker and C-terminal tail regions, respectively. This suggests that *tfam* arose by gene duplication of an ancestral gene covering either exons II to IV, which show a higher sequence variation between species, or exons V to VII.

Chromosomal assignment of *tfam* was carried out by fluorescence *in situ* hybridization using a 15-kb genomic fragment as probe. Direct R-banding localized the signal to chicken chromosome 6q1.1-q1.2 (not shown).

Targeted Disruption of c-tfam in DT40 Cells—DT40 is a chicken lymphoma cell line established by infection of chicken with avian leucosis virus. Because this cell line has a high efficiency of targeted integration of foreign DNA (33, 48), it provides a convenient system for targeted gene disruption at the cellular level. We prepared the targeting vectors in which *MPA* (*MPA-Tfam*) or *neo*^{r} (*Neo-Tfam*) were sandwiched by a 1.8-kb sequence upstream of exon I^M and a 4.6-kb sequence spanning the 3′ terminus of exons II through intron VI as left and right arms, respectively (Fig. 3*A*). In these constructs, exon I^M and the 5′-terminus of exon I^N including the ATGs for the nuclear and the mt c-TFAMs, respectively, were deleted together with the upstream sequence. Probe 1 (Fig. 3*A*) was prepared for monitoring of the targeted allele by Southern blotting of *Sal*I-*Eco*RI-digested DNA from drug-resistant clones. This probe is expected to show a 9- and 5-kb fragment for the wild type (*tfam*$^+$) and targeted (*tfam*$^-$) allele, respectively, for either of the constructs, whereas no signal was expected for any random integration.

After introduction of *MPA-Tfam* by electroporation, 30 mycophenolic acid-resistant clones could be isolated, of which 12 were targeted at either of the *tfam* alleles. Fig. 3*B* shows the Southern blotting on a half of mycophenolic acid-resistant clones. Additional integration of *Neo-Tfam* into three expanded clones resulted in 0 of 100 neomycin-resistant clones showing a Southern blotting pattern consistent with a heterozygous *tfam* deletion. These results were essentially the same when the cells were targeted first with *Neo-Tfam* followed by *MPA-Tfam*. With a 40% efficiency for targeted disruption of either of the two *tfam* alleles, it is unlikely that the second disruption should be difficult to be achieved. This demonstrates that a functional *tfam* allele is essential for the proliferation of DT40 cells. Although DT40 cells with a heterozygous deletion of *Tfam* showed no apparent phenotype, the *tfam* transcript level (see Fig. 5) as well as the amount of c-TFAM was reduced to about half that of wild-type cells (Fig. 3*C*).

The level of mtDNA was significantly reduced in *tfam*$^+$/*tfam*$^-$ DT40 cells, which is in agreement with the data found for heterozygous mice (49). Quantification of the Southern blotting signals of mtDNA in three independent *tfam*$^+$/*tfam*$^-$ clones showed a reduction of ~50% (Fig. 4). Although the results show a direct influence of c-TFAM on the mtDNA copy number, heterozygous DT40 cells showed no sign of an opposite influence on the level of c-TFAM (see Fig. 3*C*) as was observed in cells that have been artificially depleted of mtDNA (50, 51). In that case, cells exhibited low levels of TFAM concomitant with artificial DNA depletion without any decrease of *tfam* mRNA, which might be due to rapid degradation of the protein when not bound to DNA.

In *tfam*$^+$/*tfam*$^-$ DT40 cells, levels of mtDNA transcripts (ATPase 6/8 mRNA, ND6 (NADH dehydrogenase subunit VI) mRNA, and 12 S/16 S rRNA) tended to be reduced, whereas levels of ATPase α and γ mRNA encoded in the nuclear genome

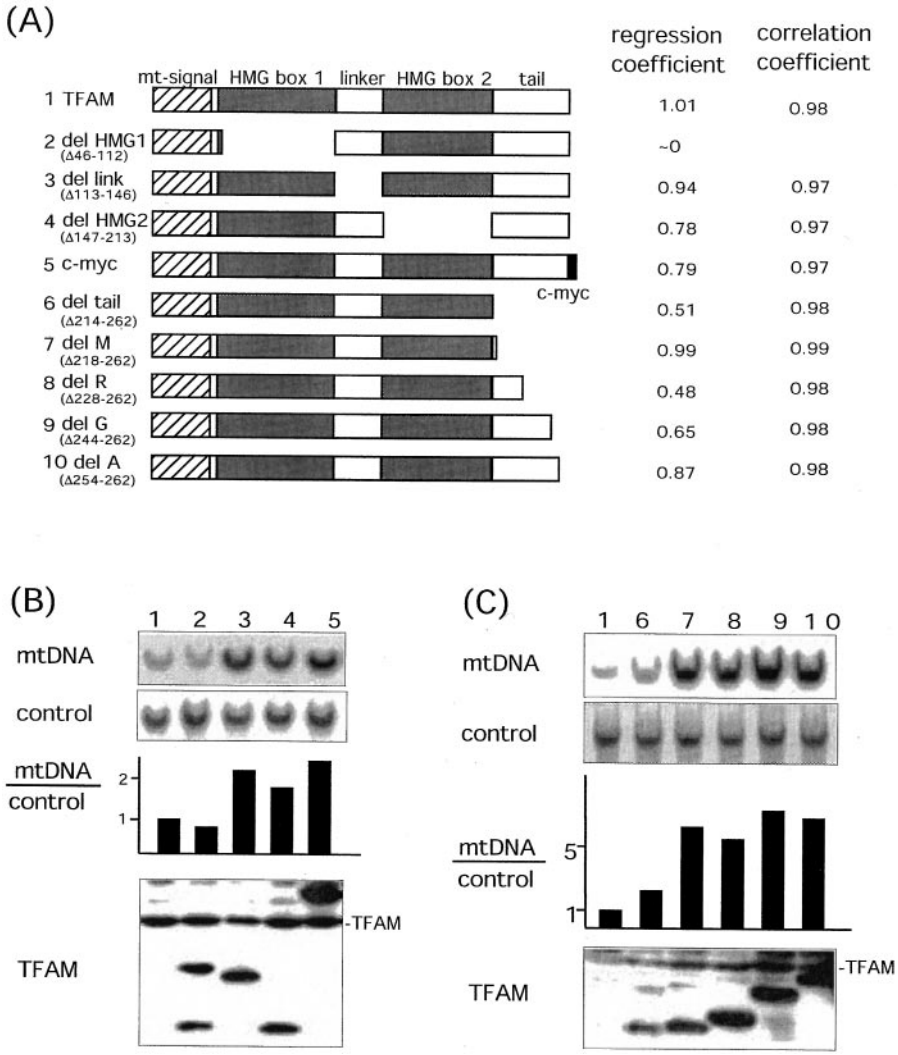

FIG. 7. **Functional domains of c-TFAM for the maintenance of mtDNA copy number.** *A* series of cDNA constructs encoding c-TFAM deleted at various domains was prepared and inserted downstream of β-actin promoter. These constructs were *TFAM*, the wild type cDNA; *del HMG1*, *tfam* cDNA lacking sequence encoding the first HMG box; *del link*, lacking sequence for the linker between HMG boxes; *del HMG2*, lacking sequence for the second HMG box; *c-myc*, *tfam* cDNA tagged with *c-myc* sequence at 3′-end; *del A*, lacking the C-terminal tail downstream of 253A; *del G*, lacking downstream of 243G; *del R*, lacking downstream of 227R; *del M*, lacking downstream of 217M; *del tail*, lacking the tail sequence entirely. The *c-tfam* cDNA constructs were introduced into the $tfam^{+}/tfam^{-}$ DT40 cells together with neo^{r}, and the drug-resistant clones were selected. Assays detailed in Fig. 8 for del M cDNA construct were carried out using three independent colonies of $tfam^{+}/tfam^{-}$ DT40 cells stably expressing individual mutant at different level, and regression coefficient (slope) and correlation efficient (ρ) obtained by drawing regression lines are listed at the right margin. In the case of del HMG1 construct, the slope of the regression line gave minus value since the mtDNA/control values for three independent clones were equal to or slightly smaller than 1. We, thus, denoted its regression coefficient as ~0. *B*, Southern blot analysis on DNA extracted from the clones introduced with none, the deletion c-TFAM mutants at HMG box 1, linker, HMG box 2, or c-TFAM tagged with c-Myc using ^{32}P-labeled mtDNA fragment (*top panel*) or ^{32}P-labeled control probe (*second panel*) was carried out as in Fig. 4. Relative mtDNA signals of individual clones (*third panel*) quantified and normalized by the internal control in Fig. 4*C* are plotted. Western blot analysis on c-TFAM and its deletion mutants in the proteins extracted from individual clones (*bottom panel*) was carried out as in Fig. 3*C*. Migration position of the endogenous c-TFAM is indicated in the right margin. *C*, results for the deletion series at the C-terminal tail are summarized.

remained unchanged (Fig. 5). Reproducible reduction of mtDNA transcripts by 40–60% together with unchanged levels of the transcripts encoded in the nuclear genome was observed in independently repeated Northern blotting (not shown). This suggested an apparent absence of nuclear-mitochondrial communication for the adjustment of the level of respiratory chain components, which are of dual origin. Thus, our results are in contrast with the insignificant changes in transcript levels observed in the mitochondria of heart, kidney, and muscle of heterozygous knock-out mice of *m-tfam* (49).

In experiments not shown, wild type DT40 cells were cultured in the presence of ethidium bromide (50 ng/ml) using a medium supplemented with 50 μg/ml uridine and 110 μg/ml sodium pyruvate. Retardation of cell growth was observed 48 h after the addition of ethidium bromide, and the cells died eventually. Southern blotting of mtDNA showed a reduction of 60% after 48 h, whereas Northern blotting of the same cells showed no change and only a 30% reduction of *c-tfam* and ATPase 6/8 mRNAs, respectively. These results suggest that maintenance of mtDNA copy number is essential for the survival of DT40 cells.

Dosage-dependent Increase of mtDNA Copy Number by Exogenous c-TFAM—We found that the reduced mtDNA level in $tfam^{+}/tfam^{-}$ DT40 cells could be restored in a dosage-dependent manner by expression of exogenous mt c-TFAM (Fig. 6*A*). For this, a plasmid having cDNA coding for mt c-TFAM and neo^{r} in tandem both under the control of β-actin promoters was transfected to DT40 cells of which either *tfam* allele was targeted by *MPA*, and the G418 resistant colonies were selected. The level of mt c-TFAM detected by Western blotting of the cell

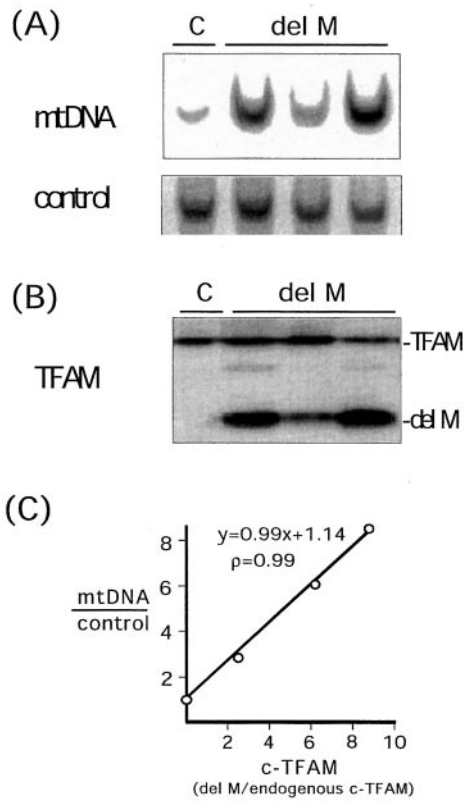

FIG. 8. **Dosage-dependent increase of mtDNA copy number in DT40 cells by exogenous c-TFAM.** Del M cDNA construct was introduced into the *tfam*$^+$/*tfam*$^-$ DT40 cells, and the drug-resistant clones were selected. *A*, Southern blot analysis on DNA extracted from three independent clones (*del M*) together with a control clone (*c*) with ^{32}P-labeled mtDNA fragment (*top panel*) or ^{32}P-labeled control probe (*lower panel*) was carried out as in Fig. 6. *B*, Western blot analysis on endogenous c-TFAM and del M mutant in the proteins extracted from individual clones was carried out as in Fig. 6. *C*, normalized mtDNA signal of an individual clone quantified as in Fig. 6 is plotted against del M signal divided by endogenous c-TFAM signal. A regression line with regression coefficient (slope) and cutting point of the vertical axis of 0.99 and 1.14, respectively, is drawn to connect the plots by the least squares method. The correlation coefficient (ρ) of the line was 0.99.

lysate was variable, probably depending on the context of the insertion site of the exogenous *c-tfam* in chromosome (Fig. 6*B*). Interestingly, the copy number of mtDNA was linearly proportional to the amount of mt c-TFAM (endogenous plus exogenous), as demonstrated in Fig. 6*C* for three such colonies. Restoration of mtDNA copy number was more than 4-fold, which is beyond the level in the *tfam*$^+$/*tfam*$^+$ cells, suggesting that stoichiometric binding of c-TFAM to mtDNA can be the background mechanism of increased copy number.

mtDNA transcripts increased marginally by the expression of exogenous c-TFAM and remained within the levels of 1.2–2-fold of the *tfam*$^+$/*tfam*$^-$ cells (Fig. 6*D*). The increase was too small to be correlated with the reinforced levels of c-TFAM and/or mtDNA (reaching up to 4-fold). This suggests that dosage of mtDNA as the template and amount of transcription factor are not the primary factors, but some post-transcriptional regulation (for example, stability of mt mRNAs) is more important for the maintenance of the transcript levels.

For the molecular dissection of functional domains in c-TFAM, a series of deletion mutants (Fig. 7*A*) was stably expressed in the *tfam*$^+$/*tfam*$^-$ DT40 cells, and the level of mtDNA was quantitated by Southern blotting. In the case of the mutant deleted at the first HMG box, considerable amounts of the protein remained with the size of precursor before localization into mitochondria, but the mature protein was also observed in the amount comparable with the endogenous c-TFAM (the *bottom panel* of Fig. 7*B*). Nonetheless, the reduced level of mtDNA in the *tfam*$^+$/*tfam*$^-$ was not restored by this mutant. Interestingly, deletion at the second HMG box impaired only marginally (see below) the ability of c-TFAM to increase the copy number of mtDNA (Fig. 7*B*). Compared with close similarities of the second HMG box to those of other species, the first HMG box of c-TFAM has a lower similarity. Almost complete loss of the activity by the deletion of the first HMG box implies that the mtDNA-maintaining activity is unique to TFAMs. More careful assay described below suggested that the deletion mutant at linker region retains almost full activity of mtDNA maintenance (Fig. 7, *A* and *B*). Surprised at the considerable activity of the c-TFAM to which the c-Myc tag was added at the C terminus (Fig. 7*B*), we also tested a deletion series of the C-terminal tail region (Fig. 7*C*) and found that most part of the C-terminal tail region is dispensable for maintaining mtDNA activity.

As was the case for exogenously expressed wild type c-TFAM (Fig. 6), mtDNA increased proportionally to the amount of expressed mutant protein, as shown in Fig. 8 for three colonies of the *tfam*$^+$/*tfam*$^-$ cells expressing the del M mutant at different levels. This indicated that evaluation of functional domain using only one colony for each deletion mutant as in Fig. 7, *B* and *C*, is not quantitative and that the assay needs to be normalized by the amount of mutant protein expressed in each colony. When the ratio of mtDNA/control (mtDNA level in each colony divided by that in the control cell) was plotted against exogenous TFAM/endogenous TFAM for the del M mutant (Fig. 8*C*), a regression line cutting the vertical axis at 1.14 (very close to 1) with a slope (regression coefficient) of 0.99 could be drawn by the least square method. This implies that the value of the regression coefficient gives relative activity of the mutants having that of endogenous c-TFAM to be 1, and the value of correlation coefficient (ρ) gives the statistic reliability of this assay.

We repeated this assay for all deletion mutants of c-TFAM and listed the values of regression coefficients and correlation coefficients at the right margin of Fig. 7*A*. It is now clear that deletion at the linker region does not impair the activity. Activity of the mutant deleted at the whole tail region was 51% of the wild type but was restored by the addition of amino acid residues 214–217 (EAKM) as observed in the del M mutant. The addition of the residues 218–227 (VELGREDLIR) was suppressive as in the del R mutant, but it was recovered to the wild type level by further addition of the tail sequence. It is worth noting that the addition of c-Myc tag to the tail sequence impaired the activity (the c-Myc mutant).

Even if the mtDNA copy number was considerably increased by the expression of some deletion mutants, the level of mtDNA transcripts remained within 1.2–1.8-fold of the *tfam*$^+$/*tfam*$^-$ cells (not shown), as it was for the expression of wild type c-TFAM (Fig. 6*D*). We thus demonstrated that the structural requisite of c-TFAM for its mtDNA maintenance activity is different from that of h-TFAM established for the transcriptional activation of mtDNA *in vitro* (21).

Conclusions—Vertebrate mtDNAs have variation mainly in the control region, which is the sequence between the genes encoding tRNA^{Pro} and tRNA^{Phe} and contains the H-strand replication origin and the binding domains of TFAMs for transcriptional activation of H- and L-strands (52). *Xenopus* and chicken mt genomes are similar in the distance of transcription start sites of L- and H-strands. They are separated by ~150 bp in human (26), ~100 bp in bovine (53) and mouse (54), ~50 bp in *Xenopus* (25), and 0 bp in chicken (28). c-TFAM cloned in this study has higher similarity to *xl*-TFAM than to h- and m-

TFAMs. It is possible that the structural similarity between xl- and c-TFAMs is related to the proximity of two start sites in *xl*- and c-mtDNAs. The binding of *xl*-TFAM to a single binding domain between the two start sites of *xl*-mtDNA was sufficient for activation of bi-directional transcription (23). The mechanisms of c-mtDNA transcription from the L- and H-strand start sites without any intervening base have not been studied, but future comparison of TFAMs for their transcriptional activation of c-mtDNA may reveal the reason for the structural similarity of *xl*- and c-TFAMs.

Disruption of *tfam* in DT40 cells resulted in essentially the same consequence as *tfam* mutant mice (49). The $tfam^{-}/tfam^{-}$ cells could not proliferate, and mtDNA copy number was reduced in the $tfam^{+}/tfam^{-}$ cells. Minor differences were that the reduction of mtDNA was ~50% in the $tfam^{+}/tfam^{-}$ cells compared with 34% in the $tfam^{+}/tfam^{-}$ mice, and mitochondrial transcripts reduced to 40–60% in the $tfam^{+}/tfam^{-}$ cells, whereas there was small change in the tissues of mutant mice. Thus, TFAM appears to have dual functions. Minimum expression of *tfam* is essential for the proliferation of DT40 cells, whereas massive expression supports the normal mtDNA copy number.

For the regulation of mtDNA copy number, many factors such as DNA polymerase, RNA polymerase and transcription factors, which create the replication primer, single-stranded DNA-binding proteins, and RNA-processing enzymes may be involved. Identification of human diseases associated with drastically reduced levels of mtDNA suggested the significance of nucleotide pool size in the organelle for the maintenance of normal level of mtDNA (55). There has been no report that increases in TFAM would lead to increases in mtDNA levels. Our surprising observation was that expression of *c-tfam* as an exogenous gene under the background of $tfam^{+}/tfam^{-}$ resulted in 4–8-fold increases in mtDNA number. The increase was proportional to the total amount of c-TFAM expressed in the cell. Considering the general ability of vertebrate TFAM and also its yeast homologue (*sc*-mtTFA) to wrap, bend, and condense DNA by HMG-boxes (18, 23, 56), the effect of exogenous c-TFAM was probably due to its activity of stabilizing mtDNA. Consistent with this understanding, deletion of the first HMG box from the exogenous c-TFAM canceled the effect. Because *sc*-mtTFA, which lacks the C-terminal tail, could bind and bend double-stranded DNA (56), it is not surprising that exogenous c-TFAMs deleted at the C-terminal tail differentially could support the increased mtDNA copy number. Considering the essential role of the C-terminal tail in supporting the *in vitro* transcription of mtDNA (21), however, the consequence might be different if these deletion mutants of c-TFAM could be introduced to the $tfam^{-}/tfam^{-}$ cells to rescue the lethal phenotype.

Acknowledgments—We thank Dr. Atsushi Kuroiwa (Graduate School of Science, Nagoya University) for the chicken cDNA library, Dr. Tatsuo Nakayama (Miyazaki Medical University) for the DT40 genomic library, and Dr. Shunichi Takeda (Graduate School of Medicine, Kyoto University) for the DT40 cell line and drug-resistant genes. We appreciate discussions with Dr. Tatsuhiko Kadowaki (Graduate School of Bioagricultural Sciences, Nagoya University).

REFERENCES

1. Jaehning, J. A. (1993) *Mol. Microbiol.* **8,** 1–4
2. Shadel, G. S., and Clayton, D. A. (1993) *J. Biol. Chem.* **268,** 16083–16086
3. Masters, B. S., Stohl, L. L., and Clayton, D. A. (1987) *Cell* **51,** 89–99
4. Schinkel, A. H., Groot Koerkamp, M. J. A., and Tabak, H. F. (1988) *EMBO J.* **7,** 3255–3262
5. Jang, S. H., and Jaehning, J. A. (1991) *J. Biol. Chem.* **266,** 22671–22677
6. Cliften, P. F., Park, J. Y., Davis, B. P., Jang S. H., and Jaehning, J. A. (1997) *Genes Dev.* **11,** 2897–2909
7. Cliften, P. F., Jang S. H., and Jaehning, J. A. (2000) *Mol. Cell. Biol.* **20,** 7013–7023
8. Mangus, D. A., Jang, S. H., and Jaehning, J. A. (1994) *J. Biol. Chem.* **269,** 26568–26574
9. Carrodeguas, J. A., Yun, S., Shadel, G. S., Clayton, D. A., and Bogen-hagen, D. F. (1996) *Gene Expr.* **6,** 219–230
10. Shadel, G. S., and Clayton, D. A. (1995) *Mol. Cell. Biol.* **15,** 2101–2108
11. Bogenhagen, D. F. (1996) *J. Biol. Chem.* **271,** 12036–12041
12. Bogenhagen, D. F., and Insdorf, N. F. (1988) *Mol. Cell. Biol.* **8,** 2910–2916
13. McCulloch, V., Seidel-Rogol, L., and Shadel, G. S. (2002) *Mol. Cell. Biol.* **22,** 1116–1125
14. Schinkel, A. H., Groot Koerkamp, M. J. A., Touw, E. P. W., and Tabak, H. F. (1987) *J. Biol. Chem.* **262,** 12785–12791
15. Christianson, T., and Rabinowitz, M. (1983) *J. Biol. Chem.* **258,** 14025–14033
16. Fisher, R. P., and Clayton, D. A. (1985) *J. Biol. Chem.* **260,** 11330–11338
17. Parisi, M. A., and Clayton, D. A. (1991) *Science* **252,** 965–969
18. Fisher, R. P., Lisowsky, T., Parisi, M. A., and Clayton, D. A. (1992) *J. Biol. Chem.* **267,** 3358–3367
19. Diffley, J. F., and Stillman, B. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7864–7868
20. Parisi, M. A., Xu, B., and Clayton, D. A. (1993) *Mol. Cell. Biol.* **13,** 1951–1961
21. Dairaghi, D. J., Shadel, G. S., and Clayton, D. A. (1995) *J. Mol. Biol.* **249,** 11–28
22. Larsson, N.-G., Garman, J. D., Oldfors, A., Barsh, G. S., and Clayton, D. A. (1996) *Nat. Genet.* **13,** 296–302
23. Antoshechkin, I., and Bogenhagen, D. F. (1995) *Mol. Cell. Biol.* **15,** 7032–7042
24. Clayton, D. A. (1991) *Annu. Rev. Cell Biol.* **7,** 453–478
25. Bogenhagen, D. F., and Romanelli, M. F. (1988) *Mol. Cell. Biol.* **8,** 2917–2924
26. Montoya, J., Christianson, T., Levens, D., Rabinowitz, M., and Attardi, G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7195–7199
27. Desjardins, P., and Morais, R. (1990) *J. Mol. Biol.* **212,** 599–634
28. L'Abbé, D., Duhaime, J.-F., Lang, B. F., and Morais, R. (1991) *J. Biol. Chem.* **266,** 10844–10850
29. Matsuda, Y., Harada, Y. N., Natsuume-Sakai, S., Lee, K., Shiomi, T., and Chapman, V. M. (1992) *Cytogenet. Cell Genet.* **61,** 282–285
30. Matsuda, Y., and Chapman, V. M. (1995) *Electrophoresis* **16,** 261–272
31. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
32. Mulligan, R. C., and Berg, P. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 2072–2076
33. Buerstedde, J. M., and Takeda, S. (1991) *Cell* **67,** 179–188
34. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
35. Bateman, A., Birney, E., Durbin, R., Eddy, S. R., Finn, R. D., and Sonnhammer, E. L. (1999) *Nucleic Acids Res.* **27,** 260–262
36. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
37. Frohman, M. A. (1993) *Methods Enzymol.* **218,** 340–356
38. Antoshechkin, I., Bogenhagen, D. F., and Mastrangelo, I. A. (1997) *EMBO J.* **16,** 3198–3206
39. Lilley, D. M. J. (1992) *Nature* **357,** 282–283
40. Margolis, R. L., Abraham, M. R., Gatchell, S. B., Li, S.-H., Kidwai, A. S., Breschel, T. S., Stine, O. C., Callahan, C., McInnis, M. G., and Ross, C. A. (1997) *Hum. Genet.* **100,** 114–122
41. Brown, S. J., Kellett, P. J., and Lippard, S. (1993) *Science* **261,** 603–605
42. Stolzenburg, F., Dinkl, E., and Grummt, F. (1992) *Nucleic Acids Res.* **20,** 4927
43. Weir, H. M., Kravlis, P. J., Hill, C. S., Raine, A. R. C., Lave, E. D., and Thomas, J. O. (1993) *EMBO J.* **12,** 1311–1319
44. Read, C. M., Cary, P. D., Crane-Robison. C., Driscoll, P. C., and Norman, D. G. (1993) *Nucleic Acids Res.* **21,** 3427–3436
45. Giese, K., Amsterdam, A., and Grosschedl, R. (1992) *Cell* **69,** 185–195
46. Nasrin, N., Buggs, C., Kong, X. F., Carnazza, J., Goebl, M., and Alexander-Bridges, M. (1991) *Nature* **354,** 317–320
47. Harley, V. R., Jackson, D. I., Hextall, P. J., Hawkins, J. R., Berkovitz, G. D., Sockanathan, S., Lovell-Badge, R., and Goodfellow, P. N. (1992) *Science* **255,** 453–456
48. Takeda, S., Masteller, E. L., Thompson, C. B., and Buerstedde, J.-M. (1992) *Proc. Natl. Acad. Sci. U. S. A.*, **89,** 4023–4027
49. Larsson, N. G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S., and Clayton, D. A. (1998) *Nat. Genet.* **18,** 231–236
50. Poulton, J., Morten, K., Freeman-Emmerson, C., Potter, C., Sewry, C., Dubowitz, V., Kidd, H., Stephenson, J., Whitehouse, W., Hansen, F. J., Parisi, M., and Brown, G. (1994) *Hum. Mol. Genet.* **3,** 1763–1769
51. Larsson, N.-G., Oldfors, A., Holme, E., and Clayton, D. A. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1374–1381
52. Clayton, D. A. (1984) *Annu. Rev. Biochem.* **53,** 573–594
53. King, T. C., and Low, R. L. (1987) *J. Biol. Chem.* **262,** 6204–6213
54. Chang, D. D., and Clayton, D. A. (1986) *Mol. Cell. Biol.* **6,** 1446–1453
55. Moraes, C. T. (2001) *Trends Genet.* **17,** 199–205
56. Diffley, J. F., and Stillman, B. (1992) *J. Biol. Chem.* **267,** 3368–3374