Regulation of Mitochondrial Single-stranded DNA-binding Protein Gene Expression Links Nuclear and Mitochondrial DNA Replication in Drosophila

Received for publication, August 27, 1999, and in revised form, December 28, 1999

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The structural organization of the Drosophila melanogaster gene encoding mitochondrial single-stranded DNA-binding protein (mtSSB) has been determined and its pattern of expression evaluated during Drosophila development. The D. melanogaster mtSSB gene contains four exons and three small introns. The transcriptional initiation site is located 22 nucleotides upstream from the initiator translation codon in adults, whereas several initiation sites are found in embryos. No consensus TATA or CAAT sequences are located at canonical positions, although an AT-rich sequence was identified flanking the major transcriptional initiation site. Northern analyses indicated that the mtSSB transcript is present at variable levels throughout development. In situ hybridization analysis shows that maternally deposited mtSSB mRNA is distributed homogeneously in the early embryo, whereas de novo transcript is produced specifically at an elevated level in the developing midgut. Transfection assays in cultured Schneider cells with promoter region deletion constructs revealed that the proximal 230 nucleotides contain cis-acting elements required for efficient gene expression. Putative transcription factor binding sites clustered within this region include two Drosophila DNA replication-related elements (DRE) and a single putative E2F binding site. Deletion and base substitution mutagenesis of the DRE sites demonstrated that they are required for efficient promoter activity, and gel electrophoretic mobility shift analyses showed that DRE binding factor (DREF) binds to these sites. Our data suggest strongly that the Drosophila mtSSB gene is regulated by the DRE/DREF system. This finding represents a first link between nuclear and mitochondrial DNA replication.

Animal mitochondria are essential energy-producing organelles that contain multiple copies of their small double-stranded DNA genome (1). Despite its limited coding capacity, mtDNA is critical because it encodes 13 polypeptides that are essential components of mitochondrial respiratory complexes. Biogenesis of mitochondria requires expression and duplication of the mtDNA genome and relies heavily on the nuclear genome, which provides all of the protein components required for these processes and also those involved in their regulation. Mitochondrial DNA polymerase (DNA polymerase γ) and mitochondrial single-stranded DNA-binding protein (mtSSB) are key nuclear encoded components of the mtDNA replication apparatus: DNA polymerase γ is the replicative DNA polymerase (2-4), and mtSSB functions in helix destabilization (5, 6) and in enhancing both the activity and processivity of DNA polymerase γ (7, 8). In vivo, mtSSB is associated with the mitochondrial nucleoid (9) and is concentrated within the perinuclear mitochondria that constitute active sites of mtDNA replication (10, 11). Its role is critical in replication because deletion of the yeast protein causes loss of mtDNA (12).

mtSSB shares both structural and functional similarities with E. coli SSB (13, 14). Both are homotetrameric proteins composed of 13–16 kDa polypeptides with similar DNA binding and replication properties. mtSSB has been purified from several sources including rat (15, 16), Xenopus (17), yeast (12), and Drosophila (7). cDNAs have been cloned and characterized from Xenopus (18, 19), yeast (12), rat and man (20), Drosophila (21), and mouse (22). Comparatively little is known about the structure of the mtSSB gene and its regulation. mtSSB genes in Xenopus (23) and rat (24) have been isolated and their promoters shown to share some regulatory elements including those that bind nuclear respiratory factors and the Sp1 transcription factor. mtSSB gene expression correlates with the mtDNA level in mammalian tissues and is up-regulated during mitochondrial biogenesis (10), but the molecular mechanisms orchestrating this regulation are unknown.

To understand the molecular mechanism of mtSSB expression, we have cloned and determined the organization of the Drosophila mtSSB gene. We have carried out a functional analysis of the promoter region and discovered a link between mtSSB expression and that of several nuclear replication genes. The use of Drosophila as an animal model also gave us the opportunity to study the expression of mtSSB during development and spatial distribution of its transcript during embryogenesis. This opens several new avenues for evaluation of the in vivo regulation of the mtSSB gene.

* This work was supported in part by National Institutes of Health Grants GM45295 and HL59656 (to L. S. K.), Grant PB94-0088 from the Ministerio de Educacion y Ciencia, Spain, and European Union Human Capital and Mobility Program Network Grant CHRX-CT94-0494 (to R. G.). 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) AF181084.

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1 The abbreviations used are: mtSSB, mitochondrial single-stranded DNA-binding protein; AMV, avian myeloblastosis virus; PCR, polymerase chain reaction; kb, kilobase pairs; DRE, DNA replication-related element(s); DREF, DRE-binding factor; bp, base pair(s); RSV, Rous sarcoma virus; β-GAL, β-galactosidase.
EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids—Unlabeled deoxyribonucleoside triphosphates were purchased from Amersham Pharmacia Biotech. [α-32P]dATP and [γ-32P]ATP were purchased from ICN Biochemicals. Plasmid DNAs were prepared by standard laboratory methods. Synthetic oligodeoxynucleotides were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer.

Bacterial Strains and Bacteriophage—E. coli NM621 (25) was used for screening an EMBL3 genomic DNA library from Drosophila melanogaster. E. coli DH15a (25) was used to subclone the mtSSB gene for DNA sequence analysis.

Drosophila Cells and Tissue Culture Medium—Schneider S2 cells, cell culture medium, and fetal bovine serum were purchased from Life Technologies, Inc. Penicillin G and streptomycin sulfate were from Sigma.

Enzymes—E. coli DNA polymerase I and its Kløen fragment were purchased from New England Biolabs. T4 poly nucleotide kinase, RNase-free DNase I, T7 RNA polymerase, and T3 RNA polymerase were from Roche Molecular Biochemicals. T4 DNA ligase and avian myeloblastosis virus (AMV) reverse transcriptase were from Life Technologies, Inc. and Promega, respectively.

Chemicals—Isopropanol-1-thio-β-D-galactopyranoside, 5-bromo-4-chloro-3-indolyl-β-D-galactoside, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Research Organics, Inc. o-Nitrophenyl-β-D-galactoside and RNase inhibitor were from Roche Molecular Biochemicals.

Methods

Cloning of the D. melanogaster mtSSB Gene—A λEMBL3 D. melanogaster gt1 race DNA library was screened using a D. melanogaster mtSSB cDNA as a probe. The probe was amplified by PCR using as a template pET11a containing the mtSSB cDNA and the primers 5′-GCCGCGCATATGGCAACAAACG-3′ (corresponding to positions 46 to 74, as numbered (21)) and 5′-GGTTGTCATATGGCGTAAAAATTAGG-3′ (corresponding to positions 456 to 428). The PCR-amplified DNA fragment was purified by gel electrophoresis and 5′-end labeled with [γ-32P]ATP using T4 polynucleotide kinase. Approximately 5 × 10^8 plaques were transferred to Zeta-Probe filters (Bio-Rad), hybridized at 65 °C with the radiolabeled probe in ZAP buffer (50 mM Tris-HCl, pH 8.0, 120 mM KCl, 66 mM EGTA, 25 mM EDTA, 0.02% xylene cyanol, 0.02% bromphenol blue), and washed in 0.5% SDS, 0.02% streptomycin, and 0.02% xylene cyanol (pH 7.2). Filters were autoradiographed with intensifying screens at 65 °C, and autoradiographed with intensifying screens at 37 °C. The riboprobe was heated for 2 min at 37 °C before use. Hybridization was carried out overnight at 55 °C in a buffer containing 50% deionized formamide, 5 × SSC, 100 μg/ml sonicated salmon sperm DNA, 50 μg/ml heparin, and 0.1% SDS in hybridization buffer and digoxigenin-labeled UTP (Roche Molecular Biochemicals). The transcription reaction (10 μl) contained 4 μl of transcription buffer and digoxigenin-labeled UTP (Roche Molecular Biochemicals), DNA (1 μg), 40 units of RNase inhibitor, and 20 units of T7 RNA polymerase (antisense) or T3 RNA polymerase (sense) was incubated for 2 h at 37 °C. Water (15 μl) was added followed by hybridization with digoxigenin-UTP antibody (Roche Molecular Biochemicals) coupled to alkaline phosphatase, and the reaction was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Primer Extension Analysis—The oligonucleotide 5′-CGCCAACCTGCTCCAAAGAT-3′ (5 pmol), complementary to nucleotide positions 274 to 285 (see Fig. 1), was 5′-end labeled with T4 polynucleotide kinase and annealed to 30 μg of total RNA isolated from 0–20 h embryos and adults, by heating the reaction mixture for 20 min at 65 °C in 10 μl of 10 mM Tris-HCl, pH 8.5, 120 mM KCl. The extension reaction was performed in buffer containing 10 μl Tris-HCl, pH 8.5, 120 mM KCl, 10 mM MgCl2, and 5 mM dithiothreitol, in the presence of 0.5 μm dNTPs, 20 units of AMV reverse transcriptase, and 40 units of RNase inhibitor. After incubation for 2 h at 42 °C, the RNA-DNA hybrid was ethanol precipitated and resuspended in loading buffer (98% formamide, 0.5% SDS, 25 mM EDTA, 0.02% xylene cyanol, 0.02% bromphenol blue), heated for 10 min at 70 °C, and electrophoresed in a 6% polyacrylamide, 7 M urea sequencing gel. The product sizes were determined by comparison with a DNA sequencing ladder generated with the same oligonucleotide primer and mtSSB cDNA cloned in Bluescript as the substrate.

Construction of Promoter Deletion Mutants—A set of 5′- and 3′-end deletion mutants was fused to the luciferase reporter gene in the vector pXp1. A DNA fragment containing the upstream region from positions −1141 to +22 of the mtSSB gene was generated by PCR using as a template pUC3.5-SalI and the primers 5′-GTGTCATCTCCTCTTGACC-3′ and 5′-GAAGATCTTATAACATAC-3′, corresponding to positions 117 to 1150, see Fig. 1) and 5′-ATTAGCTTATGCGCTTGTGTACATTTT-3′ (lisk 17; corresponding to positions 22 to 2, and containing a HindIII cleavage site at its 5′-end). The PCR product was purified by gel electrophoresis, digested with SalI and HindIII, and cloned into Bluescript to generate pBS mtSSB-S/H. A set of deletion fragment derivatives of plasmid pBS mtSSB-S/H was then generated by digestion with EcoRI, double HindIII, and then cloned into Bluescript as the substrate. Site-directed and Deletion Mutagenesis of the DRE Binding Sites in the mtSSB Promoter—Four oligonucleotides were used as primers for PCR-based mutagenesis reactions (base substitution mutations in the DRE sequences are underlined): −23 mut, 5′-TATCATGTATACATTAGTATTCTTAAATAACATAC-3′; −23 mut, 5′-AATTAAATACATGTAT- GATGATTTACGTGAACTAATGG-3′; −31 mut, 5′-TATGTAAATATACATTAGTACATCATATTATTACATATGATTACATATA-3′; and −31 mut, 5′-ATTAGCTTATGCGCTTGTGTACATTTT-3′. To construct the plasmid containing a substitution mutation at the DRE −23 site, two PCRs were performed using as template pBS mtSSB-S/H. The first reaction used the primers 5′-GTCAAGTGCGGCCCGCCTAGAC-3′ (lisk 116; corresponding to positions −470 to −450, see Fig. 1) and 23 mut (corresponding to positions −9 to −43); the second reaction used primers lisk 117 and −23 mut (corresponding to positions −34 to +1). The two PCR products were purified by gel electrophoresis and then used as template

In Situ Hybridization—In situ hybridization to yu67 embryos was carried out as described by Tautz and Pfeilfe (27). Staged embryos were dechorionated in bleach for 2.5 min and then put into a tube containing 3 ml of fixing buffer (phosphate-buffered saline, pH 8, 66 mM EDTA), 1 ml of 37% formaldehyde, and 4 ml of heptane. After vigorous shaking for 25 min, the lower aqueous phase was aspirated, and methanol was added to remove the vitelline membrane, followed by vigorous agitation for 60 s. The solution was then aspirated, and the embryos were washed several times with methanol and stored at −20 °C.

Prehybridization and hybridization treatments followed by staining and mounting were performed as described (27). Antisense and sense mtSSB riboprobe were prepared by in vitro transcription using as template pBluescript containing the mtSSB cDNA linearized by digestion with SalI (antisense) or SaeI (sense), and digoxigenin-labeled UTP (Roche Molecular Biochemicals). The transcription reaction (10 μl) contained 4 μl of transcription buffer and digoxigenin-labeled UTP (Roche Molecular Biochemicals), DNA (1 μg), 40 units of RNase inhibitor, and 20 units of T7 RNA polymerase. A 10 μl reaction (sense) was incubated for 2 h at 37 °C. Water (15 μl) was added followed by hybridization with digoxigenin-UTP antibody (Roche Molecular Biochemicals) coupled to alkaline phosphatase, and the reaction was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

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for a subsequent PCR with primers lsk 117 and lsk 116. The resulting PCR product was digested with EcoRI, rendered blunt ended, cleaved with HindIII, and then purified by gel electrophoresis and cloned into pXp1 to generate ppx mtSSB-E/DRE-23*.

The construct ppx mtSSB-/E/DRE containing only one DRE site, or they were rendered blunt ended to generate a 23-bp insertion mutation at the center of the DRE site following self-ligation, generating pBS mtSSB/S/HADRE/E/DRE* containing no DRE sites. Both plasmids were digested with EcoRI, and the resulting fragments were rendered blunt ended and then cleaved with HindIII, gel purified, and cloned into pXp1 to generate ppx mtSSB/E/DRE and ppx mtSSB/E/DRE*-DRE*.

DNA sequence analysis of the various plasmid constructs was performed to confirm their structure and sequence integrity.

**Transient Transfection Assays—**Transient transfection in Schneider S2 cells was performed as described by Soeller et al. (28) with some modifications. Streptomycin (100 μg/ml) and penicillin G (100 IU/ml) were added to the medium. Transfection reactions contained the control vector pRSV-b-gal (15 μg) with the various pXp1 constructs (5 μg). After transfection reactions were incubated for 24 h at 25 °C, washed twice with phosphate-buffered saline, resuspended in fresh medium (5 ml), and incubated for 48 h at 25 °C.

To prepare extracts, cells were harvested by centrifugation, washed with phosphate-buffered saline followed by TEN buffer (40 mM Tris-HCl, pH 7.3, 1 mM EDTA, 150 mM NaCl, resuspended in 67 mM Tris-HCl, pH 7.5 (0.1 ml), and lysed by four cycles of freezing for 60 s at −70 °C followed by heating for 60 s at 37 °C. Cell debris was removed by centrifugation at 16,000 × g.

To normalize the luciferase activity among transfection reactions, the β-galactosidase activity of the cotransfected plasmid was measured. To do so, the cell lysates (30 μl) were incubated in 0.1 mM sodium phosphate, pH 7.5, 1 mM MgCl₂, 45 mM β-mercaptoethanol, and 0.88 mg/ml o-nitrophenyl-β-D-galactoside at 37 °C, and stopped by the addition of 0.5 ml of 1 M Na₂CO₃. Promega substrate was then used for determination of 0.5 ml of 1 M Na₂CO₃. Promega substrate was then used for -D-galactosidase activity of the cotransfected plasmid which was measured.

Developmental Regulation of mtSSB Gene Expression—To initiate our studies of the transcriptional regulation of Droso- phila mtSSB, we examined the developmental pattern of gene expression by Northern and in situ hybridization analyses. Northern analysis was performed using total RNA derived from various embryonic, larval, and pupal stages, and from adult flies. We detected a single transcript of 0.5 kb in length, corresponding in size to that expected from the single copy gene (Fig. 2). The mtSSB transcript is abundant in the earliest embryos, most likely because of active ovarian transcription that results in deposition of a high level of maternal transcript in the oocyte. The steady-state level decreases slightly during early embryonic development and increases in later embryos (9–12 h) when mtDNA replication resumes (32). An elevated transcript level will also be observed in the larval stages, where active cell proliferation occurs in various tissues such as the digestive tract. In contrast, the pupal stages show a relatively low level of the mtSSB transcript which increases in adults, consistent with a high level in eggs.

The spatial and temporal expression pattern of the Droso- phila mtSSB gene was explored by whole-mount in situ hybridization using antisense RNA as a probe.
This revealed that mtSSB mRNA is distributed uniformly throughout the early embryo (Fig. 3A); the mRNA is of maternal origin because zygotic transcription begins only 1.5–2 h after egg deposition. The maternal mRNA disappears by cellular blastoderm (Fig. 3B), and by early stage 12, high levels of mtSSB expression occur in the anterior and posterior midgut primordia (Fig. 3C). During germ band retraction, the primordia extend toward each other and meet laterally on both sides of the yolk and maintain a high steady-state level of mtSSB mRNA (Fig. 3D). As the midgut closes, the gut assumes a triangular shape, with the anterior region wider than the posterior (Fig. 3E); thereafter, contractions appear, and the midgut gradually takes up its mature configuration (Fig. 3F). These regions contain moderate levels of mtSSB mRNA staining. At the same time, embryos hybridized to control RNA probe corresponding to the sense strand showed no staining at any embryonic stage (data not shown).

Functional Analysis of the mtSSB Promoter Establishes a Link between Nuclear and Mitochondrial DNA Replication—Promoter analysis of the Drosophila mtSSB gene was initiated by the determination of the transcriptional start site by primer extension. A radiolabeled antisense primer was annealed to total RNA extracted from 0–20-h embryos and adults and extended with AMV reverse transcriptase. cDNA products analyzed by gel electrophoresis identified a single transcriptional start site in adults which is located 22 nucleotides upstream from the translational initiator (Fig. 4). In embryos, multiple extension products were observed, corresponding to transcriptional start sites at positions −22, −31, and −33. The sequences flanking the transcriptional start sites showed no homology with any known initiator sequences. Although no consensus TATA box or CAAT boxes at the canonical positions were found, an AT-rich sequence (TAAATAT) is located 38 bp upstream of the transcriptional start site that lies at position −22, which might function as a TATA-like element.

Functional analysis of the promoter was pursued by deletion mutagenesis in conjunction with transient transfection analysis in Drosophila Schneider cells. A set of deletions of the 1.2-kb proximal region was constructed and linked to a luciferase reporter gene in the vector pXP1 and then tested for reporter gene expression. The structure of the deletion constructs and their relative promoter activities are shown in Fig. 5. To correct for variations in the efficiency of transfection, cells were co-transfected with the plasmid pRSV-βGAL, and luciferase activity was normalized to β-galactosidase activity. The 1.163-kb SacI-HindIII fragment in pXP mtSSB-S/H promoted ≈1,000-fold higher luciferase activity than the promoter-less pXP1 vector. This activity was defined as 100% and was used as the standard to which the luciferase activity produced by other constructs was compared. Cells transfected with a construct on which the same SacI-HindIII fragment was present in the opposite orientation showed no luciferase activity, demonstrating the requirement for the correct orientation of the mtSSB promoter (data not shown). The construct pXP mtSSB-E

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\begin{align*}
\text{FIG. 1. Nucleotide sequence of the Drosophila mtSSB gene.} & \quad \text{The deduced amino acid sequence is shown below the nucleotide sequence in one-letter codes.} \\
\text{Nucleotide +1 is the transcriptional initiation site located 22 bp upstream from the initiator methionine codon.}
\end{align*}
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\begin{align*}
\text{FIG. 2. Developmental regulation of Drosophila mtSSB gene expression.} & \quad \text{Panel A, total RNA from 0–20-h embryos, larvae (L1 and L3: first and third instar, respectively), pupae (P1 and P3), and adults (A) of Drosophila was analyzed by Northern blot using mtSSB cDNA as a probe (see "Experimental Procedures"). RNAs were fractionated by denaturing gel electrophoresis, transferred to a nylon membrane, hybridized with radiolabeled probe, and the blot was autoradiographed. Panel B, levels of RNA loaded were evaluated by ethidium bromide staining. Panel C, quantitation of the Northern data. The autoradiograph in panel A and the stained gel in panel B were scanned, and the intensities of the bands were determined by computer integration analysis; the bands in panel A were normalized to total RNA in panel B in three independent experiments, and the composite quantitation is presented in panel C, taking the value in eggs (0) as 100%. Values are means ± S.D.}
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showed 100% of luciferase activity, indicating that deletions of up to 754 bp of the 5' region had no effect on promoter function. In contrast, the construct pxp mtSSB-C, containing only a 20-bp proximal promoter fragment, showed only 0.8% of maximal luciferase activity, suggesting that the region from 20 to 208 is critical for mtSSB promoter activity. Furthermore, a set of 3'-deletion constructs that lack this region was devoid of promoter activity (data not shown). Taken together, these data demonstrate that the proximal 230 bp are responsible for directing high level expression of the D. melanogaster mtSSB gene in Schneider cells.

Several potential transcription factor binding sites within the critical proximal region were identified by their similarity to consensus binding sequences for known transcription factors (Fig. 6). In particular, two palindromic sequences for the Drosophila DRE (33), with a single-base mismatch to the consensus sequence TATCGATA, are located at positions 24 (DRE-24), and 19 to 24 (DRE-24). A potential E2F binding site, containing a 2-base mismatch compared with the consensus E2F sequence TTTCGGGC found in mammals (34), is situated at position 63 to 65.

To examine the involvement of the potential DRE sites in the promoter activity of the mtSSB gene, we made a series of constructs with specific mutations in the target sequences and analyzed their promoter activity in transient transfection assays. The luciferase activity of the mutant constructs was expressed relative to the pxp mtSSB-E construct, which was shown to elicit full promoter activity, and assigned a value of 100% (Fig. 7). The constructs pxp mtSSB-E/DRE-23 and pxp mtSSB/E/DRE-31, both carrying 3-base substitutions within the 8-bp consensus sequence, showed luciferase activities of 20 and 10%, respectively, indicating that mutagenesis of either DRE site reduces promoter activity substantially. Likewise, deletion of one of the 8-bp DRE consensus sequences, pxp mtSSB/E/DRE*, reduced reporter gene expression to 20%. Furthermore, upon introduction of a 2-bp insertionial mutation in the remaining DRE site in pxp mtSSB-E/DRE*, reporter luciferase activity was reduced to 7%. These results lead us to conclude that both DRE sites are important for the promoter activity of the mtSSB gene in Schneider cells.

To determine whether or not the DRE sequences present in the mtSSB promoter are recognized by DREF (35) we performed gel mobility shift analyses using recombinant DREF protein and nuclear extracts from Schneider cells. A radiolabeled double-stranded oligonucleotide (mtSSB-DRE) containing the DRE sites at positions 23 and 24 was incubated with recombinant DREF, either alone or in the presence of unlabeled competitor oligonucleotide. Upon native gel electrophoresis and autoradiography, a single retarded protein-DNA complex was observed which could be competed by the addition of a 150-fold excess of unlabeled mtSSB-DRE (Fig. 8). Furthermore, using a nuclear extract from Schneider cells, two protein-DNA complexes were observed (Fig. 9A), both of which were competed specifically by the addition of an excess of the same unlabeled wild type oligonucleotide (lanes 3–6). In con-
A mutant oligonucleotide (mut 223/231) containing a 3-base substitution within each of the DRE sites failed to compete (lanes 15–18). To examine the relative contributions of the two DRE sites to DREF binding, oligonucleotides containing 3-base substitutions within either DRE site at position 223 or 231 were used. Both were less effective competitors than the wild type oligonucleotide but more effective than the double mutant, indicating that both sites are functional in DREF binding (Fig. 9A, lanes 3–14).

To establish the presence of DREF in the protein-DNA complex, we carried out a supershift analysis (Fig. 9B). Inclusion of rabbit antiserum against recombinant DREF in the binding reaction resulted in the appearance of a supershifted complex and reduced the intensity of the slower migrating retarded complex that is competed specifically by excess wild type oligonucleotide, demonstrating that DREF is a component of that complex. The addition of the DREF antiserum had no effect on the faster migrating retarded complex, suggesting that DREF is either not part of that complex or that its conformation in the complex is not accessible to the antibody. Rabbit antiserum against Drosophila mtSSB included as a negative control had no effect on either complex. Taken together, the Schneider cell transfection studies and the gel mobility shift analyses demonstrate DREF binding to the DRE sites present in the mtSSB promoter and the importance of these sites in Drosophila mtSSB promoter function.

FIG. 5. **Functional analysis of the mtSSB promoter.** A schematic showing restriction endonuclease sites in the upstream region of the *Drosophila mtSSB* gene is presented at upper left. Below it are schematics of the promoter constructs used in transient transfection assays in Schneider cells (see "Experimental Procedures"). The promoter regions are represented by solid lines, and the luciferase reporter gene is shown by solid boxes. The numbers above each construct indicate the fragment size. The relative promoter activities of the constructs, as measured in the luciferase assay, are indicated on the right. The luciferase activity of the promoter construct containing the 1,163-bp fragment was defined as 100% and used as the standard for comparison. Luciferase activities were normalized to β-galactosidase activities of cotransfected control plasmids. Values are the means ± S.D. of at least three independent experiments.

![Diagram](image)

**FIG. 6. Sequence of the promoter region of the Drosophila mtSSB gene.** The sequence presented corresponds to the 230-nucleotide upstream sequence responsible for maximal promoter activity in Schneider cells. Exon nucleotides are shown in boldface. The sequence is numbered from +1 as the transcriptional initiation site located 22 nucleotides upstream from the initiator methionine codon (M). Putative transcription factor binding sites are boxed. Transcriptional start sites are indicated in adults (closed arrowhead) and in embryos (open arrowheads), respectively. The AT-rich region is underlined.
Regulation of mtSSB Gene Expression

Fig. 7. Mutations in the DRE sequences diminish the promoter activity of the Drosophila mtSSB gene. A schematic representation of the pxp mtSSB-E constructs containing mutations in the two DRE sites at positions −23 and −31 is shown on the left. 3-base substitutions are underlined. The 2-bp insertional mutation is shown in lowercase, and deletions are indicated by dashed lines. The relative luciferase activities of the mutant constructs are shown on the right. The values are presented as percentages of the promoter activity of the wild type construct pxp mtSSB-E. Luciferase activities were normalized to β-galactosidase activities of cotransfected control plasmids. Values are the means ± S.D. of at least three independent experiments.

Fig. 8. Recombinant DREF protein binds to the DRE sites in the promoter of the Drosophila mtSSB gene. DREF protein binding was evaluated by gel mobility shift analysis as described under “Experimental Procedures.” A radiolabeled double-stranded oligonucleotide containing both DRE sites at positions −23 and −31 was incubated with rabbit reticulocyte lysate in the absence (C, lane 2) or presence (lane 3) of recombinant DREF protein, either alone (lane 3) or with a 150-fold molar excess of unlabeled oligonucleotide (lane 4). Protein-DNA complexes were electrophoresed, and the gel was autoradiographed. Lane 1 represents a no-protein control.

Genomic region; 2) Southern analysis detected only fragments from this region; and 3) Northern analysis revealed the presence of a single mRNA in embryos and adults. Localization of the transcriptional start site identified a unique position 22 bp upstream from the initiator methionine codon in adults and multiple start sites in embryos. This difference in transcriptional initiation site usage was also observed in the gene encoding the rate-limiting heme biosynthetic enzyme, δ-aminolevulinate synthase (43). In both cases, the proximal promoter lacks typical TATA and CCAAT boxes in canonical positions. The absence of a typical TATA box has also been found in other nuclear genes encoding mitochondrial proteins (29, 44).

Functional analysis of the promoter region in transient transfection assays delimited the minimal promoter region to the proximal 230 bp. Several putative transcription factor binding sites were found within this region, including two palindromic sequences for the Drosophila DRE and a single presumptive E2F binding site. In Drosophila, DRE and DREF are responsible for activating the promoters of nuclear replication genes, including those for proliferating cell nuclear antigen, which is an auxiliary protein for DNA polymerase δ, the 180- and 73-kDa subunits of DNA polymerase α, and cyclin A, which is required for entry into the S and M phases of the cell cycle (33, 45, 46). Regulation of the Drosophila E2F gene by the DRE/DREF system has been also reported recently (47). We show that the DRE sites in the mtSSB gene, investigated by deletion and base substitution mutagenesis in transient transfection assays, are required for promoter activity. Furthermore, gel shift analyses demonstrated that both recombinant DREF and DREF present in nuclear extracts bind to the DRE sites. Finally, competition assays indicated that mutant DRE sites are inefficient competitors of DREF/DREF complex formation. Taken together, our data suggest strongly that the Drosophila mtSSB gene is regulated by the DRE/DREF system. This represents the first example of such control for a gene involved in mtDNA replication. Thus, although mitochondrial biogenesis is not strictly linked to the cell cycle or to cell division, the regulated expression of replication gene products may link nuclear and mitochondrial DNA transactions.

E2F is a transcription factor that has been implicated in cell cycle control in many organisms via transcriptional activation of a group of genes that encode activities important for cellular DNA replication (47–49). In Drosophila, studies performed both in vitro and in vivo suggest that DREF in combination with E2F activates coordinately the transcription of DRE-containing genes (26, 50). The finding of a putative E2F binding site in the region critical for the promoter activity in the mtSSB gene suggests the possibility that DRE and E2F may also function coordinately to activate the mtSSB promoter. Supporting this hypothesis is the finding that the DRE/DREF system is apparently also critical in the regulation of another gene involved in mtDNA replication, that encoding the accessory subunit of DNA polymerase γ2 and of the presence of a putative E2F binding site near that DRE site. Indeed, if this is the case, there may exist a common regulatory mechanism for the expression of mitochondrial replication-related proteins.

Both Northern and RNA in situ hybridization analyses indicated that the Drosophila mtSSB gene is developmentally regulated. A single mRNA of 0.5 kb in length is present at varying
levels throughout development, with elevated levels in early embryos, larvae, and adults. The mtSSB transcript is distributed uniformly during the earliest stages of embryogenesis (0–2 h), where there is rapid cell proliferation. This maternal mRNA disappears rapidly around cellularization, where it falls below the level of detection by this method. After the activated expression of zygotic genes, an elevated level of mtSSB mRNA is seen later in embryogenesis, concomitant with the development of the midgut. The abundance of the mtSSB transcript in the midgut suggests that mtDNA replication is very active in this tissue. Correspondingly, it has been shown clearly in mammals that mtSSB gene expression correlates directly with mtDNA content.

The pattern of mitosis during Drosophila embryogenesis has been described in detail (51). There are 13 rapid cleavage divisions that occur in a syncytial blastoderm; following cellularization, most cells that will give rise to larval tissues undergo only three additional rounds of mitosis which are completed by the 6th hour of embryogenesis (stage 11). Notable exceptions are neural cells, in which DNA synthesis persists after mitosis 16 (52), and malpighian tubule cells (53). Although cell proliferation ceases at this time in the Drosophila embryo, some cells, including those of the developing midgut, undergo DNA replication uncoupled from mitosis (endoreplication). Interestingly, our finding of the accumulation of mtSSB transcript in the midgut suggests that it is not the consequence of active cell proliferation but may be related to the high DNA endoreplication activity of this tissue.

The spatio-temporal pattern of expression of several Drosophila genes encoding enzymes involved in nuclear DNA synthesis, including DNA polymerase α, proliferating cell nuclear antigen, and the two ribonucleotide reductase subunits RNR1 and RNR2, are highly similar (54). In particular, during the later stages of embryogenesis, their expression is limited to tissues with high DNA replication activity (e.g., proliferating neural tissue) and tissues undergoing endoreplication. Furthermore, the pattern of expression of genes involved in the regulation of the activity of DNA replication origins, such as ORC1, a member of the origin recognition complex (55) and MCM proteins (56, 57), resembles that of genes encoding replication factors, suggesting coordinate control of the expression of genes involved in nuclear DNA replication. Although the regulation of mtSSB expression by the DRE/DREF system establishes a link between nuclear and mitochondrial DNA replication, only endoreplicating tissues (the midgut), but not proliferating tissues (neural cells), show a large accumulation of the mtSSB transcript. Thus, mtDNA replication may be highly active only in a subset of cells undergoing chromosomal DNA synthesis. This suggests that additional unidentified factors are involved in the nucleo-mitochondrial communication that regulates mtDNA transactions.

Acknowledgments—We thank Carol Farr for help in the preparation of the figures.

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J. Biol. Chem. 2000, 275:13628-13636.
doi: 10.1074/jbc.275.18.13628

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