Differential Regulation of the Catalytic and Accessory Subunit Genes of *Drosophila* Mitochondrial DNA Polymerase*

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The developmental pattern of expression of the genes encoding the catalytic (α) and accessory (β) subunits of mitochondrial DNA polymerase (pol γ) has been examined in *Drosophila melanogaster*. The steady-state level of pol γ-β mRNA increases during the first hours of development, reaching its maximum value at the start of mtDNA replication in *Drosophila* embryos. In contrast, the steady-state level of pol γ-α mRNA decreases as development proceeds and is low in stages of active mtDNA replication. This difference in mRNA abundance results at least in part from differences in the rates of mRNA synthesis. The pol γ genes are located in a compact cluster of five genes that contains three promoter regions (P1–P3). The P1 region directs divergent transcription of the pol γ-β gene and the adjacent *rpII33* gene. P1 contains a DNA replication-related element (DRE) that is essential for pol γ-β promoter activity, but not for *rpII33* promoter activity in Schneider’s cells. A second divergent promoter region (P2) controls the expression of the *orc5* and *sop2* genes. The P2 region contains two DREs that are essential for *orc5* promoter activity, but not for *sop2* promoter activity. The expression of the pol γ-α gene is directed by P3, a weak promoter that does not contain DREs. Electrophoretic mobility shift experiments demonstrate that the DRE-binding factor (DREF) regulatory protein binds to the DREs in P1 and P2. DREF regulates the expression of several genes encoding key factors involved in nuclear DNA replication. Its role in controlling the expression of the pol γ-β and *orc5* genes establishes a common regulatory mechanism linking nuclear and mitochondrial DNA replication. Overall, our results suggest that the accessory subunit of mtDNA polymerase plays an important role in the control of mtDNA replication in *Drosophila*.

Animal mitochondrial DNAs, with few exceptions, encode 13 subunits of the respiratory complexes I and III–V located in the inner mitochondrial membrane. The remainder of the –1000 protein; PCR, polymerase chain reaction; bp, base pair(s); EWG, Erect Wing; EMSA, electrophoretic mobility shift assay; DRE, DNA replication-related element; AEL, after egg laying; UTR, untranslated region; DREF, DRE-binding factor.

1 The abbreviations used are: NRF1, nuclear respiratory factor; pol γ, DNA polymerase; mtSSB, mitochondrial single-stranded DNA-binding protein; PCR, polymerase chain reaction; bp, base pair(s); EWG, Erect Wing; EMSA, electrophoretic mobility shift assay; DRE, DNA replication-related element; AEL, after egg laying; UTR, untranslated region; DREF, DRE-binding factor.
toskeleton plays an active role in the mitochondrial dynamics of the cell (for a review, see Ref. 15) and that the replication of the mitochondrial genome occurs in the perinuclear space (16). The key enzyme in mtDNA replication is DNA polymerase \( \gamma \) (pol \( \gamma \)), which constitutes only 1% of the total cellular DNA polymerase activity (17, 18). The pol \( \gamma \) holoenzyme has been characterized extensively in *Drosophila* (19); it is a heterodimer of a 125-kDa \( \alpha \) subunit containing DNA polymerase and a 3–5’ exonuclease catalytic activities (20) and a 35-kDa accessory subunit that increases the catalytic efficiency of the holoenzyme and is likely involved in primer recognition and in enhancing processivity (21, 22). Both subunits have also been identified in other systems, and their structures are evolutionarily conserved. The catalytic subunit shares homology with the family A DNA polymerases (20, 23, 24), whereas the accessory subunit is related to aminocyl-tRNA synthetases (22, 25).

The key enzyme in mtDNA replication is DNA polymerase \( \gamma \) (pol \( \gamma \)), which is expressed constitutively at a low level, the developmental pattern of expression of the pol \( \gamma \) subunits is related to aminoacyl-tRNA synthetases (22, 25). the family A DNA polymerases (20, 23, 24), whereas the accessory subunit is also critical for the activity of the mitochondrial single-stranded DNA-binding protein (mtSSB) promoter (30), establishing firmly a molecular link between nuclear and mitochondrial DNA replication.

**EXPERIMENTAL PROCEDURES**

**RNA Extraction and Northern Analysis**

Total RNAs from staged embryos, first-, second-, and third-instar larvae; early and late pupae; and adults of *Drosophila melanogaster* Oregon R were extracted using the Trizol kit (Life Technologies, Inc.) according to the manufacturer’s directions. For Northern analysis, 30 \( \mu \)g of total RNA was electrophoresed on 1.8 % formaldehyde and 1.2 % agarose gels, blotted on a Zeta Probe membrane (Bio-Rad), and probed in ZAP buffer (0.25 % sialic acid buffer, pH 7.2, 7% SDS) at 65 °C using various \([\alpha^{32}P]dCTP\)-labeled cDNA clones as probes. Filters were washed in 0.1% SDS and 0.2 x SSC at 65 °C and autoradiographed with intensifying screens at -70 °C.

**Nuclear Run-on Transcription Analysis**

To obtain nuclei from *Drosophila* adults, flies (100 in a typical experiment) were homogenized in 1 ml of buffer containing 10 mM KCl, 15 mM HEPES (pH 7.6), 5 mM MgCl\(_2\), 0.35 mM succrose, 0.5 mM EDTA, 0.1 mM EDTA, 1 mM diethiothreitol, and 10 mM phenylmethylsulfonyl fluoride. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 400 \( \times \) g for 5 min. The supernatant was centrifuged at 700 \( \times \) g for 10 min, and the nuclear pellet was resuspended in buffer containing 50 mM Tris-HCl (pH 8.3), 5 mM MgCl\(_2\), 0.1 mM EDTA, and 40% glycerol. Embryos were dechorionated by sodium hypochlorite treatment and washed extensively before homogenization. Transcriptional run-on reactions were performed essentially as described by Linial et al. (31) using 150 \( \mu \)Ci of \([\alpha^{32}P]UTP\) and \( 10^{-10} \) nuclei. Radiolabeled RNA was purified by phenol/chloroform extraction followed by precipitation with isopropanol alcohol and hybridized in ZAP buffer at 65 °C to 5 \( \mu \)g of 18 S rDNA and pol \( \gamma \) and pol \( \gamma \)-encoded cDNAs that were immobilized on nylon filters using the Manifold II system (Schleicher & Schull) and denatured by NaOH treatment. Filters were washed in 0.1% SDS and 0.2 x SSC at 65 °C and autoradiographed with intensifying screens at -70 °C.

**Promoter Constructs**

DNA fragments containing the promoter regions (P1–P3) of the five genes were amplified by PCR using several sets of oligonucleotide primers (Table I). The resulting parental DNA fragments were used as substrates for generation of deletion constructs. After digestion with restriction endonucleases, DNA fragments were excised from agarose gels and inserted into the pxp2 vector, which contains the luciferase gene as reporter. The nucleotide sequences of the parental DNA fragments and each of the promoter constructs were confirmed by DNA sequence analysis.

**pol α-γ Promoter (P3)—**Deletions of the parental DNA fragment were generated as follows. *EcoRI*/*XhoI* and *XmnI*/*XhoI* fragments were cloned into the pxp2 vector that was digested with *Smal*I to generate constructs a–1710/+113 and a–1197/+113 (where +1 corresponds to the transcriptional start site). The *XmnI*/*XhoI* fragment was also digested with DraI, and the *XmnI*/*DraI* fragment was cloned into pxp2 that was digested with *SmaI* to generate construct a–1197/+183. To obtain construct a–407/+113, a 520-bp *BglII*/*XhoI* fragment was cloned into pxp2 that was digested with *BglII*/*XhoI* to mutate the putative NRF1-like binding site in P3. We used the oligonucleotide 5’-GCCAGCTGATTTTTTTGATGCCCGC3’ (from positions +42 to +8 in the template strand for transcription); the lowercase letters represent the nucleotides that were changed from the original sequence (5’-GCCAGCTGATTTTTTTGATGCCCGC3’), and those that are underlined represent the mutated NRF1 site. After several steps of subcloning, PCR, and additional subcloning, construct a–407/+113mut was obtained. This plasmid is identical to its parental construct, a–407/+113, except for the six nucleotides changes within the NRF1-like site.

**rpH331/pol γ-β Promoter (P1)—**Deletions in the parental PCR fragment were generated as follows. *HindIII*/*XhoI* and *PvuII*/*XhoI* fragments were cloned into the pxp2 vector that was digested with *HindIII*/*XhoI* and *Smal*I, respectively, to generate constructs b–1806/+467 and b–1351/+467. To construct b–589/+467 and b–212/+467, the parental DNA fragment was digested with *PstI* and then with *Clal*, rendered blunt-ended with *T4* DNA polymerase, and cut with *XhoI*. Purified fragments were then ligated into the pxp2 vector that was digested with *Smal*I to generate construct b–467/+113mut. A 797-bp DNA fragment from positions –326 to +473 was also amplified by PCR using two oligonucleotides as primers: the same reverse primer described above (Table I) and a new forward primer (5’-GACCTGACATTGGTGTTGATGAA-3’) containing a *PstI* site at position –318. The amplified product was digested with *PstI* and cloned into the *PvuII* vector that was digested with the same restriction enzymes, generating pBS-PI. The construct was then digested with *ClaI*, end-filled with *Klenow* DNA polymerase; and religated to obtain pBS-Plmut, in which the DREF-binding site is changed from TATCGATT to TATCgGATT. The *BamHI*/*XhoI* fragments of pBS-PI and pBS-Plmut were cloned into pxp2 that was digested with *BamHI*/*XhoI* to generate the β–318/+467 and β–318/+467mut constructs, respectively.

**Promoter (P1) Deletions of the parental DNA fragment were cloned into pxp2 vector that was digested with *HindIII*/*XhoI* and *Smal*I, respectively, to generate constructs b–1806/+467 and b–1351/+467. To construct b–589/+467 and b–212/+467, the parental DNA fragment was digested with *PstI* and then with *Clal*, rendered blunt-ended with *T4* DNA polymerase, and cut with *XhoI*. Purified fragments were then ligated into the pxp2 vector that was digested with *Smal*I to generate construct b–467/+113mut. A 797-bp DNA fragment from positions –326 to +473 was also amplified by PCR using two oligonucleotides as primers: the same reverse primer described above (Table I) and a new forward primer (5’-GACCTGACATTGGTGTTGATGAA-3’) containing a *PstI* site at position –318. The amplified product was digested with *PstI* and cloned into the *PvuII* vector that was digested with the same restriction enzymes, generating pBS-PI. The construct was then digested with *ClaI*, end-filled with *Klenow* DNA polymerase; and religated to obtain pBS-Plmut, in which the DREF-binding site is changed from TATCGATT to TATCgGATT. The *BamHI*/*XhoI* fragments of pBS-PI and pBS-Plmut were cloned into pxp2 that was digested with *BamHI*/*XhoI* to generate constructs b–318/+71 and b–318/+71mut, with the inverse orientation of the DNA fragments. These constructs are numbered with respect to the transcriptional start site (t + 1) of the reference genome.

The *orc5*/*sop2* Promoter (P2)—Deletions of P2 were generated by digestion with restriction endonucleases, DNA fragments were excised from agarose gels and inserted into the pxp2 vector, which contains the luciferase gene as reporter. The nucleotide sequences of the parental DNA fragments and each of the promoter constructs were confirmed by DNA sequence analysis. The *HindIII*/*XhoI* and *SalI*/*XhoI* fragments were cloned into pxp2 that was digested with *BamHI*/*XhoI* to generate the β–318/+467 and β–318/+467mut constructs, respectively.

| Promoter region | Positions | Forward oligonucleotide (5’→3’) | Reverse oligonucleotide (5’→3’) |
|-----------------|-----------|--------------------------------|-------------------------------|
| P1              | −2270/+473| ACGTACATGAGATTGCGACG          | TGGTCGAGCGGAAAGTGGTCGGCCG    |
| P2              | −1855/+316| AAGGAGTGCAGCTGATTGCCC         | GGCCCTCGAGGGTCCTAATGCG       |
| P3              | −2013/+119| AACATCGTCAGACGCACTGG          | TCCCTAGGTTAATAGGAAACAT       |

**TABLE I**

Oligonucleotide primers used in this study

+1 represents the transcriptional start site of pol \( \gamma \)- and orc5, and pol \( \gamma \)-α, respectively. The *XhoI* restriction sites included in the reverse oligonucleotides are underlined. Their positions are according to +1.
Drosophila pol γ Gene Expression

Fig. 1. Schematic representation of the Drosophila pol γ gene cluster. The structure and direction of transcription of the five genes in the pol γ cluster are shown schematically. Thick arrows represent the coding regions, and thin arrows indicate the transcriptional start sites. Stippled boxes represent 5'- and 3'-untranslated regions, and open boxes represent introns. P1–P3 correspond to the three promoter regions that direct transcription within the cluster. KB, kilobases.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) were carried out using protein prepared from two sources: the in vitro TNT transcription/translation system (Promega) for DREF binding assays and Schneider’s cell nuclear extracts for DREF and NRF1 binding assays. The binding reactions were carried out using 2 μl of in vitro transcription and translation product or 5 μg of nuclear protein and 50,000 cpm of the DNA probe in binding buffer containing 20% glycerol, 1 mM dithiothreitol, 20 mM HEPES (pH 9.0), 5 mM MgCl₂, 0.2 mM EDTA, and 200 mM KCl. After incubation for 30 min at 4 °C, reaction products were electrophoresed on 4.5% polyacrylamide gels (for PCR probes) or 6% polyacrylamide gels (for oligonucleotide probes) containing 0.5x Tris borate/EDTA. DNA probes were labeled using [γ-³²P]ATP and T4 polynucleotide kinase. For the P1 probes, two DNA fragments containing the DRE were used as probes: a double-stranded 30-mer oligonucleotide from positions −225 to −201 and a 129-bp fragment from positions −325 to −153 that was obtained by PCR amplification (where +1 corresponds to the transcriptional start site for pol γ-β). For the P2 probe, a 120-bp DNA fragment from positions −132 to −13 containing the DREs was amplified by PCR (where +1 corresponds to the transcriptional start site for sop2). In DREF EMSA experiments, a 100-fold molar excess of various oligonucleotides was used as competitor, including unlabeled DNA probes and oligonucleotides from the mtSSB promoter containing wild-type or mutated DRE sites (30). For the P3 probe, a double-stranded 35-mer oligonucleotide from positions +42 to +8 containing the NFR1-like site and its mutated version (described above) was used. DREF antiserum was prepared as described (30). Antiserum against cEG was kindly provided by Dr. Kalpana White. In supershift assays, 2 μl of a 1:100 dilution of the relevant antiserum was used.

RESULTS

Differential Expression of the Genes Encoding the pol γ Catalytic and Accessory Subunits during D. melanogaster Development—The genes encoding the two subunits of the mitochondrial DNA polymerase map within a cluster of genes, the pol γ cluster, that extends over a region of 10.5 kilobases in the alcohol dehydrogenase region (34D–E) of the second chromosome of D. melanogaster (27, 28). The cluster contains five genes that encode proteins involved in essential cellular processes, including the two pol γ subunits (pol γ-α and pol γ-β), one subunit of RNA polymerase II (RP1133), one subunit of the origin recognition complex (ORC5), and one subunit of the actin-related protein 2/3 complex (SOP2/ARC41). The pol γ cluster is extremely compact: the genes are tightly packed with very little intergenic space, with some overlap in their 5’- and 3’-ends (Fig. 1). The cluster contains three promoter regions (P1–P3), and two of them direct bidirectional transcription (28). This tight organization suggests that the structure of the pol γ cluster plays an important role in regulating the expression of its genes. This is a particularly important consideration given that two of the genes encode the subunits of the key enzyme in the mitochondrial DNA replication process (pol γ-α and pol γ-β), representing a rare example in eukaryotes of genes encoding functionally related proteins that are linked in the genome. Moreover, a third gene of the pol γ cluster, orc5, encodes an essential component of the nuclear DNA replication machinery, suggesting the presence of potential common regulatory
mechanisms for nuclear and mitochondrial DNA replication.

As a first step to study the factors involved in regulating the expression of the pol γ genes, we determined (by Northern analysis) their patterns of expression during Drosophila development and compared them with the expression profiles of the other genes encoded in the pol γ cluster. The level of pol γα mRNA was relatively high in eggs and decreased rapidly during embryonic development (Fig. 2A). This result is perhaps surprising because mtDNA replication started ~10 h after egg laying (AEL); and at this developmental stage, the level of pol γα mRNA has declined substantially. During the larval stages, a period characterized by increases in body weight and cell ploidy, the steady-state level of pol γα mRNA remained very low, whereas that in adults was comparatively higher. The spatiotemporal pattern of expression of pol γα mRNA, examined by whole-mount RNA in situ hybridization, is in good agreement with the Northern results. pol γα mRNA was distributed homogeneously in early blastoderm, and the intensity of the signal decreased through gastrulation and was very faint or undetectable in older embryos (data not shown).

pol γβ mRNA showed a different developmental profile (Fig. 2A). The level present in eggs was relatively low, but its steady-state level increased in early embryonic stages, reaching its maximum between 6 and 12 h AEL. In late embryos, its steady-state level decreased; and in first-instar larvae, there was a moderate increase. These results suggest that the expression of the pol γβ subunit is regulated precisely during Drosophila development. Moreover, the steady-state level of pol γβ reached its maximum just before the start of mtDNA synthesis that occurred 10–12 h AEL.

To determine whether the differences observed in the pattern of expression of pol γα and pol γβ mRNAs are due to different rates of transcription, we performed a series of run-on transcription experiments using nuclei extracted from 8–10-h AEL embryos and adults. A higher rate of mRNA synthesis was detected in the pol γβ gene in both embryos and adults (Fig. 3). Although we have not evaluated possible differences in mRNA stability, the different rates of mRNA synthesis correspond well with the mRNA steady-state levels detected by Northern analysis (Fig. 2A), documenting a differential regulation of the pol γα and pol γβ genes at the transcriptional level.

Unfortunately, we were unable to detect pol γβ mRNA by in situ hybridization despite systematic attempts using a variety of experimental conditions (data not shown). A lack of sensitivity of the technique is an unlikely explanation because Northern analysis showed that the steady-state level of pol γβ mRNA was similar or higher than that of pol γα mRNA. We have also used in parallel several control probes that detect other unrelated mRNAs, obtaining in each case the expected pattern. Although the reason for the lack of in situ detection of pol γβ mRNA is presently unknown, our data suggest the possibility of some type of sequestration of pol γβ mRNA that does not occur with pol γα mRNA.

The rpl33 gene is divergently transcribed from the same promoter as the pol γβ gene, yet its developmental pattern of expression is entirely different (Fig. 2B). rpl33 mRNA was accumulated in eggs, and its level was maintained fairly con-

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**Fig. 2. Developmental pattern of expression of the five genes encoded in the Drosophila pol γ cluster.** Total RNAs from 0–18-h embryos, larvae (first-, second-, and third-instar (L1–L3, respectively)), pupae (P1–P3), and adults (A) of D. melanogaster were analyzed by Northern blotting using the corresponding cDNAs as probes. RNAs were fractionated on formaldehyde-containing 1.2% agarose gels, transferred to nylon membrane, and hybridized with the corresponding radiolabeled probes, and the blots were autoradiographed. Levels of RNA loaded were evaluated by ethidium bromide (EthBr) staining. The expression patterns of the pol γ genes are shown in A, and those of rpl33, sop2, and orc5 are shown in B.

**Fig. 3. Nuclear run-on transcription analysis of the pol γα and pol γβ genes.** The relative rates of transcription of the pol γα and pol γβ genes were evaluated in run-on experiments as described under “Experimental Procedures.” A, nuclei obtained from adults and 8–10-h AEL embryos were labeled with [α-32P]UTP. The RNA synthesized de novo was hybridized to 5 μg of 18 S rDNA (as a control), pol γα cDNA, and pol γβ cDNA immobilized on a nylon membrane. B, shown is the densitometric quantitation of the data obtained in four independent experiments. Values are expressed in arbitrary units.
Drosophila pol γ Gene Expression

FIG. 4. Functional analysis of the P1 region. A schematic representation of the divergent P1 region and the adjacent pol γ-β and rpII33 genes is shown in the middle. Thick arrows represent the protein coding regions; stippled boxes represent 5′-untranslated regions; and open boxes represent introns. Transcriptional initiation sites (Δ+1) are indicated by thin arrows, and the positions of translational initiation sites are indicated as ATG with a corresponding number that indicates the length of the 5′-untranslated region. The DRE sequence is represented as an ellipse. DNA fragments cloned upstream of the luciferase reporter gene and used in the analysis of the pol γ-β promoter activity are shown above the schematic, and those used in the analysis of the rpII33 promoter activity are shown below. Plasmid designations indicate the specific DNA sequences present in the constructs, with numbers relative to the position of the transcriptional start site. The internal X in the DRE sequence indicates that the site is mutated. The relative promoter activities of the constructs, as measured in the luciferase assay, are indicated to the right. The luciferase activity of the pxp2 vector was defined as 1.0 and was used as the standard for comparison. Luciferase activity was normalized to β-galactosidase activity for each construct. Values are means ± S.D. of at least three independent experiments. Statistical analysis was carried out using the Wilcoxon signed-rank test. * indicates p < 0.05.

constant during early embryogenesis up to 6 h AEL. From 9 h AEL onward, its steady-state level decreased and was very low during the larval and pupal periods. Adults also contained a relatively low amount of the rpII33 transcript. A similar result was observed in the case of the orc5/sop2 genes, and the pattern of expression was also different for each gene (Fig. 2B). sop2 mRNA was present at relatively high levels in all developmental stages, whereas the steady-state level of orc5 mRNA was very high in eggs and decreased sharply as development proceeded. In larvae and pupae, its concentration was low and increased again in adults. This pattern is generally similar to that for pol γ. Our results indicate clearly that the pairs of genes transcribed from the bidirectional promoters P1 and P2 are differentially regulated during development.

Analysis of the rpII33/pol γ-β Promoter Region (P1)—The developmental pattern of expression of the pol γ-β gene suggests that the accessory subunit of the pol γ enzyme may play an important role in regulating mtDNA replication. The pol γ-β gene is divergently transcribed from the rpII33 gene, and their 5′-ends are separated by only 245 bp (Fig. 1). To identify and characterize transcriptional regulatory elements in the rpII33/pol γ-β intergenic promoter region (P1), we cloned defined DNA fragments in the pxp2 vector and determined their promoter activities in transient transfection experiments in Schneider’s S2 cells, measuring luciferase activity in cell extracts (see “Experimental Procedures”).

A construct containing the 5′-UTR and 1.8 kilobases of the proximal 5′-upstream region of the pol γ-β gene (β-1806/+467, where +1 corresponds to the pol γ-β transcriptional start site), which includes the complete adjacent rpII33 gene, directed a substantial level of luciferase activity in Schneider’s cells, >1000-fold as compared with the pxp2 vector (Fig. 4). A similar level of activity was maintained in shorter constructs, including β-318/+467, which contains the rpII33/pol γ-β intergenic region and the 5′-UTRs of both genes. Computer analysis of the DNA sequence spanning positions −318 to +467 revealed the presence of a DRE motif (TATCGATT) located at position −216 within the rpII33/pol γ-β intergenic region, close (−30 bp) to the transcriptional initiation site of the rpII33 gene. The DRE is recognized by DREF, a transcription factor critical in the regulated expression of several genes encoding proteins involved in nuclear DNA replication (29). We have also demonstrated recently that it is essential for the activity of the D. melanogaster mtSSB promoter (30). Mutation of the DRE-binding site reduced the activity of construct β-318/+467 by 80% (p < 0.05), suggesting strongly that DREF is critical to
achieve full pol γ-β promoter activity. Furthermore, construct β-212/+417, with the DRE site eliminated, abolished promoter activity in Schneider’s cells.

To determine whether the DRE sequence is also important for the activity of the rpII33 promoter, we determined the luciferase activity of the b2318/1467 fragment cloned in the pxp2 vector in the opposite orientation (rpII2715/171). An 800-fold induction in luciferase activity was detected, indicating that the rpII33/pol γ-b intergenic region divergently directs the transcription of the two genes. Importantly, when the assay was carried out with a construct containing the same DNA fragment with the DRE site mutated (rpII2715/171mut), no significant change in luciferase activity was observed. This result demonstrates that the DRE site located in the P1 region is essential only for the promoter activity of the pol γ-b gene.

Analysis of the orc5/sop2 Promoter Region (P2)—Two of the genes in the pol γ cluster, orc5 and sop2, overlap in their 5'-ends (Fig. 1). We found that a DNA fragment encompassing the 5'-upstream region of the two genes contains potent bidirectional promoter activity. Construct sop-1616/+307, which contains the sop2 5'-UTR and most of the orc5 gene, directed a high level of luciferase activity, >9000-fold as compared with the pxp2 vector. A high level of activity was maintained in the sop-303/+307 construct, which contains the complete 5'-UTRs of the two overlapping genes. The 5'-UTR of the orc5 gene contains two DRE sites. To examine the functional relevance of the DRE sites in sop2 promoter activity, we mutated both sites in construct sop-303/+307mut. We observed no change in the level of luciferase activity, indicating that the DRE sites do not play an essential role in the sop2 promoter activity in Schneider’s cells.

To evaluate the orc5 promoter, we assayed the activity of the orc2170/1346 construct and observed a high increase in luciferase activity, >8000-fold as compared with the pxp2 vector. The same region containing mutated DRE sites (orc-170/+346mut) directed significantly reduced activity, only 30% of that detected in the orc-170/+346 construct (p < 0.05). Thus, similar to the results obtained in the bidirectional rpII33/pol γ-β promoter region, the DRE sites located in the 5'-UTR of the orc5 gene are essential for the activity of one promoter (orc5), but have no effect on the activity of the other (sop2). More importantly, these data establish a common molecular mechanism involved in the regulated expression of two genes in the pol γ complex that are involved in nuclear and mitochondrial DNA replication, orc5 and pol γ-β, respectively.

DREF Binds to the pol γ-β and orc5 Promoter Regions—To characterize further the DRE sequences in P1 and P2, we carried out electrophoretic mobility shift assays using probes covering the P1 and P2 regions that contain the DRE sites. We used either DREF produced by in vitro transcription/translation or nuclear extracts prepared from cultured Schneider’s cells as the protein source. When a radiolabeled 173-bp frag-
ment containing the single DRE site present in the P1 region (P1-DRE) was incubated with recombinant DREF protein, a single retarded protein-DNA complex was detected, which was eliminated when a 100-fold molar excess of unlabeled probe was included in the binding reaction (Fig. 6A). Inclusion of rabbit anti-DREF serum produced a clear supershift, demonstrating that DREF interacts with the DRE site in the pol γ-β promoter region. The supershift was also eliminated by inclusion of a 100-fold molar excess of unlabeled probe. When a similar experiment was carried out in which a 120-bp fragment containing the two DRE sites present in the P2 region (P2-DRE) was used as probe, similar results were obtained (Fig. 6B). In this case, two retarded protein-DNA complexes were apparent, and both were supershifted by the anti-DREF antiserum. As a control, we included preimmune serum in the binding reaction, and no supershift was produced. The same EMSA patterns were observed with both promoter fragments when the source of DREF was a nuclear extract prepared from Schneider’s cells (Fig. 6, A and B).

Additional EMSA experiments were carried out with Schneider’s cell nuclear extracts and functional DRE sequences that were characterized previously in the Drosophila mtSSB promoter as competitors (30). Here the probe for P1 was a double-stranded 30-mer oligonucleotide spanning positions −225 to −204 (Fig. 7A), and that for P2 was as described above (Fig. 7B). Both probes gave rise to shifted bands, and inclusion of anti-DREF antiserum in the binding reaction produced supershifts not observed upon inclusion of preimmune serum. When competition of DREF binding to P1 and the corresponding anti-DREF antiserum-mediated supershift was carried out using a 100-fold molar excess of unlabeled P1, P2, or ssb (a double-stranded oligonucleotide containing the two functional DRE sites in the mtSSB promoter (30)) DNA, the retarded bands and supershifts were abolished. However, if the competition was performed with ssb DNA (a double-stranded oligonucleotide containing mutated DRE sites from the ssb promoter), neither the retarded bands nor the supershifts were eliminated, providing further support for the binding of DREF to P1. Similar results were obtained with DREF binding to P2. A 100-fold molar excess of P2 or ssb DNA eliminated the retarded bands and supershifts, but competition with ssb DNA had no effect. Notably, however, only partial competition of DREF binding to the P2 region was observed with P1 DNA, likely due to the fact that P1 contains a single DRE site, whereas P2 contains two. Taken together, the EMSA experiments demonstrate that the DRE sites present in the pol γ-β and orc5 promoters are bound specifically by DREF.

**Analysis of the pol γ-a Promoter Region (P3)**—A construct (α−1710/+113) containing the complete coding sequence of the sop2 gene, the −140-bp sop2/pol γ-a intergenic region, and 119 bp of the 5′-UTR of the pol γ-a gene directed 300-fold higher luciferase activity than the pxp2 vector (Fig. 8). Constructs containing shorter DNA fragments maintained a similar level of activity, including construct α−407/+113, which contains only the sop2/pol γ-a intergenic region and the pol γ-a 5′-UTR. Luciferase activity was abolished in construct α−1197−183, which has the transcriptional start site eliminated. These data indicate that the proximal 5′-upstream region of the pol γ-a gene has weak but significant promoter activity in Schneider’s cells.

The proximal 5′-upstream region of the pol γ-a gene does not contain DNA sequence motifs with significant homology to the DRE site. However, its 5′-UTR contains a proximal binding motif potentially recognized by the transcription factor NRF1.
To evaluate its functional relevance, we mutated the NRF1-like site. We observed a moderate but significant decrease (50%; \( p < 0.01 \)) in the promoter activity of construct \( a2_{113}^{407} \) compared with the native construct \( a2_{113}^{407} \), suggesting that the NRF1-like site might be functional in Schneider’s cells.

NRF1 likely plays a key role in nucleo-mitochondrial communications in mammals (12). Interestingly, its DNA-binding motif is conserved in two regulatory proteins identified in Drosophila (Erect Wing) and sea urchin (P3A2). To study the binding of putative regulatory proteins to the NRF1-like site, we pursued EMSA experiments using, as probes, a double-stranded 35-mer oligonucleotide spanning positions +142 to +18 from the P3 region that contains the NRF1-like site (NRF1\(^{1}\)) and a similar oligonucleotide containing a mutated NRF1-like site (NRF1\(^{2}\)). When Schneider’s cell nuclear extracts were incubated with the NRF1\(^{1}\) probe, several retarded bands were observed that were eliminated specifically by a 250-fold molar excess of unlabeled NRF1\(^{1}\) oligonucleotide as competitor (Fig. 9, left panel). Inclusion of anti-EWG antiserum in the binding reaction did not produce a supershift, suggesting that the EWG protein is not present in the retarded complexes. Competition with a 250-fold molar excess of the NRF1\(^{2}\) oligonucleotide eliminated the retarded fragments, indicating that the protein bound to the probe likely interacts with sequence elements other than the NRF1-like site. Furthermore, when the NRF1\(^{1}\) oligonucleotide was used as probe, several retarded bands were clearly visible, including an extra band not observed in the EMSAs with NRF1\(^{1}\) (Fig. 9, right panel). Whereas an excess of the NRF1\(^{1}\) probe competed completely, only the shared retarded complexes were eliminated by a 250-fold excess of the NRF1\(^{1}\) oligonucleotide. These results suggest that there is protein binding to NRF1\(^{1}\) that is mediated specifically through the mutant sequence, possibly explaining the inhibition of promoter activity detected in transient transfection experiments with the mutant NRF1-like construct (Fig. 8). This protein is not EWG because no supershift was observed in the EMSAs in the presence of anti-EWG antiserum. Overall, the data suggest that neither the EWG protein nor the native NRF1-like site per se is likely involved in the control of pol \( \gamma - \alpha \) promoter activity. Consistent with this interpretation, overexpression of EWG in Schneider’s cells had no effect on the activity of the pol \( \gamma - \alpha \) constructs examined in transient transfection assays (data not shown).

DISCUSSION

Eggs are highly enriched in mitochondria that are deposited by the mother during oogenesis. As development proceeds, important changes in the morphological and functional maturation of mitochondria occur (3) in a process that requires a careful orchestration of nucleo-mitochondrial interactions. Evidence from studies in different systems including mammals shows that the mitochondria stored in the egg are sufficient for the first stages of embryonic development (35). Later on, mitochondria are distributed throughout the various embryonic territories, which exhibit specific patterns of organelle differentiation (36). In Drosophila, the developmental pattern of expression of several genes encoding mitochondrial proteins, including the nuclearly encoded \( \alpha \)- and \( \beta \-H^+\)-ATP synthase subunits and the mitochondrially encoded ATPase-6 and -8 subunits, has been analyzed (37, 38). In each of these cases,
gene expression is activated coordinately 6–12 h AEL, suggesting that during this period, the maternal contribution becomes limiting, and there is an activation of mitochondrial biogenesis in the embryo. Accordingly, it has been shown that mtDNA replication and likely mitochondrial proliferation start around this time of development (39).

In this work, we have evaluated the expression of the genes encoded in the pol \( \gamma \alpha \) cluster during Drosophila development and, in particular, those encoding the two subunits of the pol \( \gamma \alpha \) holoenzyme. Both pol \( \gamma \alpha \) and pol \( \gamma \beta \) mRNAs are stored in the egg, probably as a result of the high level of mtDNA replication occurring during oogenesis. The steady-state level of pol \( \gamma \alpha \) mRNA declines rapidly during development, reaching a minimum level 10 h AEL and onward. In contrast, the pol \( \gamma \beta \) mRNA level increases during the first hours of development and reaches its maximum level 6–10 h AEL. Nuclear run-on transcription analyses indicate that the expression of the genes encoding the two pol \( \gamma \) subunits is regulated differentially at the transcriptional level during Drosophila development. Notably, only the pol \( \gamma \beta \) mRNA level is high in the period of active mtDNA replication. This might seem surprising because the pol \( \gamma \alpha \) subunit contains the catalytic activities of the enzyme. However, the lack of correlation between the pol \( \gamma \alpha \) mRNA level and mtDNA replication during Drosophila development. Notably, only the pol \( \gamma \beta \) mRNA level is high in the period of active mtDNA replication. This might seem surprising because the pol \( \gamma \alpha \) subunit contains the catalytic activities of the enzyme. However, the lack of correlation between the pol \( \gamma \alpha \) mRNA level and mtDNA replication during Drosophila development.

Fig. 8. Functional analysis of the P3 region. A schematic representation of the P3 region, the adjacent sop2 gene, and the 5’-region of the pol \( \gamma \alpha \) gene is shown. The NRF1-like sequence element is shown as an ellipse. DNA fragments cloned upstream of the luciferase reporter gene and used in the analysis of the pol \( \gamma \alpha \) promoter activity are shown below. The relative promoter activities of the constructs, as measured in the luciferase assay, are indicated to the right. The luciferase activity of the pxp2 vector was defined as 1.0 and was used as the standard for comparison. Luciferase activity was normalized to \( \beta \)-galactosidase activity for each construct. Values are means ± S.D. of at least three independent experiments. Statistical analysis was carried out using the Wilcoxon signed-rank test. * indicates \( p < 0.01 \).

Fig. 9. Regulatory protein binding in the P3 region. Protein binding to the P3 region was examined by EMSAs. The probes were a double-stranded 35-mer oligonucleotide containing the NRF1-like site (NRF1\(^{-}\)) and a derivative in which the NRF1-like site was mutated (NRF1\(^{+}\)). Arrowheads indicate the positions of shared retarded bands, and the arrow indicates the position of the retarded band produced only with the NRF1\(^{-}\) probe. The competitors used were a 250-fold molar excess of the unlabeled NRF1\(^{-}\) (1) and NRF1\(^{+}\) (2) probes as indicated. Ab, antibody.

gene expression is activated coordinately 6–12 h AEL, suggesting that during this period, the maternal contribution becomes limiting, and there is an activation of mitochondrial biogenesis in the embryo. Accordingly, it has been shown that mtDNA replication and likely mitochondrial proliferation start around this time of development (39).
of pol γ-β mRNA has not been determined in mammals, its developmental profile in Drosophila suggests that the pol γ-β subunit participates in the reactivation of mtDNA synthesis during development. Interestingly, the pol γ accessory subunit shares conserved protein domains with aminoacyl-tRNA synthetases (22, 25) and has been suggested to participate in primer recognition (22) and demonstrated to increase the template-primer binding affinity, catalytic efficiency, and processivity of the holoenzyme (21, 25, 42–44). Together, the transcriptional regulation shown here and the biochemical data regarding accessory subunit function in several animal systems including Drosophila support the conclusion that the accessory subunit of pol γ plays a key role in the control of mtDNA replication.

We have analyzed the promoter region of the pol γ-β gene and shown that its expression is controlled by DREF, a regulatory protein essential for the transcriptional regulation of a set of genes encoding proteins involved in nuclear DNA replication and cell cycle control in Drosophila. These include proliferating cell nuclear antigen (which is an auxiliary protein for nuclear replication accessory DNA polymerase δ), the 180- and 73-kDa subunits of nuclear initiator DNA polymerase α, cyclin E2F, and the proto-oncogene draf (45–49). Transient transfection assays and EMSA experiments demonstrate that the DRE site present in the pol γ-β promoter is functional in Schneider’s cells and that DREF binds to this site. Furthermore, DREF is expressed throughout Drosophila development, reaching its maximum level 2–8 h AEL (29), suggesting that it may contribute significantly to the expression of the pol γ-β gene in vivo. During the cell cycle, nuclear DNA synthesis is extremely well controlled through complex regulatory mechanisms that have been characterized extensively during the last few years (50). In contrast, although mtDNA content, maintenance, and partitioning are also strictly controlled during cell proliferation, very little is known about the molecular mechanisms involved in the regulation of these processes. With regard to the control of nuclear and mitochondrial DNA replication, it is notable that mutations in the RPN11/MPR1 gene of Saccharomyces cerevisiae, which encodes a subunit of the proteasome, produce a cell cycle arrest phenotype with over-replication of the nuclear and mitochondrial DNA genomes coupled with severe alterations in mitochondrial morphology (51). The results presented in this report, in addition to our recent finding that the expression of the mtSSB gene is also controlled by the DRE/DREF system (30), establish in molecular terms a link between mitochondrial and nuclear DNA replication.

Interestingly, the pol γ-β gene is transcribed from a promoter region that also directs the divergent transcription of the rpl133 gene, yet the DRE site is important only for the expression of the pol γ-β gene. Remarkably, a second promoter region in the pol γ complex that directs the divergent transcription of two genes that overlap in their 5'-ends, orc5 and sop2, contains two copies of the DRE motif. When both DRE sites are mutated, there is a substantial reduction in orc5 promoter activity, consistent with the essential role of the origin recognition complex in nuclear DNA replication. In contrast, the same mutation has no effect on sop2 promoter activity. Although we do not know at present the physiological significance of the juxtaposition of the pol γ-β and orc5 genes, it is clear that the DRE/DREF system provides an elegant mechanism to orchestrate nuclear and mitochondrial DNA replication during cell proliferation.

We have also delimited the promoter region of the gene encoding the pol γ catalytic subunit. This region does not contain DRE sites, suggesting that the expression of the pol γ-α gene may not be cell proliferation-dependent, consistent with its developmental pattern of expression. However, in its 5'-UTR, there is a DNA element potentially recognized by NRF1, a transcription factor that likely plays a key role in nucleo-mitochondrial communication in mammals (7, 8). Mutation of this site significantly reduces the activity of the pol γ-α promoter in Schneider’s cells, suggesting that the NRF1-like element could be involved in the transcriptional regulation of the gene. NRF1 belongs to a family of transcriptional regulatory proteins with a DNA-binding domain conserved in two transcription factors, P3A2 and EWG, which play an essential role in the development of the sea urchin and Drosophila, respectively (52, 53). Although the erect wing gene is expressed in all cell types, it is essential only in neurons and myoblasts (54); and thus, erect wing mutants exhibit neural and muscle defects. However, EMSA experiments do not support the involvement of the Erect Wing protein in the control of pol γ-α gene expression. Nonetheless, because our transient transfection data demonstrate that the proximal 5'-upstream region of the gene is critical for promoter activity, the identification and characterization of protein factors that bind to this region and that regulate the expression of the gene remain an avenue for future studies.

In summary, we have shown that the expression of the catalytic and accessory subunit genes of the Drosophila mitochondrial DNA polymerase is regulated by different mechanisms. In particular, the expression of the pol γ-β gene is regulated by the transcription factor DREF, thereby linking mitochondrial and nuclear DNA replication. Parallel studies on the biochemical roles of the accessory subunit and the developmental control of its synthesis should increase our understanding of the regulation of mtDNA replication under both physiological and pathological conditions.

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