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Structure and expression during development of *Drosophila melanogaster* gene for DNA polymerase α

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ABSTRACT

The *Drosophila melanogaster* gene and cDNA which span the entire open reading frame for DNA polymerase α, were cloned, and their nucleotide sequences were determined. The gene consists of 6 exons separated by 5 short introns. The major transcription initiation site was localized 85 bp upstream from the initiation codon. The nucleotide sequence of the open reading frame revealed a polypeptide of 1,505 amino acid residues with a molecular weight of 170,796. The amino acid sequence of the polypeptide was 37% homologous with that of the catalytic subunit of human DNA polymerase α. This sequence contains six regions, the orders and amino acid sequences of which are highly conserved among a number of other viral and eukaryotic DNA polymerases. We found 7 amino acid residues in the region between the 639th and 758th positions, identical to those essential for the active site of *Escherichia coli* DNA polymerase I-associated 3′→5′exonuclease. Thus, the exonuclease activity may be associated with *Drosophila* DNA polymerase α. Levels of the DNA polymerase α mRNA were high in unfertilized eggs and early embryos, relatively high in adult female flies and second-instar larva, and low in bodies at other stages of development. This feature of the expression is similar to that of the proliferating cell nuclear antigen (an auxiliary protein of DNA polymerase δ) and seems to coincide with the proportions of proliferating cells in various developmental stages. As the half life of the mRNA for DNA polymerase α in cultured *Drosophila* Kc cells was 15 min, expression of the DNA polymerase α gene is probably strictly regulated at the step of transcription.

INTRODUCTION

DNA polymerase α is an enzyme essential for replication of the eukaryotic chromosomal DNA and of some viral DNA's. A monoclonal antibody against human DNA polymerase α inhibited DNA replication of permeabilized cells in a dose-dependent manner (1). A mutant mouse cell (tsFT20) which carries DNA polymerase α with heat-labile activity was arrested at the G_1/S boundary or in the S phase at non-permissive temperature (2, 3). It has been demonstrated that DNA polymerase α, in association with primase mainly plays a role in synthesizing lagging strand DNA as well as for initiating at the replication origin in the *in vitro* systems which supported the SV40 DNA replication (4–9).

Wong *et al.* (10) have reported that the enzyme activity, steady-state mRNA level and translation rate of human DNA polymerase α were fairly constant throughout the cell cycle with only a modest elevation prior to the S phase in exponentially growing cells. However, quantities of the mRNA and protein for this enzyme increased after stimulation of quiescent cells with serum (11). Similar features of expression have been noted with the small subunit p49 of the primase from mouse cells (12). These observations suggest that expressions of genes for mammalian DNA replication enzymes correlate with proliferation rather than with the cell cycle. In contrast, expression of the yeast gene for DNA polymerase I (α) changes during the cell cycle, and the level of mRNA reached maximum at the G_1/S boundary (13–15). The specific nucleotide sequence (ACGCGT) commonly present in regulatory regions of many yeast DNA replication-related genes was thought to be responsible to cell cycle-dependent expression (16–21).

Using specific monoclonal antibodies, we found that expression of the DNA polymerase α and primase was low in quiescent cells in serum-depleted cultures of chicken fibroblasts, and was prominent after serum stimulation to introduce cells to proliferate (22). Furthermore, a rapid repression of DNA polymerase α expression occurred concomitantly with the onset of cell differentiations in chick embryonic tissues such as the lens and neural tube (23). These observations suggest that the expression of DNA polymerase α is regulated in correlation with differentiation as well as proliferation.

We isolated the *Drosophila* gene for DNA polymerase α and determined the nucleotide sequences including the entire coding

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region and the 5'-flanking region. Expression of this enzyme seems to be closely related to cell proliferation during development. Various amino acid sequences of this enzyme including a putative domain that may be responsible for the 3'→5' exonulease activity are discussed.

MATERIALS AND METHODS

Isolations of the Drosophila gene and cDNA for DNA polymerase α

A genomic library was constructed by inserting the partially Sau3AI-digested DNA from *Drosophila melanogaster* Oregon-R into the BamHI site of λEMBL3. Clones containing the DNA polymerase α gene sequence were first isolated by screening the library using as a probe the 994 bp SalI-HinfI fragment derived from the cDNA for the human DNA polymerase α. This human cDNA fragment contains the sequences encoding three oligopeptide domains (II, III, IV) which are highly conserved among a number of viral DNA polymerases and other α-type DNA polymerases (24, 10). Plaque hybridizations were carried out under conditions at 42°C in solution containing 20% formamide, 3×SSC, 5×Denhardt's solution, 10% dextran sulfate, 1% SDS, 1 mM sodium pyrophospate, 100 μg/ml yeast tRNA, 100 μg/ml heat denatured salmon sperm DNA and the probe, as described previously but with minor modifications (25). The probe was labeled with [α-^{32}P]dCTP using a random priming method (26).

DNAs from two positive clones were analyzed by restriction enzyme site-mapping and the partial nucleotide-sequencing. Since these clones (λDGDPA01 and λDGDPA02) contained only regions from the 3'-half of the gene, re-screening was performed using as a probe the 1.3 kb SalI-HindIII fragment (SalI site is present in the polylinker sequence of λEMBL3) derived from the 5'-region of λDGDPA02 under a highly stringent conditions of hybridization at 65°C in 1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml heat-denatured salmon sperm DNA and the probe as described (27). Four additional clones (λDGDPA03, λDGDPA07, λDGDPA09, λDGDPA11) were isolated by re-screening. The 36 kb DNA regions spanning the entire DNA polymerase α gene were subjected to structural analysis.

Two kinds of λgt10 cDNA libraries constructed from mRNA preparations of 0−3 h and 3−12 h old embryos were kindly provided by L.M.Kauvar (28). Using as a probe a 4.2 kb BamHI-EcoRI genomic fragment (see below) as a probe under a highly stringent conditions of hybridization, six independent positive clones were isolated from the cDNA library from 3−12 h embryos.

Restriction endonuclease site-mapping of genomic and cDNA clones and subcloning of restriction fragments

Two genomic clones (λDGDPA02 and λDGDPA11, see Fig. 2) were analyzed. Positions of sites for EcoRI, HindIII, BamHI, PstI and NcoI in these clones were determined. The 3.5 kb HindIII-EcoRI fragment, the 2.4 kb BamHI fragment and the 2.0 kb EcoRI-BamHI fragment derived from λDGDPA11 were independently subcloned into compatible restriction sites of Bluescript sk(−) (Stratagene). The 1.2 kb BamHI-HindIII fragment, the 2.3kb HindIII-EcoRI fragment, the 1.5 kb PstI fragment and the 4.2 kb EcoRI fragment derived from λDGDPA02 were also subcloned into compatible restriction sites of pUC19. A 0.7 kb NcoI-BamHI fragment was isolated from λDGDPA11, then after converting the NcoI site into SalI site by linker ligation, ligated with a 3.5 kb BamHI-EcoRI fragment derived from λDGDPA02. The resultant 4.2 kb SalI-EcoRI fragment was subcloned into Bluescript sk(−).

The cDNA fragments excised from all the isolated λgt10 clones were subcloned into EcoRI sites of Bluescript sk(−).

Southern and Northern hybridization analysis

DNA was extracted from adult flies (Canton S) as described elsewhere (29). Human and mouse DNAs were prepared from HeLa cells and mouse spleens, respectively. These DNAs were digested with restriction enzymes, separated by agarose gel electrophoresis and blotted onto a Gene Screen Plus membrane (New England Nuclear) using a VacuGene blotting apparatus (LKB). The blot was then, subjected to hybridization analysis, under the conditions described above.

Total RNA was extracted from *Drosophila* bodies at various stages of development or from Kc cells by the method of Chomczynski and Sacchi (30), with minor modifications. Frozen materials were manually ground into powder with a pestle in a ceramic mortar filled with liquid nitrogen, then RNA was extracted by mixing the frozen powder with 4 M guanidinium thiocyanate solution containing 0.3 M sodium acetate (pH 4.8), 5 mM dithiothreitol, 0.5% sodium lauryl sarcosinate. After further extraction with acid-phenol and chloroform, RNA was recovered from the aqueous phase. RNA was precipitated using iso-propanol, re-dissolved in RNase-free water, then ethanol-precipitated and stored at −20°C. Total RNA was electrophoretically separated in an agarose gel containing 2.2 M formaldehyde and then blotted onto a Gene Screen Plus membrane by a conventional capillary transfer method (27). The blot was sequentially hybridized with radioactive probes for DNA polymerase α, proliferating cell nuclear antigen (PCNA) (31), and actin 5C (32) at 42°C in a solution containing 50% formamide, 1 M NaCl, 1% SDS, 100 μg/ml heat denatured salmon sperm DNA, 100 μg/ml yeast tRNA.

To determine the half life of mRNAs for the DNA polymerase α and PCNA, RNA was extracted from Kc cells at various times after the addition of 5 μg/ml actinomycin D to cell cultures in M(3)BF medium supplemented with 2% fetal calf serum, and subjected to the Northern hybridization analysis. The relative amounts of mRNAs for the DNA polymerase α, PCNA and actin 5C were quantified by scanning the autoradiogram, using a Shimadzu film scanner.

DNA sequencing

Nested sets of subclones for the genomic and cDNA sequences were created in both orientations using *Escherichia coli* exonuclease III and S1 nuclease (33), and the nucleotide sequences were determined by the dideoxy-sequencing method (34) using a Sequenase kit supplied by United States Biochemical Co. When necessary, chemically synthesized oligonucleotides (17mer) with sequences derived from the gene or cDNA were used as sequencing primers.

Determination of transcription initiation site by RNase protection assay

A 330 bp HincII-PstI fragment and a 542 bp EcoRI-PvuII fragment containing a part of the exon 1 and the 5'-flanking region of the *Drosophila* DNA polymerase α gene were subcloned into the SmaI-PstI or EcoRI-SmaI sites of Bluescript sk(−), respectively. The plasmids were linearized at the XhoI or HindIII site, and transcribed to obtain RNA probes with the sequence

complementary to mRNA in the reaction containing T3 or T7 RNA polymerase and [^{32}P]UTP, as described by Melton *et al* (35). About 100 μg total RNA from embryos in hybridization solution containing 80% formamide, 0.5 M NaCl, 0.5 M PIPES (pH 6.4) was mixed with an excess amount of the ^{32}P-labeled probe RNA (5×10^5 cpm). Hybridization was carried out at 45°C for 16 h, and single-stranded RNA was digested with 40 μg/ml RNase A and 2 μg/ml RNase T1 37°C for 90 min. After precipitation with ethanol in the presence of 20 μg glycogen as a carrier, RNase-resistant fragments were electrophoretically resolved in a 6% polyacrylamide sequencing gel containing 8 M urea. HpaII fragments of pUC19 were ^{32}P-labeled with Klenow fragment and used for size markers.

Determination of DNA polymerase activities

Crude extracts were prepared by sonicating embryos at various stages of development in extraction solution containing 50 mM Tris-HCl (pH7.4), 10% glycerol, 0.1 mM EDTA, 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 1 μg/ml aprotinin and 0.1% Triton X-100. The extracts were centrifuged at 100,000 rpm for 1 h at 4°C in Beckman TLA100.1 rotor, and DNA polymerase activities in the resultant supernatants were measured.

The assay for DNA polymerase activity was performed as described elsewhere (22). A standard reaction mixture of a final volume of 25 μl consisted of 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 10 mM $MgCl_2$, 80 μg/ml activated calf thymus DNA, 20 mM KCl, 15% glycerol, 0.1 mM each of dATP, dGTP, dCTP and [^{3}H]dTTP (2,220 bec/mmol) and the extract. Incubation was for 30 min at 37°C. To distinguish the DNA polymerase α activity from activities of DNA polymerase β and δ, specific inhibitors, aphidicolin (an inhibitor of DNA polymerase α and δ) and butyl-phenyldeoxyguanosine triphosphate (BuPdGTP) (an inhibitor of DNA polymerase α) were added to some reactions, at final concentrations of 20 μg/ml and 50 μM, respectively (36, 37). Thus, the DNA polymerase α activity was defined as that sensitive to both inhibitors while DNA polymerase δ was that sensitive to aphidicolin but resistant to BuPdGTP, and DNA polymerase β was that resistant to both inhibitors.

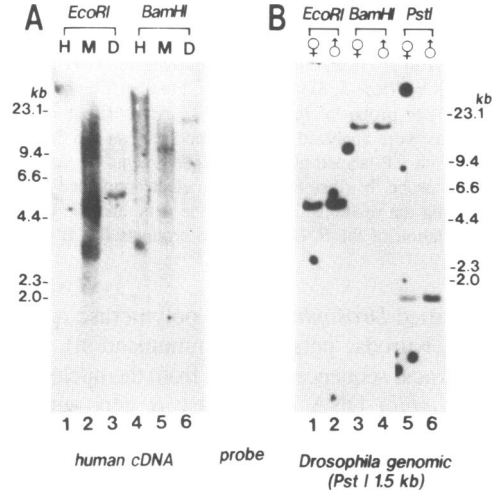

Fig. 1. Southern hybridization analysis of DNAs from human, mouse, and *D. melanogaster*. DNA's were purified from HeLa cells (H), mouse spleen (M), and *Drosophila* adults (D) and digested with the indicated restriction endonucleases. A 20 μg sample of DNA from each digestion was electrophoresed in a 0.7% agarose gel and transferred to a Gene Screen Plus filter. (A) The blot was hybridized with the ^{32}P-labeled human cDNA probe for DNA polymerase α under conditions of low-stringency. (B) The blot of DNA from *Drosophila* was hybridized with the ^{32}P-labeled 1.5 kb DNA fragment derived from λDGDPA02 as a probe under conditions of high-stringency. The size markers (in kilobases) were HindIII-digested λ phage DNA.

RESULTS

Isolation of the Drosophila gene and cDNA for DNA polymerase α

The Southern blot hybridization analysis of DNAs from human, mouse and *Drosophila* cells indicated that the cDNA fragment encoding the amino-terminal quarter of human DNA polymerase α did not hybridize to *Drosophila* DNA, even under conditions of low-stringency (data not shown). However, the other part of the human cDNA fragment of about 1 kb, which encodes highly conserved amino acid sequences encompassing domains II, III and IV (10), was successfully used as a probe. As shown in Fig. 1A, with this human cDNA fragment as a probe, single bands of 5.2 kb and 18.0 kb were detected in the DNA blots digested with EcoRI and BamHI, respectively. Unexpectedly, we failed to detect clear bands in the blots of human and mouse DNA (Fig. 1A, lane 1, 2, 4, 5), possibly because of the complicated structures of human and mouse DNA polymerase α genes, in which relatively short exons are separated by long introns (our unpublished data). A *Drosophila* genomic library was screened using this human cDNA fragment. The gene and followingly the cDNA for *Drosophila* DNA polymerase α were isolated. Restriction enzyme map of the gene and the relationship between the gene and cDNA are summarized in Fig. 2.

The isolated clones, λDGDPA01 and λDGDPA02 were also suggested to contain the sequence of the gene for *Drosophila* DNA polymerase α, because unique bands detected with the 1.5 kb PstI fragment derived from λDGDPA01 and λDGDPA02 were compatible with those detected by the human cDNA. Southern blot analysis using other restriction enzymes revealed single bands (data not shown), suggesting that the DNA polymerase α gene exists as a single copy per genome.

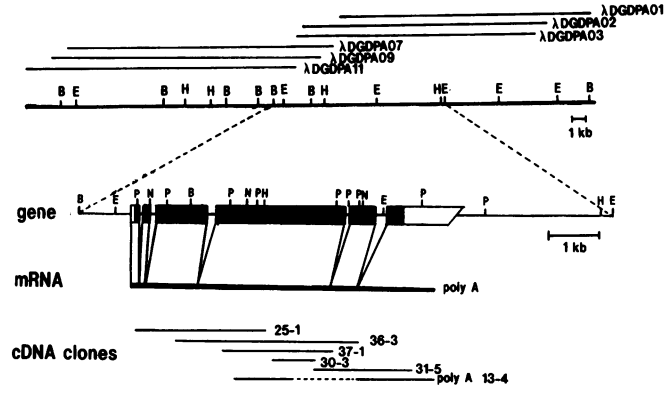

Fig. 2. Comparison of structures of the gene and cDNA for DNA polymerase α. Using the cDNA for the human DNA polymerase α, the 6 genomic clones shown at the top of the Figure were obtained. The restriction map is that of the *Drosophila* DNA polymerase α gene and the physical relationship among the gene, mRNA and isolated cDNA clones is shown. The closed box represents the coding region of *Drosophila* DNA polymerase α gene, and the open boxes indicate the 5′ and 3′ non-coding regions. The broken line of cDNA clone 13−4 represents an internal deletion. B, BamHI; E, EcoRI; H, HindIII; P, PstI; N, NcoI.

The 1.3 kb SalI-HindIII fragment from λDGDPA02 was used as a probe to obtain genomic clones containing the further 5′-region of the gene. Four additional clones (λDGDPA03, λDGDPA07, λDGDPA09 and λDGDPA11) were isolated by rescreening the genomic library so that we obtained clones covering over the 36 kb region containing the entire DNA polymerase a gene.

The 4.2 kb BamHI-EcoRI fragment which was generated by ligating the 0.7 kb BamHI fragment from λDGDPA11 with the 3.5 kb BamHI-EcoRI fragment from λDGDPA02, was used as a probe to screen *Drosophila* cDNA libraries. Six positive clones with cDNA inserts of 2.8 kb (25−1), 3.8 kb (36−3), 2.2 kb (37−1), 0.8 kb (30−3), 1.5 kb (31−5) and 3.5 kb (13−4) were obtained.

Structure of the Drosophila DNA polymerase α gene

The nucleotide sequence of about 5.5 kb from the cloned genomic DNA containing the entire open reading frame and the proximal 5′-flanking region of the DNA polymerase α gene was determined. The exons and introns were confirmed by comparing the nucleotide sequences of the gene and the cDNA and by identification of the transcription initiation site (see below). The nucleotide sequences at the splicing junctions agreed with the consensus sequences for the splicing donor and acceptor.

The gene consists of 6 exons separated by 5 short introns. The first exon (101 bp) contains an 85 bp untranslated region and a unique ATG initiation codon in the correct frame. An open reading frame of 4,515 bp is thought to start from the second A- residue of the 5′-GCAATG sequence, which is similar to the Kozak's consensus sequence A/GNNATG (38).

The transcription initiation site(s) was determined by an RNase protection method (Fig. 3). Two kinds of RNA probes were prepared, as shown in Fig. 3B. Lengths of major protected bands were estimated as 45 nucleotides from the probe 'a' (lanes 4 and 5) and as 103 nucleotides from probe 'b' (lanes 9 and 10), by assuming that RNA migrated in a denaturing polyacrylamide gel by approximately 10% behind single stranded DNA fragments of the same length (27). From the results obtained using two probes, the major transcription initiation site was mapped at the adenine residue 85 bp upstream from the initiation codon, and this position was defined as the +1 nucleotide position. The primer extension analysis using a primer complementary to +56−+91 sequences confirmed the result obtained by the RNase protection method (data not shown).The sequence around +1 (ATCATCC) completely matches with the signal for transcription initiation of a number of *Drosophila* genes. Additional minor protected RNA bands were detected, hence transcription might start at sites corresponding to positions +4 and +11 at low frequency. Furthermore, it seems that the same transcription initiation sites were used for both the maternally transcribed mRNA (lanes 4 and 9) and the zygotically transcribed mRNA (lanes 5 and 10). The promoter activity was found to reside in the adjacent 5′-upstream region of the transcription initiation site (our unpublished data).

Amino acid sequences of Drosophila DNA polymerase α

The open reading frame predicts a polypeptide of 1,505 amino acid residues with a molecular weight of 170,796, a size close to that estimated for the catalytic polypeptide of the purified *Drosophila* DNA polymerase α (40, 41). It should be noted that the predicted polypeptide contains oligopeptide sequences completely matching to partial amino acid sequences of the

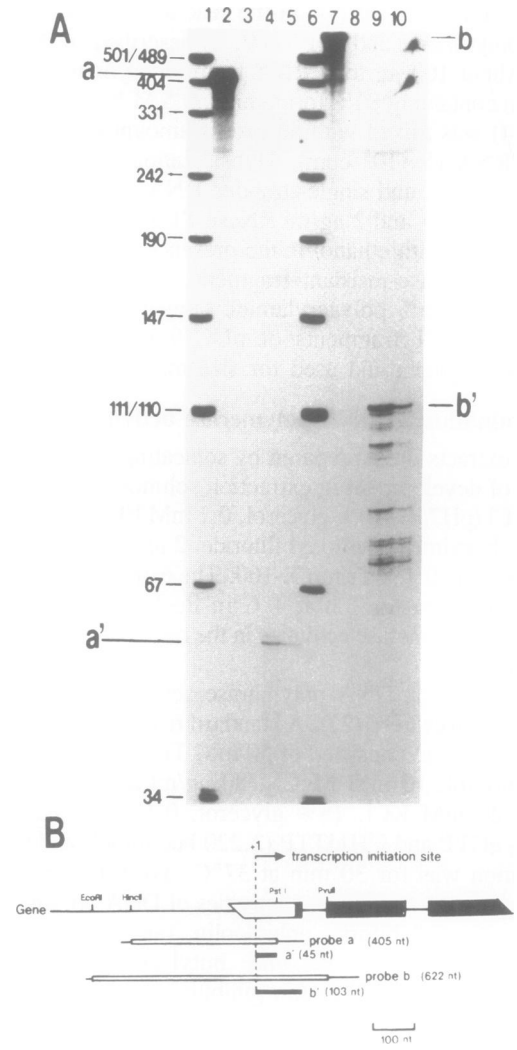

Fig. 3. Mapping of transcription initiation sites by RNase protection analysis. (A) Total RNA (100 μg) from unfertilized egg (lane 4, 9), 8−12 h embryo (lane 5, 10) or yeast tRNA (lane 3, 8) was hybridized to 5×10^5 cpm of the RNA probe 'a' (lane 3, 4, 5) or probe 'b' (lane 8, 9, 10). After RNase digestion, RNase-resistant products were analyzed by electrophoresis on a 6 % acrylamide/8 M urea gel along with ^{32}P-labeled pUC19 HpaII fragments for size standard. Probe 'a' (lane 2) and probe 'b' (lane 6) were also electrophoresed in parallel. (B) A diagram depicting the relationships among the gene and the two RNA probes, and an interpretation of the RNase-resistant products (a′, b′).

immuno-purified *Drosophila* DNA polymerase α as underlined in Fig. 4 (K.Kuroda, personal communication).

The amino acid sequence deduced from the nucleotide sequence of the *Drosophila* DNA polymerase α gene and cDNA was compared with the sequence of the human homologue (10) (Fig. 4). The sequences for the *Drosophila* and human enzymes share 37% identical amino acid residues, and a 75% similarity is defined when conservative amino acid substitutions are included. The six highly conserved regions which were identified by Wong *et al* (10) in human DNA polymerase α, yeast DNA polymerase I (α), DNA polymerases of herpes simplex virus, Epstein-Barr virus, cytomegalovirus, vaccinia virus, human adenoviruses and bacteriophage T4, are present in the same linear and spatial arrangements in *Drosophila* DNA polymerase α.

The sequence homologies between *Drosophila* and human proteins were observed outside of the segment delimited by the

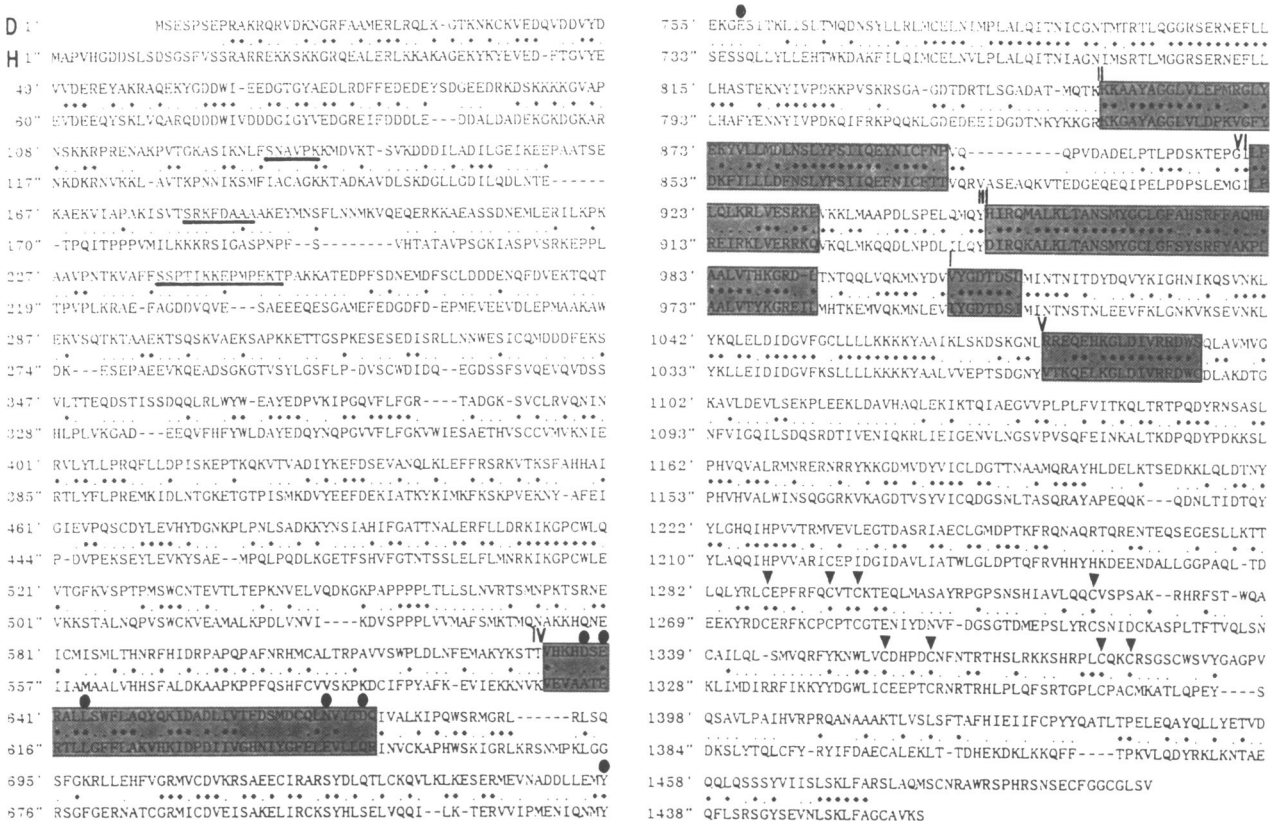

Fig. 4. Comparison of amino acid sequences of *Drosophila* and human DNA polymerase α. The deduced amino acid sequence of *Drosophila* DNA polymerase α is compared with that of human (10). Identical amino acid residues are indicated (*). Conservative amino acid substitutions are shown (.). The six conserved regions are shown in shaded boxes. Closed triangles represent cysteine residues conserved between two DNA polymerase α's. Closed circles indicate residues identical to those involved in the 3′→5′ exonuclease activity in *E. coli* DNA polymerase I. The amino acid sequences corresponding to those determined by an Edman analysis of the immuno-affinity-purified *Drosophila* DNA polymerase α (Kuroda, personal communication) are underlined.

six conserved regions. Eight cysteine residues in the carboxy-terminal region (residues 1288−1383), in which four cysteine residues in the C-terminal most region may be capable of binding metal ions by forming tetrahedral box structures named zinc fingers and interacting with DNA, are completely conserved in human and *Drosophila* sequences (42−44).

It should be noted that seven amino acids residues which are essential for 3′→5′ exonuclease activities associated with *E. coli* DNA polymerase I and φ29 phage DNA polymerase (45) are conserved in the region from 638th to 758th residues of *Drosophila* DNA polymerase α (Fig. 5).

The amino acid sequence starting at position 96 of *Drosophila* DNA polymerase α is rich in basic amino acids (lysine and arginine), and this sequence (RKDSKKKK) is similar to nuclear localization signals found in various steroid hormone receptors (46) and the SV40 large T antigen (47).

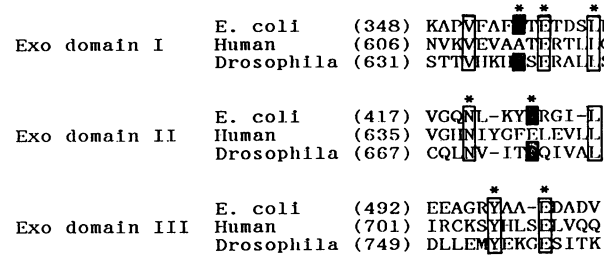

Fig. 5. Comparison of amino acid sequences responsible for 3′→5′ exonuclease activities. Based on the proposal by Bernad (45), The amino acid sequences of *Drosophila* DNA polymerase α homologous to the 3′→5′ exonuclease domains I, II and III of *E. coli* DNA polymerase I were aligned with those of the human sequences. Residues conserved in the three DNA polymerases are boxed. The amino acid residues which are essential for 3′→5′ exonuclease activity of *E. coli* DNA polymerase I are indicated by asterisks. Two amino acid residues identical between *Drosophila* and *E. coli* DNA polymerases, but different in the human enzyme are indicated by shaded boxes. Numbers in brackets indicate the amino acid positions from amino-termini of these enzymes.

Changes in the DNA polymerase α gene expression during development

We measured levels of DNA polymerase α expression during *Drosophila* development by Northern hybridization analysis and DNA polymerase α assay. Results of the Northern hybridization analysis using RNA extracted from *Drosophila* bodies at various stages are shown in Fig. 6. Using as a probe the DNA polymerase α cDNA, a single transcript of 5.7 kb was detected. A cDNA for the PCNA was also used as a reference probe, since the *Drosophila* PCNA mRNA of 1.1 kb was expressed in correlation to cell proliferation during development (31). The cytoplasmic actin 5C cDNA was also used as another reference probe to monitor the integrity of mRNA (32).

As shown in Fig. 6C, the DNA polymerase α mRNA was detected at the highest level in 2−4 h embryos, and at the relatively high level in unfertilized eggs, 12−16 h embryos, second instar larva, and adult female flies. Low levels of the mRNA were detected in 16−20 h embryos, pupa and adult male flies.

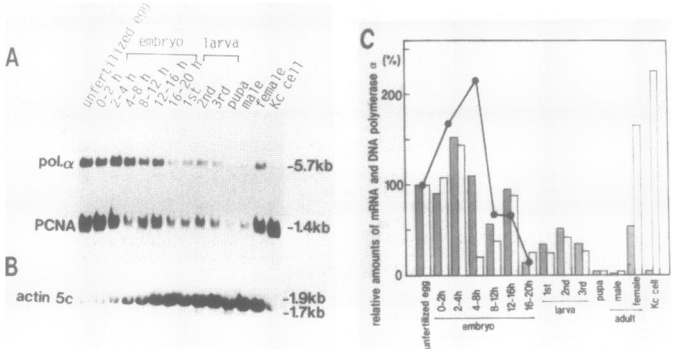

Fig. 6. Changes in the mRNA content and enzyme activity for DNA polymerase α during development. Total RNA were prepared from *Drosophila* bodies at various stages of development. Twenty micrograms of RNAs were fractionated in a 1.2% agarose gel containing formaldehyde, transferred to a Gene Screen Plus filter and subjected to hybridization with the ^{32}P-labeled probe. Sizes of transcripts were estimated by comparing with a commercially available RNA size marker (purchased from BRL) as a standard. (A) The RNA blot was hybridized with the mixture of a 1.5 kb PstI fragment derived from λDGDPA02 for DNA polymerase α and a 1.1 kb cDNA for *Drosophila* PCNA (31). (B) The same filter used in (A) was rehybridized with cDNA for cytoplasmic actin 5C as a probe (3). (C) The amounts of DNA polymerase α mRNA (shaded bars) and PCNA mRNA (open bars) were determined by a densitometrical scanning of the autoradiograms shown in (A), and expressed in a relative value to the amount of the mRNAs in unfertilized eggs. The DNA polymerase α activities from embryos at various stages are expressed as relative values (●). One hundred percent value of the DNA polymerase α activity from unfertilized eggs corresponds to 22.5 units/mg protein.

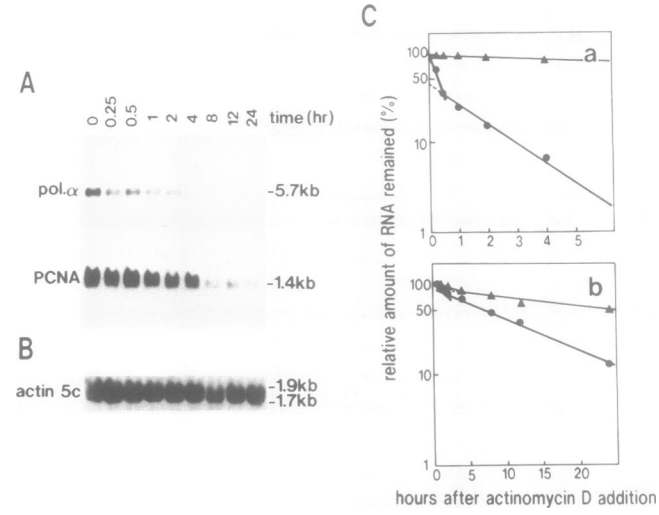

Fig. 7. Half life of *Drosophila* DNA polymerase α and PCNA mRNA's. Actinomycin D was added to culture media of Kc cells to the final concentration of 5 μg/ml. (A)(B) RNAs were extracted after 0, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after the addition of actinomycin D and subjected to a Northern hybridization analysis as in Fig. 6 with exception using a 1.2% agarose gel. (C) Degradation kinetics of mRNAs for DNA polymerase α (a) and PCNA (b). The amounts of mRNAs were measured by densitometrical scanning of the autoradiograms like those represented in (A). The plot of each time point was made by taking an average of results obtained from three independent experiments, and represented as a relative value to the amount of the mRNA at time zero. In a, DNA polymerase α mRNA (●); in b, PCNA mRNA (●); in a and b, actin 5C mRNA (▲).

We determined the DNA polymerase activities at various stages of development of *Drosophila*. To distinguish DNA polymerase α activity from activities of DNA polymerases β and δ in crude extracts, specific inhibitors, aphidicolin and butylphenyldGTP (BuPdGTP) were used in the enzymatic reactions. Under our assay conditions, relative activities of DNA polymerase α, β and δ in the extract of unfertilized eggs accounted for about 70, 2 and 28%, respectively. However, these values might not necessarily reflect absolute enzyme contents, because the reaction condition favorable for DNA polymerase α was used. Fluctuations of DNA polymerase α activity in early stage embryos was similar to that of mRNA shown by a Northern hybridization analysis (Fig. 6C). A large amount of the DNA polymerase α was present in unfertilized eggs, as was the case with mRNA.

Stability of the DNA polymerase α mRNA in cultured cells

The amount of the DNA polymerase α mRNA in *Drosophila* bodies changed rapidly during progression of development, perhaps because of the short life of the DNA polymerase α mRNA. Half-life of mRNAs for the DNA polymerase α and PCNA in cultured *Drosophila* Kc cells was determined. RNA was extracted from cells at various times after addition of actinomycin D to the cell cultures and analyzed by the Northern hybridization method (Fig. 7). Half life of mRNAs for the DNA polymerase α and PCNA was estimated to be 15 min and 4 h, respectively. This extremely short half life of the DNA polymerase α mRNA might be caused by the mRNA-destabilizing sequence, AUUUA (48). Thirteen copies of this sequence are present in the 3'-untranslated region of DNA polymerase α mRNA (data not shown), while only one AUUUA sequence was found in the PCNA mRNA (31).

Table I. Sequences homologous to the homeodomain protein-binding consensus in the 5'-upstream region of the *Drosophila* DNA polymerase α gene

Consensus sequences	Positions	Sequences	Homology
Binding sequence for homeodomain-proteins (TCAATTAAAT or ATTTAATTGA)	−901	AaaTAATTGt	7/10
	−851	aCtATTAAAT	8/10
	−813	aCAATTAAAc	8/10
	−713	atAATTAtAT	7/10
	−670	tTTTAATcGA	8/10
	−584	aCgATTAAAa	7/10
	−557	tTTTAATTtg	7/10
	−461	gCAATTAAAt	8/10
	−370	TCAATTAtca	7/10
	−288	cTgTAATcGA	7/10
	−121	tTTTAATTtc	7/10
Binding sequence for *Antennapedia* (ANNNNCATTA)	−529	AAGCACATTA	10/10

DISCUSSION

DNA polymerase α with its intricate catalytic properties binds deoxynucleoside 5'-triphosphate, recognizes template and primer molecules and interacts with proteins such as primase in the enzyme complex for DNA replication. Structures responsible for these subfunctions are expected to be conserved among various DNA polymerases. Comparison of the primary amino acid sequence of the *Drosophila* DNA polymerase α with that of the human enzyme revealed high conservation over a long evolutional distance between insect and mammal. In addition to six regions which are highly conserved in a number of DNA polymerases, we found several regions sharing homologies with other α-type DNA polymerases. One is a cysteine-rich potential metal-binding

domain present near the carboxy-terminal, that might be involved in the interaction with nucleic acids. Furthermore, sequences well-conserved among α-like polymerases started with the 511th position (RKIKGPCWL) preceding the conserved region IV. This sequence corresponds to the region around the 493rd position of yeast DNA polymerase I (α) which was reported to be important for interaction with the primase, as based on evidence that a single amino acid substitution (from glycine to arginine) within this sequence resulted in a temperature-sensitive phenotype of the DNA polymerase I (α)-primase complex formation in budding yeast (49). The evidence strongly supports the possibility that this region of *Drosophila* DNA polymerase α is responsible for interaction with primase.

One characteristic feature of *Drosophila* DNA polymerase α which differs from mammalian homologues is an associated 3′→5′ exonuclease activity (50, 51). A comparison of the sequence from 638th to 758th residues of *Drosophila* DNA polymerase α with sequences of DNA polymerase-associated exonucleases reported by Bernad *et al.* (45), revealed the complete conservation in amino acid residues essential for exonuclease activity. On the other hand, the residue corresponding to the aspartic acid which might be one of the essential amino acid residues in the domain I for exonuclease activity is alanine in the human DNA polymerase α. To verify the relationships between structural subdomains and their functions in the DNA polymerase α polypeptide, we are preparing recombinant *Drosophila* DNA polymerase α using a baculovirus expression system. This will be done in conjunction with *in vitro* mutagenesis, as our studies on the recombinant rat DNA polymerase β (52, 53).

Drosophila is a suitable organism with which to investigate regulatory mechanisms of DNA replication during oogenesis, embryogenesis and following stages of development. The levels of expression of DNA polymerase α seemed to be closely correlated with cell proliferation in *Drosophila* bodies. In particular, we demonstrated that a large amount of mRNA as well as DNA polymerase α accumulated in unfertilized eggs, hence, both mRNA and the enzyme were synthesized during oogenesis for subsequent use in DNA replication occurring every 10 min during early embryogenesis.

The level of the DNA polymerase α mRNA fluctuated during developmental stages. The increase of the mRNA through the first instar to second instar larva stages was reproductively observed, indicating that DNA replication in imaginal discs in which cells for adult body formation proliferate occurs in this period. These observations indicate that the expression of DNA polymerase α is precisely regulated by control mechanisms closely related to cell proliferation and/or cell differentiation. Furthermore, the short half life of mRNA for this enzyme in comparison with that for PCNA suggests that a transcriptional step is primarily important for regulation of DNA polymerase α expression. However, another possibility that a specific posttranscriptional mechanism such as one for stabilizing the mRNA is present in *Drosophila* body during development, should be considered.

Our result suggests that the minimal promoter function present within a 109 bp region from the nucleotide position −67 to +42 (our unpublished data). Analysis of the nucleotide sequence in and around the promoter region revealed that well known transcription-regulatory elements such as TATA or CAAT boxes are absent within a proper distance from the CAP site. Within the 0.9 kb upstream region from the transcription initiation site, the sequences similar to the binding sites (5′-TCAATTAAAT) for several homeodomain-containing proteins (54, 55) are present in a cluster between the nucleotide positions −901 and −121. Furthermore, there is another sequence 5′-AAGCACATT at position −584 which perfectly matches the binding sequence for the homeodomain-containing protein encoded by the *Antennapedia* gene (Table 1). It is striking, because we have also found similar sequences in the 5′-flanking region of the promoter of the *Drosophila* PCNA gene and we demonstrated by DNase I-footprinting analysis that recombinant homeodomain proteins coded by *even-skipped* and *zerknullt* specifically bound to these sequences (31). These lines of evidence suggest that genes for DNA replication enzymes are under a common regulatory mechanism governed by homeodomain-containing proteins, whose expressions are spatially and temporally regulated during morphogenesis. We are now analyzing functions of upstream regulatory sequences of DNA polymerase α and PCNA genes and obtained preliminary results suggesting that these elements are regulated by several homeodomain-containing proteins.

It has been reported that the levels of mRNAs for the human DNA polymerase α and murine primase increased during activation of quiescent cells to proliferate by the addition of serum (11, 12). These observations suggest that there exists a mechanism(s) which induces expressions of genes coding for enzymes required for DNA replication in the course of a shift from the quiescent state into the mitotic state, and this mechanism differs from that governing the cell cycle regulation in mitotic cells. It will be of interest to clarify common molecular mechanisms that control the expressions of *Drosophila* DNA polymerase α and PCNA genes by investigating regulatory elements.

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REFERENCES

1. Miller, M. R., Ulrich, R. G., Wang, T. S.-F., and Korn, D. (1985) J. Biol. Chem. 260, 134−138.
2. Eki, T., Enomoto, T., Murakami, Y., Hanaoka, F., and Yamada, M. (1988) Arch. Biochem. Biophys. 260, 552−560.
3. Murakami, Y., Yasuda, H. Miyazawa, H., Hanaoka, F., and Yamada, M. (1985) Proc. Natl. Acad. Sci. USA 82, 1761−1765.
4. Tsurimoto, T., and Stillman, B. (1989) Mol. Cell. Biol. 9, 609−619.
5. Wold, M. S., Weinberg, D. W., Virshup, D. M. Li, J. J., and Kelly, T. J. (1989) J. Biol. Chem. 264, 2801−2809.
6. Lee, S.-H., Eki, T., and Hurwitz, J. (1989) Proc. Natl. Acad. Sci. USA 86, 7361−7365.
7. Tsurimoto, T., and Stillman, B. (1989) EMBO J. 8, 3883−3889.
8. Tsurimoto, T., Melendy, T., and Stillman, B. (1990) Nature 346, 534−539.
9. Weinberg, D. H., and Kelly, T. J. (1989) Proc. Natl. Acad. Sci USA 86, 9742−9746.
10. Wong, S. W., Wahl, A. F., Yuan, P.-M. Arai, N. Pearson, B. E., Arai, K., Korn, D., Hunkapiller, M. W., and Wang, T. S.-F. (1988) EMBO J. 7, 37−47.
11. Wahl, A., Geis, A. M., Spain, B. H., Wong, S. W. and Wang, T. S.-F. (1988) Mol. Cell. Biol. 8, 5016−5025.
12. Tseng, B. Y., Prussak, C. E., and Almazan, M. T. (1989) Mol. Cell. Biol. 9, 1940−1945.

13. Budd, M., and Campbell, J. L. (1987) Proc. Natl. Acad. Sci. USA 84, 2838–2842.
14. Johnson, L. M., Snyder, M., Chang, L. M. S., Davis, R. W., and Campbell, J. L. (1985) Cell 43, 369–377.
15. Johnston, L. H., White, J. H., Johnson, A. L., Lucchini, G. and Plevani, P. (1987) Nucleic Acids Res. 15, 5017–5029.
16. Boulet, A., Simon, M., Faye, G., Bauer, G. A., and Burgers, P. M.-J. (1989) EMBO J. 8, 1849–1854.
17. Foiani, M., Santocanale, C., Plevani, P., and Lucchini, G. (1989) Mol. Cell. Biol. 9, 3081–3087.
18. Giaver, G., Lynn, R., Goto, T., and Wang, J. C. (1986) J. Biol. Chem. 261, 12448–12454.
19. Johnston, L. H., White, J. H. M., Johnson, A. L., Lucchini, G., and Plevani, P. (1990) Mol. Gen. Genet. 221, 44–48.
20. Morrison, A., Araki, H., Clark, A. B., Hamatake, R. H., Sugino, A. (1990) Cell 62, 1143–1151.
21. Lowndes, N. F., Johnson, A. L., and Johnston, L. H. (1991) Nature 350, 247–250.
22. Hirose, F., Yamamoto, S. Yamaguchi, S., and Matsukage, A. (1988) J. Biol. Chem. 263, 2925–2933.
23. Matsukage, A., Kitani, H., Yamaguchi, M., Kusakabe, M., Morita, T., and Koshida, Y. (1986) Dev. Biol. 117, 226–232.
24. Wang, T. S.-F., Wong, S. W., and Korn, D. (1989) The FASEB J. 3, 14–21.
25. Miller, M. A., Korn, D., and Wang, T. S.-F. (1988) Nucleic Acids Res. 16, 7961–7973.
26. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. Poole, S., Kauvar, L. M., Dress, B., and Kornberg, T. (1985) Cell 40, 37–43.
29. McGinnis, K., Shermoen, A. W., and Beckendorf, S. K. (1983) Cell 34, 75–84.
30. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
31. Yamaguchi, M., Nishida, Y., Moriuchi, T. Hirose, F. Hui, C.-c., Suzuki, Y., and Matsukage, A. (1990). Mol. Cell. Biol. 10, 872–879.
32. Bond, B. J., and Davidson, N. (1986) Mol. Cell. Biol. 6, 2080–2088.
33. Henikoff, S. (1984) Gene 28, 351–359.
34. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA. 74, 5463–5467.
35. Melton, D. A., Krieg, P. A., Rebagliati, M. R. Maniatis, T., Zinn, K., and Green, M. R. (1984) Nucleic Acids Res. 12, 7035–7056.
36. Ikegami, S., Taguchi, T., Ohashi, M., Nagano, H., and Mano, Y. (1978) Nature 275, 458–460.
37. Lee, M. Y. W. T., Toomy, N. L., and Wright, G. E. (1985) Nucleic Acids Res. 13, 8623–8630.
38. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148.
39. Hultmark, D., Klemens, R., and Gehling, W. J. (1986) Cell 44, 429–438.
40. Kaguni, L. S., Rossignol, J.-M., Connaway, R. C., and Lehman, I. R. (1983) Proc. Natl. Acad. Sci. USA 80, 2221–2225.
41. Kuroda, K., Kagiyama-Takahashi, R., and Shinomiya, T. (1990) J. Biochem. (Tokyo) 108, 926–933.
42. Berg, J. M. (1986) Science 232, 485–487.
43. Evans, R. M., and Hollenberg, S. M. (1988) Cell 52, 1–3.
44. Miller, J., McLachlan, A. D., and Klug, A. (1985) EMBO J. 4, 1609–1614.
45. Bernad, A., Blanco, L. Lazaro, J. M. Martin, G., and Salas, M (1989) Cell 59, 219–228.
46. Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S. Atger, M., Perrot-Applanat, M., and Milgrom, E. (1989) Cell 57, 1147–1154.
47. Kalderson, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984) Nature 311, 33–38.
48. Shaw, G., and Kamen, R. (1986) Cell 46, 659–667.
49. Pizagalli, A., Valsasnini, P. Plevani, P., and Lucchini, G. (1988) Proc. Natl. Acad. Sci. USA 85, 3772–3776.
50. Cotterill, S. M., Reyland, M. E., Loeb, L. A., and Lehman, I. R. (1987) Proc. Natl. Acad. Sci. USA 84, 5635–5639.
51. Kunkel, T. A. (1988) Cell 53, 837–840.
52. Date, T., Yamamoto, S., Tanihara, K., Nishimoto, Y., Liu, N., and Matsukage, A. (1990) Biochemistry 29, 5027–5034.
53. Date, T., Yamamoto. S., Tanihara, K., Nishimoto, Y., and Matsukage, A. (1991) Biochemistry 30, 5286–5292.
54. Desplan, C., Theis, J., and O'Farrell, P. H. (1988) Cell 54, 1081–1090.
55. Hoey, T., and Levine, M. (1988) Nature 332, 858–861.