Synthesis by the DNA primase of *Drosophila melanogaster* of a primer with a unique chain length

(DNA polymerase α/DNA replication/Okazaki fragments/M13 DNA)

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ABSTRACT   The primase associated with the DNA polymerase α from embryos of *Drosophila melanogaster* catalyzes the synthesis of ribo-oligonucleotide primers on single-stranded M13 DNA or polydeoxythymidylate templates, which can be elongated by DNA polymerase action [Conaway, R. C. & Lehman, I. R. (1982) *Proc. Natl. Acad. Sci. USA* 79, 2523–2527]. The primers synthesized in a coupled primase–DNA polymerase α reaction with an M13 DNA template are of a unique size (15 residues); those synthesized with poly(dT) range from 8 to 15 nucleotides. Primer synthesis is initiated at multiple but nonrandom sites. Like the DNA primase of *Escherichia coli* and the comparable activity in intact nuclei of polyoma-infected mouse cells, the DNA primase of *D. melanogaster* can substitute deoxynucleotides for ribonucleotides during primer synthesis.

We recently described a DNA primase in embryos of *Drosophila melanogaster* that catalyzes the synthesis of a ribo-oligonucleotide primer that can be elongated by DNA polymerase (1). Purification of the primase activity showed it to be closely associated with the DNA polymerase α of *D. melanogaster*, possibly part of the multisubunit DNA polymerase α itself. A similar finding has been reported by Yagura *et al.* (2) for a DNA polymerase α subspecies of Ehrlich ascites tumor cells.

We have now characterized the primers synthesized in a coupled primase–DNA polymerase α reaction that uses single-stranded M13 DNA and poly(dT) as templates. We find that primer synthesis is initiated at multiple but preferred sites on M13 DNA to produce primers whose chain length is 15 nucleotides. In the case of poly(dT), a series of primer chain lengths ranging from 8 to 15 residues is synthesized. In both instances, deoxynucleotides can be incorporated in place of ribonucleotides to generate a mixed ribo-deoxyribonucleotide primer. The DNA primase of *D. melanogaster* thus resembles a similar activity observed by Reichard and Eliasson in intact nuclei of polyoma-infected mouse fibroblasts (3).

MATERIALS AND METHODS

Materials. Unlabeled ribonucleoside and deoxyribonucleoside 5'-triphosphates and ribonucleoside and deoxyribonucleoside 3'-monophosphates were purchased from P-L Biochemicals. [α-^{32}P]ATP and [α-^{32}P]CTP were from Amersham. Acrylamide, N,N'-methylenebis(acrylamide), N,N,N',N'-tetramethylethylenediamine, ammonium persulfate, Bio-Gel A-1.5m, and Bio-Gel A-0.5m were obtained from Bio-Rad. Dithiothreitol was purchased from Calbiochem–Behring. Ultrapure urea was from Schwarz/Mann. Agarose was purchased from Matheson, Coleman, and Bell. Bovine serum albumin was obtained from Pentex and PEI-cellulose sheets were from Brinkmann.

Nucleic acids. Poly(dT), ≈1,000 residues long, was purchased from P-L Biochemicals. Single-stranded M13 DNA (M13H *ori4* and M13H *ori8*) and their double-stranded replicative forms (M13H *ori10*) were gifts from Laurie Kaguni of this department (4).

Enzymes. DNA polymerase α (fraction VI) was purified according to Banks *et al.* (5). Pancreatic DNase I (Worthington) that had been purified further by chromatography on agarose-5'-(4-aminophenylphosphoryl) uridine 2'(3') phosphate (Miles) was a gift from M. Fromm of this department. DNA polymerase III holoenzyme (6) was kindly provided by S. B. Biswas of this department. Restriction endonuclease *Hinf*I was purchased from New England BioLabs and restriction endonuclease *Hpa* II was from Bethesda Research Laboratories. Micrococcal nuclease was a gift from J. Weliky (Stanford University) and spleen phosphodiesterase was from Worthington.

Methods. *Analysis by nuclease digestion of products of DNA synthesis.* Reaction mixtures for DNA synthesis (100 µl) contained 50 mM Tris·HCl (pH 8.5), 10 mM MgCl$_2$, bovine serum albumin at 200 µg/ml, 4 mM dithiothreitol, 600 pmol of single-stranded M13 DNA or 4,000 pmol of poly(dT) (nucleotides), and 1.0 µg of DNA polymerase α. With M13 DNA as template, reactions were performed with 100 µM ATP or with 100 µM each of the four ribonucleoside triphosphates in the presence or absence of 100 µM each of the four deoxynucleoside triphosphates. [α-^{32}P]ATP or [α-^{32}P]CTP was present at 200 µCi per reaction (1 Ci = 3.7 × 10^{10} becquerels). With poly(dT), reactions were performed with 100 µM ATP in the presence or absence of 100 µM dATP. [α-^{32}P]ATP was added at 200 µCi per reaction. After incubation for 30 min at 30°C, the reaction mixtures were made 1% in sodium dodecyl sulfate and 15 mM in EDTA. The reaction mixtures containing M13 DNA were filtered through a 2-ml column of Bio-Gel A-1.5m; those with poly(dT) were filtered through a 2-ml column of Bio-Gel A-0.5m. Both were equilibrated and eluted with 20 mM Tris·HCl, pH 8.5/1 mM EDTA. The DNA in the excluded volume was collected, precipitated with ethanol, dissolved in 30–60 µl of 20 mM Tris·HCl, pH 8.5/1 mM EDTA, and, if necessary, stored at −20°C.

DNase I digestion of the products of DNA synthesis was performed in reaction mixtures (10 µl) that contained, in addition to the DNA product, 20 mM Tris·HCl (pH 8.5), 10 mM MgCl$_2$, 1 mM EDTA, and 0.8–1.6 units of DNase I. The reaction mixtures were incubated for 30 min at 37°C and then were made to 15% in glycerol, 0.05% in bromphenol blue, and 0.05% in xylene cyanol. After heating at 100°C for 4 min, the digest was analyzed by polyacrylamide gel electrophoresis.

Micrococcal nuclease and spleen phosphodiesterase digestion of the products of DNA synthesis was performed in reaction mixtures (20 µl) that contained 20 mM Tris·HCl (pH 8.5), 10 mM MgCl$_2$, 1 mM EDTA, 1 mM CaCl$_2$, and 20 units of micrococcal nuclease. After 2 hr at 37°C, sodium acetate (pH 4.8)

to 45 mM and 0.06 unit of spleen phosphodiesterase were added
and the mixture was incubated for 1 hr at 37°C. The digestion
products were then separated by PEI-cellulose thin-layer
chromatography.

When the DNA products were digested with restriction en-
donucleases, M13H *ori8* was the single-stranded M13 DNA
template, and *E. coli* DNA polymerase III holoenzyme was
used to convert the partially replicated molecules to the circu-
lar double-stranded form prior to restriction endonuclease di-
gestion. The DNA polymerase III holoenzyme reaction mix-
tures (10 μl) contained 20 mM Tris·HCl (pH 8.5), 10 mM
MgCl$_2$, 1 mM EDTA, 4 mM dithiothreitol, 1 mM ATP, 100 μM
each of the four deoxynucleoside triphosphates, the products
of the coupled primase–DNA polymerase α reaction, and 3
units of DNA polymerase III holoenzyme. After incubation for
30 min at 30°C, 1.5 μg of carrier double-stranded M13H *ori10*
DNA and either 3 units of *Hin*fI or 1.5 units of *Hpa* II was
added. Incubation was continued for 1 hr at 37°C. In the case
of *Hin*fI reaction, NaCl was added to 100 mM prior to incu-
bation. After incubation, the samples were made 1% in sodium
dodecyl sulfate, 15 mM in EDTA, and 0.05% in bromphenol
blue, and analyzed by agarose gel electrophoresis.

Gel electrophoresis. Gel electrophoresis was performed in
20% polyacrylamide slab gels (13 × 23 × 0.15 cm) containing
7.0 M urea in 90 mM Tris·borate, pH 8.3/2.5 mM EDTA or
in 1.8% agarose gels in the same buffer. Electrophoresis was
performed at 300 V for polyacrylamide gels and 120 V (constant
voltage) for agarose gels. After electrophoresis, the polyacryl-
amide gels were autoradiographed with XAR-5 x-ray film (Ko-
dak) at −70°C in the presence of an intensifying screen. The
agarose gels were stained with ethidium bromide, photo-
graphed, dried, and autoradiographed as described above.

PEI-cellulose thin-layer chromatography. One-dimensional
PEI-cellulose thin-layer chromatography was performed with
1.0 M acetic acid as solvent. Two-dimensional PEI-cellulose
thin-layer chromatography was performed in the first dimen-
sion with 0.38 M LiCl$_2$ and 20 mM Tris·HCl (pH 6.8). Chro-
matography in the second dimension was accomplished by com-
plete development with 1.0 M acetic acid followed by devel-
opment to the level of 3′-AMP with 1.0 M formic acid.

RESULTS

**DNase I Digestion of the Products of the Coupled Pri-
mase–DNA Polymerase α Reaction Yields an Oligonucleotide
of Unique Length.** When DNA polymerase α was incubated
with single-stranded M13 DNA, the four ribonucleoside tri-
phosphates including [α-^{32}P]ATP, and the four deoxynucleo-
side triphosphates and the products of the reaction degraded
with DNase I, a labeled oligonucleotide 15 residues in length
was observed after polyacrylamide gel electrophoresis (Fig. 1,
lanes E and F). A similar result was obtained with [α-^{32}P]CTP
(data not shown).

When the products of the coupled primase–DNA polymerase
α reaction performed with single-stranded M13 DNA, [α-^{32}P]-
ATP, and the four deoxynucleoside triphosphates were digested
with DNase I and subjected to polyacrylamide gel electropho-
resis, a graded series of oligonucleotides was observed (Fig. 1,
lanes C and D) with a maximum length of 15 residues. The dis-
tribution of oligonucleotide chain lengths in this instance is a
consequence of the incorporation of deoxynucleotides in place
of ribonucleotides during primer synthesis (see below).

Incubation of DNA polymerase α with single-stranded M13
DNA and the four ribonucleoside triphosphates including
[α-^{32}P]ATP in the absence of the four deoxynucleoside tri-
phosphates resulted in the synthesis of an oligonucleotide of
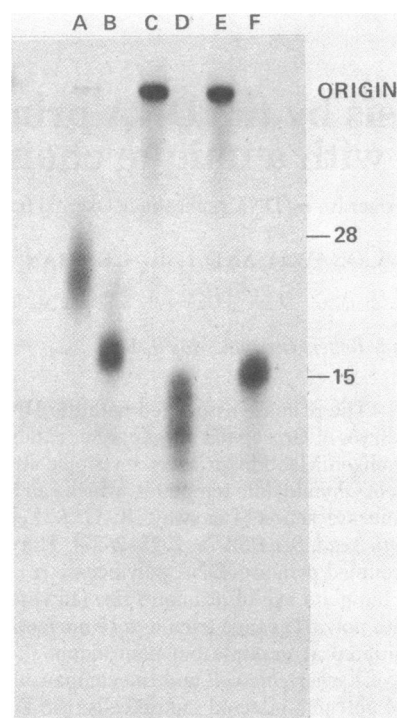approximately 28 residues (Fig. 1, lane A). After digestion with

FIG. 1.   Polyacrylamide gel electrophoresis of DNase I digests of
products synthesized with single-stranded M13 DNA. DNA synthesis
with [α-^{32}P]ATP as the radioactive nucleotide, DNase I digestion, and
20% polyacrylamide gel electrophoresis in 7.0 M urea were performed
as described. The nucleotide composition of the reaction mixtures was
as follows: lanes A and B, the four ribonucleoside triphosphates at 100
μM each; lanes C and D, ATP at 100 μM and the four deoxynucleoside
triphosphates at 100 μM each; lanes E and F, the four ribonucleoside
triphosphates at 100 μM each and the four deoxynucleoside triphos-
phates at 100 μM each. Lane B contained the DNase I (0.6 unit) digest
of the reaction products appearing in lane A. Lane D contained the
DNase I digest of the products in lane C, and lane F contained the
DNase I digest of the products in lane E.

DNase I, an oligonucleotide 15 residues in length was again
generated (Fig. 1, lane B). A similar result was obtained with
[α-^{32}P]CTP (data not shown). The decrease in chain length after
digestion with DNase I is presumably a consequence of incor-
poration into the primer of deoxynucleotides present as con-
taminants in the mixture of the four ribonucleoside triphos-
phates. In fact, when this reaction was performed in the
presence of aphidicolin, an inhibitor of DNA polymerase α (7),
a 15-residue oligonucleotide was synthesized whose chain
length was unaffected by DNase I digestion (data not shown).

When poly(dT) was used as template with [α-^{32}P]ATP in the
presence or absence of dATP and the products digested with
DNase I, the results were similar to those obtained with single-
stranded M13 DNA. With both ATP and dATP a size distri-
bution of oligonucleotides with maximum length of 15 residues
was obtained (Fig. 2, lanes D, E, and F). This distribution re-
sembles that observed after DNase I digestion of the DNA syn-
thesized with single-stranded M13 DNA in the presence of ATP
and the four deoxynucleoside triphosphates (compare Fig. 2,
lanes D, E, and F with Fig. 1, lanes C and D). When DNA
polymerase α was incubated with poly(dT) and [α-^{32}P]ATP in
the absence of dATP, a 28-residue oligonucleotide was synthe-
sized (Fig. 2, lane A). After DNase I digestion the length of the
oligonucleotide was reduced to 15 residues (Fig. 2, lanes B and
C). This result is similar to that obtained with single-stranded
M13 DNA in the presence of the [α-^{32}P]ATP and the other three
ribonucleoside triphosphates (compare Fig. 2, lanes A, B, and

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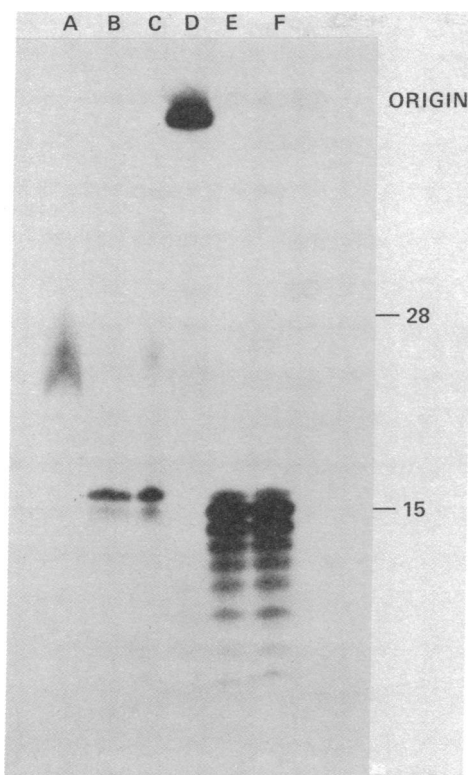*Proc. Natl. Acad. Sci. USA 79 (1982)*   4587

FIG. 2. Polyacrylamide gel electrophoresis of DNase I digests of products synthesized with poly(dT). DNA synthesis with [α-^{32}P]ATP, DNase I digestion, and 20% polyacrylamide gel electrophoresis in 7.0 M urea were performed as described. The nucleotide composition of the reaction mixtures was as follows: Lanes A, B, and C, ATP at 100 μM in the absence of dATP; lanes D, E, and F, ATP at 100 μM and dATP at 100 μM. Lanes B and C contained DNase I digests (0.8 and 1.6 units, respectively) of the reaction products appearing in lane A. Lanes E and F contained DNase I digests (0.8 and 1.6 units, respectively) of the reaction products appearing in lane D.

C with Fig. 1, lanes A and B). Thus, with both single-stranded M13 DNA and poly(dT) templates primase generates an oligonucleotide 15 residues in length which serves as a primer for DNA synthesis.

Deoxynucleotides Can Be Incorporated in Place of Ribonucleotides During Primer Synthesis. DNA polymerase α was

incubated with single-stranded M13 DNA, [α-^{32}P]ATP, and the four deoxynucleoside triphosphates. Upon digestion of the products with micrococcal nuclease and spleen phosphodiesterase, ^{32}P-labeled deoxynucleoside 3'-monophosphates were observed (Fig. 3A). Covalent ribo-deoxyribonucleotide linkages were therefore formed with the deoxynucleoside monophosphates positioned 5' to ^{32}P-labeled AMP. When DNA synthesis was performed under the same conditions except that the four ribonucleoside triphosphates were included in the reaction, the micrococcal nuclease–spleen phosphodiesterase digestion products again contained both ^{32}P-labeled deoxy- and ribonucleoside 3'-monophosphates (Fig. 3B). Covalent ribo-deoxyribonucleotide linkages are therefore formed even in the presence of the four ribonucleoside triphosphates. Thus, it is clear that deoxynucleotides can be incorporated in place of ribonucleotides during primer synthesis.

When poly(dT) was used as template for DNA synthesis in the presence of [α-^{32}P]ATP and dATP and the products of the reaction digested with micrococcal nuclease and spleen phosphodiesterase, the predominant ^{32}P-labeled nucleotide was 3'-AMP (Fig. 4, lane B). However, a small amount of labeled 3'-dAMP was also formed. Thus, with poly(dT) as template dAMP can replace AMP during primer synthesis; however, the primers synthesized are predominantly oligo-riboadenylates.

Primer Synthesis Occurs at Multiple but Preferred Sites on Single-Stranded M13 DNA. DNA polymerase α was incubated with single-stranded M13 DNA and either [α-^{32}P]ATP and the four deoxynucleoside triphosphates or the four ribonucleoside triphosphates, including [α-^{32}P]ATP, and the four deoxynucleoside triphosphates. The reaction products were treated with DNA polymerase III holoenzyme to produce full-length DNA strands, digested with the restriction endonucleases HinfI or Hpa II, and subjected to agarose gel electrophoresis. Multiple ^{32}P-labeled restriction fragments were generated indicating that primer synthesis occurs at many sites on the single-stranded M13 DNA template (Fig. 5, lanes A–D). Inasmuch as the restriction fragments contained different levels of radioactivity, primer synthesis was not initiated at random, but rather at preferred sites on the template.

DISCUSSION

The DNA primase from *Drosophila* embryos shows a striking resemblance to the primase activity identified by Reichard and Eliasson (3) in intact nuclei of polyoma-infected mouse fibro-
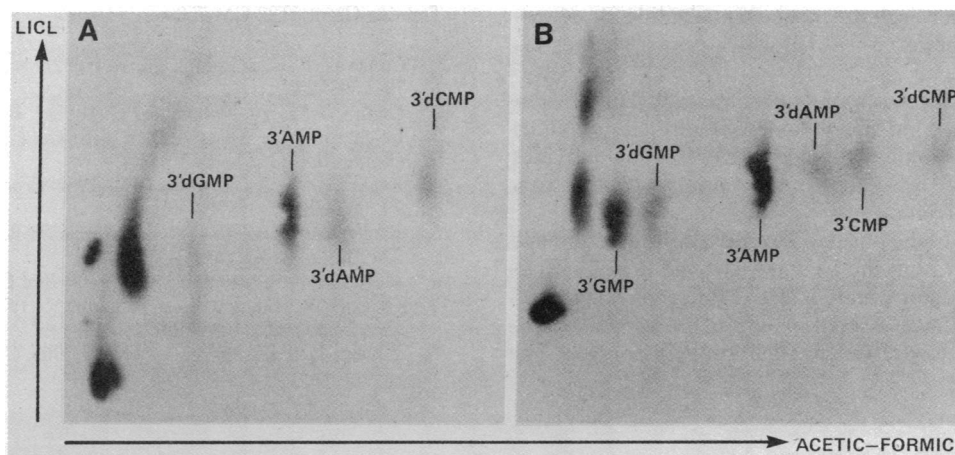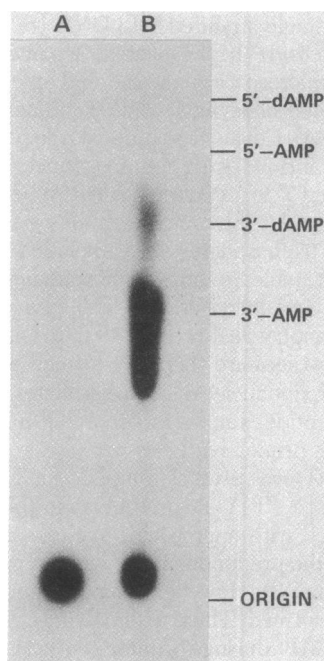

FIG. 3. Two-dimensional PEI-cellulose thin-layer chromatography of micrococcal nuclease and spleen phosphodiesterase digests of products synthesized with single-stranded M13 DNA template. DNA synthesis with [α-^{32}P]ATP, nuclease digestion, and thin-layer chromatography were performed as described. The nucleotide composition of the reaction mixtures was as follows: (A) ATP at 100 μM and the four deoxynucleoside triphosphates at 100 μM each; (B) the four ribonucleoside triphosphates and the four deoxynucleoside triphosphates at 100 μM each.

FIG. 4. One-dimensional PEI-cellulose thin-layer chromatography of micrococcal nuclease and spleen phosphodiesterase digests of products synthesized with poly(dT) as template in the presence of [α-^{32}P]ATP and dATP at 100 μM each. DNA synthesis, nuclease digestion, and thin-layer chromatography were performed as described. Lane A contained the untreated reaction product and lane B contained the reaction product after nuclease treatment.

blasts. (*i*) Initiation of primer synthesis requires ATP or GTP, with ATP the preferred substrate; UTP and CTP are inactive. (*ii*) The primer synthesized is of unique length (10 nucleotides in the case of mouse nuclei; 15 for the *Drosophila* enzyme). A similar chain length specificity has recently been observed in nuclear extracts of simian virus 40-infected monkey cells (G. Kaufman, personal communication) and in extracts of cultured human lymphoblasts (B. Y. Tseng, personal communication). (*iii*) Deoxyribonucleotides can be incorporated into the primer in place of ribonucleotides. (*iv*) Initiation of primer synthesis occurs at multiple, but nonrandom sites on the template. In the latter two respects the *Drosophila* primase also resembles the DNA primase (*dnaG* protein) of *E. coli* (8). Analysis of the primers synthesized on a single-stranded M13 DNA template should reveal whether there are common nucleotide sequences that correspond to preferred sites at which primer synthesis is initiated.

The finding that the *Drosophila* primase catalyzes the synthesis of mixed ribo-deoxyribo-oligonucleotides *with a unique chain length* in an α-amanitin-insensitive reaction provides strong support for the existence of a DNA primase distinct from the known RNA polymerases.

A unique feature of the purified *Drosophila* primase and one that distinguishes it from the bacterial enzyme is its close association with DNA polymerase α (1). Yagura *et al.* (2) have recently reported a similar association of primase with a subspecies of DNA polymerase α from Ehrlich ascites tumor cells. The
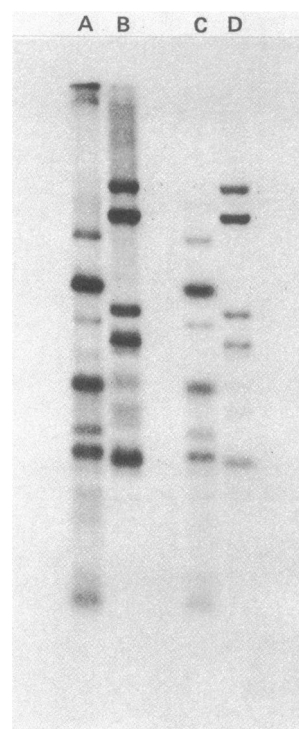

FIG. 5. Agarose gel electrophoresis of restriction endonuclease digests of products synthesized with single-stranded M13 DNA. DNA synthesis in the presence of ATP at 100 μM and the four deoxynucleoside triphosphates at 100 μM each (lanes A and B) or DNA synthesis in the presence of the four ribonucleoside triphosphates at 100 μM each and the four deoxynucleoside triphosphates at 100 μM each (lanes C and D) was performed as described with [α-^{32}P]ATP as the radioactive nucleotide. *Hinf*I (lanes A and C) and *Hpa* II (lanes B and D) restriction endonuclease digestion and 1.8% agarose gel electrophoresis were also performed as described.

finding that the association of primase with polymerase occurs in eukaryotic cells as diverse as those from *D. melanogaster* and the Ehrlich ascites tumor of mice suggests that such an association, should it persist, may be a general feature of eukaryotic DNA replication complexes.

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