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 polydeoxynucleotide templates, which can be elongated by DNA polymerase action (Conaway, R. C. & Lehman, I. R. (1982) Proc. Natl. Acad. Sci. USA 79, 2553–2557). 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-32P]ATP and [α-32P]CTP were from Amersham. Acrylamide, N,N’,methylenebis(acrylamide), N,N’,N’,N”-tetra-methylethylenediamine, ammonium persulfate, Bio-Gel A-1.5m, and Bio-Gel A-0.5m were obtained from Bio-Rad. Dithiothreitol was purchased from Calbiochem–Behring. Ultra-pure 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. Biswas of this department. Restriction endonuclease Hinfl was purchased from New England BioLabs and restriction endonuclease Hpa II was from Bethesda Research Laboratories. Micrococcal nu- clease 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 MgCl2, 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 deoxyribonucleoside triphosphates. [α-32P]ATP or [α-32P]CTP was present at 200 μCi per reaction (1 Ci = 3.7 × 1010 becquerels). With poly(dT), reactions were performed with 100 μM ATP in the presence or absence of 100 μM DATP. [α-32P]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 MgCl2, 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 MgCl2, 1 mM EDTA, 1 mM CaCl2, 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 endonucleases, 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 circular double-stranded form prior to restriction endonuclease digestion. The DNA polymerase III holoenzyme reaction mixtures (10 µl) contained 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 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 HindI or 1.5 units of Hpa II was added. Incubation was continued for 1 hr at 37°C. In the case of HindI reaction, NaCl was added to 100 mM prior to incubation. 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 polyacrylamide gels were autoradiographed with XAR-5 X-ray film (Kodak) at −70°C in the presence of an intensifying screen. The agarose gels were stained with ethidium bromide, photographed, 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 dimension with 0.38 M LiCl₂ and 20 mM Tris-HCl (pH 6.8). Chromatography in the second dimension was accomplished by complete development with 1.0 M acetic acid followed by development to the level of 3'-AMP with 1.0 M formic acid.

**RESULTS**

**DNase I Digestion of the Products of the Coupled Primase–DNA Polymerase α Reaction Yields an Oligonucleotide of Unique Length.** When DNA polymerase α was incubated with single-stranded M13 DNA, the four ribonucleoside triphosphates including [α-³²P]ATP, and the four deoxynucleoside 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-³²P]CTP (data not shown).

When the products of the coupled primase–DNA polymerase α reaction performed with single-stranded M13 DNA, [α-³²P]-ATP, and the four deoxynucleoside triphosphates were digested with DNase I and subjected to polyacrylamide gel electrophoresis, a graded series of oligonucleotides was observed (Fig. 1, lanes C and D) with a maximum length of 15 residues. The distribution 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-³²P]ATP in the absence of the four deoxynucleoside triphosphates resulted in the synthesis of an oligonucleotide of approximately 28 residues (Fig. 1, lane A). After digestion with DNase I, an oligonucleotide 15 residues in length was again generated (Fig. 1, lane B). A similar result was obtained with [α-³²P]CTP (data not shown). The decrease in chain length after digestion with DNase I is presumably a consequence of incorporation into the primer of deoxynucleotides present as contaminants in the mixture of the four ribonucleoside triphosphates. In fact, when this reaction was performed in the absence 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-³²P]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 distribution of oligonucleotides with maximum length of 15 residues was obtained (Fig. 2, lanes D, E, and F). This distribution resembles that observed after DNase I digestion of the DNA synthesized 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-³²P]ATP in the absence of dATP, a 28-residue oligonucleotide was synthesized (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-³²P]ATP and the other three ribonucleoside triphosphates (compare Fig. 2, lanes A, B, and
incubated with single-stranded M13 DNA, \([\alpha-32P]ATP\), 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 \([\alpha-32P]ATP\) 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 \(\alpha\) was incubated with single-stranded M13 DNA and either \([\alpha-32P]ATP\) and the four deoxynucleoside triphosphates or the four ribonucleoside triphosphates, including \([\alpha-32P]ATP\), 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 *Hinf I* 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-

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**Fig. 2.** Polyacrylamide gel electrophoresis of DNase I digests of products synthesized with poly(dT). DNA synthesis with \([\alpha-32P]ATP\), 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 \(\mu\)M in the absence of dATP; lanes D, E, and F, ATP at 100 \(\mu\)M and dATP at 100 \(\mu\)M. 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 \(\alpha\) was

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**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 \([\alpha-32P]ATP\), nuclease digestion, and thin-layer chromatography were performed as described. The nucleotide composition of the reaction mixtures was as follows: (A) ATP at 100 \(\mu\)M and the four deoxynucleoside triphosphates at 100 \(\mu\)M each; (B) the four ribonucleoside triphosphates and the four deoxynucleoside triphosphates at 100 \(\mu\)M each.
bleasts. (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 subpopulation of DNA polymerase α from Ehrlich ascites tumor cells. The 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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