Interaction of Ribonuclease H from Drosophila melanogaster with DNA Polymerase-Primase*

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An RNase H was purified 2,500-fold to near homogeneity from early embryos of Drosophila melanogaster. The purified enzyme has an approximate molecular weight of 180,000 and appears to consist of two 49,000- and two 39,000-dalton polypeptides. The enzyme specifically hydrolyzes RNA-DNA hybrids and releases oligoribonucleotides ranging in size from 2–9 residues. The RNase H can also remove RNA primers that are synthesized and subsequently elongated by the Drosophila polymerase-primase.

Preincubation of the RNase H from D. melanogaster embryos with the homologous DNA polymerase-primase results in an increased rate of DNA synthesis. The DNA chains synthesized under these conditions are shorter than those synthesized in the absence of the RNase H, and the rate of primer synthesis is increased significantly. These findings suggest that the RNase H forms a complex with the polymerase-primase, increasing its recycling capacity and thereby increasing the frequency of chain initiation.

We have undertaken the analysis of DNA replication in embryos of Drosophila melanogaster. Our approach has been to purify enzymes analogous to those known to be essential for DNA replication in prokaryotes, and with the purified enzymes, to reconstitute a replication complex or “replisome” that can function at a replication fork in vitro.

These studies began with the purification of the DNA polymerase α from D. melanogaster embryos (1, 2). The near-homogeneous enzyme was found to contain a DNA primase activity, an indispensable component of a eukaryotic replication complex (3). Further analysis of the Drosophila polymerase-primase showed it to lack other, adjunctive, replication enzymes, for example, 3’–5’ exonuclease, DNA helicase, and polymerase, increasing its recycling capacity and thereby increasing the frequency of chain initiation.

The DNA chains synthesized under these conditions are shorter than those synthesized in the absence of the RNase H, and the rate of primer synthesis is increased significantly. These findings suggest that the RNase H forms a complex with the polymerase-primase, increasing its recycling capacity and thereby increasing the frequency of chain initiation.

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RNase H, a ribonuclease that specifically degrades the RNA component of an RNA-DNA hybrid was first described by Okazaki (5) and Stein (6) in calf thymus extracts and subsequently found to be widely distributed among pro- and eukaryotes (6). Since RNA serves as a primer for the synthesis of Okazaki fragments (6), a plausible role for RNase H in DNA replication is to remove the RNA primers from Okazaki fragments prior to their ligation (6). The involvement of RNase H in the initiation of the plasmid ColEl and bacterial chromosomal replication has also been reported (7, 8).

An examination of side fractions obtained during purification of the Drosophila polymerase-primase showed one of them to be highly enriched in RNase H activity. We describe here the purification of the RNase H to near homogeneity, and consider its structure and catalytic properties. We also describe a novel feature of the Drosophila RNase H: its ability to stimulate DNA synthesis catalyzed by the Drosophila polymerase-primase.

**EXPERIMENTAL PROCEDURES**

**Materials**

Nucleotides and Homopolymers—Unlabeled deoxy- and ribonucleoside triphosphates and poly- and oligonucleotides were purchased from P-L Biochemicals. [3H]dTTP was purchased from New England Nuclear. [γ-32P]ATP, [γ-32P]dTTP, and [α-32P]poly(A) (chain length 40–140 nucleotides; specific activity 574 mCi/mmol) were purchased from Amersham Corp.

Nucleic Acids—Poly(dT)1000 or poly(U)1000 was annealed to [3H]poly(A) for 25 min at 65 °C at a 2:1 molar ratio of thymine or uracil to adenine in a buffer consisting of 10 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, and 0.02 mM sodium citrate. [3H]labeled double-stranded DNA was prepared by incubating activated calf thymus DNA (9) with Escherichia coli DNA polymerase I in the presence of [3H]dTTP. 2°H-labeled ssDNA refers to a solution of [3H]labeled double-stranded DNA made 0.1 n in NaOH, then neutralized. M13mp8 and M13GoriI ssDNAs were gifts from D. Sollits of this department.

Chromatography—Phosphocellulose P11, DEAE-Sephadex A-50, and Bio-Sil TSK-250 were purchased from Whatman, Pharmacia Fine Chemicals, and Bio-Rad, respectively. Single-stranded DNA-cellulose was prepared according to the procedure of Alberts and Herrick (10). Collodion membranes were purchased from Schleicher & Schuell.

Enzymes—The Drosophila DNA polymerase-primase was prepared as described previously (2). E. coli DNA polymerase III holoenzyme, RNase H, and single-stranded DNA binding protein were the gifts of M. O'Donnell, L. Bertsch, and D. Sollits of this department, respectively. E. coli DNA ligase and DNA polymerase I were purchased from United States Biochemical Corp. and P-L Biochemicals, respectively. Bovine serum albumin was purchased from Pentex. Thyroglobulin, bovine γ-globulin, chicken ovalbumin, and bovine myoglobin were obtained from Bio-Rad. Rabbit muscle myosin, E. coli β-galactosidase, rabbit muscle phosphorylase B, and bovine carbonic anhydrase were purchased from Sigma.

Buffers—All potassium phosphate buffers were at pH 7.6 and contained 1 mM 2-mercaptoethanol, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium bisulfite, 2 μg/ml leupeptin, and 10% glycerol. The ionic strength of buffers was checked with a radiometer conductivity meter.

**Methods**

**RNase H Assay**—Reaction mixtures (0.05 ml) contained 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 10 mM (NH₄)₂SO₄, 10 mM MgCl₂, 200 μg of bovine serum albumin, [3H]poly(A) (19 μM, 40 cpm/μmol) annealed to poly(dT) (20 μM), and enzyme. Incubation was for 15 min at 37 °C. Reactions were terminated by the addition of 0.05 ml of calf thymus DNA (1 mg/ml) and 0.2 ml of 10% SDS.
trichloroacetic acid. The tubes were kept on ice for 10 min and then centrifuged for 10 min at 7000 rpm. Aliquots (0.2 ml) of the supernatant fluid and the resulting precipitate collected by centrifugation at 32,000 g for 20 min, and the supernatant fluid was loaded onto a column (6.6 cm x 6 cm) of single-stranded DNA cellulose equilibrated with 20 mM potassium phosphate containing 60 mM NaCl, at the rate of 40 ml/h. The column was washed (290 ml/h) with 160 ml of buffer containing 120 mM NaCl, followed by 1 ml of buffer containing 300 mM NaCl. Active fractions, eluted with the latter buffer, were pooled (Fraction IV) and solid (NH4)2SO4 was added (0.472 g/ml). The precipitate was collected and dried before centrifugation at 80 °C before resuspension in T buffer (50 mM Tris-HCl (pH 8.5), 10% glycerol, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol (Fraction IVb).

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. S100a</td>
<td>1,887</td>
<td>11,184</td>
<td>7,133</td>
<td>63.9%</td>
<td>100</td>
</tr>
<tr>
<td>II. Phosphocellulose</td>
<td>2,734</td>
<td>1,121</td>
<td>2,406</td>
<td>214%</td>
<td>33.7</td>
</tr>
<tr>
<td>III. Ammonium sulfate</td>
<td>162</td>
<td>670</td>
<td>1,704</td>
<td>254%</td>
<td>23.8</td>
</tr>
<tr>
<td>IV. DNA-cellulose</td>
<td>24</td>
<td>8.4</td>
<td>302</td>
<td>15,500</td>
<td>18.3</td>
</tr>
<tr>
<td>V. DEAE-Sephadex</td>
<td>11.8</td>
<td>11.1</td>
<td>564</td>
<td>52,700</td>
<td>7.9</td>
</tr>
<tr>
<td>VI. HPLC</td>
<td>1.0</td>
<td>0.16</td>
<td>240</td>
<td>150,000</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*a Fraction I was prepared from 432 g of freshly harvested embryos.

**RESULTS**

Physical Properties of D. melanogaster RNase H

NaDodSO4-polyacrylamide gel electrophoresis of Fraction VI yielded two major polypeptides, with Mr values of 49,000 and 39,000 (Fig. 1). Densitometric scanning of the gel stained with Coomassie Blue showed the relative abundance of the Mr 49,000 and 39,000 polypeptides to be 1.0/1.5 (Fig. 1, right). The two polypeptides together accounted for approximately 75% of the protein applied to the gel.

When the peak fractions obtained by the high performance liquid chromatography were subjected to NaDodSO4-polyacrylamide gel electrophoresis and stained with Coomassie Blue, the Mr 49,000 and 39,000 polypeptides corresponded closely to the RNase H activity (data not shown).

The molecular weight of the native RNase H was determined by Bio-Sil TSK-250 gel filtration in the presence of 0.2 M (NH4)2SO4. RNase H activity eluted as a major peak with a molecular weight of 186,000 (Fig. 2). Based on its behavior during NaDodSO4-polyacrylamide gel electrophoresis and Bio-Sil TSK-250 gel filtration, the Drosophila RNase H appears to be a heterotetramer composed of two 49,000-dalton and two 39,000-dalton subunits.

**Characterization of RNase H Activity**

**Reaction Requirements**—The pH optimum in 50 mM Tris-HCl was 7.5; the reaction rates at pH 7.2 and 8.5 were 38 and 45%, respectively, of the rate at pH 7.5. A divalent cation was absolutely required; the optima for MgCl2 and MnCl2 were 10 mm and 0.4 mm, respectively; the former stimulating 3-fold more efficiently than the latter. RNase H activity was optimal at 10 mm (NH4)2SO4; however, varying the concentration of (NH4)2SO4 from 0–40 mM resulted in less than a 30% change in the rate of reaction.

**Substrate Specificity**—The substrate specificity of the Drosophila RNase H is summarized in Table II. The enzyme was specific for an RNA-DNA hybrid. There was a linear depend-
**RNase H from D. melanogaster**

**FIG. 1.**  NaDodSO₄-polyacrylamide gel electrophoresis of Drosophila RNase H. Left, Fraction VI (4 µg) was denatured and electrophoresed in a 5-10% linear gradient NaDodSO₄-polyacrylamide slab gel. Marker proteins run in adjacent lanes and indicated by their molecular weights (× 10⁻³) were myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase. Right, densitometric scan of lane shown on the left. The molecular weights (× 10⁻³) of the two major polypeptides are indicated.

**TABLE II**  Substrate specificity of RNase H from D. melanogaster

<table>
<thead>
<tr>
<th>Substrate</th>
<th>RNase H added</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]Poly(A) - poly(dT)</td>
<td>0.075</td>
<td>0.225</td>
</tr>
<tr>
<td>[³H]Poly(A)</td>
<td>0.075</td>
<td>7.5</td>
</tr>
<tr>
<td>[³H]Poly(A) - poly(U)</td>
<td>0.075</td>
<td>7.5</td>
</tr>
<tr>
<td>³H-labeled double-stranded DNA</td>
<td>0.075</td>
<td>7.5</td>
</tr>
<tr>
<td>³H-labeled single-stranded DNA</td>
<td>0.075</td>
<td>7.5</td>
</tr>
</tbody>
</table>

The presumed substrate for the Drosophila RNase H in vivo is an RNA primer that had initiated Okazaki fragment synthesis. Circular ssDNA, partially replicated by the Drosophila polymerase-primase in the presence of [α³²P]ATP, was treated with increasing levels of RNase H, and the products were analyzed by neutral agarose gel electrophoresis (Fig. 3A). In the absence of RNase H (1st lane), there was a distribution of partially replicated ssDNA circles. Upon addition of increasing levels of RNase H, the number of labeled molecules decreased (2nd through 4th lanes). In contrast, RNase H treatment of ssDNA that was partially replicated by the polymerase-primase in the presence of [³²P]dTTP, had no effect on the size distribution of the newly synthesized DNA chains (Fig. 3B). Thus, the Drosophila RNase H specifically
RNase H from D. melanogaster

Fig. 3. Hydrolysis by RNase H of RNA primers synthesized by Drosophila polymerase-primase. A, M13mp8 ssDNA was replicated by the Drosophila polymerase-primase in the presence of [α-32P]ATP, using conditions previously described (14). The replicated DNA was treated with increasing levels of RNase H and analyzed by neutral agarose gel electrophoresis. The gel was stained with ethidium bromide (0.5 µg/ml) to visualize DNA markers, dried under vacuum, and exposed to Kodak XAR-5 x-ray film. B, DNA synthesis was carried out as described above except that [α-32P]dTTP was the labeled nucleotide. The replicated DNA was treated with increasing levels of RNase H and analyzed by alkaline agarose gel electrophoresis. Molecular weight markers, whose sizes are indicated in nucleotide residues, were produced by HaeIII digestion of φX174 supercoiled duplex DNA. They were run in an adjacent lane and visualized by ethidium bromide staining.

Table III

Products of digestion of [3H]poly(A)-poly(dT) by RNase H

<table>
<thead>
<tr>
<th>Products</th>
<th>0 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(A)</td>
<td>417</td>
<td>60</td>
</tr>
<tr>
<td>rA4</td>
<td>6.7</td>
<td>58.8</td>
</tr>
<tr>
<td>rA3</td>
<td>4.3</td>
<td>68.8</td>
</tr>
<tr>
<td>rA2</td>
<td>8.6</td>
<td>59.1</td>
</tr>
<tr>
<td>rA</td>
<td>7.7</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Hydrolyzes RNA primers synthesized by the polymerase-primase, leaving the newly synthesized DNA chains intact.

The RNase H can remove the last ribonucleotide of an RNA primer attached to the 5' end of the DNA, as judged by its ability to generate a suitable substrate for E. coli DNA ligase (15). Hydrolysis of the last ribonucleotide does, however, appear to be rather inefficient (approximately 10% the rate at which the bulk of the ribonucleotide residues are cleaved).

Analysis of Products—The products in an exhaustive digest of [3H]poly(A)-poly(dT) were analyzed by descending paper chromatography. As summarized in Table III, a distribution of oligonucleotides ranging from 2–9 residues was generated. Although 85% of the [3H]poly(A) was degraded to acid-soluble products by the Drosophila RNase H, like similar enzymes (16–23), less than 0.5% was in the form of [3H]AMP.

Stimulation of Polymerase-Primase by RNase H

Preincubation of the Drosophila polymerase-primase for 20 min at 0 °C with the purified RNase H and M13mp8 ssDNA produced a significant stimulation in DNA synthesis upon subsequent incubation at 30 °C (Fig. 4). Stoichiometric amounts of RNase H were required for the stimulation. Thus, an increase in the molar ratio of RNase H to polymerase-primase from 0.8 to 8.0 produced an approximate 5-fold stimulation in DNA synthesis. The stimulation occurred only with unprimed ssDNA and was not observed with either singly primed φX174 ssDNA or with activated duplex DNA (data not shown). The stimulation was also specific for the Drosophila RNase H. Under the same conditions, RNase H from E. coli at molar ratios ranging from 1 to 10 had no effect (data not shown).

Identity of RNase H and Stimulatory Activity

To determine whether the RNase H and stimulatory activities resided in the same protein, the purified RNase H (Frac-
column. As shown in Fig. 2, RNase H and stimulatory activity was observed without preincubation of the RNase H together with the fact that the RNase H has been purified co-eluted perfectly as a 186,000-dalton protein. This finding, the two activities reside in different proteins.

Preincubation—Less than 2-fold stimulation in DNA synthesis was observed without preincubation of the RNase H and polymerase-primase (Fig. 5). As the time of preincubation was increased the level of stimulation increased correspondingly, so that at 30 min there was an approximate 8-fold stimulation. The requirement for preincubation suggests that there is a time-dependent formation of a complex between the RNase H and polymerase-primase. In fact, a fraction of the Drosophila RNase H remains associated with polymerase-primase during purification of the RNase H. When the DEAE-Sephadex fraction (Fraction V) of RNase H was applied to a high performance liquid chromatography gel filtration column, approximately 10-15% of the RNase H activity recovered eluted as a protein of greater than 380,000 daltons, in association with the polymerase-primase.3

Ionic Strength—Increasing the concentration of (NH₄)₂SO₄ from 1 to 41 mM had only a slight (inhibitory) effect on DNA synthesis catalyzed by the polymerase-primase. In contrast, there was a significant effect of ionic strength during the preincubation. At low (NH₄)₂SO₄ concentrations (1-11 mM) there was an approximate 2-fold stimulation in DNA synthesis; maximum stimulation occurred at 21 mM (approximately 4-fold). However, at 41 mM (NH₄)₂SO₄ the stimulation fell to 2-fold. The interaction between the RNase H and polymerase-primase thus appears to be influenced by ionic strength.

Effect of RNase H on DNA Products Synthesized by the Polymerase-Primase

To investigate the site (chain initiation or elongation) at which the RNase H exerts its stimulatory effect, the products of DNA synthesis were examined by alkaline agarose gel electrophoresis (Fig. 6). At 15 min in the absence of RNase H, the newly synthesized DNA chains were 250-900 nucleotides in length (Fig. 6, lane 1). As the reaction proceeded, the size of the products increased, and at 60 min they ranged from 500 to 1600 nucleotides (Fig. 6, lanes 2-4). In the presence of RNase H, DNA chains in the size range 100-500 nucleotides were observed at 15 min (Fig. 6, lane 5). This size distribution is approximately 2-fold smaller than that seen with the polymerase-primase alone (compare lanes 1 and 5). After 60 min, the products increased to 400-1000 nucleotides (Fig. 6, lanes 6-8), but were still smaller than those synthesized by the polymerase-primase alone. Thus, under conditions where the

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RNase H stimulates the polymerase-primase, the DNA chains are significantly shorter than those synthesized by the polymerase-primase alone. This finding suggests that the RNase H stimulates DNA synthesis by increasing the number rather than the length of DNA chains synthesized (i.e., by increasing the number of primers).

**Stimulation of Primer Synthesis by RNase H**

Since degradation of RNA primers occurs in the coupled primase-polymerase reaction carried out in the presence of RNase H (data not shown), we were unable to measure primer synthesis directly. However, the coupled assay for primer synthesis, which scores the number of primed M13mp8 ssDNA circles synthesized by the polymerase-primase, does permit accurate measurement of the rate of primer synthesis. As shown in Fig. 7, a burst of primer synthesis occurred in the first 10 min of the reaction performed with polymerase-primase alone. However, during the course of the next 50 min the number of primers increased by only 50%. When primer synthesis was carried out in the presence of RNase H, there was also an initial burst of primer synthesis. In this case, however, synthesis continued at a linear rate for approximately 30 min. Thus, the RNase H appears to increase the recycling capability of the polymerase-primase, thereby allowing it to increase the number of primers synthesized.

To test this hypothesis, a DNA challenge experiment using the coupled assay was performed. Primer synthesis was initiated with M13mp8 ssDNA as template; at 10 min, M13Goril ssDNA was added and the reaction allowed to proceed for an additional 60 min. As shown in Fig. 8a, RNase H stimulated primer synthesis approximately 3-fold. To determine which of the ssDNA templates was utilized by the polymerase-primase, aliquots were removed from the two reactions (+ RNase H) and analyzed by agarose gel electrophoresis (Fig. 8b). With polymerase-primase alone, the M13mp8 ssDNA was replicated preferentially; however, a low level of the challenging M13Goril ssDNA also underwent replication (Fig. 8b, 1st through 6th lanes). A densitometric scan of the autoradiograph revealed that 10 min after addition of the challenge DNA, 10% of the replicated DNA was M13Goril, which increased to approximately 25% after 60 min. In priming reactions carried out in the presence of RNase H, approximately 20% of the replicated DNA was M13Goril at 10 min after the addition of the challenge ssDNA, which increased to 50% after 60 min (Fig. 8b, 7th through 12th lanes). Thus, the RNase H increases the recycling capacity of the polymerase-primase, thereby increasing the number of primers synthesized.

**DISCUSSION**

We have purified an RNase H from early embryos of *D. melanogaster* approximately 2,500-fold, to near homogeneity. It has an approximate molecular weight of 180,000 and appears to consist of two 49,000- and two 39,000-dalton polypeptides. The eukaryotes that have been examined thus far (e.g., yeast, calf thymus, and rat liver) possess several species of RNase H, which fall into two size classes: a low molecular weight form with *M* of 70,000 to 120,000, and a high molecular weight form with *M* values ranging from 20,000 to 40,000, and a high molecular weight form with *M* values of 70,000 to 90,000 (17-23). In contrast, we have observed only a single RNase H in *Drosophila* embryos which is considerably higher than even the high molecular forms found in other eukaryotes.

Like the analogous enzymes from yeast, calf thymus, and KB cells, the *Drosophila* RNase H is specific for RNA-DNA hybrids. Activity with either single- or double-stranded RNA is approximately 3 orders of magnitude lower than that with the hybrid substrate. Moreover, neither single- nor double-stranded DNA are susceptible to hydrolysis by the *Drosophila*
enzyme. Similarly, upon digestion of an RNA-DNA hybrid the enzyme releases oligoribonucleotides ranging in size from 2-9 residues.

The physiological role of RNase H in eukaryotes has yet to be firmly established. It has been suggested that the multiple forms of RNase H have different functions in vivo. Biesni et al. (24) have proposed that RNase H IIb from calf thymus, a low molecular weight form, is involved in "RNA metabolism", while the RNase H1, an 80,000-dalton protein, is required for DNA synthesis. In the case of yeast, an RNase H activity has been identified that is associated with RNA polymerase I, suggesting that it is involved in transcription of ribosomal RNA genes (25). A yeast RNase H activity has also been described that stimulates DNA polymerase I activity (19). We have shown that the Drosophila RNase H is capable of removing RNA primers that were synthesized and subsequently elongated by the Drosophila polymerase-primase, suggesting that it may play a role in DNA replication.

A novel feature of the purified RNase H from Drosophila is its ability to stimulate DNA synthesis by the homologous DNA polymerase-primase. The stimulation is unique in that it is specific for a coupled reaction in which both priming and subsequent chain elongation are catalyzed by the polymerase-primase. Karwan et al. (19) recently reported that an RNase H activity purified from yeast can stimulate the yeast DNA polymerase-primase. The stimulation is unique in that it is specific for a coupled reaction in which both priming and chain elongation are catalyzed by the polymerase-primase complex. In both the rate and processivity of the polymerase are markedly increased (51) despite their relatively weak interaction. Similarly, the C1C3 primer recognition proteins isolated from yeast cells form a complex with the homologous DNA polymerase α, thereby enhancing the ability of the polymerase to locate primers by eliminating nonproductive binding to ssDNA (27, 28).

In our efforts to reconstitute a complex of enzymes from D. melanogaster embryos that can catalyze the efficient synthesis of Okazaki fragments, we have isolated a DNA polymerase-primase and an RNase H, activities that should be essential components of such a complex. The DNA polymerase-primase is essential for Okazaki fragment synthesis; the synthesis and subsequent extension of an RNA primer. The RNase H should then remove the RNA primers, permitting joining of the Okazaki fragments by DNA ligase. Although these proteins can catalyze the basic reactions required for Okazaki fragment synthesis, other replication factors (e.g. helix destabilizing proteins, processivity factors, etc.) that should increase the efficiency of this reaction must also be involved. Efforts are currently under way to identify such proteins in Drosophila embryos.

REFERENCES