Interaction of Ribonuclease H from Drosophila melanogaster Embryos 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.

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 RNase H. RNase H forms a complex with the polymerase-primase, increasing its recycling capacity and thereby increasing the frequency of chain initiation.

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.

**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. [3H]ATP, [3H]dTTP, and [3H]poly(A) (chain length 40-140 nucleotides; specific activity 574 mCi/mmol) were purchased from Amersham Corp.

Nuclease Activities—Poly(dT)1000 or poly(U)1000 was annealed to [3H]poly(A) for 20 min at 65 °C in a 2:1 molar ratio of thymine or uracil to adenine in a buffer consisting of 10 mM Tris.HCl (pH 8.0), 0.3 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. [3H]labeled ssDNA refers to a solution of [3H]labeled double-stranded DNA made 0.1 mM NaOH, then neutralized. M13mp8 and M13GoriI ssDNAs were gifts from D. Sollis 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. Solis 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. Thryoglobulin, 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 cpn/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% 

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The abbreviations used are: ssDNA, single-stranded DNA; Na-DodSO₄, sodium dodecyl sulfate.
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 were counted in a Tricoros-based scintillator. One unit is defined as 1 nmol of [3H]poly(A) hydrolyzed in 15 min at 37 °C.  

**Assay for DNA Synthesis**—Reaction mixtures (0.05 ml) contained 50 mM Tris-HCl (pH 8.5), 5 mM 2-mercaptoethanol; 10 mM MgCl$_2$; 20 mM (NH$_4$)$_2$SO$_4$; 200 µg of bovine serum albumin; 500 µM (each) ATP, GTP, CTP, and UTP; 100 µM (each) dATP, dGTP, dCTP, and dUTP; and [32P]dGTP ([3H]poly(A) was 200 cpm/pmol. Following incubation at 37 °C, aliquots (20 µl) were withdrawn and added to a solution (5 µl) containing 1.3 µg of E. coli single-stranded DNA binding protein and 30 units of DNA polymerase III holoenzyme. After 15 min at 30 °C, [32P]dTTP incorporated into acid-insoluble material was determined by scintillation counting.

**Assay for Primer Synthesis**—Primer synthesis was measured in a coupled assay with a full-length circle by the DNA polymerase I11. The rate of reaction was measured by centrifugation of 24,000 × g for 20 min, and the supernatant fluid was loaded onto a column (6.6 cm × 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 (240 ml/h) with 160 ml of buffer containing 120 mM NaCl, followed by 80 ml of buffer containing 300 mM NaCl. Active fractions, eluted with the latter buffer, were pooled (Fraction IV eod and solid (NH$_4$)$_2$SO$_4$ was added (0.472 g/ml). The precipitate was collected by centrifugation and Bio-Sil TSK-250 gel filtration, the Drosophila RNase H activity eluted as a major peak at 100 mM (NH$_4$)$_2$SO$_4$ and 1 mM 2-mercaptopethanol, 1 mM EDTA, and 10% glycerol (Fraction Vb).
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 (X 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 (× 10⁴) of the two major polypeptides are indicated.

**TABLE II**

Substrate specificity of RNase H from D. melanogaster

| Substrate                     | RNase H added | Activity units | pmol/15 min |
|-------------------------------|---------------|----------------|-------------|
| [³H]Poly(A)-poly(dT)          | 0.075         | 71.6           |             |
| [³H]Poly(A)                   | 0.225         | 244            |             |
| [³H]Poly(A)-poly(U)           | 0.075         | <1.0           |             |
| ³H-labeled double-stranded DNA| 7.5           | 9.0            |             |
| ³H-labeled single-stranded DNA| 0.075         | <1.0           |             |

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
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 of DNA synthesis was observed without preincubation of the RNase H. However, together with the fact that the RNase H has been purified to co-elute perfectly as a 186,000-dalton protein. This finding, the two activities reside in different proteins.

The legend to Fig. 4 describes the method by which the RNase H was preincubated in the absence of (NH₄)₂SO₄, stimulation of DNA synthesis by the polymerase-primase. In fact, a fraction of the Drosophila RNase H remains associated with 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.

requirement for stimulation of the RNase H by RNase H. Polymerase-primase (90 ng) was preincubated in the absence or presence of RNase H (400 ng) for increasing periods of time at 0 °C, using the conditions described in the legend to Fig. 4. After preincubation, ribo- and deoxyribonucleoside triphosphates were added. Incubation was for 30 min at 30 °C and [³²P]dTMP incorporated into acid-insoluble material was determined.

**Requirements for RNase H Stimulation of DNA Synthesis by Polymerase-Primase**

**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.

**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

![Fig. 5. Preincubation requirement for stimulation of the polymerase-primase by RNase H.](image)

![Fig. 6. Alkaline agarose gel electrophoresis of products of DNA synthesis.](image)

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3 R. DiFrancesco and I. R. Lehman, unpublished observation.
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, 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. Bisson 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 I. However, in contrast to the Drosophila enzyme, stimulation was observed with such primed templates as poly(dA)-oligo(dT) and activated DNA. The specificity of stimulation by the Drosophila RNase H also differs from that observed with the primer recognition factors that have been identified in yeast, human, and monkey cells, as well as in Drosophila embryos (26–30).

The Drosophila RNase H appears to stimulate DNA synthesis by forming a complex with the polymerase-primase, enhancing its recycling capacity, and thereby increasing the rate of primer synthesis. Several lines of evidence support this hypothesis. Thus, preincubation of stoichiometric amounts of the homologous RNase H and polymerase-primase are required to observe stimulation. Furthermore, a fraction of the RNase H remains in association with the polymerase-primase during RNase H purification. This association may reflect an intact RNase H-polymerase-primase complex that had not undergone dissociation during purification. The fact that only a relatively small fraction (10–15%) of the RNase H remains associated with polymerase-primase up to the final step in the purification suggests that the interaction between these proteins is relatively weak. Attempts to isolate a complex following incubation of the two enzymes under conditions that yield maximal stimulation, by either gel filtration or glycerol gradient sedimentation, have thus far been unsuccessful.

The interaction of RNase H and polymerase-primase may be analogous to the situation with the bacteriophage T4 DNA polymerase and its various polymerase-accessory factors (gene 44/62 and gene 45 proteins). In the presence of these proteins both the rate and processivity of the polymerase are markedly increased (31) despite their relatively weak interaction. Similarly, the CcCj primer recognition proteins isolated from monkey 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.

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