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Adrenal Cortical Ribonuclease H (Hybrid)[1] (38494)

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(Introduced by J. Holland)

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An enzyme that specifically degrades the RNA strand of an RNA:DNA hybrid (RNase H) was first detected in calf thymus by Hausen and Stein (1, 2) and subsequently highly purified by Stavrianopoulos and Chargaff (3). Several recent studies have indicated a general requirement for RNA synthesis in DNA replication (4–11). Because the known DNA polymerases can only extend polynucleotide chains, these observations have been interpreted as indicating a requirement for the synthesis of a short RNA chain which exists as an RNA:DNA hybrid. Sugino *et al.* (10) have shown that a short strand of 50–100 nucleotides of RNA is covalently attached to nascent "Okazaki" DNA fragments; this RNA is removed before ligation of the DNA fragments. Recent evidence indicates that a similar segment of RNA is covalently attached to mammalian DNA (12). In addition, mammalian mitochondrial DNA supercoils have been shown to be alkali and RNase H sensitive, suggesting that an RNA fragment is incorporated in a manner similar to that observed in col E_1 DNA supercoils in chloramphenicol-treated *Escherichia coli* (13, 14). The RNA primer may be removed by the $5' \rightarrow 3'$ exonuclease function of DNA polymerase I (6, 15); alternatively, RNase H may function to remove the RNA primer from DNA chains and from an integrated position in closed circular duplex DNA.

The present report describes the partial purification and characterization of the RNase H present in adrenal cortical tissue.[3] The adrenal RNase H specifically degrades the RNA strand of RNA:DNA hybrids endonucleolytically, producing oligoribonucleotides with 3′-hydroxyl and 5′-phosphate termini. ACTH administration results in increased adrenal cortical RNA synthesis followed by increased DNA synthesis (17, 18). The increase in DNA synthesis begins 10 hr following initiation of ACTH treatment and is accompanied by increases in DNA polymerase and thymidine kinase enzymatic activities. In the present studies, RNase H activity has been examined during the course of induction of DNA synthesis by ACTH. RNase H activity has also been examined under conditions of ACTH-induced inhibition of DNA synthesis (19).

Materials and Methods. Materials are those described previously (20). RNA:DNA hybrid was prepared using denatured calf thymus DNA as template for *E. Coli* RNA polymerase; [^{3}H]UMP-labeled product was isolated as described by Hausen and Stein (2). The hybrid contained 200 cpm/pmole of incorporated UMP. Double labeled hybrid was prepared in a similar manner using [α^{32}P]UTP and [^{3}H]ϕX174 single-stranded DNA. Single-stranded RNA was prepared using native calf thymus DNA in the above reaction.

Standard reaction mixtures (0.065 ml) contained 0.1 *M* $(NH_4)_2SO_4$, 0.03 *M* Tris–HCl (pH 7.9 at 25°), 0.001 *M* $MnCl_2$, and nucleic acid substrate and enzyme as indicated in the text. Assays were incubated at 37° for the indicated times and were terminated by spotting 0.05 ml aliquots directly onto Whatman DE81 filter paper discs (diam 2.4 cm). The filters were subjected to five 10 min washes in 5% dibasic sodium phosphate, rinsed twice in deionized water, once in 95% ethanol, once in ether, dried and counted in 10 ml of scintillant containing 42 ml Liquifluor (New England Nuclear) per liter of toluene. Only undegraded hybrid adheres to the filters. Enzmye activity, ex-

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[3] Some of this work has been reported earlier in abstract form (16).

pressed as pmoles of UMP released from hybrid, was calculated by subtracting the amount of hybrid retained on the filter from the amount retained in a parallel assay lacking enzyme, *i.e.* (pmoles UMP released from hybrid by enzyme) = (pmoles UMP retained on filters in control assay without enzyme) − (pmoles UMP retained on filters in assay with enzyme). All assays were performed in duplicate or triplicate.

Bovine adrenal cortices were obtained in ice from a local abattoir. Guinea pig adrenal cortices were fractionated as previously described (17, 18). The dose and time schedule of ACTH administration is indicated in the text. Y-1 cells were treated as described by Masui and Garren (19).

Results. Characterization of adrenal cortical RNase H. RNase H was partially purified from the cytosol of isolated bovine adrenal cortices by ammonium sulfate fractionation, calcium phosphate adsorption and chromatography on DEAE52 and on Sepharose 6B. The active fractions from the Sepharose columns comprised less than 0.2% of the protein present in the crude homogenate and released 50.7 nmoles of UMP from the RNA:DNA hybrid per mg of protein per min. This represents a purification of at least 100-fold with respect to specific activity; however, contaminating ribonucleases in earlier fractions render the exact extent of purification uncertain. The Sepharose 6B column fractions were used for enzyme characterization.

The isoelectric point of RNase H is at pH 4.9 ± 0.2. The estimated molecular weight of RNase H obtained from Sepharose 6B chromatography, sucrose density gradient sedimentation ($S_{20,w}$ = 5.0), and from polyacrylamide gel electrophoresis at varying concentrations of acrylamide (21) is 92,000.

RNase H specifically degrades the RNA strand of RNA:DNA hybrids (Fig. 1). After denaturation of the hybrid by heating and rapid cooling, RNase is not effective but the single-stranded RNA which is produced becomes sensitive to RNase A (Fig. 1B). RNase H was inactive on other forms of single- and double-stranded RNA (Fig. 1C, D). Hybrid labeled in both the RNA and DNA strands was synthesized with RNA polymerase using [α^{32}P]UTP with [^{3}H]ϕX174

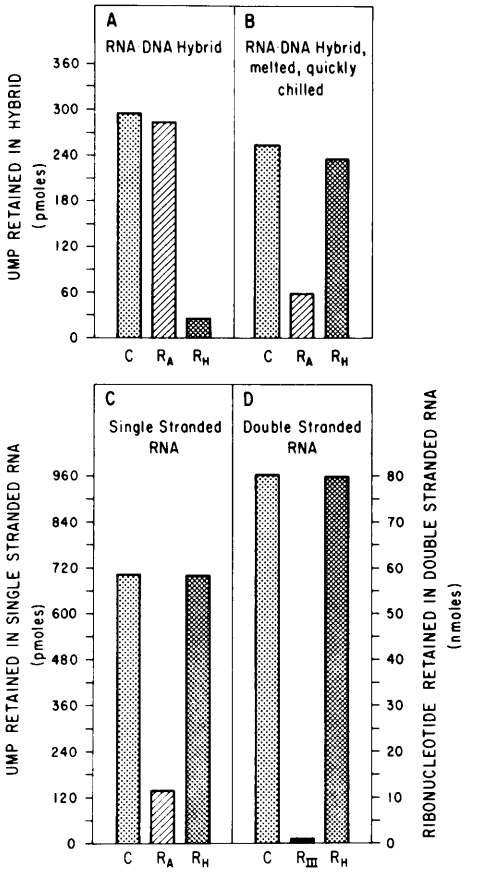

FIG. 1. Substrate specificity of RNase H. All assays were carried out in duplicate under standard assay conditions (see Materials and Methods) for 30 min at 37° with nucleic acids and enzymes as follows: (A) individual assays contained 294 pmoles of UMP in [^{3}H]-RNA:DNA hybrid and no enzyme (column C), pancreatic RNase A to 5 μg/ml (column R_A), or 20 μg RNase H (pooled peak Sepharose column fractions) (column R_H); (B) individual assays contained 253 pmoles of UMP in denatured [^{3}H]RNA:DNA hybrid prepared by heating at 100° for 20 min and then chilling in a salt-ice slurry and enzymes as in (A); (C) individual assays contained 707 pmoles of UMP in single-stranded [^{3}H]RNA and enzymes as in (A); and (D) individual assays contained 80 nmoles of double-stranded Sindbis virus [^{3}H]RNA and no enzyme (column C), an aliquot of *E. coli* K12 strain 514 lysate as a source of RNase III (column R_{III}), or a large excess (250 μg) of RNase H (pooled peak Sepharose column fractions) (column R_H).

single-stranded DNA as template. RNase H released the [^{32}P]ribonucleotides from this hybrid but did not reduce the amount

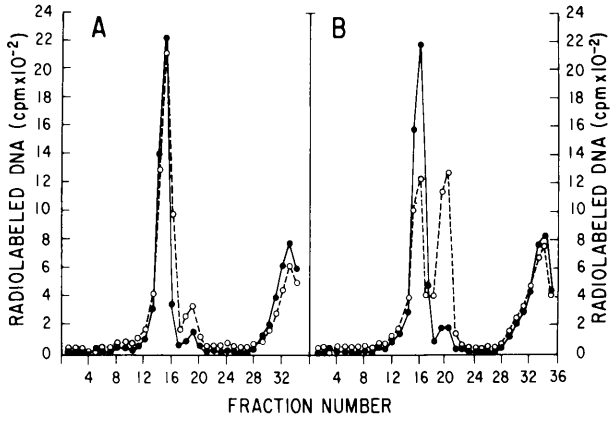

FIG. 2. Sensitivity of covalently closed col E_1 DNA to RNase H. Mixtures of col E_1 DNA supercoils purified from chloramphenicol-treated (labeled with ^{32}P) and those purified from untreated *E. coli* K12 JC411 (labeled with ^{3}H) were incubated under standard assay conditions (see Materials and Methods) at 37° for 15 min with 5.4 μg RNase H (pooled peak Sepharose column fractions). The reaction was terminated by addition of pronase and incubation was continued for another 10 min. The entire reaction mixture was then analyzed by centrifugation in a 5 ml, 5–20% sucrose gradient, for 160 min in a Spinco SW50 1 rotor. The sucrose gradients contained 0.05 *M* Tris–HCl pH 8, 0.55 *M* NaCl, and 0.005 *M* EDTA. (A) Shows the profile obtained from a sample incubated without RNase H, and (B) shows the profile obtained after the 15 min RNase H treatment. Recoveries were greater than 80% in both cases (●——●, [^{3}H]-labeled non-chloramphenicol col E_1; O-----O, [^{32}P]-labeled chloramphenicol col E_1).

of[^{3}H] deoxyribonucleotides retained on the DE81 filters nor that precipitable with TCA, indicating specific degradation of the RNA strand.

RNase H has an absolute divalent cation requirement; Mn^{2+} is the most effective divalent cation with optimal activity occurring at 0.5 m*M*. Reduced sulfhydryl groups are required for maximum activity. As estimated by the technique of Scolnick *et al.* (22), RNase H contains no detectable DNA- or RNA-dependent DNA polymerase activity.

The mode of action of the adrenal cortical RNase H was examined by two methods. First, the effect of RNase H on col E_1 DNA supercoils synthesized in the presence of chloramphenicol was examined. Col E_1 DNA supercoils synthesized in the presence, but not in the absence of chloramphenicol, appear to contain a small segment of RNA (14). The supercoils from the chloramphenicol-treated *E. coli* are converted to open circles by alkali and bacterial RNase H but not by AMV RNase H (14, 20). Incubation of col E_1 DNA supercoils from chloramphenicol-treated *E. coli* with adrenal cortical RNase H resulted in a 40–45% conversion to open circles, indicating an endonucleolytic mode of cleavage (Fig. 2). There is no effect of the RNase H on col E_1 DNA supercoils from untreated E. coli (Fig. 2) nor on SV-40 DNA supercoils, confirming the lack of DNase activity in the enzyme preparation.

Second, the digestion products from the standard RNA:DNA hybrid were examined on chromatography under conditions where only small oligonucleotides migrate from the origin (23). The RNA:DNA hybrid was digested to varying extents as determined by the standard assay and the digestion products were examined. No oligonucleotides of chain length less than eight are detected until extensive degradation has occurred. After exhaustive digestion, smaller oligomers as well as a small amount of free UMP appear although the bulk of the product remains large. The production of primarily large oligomers and the absence of small digestion products again indicates an endonucleolytic mode of cleavage.

Oligonucleotides terminating in 3′-phosphates are relatively resistant to snake venom phosphodiesterase (24), whereas oligonucleotides terminating in 5′-phosphates are relatively resistant to bovine spleen phosphodiesterase (25). The products

of RNase H digestion were examined for sensitivity to these enzymes to determine the site of bond cleavage. Venom phosphodiesterase quantitatively converts to oligonucleotide digestion products to 5′UMP, indicating a terminal 3′-OH group. No uridine was formed, confirming the absence of contaminating phosphatase activity. Bovine spleen phosphodiesterase was completely without effect, indicating the presence of 5′-PO_4^{-3} groups in the reaction products. The oligonucleotide digestion products of RNase H thus terminate in 3′-hydroxyl and 5′-phosphate groups.

Lack of effect of ACTH on RNase H activity. RNase H activity was examined in guinea pig adrenal cortices following ACTH administration (Table I). At 14 hr following initiation of ACTH treatment, RNase H specific activity remained constant. The 14 hr time point coincides with a two-fold increase in thymidine incorporation into DNA (18). The lack of correlation of RNase H activity with ACTH-induced changes in DNA synthesis was observed also in functional adrenal tumor cells in tissue culture. In Y-1 cells, ACTH or cAMP inhibit DNA synthesis by more than 90% after 24 hr of treatment (19). RNase H specific activity is unchanged (Table I). RNase H activity thus remains constant under conditions of ACTH stimulation or inhibition of DNA synthesis.

TABLE I. EFFECT OF ACTH TREATMENT ON RNASE H ACTIVITY.[a]

Experiment	Source of enzyme	UMP released from hybrid (pmol/min/mg[b])
(1) Control	Guinea pig adrenal cortical cytoplasm	116
ACTH-treated		103
(2) Control	Guinea pig adrenal cortical cytoplasm	210
ACTH-treated		200
(3) Control	Y-1 cytoplasm	32.6
ACTH-treated		36.3
(4) Control	Y-1 nuclei	13.2
ACTH-treated		14.4

[a] Guinea pigs (experiments 1 and 2) received 20 U of depot ACTH every 12 hr beginning at time zero. Animals were sacrificed at 14 hr; adrenals from 10 animals were pooled, cytosol isolated (26) and assayed in duplicate. Experiments 1 and 2 represent separate experiments. Y-1 cells (experiments 3 and 4), grown as previously described (19), were incubated with 100 miliunits of ACTH per ml of media for 24 hr. Cells from five plates (approximately 10^7 cells/plate) were harvested, pooled, and fractionated into nuclear and cytosol fractions (26) which were assayed in duplicate.

[b] For assays of cytosol fractions, enzymatic activity is normalized to mg protein; for assays of nuclear fractions, activity is normalized to mg DNA. Protein and DNA content were determined as described (17).

Discussion. The adrenal cortical RNase H resembles the enzyme from other mammalian sources such as calf thymus and KB cells but is distinct from the AMV RNase H, a processive exonuclease associated with reverse transcriptase activity.

Though the ubiquitousness of this enzyme suggests an important conserved action, the nature of its function remains obscure. Several lines of evidence suggest that RNase H may function in DNA replication. RNase H may serve to remove the RNA moiety from Okazaki fragments (with subsequent repair and action of DNA ligase) or, alternatively, may, by generating multiple 3′-hydroxyl ends from an RNA strand hybridized to DNA template, provide a proper substrate for initiation of replication.

The current studies of the RNase H from bovine adrenal cortical tissue were instituted during studies of DNA replication induced by ACTH. RNase H with similar properties is present in guinea pig adrenal cortices and in Y-1 mouse adrenal tumor cells in tissue culture. The effect of ACTH administration to guinea pigs on the level of RNase H was examined at 14 hr, a time when an increase in DNA synthesis is evident (18). RNase H activity was also examined in Y-1 cells at 24 hr, a time when marked inhibition of DNA synthesis is evident (19). No change in RNase H activity was detectable. These studies suggest that changes in RNase H activity are not a requirement for changes in DNA synthesis observed after hormonal administration.

Summary. Ribonuclease Hybrid (RNase

H) from adrenal cortical tissue has been characterized. RNase H specifically degrades the RNA strand of purified RNA:DNA hybrids but is inactive on single- or double-stranded RNA or on DNA. The mode of cleavage by RNase H is endonucleolytic, producing oligoribonucleotides with 3′-hydroxyl and 5′-phosphate termini. ACTH administration to guinea pigs results in no detectable change in adrenal cortical RNase H activity at times when changes in DNA synthesis are marked.

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