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Physical Association of a DNA Polymerase Stimulating Activity with a Ribonuclease H Purified from Yeast†

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ABSTRACT: From the yeast *Saccharomyces cerevisiae* we have purified by three consecutive column-chromatographic steps a ribonuclease H activity to apparent homogeneity. The enzyme, a single polypeptide chain of molecular weight around 68 000, is optimally active at neutral pH and at a magnesium ion concentration of 10 mM and is sensitive to *N*-ethylmaleimide. It degrades the RNA strand of a DNA-RNA hybrid in an endonucleolytic mode and hydrolyzes neither double- or single-stranded DNA nor single-stranded RNA. The enzyme is capable of stimulating in vitro DNA synthesis by yeast DNA polymerase A up to more than 50-fold. This effect is strongly dependent on the relative amounts of primer template, DNA polymerase, and ribonuclease H in the assay mixtures. Yeast DNA polymerase B and *Escherichia coli* DNA polymerase I are barely stimulated under our assay conditions.

Whereas for bacteria and their phages combined biochemical and genetic efforts have led to a fairly clear picture of the process of DNA replication, our knowledge of this fundamental mechanism in eukaryotes is less developed (Kornberg, 1980, 1982). In vitro studies are most advanced for viral DNA synthesis in animal cells [see, e.g., Winnacker (1978) and Waldeck et al. (1979)] although the proteins that are involved are only partly characterized. Because of the accessibility of yeast to simple genetic methods, we have earlier chosen this eukaryotic microorganism for biochemical studies of proteins involved in DNA synthesis. Three DNA polymerases had been characterized (Wintersberger, 1978), and DNA synthesis had been studied in isolated nuclei (Wintersberger, 1976). In order to find further proteins participating in DNA replication, repair, or recombination of yeast, we have undertaken a search for DNA-binding proteins able to stimulate in vitro DNA synthesis by yeast DNA polymerase A. Several such proteins from other organisms had been described (Hubermann et al., 1971; Banks & Spanos, 1975; Herrick et al., 1976; Otto et al., 1977; Duguet et al., 1977; Blue & Weissbach, 1978; Ganz & Pearlman, 1980; Riva et al., 1980). From yeast, a factor with a molecular weight of 37 000 that stimulated DNA synthesis on single-stranded DNA in the absence of a primer was isolated by Chang and co-workers (Chang et al., 1978). We describe here another DNA-binding protein from *Saccharomyces cerevisiae* that stimulates in vitro DNA synthesis on gapped double-stranded templates by DNA polymerase A.

During our attempts to isolate this protein, we noticed the copurification of a ribonuclease activity, which specifically degraded RNA in DNA-RNA hybrid structures, a ribonuclease H (RNase-H).[1] Thus, the question arose whether

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[1] Abbreviations: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid disodium salt; GF/C, glass microfiber filters; NEM, *N*-ethylmaleimide; PEI, poly(ethylenimine); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; RNase-H, ribonuclease H; DNase I, deoxyribonuclease I.

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the two activities were fortuitously associated during several purification steps or whether they were integral parts of a single polypeptide. To resolve this problem, we purified first the RNase-H activity to apparent homogeneity, because of the availability of a more clear-cut enzyme assay. This enzyme did not resemble any of the RNase-H activities described earlier by the Fromageot group for yeast cells (Wyers et al., 1976a,b). The ability to stimulate in vitro DNA synthesis accompanied also the most purified preparation of our enzyme and could not be separated from the RNase-H activity by any method employed.

Experimental Procedures

Yeast Cells. The two strains of *Saccharomyces cerevisiae* used were strain 419, obtained from G. Simchen, and the protease deficient mutant 20B-12 from the Yeast Stock Center, University of California. The cells were grown at 28 °C in full medium (0.3% yeast extract, 0.5% bactopeptone, 2% glucose) in a New Brunswick fermentor under vigorous aeration until they reached the late logarithmic phase of growth (around 1.8×10^8 cells/mL).

Substrates and Enzymes. A DNA–[^{3}H]RNA hybrid was prepared as substrate for RNase-H according to a published procedure (Hausen & Stein, 1970; Büsen & Hausen, 1975) with some modifications: Calf thymus DNA (Sigma, type I) was heat denatured, and 100 nmol/mL was incubated at 37 °C with 3 units/mL *Escherichia coli* RNA polymerase (Boehringer, Mannheim) in buffer containing 50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 5 mM Mn^{2+}, 0.15 M KCl, 0.5 mg/mL bovine serum albumin, 0.8 mM each of the four ribonucleoside 5′-triphosphates, and 40 μCi/mL [^{3}H]UTP (46 Ci/mmol). RNA synthesis was monitored by determining the acid-insoluble radioactivity. When the molar ratio of DNA to RNA in the hybrid had reached about 1:0.7, the incubation mixture was cooled, and the hybrid was freed from the reactants by chromatography on Sephadex G-50. The specific activity was around 7000 cpm/nmol of ribonucleotide in the hybrid.

The DNA–[^{3}H]RNA hybrid was labeled at the 5′-end with radioactive phosphate as follows: The unlabeled phosphate groups were removed from the 5′-ends by incubating the hybrid for 1 h at 37 °C with calf intestine alkaline phosphatase (Boehringer, Mannheim, grade I). Before use, this enzyme was purified to a form free of any detectable activity of endo- or exonuclease by Sephadex G-75 chromatography as suggested by Efstratiatis et al. (1977). The reaction mixture (55 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) contained in 1 mL 1 unit of enzyme and 130 nmol of DNA–RNA hybrid. After the reaction was stopped by addition of potassium phosphate buffer (pH 9.2) to a final concentration of 1 mM (Chaconas & van de Sande, 1980), the mixture was made 50 mM in glycine, 10 mM in dithiothreitol, and 5 mM in $MgCl_2$. Then [γ-^{32}P]ATP (700 Ci/mmol; final concentration 30 μM) and 15 units of T4 polynucleotide kinase (nuclease free, P-L Biochemicals) were added, and the incubation was continued for 45 min at 37 °C (Lillehaug et al., 1976). After removal of low molecular weight reactants by Sephadex G-75 chromatography, the fractions containing acid-precipitable radioactivity were pooled. Ammonium acetate (to a concentration of 0.5 M) and calf thymus DNA (10 μg/mL) were added (Maxam & Gilbert, 1977), and the hybrid was isolated by ethanol precipitation and resuspension in 10 mM Tris-HCl buffer, pH 7.1. It had a specific activity of 3×10^7 cpm ^{32}P and 3700 cpm ^{3}H per nmol of ribonucleotide. When incubated with alkaline phosphatase, 99% of the ^{32}P counts could be rendered acid soluble.

Preparation of Substrate for Determination of 3′ → 5′ Exodeoxyribonuclease Activity. The reaction mixture contained within 8.75 mL 210 μg of poly(dA)·oligo(dT)$_{12-18}$ (5:1), 50 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 50 μM dTTP, 4 μCi of [^{3}H]dTTP, and 80.5 units of DNA polymerase A from yeast. After incubation for 30 min at 30 °C, the polynucleotide containing oligo(dT)$_{\sim 30}$ pieces ^{3}H-labeled at the 3′-parts was purified by Sephadex G-50 chromatography. It had a specific activity around 7000 cpm/nmol of dTMP.

Yeast DNA polymerases A and B were prepared as described earlier (Wintersberger, 1974). Calf thymus DNA (Sigma) was activated according to Aposhian & Kornberg (1962). Poly(dA), oligo(dT)$_{8-12}$, oligo(dT)$_{12-18}$, *E. coli* DNA polymerase I, DNase I from bovine pancreas, and unlabeled ribo- and deoxyribonucleoside triphosphates were purchased from Boehringer, Mannheim; *E. coli* exonuclease III was from Biolabs; bovine pancreatic ribonuclease A was from Worthington. ^{3}H-Labeled poly(U), [^{3}H]UTP, and [^{3}H]dTTP came from Amersham International. Proteins used as molecular weight standards were from Boehringer, Mannheim. Labeled *E. coli* DNA was a gift of E. Wintersberger (sp act. 2000 cpm/nmol).

Enzyme Assays. The assay mixture for RNase-H (total volume 0.125 mL for characterization assays, 0.05 mL during the purification procedure) contained 50 mM Tris-HCl (pH 7.1), 10 mM $MgCl_2$, 50 mM KCl, 1 mM β-mercaptoethanol, 0.2 mg/mL bovine serum albumin, 1 μg/100 μL DNA–[^{3}H]RNA hybrid, corresponding to 11.2 μM ribonucleotide, and various amounts of RNase-H fractions. After incubation for 10 min at 30 °C, an aliquot of the assay mixture was applied to a GF/C filter (Whatman), precipitated in 10% trichloroacetic acid, washed extensively with 5% trichloroacetic acid, and subsequently washed with ethanol–ether (1:1). The dried filters were counted in a toluene-based scintillator. The activity of RNase-H was calculated from the portion of RNA in the hybrid remaining acid insoluble. This assay exhibited linearity with respect to the amount of RNase-H present if less than 40% of the substrate was degraded during the incubation period. A unit is defined as the amount of RNase-H that converts 1 nmol of ribonucleotide of the DNA–RNA hybrid into acid-soluble form within 10 min at 30 °C.

DNA polymerase activity was determined as described earlier (Wintersberger & Wintersberger, 1970); for the assay measuring the stimulation of yeast DNA polymerase A by RNase-H fractions, the incubation mixtures (0.125 mL) contained 66 mM Tris-HCl (pH 7.7), 2 mM EDTA, 5 mM Mg^{2+}, 3 mM β-mercaptoethanol, 50 μM dATP, dGTP, dCTP, and dTTP each, 0.5 μCi of [^{3}H]dTTP (41 Ci/mmol), 0.31 μg of poly(dA)·oligo(dT)$_{8-12}$ (5:1), various amounts of DNA polymerase A, and RNase-H, as well as 40% glycerol (added with the enzymes or separately in case of the controls).

Tracing the Products Generated by RNase-H. (*a*) *Kinetics of Generation of Acid-Soluble 5′-^{32}P As Compared to Internal ^{3}H.* Standard assay mixtures (0.125 mL each) containing 3 μg of DNA–[^{3}H]RNA hybrid labeled with ^{32}P at the 5′-end (prepared as described above) were incubated for increasing lengths of time at 30 °C after which the amounts of acid-soluble ^{32}P and ^{3}H were determined.

(*b*) *Analysis of Degradation Products.* Reaction mixtures as above were incubated at 30 °C for various periods of time, and aliquots were applied to thin-layer chromatographic PEI–cellulose-F strips (2 × 20 cm, Merck). Ascending chromatography was carried out in 1 M formic acid, containing 0.5 M LiCl (Kornberg et al., 1978) at room temperature for about 1 h. Under these conditions, oligoribo-

nucleotides containing eight or more ribonucleotides stuck to the origin as tested with an oligo$(U)_8$ standard; monoribonucleotides (standards used: 5′-AMP, 5′-UMP, 3′-UMP, and uridine) added to each sample were visualized after chromatography by inspection under UV light. They accumulated at the upper ends of the strips. Cellulose strips were cut into 1-cm pieces; ^{32}P and ^{3}H were determined in each one.

SDS–Polyacrylamide Gel Electrophoresis. Slab gels (12.6%) with a stacking gel (4%) were run according to the method of Laemmli (1970). Proteins to be analyzed were precipitated with 5% trichloroacetic acid, washed with acetone, and boiled in sample buffer for 5 min before being applied to the gel. After electrophoresis, the gels were stained with Coomassie brilliant blue, destained, and scanned with a Beckman DU-8 spectrophotometer. Molecular weights were computed relative to standards.

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Purification of RNase-H Activity. All procedures were carried out at 0–4 °C and with as little interruption as possible.

Cell Extract. The yeast cells were harvested, washed with water, and suspended in buffer A (20 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM β-mercaptoethanol and 10% glycerol; the protease inhibitors 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A, 0.1 mM sodium tetrathionate, and 1 mM sodium hydrogen sulfite were added immediately before use). The suspension was adjusted to 2 M NaCl, and the cells were broken by a shaking of the suspension with glass beads in a Braun homogenizer (3 times for 30 s under CO_2 cooling). After removal of glass beads and cell debris by centrifugation at 6000*g*, the homogenate was centrifuged for 90 min at 170000*g* (fraction I). The extract was freed from the major part of nucleic acids by treatment with 10% poly(ethylene glycol) 6000 (British Drug House) for 30 min, similarly as described by Herrick & Alberts (1976), centrifuged at 20000*g*, quickly dialyzed against buffer A until the conductivity corresponded exactly to 0.1 M NaCl in buffer A, and cleared by another short centrifugation step (fraction II).

DNA–Cellulose Column Chromatography. DNA–cellulose was prepared as described by Litman (1968) with undenatured calf thymus DNA (Sigma, type I). The column (8.6 cm^2 × 58 cm), containing 13 mg of DNA/g of dry cellulose, was equilibrated with buffer A made 0.1 M NaCl and was loaded with the extract to about 2 mg of protein/mg of cellulose-bound DNA. After all proteins not binding to DNA were washed off with the equilibration buffer, the column was eluted with buffer A containing 1.3 M NaCl. Fractions exhibiting RNase-H activity were pooled, dialyzed against buffer A containing 0.001 M KH_2PO_4/K_2HPO_4, and centrifuged for 20 min at 20000*g* (fraction III).

Hydroxylapatite Column Chromatography. Fraction III was applied to a hydroxylapatite (Bio-Gel HT, Bio-Rad) column (3.5 cm^2 × 29 cm) equilibrated with buffer A and 0.001 M KH_2PO_4/K_2HPO_4 to about 1 mg of protein/2 mL of column volume. The column was washed with the same buffer until the first protein peak was completely eluted; then, 8 column volumes of a linear gradient (0.001–0.4 M KH_2PO_4/K_2HPO_4 in buffer A) was applied. The activity eluted in a single peak around 0.3 M KH_2PO_4/K_2HPO_4, the fractions of which were pooled and quickly dialyzed against buffer A containing 0.01 M NaCl (fraction IV).

DEAE-cellulose Column Chromatography. A DE-52 cellulose (Whatman) column (1.5 cm^2 × 10 cm), equilibrated with buffer A (0.01 M NaCl), was loaded with about 0.5–2 mg of protein/mL of column volume of fraction IV. It was washed free of unbound proteins with equilibration buffer and then eluted with 8 volumes of a 0.01–0.3 M NaCl linear gradient in buffer A. RNase-H activity was recovered between 0.09 and 0.15 M NaCl, pooled, dialyzed against buffer A (fraction V), and stored in liquid nitrogen. The enzyme could be kept for several weeks without loss of activity when dialyzed against buffer A containing 50% glycerol and stored at −20 °C.

Determination of Molecular Weight by Gel Filtration. A column (diameter 1.6 cm, height 80 cm) was filled with Sephacryl S-300 (Pharmacia, Uppsala) and equilibrated with buffer A containing 0.1 M NaCl. Forty micrograms of protein of fraction V was applied and eluted at the velocity of 1.5 $mL \cdot cm^{-2} \cdot h^{-1}$. Fractions of 2-mL volume were collected and tested for RNase-H, as well as DNA polymerase stimulating activity. A mixture of proteins dissolved in buffer A was used as molecular weight standard and chromatographed in essentially the same manner.

Determination of Relative Sedimentation Value by Glycerol Gradient Centrifugation. Gradients of 12–17% glycerol in buffer A without salt or with 0.5 M NaCl were prepared and loaded with 1 mL of enzyme after DEAE-cellulose chromatography. Markers with known sedimentation values were added, and centrifugation was carried out in a Beckman SW 41 rotor at 40000 rpm for 21 h.

Results

Purification of a Ribonuclease H Activity from Yeast. Chromatography of a crude yeast cell extract on DNA–cellulose prepared according to Litman (1968) leads to the separation of a heterogeneous group of proteins, binding to single- or double-stranded DNA, from the bulk of extract constituents. Upon further separation of these proteins, we found a fraction containing a factor that stimulated in vitro DNA synthesis by yeast DNA polymerase A. As the same fraction also showed RNase-H activity, we became interested in the question of whether the observed stimulation of DNA synthesis was a property of one of the yeast RNases-H described earlier (Wyers et al., 1976a,b). Unfortunately however, we were not able to reproduce the published procedure for purifying these enzymes. The initial attempts to work out our own method for the isolation of RNase-H from the yeast strain 419 (from G. Simchen) were hampered by the instability of the enzyme during purification and by low and extremely variable yields. We therefore turned to a mutant deficient in several proteases [strain 20B-12, isolated by Jones (1977)] and in addition used a battery of protease inhibitors during the isolation procedure.

These precautions led to the highly reproducible enzyme-purification method described under Experimental Procedures and summarized in Table I. Figure 1 shows the pattern obtained by SDS–polyacrylamide gel electrophoresis of the fractions from the different purification steps. It should be mentioned that identical enzyme preparations could be obtained from both yeast strains; the protease deficiency of strain 20B-12, however, greatly facilitated the task and made possible a final yield of more than 20%.

All the RNase-H activity present in the yeast extract was bound to the DNA–cellulose column under the conditions indicated. Whereas during the following step of hydroxylapatite chromatography no splitting of enzyme activity into different fractions was observed, about 10–30% of the activity applied to the DEAE-cellulose was not bound at the salt concentration employed. As the ability to stimulate the DNA synthesis in vitro accompanied the bound fraction of RNase-H activity, we concentrated on that enzyme and have not further studied the flowthrough of the DEAE-cellulose column, which

Table I: Purification of Ribonuclease H from *S. cerevisiae*[a]

fraction	volume (mL)	total protein (mg)	total act. (units)	sp act. (units/mg)
I (crude cell extract)	65	4550	32174	7.1
II [poly(ethylene glycol) supernatant]	54	1220	31000	25.4
III (DNA–cellulose)	49	78.4	27446	350.1
IV (hydroxylapatite)	60	7.2	11554	1604.7
V (DEAE-cellulose)	23	0.92	7130	7750.0

[a] Purification was carried out as described in the text, starting from 2.1×10^{12} cells (70 g wet wt). A unit is defined as the amount of enzyme catalyzing the conversion of 1 nmol of RNA from the DNA–RNA hybrid to an acid-soluble form in 10 min at 30 °C and standard assay conditions as described.

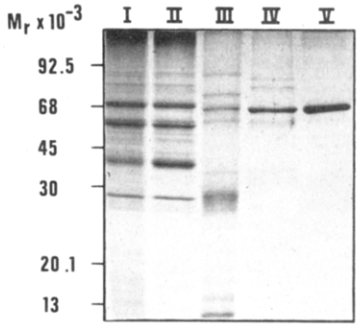

FIGURE 1: SDS–polyacrylamide gel electrophoresis pattern of fractions obtained during purification of RNase-H: (I) crude cell extract (84 μg); (II) extract before application to DNA–cellulose (57 μg); (III) pooled DNA–cellulose peak fractions (40 μg); (IV) pooled hydroxylapatite peak fractions (40 μg); (V) pooled DEAE-cellulose peak fractions (10 μg). The molecular weight standards used were phosphorylase A (92500), bovine serum albumin (68000), ovalbumin (45000), carbonic anhydrase (30000), trypsin inhibitor (20000), and cytochrome *c* (13000).

might contain an additional RNase-H with different properties. The pooled RNase-H activity containing fractions from the DEAE-cellulose column showed one band when analyzed by SDS–polyacrylamide gel electrophoresis (Figure 1, lane V). According to a calculation using the scan of this electrophoresis pattern, the enzyme was more than 95% pure.

Properties of Purified Enzyme. (*a*) *Molecular Weight.* The molecular weight was determined by three different methods. In SDS–polyacrylamide gel electrophoresis, the RNase-H band was found at a position very similar to that of bovine serum albumin with a molecular weight of 68000 (Figure 1). With gel filtration on Sephacryl S-300, a nearly identical result, namely, a molecular weight of 70000, was obtained (Figure 2). This nondenaturing method allowed one to test for enzyme activities, and therefore, RNase-H as well as the stimulation of DNA polymerase A from yeast were determined for each fraction. It can be seen from Figure 2 that the two activity peaks coincided. In addition, the purified enzyme was subjected to glycerol gradient centrifugation (Figure 3). When the centrifugation was carried out in buffer A without further addition of salt, a major peak of RNase-H activity with a relative sedimentation value of 4.6 S was obtained. During different experiments, this peak was accompanied by a faster sedimenting activity to a varying extent, indicating some aggregation of the enzyme–protein. Addition of 0.5 M NaCl to the gradient buffer did not change the position of the main peak but reduced the faster sedimenting one (Figure 3). The pooled peak fractions showed one band at a molecular weight of 68000 in SDS–polyacrylamide gel electrophoresis and stimulated DNA synthesis in vitro (data not shown).

(*b*) *Requirements for RNase-H Activity.* The pH optimum for the enzyme reaction was observed at pH 7.1 in Tris-HCl buffer. Less than 10% of enzyme activity was left above a pH of 8 and below a pH of 6.5. Activity depended on the addition

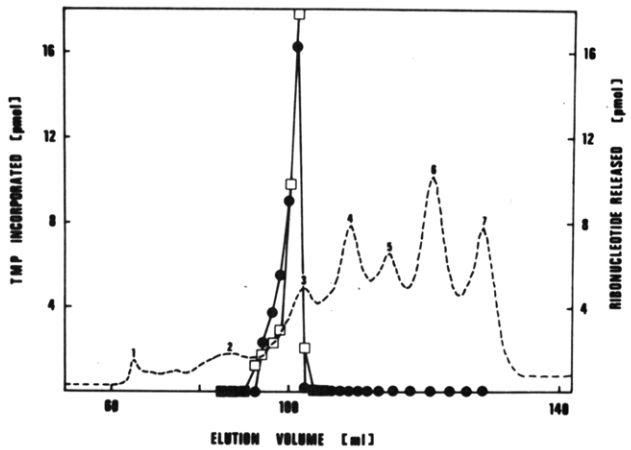

FIGURE 2: Gel filtration of RNase-H, fraction V, from purification procedure. A column containing Sephacryl S-300 was calibrated with proteins as standards (dotted line). According to the suppliers of the gel, the molecular weight standards were (1) catalase (210000), (2) aldolase (158000), (3) bovine serum albumin (67000), (4) ovalbumin (43000), (5) carbonic anhydrase (30000), (6) trypsin inhibitor (20000), and (7) cytochrome *c* (13000). (●) RNase-H activity; (□) stimulation activity.

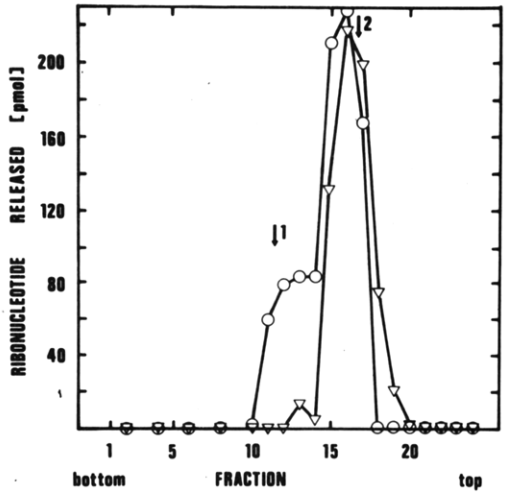

FIGURE 3: Glycerol gradient centrifugation of fraction V from purification procedure. RNase-H activity from the salt-free (○) and from the NaCl-containing gradient (▽). Arrows indicate marker enzymes with known sedimentation values: (1) alcohol dehydrogenase, 7.6 S; (2) malate dehydrogenase, 4.3 S.

of divalent cations; the optimum for Mg^{2+} was at a concentration of 10 mM whereas higher amounts led to inhibition that was complete at 20 mM. Mn^{2+} ions did not substitute Mg^{2+} ions (Table II). The SH-blocking reagent *N*-ethylmaleimide strongly inhibited the RNase-H activity (Table II). Addition of 50 mM KCl to the reaction mixture ensured the integrity of the hybrid substrate and was optimal for the degradation reaction.

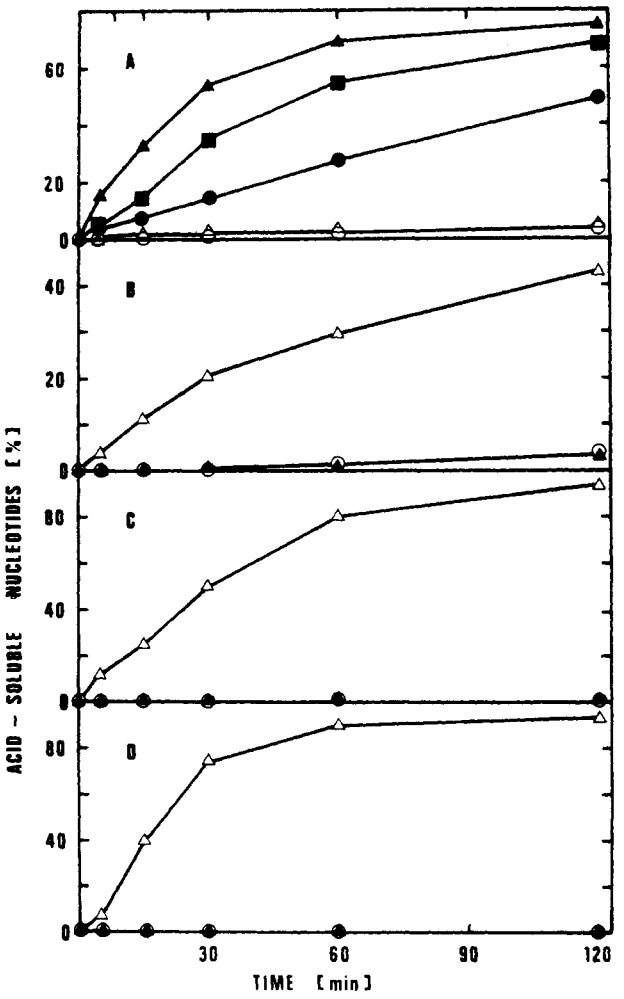

FIGURE 4: Action of RNase-H and other nucleases on different nucleic acid substrates: (○) nucleic acids incubated without enzymes; (A) (●) 40 ng, (■) 80 ng, and (▲) 160 ng of RNase-H or (△) 0.4 ng of ribonuclease A incubated with native DNA–[^{3}H]RNA hybrid; (B) (▲) 160 ng of RNase-H or (△) 0.4 ng of ribonuclease A incubated with heat-denatured DNA–[^{3}H]RNA hybrid; (C) (▲) 8 μg of RNase-H (values obtained with 0.8 and 0.08 μg of RNase-H coincided) or (△) 0.05 μg of deoxyribonuclease I incubated with native [^{3}H]DNA from *E. coli*; (D) (▲) 2 μg (or 0.02 μg) of RNase-H (using 10 or 0.66 mM Mg^{2+}) or (△) 1 μg of exonuclease III from *E. coli* (0.66 mM Mg^{2+}) incubated with poly(dA)·^{3}H-labeled oligo(dT)$_{\sim 30}$ (2:1).

(*c*) *Specificity of RNase-H Activity.* Figure 4 shows the results obtained when different nucleic acids were tested as substrates for our yeast RNase-H preparation. Whereas the native calf thymus DNA–RNA hybrid proved to be a specific substrate for RNase-H, which was not degraded by pancreatic ribonuclease A (Figure 4A) after denaturation (100 °C, 10 min), the RNA part of the hybrid was no longer degradable by RNase-H but became a substrate for pancreatic ribonuclease A (Figure 4B). The apparent K_m for the RNase-H reaction on the calf thymus DNA–RNA hybrid was determined as 1.6×10^{-5} M. We also tested single-stranded ^{3}H-labeled poly(U) and found that ribonuclease A but not yeast RNase-H hydrolyzed this synthetic polyribonucleotide (data not shown). Double-stranded DNA substrates sensitive to DNase I or exonuclease III were resistent to the enzyme (Figure 4C,D).

(*d*) *Mode of Cleavage.* In order to get information on the specificity of RNase-H, the ^{3}H-labeled DNA–RNA hybrid was labeled with [^{32}P]phosphate groups at the 5′-ends (see Experimental Procedures). This substrate was digested with RNase-H for varying periods of time, and ^{3}H—as well as ^{32}P—counts made acid soluble were determined. From the results depicted in Figure 5, nearly parallel solubilization of the two kinds of labels is apparent. We suspected therefore that the mode of action of the yeast RNase-H was endonucleolytic. An exonuclease activity cleaving in the 5′ → 3′ direction would have solubilized the ^{32}P label preferentially at the beginning of the reaction, a 3′ → 5′ exonuclease only after prolonged incubation periods. To further substantiate this, we chromatographed the reaction products under conditions under which only mononucleotides and small oligonucleotides of chain length less than eight would migrate. After 10 min of incubation, when, with the amount of RNase-H used in this experiment, more than 50% of radioactivity was soluble in our enzyme assay, only around 1% of the ^{3}H and the ^{32}P counts were found at the spot of the chromatogram where the standard mononucleotides migrated (Table III). Only later during the digestion reaction did small oligonucleotides (chromatogram not shown) and free mononucleotides (Table III) appear. Thus, the mechanism of action of the yeast enzyme described here probably is endonucleolytic.

Table II: Reaction Requirements for Ribonuclease H

additions or deletions	activity (%)
complete	100
minus $MgCl_2$ [a]	<1
minus $MgCl_2$ and plus $MnCl_2$ [a]	
0.5 mM	3
1 mM	6
2 mM	3
6 mM	<1
10 mM	<1
20 mM	<1
minus KCl	35
plus 100 mM KCl	30
minus β-mercaptoethanol	80
minus β-mercaptoethanol and plus *N*-ethylmaleimide	
1 μM	46
5 μM	25
10 μM	5
20 μM	<1

[a] Assay mixtures were preincubated for 30 min at 0 °C before the addition of the enzyme. This appears to reduce the scattering of the values obtained under conditions of very low enzyme activity.

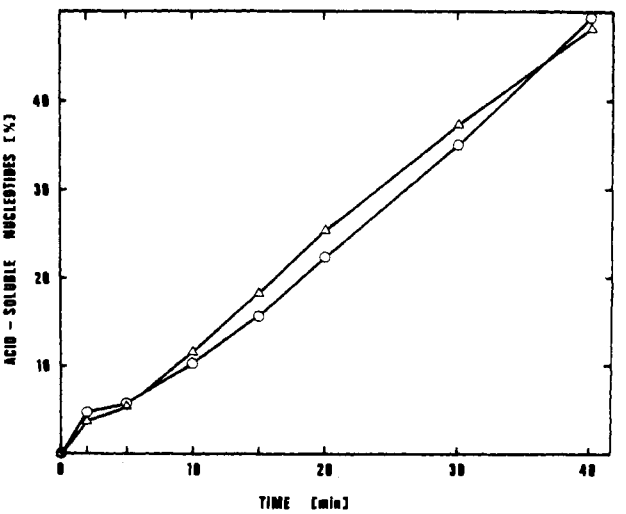

FIGURE 5: Kinetics of degradation of 5′-end ^{32}P-labeled DNA–[^{3}H]RNA by RNase-H: (○) ^{3}H counts or (△) ^{32}P counts released by 100 ng of RNase-H in 125 μL of standard assays after various incubation periods (100% ^{3}H amounts to 2300 cpm; 100% ^{32}P amounts to 3800 cpm).

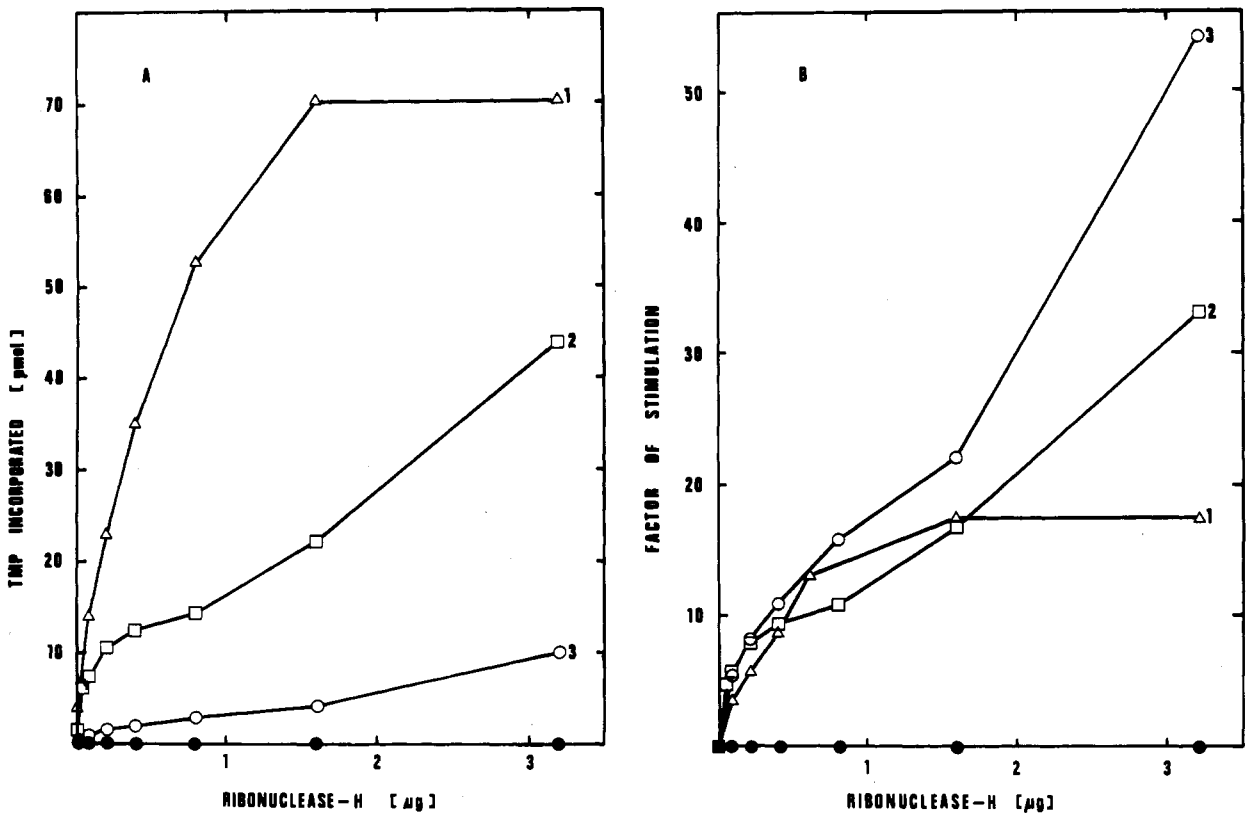

FIGURE 6: Stimulation of yeast DNA polymerase A in vitro by RNase-H. The template primer, poly(dA)·oligo(dT)$_{8-12}$ (5:1), was incubated for 30 min at 30 °C (●) without or with (○) 3, (□) 6, or (△) 12 milliunits of DNA polymerase A and increasing amounts of RNase-H, fraction V. Effect of RNase-H plotted as (A) amount of deoxyribonucleotides incorporated or as (B) degree of stimulation.

Table III: Generation of Ribonucleoside Monophosphates by Ribonuclease H[a]

incubation at 30 °C (min)	acid-soluble part of substrate (%)		ribonucleoside monophosphates (%)	
	^{3}H	^{32}P	[^{3}H]UMP	[^{32}P]NMP
0	0	0	0	0
10	52	61	1.0	1.7
30	81	74	2.9	4.5
300	nd	nd	17.2	21.9

[a] The 5′-end ^{32}P-labeled DNA-[^{3}H]RNA hybrid was incubated in standard assay mixtures (100 µL each) with 200 ng of RNase-H (fraction V). After the incubation periods indicated, 40-µL aliquots each were used for determination of acid-precipitable part of the substrate and for PEI-cellulose chromatography. 100% ^{3}H corresponds to 1530 cpm; 100% ^{32}P corresponds to 1890 cpm.

(*e*) *Stimulation of DNA Polymerase A from Yeast by RNase-H.* Yeast cells contain—besides a mitochondrial DNA polymerase (Wintersberger & Blutsch, 1976)—two high molecular weight DNA-polymerizing enzymes (Wintersberger, 1974; Chang, 1977), DNA polymerase A, which probably is involved in DNA replication during the S phase of the cell cycle, and DNA polymerase B, the physiological role of which is not yet clear. When increasing amounts of RNase-H were added to incubation mixtures for DNA polymerase A operating on the gapped template, poly(dA)·oligo(dT)$_{8-12}$ (5:1), incorporation of TMP could be enhanced up to about 50-fold (Figure 6). The stimulation factor strongly depended on the proportions of template primer and the two enzymes in the reaction mixture. When the DNA concentration was held constant and high amounts of DNA polymerase A were used, saturation of stimulation was reached above a certain amount of added RNase-H (curves 1 in Figure 6). If, for the same template primer concentration, only half the amount of DNA polymerase was available, stimulation of the polymerization reaction was more exaggerated and did not reach saturation with the highest amount of RNase-H that could be added without changing the volume of the assay mixture (curves 2 in Figure 6). RNase-H alone had no DNA-polymerizing activity. If native DNA, activated by DNAse I according to Aposhian & Kornberg (1962), was used as template primer, polymerization was likewise enhanced by RNase-H, although not as highly as with the synthetic template (up to 12-fold, data not shown). We also tried to stimulate the polymerization reaction catalyzed by yeast DNA polymerase B but only found some stimulation when extremely low concentrations of polymerase were used. The same was true for commercially available *E. coli* DNA polymerase I (data not shown).

Discussion

Since the first discovery of a nuclease that specifically degrades the RNA moiety of a DNA–RNA hybrid by Stein & Hausen (1969), such enzymes have been found in many different organisms. When it became evident that RNA synthesis might be involved in the initiation of viral (Brutlag et al., 1971) and chromosomal (Lark, 1972) DNA replication as well as in the priming of Okazaki fragments in the course of ongoing, discontinuous DNA replication (Sugino et al., 1972), a possible physiological role for the enzymes hydrolyzing RNA from a DNA–RNA hybrid, namely, the removal of RNA primers during DNA synthesis, was anticipated. Today it is known that in E. *coli* the 5′ → 3′ exonucleolytic function of DNA polymerase I is responsible for the excision of the RNA primers from the Okazaki fragments (Kornberg, 1980). A role for the RNase-H from this organism exhibiting endonucleolytic activity (Miller et al., 1973; Berkower et al., 1973; Henry et al., 1973) has been described only for in vitro DNA replication systems of ColE1-type plasmids (Hillenbrand & Staudenbauer, 1982; Selzer & Tomizawa, 1982) but not for *E. coli* chro-

mosomal DNA replication. However, synthetic plasmids, containing the chromosomal origin from *E. coli*, are now available and can be replicated in a cell-free system (Fuller et al., 1981; Kaguni et al., 1982). Therefore, the mechanism of initiation of the bacterial DNA replication as well as the proteins involved will soon be elucidated.

For yeast, our knowledge of this important process is less developed. Concerning the RNases-H, purification of proteins exhibiting such activity was reported (Wyers et al., 1976a,b). The possible role of these enzymes in DNA replication or in other cellular processes, as well as of similar enzymes later described as being associated with yeast RNA polymerase A (Huet et al., 1976, 1977; Iborra et al., 1979), was discussed by the investigators. The RNase-H activity isolated in apparently homogeneous form from a protease-deficient yeast strain (Figure 1, Table I) and partly characterized by us was found different from all these proteins. It is a single polypeptide chain of molecular weight around 70 000 (which is higher than that of the RNases-H described before from yeast), requires magnesium ions (which cannot be replaced by manganese), and requires a sulfhydryl reagent for maximal activity (Table II). It releases preferentially oligoribonucleotides from a calf thymus DNA–RNA hybrid and therefore is most likely an endonuclease (Table III). After denaturation, the RNA strand of the hybrid becomes insensitive to the enzyme. Single- or double-stranded DNA (intact or gapped) is not susceptible to degradation (Figure 4). Concerning the specificity, this yeast RNase-H resembles the *E. coli* RNase-H (see above) and several enzymes isolated from eukaryotic sources, e.g., RNase-H from *Ustilago maydis* (Banks, 1974), RNase-H I from calf thymus (Büsen, 1980), RNase-H from KB cells (Keller & Crouch, 1972), and the Mg^{2+}-dependent enzyme from rat liver (Roewekamp & Sekeris, 1974; Sawai et al., 1978). The physiological role remains to be determined for our, as well as for all, above-mentioned enzymes. A very interesting property of the yeast enzyme, namely, the ability to stimulate DNA synthesis catalyzed by yeast DNA polymerase A with a gapped template primer, has not been reported for any other RNase-H. The degree of this stimulation was found highly dependent on the proportions of the two enzymes, DNA polymerase A and RNase-H, participating in the DNA-polymerizing reaction. Although RNase-H alone exhibits no polymerizing activity at all, it is most effective with relative small amounts of DNA polymerase (Figure 6B). As the template primer used is not cleaved by the RNase-H (Figure 4C), the effect on the polymerization reaction cannot be explained by making available more starting points for the DNA polymerase. We rather suspect that the stimulation of the DNA polymerization by RNase-H is somehow due to its ability to bind to single-stranded DNA, which we have observed with filter-binding assays (R. Karwan and U. Wintersberger, unpublished data). Thus if a very limited number of DNA polymerase molecules has to react with many primer sites, RNase-H molecules might facilitate the polymerization reaction because they possibly bind to the single-stranded gaps and hold the template strands in a conformation favorable for the polymerization reaction. Obviously, a greater and greater excess of DNA polymerase molecules, by binding themselves to DNA, renders the action of RNase-H more and more superfluous. It could also be that the mode of action of DNA polymerase A in presence of RNase-H changes from a distributive to a processive manner. The stimulation effect is not dependent on the synthetic template primer that was used for the experiments depicted in Figure 6 but can also be observed with "activated" calf thymus DNA. When tested with DNA polymerase B from yeast or *E. coli* DNA polymerase I, RNase-H showed low stimulating activity but only with extremely small polymerase concentrations. Clearly, RNase-H acts differently from the yeast factor described by Chang et al. (1978; see the introduction).

The elucidation of the stimulation mechanism, especially in connection with the RNase-H activity of the protein, a function that has not been reported for any other DNA synthesis stimulating factor, will be the aim of our further studies. The possibility that the protein isolated by us might eventually also be involved in a cellular process different from DNA replication (e.g., maintenance or variation of chromatin structure) will also have to be considered.

Acknowledgments

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Registry No. RNase-H, 9050-76-4; DNA polymerase, 9012-90-2.

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Lanthanide–Adenosine 5′-Triphosphate Complexes: Determination of Their Dissociation Constants and Mechanism of Action as Inhibitors of Yeast Hexokinase†

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ABSTRACT: A kinetic procedure has been used to determine values for the dissociation constants of the complexes that ATP forms with lanthanum, 13 lanthanides, and scandium (Ln). The data indicate that the strength of the interaction increases as the ionic radius of the Ln(III) ion decreases and is much greater than that with Mg(II) and Mn(II). The LnATP complexes act as inhibitors of the reaction catalyzed by hexokinase, and the inhibition increases with the decreasing size of the Ln ion. While the ATP complexes of La, Ce, Pr, and Nd act as classical inhibitors, the nucleotide complexes formed with the other lanthanide ions behave as slow-binding inhibitors. The slow-binding inhibition with each LnATP complex conforms to a mechanism which involves rapid formation of an enzyme–glucose–LnATP complex which then undergoes a slow, reversible isomerization reaction. The reasons for the thermodynamic and kinetic behavior of the LnATP complexes are discussed.

Several years ago, it was suggested that Ln(III) ions[1] may function as good activators of phosphotransferases (Morrison & Heyde, 1972). It was pointed out that, while the electronic properties and ionic sizes of Ln ions were similar to those of the bivalent metal ions that function as activators, they possess a higher charge density. Subsequent investigations showed that, rather than functioning as activators, Ln ions acted as potent inhibitors of creatine kinase (Ellis & Morrison, 1974; Williams & Morrison, 1979) and several other phosphotransferases including hexokinase.

Ln ions undergo nonenzymic interactions with nucleotides to form Ln–nucleotide complexes (Ellis & Morrison, 1974; Morrison & Cleland, 1980), and it has been demonstrated that such complexes act as inhibitory analogues of MgATP. Thus,

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[1] To facilitate discussion, lanthanum will be regarded as a member of the lanthanide series, and the abbreviation Ln will be used for the entire group of trivalent ions.

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