Yeast ribonuclease H(70) cleaves RNA-DNA junctions

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Received 7 August 1986

A specific substrate, M13 DNA:RNA-[^{32}P]DNA, was synthesized to investigate the mode of cleavage of enzymes with RNase H activity. RNase H(70) from *Saccharomyces cerevisiae* hydrolyzes the phosphodiester bond at the RNA-DNA junction of this substrate, thereby producing a 5′-monophosphate-terminated polydeoxyribonucleotide and 3′-hydroxyl-terminated oligoribonucleotides.

RNase H *DNA-RNA hybrid* *DNA replication* *RNA primer removal* (Saccharomyces cerevisiae)

1. INTRODUCTION

Recently we have purified a ribonuclease H (RNase H, i.e. an enzyme which specifically hydrolyzes the RNA strand of a DNA-RNA hybrid) from the yeast, *Saccharomyces cerevisiae*, which stimulates the in vitro DNA synthesis by DNA polymerase A from the same organism [1,2]. Under certain conditions this protein also exhibits reverse transcriptase activity [3]. As with RNases H from other organisms [4] no definite physiological role could be attributed to the enzyme until now. DNA-RNA hybrids occur during cellular DNA replication [5], both during the initiation of leading strand synthesis as well as during the discontinuous polymerization of the lagging strand whereby each nascent Okazaki fragment contains a short RNA primer covalently attached to the 5′-end of the DNA piece. A plausible role for an RNase H would be its responsibility for the removal of these RNA primers. To determine whether the enzyme isolated by us [designated yeast RNase H(70)] is, at least in vitro, capable of such a task we have tested it with a substrate specifically constructed for this purpose. The substrate (which we term M13 DNA: RNA[P*]DNA) consists of a closed circle of M13 DNA with complementary DNA strands containing short RNA primers. The phosphorus atom of the phosphate bridging the RNA and DNA parts of this polynucleotide is radiolabeled (see fig.1). Here we show that, in vitro the yeast RNase H(70) preferentially hydrolyzes the phosphodiester bond between the ribonucleotide and the deoxyribonucleotide at the RNA-DNA junction.

2. MATERIALS AND METHODS

RNase H(70) and DNA polymerase A from *S. cerevisiae* as well as M13mp9 DNA were purified as described [1,6,7]. DNA polymerase I (Klenow fragment) and RNA polymerase from *E. coli* were from Boehringer/Mannheim.

2.1. *Preparation of the M13 DNA : RNA[P*]DNA substrate*

Single-stranded M13mp9 DNA was incubated for 10 min with all 4 ribonucleoside triphosphates in presence of $MnCl_2$ (4 mM) to favor nonspecific transcription initiation (for detailed reaction conditions see [1]). The reaction was stopped by addition of EDTA (50 mM). The hybrids were purified by 3 successive spun columns [7], phenol extraction and ethanol precipitation. Then they were reacted for 2 min at 30°C with Klenow polymerase (0.1 pmol/0.6 pmol M13 DNA) and 5 μM

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00145793/86/$3.50

[α-^{32}P]dATP (5 Ci/mmol) [7]. The reaction products were purified as above and further elongated by incubation with 25 units/ml of yeast DNA polymerase A [6] and all 4 unlabeled deoxyribonucleoside triphosphates for 30 min at 30°C.

2.2. *Nearest-neighbour analysis*

M13 DNA:RNA[P*]DNA (10 μM ribonucleotides/150 μl assay mixture) was incubated with 0.1 μg RNase H(70) at 30°C and aliquots (20 μl) were removed after different time intervals. These were made 50 mM in EDTA and trichloroacetic acid was added to a final concentration of 10%. After centrifugation the precipitates were repeatedly solubilized in 0.5 M KOH and precipitated in 5% trichloroacetic acid; finally 10 μl aliquots were hydrolyzed with 0.3 M KOH for 20 h at 37°C. The remaining polydeoxyribonucleotides were again precipitated (5% trichloroacetic acid), washed and analyzed by electrophoresis in denaturing agarose gels (0.7%) [8]. Size standards were *Hin*dIII fragments of λDNA [7]; autoradiography was carried out for 48 h [7]. The supernatants from the first trichloroacetic acid precipitations were lyophilized, solubilized in water, neutralized and analyzed by ascending thin-layer chromatography on polyethyleneimine-cellulose in 1 M acetic acid/3 M LiCl (9:1, v/v).

3. RESULTS

The rationale of the nearest-neighbour analysis for testing the specificity of RNase H(70) is described in fig.1. If M13 DNA:RNA[P*]DNA (see sections 1 and 2) is reacted with a nuclease able to hydrolyze the bond between the last ribonucleotide and the labeled 5′-phosphorus of the first deoxyribonucleotide the label will stay with the acid-precipitable reaction products. No acid-soluble radioactive ribonucleoside monophosphates should be detectable after subsequent alkaline hydrolysis (reaction a + $(OH)^-$ in fig.1). By contrast, alkaline hydrolysis alone (experimentally or, because the RNase H cannot cleave a junction between RNA and DNA, expt b in fig.1) will result in labeled ribonucleoside monophosphates. Cleavage of the bond between the phosphorus and the 5′-end of the DNA part at the RNA-DNA junction (reaction c in fig.1) would lead to labeled products only in the acid-soluble fraction

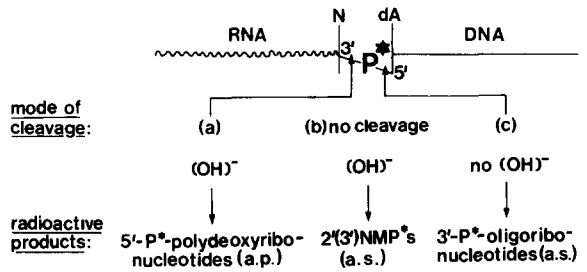

Fig.1. Scheme of experiments to analyze the mode of cleavage of an RNA-DNA junction by an RNase H (see text for explanation; a.p., acid-precipitable; a.s., acid-soluble). The complementary DNA strand of the substrate is omitted in the drawing.

(3′-[P*]oligoribonucleotides which can be discriminated from mononucleotides chromatographically). Thus, by reacting M13 DNA:RNA[P*]DNA with RNase H(70) and alkali, and by analyzing the reaction products one can distinguish between all possible modes of cleavage.

The results of our tests are depicted in figs 2 and 3. Incubation of M13 DNA:RNA[P*]DNA with RNase H(70) alone (reaction a of fig.1) does not noticeably change the size distribution of the acid-precipitable polymers (cf. lanes 1 and 2 of the denaturing gel electrophoresis in fig.2). This means that the substrate contains DNA chains of between 2 and 4 kb primed by short oligoribonucleotides, hence reflecting the in vivo situation during lagging strand DNA replication. It may be interesting to note that yeast DNA polymerase A which was used for the RNA primer elongation reaction must have behaved quite processively during the polymerisation process. No radioactivity is made acid soluble during incubation of the substrate with RNase H(70) alone (lane 2 of fig.3) indicating that RNase H(70) cannot have cleaved the ester bond between the labeled phosphate and the 5′-end of the DNA part of the substrate (a reaction which would correspond to proposal c of fig.1). The acid-soluble products of the reaction of M13 DNA:RNA[P*]DNA with alkali alone (reaction b of fig.1) consist, as expected, of 2′(3′)-ribonucleoside monophosphates (fig.3, lane 3) and no radioactivity is left to the DNA part of the substrate (fig.2, lane 3). If the substrate was incubated with RNase H(70) for increasing periods of time before alkaline hydrolysis the radioactivity was increasingly found with the acid-precipitable

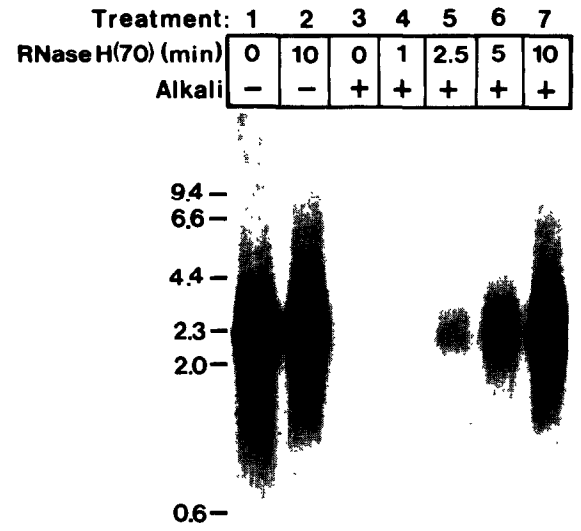

Fig.2. Denaturing agarose gel electrophoresis of acid-precipitable products from the reaction of M13 DNA:RNA[P*]DNA with RNase H(70) followed by treatment with alkali. Numbers on the left: size markers in kb.

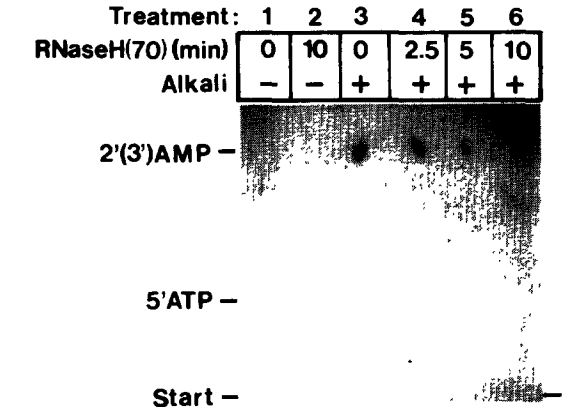

Fig.3. Polyethyleneimine-cellulose thin-layer chromatography of acid-soluble products from the reaction of M13 DNA:RNA[P*]DNA with RNase H(70) followed by treatment with alkali. On the left the positions of reference molecules are shown.

reaction products (lanes 4–7 of fig.2 and lanes 4–6 of fig.3). This can only be interpreted by assuming hydrolysis of the ester bond between the 3′-terminus of the RNA primer and the labeled phosphorus at the RNA-DNA junction by the action of RNase H(70) as proposed by reaction a of fig.1. An identical experiment using the same substrate, but labeled throughout the RNA primer part, was carried out in parallel. The amounts of RNA counts rendered soluble by RNase H(70) alone were 2, 7, 16 and 30% after 1, 2.5, 5 and 10 min incubation, respectively (not shown). As complete cleavage of the RNA-DNA junction is obtained after 10 min this reaction obviously takes a faster course than total RNA hydrolysis.

4. DISCUSSION

Our data show that RNase H(70) from yeast is able to hydrolyze the phosphodiester bond between the 3′-oxygen and the phosphorus preferentially at a RNA-DNA junction. The products of this reaction are a 3′-OH-terminated oligoribonucleotide and a 5′-monophosphate-terminated polydeoxyribonucleotide, the latter being a substrate for DNA ligase. Thus, RNAse H(70) is suited for and might well be involved in primer removal during in vivo DNA replication. In yeast the situation is different from *E. coli* because, in contrast to *E. coli* DNA polymerase I, there is no yeast DNA polymerase exhibiting a 3′–5′-exonuclease activity [9] which could be used for primer degradation. Therefore, a separate protein must be assumed for this function in yeast and possibly in other eukaryotes. Interestingly, yeast RNase H(70) resembles more closely the proteins from retroviruses which exhibit RNase H activity [3] than the RNase H from *E. coli* [4,10]. The RNase H activity, e.g. of AMV reverse transcriptase, does remove RNA primers and is able to hydrolyze precisely and preferentially the junction between RNA and DNA at the origin for plus-strand synthesis of proviral DNA [11]. One other eukaryotic RNase H, namely that from chicken embryos, was also shown to hydrolyze the diester bond between RNA and DNA in vitro [12]. However, the authors used a synthetic homopolymer as substrate poly(dT):poly(A)-poly(dA) which may be conformationally different from double helices containing all 4 nucleotides [13]. RNase H activities may specifically recognize the junction between the B-form of a DNA double helix and the A-form of an RNA-DNA hybrid [4] which most probably is present in the substrate prepared by us.

The function of the *E. coli* RNase H is not clear and is even dispensable as long as the cells grow slowly [10]. Therefore, and because this enzyme is not capable of cutting RNA-DNA junctions [14], its role in primer removal might be only an auxiliary one and an involvement in the proper initiation of DNA replication at oriC was suggested as the predominant function of *E. coli* RNase H [10]. So far there is no indication for a similar function of any eukaryotic RNase H.

ACKNOWLEDGEMENTS

We are grateful to the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich for support (Grant 5508), to Dr M. Binder for critically reading the manuscript and to Mrs D. Büchinger for word processing.

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