DNA Binding, Condensing and Unwinding Properties of Yeast RNase H₁*

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Two distinct RNases H have recently been purified from yeast cells. One of these proteins, very abundant in the cell, called RNase H₁, is shown here to be an interesting nucleic acid binding protein. The affinity of RNase H₁ for various nucleic acids was compared by membrane filtration and sedimentation techniques. Competition experiments indicated the following order of affinity: single-stranded RNA > single-stranded DNA > double-stranded DNA > DNA-RNA hybrid. Binding of RNase H₁ to T₃ DNA or SV40 DNA resulted both in the condensation of the DNA, observed by electron microscopy, and in the destabilization of the double helix. However, there was no permanent modification of the DNA structure. Concomitant with these structural changes, a drastic stimulation of transcription of yeast RNA polymerases A or B. As evidenced by electron microscopy and sedimentation studies of the transcription complex, there was a competition between the DNA template and the RNA product for binding RNase H₁ during the course of the transcription. These results suggest the involvement of RNase H₁ in chromatin structure and function.

In the present work, it is shown that RNase H₁ binds preferentially to single-stranded nucleic acids. This protein alters the conformation of double-stranded DNA as evidenced by electron microscopy, and causes a destabilization of the DNA double helix. Concomitant with these DNA structural changes, a drastic stimulation of transcription was observed with yeast RNA polymerases.

EXPERIMENTAL PROCEDURES

Materials

Yeast RNA polymerases A and B (3, 4) and yeast RNase H₁ (1) were purified as previously described. S₁ nuclease from Aspergillus oryzae (5) and relaxation enzyme from KB cells (6) were obtained from P. F. Spahr (Geneve) and B. Hirt (Lausanne), respectively. SV40 DNA form I was provided by P. Nardeux (Villejuif). T₃ DNA, [³²P]DNA, [³²P]RNA and DNA-RNA hybrids, and other nucleic acids were prepared or obtained as previously described (7, 8). Other materials were as previously described (8).

Methods

Polyacrylamide Gel Electrophoresis—Polyacrylamide slab gel electrophoresis of proteins were carried out on 12.5% acrylamide gel according to Laemmli (9). Electrophoretic separation of DNA was performed on polyacrylamide gradient gel slab from 2.5 to 8% acrylamide as described by Allet (10) and Jeppesen (11). Mixed slab gels of polyacrylamide (1.5%) and agarose (0.6%) for separation of SV40 DNA were prepared and run according to Germond et al. (6).

Electron Microscopy—Samples for electron microscopy were diluted to a concentration of 0.5 to 0.6 μg/ml of nucleic acid with the incubation buffer at 30°C (20 mm Tris/HCl (pH 8.0), 2 mm MnCl₂, 25 mm ammonium sulfate, and 5 mm dithiothreitol) and the DNA complexes were directly adsorbed to positively charged carbon film according to Dubochet et al. (12). The specimens were stained for 30 s with a 2% aqueous solution of uranyl acetate for dark field examination. Observations were made with a Siemens Elmiskop 101 electron microscope. The dark field was obtained by the tilted beam method. A thin film objective aperture of 20 μm was used. Micrographs were recorded on Kodak film (95 x 90 mm) at magnifications ranging from 20,000 to 60,000.

Binding of RNase H₁ to Nucleic Acids—RNase H₁ was incubated with the labeled polynucleotide as described under the figure legends and complex formation was estimated by filtration on nitrocellulose membranes according to a method previously described (13) or by sedimentation on glycerol gradient. Fractions from the gradient were analyzed for the presence of RNase H₁ by gel electrophoresis with dodecyl sulfate, and for DNA by radioactivity measurements.

RESULTS

Binding of RNase H₁ to Double Stranded DNA—Optimal hybridase activity of RNase H₁ takes place between pH 6 and
pH 8, in the presence of Mg"+ and Na' ions, at a protein to nucleic acid ratio around 0.5 (2). When incubated under these conditions with a labeled polynucleotide, RNase H, was able to form a complex which could be recovered on nitrocellulose membrane (Fig. 1). Binding of RNase H, to T7 [3H]DNA occurred under a wide variety of conditions between pH 6 and pH 8, with or without divalent cations or salt, but this depended on the ratio of RNase H, to DNA. At protein to DNA ratios higher than 1 (w/w) complex formation was maximal and unaffected by the addition of divalent cations (10 mm Mg"+) and salt up to 0.3 M NaCl (Figs. 1 and 2). At lower protein to DNA ratios, however, the presence of divalent cations, as well as the addition of salt, was inhibitory. Practically no retention of DNA was observed at pH 8 with 5 mm Mg"+ or 0.1 M NaCl at ratios lower than 0.5 (by weight). Retention of DNA at high salt was more efficient at pH 6 than pH 8 (Figs. 1 and 2).

Formation of the complex does not appear to be temperature-dependent between 0 and 50°C (results not shown). Assuming a molecular weight for native RNase H, of 48,000 (2), it can be calculated from the above results that 1 molecule of RNase H, can cause the retention of 250 to 300 base pairs under optimal conditions.

**Binding of RNase H, to Various Polynucleotides and Competition with DNA — RNase H, also formed a complex with RNA (Escherichia coli RNA) or with RNA-DNA hybrids which were retained on membrane filters. In this case, a 10-fold excess of protein was required when compared with T7 DNA. However, these figures were difficult to compare since the size of the labeled polynucleotides was much different. Furthermore, the direct filtration technique could not be applied to single-stranded DNA. Therefore, competition experiments were performed to compare the affinity of RNase H, for various nucleic acids. RNase H, was incubated under optimal conditions with 3H-labeled T7 DNA in the presence of increasing amounts of unlabeled competitor. As shown in Fig. 3, RNase H, was preferentially bound to single-stranded nucleic acids, RNA or DNA. Especially single-stranded RNA displaced very efficiently RNase H, from double-stranded DNA. Surprisingly, the RNA-DNA hybrid was the least efficient competitor (Fig. 3). The order of affinity inferred from the competition experiments was: single-stranded RNA > single-stranded DNA > DNA duplex > DNA-RNA hybrid.

To explore further the complex formation, a mixture of RNase H, and T7 [3H]DNA was sedimented through a glycerol gradient and the fractions were analyzed for the presence of DNA and protein. At a protein to DNA weight ratio of 2, sedimentation of T7 DNA was drastically modified (Fig. 4). One-third of the DNA was found as a pellet, in spite of the presence of a 80% glycerol cushion, one-half migrated approximately twice as fast as T7 DNA alone, and only 10% migrated as free DNA. RNase H, co-sedimented with the DNA in the pellet and at the level of the rapid sedimenting fraction. Practically no protein was detected on top of the gradient, at the level where the protein alone stayed under the same conditions. At an RNase H, to DNA ratio of 0.5, sedimentation of the DNA was not significantly affected, but again the protein co-migrated with the DNA in the gradient.

These experiments clearly show that stable complexes were formed with probably more or less compact structures, depending on the amount of ligand. In a parallel experiment, RNase H, was preincubated with T7 [3H]DNA for 2 min at 30°C, then competitor RNA was added, and the mixture was centrifuged in glycerol gradient. Under these conditions, the DNA migrated like free T7 DNA. This displacement reaction occurred despite a 2-fold excess of DNA versus competitor RNA. This observation illustrated the strong affinity of RNase H, for single-stranded RNA, already evidenced by the filtration experiments.

**Structure of Protein-DNA Complexes as Seen by Electron Microscopy — The nature of the interaction between RNase H, and DNA was visualized in the electron microscope by dark field observation of specimens prepared by adhesion on positively charged carbon films (12). Complexes with T7 DNA or...
regions of naked DNA alternated with parts where proteins accumulated and created multiloops, hairpins, cloverleaf, and more complicated structures. Bubbles were also seen in these dense entanglements, which might correspond to denatured DNA with bound RNAse H,

The apparent complexity of RNAse H, induced structures increased with the weight ratio of protein to DNA. The central core seen in some complexes probably arose with protein-protein interactions. Although this suggested some cooperative binding, all DNA molecules had some protein bound even at a low ratio of protein to DNA. Fifty per cent of the complexes appeared with a central core at a ratio of 1 and at pH 6. At higher protein to DNA ratio, every DNA molecule was highly collapsed and the regular dense nucleoprotein core was surrounded by very small DNA loops. The association of several such condensed complexes was also observed. These collapsed structures probably corresponded to the RNAse H, DNA complexes found in the pellet after centrifugation (see Fig. 4). Within these nucleoprotein cores, the length of the DNA was reduced 50-fold as compared with fully extended T7 DNA. Observations made at pH 8 were very similar except that the corresponding figures were observed at a higher protein to DNA ratio. With SV40 DNA (3.5×10^6 daltons), complexes had a similar shape but were considerably smaller than those formed with T7 DNA, suggesting that most of the complex appeared with a central core at a ratio of 1 and at pH 6.

To summarize these observations, at a protein to DNA ratio around 2 or lower, a variety of structures were induced by RNAse H,. Furthermore, these bubbles, loops, and hairpin-like structures were not regularly spaced along the DNA molecules. At a ratio higher than 2, only compact structures with a central core were seen. It should be added that similar figures were observed when RNAse H, formed complexes with high molecular weight T7 DNA-DNA hybrids (results not shown).

Unwinding of DNA by RNAse H,. The observation of DNA bubbles under the electron microscope, together with the fact that RNAse H, has a high affinity for single-stranded polymers prompted us to investigate the possibility that this protein could induce the formation of unpaired DNA. The presence of unpaired DNA regions was sought by investigating their susceptibility to nuclease S, under conditions where RNAse H, binds to DNA. In the presence of RNAse H, T7 DNA complexes formed at pH 6 with 0.2 µg of RNAse H, (r = 1) (× 125,000); b, with 0.4 µg of RNAse H, (r = 2) (× 110,000); c, with 1.2 µg of RNAse H, (r = 6) (× 100,000); d, control T7 DNA (× 50,000); e, T7 DNA-RNA complex formed at pH 8 with 0.4 µg of RNAse H, (r = 2) (× 125,000), f, with 0.8 µg of RNAse H, (r = 4) (× 100,000); g, SV40 DNA. RNAse H, complexes formed at pH 8 with r = 2 (× 170,000) (upper part), and control SV40 DNA (lower part).
DNA Binding Properties of Yeast RNase HI

TABLE I

| Template | RNA polymerase A | RNA polymerase B |
|---------|-----------------|-----------------|
|          | -RNase H1 | +RNase H1 | -RNase H1 | +RNase H1 |
| T7 DNA   | 1,100     | 12,800 (11) | 220       | 11,000 (50) |
| T4 DNA   | 700       | 4,200 (6)   | 400       | 3,600 (11)  |
| λ DNA    | 180       | 4,200 (6)   | 400       | 3,600 (11)  |
| SV40 DNA | 2,900     | 8,800 (3.5) | 2,500     | 8,800 (3.5) |
| Yeast DNA | 800     | 6,150 (3)   | 650       | 3,800 (6)   |
| Denatured T7 DNA | 860 | 21,200 | 21,800 (0) |
| (dC)4  | 24        | 24 (0)      | 24        | 24 (0)      |
| (dG)4  | 760       | 900 (0)     | 1,050     | 2,000 (0)   |
| (dA)4  | 580       | 520 (0)     | 520       | 520 (0)     |
| (dT)4  | 120       | 80 (0)      | 120       | 80 (0)      |
| (dA-T)4 | 1,080     | 740 (0)     | 3,080     | 2,200 (0)   |

was hydrolyzed by the nuclease (Fig. 6). The relative amount of DNA rendered acid-soluble was roughly proportional to the amount of protein added. At a weight ratio of 2, up to 30% of the radioactive DNA was solubilized in 10 min. There was no additional degradation during a prolonged incubation of 20 min, with a 6-fold excess of RNase H1 over DNA (w/w). The experiment shown in Fig. 6 was performed at neutral pH but also at pH 5 and 4.3, with essentially the same results. Such an extensive degradation of the DNA by nuclease S1 was surprising, however, in view of the comparatively small amount of possibly denatured DNA bubbles seen under the electron microscope.

The effect of RNase H1 on supercoiled SV40 DNA was further investigated by the electrophoretic technique which allows the resolution of the different supercoiled states as well as the relaxed and linear forms of the DNA (Fig. 7). Using this highly sensitive technique, one can estimate the degree of contamination of RNase H1 with endonucleases but also investigate whether RNase H1 caused some structural constraint on the DNA molecule which might interfere with the activity of a relaxing enzyme (6) or change the final DNA products.

Preincubation of RNase H1 with supercoiled SV40 DNA at a weight ratio of 1 did not change the migration pattern of the supercoiled bands. With increasing amounts of RNase H1, the band of relaxed form seemed to increase a little, suggesting the presence of trace amounts of nuclease. However, another possibility was that the RNase H1 preparation con-
DNA Binding Properties of Yeast RNase H1

Effect of RNase H1 on Transcription of Double-stranded Templates—Yeast RNA polymerases, like the corresponding mammalian enzymes, have a very low activity with intact DNA duplexes. Unpaired DNA regions are required in vitro by the yeast enzymes to perform the initiation step (14). Therefore, we investigated whether a structural alteration of the DNA brought about by RNase H1 could stimulate transcription by yeast RNA polymerases.

The effect of RNase H1 on transcription of a variety of natural and synthetic templates was investigated using a weight ratio of 2 RNase H1/DNA, since it was under these conditions that DNA was most susceptible to nuclease S (see Fig. 6). Table 1 shows that transcription of all double-stranded natural DNA, either phage DNA, calf thymus, or yeast DNA, was indeed markedly stimulated. The most active template in the presence of RNase H1 was T7 DNA. The template activity was increased to the extent that yeast RNA polymerases reached a specific activity approaching that of the bacterial enzyme with the corresponding templates. On the other hand, no effect of RNase H1 was observed on transcription of the double-stranded homopolymer (dG), or with the alternated copolymer d(A-T), or d(G-C). The template activity of denatured T7 DNA and (rC), was also practically unaffected. It should be noted here that no change in the sedimentation of T7 [3H]DNA in alkaline sucrose gradient was observed after a prolonged incubation of native DNA with RNase H1 in transcription buffer, and no acid-soluble radioactivity could be detected (less than 0.01%). These controls were performed to exclude the artifact of template activation by nucleases.

Fate of RNase H1 during Transcription—In view of its strong affinity for the RNA, it was interesting to study the distribution of RNase H1 during transcription. T7 DNA-directed RNA synthesis was carried out in the presence of RNase H1, and the transcription complex was sedimented through a glycerol gradient. Under the conditions chosen, free RNase H1 could be separated from free T7 DNA and from the ternary transcription complex, made of T7 DNA, RNA polymerase, and RNA product. The fractions were analyzed for radioactivity to detect the [32P]-labeled RNA product and...
The sedimentation properties of T₁ DNA template at the beginning of the transcription process were similar to that observed with RNase H₁ alone. But as soon as the RNA product accumulated, the DNA progressively sedimented more like free DNA, whereas most of the RNA was released. RNase H₁, initially bound in part to the DNA, dissociated (Fig. 9) after 30 min transcription and sedimented mainly at the level of RNA or free RNase H₁, which were not well separated here. The results were somewhat different with RNA polymerase A (Fig. 9A). Although after 30 min or 90 min, there was clearly a shift in the sedimentation of T₁ DNA towards the top of the gradient, there still remained a considerable amount of rapidly sedimenting molecules of DNA and RNA throughout the gradient and in the pellet. RNase H₁ also remained distributed along the gradient together with the nucleic acid (Fig. 9).

Transcription of RNase H₁ DNA complexes by RNA polymerase B was also investigated by electron microscopy. Typical transcription complexes are shown in Fig. 10. The compact DNA structures and DNA bubbles have completely disappeared. Most of RNase H₁ has dissociated from the DNA. Large and expanded RNA bushes are now present, either free or bound to the DNA template (Fig. 10, B and C). A comparison of these RNA bushes with those, highly collapsed, obtained upon transcription of T₁ DNA by Escherichia coli RNA polymerase (Fig. 10D) (15), leads to the conclusion that the expanded and granular aspect of the RNA made with yeast RNA polymerase B is due to the binding of large amounts of protein to the RNA. Apparently, RNase H₁ was displaced from the DNA by the newly synthesized RNA, which is in keeping with the competition experiment shown in Fig. 8. On the other hand, the small and regular spot in the center of the ribonucleoprotein complexes bound to the DNA may well be RNA polymerase.

**Discussion**

Present studies indicate that yeast RNase H₁ binds to nucleic acids, preferentially to single-stranded polymers, and also alters the conformation of double-stranded DNA. Upon binding of RNase H₁ to T₁ DNA or SV40 DNA, loops, hairpin structures, bubbles, and dense cores were seen under the electron microscope. The formation of DNA bubbles suggested that RNase H₁ could open the DNA double helix and this was evidenced using nuclease S₁, which digests specifically unpaired DNA regions, but also DNA folding and kinking by RNase H₁ as well as other locally altered structures. The condensation of the DNA molecule could be explained by the neutralization of the DNA phosphate groups by the basic protein (18) and by protein-protein interactions. A cooperative effect of RNase H₁ is supported by the fact that this protein has a strong tendency to self-associate (2) and also by the increased resistance of the DNA-RNase H₁ complex to high salt and divalent cation concentrations at high protein to DNA ratios (see Figs. 1 and 2). It might be argued that any basic protein is expected to bind nucleic acids, especially nucleases. For instance, under restricted ionic conditions, pancreatic RNase binds to double-stranded DNA and destabilizes some extent the double helix (19) but without condensing the DNA. DNA collapse can be induced by basic polypeptides as well as by highly acidic polymers. Interestingly, however, only acidic polymers, such as polyethylene oxide favor the formation within the collapsed structure, of DNA regions vulnerable to single-strand-specific nuclease, like RNase H₁. Condensation of DNA by polylysine or histone rather resulted in the stabilization of the double helix (18, 20). Therefore, compared to other basic proteins, RNase H₁ appears to interact with DNA in a rather original way, suggesting its participation in chromatin structure.

**Probability as a consequence of the structural alternations of the DNA, yeast RNA polymerases, which are practically unable to initiate on intact DNA duplexes (14), could now transcribe efficiently a linear DNA, or a circular supercoiled DNA. One might be surprised that the collapsed DNA structures induced by RNase H₁ are transcribed so efficiently. A rather similar observation has been made with the highly condensed bacterial nucleoid. The folded chromosome from *Escherichia coli* is a more efficient template than the unfolded form, essentially due to an increased rate of chain initiation (21). In fact, the effect of the three-dimensional structure of the chromosome on gene expression remains largely unknown. The strong affinity of RNase H₁ for RNA compared to DNA or to RNA-DNA hybrids suggested that this protein could bind to the RNA product during transcription, thereby participating in the dissociation of the RNA from the transcription complex at termination sites, a process which might also involve its RNase H activity. It is difficult though to predict the target of RNase H₁ in the cell, since a number of other nucleic acid-binding proteins will compete with it for binding RNA or DNA.

Stimulation of transcription by RNase H₁ was observed at protein to DNA ratios similar to those which induced conformational changes of the template. Therefore, in view of the relatively large amounts of protein used, the possibility remains that it is a contaminant of the preparation which has
the stimulatory activity. However, the two activities, RNase H, and the stimulatory activity, were, until now, inseparable by various fractionation procedures. Furthermore, the recent finding of an RNase H activity tightly associated with yeast RNA polymerase A (22) adds additional weight to the consideration of the role of RNase H, in transcription. We have also accumulated evidence in favor of the identity between RNase H, and one of the polypeptides present in RNA polymerase A (M, = 48,000) (4). These observations and further work characterizing the stimulatory activity will be presented elsewhere.

The question which remains, at this point of the in vitro studies, concerns the in vivo significance of all these observations. It should be noted that all the properties of RNase H, stimulation of transcription, DNA binding, and hybridase activity, can occur under similar ionic environment. Furthermore, from the high amount of RNase H, which can be extracted from yeast cells (1), a 2:1 ratio, by weight, of RNase H, to DNA does not seem unreasonable. Accurate determination of the number of RNase H, molecules per yeast genome are required to clearly establish this point. At any rate, it is clear that the biological role of the RNases H found in yeast (1, 2), and specially of RNase H, will remain uncertain and speculative until defective proteins are isolated and the phenotype of the mutants are investigated in detail.

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