Volume 12 Number 6 1984 Nucleic Acids Research

Binding of *Xenopus* transcription factor A to 5S RNA and to single stranded DNA

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Received 19 December 1983; Revised and Accepted 27 February 1984

ABSTRACT

Footprint competition assays are utilized to study the binding of Xenopus transcription factor A to a variety of single-stranded nucleic acids. The addition of Xenopus oocyte, yeast, or wheat germ 5S RNA as footprint competitors reveals that factor A binds these 5S RNAs with similar affinity. In contrast, factor A does not bind to E.coli 5S RNA or wheat germ tRNA in this assay. Factor A binding to single stranded DNA is also examined using footprint competition. Factor A binds preferentially to non-specific single stranded (M13) DNA versus double stranded (pBR322) DNA. Factor A binds equally well to single stranded DNA fragments containing either the coding or non-coding strands of the 5S RNA gene. Using single stranded M13 DNA as a competitor, the factor A-5S RNA gene complex is found to dissociate with a half-life of 5-6 min.

INTRODUCTION

Xenopus 5S RNA genes contain an intragenic control region of approximately 50 bp which is required for accurate transcription initiation (1,2). A 38,000 dalton protein, designated TFIIIA or factor A, binds to this region of 5S RNA genes and directs accurate transcription initiation by RNA polymerase III (3). The binding of factor A to the 5S RNA gene appears to be an early event in the formation of stable transcription complexes (4).

Factor A binds to the 5S RNA gene with an apparent dissociation constant of about 10^{-9} M. The stoichiometry of factor A binding is low. We have previously reported experiments that suggest that two proteins bind per gene (5). Strong contact points between factor A and the 5S RNA gene have been observed clustered near the 3' end of the control region on the non-coding strand (6).

In vivo, factor A is found in a specific association with oocyte 5S RNA in a stable 7S nucleoprotein particle (7). Xenopus

5S RNA but not E.coli tRNA inhibits both factor A-dependent in vitro 5S RNA synthesis and DNase I protection of the ^{32}P end-labeled 5S RNA gene (7). The ability of factor A to bind either the 5S RNA gene or 5S RNA suggests a feedback inhibition mechanism for the control of Xenopus 5S RNA synthesis (7). Sakonju and Brown have suggested that factor A may recognize a common structural feature in the 5S RNA gene and 5S RNA (6).

It is apparent that the ability of factor A to bind RNA and DNA has important consequences for the regulation of Xenopus 5S RNA synthesis by this protein. In the present study we examine a number of aspects concerning the interaction of Xenopus transcription factor A with various RNAs and DNAs in order to better define this regulation. The binding studies utilize the DNase I protection or footprinting technique (8) which affords a direct measure of factor A binding to the ^{32}P end-labeled 5S RNA gene. The addition of unlabeled competing nucleic acids in the footprinting reaction provides an indirect measure of factor A binding to the competitor (6).

MATERIALS AND METHODS

7S Particle and factor A isolation

7S particles from Xenopus immature oocytes were isolated by glycerol gradient centrifugation (7). The particles were then fractionated by DEAE cellulose chromatography resulting in preparations at least 90% pure (5). Factor A was isolated by digesting 200 µl of 7S particles (1.5 mg/ml in 20 mM Tris-HCl, pH 7.5, 320 mM KCl, 250 mM NaCl, 1.5 mM $MgCl_2$, and 1 mM DTT (dithiothreitol) with RNase A (Calbiochem) at a final concentration of 150 µg/ml. Digestion proceeded for 1-1.5 hrs at 22°C. The mixture was then chromatographed on a Sephadex G-50 (Pharmacia) column equilibrated with 20 mM Tris-HCl, pH 7.5, 280 mM NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol. Factor A was collected in the void volume. This procedure served to remove RNase A as well as greater than 95 % of the A_{260} absorbing material present in the original 7S particle. Factor A fractions (0.2-0.25 mg/ml) were stable for several weeks at 0° C. Factor A concentrations were routinely determined by the method of Bradford (9) which is consistent with quantitative amino acid

analysis (5).

Preparation of DNA and RNA

The 5S RNA gene-containing plasmids, pXbs -73x201 (with a 5' deletion end-point at residue -73) and pXbs 201 (with a 5' deletion end-point at residue -48, reference 2), were purified by ethidium bromide-CsCl gradient centrifugation. Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories. To prepare large quantities of specific single stranded fragments, the coding and non-coding strands of the Xbs 5S RNA gene (258b) as well as an Alu I fragment (480b) of pBR322 were cloned into the HincII site of M13mp7 as described previously (10). The single stranded DNA clones containing the desired inserts were identified directly by DNA sequencing using the chain-termination method (11). The coding and non-coding strands of the 5S RNA gene as well as the Alu fragment were isolated from their respective clones in single stranded form by BamH1 digestion followed by agarose gel electrophoresis (10). Xenopus oocyte 5S RNA, yeast 5S RNA and E.coli 5S RNA were purified from cellular RNA by electrophoresis in an acrylamide/urea gel (12). Wheat germ 5S RNA and tRNA (gly I) were provided by Dr. Bernard Dudock. Single stranded DNA and RNA concentrations were determined using an A_{260} unit for a nucleic acid concentration of 40 μg/ml at one cm pathlength.

DNase I footprinting assays

The DNase I footprinting method of Galas and Schmitz (8) was performed as described previously (5). A 276 bp DNA fragment containing the Xenopus borealis somatic (Xbs) 5S RNA gene was prepared from plasmid pXbs201 and end-labeled with [α-^{32}P] dATP (5). Footprint competition assays were conducted and quantitated essentially as described by Sakonju and Brown (6). Quantitation of the footprint competition assays at the various competitor concentrations involved autoradiogram densitometry of the major DNase I cleavage band of the coding strand within the control region of the Xenopus 5S RNA gene. This band intensity, relative to the band intensity in the absence of factor A, is proportional to the fraction of factor A displaced from the control region (θ) by the competitor. In footprint competition experiments utilizing RNA as the competitor, factor A at a

concentration of $1x10^{-7}$ M was preincubated with RNasin (1000 units/ml, Promega Biotec), a specific RNase A inhibitor (13), for 5 min at 22°C. The final concentration of RNasin in the footprint reactions was 1000 units/ml.

RESULTS

Binding of Xenopus transcription factor A to various RNAs

Footprint competition experiments utilize a ^{32}P end-labeled 276bp duplex DNA fragment containing the 5S RNA gene as a factor A binding indicator. Upon varying the concentration of competing, unlabeled nucleic acid in the reaction, binding of factor A to the competitor is seen as a decrease in DNase I protection of the ^{32}P end-labeled 5S RNA gene, or loss of the

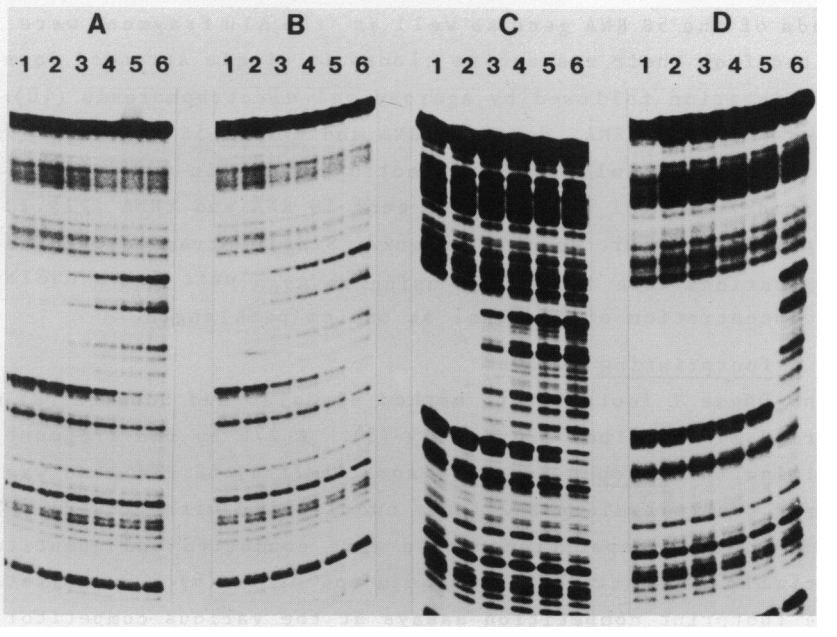

Figure 1. Loss of the factor A-dependent footprint on the 5S RNA gene in the presence of increasing concentrations of Xenopus, wheat germ, yeast and E.coli 5S RNA. Factor A ($1x10^{-8}$M) was incubated for 5 min at 22°C with ^{32}P end-labeled 5S DNA ($1x10^{-9}$M) and competitor RNAs. Samples were processed and electrophoresed as described in Materials and Methods. A, lanes 1-5: 0, 0.60, 1.2, 2.5, 5.0 $x10^{-8}$ M Xenopus oocyte 5S RNA; lane 6: $5x10^{-8}$M RNA and no added factor A. B, C, and D: results of experiments identical to that in panel A but with wheat germ, yeast, and E.coli 5S RNA.

footprint. Thus, this method is an indirect approach to measuring relative binding affinities of competitor nucleic acids (6). The abilities of Xenopus 5S RNA and a number of other 5S RNA molecules to compete in factor A-dependent, DNase I protection of the ^{32}P end-labeled 5S RNA gene are illustrated in Figure 1. Oocyte 5S RNA (Figure 1A, lanes 2-5), wheat germ 5S RNA (Figure 1B, lanes 2-5) and yeast 5S RNA (Figure 1C, lanes 2-5) are equally effective in the footprint inhibition assay. E. coli 5S RNA is not an effective binding competitor for factor A in this assay (Figure 1D, lanes 2-5). This lack of footprint inhibition by E.coli 5S RNA is apparently not due to degradation of the RNA under footprinting conditions since gel electrophoresis of the reaction mixtures containing Xenopus oocyte or E.coli 5S RNA reveals no degradation of RNA (data not shown). Wheat germ tRNA also does not compete for factor A binding (see Figure below).

The footprint competition observed in Figure 1 with these RNAs is graphed in Figure 2. The 50% inhibition of factor A-dependent footprinting by Xenopus 5S RNA, wheat germ 5S RNA, or yeast 5S RNA is observed at about $2x10^{-8}M$ (curve A, Xenopus;

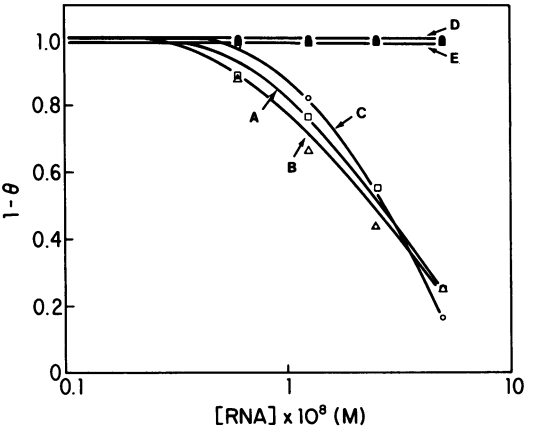

Figure 2. Graph of the relative binding of factor A to various RNAs. Curves A, B, C, D and E illustrate the competitive strengths of Xenopus 5S RNA, wheat germ 5S RNA, yeast 5S RNA, E.coli 5S RNA and wheat germ tRNA respectively, in factor A-dependent footprinting reactions (see Figure 1 legend). Samples were processed, electophoresed, and quantitated as described in Materials and Methods. θ is the fraction of factor A displaced from the 5S RNA gene by competitor DNA.

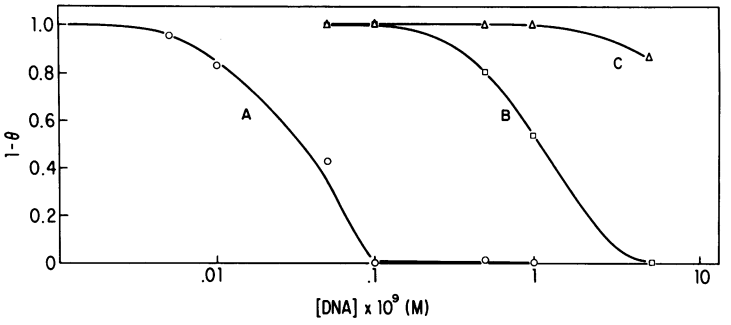

Figure 3. Competition of factor A binding to the 5S RNA gene by increasing concentrations of various DNAs. Factor A ($1x10^{-8}$ M) was incubated for 10 min at 22° C with ^{32}P end-labeled 5S DNA ($1x10^{-9}$ M) and competitor DNAs at the concentrations indicated. DNase I, at a final concentration of 15 µg/ml, was then added and incubated for an additional min. Footprints were quantitated as described in the Figure 2 legend. Curves A, B, and C represent the competition observed with M13mp9, pXbs 201, and pBR322,respectively.

curve B, wheat germ; curve C, yeast), a concentration at which E.coli 5S RNA or wheat germ tRNA fail to compete (D, E).

Xenopus transcription factor A binds preferentially to single stranded DNA

Factor A has been proposed to bind to single stranded DNA during transcription of Xenopus 5S RNA genes (5,6). Footprint competition experiments were conducted in order to examine the binding of factor A to single stranded DNA. The use of various double stranded and single stranded DNAs of similar molecular weight as competitors in footprint reactions reveals that single stranded phage M13mp9 DNA is a very effective competitior of factor A binding to the 5S RNA gene (Figure 3, curve A). The concentration of M13mp9 DNA required for 50% inhibition is near $3X10^{-11}$ M. This value is about 30-fold lower than that observed with the 5S RNA gene-containing pXbs201 plasmid of similar molecular weight ($1X10^{-9}$ M; Figure 3, curve B). Substantially less footprint competition is observed by the non-specific double stranded plasmid, pBR322 (Figure 3, curve C). These results demonstrate that factor A binds preferentially to non-specific single stranded DNA (curve A) versus double stranded DNA (curve C) by at least two orders of magnitude.

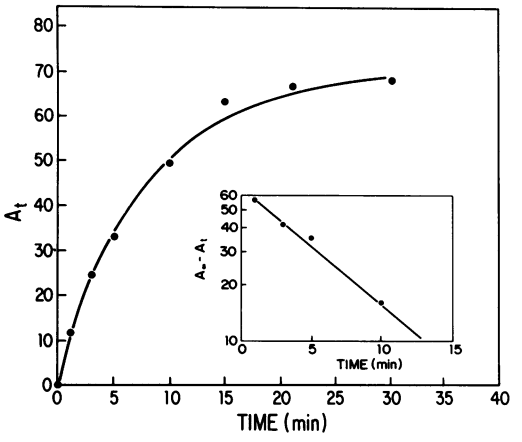

Figure 4. Kinetics of dissociation of factor A from the 5S RNA gene. Factor A ($1x10^{-8}$ M) and ^{32}P end-labeled 5S DNA were preincubated for 10 min at 22° C. M13mp9 viral DNA was then added ($1x10^{-9}$ M) and aliquots were removed at the times indicated and subjected to DNase I treatment (final concentration, 15 μg/ml) for an additional min. Samples were processed, electrophoresed, and quantitated as described in Materials and Methods. A_t values are relative amounts of displaced factor A from the 5S RNA gene. Insert: a semilog plot of $A_\infty - A_t$ versus time, where A_∞ represents the maximum amount of factor A displaced.

Rate of dissociation of factor A from the 5S RNA gene

The preferential binding of factor A to single stranded M13mp9 DNA shown in Figure 3 allows this DNA template to be utilized for determining the dissociation of factor A from the 5S RNA gene. The rate at which a protein dissociates from DNA can be obtained by adding an excess of binding competitor to a preformed complex and following the kinetics of complex dissociation, assuming that this step is rate limiting. Figure 4 illustrates the loss of the factor A footprint on the 5S RNA gene as a function of time, A_t, in the presence of unlabeled single stranded M13mp9 DNA. The data can be analyzed by first order kinetics which is indicative of a dissociation process (see insert). A half-life ($t_{1/2}$) of 5-6 min is obtained for the factor A-5S RNA gene complex. The first order rate constant (k) for dissociation of this complex is estimated to be 10^{-3} sec^{-1}.

Factor A binds equally well to the coding and non-coding strands of the 5S RNA gene

Figure 3 demonstrates that factor A can bind single stranded

DNA in non-specfic fashion. One approach to determine if factor A can interact specifically with single stranded DNA is to ask whether the protein can discriminate between 5S DNA and non-specific fragments or between the coding and non-coding strands of the 5S RNA gene in footprint competition experiments. This approach seems promising because factor A has been shown to make strong contacts primarily with the non-coding strand of the duplex 5S RNA gene (6) which may serve as a basis for discrimination. In our first experiments of this sort, single stranded DNAs from M13 mp8 clones containing either the coding or non-coding strand of the Xenopus 5S RNA gene were found to compete to the same extent in the footprint assays as the M13 vector alone (data not shown). Although this result was consistent with the non-specific single strand binding ability of factor A demonstrated in Figure 3, the presence of a large excess of non-specific M13 DNA in these clones hampers interpretation of the results. In order to obtain well defined, short, single stranded fragments of 5S DNA, we took advantage of the ability to clone and isolate specific single stranded DNA fragments from the mp7 variant of phage M13 DNA. Patton and Chae (10) have shown that duplex DNA fragments inserted into the poly-linker site of the replicative form of M13mp7 can be excised with Bam H1 from isolated phage DNA (single stranded), presumably due to the formation of a short duplex involving the repeat sequences in the poly-linker region. Employing this methodology (see Figure 5), we obtained DNA fragments containing the coding or non-coding strands of the Xenopus 5S RNA gene and a DNA fragment containing non-specific sequences from pBR322. This biological approach to purification of single stranded DNA segments results in more highly purified DNA than that obtained from strand separation by gel electrophoresis.

Results of footprint competition experiments utilizing single-stranded coding and non-coding 5S DNA fragments as well as a non-specific DNA fragment are shown in Figure 6. The competitors (coding strand, curve A; non-coding strand, curve B; pBR322 fragment, curve C) acheive 50% inhibition of the footprint reaction at about the same concentration, 5×10^{-9} M. Thus, as measured by footprint competition, factor A binds equally well to

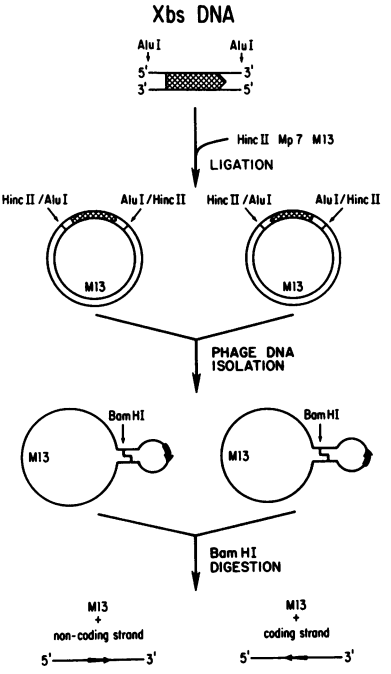

Figure 5. Cloning of the Xenopus 5S RNA gene coding and non-coding strands in M13 mp7. The Xbs 5S RNA gene with Alu I-generated blunt ends was ligated into M13 mp7 replicative form which had been digested with HincII. The blunt-end ligation products included both orientations of the Xbs 5S RNA gene. Individual phage DNA clones containing either the coding or non-coding strand of the 5S RNA gene were identified by DNA sequencing. The respective DNA fragments were isolated by BamHI digestion and agarose gel electorophoresis as described in Materials and Methods.

the coding and non-coding strands of the 5S RNA gene as well as to a non-specific DNA fragment of approximately the same size. The value of 50% inhibition observed in Figure 6 is approximately 5-fold higher than that observed for the double stranded 5S RNA gene and 150-fold larger than viral M13mp9 DNA (Figure 3, curves A and B). In addition, factor A apparently requires a minimum length of single stranded DNA to bind since an oligonucleotide of 15 bases does not compete in the footprint reaction (Fig. 6, curve D).

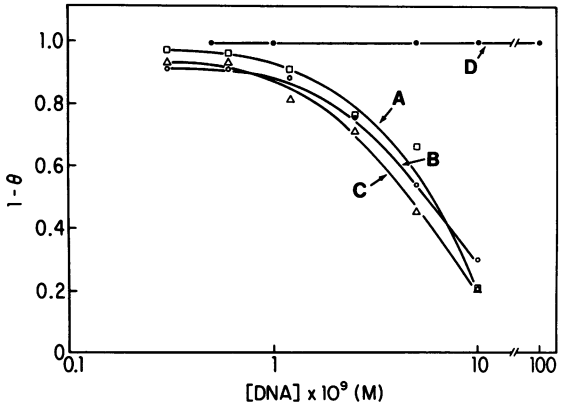

Figure 6. Competition of factor A binding to the 5S RNA gene by single stranded DNA fragments. Factor A ($1x10^{-8}$ M) was incubated for 10 min at 22°C with ^{32}P end-labeled 5S DNA ($1x10^{-9}$M) and competitor DNAs at the concentrations indicated. The reactions were processed, electrophoresed, and analyzed as described in the Figure 2 legend. Curves A,B, and C represent the competition observed for non-coding (A) and coding (B) strands of the 5S RNA gene and a non-specific single stranded DNA fragment from pBR322 (C), respectively. Curve D represents the competition observed with the M13 sequencing primer from Biolabs, a DNA oligonucleotide 15 bases in length.

DISCUSSION

The use of the DNase I footprinting method has permitted rapid progress in understanding the binding of transcription factor A to the Xenopus 5S RNA gene. This assay is limited, however, to the study of highly specific binding of proteins to ^{32}P end-labeled nucleic acids. We have made use of a variation of this footprinting method to study the binding of factor A to unlabeled RNA and DNAs that may lack specific binding sites. The competition assay employed simply involves the addition of various concentraions of competitor nucleic acids to a footprint reaction containing an end-labeled 5S DNA fragment whose factor A binding affinity has been characterized. This assay is capable of providing estimates of relative binding affinities. Sakonju and Brown (6) have previously used this assay to detect a four-fold difference between the affinities of the transcription factor for oocyte and somatic 5S RNA genes. In the present study, we have applied this assay to an analysis of factor A binding to a

variety of RNA and DNA molecules.

The examination of factor A-RNA interactions using footprint competition reveals several interesting observations. Pelham and Brown (7) showed previously that Xenopus 5S RNA could inhibit factor A-dependent footprinting of the 5S RNA gene. This inhibition was assumed to be a result of a specific interaction between factor A and Xenopus 5S RNA since E.coli tRNA was unable to compete in this assay (7). We demonstrate (Figure 1 and 2) that wheat germ or yeast 5S RNA compete as well as oocyte 5S RNA in the footprint competition assay. The sequence homology between Xenopus oocyte and wheat germ or yeast 5S RNA is 64% and 56% respectively (14,15,16). This homology along with similarities in secondary structure may account for the RNA binding to factor A observed in the footprint competition assays. In contrast, the lack of competition by E.coli 5S RNA in this assay may reflect the low sequence homology (28%) between Xenopus laevis oocyte (14) and E.coli 5S RNA (17), as well as possible secondary structure differences. Thus, the observations that factor A binds to wheat germ, yeast or Xenopus 5S RNA but not to E.coli 5S RNA or wheat germ tRNA indicate that the protein is able to recognize some features of the eukaryotic 5S RNA sequence and/or structure. Further studies, possibly utilizing a variety of RNA molecules in the footprint competition assay described here, may contribute to the identification of specific RNA sequences recognized by factor A.

The estimated dissociation constant for the factor A-5S RNA interaction as measured by the footprint competition assay is about 2×10^{-8}M (Figure 2). The factor A-5S RNA interaction observed in the 7S particle isolated in vivo is considerably more stable since the 5S RNA gene, which binds factor A in a complex with a dissociation constant of about 10^{-9}M (5), is unable to compete with 5S RNA for factor A binding in the 7S particle (7). Thus, although the footprint competition assay employed in this study is able to detect specific binding of factor A to RNA to a certain degree, the interaction observed with Xenopus 5S RNA probably does not mimic 7S particle formation in vivo. Similarly, it is not known whether the 5S RNA-factor A interaction which results in RNA inhibition of in vitro 5S RNA

transcription (7) involves formation of authentic 7S particles. Detailed knowledge of the structure of the 7S particle will be required to evaluate whether the factor A-RNA interaction occurring in footprint competition or transcription inhibition can be used to examine 7S particle formation.

Using the footprint competition assay, we demonstrate that isolated factor A binds to single stranded DNA as well as to RNA. The binding of factor A to non-specific single stranded DNA (M13mp9 viral DNA) is several orders of magnitude stronger than to non-specific duplex DNA (pBR322). Thus, Xenopus transcription factor A can be classified as a single-stranded DNA binding protein (18). The competitive strength of single stranded DNA for factor A binding is dependent on the size of the DNA. At a concentration of $1x10^{-10}$ M, M13mp9 DNA completely inhibits the binding of $1x10^{-8}$ M factor A to the 5S RNA gene (Fig. 3). It appears, therefore, that each M13mp9 molecule binds about 100 molecules of factor A. Furthermore, a single stranded DNA which is 30 times smaller than M13mp9 DNA is 150-fold less competitive in the footprint competition assay (Fig. 6). One possible explaination for the higher apparent affinity for the larger DNA (5 fold after normalizing the size difference) would be positive cooperativity in the protein-DNA interaction. However, a more complete analysis of this phenomenon is not possible at this time because the exact size of the factor A binding site on single stranded DNA is not known. Without knowing the number and nature of binding sites, direct comparison of the apparent binding constants is inappropriate since the effective concentration of binding sites may be quite different from the molar concentration of DNA molecules.

The sequence specificity of the single stranded DNA binding behavior of factor A is unknown at present. As mentioned previously, a number of strong factor A contact points are clustered near the 3' end of the intragenic control region predominantly on the non-coding strand and factor A has been proposed to specifically bind to this region of the non-coding strand (6). Footprinting competition assays with the non-coding or coding DNA strand or with a non-specific single stranded DNA fragment have shown no evidence that factor A can preferentially bind the non-coding strand in single stranded form.

Despite the apparent lack of specificity, single stranded DNA binding by factor A may play a role in transcription of the 5S RNA gene. Although factor A itself does not appreciably unwind the DNA helix (19,20), either RNA polymerase III and/or some other transcription factor must cause separation of the DNA daughter strands during transcription initiation and elongation. Since factor A-5S RNA gene complex formation precedes these events (4) and because this complex is quite stable (half-life of 5-6 minutes, Figure 4) relative to the rate of transcription (21), some sort of displacement of factor A from duplex DNA is required for transcription of the center of the gene. It is conceivable that factor A may bind to the transiently separated strands of 5S DNA during some stages of elongation. The length of single stranded DNA which may become accessible during transcription is unknown. Our results would suggest that there is no one specific site for single stranded DNA binding by factor A. Further studies to determine the size of the single stranded DNA binding site for factor A and to investigate the possible existence of single stranded 5S DNA in transcription complexes should shed light on mechanisms by which factor A regulates 5S RNA synthesis.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (GM 28069 to C.-W.W. and GM 29861 to D.F.B.) and the American Cancer Society (NP 3091 to C.-W.W.).

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