DNA unwinding ability of *Xenopus* transcription factor A

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ABSTRACT

The duplex DNA unwinding ability of *Xenopus* transcription factor A was examined by treatment of plasmid-protein complexes with calf thymus topoisomerase I and resolution of the topoisomer distributions by agarose gel electrophoresis. Factor A binding to plasmids does result in a small degree of unwinding as evidenced by a shift in topoisomer distributions upon relaxation. Slightly more unwinding by factor A was observed on a specific plasmid, containing four tandemly repeated 5S RNA genes, when compared to a non-specific plasmid of nearly identical size containing no 5S specific sequences. The amount of unwinding observed on the specific plasmid, about one base-pair per gene, suggests that unwinding of the DNA duplex is not an important aspect of binding of factor A to the 5S RNA gene.

INTRODUCTION

A number of proteins control gene transcription by binding to specific DNA sequences at or near RNA polymerase initiation sites. In vitro mutagenesis of the *Xenopus* 5S RNA gene has identified an intragenic DNA sequence of about 50 bp which is required for transcription initiation (1,2). Engelke et al. (3) described a 40,000 dalton protein, designated TFIIIA or factor A, which binds to this region of the 5S RNA gene and directs accurate transcription initiation by RNA polymerase III. Deletion of the control region in the 5S RNA gene results in the concomitant loss of factor A binding and transcriptional activity (4).

The role factor A plays in transcription is unknown at present. It clearly acts at an early stage in the formation of active transcription complexes (5). Because of its novel intragenic binding mode, one attractive possibility for its role would be to destabilize or unwind the duplex DNA helix and

thus facilitate the entry of RNA polymerase III and other factors into the 5S RNA gene.

A recent report has addressed the ability of Xenopus transcription factor A to unwind the 5S RNA gene (6). In this study, factor A was found to unwind the 5S RNA gene to a limited extent, in a range of two to four base pairs. In the present study, we have examined the helix destabilizing ability of factor A and find that the protein unwinds the 5S RNA gene to an extent of about one base pair per gene. Thus, we conclude from the previous report and the present study that the binding of factor A to the 5S RNA gene does not cause an appreciable change in the helical structure of the duplex DNA.

MATERIALS AND METHODS

7S particle and factor A isolation

7S particles from Xenopus laevis 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 (8). Factor A was isolated by digesting 150 μ l of 7S particles (1.5 mg/ml in 20 mM Tris-HCl, pH 7.5, 320 mM KCl, 250 mM NaCl, 1.5 mM MgCl₂, 1 mM dithiothreitol) with RNase A (Calbiochem) at a final concentration of 150 μ g/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 MgCl₂, 1 mM dithiothreitol. Factor A was collected in the void volume. This procedure serves to remove RNase A as well as greater than 95 % of the A₂₆₀ 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. Protein concentrations were routinely determined by the method of Bradford (9) which agrees well with amino acid analysis (8).

DNA preparation and DNase I footprinting

A 276 bp fragment containing the Xenopus borealis somatic (Xbs) 5S RNA gene was prepared from plasmid pXbs 201 (2) and end-labeled with [α -³²P] dATP (8). This fragment was used in all footprinting assays. The DNase I footprinting method of Galas and Schmitz (10) was performed as described previously

(9). Footprint competition assays were conducted and quantitated as described by Sakonju and Brown (11). DNA templates employed in this study and their approximate sizes are as follows: pXbs201 (4262 bp), pBR322 (4362 bp), pXbs Δ 5'-73HX4 (5742 bp), pXbs Δ 5'-73HX4' (~3300bp), and pKA2 (~3300bp), a smaller version of pKC7 (12). All plasmids were purified by ethidium bromide-CsCl gradient centrifugation.

Assay for DNA unwinding

DNA plasmid-protein complexes were formed in J buffer (20 mM Tris-HCl, pH 7.5, 70 mM NH₄Cl, 7 mM MgCl₂, 1 mM dithiothreitol) supplemented with 0.1% Nonidet P40 for 10 min at 22° C. Calf thymus topoisomerase I (a gift from Dr. Leroy Liu) was then added and incubated for an additional 15 min. The reaction was terminated with 0.2 volume of dye buffer (25% glycerol, 50 mM Tris-HCl, pH 7.5, 50 mM EDTA, 2% NaDodSO₄, 0.01% bromphenol blue). The samples were mixed briefly and applied to a 1.1% agarose gel in Tris-acetate buffer (13). Electrophoresis was conducted at 40 V for 12 hrs at 22°C. Gels were stained with 1 µg/ml ethidium bromide for 20 min and rinsed with distilled water for an additional 30 min. Gels were photographed on a short-wavelength UV illuminator with Kodak type 55 positive-negative Polaroid film. A film negative of topoisomer distributions was scanned with a Biomed densitometer. The mean-weighted averages of the respective distributions were calculated assuming that individual peak heights were proportional to DNA topoisomer concentrations (14).

RESULTS

Each of four *Xenopus* 5S RNA genes in a "tetrameric" plasmid binds factor A

The method we employ to attempt to detect specific unwinding of the DNA helix by factor A is that of Wang et al (15). This method compares the distributions of DNA topoisomers resulting from nicking and closing the DNA in the presence and absence of a DNA binding protein (or other ligand). This method detects unwinding most sensitively when the plasmid contains several specific binding sites and is of minimal size. Therefore, we constructed a plasmid containing four tandem copies of an Xbs 5S

Figure 1. Relative binding of factor A to plasmids containing one and four copies of the 5S RNA gene. Factor A (1×10^{-8} M) was incubated for 10 min at 22° C with 32 P end-labeled 5S DNA (1×10^{-9} M) plus pXbs Δ 5'-73HX4 plasmid (curve a) or pXbs201 (curve b) at the concentrations indicated. DNase I, at a final concentration of $15 \mu\text{g/ml}$, was added and incubated for an additional min. Samples were then processed, electrophoresed, and quantitated as described in Materials and Methods. θ is the fraction of factor A displaced from the 32 P end-labeled 5S RNA gene by competitor DNA.

RNA gene. We selected a somatic-type 5S RNA gene for this construction because most of the previous studies of binding of factor A to 5S DNA have employed the Xbs 5S RNA gene, and because the factor binds more tightly to somatic than to oocyte 5S RNA genes (11).

In order to determine that all four copies of the 5S RNA gene are active in binding factor A, the ability of this tetrameric plasmid to compete for factor A binding with a linear 5S DNA, end-labeled fragment was compared to the competitive strength of a plasmid containing one 5S RNA gene. Figure 1 shows the competitive strength of two plasmids containing one and four copies of the Xbs 5S RNA gene in the footprint competition assay. The competitive strength of a tetramer plasmid (pXbs Δ 5'-73HX4, Figure 1, curve A) is four times as great as that of the monomer plasmid (pXbs201, Figure 1, curve B) as judged by the competitor concentrations at which inhibition is first observed (0.1 nM-tetramer, 0.5 nM-monomer), at the 50 % inhibition value (0.35 nM versus 1.2 nM), and at the inhibition end point (1 nM versus 4 nM). Therefore, all four 5S RNA genes in the tetramer plasmid participate in binding factor A.

Figure 2. A: Unwinding by factor A and *E. coli* RNA polymerase of a plasmid containing four *Xenopus* 5S RNA genes. The pXbs Δ 5'-73HX4 plasmid (1×10^{-8} M) was incubated for 10 min at 22° C with various amounts of protein as follows: lanes 1, 2 and 7--no protein; lanes 3, 4, 5 and 6-- $8, 20, 30, 50 \times 10^{-8}$ M factor A; lanes 8, 9 and 10-- $2, 4, 6 \times 10^{-8}$ M *E. coli* RNA polymerase. Calf thymus topoisomerase I was then added and incubated for an additional 15 min (lanes 2-10). After addition of 0.2 volumes of 5X sample buffer (2% NaDodSO₄, 10 mM EDTA, 20 % glycerol and 0.05 % bromphenol blue), samples were mixed briefly and subjected to agarose gel electrophoresis as described in Materials and Methods. B: A plot of the unwinding angle (α) of *E. coli* RNA polymerase (curve A) and factor A (curve B) versus the respective protein/DNA ratios. DNA topoisomer distributions in Fig. 2A were quantitated as described in Materials and Methods.

Xenopus transcription factor A exhibits little unwinding activity when compared to E.coli RNA polymerase

Figure 2A depicts the unwinding effect of increasing concentrations of factor A and E.coli RNA polymerase on the topoisomer distribution of the tetramer plasmid, pXbs Δ 5'-73HX4. Unwinding is seen as an upward shift in the topoisomer distribution. A small upward shift is observed in lanes 3-6 with increasing concentrations of factor A (lanes 2 and 7). As a positive control, lanes 8-10 exhibit the unwinding effect of E.coli RNA polymerase holoenzyme, which induces a large upward shift. Thus, although factor A exhibits some unwinding ability, it is clearly not of the magnitude exhibited by E. coli RNA polymerase.

The unwinding ability of factor A and that of E. coli RNA polymerase are compared graphically in Figure 2B, in which the unwinding angle (α) is plotted as a function of the [protein]/[DNA] ratio. At a ratio of two RNA polymerase molecules per plasmid (Figure 2A, lane 8; Figure 2B, curve A), a full turn (360 degrees) of unwinding is observed, equal to approximately 180 degrees per protein. This number agrees reasonably well with the previously published value of about 250 degrees of unwinding given the pronounced effect of temperature on unwinding (15). The present study was performed at 22°C whereas the larger unwinding angle for E.coli RNA polymerase is observed at 37° C. The degree of unwinding caused by factor A is a great deal less than that by E. coli RNA polymerase. It is important to evaluate this unwinding at appropriate ratios of factor A to DNA. Saturation of the factor A binding sites on the four 5S RNA genes should be attained at a ratio of eight protein molecules per tetramer gene-containing plasmid since each 5S RNA gene binds no more than two factor A molecules (5,11). In some of our experiments we included a trace amount of a ³²P end-labeled 5S DNA fragment and performed appropriate footprinting controls to confirm that saturation of specific binding sites occurred at these predictable factor A concentrations. Under these conditions, factor A induces unwinding of about 100 degrees at eight proteins per tetramer plasmid (Figure 2A, lane 3; Figure 2B, curve B). However,

Figure 3. A: Agarose gel electrophoretic patterns of DNA topoisomers of specific and non-specific plasmids generated by topoisomerase I in the presence of various concentrations of factor A. Factor A binding, topoisomerase reactions, and gel electrophoresis were performed as described in Figure 2A. Lanes 1-5: topoisomer distributions obtained with factor A binding to the specific plasmid containing four 5S RNA genes (pXbs Δ 5'-73HX4') at protein/DNA ratios of 0, 8, 20, 30 and 50. Lanes 6-10: topoisomer distributions obtained from factor A binding to the non-specific plasmid pKA2 at the same protein/DNA ratios. **B:** Graphical representation of the unwinding angle (α) versus the protein/DNA ratio. Curve A: lanes 1-5; Curve B: lanes 6-10.

further unwinding at higher ratios of factor A to DNA is also observed indicating that additional unwinding can take place upon binding of factor A at non-specific sites on the DNA.

Factor A unwinding on specific and non-specific plasmids

A comparison of the ability of factor A to unwind specific and non-specific DNA plasmids is given in Figure 3A. From inspection of this agarose gel it is possible to distinguish a difference between the topoisomer distributions resulting from factor A unwinding of the specific plasmid (lanes 1-5) and non-specific plasmid (lanes 6-10). The extent of unwinding on the two plasmids is best compared graphically in Figure 3B which illustrates the difference in the unwinding by factor A of the specific and non-specific plasmids. Factor A unwinds the plasmid containing four 5S RNA genes by about 100 degrees (Figure 2A, lane 2; Figure 2B, curve A) at a protein/DNA ratio of eight where specific unwinding is occurring. At this ratio, about 20 degrees of unwinding is observed for a plasmid lacking 5S DNA (pKA 2, Figure 2A, lane 7; Figure 2B, curve B). After an initial increase in unwinding by binding of the protein to specific sites, the further unwinding at higher protein/DNA ratios observed in curve A parallels the effect of non-specific binding. The unwinding observed at a ratio of eight factor A molecules to four copies of the 5S RNA gene is about 100 degrees (Figure 2B, curve B and Figure 3B, curve A). Thus, the unwinding observed for each copy of the control region is approximately 25 degrees, or less than one base pair. Assuming that this unwinding is distributed throughout the 50 base pairs of the control region, this result indicates that only a subtle change in the pitch of the DNA helix accompanies factor A binding to the Xenopus 5S RNA gene.

Effect of elevated temperature on the binding of Xenopus transcription factor A to the 5S RNA gene

In the previous study of the DNA unwinding ability of Xenopus transcription factor A, the relaxation of factor A-plasmid complexes by topoisomerase I was conducted for 45 min at 37°C (6). We have performed our unwinding experiments at 22°C because factor A was found to bind the 5S RNA gene more readily at this temperature, as shown in Figure 4. This figure compares the footprinting ability of factor A at 37° and 22 °C. In this experiment, factor A-DNA complexes were preformed at 22°C for 5 min. The samples were then incubated at 37°C for various

Figure 4. Reduction in the ability of Xenopus transcription factor A to bind the 5S RNA gene at 37°C. Factor A (1×10^{-8} M) was incubated for 5 min at 22°C with ^{32}P end-labeled 5S DNA (1×10^{-9} M). Samples were then transferred to 37°C for 0, 5, 10, 15, 20, and 30 min (lanes 1-6). The sample utilized in lane 7 was maintained at 22°C for 30 min. The sample in lane 8 contained no factor A. DNase I, at a final concentration of 15 $\mu\text{g}/\text{ml}$, was then added and incubated for an additional min at 22°C. Samples were processed and electrophoresed as described in Materials and Methods.

times (lanes 1-6). As can be seen in these lanes, factor A binding is reduced (loss of footprint) at 37°C when compared to a sample maintained at 22°C over this time course (lane 7).

DISCUSSION

The intragenic binding mode of Xenopus transcription factor A is a unique feature of the regulation of 5S RNA synthesis by this protein. Because intragenic melting of the 5S RNA gene is necessary for transcription elongation, it is important to determine whether factor A can unwind the 5S RNA gene and, thus,

provide this function in 5S RNA synthesis. By relaxing factor A-plasmid complexes with topoisomerase I and resolution of the resultant topoisomer distributions by agarose gel electrophoresis, we observe only a slight shift in topoisomer distribution as a result of specific factor A binding, equal to unwinding of about one base-pair per gene.

Our results are in qualitative agreement with a previously published report which stated that factor A unwinds the 5S RNA gene to a limited extent (6). Quantitatively, we observe less unwinding (one base pair) than the previous report (two-four base pairs). We believe that a number of points may contribute to this quantitative discrepancy. The factor A employed in the previous study was 30% pure compared to the 90% purity of our factor A preparations. Impurities may have contributed to the unwinding observed in the previous study. Probably a more significant experimental difference with respect to DNA melting is that the previous study was performed at 37°C while we have employed 22°C. DNA unwinding experiments with E.coli RNA polymerase indicate that unwinding, as measured by shifts in DNA topoisomer distributions on agarose gels, is very temperature dependent, i.e. elevated at higher temperatures (15). This temperature effect probably contributes to the quantitative discrepancy observed between our factor A unwinding estimate of one base-pair per gene and the previously published range of two-four base pairs. In addition, our control experiment (Figure 4) indicates that factor A is less active in specific binding at 37°C than at 22°C.

Although factor A has minimal unwinding ability, the investigators of the previously published report concluded that this unwinding by factor A may facilitate transcription of the 5S RNA gene (6). Although difficult to prove, the slight amount of unwinding we observe suggests to us that this behavior by factor A is not of physiological significance. The unwinding detected could be due to a slight alteration in the pitch of the DNA helix simply as a result of protein binding. Nominal unwinding behavior has also been observed for other transcriptional regulatory proteins, e.g., E.coli catabolite gene activator protein (16) and SV40 T antigen (13). The slight helix

destabilizing ability of these proteins is not considered to play a major role in their function.

Recently, Folk and Hofstetter (17) have presented evidence consistent with the hypothesis that intrastrand hairpin formation within the intragenic control region of a Xenopus tRNA gene is necessary for transcription of this gene by RNA polymerase III. If similar hairpin formation is required for the transcription of the Xenopus 5S RNA gene by RNA polymerase III, factor A alone would be unable to induce such cruciform structures. At least two other RNA polymerase III transcription factors, B and C, are necessary for transcription of both Xenopus tRNA and 5S RNA genes (18). RNA polymerase III, either alone or in association with the various transcription factors, may be responsible for any unwinding event necessary for the transcription of the 5S RNA gene.

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