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Bead-shift isolation of protein–DNA complexes on a 5S RNA gene

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ABSTRACT

Specific protein–DNA complexes formed on a *Xenopus* 5S RNA gene were isolated and characterized using a novel technique. A DNA template reversibly immobilized on paramagnetic beads was used to capture, affinity purify, and concentrate protein–DNA complexes formed in a whole cell extract. The complexes were then released from the beads in a soluble and transcriptionally active form via restriction enzyme digestion of the DNA. A band-shift gel was used to separate and obtain the DNase I footprints of five individual complexes. Three of the complexes resulted from the independent binding of two proteins, TFIIIA and an unidentified protein binding to a large region just downstream of the 3′ end of the gene. Two more slowly migrating complexes contained an additional large central protected region covering most of the gene. The most slowly migrating complex displayed protein interactions over the 5′ flanking sequences. The formation of two of these complexes was shown to be dependent on TFIIIC activity. The correlation between transcriptional activity and the formation of these complexes suggests that the observed protein–DNA interactions are important for transcription of 5S RNA genes.

INTRODUCTION

Transcription of 5S RNA genes by RNA polymerase III requires at least three transcription factors, TFIIIA, TFIIIB, and TFIIIC (1–3). TFIIIA binds to the essential promoter elements within the 5S RNA gene and nucleates the formation of a transcription complex formed by the sequential addition of TFIIIC, TFIIIB, and RNA polymerase III. Multiple 5S RNAs can then be transcribed from a single, remarkably stable, transcription complex. Characterization of the interactions of the transcription factors with the template DNA is essential for understanding the molecular mechanisms of this process. TFIIIA and the complex of TFIIIA with the internal control region of a 5S RNA gene have been extensively studied. However, probing the interactions of *Xenopus* TFIIIC and TFIIIB with a 5S RNA gene has been hampered by the difficulty of obtaining sufficient quantities of active purified transcription factors.

The structure of the transcription complexes on 5S RNA genes at several stages of formation and elongation have been extensively studied using highly purified yeast transcription factors (3). In contrast, the protein–DNA interactions of vertebrate transcription factors with 5S RNA genes are not well defined. Previously published reports show *Xenopus* 5S RNA gene transcription complexes with a structure which more closely resembles a simple TFIIIA–DNA complex than one with the extensive interactions observed for a transcription complex in the yeast system (4–6). Also puzzling is the report that the chromatin structure of oocyte-specific 5S RNA genes obtained from immature *Xenopus* oocytes, where the genes are active, is dramatically different from deproteinized DNA, yet shows no evidence of TFIIIA binding (7).

The nature of the interaction of vertebrate TFIIIC with a TFIIIA–5S RNA gene complex is also not well defined. Several reports show that *Xenopus* TFIIIC stabilizes the TFIIIA–5S RNA gene complex (8–12). Remarkably, many studies report that TFIIIC did not alter the gel mobility of the TFIIIA–5S RNA gene complex (6, 10–12). A more recent study using affinity purified *Xenopus* TFIIIC does report that a series of three TFIIIC–TFIIIA–5S RNA gene complexes with different gel mobilities were observed under certain conditions (13). Surprisingly, in all cases the DNase I footprint of a TFIIIA–5S RNA gene complex was essentially unchanged by the addition of TFIIIC. Two separate studies report that purified human TFIIIC (or the sub-fractionated TFIIIC2) binds to a 5S RNA gene in the absence of TFIIIA (14, 15). However, the DNase I footprints of the two complexes show binding to different regions of the 5S RNA gene. In addition, TFIIIC (or TFIIIC2) binding was independent of TFIIIA binding, and in one case (TFIIIC2) was separable from the B-box binding activity known to be essential for transcription of tRNA-like genes.

Although it is preferable to study protein–DNA interactions using highly purified components, it is not always possible to do so, either because of the difficulty of obtaining sufficient quantities of the purified proteins or because all of the components have not yet been identified. We have pursued a strategy, outlined in Fig. 1, which allows the characterization of individual protein–DNA complexes formed at low concentrations in a heterogeneous mixture. Extracts from *Xenopus* oocytes actively transcribe cloned 5S RNA genes and contain all of the essential transcription factors. Like many *in vitro* transcription systems, only a fraction of the template DNA molecules included in a standard reaction form active transcription complexes and are used as templates for RNA synthesis. Therefore, multiple complexes, both active and inactive, are expected to form on 5S RNA genes incubated in oocyte extracts. We have used a

reversibly immobilized template DNA to affinity purify and concentrate protein–DNA complexes formed on 5S RNA genes in oocyte extracts. Individual complexes were then separated using a band-shift gel. The formation of two of the newly identified complexes was correlated with TFIIIC activity. The DNase I footprints of these complexes share many features of the active transcription complexes observed in yeast and are significantly different from those previously observed using vertebrate components.

MATERIALS AND METHODS

DNA probes

The clone ps (16) containing a somatic-type 5S RNA gene from *Xenopus laevis* was used in the experiments reported here. The plasmid DNA was biotin-labeled by filling in the unique *Afl*II site with biotin-14-dATP (BRL) and radioactively labeled (either 3′ or 5′) at the unique *Ngo*M 1 site using standard techniques. The resulting linear DNA was 3043 bp long and contained a 5S RNA gene positioned roughly halfway between a unique *Eco*R I site and the *Ngo*M 1 site 420 bp away.

Bead-shift isolation of protein–DNA complexes

Extracts from *Xenopus* oocytes were prepared as described by (17) using the following buffer: 30 mM sodium–HEPES (pH 7.6), 100 mM potassium glutamate, 2 mM $MgCl_2$, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM ATP, 0.1 mM EGTA, 10 μM $ZnCl_2$, and 10% glycerol. Before use, the $MgCl_2$ concentration of the extract was adjusted to 7 mM. Addition of other components, washes, and incubations were done at 22°C in buffer T2: 30 mM sodium–HEPES (pH 7.6), 70 mM KCl, 7 mM $MgCl_2$, 1 mM DTT, 50 μM EDTA, 50 μM EGTA, 10 μM $ZnCl_2$, and 100 μg/ml acetylated BSA. Competitor DNA was included at a total concentration of 10 to 50 μg/ml and consisted of poly(dA-dT)·poly(dA-dT), poly(dG-dC)·poly(dG-dC), poly(dI-dC)·poly(dI-dC), poly(dA-dC)·poly(dG-dT), and plasmid DNA. Vector plasmid was used for non-specific competitor and plasmids containing cloned genes were used as specific competitors. The synthetic DNA polymers were present in equal amounts, with a total concentration twice that of the plasmid DNA. Typically 50 μl of paramagnetic streptavidin coated beads (Advanced Magnetics, Inc.) were washed twice with 100 μl of T2, washed once with 100 μl of T2+DNA (non-specific) and the labeled DNA probe (~0.1 pmoles) containing a 5S RNA gene was bound to the beads for 1 hour in 100 μl of T2+DNA. Unbound probe (~15%) was removed using 3 washes with 100 μl of T2+DNA. Extract (~1 ml, ~4 mg protein) was pre-incubated for 1 hour with non-specific competitor DNA and then incubated for 3 hours with the beads containing the bound probe. The protein–DNA complexes formed on the immobilized probe were purified by removal of the extract and sequential washes with 200 ml and then 100 ml of T2+DNA. The beads were resuspended in 40 ml of T2+DNA and the complexes were released from the beads via *Eco*R 1 digestion (20 to 100 units) for 1 hour. *Eco*R 1 digestion released ~50% of the radioactivity on the beads.

Band-shift gels

EDTA was added to a final concentration of 10 mM and the individual complexes were separated by electrophoresis on 1% agarose gels using 90 mM Tris–borate (pH 8.3) and 50 μM EDTA as the electrophoresis buffer. Electrophoresis was carried out for 5 hours at 80 volts.

Inactivation of TFIIIC

TFIIIC activity in the oocyte extract was eliminated by either heating the extract to 42°C for 15 min. (18, 19) or by incubating the extract in 1 mM Ca^{2+} for 1 hour and then adding EGTA to a final concentration of 2 mM (19).

In vitro transcription

The transcriptional activity of the isolated complexes was assayed in buffer T2 by the addition of radiolabeled nucleoside triphosphates for 1 hour. The final concentrations of the NTPs were: 1 mM ATP, 500 μM CTP, 200 μM UTP, 20 μM GTP, with 10 μCi α-^{32}P-GTP in each reaction. The reactions were stopped and the transcription products were isolated and separated on denaturing 8 M urea, 10% polyacrylamide gels as described (20).

DNase I footprinting

The mixture of affinity purified and soluble complexes released from the paramagnetic beads was digested with DNase I at a final concentration of 5 μg/ml for 1 min., digestion was stopped by the addition of EDTA to a final concentration of 10 mM, and the individual complexes were separated on a 1% agarose band-shift gel. The DNA contained in each of the complexes was electroeluted from the band-shift gel and equal amounts of labeled DNAs were re-run on denaturing DNA sequencing gels. An important aspect of this procedure was that each complex was digested to an equivalent extent since it was the mixture of complexes which was digested prior to the separation of the individual complexes on a band-shift gel. Chemical sequencing ladders were used to determine the positions of the DNase I cleavages.

RESULTS

Using the bead-shift technique outlined in Fig. 1 we observed several distinct complexes formed on a DNA probe containing a 5S RNA gene following incubation of the probe in an extract from *Xenopus* oocytes. Fig. 2 illustrates the pattern observed when a 1% agarose band-shift gel was used to separate the complexes following affinity purification, concentration, and release from the beads. The complex of purified TFIIIA with the DNA probe migrated more slowly than the free DNA (lane a). Four additional complexes, migrating increasingly more slowly than the TFIIIA complex, were observed to form on this DNA probe (lane c). These additional complexes were not observed when specific competitor DNA, containing the same DNA sequence as the probe, was included in the reaction (lane d). This demonstrates that these complexes were specific for the DNA sequences of the probe.

The specificity of the complexes was investigated further using different 5S RNA genes as competitors. Three different clones containing 5S RNA genes were used, each with similar but not identical coding regions surrounded by distinctly different flanking sequences. Most of the complexes formed on the somatic-type 5S RNA gene probe were competed away by each of the cloned 5S RNA genes (Fig. 3). This indicates that the formation of these complexes was dependent on the coding region of the 5S RNA gene. One complex, band 3 in Fig. 3, was only

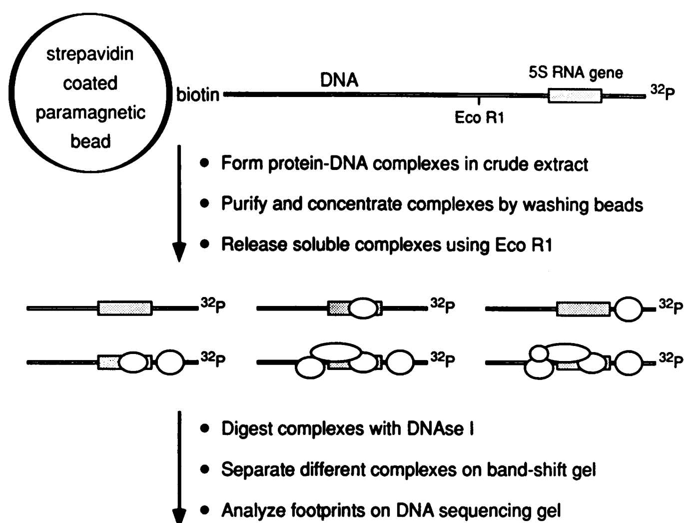

Figure 1. Strategy for bead-shift isolation of protein–DNA complexes. Plasmid DNA containing a cloned 5S RNA gene is linearized, biotin-labeled at one end, and ^{32}P-labeled at the other end. The DNA is then immobilized on streptavidin coated paramagnetic beads and protein–DNA complexes are formed during an incubation in a crude extract. The complexes are affinity purified and concentrated by washing the paramagnetic beads. Competitor DNA is included during both complex formation and washing to remove non-specific proteins. The novel aspect of this adaptation of the immobilized template technique is to use restriction enzyme digestion at a unique site to release the complexes from the beads. The soluble complexes can then be subjected to enzymatic and chemical probes and the individual complexes separated using a band-shift gel.

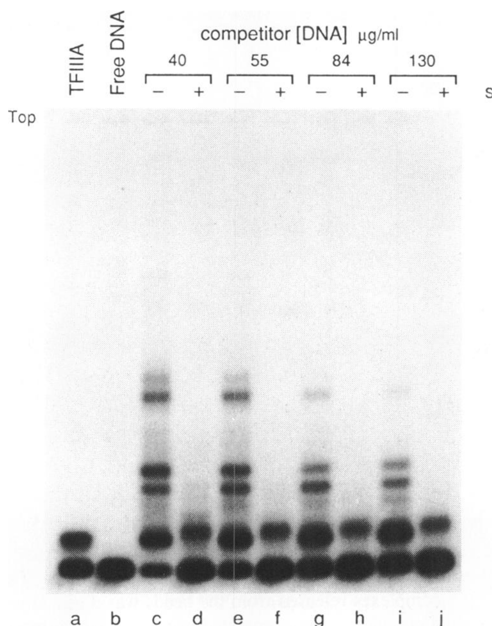

Figure 2. Separation of protein–DNA complexes formed on a 5S RNA gene. The bead-shift strategy outlined in Fig. 1 was used to isolate complexes formed on a *Xenopus* 5S RNA gene during incubation in a crude extract from *Xenopus* oocytes. An autoradiograph of a 1% agarose band-shift gel is shown. Various amounts of competitor DNA were included during the formation and washing of complexes as indicated. Alternating lanes correspond to reactions which contained specific competitor DNA (plasmid ps indicated by s). The band-shift observed with purified TFIIIA is also shown.

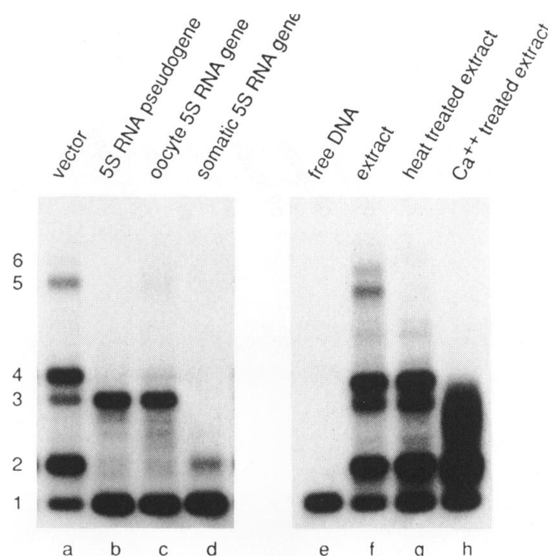

Figure 3. Specificity of individual bead-shift complexes. The left panel shows the effects of using different 5S RNA genes as specific competitors during the formation of complexes on a somatic-type 5S RNA gene probe. The right panel shows the effects of pre-treating the extract before complex formation. Both heat treatment and calcium treatment destroy TFIIIC activity in the extract and prevent the formation of transcriptionally active complexes.

competed away when the cloned 5S RNA gene used as competitor had the same flanking sequences as the 5S RNA gene probe. This implies that the formation of band 3 was dependent on the flanking sequences and in combination with the results of DNase I footprinting (see below) suggests that this complex was formed by protein binding to the 3′ flanking sequences of the somatic-type 5S RNA gene.

We tested the transcriptional activity of the mixture of affinity purified complexes isolated using a reversibly immobilized template DNA. The transcriptional activity of the isolated complexes was found to be dependent on the concentration of competitor DNA present during complex formation. When relatively low concentrations of competitor DNA were present, both immobilized complexes and complexes which were released from the beads by *Eco*R 1 digestion were transcriptionally active (Fig. 4). All of the components required for transcription of a 5S RNA gene, including RNA polymerase III, were affinity purified using this method. However, at these low concentrations of competitor DNA, the pattern of complexes observed on a band-shift gel was less distinct, i.e. smeared (data not shown). When complexes were isolated in the presence of relatively high concentrations of non-specific competitor DNA, a well-defined pattern of bands was observed on a band-shift gel (e.g. Fig. 2 lane c) but these complexes had little or no transcriptional activity (data not shown). Addition of RNA polymerase III to these complexes did not result in active transcription. However, the transcriptional activity of these isolated complexes was restored by the addition of heat treated extract (data not shown). Because a mild heat treatment of the extract selectively eliminates TFIIIC activity (18, 19), this result suggests that at least one of the observed complexes (e.g. in Fig. 2 lane c) contained TFIIIC.

In addition to a mild heat treatment, the ability of an oocyte extract to transcribe 5S RNA genes can be abolished by incubating the extract with Ca^{2+} (19). Both of these treatments

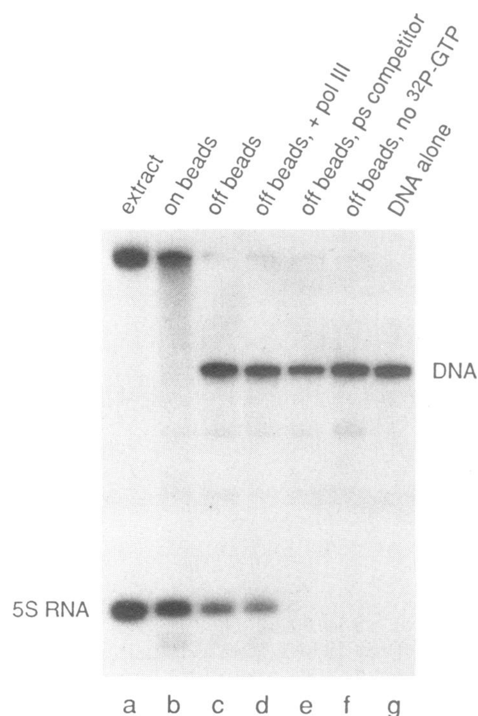

Figure 4. Transcriptional activity of affinity purified complexes. Protein–DNA complexes formed on a 5S RNA gene were isolated using the bead-shift method and were assayed for transcriptional activity by the addition of radiolabeled nucleoside triphosphates. Both immobilized complexes (lane b, on beads) and complexes released from the beads by *Eco*R 1 digestion (lanes c to e, off beads) were tested. An autoradiograph of the denaturing polyacrylamide gel used to separate the *in vitro* transcription products from the radiolabeled DNA released from the beads is shown. Control reactions defined the positions of 5S RNA and template DNA as indicated. RNA polymerase III was added to the reaction corresponding to lane d after the complexes were released from the beads. Complexes were formed in the presence of 10 μg/ml non-specific competitor DNA. A specific competitor, plasmid ps, was added to the reaction corresponding to lane e during complex formation.

selectively destroy TFIIIC activity. Fig. 3 shows the altered pattern of protein–DNA complexes observed on a 5S RNA gene probe when TFIIIC has been inactivated. In particular, bands 5 and 6 were not observed with the transcriptionally compromised extracts. These results suggest a correlation between the formation of complexes 5 and 6 and the transcriptional activity of the template DNA probe and imply that TFIIIC activity is required for the formation of bands 5 and 6.

The DNase I footprints of the individual complexes formed on a 5S RNA gene probe were obtained by the strategy outlined in Fig. 1. The footprints, as illustrated in Figs. 5 and 6, have many distinctive features. Band 1 represents the free DNA probe. Band 2 represents the complex of TFIIIA with the 5S RNA gene since both the footprint and the gel mobility of this complex matched those observed for the complex of purified TFIIIA with this DNA probe. The footprint of band 3 showed a large protected region starting near the 3′ end of the 5S RNA gene and extending well into the 3′ flanking sequences (+115 to +160) with enhanced cleavage near the middle on the top strand (+135) and at the downstream end of the protected region on both strands (+160). The localization of the band 3 footprint to the downstream sequences of the somatic-type 5S RNA gene is in agreement with the fact that this complex was not competed away

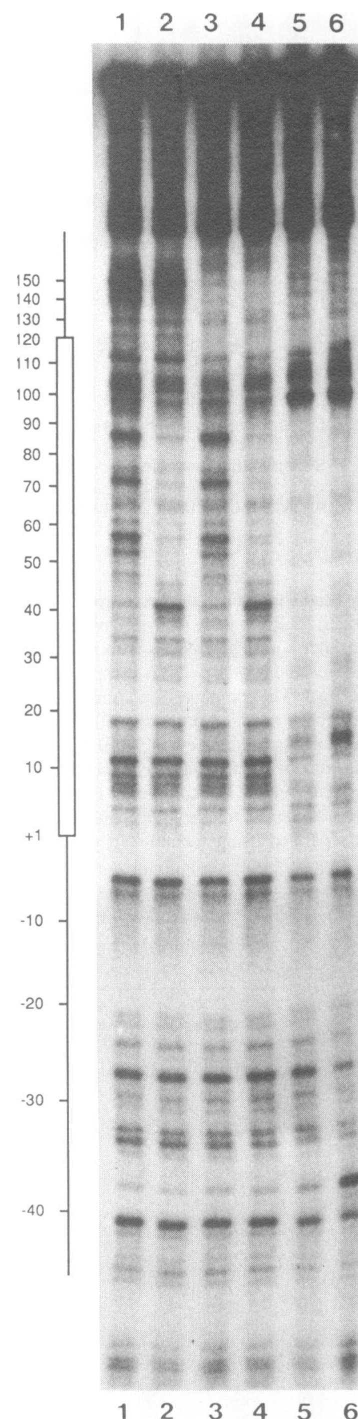

Figure 5. DNase I footprints of individual bead-shift complexes. The mixture of protein–DNA complexes released from the beads was digested with DNase I and the individual complexes were separated using an agarose band-shift gel. The DNA probe in each of the complexes was eluted from the gel and re-run on a denaturing sequencing gel. Footprints on the bottom strand (transcribed) are shown. Lanes 1 to 6 correspond to complexes 1 to 6 indicated in Fig. 3, lane a. The position of the 5S RNA gene is indicated.

by other 5S RNA genes with different 3′ flanking sequences (Fig. 3). The footprint of band 4 appears as a simple addition of the separate footprints of band 2 (TFIIIA) and band 3 (the 3′ flanking sequence protected region). The gel mobility of band 4 is also consistent with the interpretation that this complex contained both

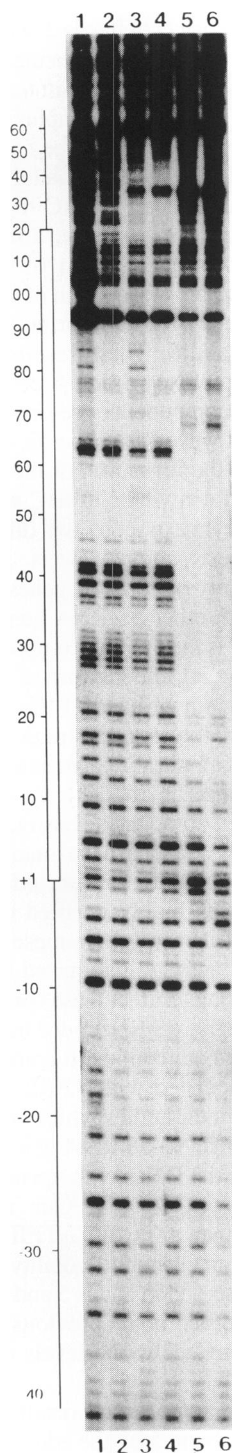

Figure 6. DNase I footprints of individual bead-shift complexes. Footprints on the top strand (nontranscribed) are shown. Lanes 1 to 6 correspond to complexes 1 to 6 indicated in Fig. 3, lane a. Note that a TFIIIA footprint is partly visible in lane 1 (free DNA) and in lane 3. Some TFIIIA complexes were probably disrupted by the EDTA added after DNase I digestion and the DNA therefore ran with the free DNA on the band-shift gel. The position of the 5S RNA gene is indicated.

TFIIIA and another, as yet unidentified, protein bound to the 3′ flanking sequences.

The footprints of bands 5 and 6 showed a large central protected region starting near +20 and extending to the 3′ boundary of a TFIIIA footprint (+95). Both bands 5 and 6 showed nearly complete protection from DNase I cleavage over this entire region. The central protected region had the same 3′ boundary as a TFIIIA footprint but the 5′ boundary was at least 20 bp further upstream. In addition to the extension of the footprint toward the 5′ end of the gene, there were several differences between a TFIIIA footprint and the footprints of bands 5 and 6 over the region of TFIIIA binding. Note that enhanced cleavage near +63 on the top strand, characteristic of a TFIIIA footprint, was not observed for bands 5 and 6. Also, enhanced cleavage on the bottom strand was observed at the 3′ boundary of the central protected region (+95). This enhanced cleavage is not characteristic of a TFIIIA footprint. Starting a bit upstream of the transcription start site (−5) and extending to the 5′ end of the central protected region (+20) partial protections and alterations of the cleavage patterns were observed. Differences between the footprints of bands 5 and 6 were seen in this region. A significant difference between the footprints of bands 5 and 6 was the partially protected region observed in the 5′ flanking region (−37 to −15) of band 6 but not on band 5. The partial protections were most pronounced on the top strand (Fig. 6 lane 6) but were also present to a lesser degree on the bottom strand. In particular, note the enhanced cleavage near −37 on the bottom strand of band 6 in Fig. 5. The partial protections upstream of the gene on complex 6, but not 5, suggest that complex 6, but not 5, contains a protein interacting with this upstream region. This would be consistent with the slower electrophoretic mobility of band 6 relative to band 5. The protection of the 3′ flanking sequences described above for bands 3 and 4 was also observed on the footprints of bands 5 and 6.

DISCUSSION

Using a reversibly immobilized DNA we have affinity purified protein−DNA complexes formed on a 5S RNA gene in an extract from *Xenopus* oocytes. Reversible immobilization takes advantage of the ability to rapidly purify and concentrate complexes afforded by the attachment of the DNA to a solid support, but at the same time, supplies the complexes in a soluble form suitable for enzymatic and chemical probing. In addition, the complexes remained under optimal buffer conditions throughout the entire procedure. Individual soluble complexes were separated and studied using a band-shift gel.

Five complexes with distinct gel mobilities were separated and their DNase I footprints were examined. The footprints of the first three complexes (bands 2, 3, and 4) contained two protected regions and can be accounted for by the binding of two proteins, TFIIIA and an unidentified 3′ flanking sequence binding protein. The binding of these two proteins appears to be independent since we observed complexes with each of the proteins bound individually as well as a complex with both proteins bound together (band 4). A similar DNase I protected region in the 3′ flanking sequences of the somatic-type 5S RNA gene has been observed before using a *Xenopus* oocyte nuclear extract (5, 6). Curiously, a partially purified TFIIIB fraction from yeast was also observed to protect a region downstream of a somatic-type 5S RNA gene from *Xenopus* (21). In addition, extended footprints over the 3′ flanking sequences of a tRNA gene have been observed using partially purified silkworm transcription factors (22). In this case, binding to the 3′ flanking sequences was shown to result from specific contacts made by essential transcription factors and was only observed together with protection over the

essential promoter elements. In contrast, the 3′ flanking sequence protection which we have observed in the *Xenopus* extract occurs independently of binding by other proteins and may not play an essential role in the transcription process. However, examination of the band-shift and footprinting gels suggests that complexes 5 and 6 (which are correlated with transcriptional activity) are built by the addition of proteins to complex 4 and do contain the 3′ flanking sequence protections. Complexes containing the additional proteins of bands 5 and 6 but without the 3′ flanking sequence protections were not observed.

The function of the protein responsible for the 3′ flanking sequence protection is currently unknown. It is possible that there is no functional connection between this protein and 5S RNA gene transcription. On the other hand, it is possible that this protein is involved in the differential transcription of different types of 5S RNA genes. The transcription of different families of 5S RNA genes in the *Xenopus* genome is developmentally regulated (23). In particular, the abundant oocyte-type 5S RNA genes are transcriptionally active during oogenesis, but repressed during early embryogenesis and in somatic cells. Another far less abundant family, the somatic-type 5S RNA genes, are transcriptionally active both during oogenesis and in somatic cells. These two types of 5S RNA genes have nearly identical coding regions but significantly different flanking sequences. We have found a protein that binds specifically to the somatic-type 3′ flanking sequences (Fig. 3). Replacing oocyte-type 3′ flanking sequences with somatic-type 3′ flanking sequences increases the rate of 5S RNA gene transcription complex assembly up to 10-fold (Seidel and Peck, unpublished results). The 3′ flanking sequence binding protein may be responsible for the increase in the rate of transcription complex assembly which could directly influence the differential expression of the different genes (16).

The footprints of the more slowly migrating complexes, bands 5 and 6, contained two identical protected regions: a large central region extending over much of the 5S RNA gene and the same region downstream of the gene protected in bands 3 and 4. The central protected region contains the entire TFIIIA binding site and includes additional adjacent sequences just upstream. This 5′ extension of the TFIIIA protection is the most distinctive feature of the footprints of these complexes. A TFIIIA specific enhanced cleavage near +63 on the top stand was protected in the footprints of bands 5 and 6. This suggests additional protein interactions over the TFIIIA binding site. Although we expect TFIIIA to be bound to the DNA in complexes 5 and 6, the footprints leave open the possibility that TFIIIA has been replaced by other proteins interacting with the same and additional sequences. Differences between the footprints of bands 5 and 6 were observed as altered cleavage patterns in an area around the transcription start site (−5 to +20). Significantly, a unique feature of the footprint of band 6, but not band 5, was an upstream region of partial protection (most pronounced on the top strand) along with an enhanced cleavage on the bottom strand nearly 40 bp upstream of the transcription start site. These features, as well as the slower electrophoretic mobility of band 6 relative to band 5, suggest that band 6 contains an additional protein bound to the 5′ flanking sequences. The extensive footprints observed for bands 5 and 6, as well as the slow gel mobilities, suggest the formation of large complexes with many components.

The footprints of bands 5 and 6 are significantly different from the previously observed footprints of 5S RNA genes complexed with *Xenopus* proteins. Published footprints of 5S RNA gene transcription complexes with *Xenopus* components have patterns which resemble the footprints of bands 2 and 4 and which contain as the most prominent and consistent feature a TFIIIA-like pattern of protection (4−6). Using the bead-shift technique we have been able to characterize individual complexes present in small amounts within a complex mixture. The footprints of the earlier experiments represented the average of all the complexes present. Using partially purified human TFIIIC together with *Xenopus* TFIIIA and a *Xenopus* 5S RNA gene, a footprint very similar to the large central protected region of bands 5 and 6 was observed (14). In particular, the addition of human TFIIIC caused a 5′ extension of the TFIIIA footprint very similar to that observed for bands 5 and 6. These results are consistent with the possibility that complexes 5 and 6 contain TFIIIC.

The identity of the components present in bands 5 and 6 is at present unknown. However, two lines of evidence suggest that TFIIIC is required for the formation of these complexes. First, bands 5 and 6 do not form when TFIIIC activity is selectively eliminated from the oocyte extract by two different methods (Fig. 3). Formation of these complexes is also eliminated by competition with a variety of 5S RNA genes (Fig. 3). We would expect competition with other 5S RNA genes to compete away TFIIIC because TFIIIC is the limiting component for transcription in this system (19). Second, the mixture of affinity purified and isolated complexes containing bands 1 through 6 was not itself transcriptionally active. However, the transcriptional activity was restored by the addition of a heat-treated extract that lacks TFIIIC activity. This implies that TFIIIC activity was present together with the affinity purified complexes. Lastly, the overall patterns of protection for bands 5 and 6 have many similarities to the structure observed for an active transcription complex formed on a 5S RNA gene using highly purified transcription factors from yeast (24, 25). Taken together these results support the conclusion that TFIIIC activity is required for the formation of complexes 5 and 6 and suggest that these complexes are involved in the formation of an active 5S RNA gene transcription complex.

These results identify both band-shift complexes and extended DNase I footprints associated with TFIIIC activity. This is in contrast to several previous studies which showed that although TFIIIC stabilizes the TFIIIA−5S RNA gene complex, it did not alter the gel mobility or the DNase I footprint of the TFIIIA−5S RNA gene complex (6, 10−13). Our results suggest the alternative possibility that a TFIIIC−TFIIIA−5S RNA gene complex has both a distinct gel-shift mobility and a characteristic DNase I footprint as observed in bands 5 and 6. These complexes may not have been observed in the previous studies because the amounts of TFIIIC were below the levels of detection for the assays used.

Both the gel-shift and footprinting results support the notion that band 6 differs from band 5 by the addition of a protein with interactions in the 5′ flanking sequences. The identity and/or functional significance of this additional protein is currently unknown. The mixture of complexes containing band 6 was not significantly transcriptionally active either by itself or upon the addition purified RNA polymerase III. Thus band 6 is lacking an essential component required for transcription in addition to purified RNA polymerase III.

Experiments are currently underway to examine the relationship between these complexes and transcriptional activity using fractionated transcription factors.

The bead-shift method described in this report should provide a key tool in efforts to refine the analysis of the functional significance of these complexes, to identify new complexes, and

to identify the corresponding proteins. The bead-shift technique should also prove useful for the isolation and characterization of protein–DNA complexes in other systems.

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