The Positive Transcription Factor of the 5S RNA Gene Proteolyses during Direct Exchange between 5S DNA Sites

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Abstract. We have examined the association, dissociation, and exchange of the 5S specific transcription factor (TFIIIA) with somatic- and oocyte-type 5S DNA. The factor associates faster with somatic than with oocyte 5S DNA, and the rate of complex formation is accelerated by vector DNA. Once formed, the TFIIIA-5S DNA complex is stable for >4 h in the absence of free 5S DNA, and its dissociation is identical for somatic and for oocyte 5S DNA. In the presence of free 5S DNA, the factor transfers promptly from the complex to the free 5S DNA site. Unexpectedly, the direct exchange of factor between 5S DNA sites leads to proteolysis at the C-terminal arm of TFIIIA.

Two types of chromatin can be assembled on DNA with a Xenopus oocyte extract (oocyte S-150; Glikin et al., 1984). One type, called conventional or static chromatin, is formed in the absence of additional factors and displays constrained supercoils (Ruberti and Worcel, 1986). The other type, called dynamic chromatin, displays torsionally strained supercoils and requires additional factors for assembly. The DNA supercoils in the static chromatin are assembled gradually after 2–4 h incubation in the oocyte S-150, and they are not relaxed by the addition of DNA topoisomerase I or novobiocin. In contrast, the DNA supercoils in the dynamic chromatin appear within 30 min of incubation, and they are fully relaxed by the addition of DNA topoisomerase I or novobiocin (Kmiec and Worcel, 1985; Kmiec et al., 1986b; Ruberti and Worcel, 1986). Novobiocin inhibits the eucaryotic DNA topoisomerase II by competing with ATP (Hsieh and Brutlag, 1980; Miller et al., 1981; Shlomai and Zadok, 1983). Similarly, the inhibition of DNA supercoiling in the oocyte S-150 by novobiocin is reversed by ATP (see Fig. 6 in Glikin et al., 1984), which suggests that the DNA topoisomerase II present in the oocyte S-150 (Benedetti et al., 1983) catalyze DNA supercoiling in these extracts.

The dynamic chromatin forms on 5S ribosomal RNA gene (5S DNA)[1] plasmids via a gyration reaction triggered by the 5S specific factor transcription factor III A (TFIIIA) (Kmiec and Worcel, 1985). In this in vitro system, the dynamic 5S DNA minichromosomes are transcriptionally active and the static 5S DNA minichromosomes are transcriptionally inactive (Kmiec et al., 1986b). Furthermore, differential 5S DNA activation can be induced at low TFIIIA concentrations because somatic 5S DNA gyrates at a TFIIIA:5S DNA ratio of 1 whereas oocyte 5S DNA requires a fivefold higher TFIIIA concentration for gyration (Kmiec et al., 1986a).

Chromatin is assembled on the DNA plasmids by an irreversible maturation process and, once assembled, both chromatin types are stable (Gargiulo et al., 1984; Kmiec et al., 1986b; Ruberti and Worcel, 1986). Moreover, the nascent chromatin is committed to either the static or the dynamic assembly pathway at an early stage. Thus, if TFIIIA is added 10 min after the onset of the assembly process, the factor can no longer induce DNA gyration and dynamic chromatin formation in spite of the fact that chromatin assembly takes >4 h for completion under our in vitro conditions (Kmiec and Worcel, 1985; Ruberti and Worcel, 1986). This result implies that the rate of association between TFIIIA and 5S DNA must play a governing role in the 5S RNA gene activation. A fast association rate constant and/or a high TFIIIA concentration assures that the factor gets to its site promptly. A slow association rate and/or a low TFIIIA concentration will have the opposite effect, and the factor will be excluded by the nascent static chromatin.

We have measured the rates of TFIIIA-5S DNA association and dissociation under our experimental conditions and find that all the somatic 5S DNA sites bind the factor within 10 min at a TFIIIA:5S DNA ratio of 1, but the oocyte 5S DNA sites take 2 h to bind the factor. This result explains why the somatic 5S DNA gyrates but the oocyte 5S DNA does not gyrate in the extract at a TFIIIA:5S DNA ratio of 1. On the other hand, the factor binds to all the oocyte 5S DNA sites within 10 min at a TFIIIA:5S DNA ratio of 5, which explains why the oocyte 5S DNA gyrates in the extract at this TFIIIA:DNA ratio.

We also present evidence for direct transfer of TFIIIA between 5S DNA sites. Surprisingly, the direct exchange leads

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1. *Abbreviations used in this paper:* 5S DNA, 5S ribosomal RNA gene; TFIIIA, transcription factor IIIA.

to proteolysis of TFIIIA, and thus, the factor is stable only when all the 5S DNA sites are occupied. Free 5S DNA sites, which are present when less than stoichiometric amounts of TFIIIA are used, trigger the exchange reaction that ends with the breakdown of TFIIIA. This novel and peculiar process further ensures that 5S RNA gene activation can only occur at TFIIIA:5S DNA ratios equal to or above 1.

Materials and Methods

Plasmid DNAs and Oocyte Supernatant

The plasmid carrying the 249-base pair (bp) somatic 5S DNA in pUC, pXbsF201 is described in Razvi et al. (1983). The plasmid carrying the 200-bp oocyte 5S DNA in pUC, pXloF3ls is described in Kmiec et al. (1986a). The plasmids were purified and labeled with ^{32}P as previously described (Razvi et al., 1983).

The oocyte S-150 was prepared by high speed centrifugation of oocytes in extraction buffer as previously described (Glikin et al., 1984).

Preparation of TFIIIA, 34,000-mol-wt and 30,000-mol-wt Proteins

The positive transcription factor of the 5S RNA gene was purified as described by Dingman et al. (1983) and was stored at no less than 150 μg protein/ml at −70°C in siliconized vials coated with BSA and containing 10 μM $ZnCl_2$. Protein concentration was determined by the method of Bradford (1976), by absorbance at 280 nm, and by the assay of Krystal et al. (1985). Protein dilutions were made into extraction buffer (Glikin et al., 1984) supplemented with 10 μM $ZnCl_2$ in siliconized tubes immediately before use.

The activity of TFIIIA was monitored by two assays: DNA gyration and 5S RNA transcription. Both assays are performed using the oocyte S-150, which is depleted of endogenous factors by preincubation with unlabeled 5S DNA (see below). We have also developed a filter binding assay that is independent of the oocyte S-150: the somatic 5S DNA plasmid is quantitatively retained by the nitrocellulose filter after 10 min incubation at a TFIIIA:5S DNA ratio of 1 (see Fig. 3 a). As measured by these three assays, TFIIIA begins to lose activity after 6 wk storage at −70°C. We have also occasionally obtained TFIIIA preparations that were not fully active. In those cases, the old TFIIIA is discarded and a new protein is prepared. Under the conditions that we describe, and within the experimental precision of our DNA and protein measurements, somatic 5S DNA gyration is triggered at a TFIIIA:DNA ratio of 1, and fivefold higher TFIIIA concentrations are required to trigger oocyte 5S DNA gyration.

The 30,000-mol-wt fragment of TFIIIA was produced by papain digestion and was purified as described by Smith et al. (1984). The 34,000-mol-wt fragment of TFIIIA was produced and purified as follows. Standard reactions containing 0.25 μg TFIIIA, 25 μg DNA (TFIIIA:DNA molar ratio of 0.5), and 2 mM dithiothreitol were incubated at 22°C for 15 min in 10 μl total volume. 20 reaction tubes were combined (5 μg total protein) and the mixture was loaded on a 0.1 ml Biorex 70 (Bio-Rad Laboratories, Richmond, CA) column equilibrated in 50 mM 4-morpholineethanesulfonic acid (7.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM $ZnCl_2$, and 20% glycerol. The column was washed slowly with this buffer plus 0.25 M KCl, and the 34,000-mol-wt protein was eluted with 0.5 M KCl in the above buffer. Dithiothreitol is required throughout this purification because TFIIIA breaks down into small peptides after a long incubation with excess 5S DNA under oxidative conditions (unpublished observations).

The proteins were electrophoresed in the SDS gel system of Laemmli (1970) using a 25% separating gel (0.3% bis-acrylamide) and a 5% stacking gel (0.025% bis-acrylamide). Molecular weight standards were obtained from Sigma Chemical Co. (St. Louis, MO) and Bio-Rad Laboratories.

Proteolytic Cleavage of the 34,000-mol-wt Protein

5 μg of isolated 34,000-mol-wt protein (see above) were incubated at room temperature with 10 U of papain (Boehringer Mannheim Diagnostics, Inc., Houston, TX) and 3 mM $CaCl_2$ for the times indicated in Fig. 5 c. The reaction was terminated by addition of 1% SDS, 10 mM EDTA, and 6 M urea, and the samples were boiled for 5 min and electrophoresed on SDS PAGE as described above. Treatment with trypsin was performed using 10 U of trypsin (L-1-p-tosylamino-2-phenylethyl chloromethyl ketone–treated, Pharmacia Fine Chemicals, Piscataway, NJ) for 15, 30, and 60 s. The reac-

tion was terminated by addition of an excess of trasylol (Boehringer Mannheim Diagnostics, Inc.), 1% SDS and 10 mM EDTA.

Analyses of Gyration and 5S RNA Transcription

All incubations for these as well as for the following binding assays were performed at 22°C. Gyration reactions were carried out using the factor-depleted extracts as described previously (Kmiec and Worcel, 1986 a, b). Briefly, 3 μl of oocyte S-150 are preincubated for 1 h with 40 ng of pXbsF201 in 8 μl total volume of a solution containing 3 mM ATP, 8 mM $MgCl_2$, 2 mM dithiothreitol, and 10% glycerol. After this preincubation the reaction is started by the addition of relaxed ^{32}P-labeled 5S DNA plasmid and transcription factor, and the mixture is incubated for an additional hour. Transcription was assayed by a pulse with $α^{32}$-P-GTP during the last 15 min of incubation as described (Kmiec et al., 1986a, b). Reactions were terminated by the addition of 5 μl of EDTA and SDS to a final concentration of 25 mM and 0.1%, respectively; 2 μl of a 10 mg/ml solution of proteinase K was then added and the mixture was incubated at 50°C for 4 h. DNA topology and RNA transcripts were analyzed in 1% agarose and in 8% acrylamide gels as previously described (Kmiec et al., 1986a, b).

Kinetics of TFIIIA Dissociation

To form the TFIIIA–5S DNA complexes, 0.12 ng TFIIIA were preincubated for 2 h in 2 μl total volume with 5 ng of ^{32}P-labeled somatic or oocyte 5S DNA plasmid (TFIIIA:5S DNA ratio, 1.2) in siliconized, BSA-coated, 0.6 ml Eppendorf vials. We used here a 1.2 ratio of TFIIIA to 5S DNA because TFIIIA breaks down after long incubations at a TFIIIA:5S DNA ratio of 1 (see Results). At this time (time 0) 1 μl containing 1.25 ng 30,000-mol-wt protein was added to half of the samples and 1 μl of buffer was added to the controls. At various times thereafter (as indicated in the left panels of Fig. 2) the samples were incubated for 1 h with 8 μl of factor-depleted S-150 to assay for gyration.

Kinetics of TFIIIA Association

0.1 or 0.5 ng TFIIIA were mixed with 5 ng of ^{32}P-labeled somatic or oocyte 5S DNA plasmid (TFIIIA:5S DNA ratio, 1 or 5) in 2 μl total volume (time 0). At various times thereafter (as indicated in the right panels of Fig. 2) 1 μl of a solution containing a 10-fold excess 30K protein (1 or 5 ng) was added, followed by incubation for 1 h with 8 μl of factor-depleted S-150 to assay for gyration.

Filter Binding Assays

TFIIIA association with the ^{32}P-labeled 5S DNA plasmids was performed as before in 2 μl total volume for various times and at various TFIIIA:5S DNA ratios, as Fig. 3 indicates. After the incubation, 50 μl of 20 mM Tris–25 mM EDTA buffer (pH 7.5) was added slowly and the solution was gently mixed and immediately filtered through nitrocellulose filters at a rate of 1 ml/min. The filters were washed twice at this slow rate with 1 ml of the Tris–EDTA buffer; they were then dried under a heat lamp, and the bound radioactivity was measured by scintillation counting. The nitrocellulose filters; (GN-6 membranes; Gelman Sciences, Inc., Ann Arbor, MI) were prepared by boiling them in 20 mM Tris and 1 mM EDTA (pH 7.5) for 10 min immediately before use, and then cooled to room temperature. About 95% of the input DNA was retained by the filters in the presence of saturating amounts of TFIIIA, and <3% of the input DNA was retained in the absence of TFIIIA.

Results

The 30,000-mol-wt Papain Fragment of TFIIIA Does Not Induce DNA Gyration

The endopeptidase papain cleaves the 5S specific factor TFIIIA producing a 30,000-mol-wt protein that still binds with high affinity to the intragenic 5S DNA site (Smith et al., 1984; Razvi, F., E. B. Kmiec, and A. Worcel, manuscript in preparation). We find that, unlike TFIIIA, this 30,000-mol-wt protein does not promote 5S DNA gyration in the oocyte S-150 (for protein purification details see Materials and Methods and Fig. 5). TFIIIA triggers somatic 5S DNA gyra-
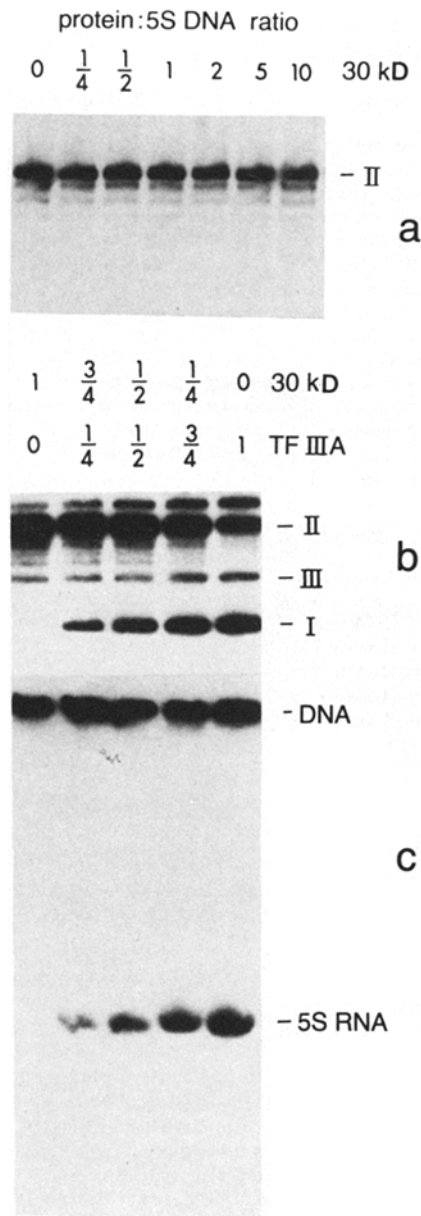
protein:5S DNA ratio

0   1/4   1/2   1   2   5   10   30 kD

— II

a

1   3/4   1/2   1/4   0   30 kD

0   1/4   1/2   3/4   1   TF IIIA

— II

b

— III

— I

- DNA

c

-5S RNA

Figure 1. The 30,000-mol-wt fragment of TFIIIA occupies the DNA binding site but does not induce gyration. (*a*) 5 ng of ³²P-labeled somatic 5S DNA plasmid (pXbsF201) were incubated with the indicated molar amounts of 30,000-mol-wt protein in the factor-depleted oocyte S-150 for 1 h. (*b* and *c*) 5 ng of ³²P-labeled pXbsF201 were incubated in the factor-depleted oocyte S-150 for 1 h with the indicated molar mixtures of 30,000-mol-wt and TFIIIA, from 80 pg 30,000-mol-wt/no TFIIIA in first lane to no 30,000-mol-wt/100 pg TFIIIA in last lane. During the last 15 min, the mixture was pulsed with α³²P-GTP. Aliquots were taken for the analysis of DNA topology and 5S RNA transcription as described in Materials and Methods. I, supercoiled DNA; II, relaxed DNA; III, linear DNA. The band marked DNA at the top of (*c*) represents the ³²P-labeled DNA molecules, which do not migrate appreciably in the 8% acrylamide gels. In this, as well as in the following figures, the variable amounts of relaxed form II DNA and linear form III DNA still remaining in the samples that contain fully supercoiled form I DNA result from unavoidable DNA breaks introduced during the experimental handling. We have observed that the gyrating DNA molecules are susceptible to DNA breakage during the SDS–proteinase K treatment of the samples, and that the extent of broken DNA molecules is a function of the age of TFIIIA, the oocyte S-150,

tion in an all-or-none manner at a protein:DNA ratio of 1 (Kmiec and Worcel, 1985; Kmiec et al., 1986a, b). In contrast, the 30,000-mol-wt protein fails to induce 5S DNA gyration, even at high protein:DNA ratios (Fig. 1 *a*). This result implies that the 10,000-mol-wt C-terminal arm of TFIIIA (Smith et al., 1984; Ginsberg et al., 1984; Miller et al., 1985) plays a key role in the TFIIIA-gyrase interaction (see Discussion).

We previously suggested that unoccupied 5S DNA sites, which are present when less than stoichiometric amounts of TFIIIA are used, block gyration because the free sites dissociate the TFIIIA-5S DNA complexes formed (Kmiec et al., 1986a). In agreement with that suggestion, we now find that the 30,000-mol-wt protein will occupy the free 5S DNA sites, allowing gyration on the fraction of 5S DNA plasmids that have bound TFIIIA. Thus, at a total protein:DNA ratio of 1 with appropriate mixtures of TFIIIA and 30,000-mol-wt protein, gyration occurs in the extract with less than stoichiometric amounts of TFIIIA, and the proportion of gyrating plasmids increases linearly with TFIIIA (Fig. 1 *b*).

Gyration of the 5S DNA plasmids in the oocyte S-150 is accompanied by transcriptional activation of the resident 5S RNA gene (Kmiec et al., 1986a, b). As expected, the rate of 5S RNA transcription is also a linear function of TFIIIA concentration when the TFIIIA/30K mixtures are used (Fig. 1 *c*). It should be noted that using fractions B and C (Shastry et al., 1982), Smith et al. (1984) found the 30,000-mol-wt protein to be one-third as effective as TFIIIA in promoting 5S RNA transcription. In contrast, using the supernatant of whole oocytes, no transcription whatsoever is seen with the 30,000-mol-wt protein (see lane *1*, Fig. 1 *c*). This difference is probably due to the fact that the whole oocyte supernatant also forms chromatin on the DNA and, in the absence of gyration, the DNA template is assembled into static, transcriptionally nonpermissive chromatin (Kmiec et al., 1986a, b; Ruberti and Worcel, 1986).

Addition of Excess 30,000-mol-wt Protein Allows Measurement of the Rates of TFIIIA-5S DNA Dissociation and Association in Solution

The method most commonly used in the past to analyze protein DNA interactions is DNA retention on nitrocellulose filters. Double-stranded DNA fragments, which are radiolabeled to facilitate the measurements, are not retained by these filters unless a protein is stably bound to them. For kinetic measurements, excess unlabeled DNA is usually added at various times after mixing the protein with the labeled DNA to trap the "unbound" protein (Riggs et al., 1970). Unfortunately, if the protein in question is able to transfer directly between DNA sites, as was recently shown to be the case for the lac repressor and for TFIIIA, excess unlabeled DNA will promote exchange of factor, and this reaction will interfere with the measurement of the actual association and dissociation rates of the soluble protein (see Fried and Crothers, 1984; Kmiec et al., 1986a).

We have developed alternative procedures to measure as-

and the ³²P-labeled DNA. Despite the variation between different experiments in the proportion of supercoiled DNA, very good reproducibility is always observed between the samples within a given experiment.

sociation and dissociation rates that do not require addition of unlabeled DNA. One method uses excess 30,000-mol-wt protein as a trap. Dissociation rates can be determined by adding the 30,000-mol-wt protein to a preformed TFIIIA–5S DNA complex followed by, at various times afterwards, the oocyte S-150. If TFIIIA is still bound to its site, the plasmid will gyrate; if TFIIIA has dissociated, the site will be occupied by the 30,000-mol-wt protein and the plasmid will not gyrate. Similarly, association rates can be determined by adding the 30,000-mol-wt protein before the equilibrium between TFIIIA and 5S DNA is reached (for details, see Materials and Methods).

The complex of TFIIIA with 5S DNA is remarkably stable and does not dissociate within the first 4 h after the addition of the 30,000-mol-wt protein (see left panels in Fig. 2). The failure to gyrate seen 5 h after the addition of 30,000-mol-wt protein must be due to dissociation and not to loss of activity of TFIIIA because the complexes are stable for >6 h in the absence of the 30,000-mol-wt protein. The dissociation reaction, which is identical for somatic and for oocyte TFIIIA–5S DNA complexes, is a nonstochastic process that we shall analyze in detail elsewhere (Razvi F., E. B. Kmiec, and A. Worcel, manuscript in preparation).

The right panels of Fig. 2 show the gradual kinetics of the association reaction between TFIIIA and 5S DNA. Under our experimental conditions and at a TFIIIA:5S DNA ratio of 1, it takes ∼8 min for the factor to stably occupy all the somatic 5S DNA sites. At a TFIIIA:5S DNA ratio of five the sites are occupied in ∼2 min, and the association is also accelerated when the reaction is performed in a smaller volume or in the presence of vector DNA (data not shown, but see later, Fig. 3). In contrast, TFIIIA associates with the oocyte 5S DNA at a much slower rate: it takes the factor ∼2 h to occupy all the oocyte 5S DNA sites at a 1:1 ratio and ∼8 min at a 5:1 ratio.

We have previously shown that the factor cannot bind productively to its site 10 min after the onset of the chromatin assembly reaction (see Fig. 5 in Kmiec and Worcel, 1985). Thus, the association rates displayed in Fig. 2 readily explain why the factor is excluded at a TFIIIA:oocyte 5S DNA ratio of 1 and included at a TFIIIA:oocyte 5S DNA ratio of 5 (Kmiec et al., 1986a). Kinetic exclusion must be the reason for our previous results because, as shown in the bottom of Fig. 2, the oocyte 5S DNA will gyrate at a 1:1 ratio of factor to DNA if the factor is preincubated with the DNA for ∼2 h before the addition of the extract.

Measurement of the TFIIIA–5S DNA Association Rate by DNA Retention on Nitrocellulose Filters

The TFIIIA–5S DNA complex, like the complexes formed between other specific DNA binding proteins and DNA (see, e.g., Riggs et al., 1970), is retained on nitrocellulose filters. After a 10-min incubation, quantitative retention of the somatic 5S DNA is observed at a TFIIIA:5S DNA ratio of 1, whereas no significant retention of vector pUC DNA is seen under these conditions (Fig. 3 a). Association rates measured by filter binding are in excellent agreement with the measurements presented above using excess 30,000-mol-wt protein. Thus, at a TFIIIA:5S DNA ratio of 1, the factor binds to all the somatic 5S DNA sites in ∼8 min (Fig. 3 b) and to all the oocyte 5S DNA sites in ∼2 h (Fig. 3 c). At
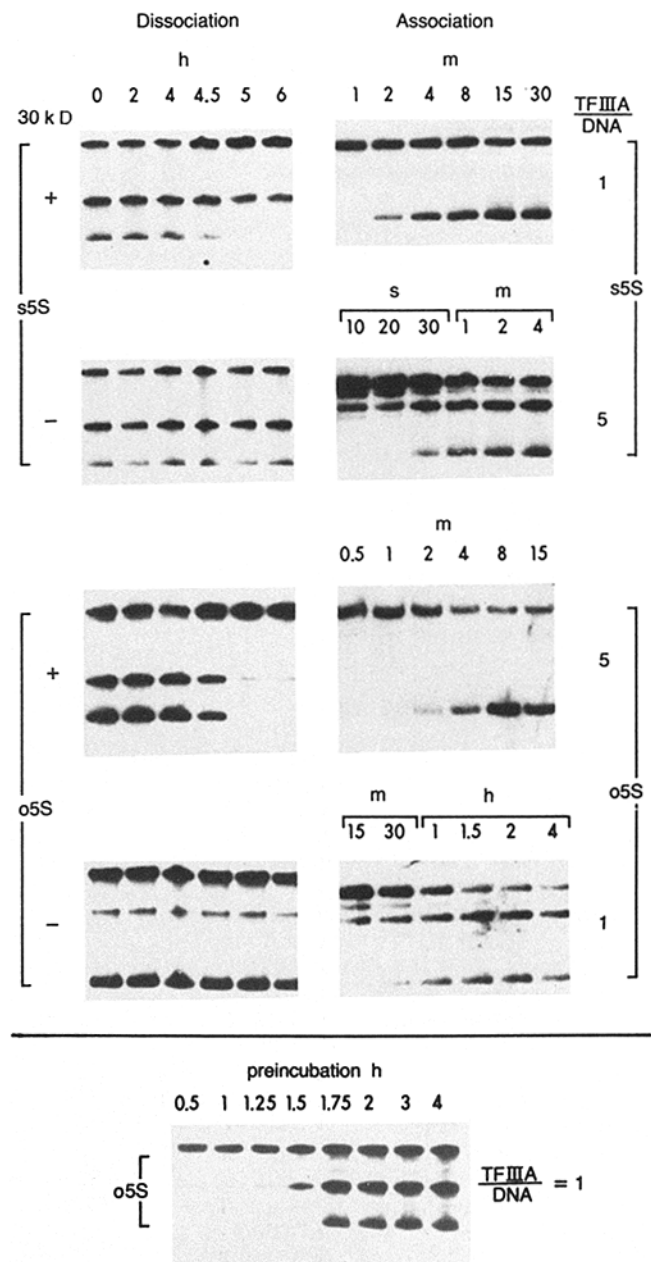

Figure 2. Analyses of TFIIIA–5S DNA dissociation and association using excess 30,000-mol-wt protein. 5 ng of ^{32}P-labeled somatic or oocyte 5S DNA plasmids were associated with TFIIIA (as described in Materials and Methods), using a 10-fold excess 30,000-mol-wt protein to trap the unoccupied 5S DNA sites. Dissociation of the TFIIIA–5S DNA complexes was followed after first forming the complexes at a TFIIIA:5S DNA ratio of 1.2. Association of TFIIIA with 5S DNA was studied at TFIIIA:5S DNA ratios of 1 and 5, as indicated. The lower panel shows the effect of preincubation of TFIIIA with oocyte 5S DNA plasmid, at a TFIIIA:5S DNA ratio of 1 and in the absence of 30,000-mol-wt protein, on subsequent gyration in the extract; oocyte 5S DNA plasmids that are preincubated with TFIIIA for 105 min or longer gyrate, whereas oocyte 5S DNA plasmids preincubated for 90 min or less do not gyrate (see text). DNA topology was analyzed as described in Materials and Methods. m, minutes.
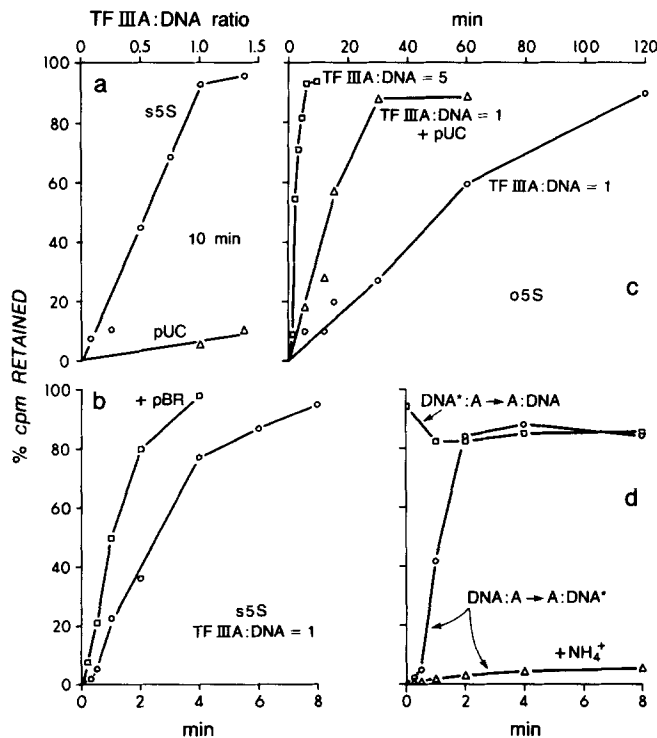
Figure 3. Analyses of TFIIIA-5S DNA association (a, b, and c) and exchange (d) using filter binding assays. (a) 5 ng of ^{32}P-labeled pXbsF201 (○) or 5 ng ^{32}P-labeled pUC (△) were incubated for 10 min with various amounts of TFIIIA, in 2 μl total volume at 22°C (standard conditions). Retention of DNA on the nitrocellulose filters was measured as described in Materials and Methods. (b) 5 ng of ^{32}P-labeled pXbsF201 were incubated under standard conditions, at a TFIIIA:5S DNA ratio of 1 for various times, without (○) or with (□) 15 ng of unlabeled pBR322. (c) 5 ng of ^{32}P-labeled pXloF31s were incubated under standard conditions, at a TFIIIA:5S DNA ratio of 1 without (○) or with (△) 15 ng of unlabeled pUC, or at a TFIIIA:5S DNA ratio of 5 without pUC (□). (d) 5 ng of ^{32}P-labeled pXbsF201 (25,000 total cpm) were incubated under standard conditions at a TFIIIA:5S DNA ratio of 1 for 15 min to form the labeled TFIIIA-5S DNA complex. At this time (time 0), 1 μl containing 0.5 ng (10%) unlabeled pXbsF201 was added (□). In the "mirror image" experiment, 5 ng of unlabeled pXbsF201 were incubated under standard conditions at a TFIIIA:5S DNA ratio of 1 for 15 min to form the unlabeled TFIIIA-5S DNA complex. At this time (time 0), 1 μl containing 0.5 ng (10%) labeled pXbsF201 (2,500 total cpm) was added without (○) or with (△) NH₄Cl (70 mM final concentration). The mixtures were then filtered at various times as indicated.

a TFIIIA:5S DNA ratio of 5, the factor binds to all the oocyte 5S DNA sites in ~8 min (Fig. 3 c).

Fig. 3, b and c also show that unlabeled vector DNA molecules accelerate the rate at which the factor finds the 5S DNA sites on the ^{32}P-labeled 5S DNA plasmids. A similar acceleration is observed when the association reaction is performed in a smaller volume (data not shown). In both cases, the higher concentration of DNA increases the probability that soluble TFIIIA will collide with DNA. As previously discussed, such a molecule of TFIIIA transiently bound to a low affinity DNA site will transfer readily and efficiently to a high affinity 5S DNA site (Kmiec et al., 1986a).

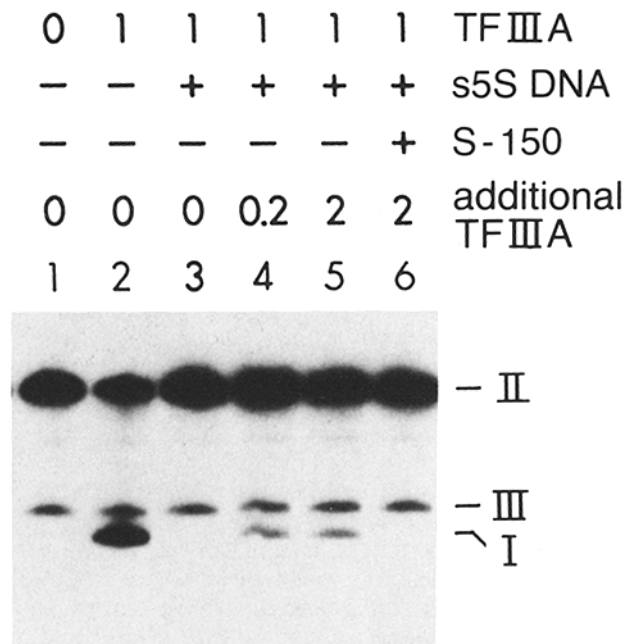The nitrocellulose binding assay can also be used to ana-

Figure 4. Gyration is blocked by free 5S DNA sites. 5 ng of ^{32}P-labeled pXbsF201 were incubated for 15 min without, lane 1, or with, lanes 2-6, 100 pg TFIIIA (TFIIIA:5S DNA ratio of 1). The samples on lanes 3-6 received afterwards at 10% molar equivalent of the 249 bp somatic 5S DNA fragment (see Kmiec et al., 1986a) and 8 min later, the sample in lane 4 received an additional 20 pg TFIIIA, and the sample in lane 5 received an additional 200 pg TFIIIA. After a further 15-min incubation, the samples were incubated in the factor-depleted oocyte S-150 for 1 h. The sample in lane 6 received the oocyte S-150 15 min before the addition of the extra 200 pg TFIIIA, and the reaction was continued for 1 h after the extra TFIIIA addition. DNA topology was analyzed as described in Materials and Methods.

lyze the direct exchange of TFIIIA between 5S DNA sites. When the unlabeled TFIIIA-5S DNA complex is challenged with protein-free, labeled 5S DNA, the TFIIIA transfers to the labeled plasmid and this plasmid is then retained by the filter. The 2-min binding curve (Fig. 3 d) must be the result of direct exchange between 5S DNA sites because, as shown above, the rate of dissociation equals 5 h and the rate of association equals 8 min under these experimental conditions. The exchange reaction, which will be analyzed in detail elsewhere (Razvi F., E. B. Kmiec, and A. Worcel, manuscript in preparation), is unaffected by ZnCl₂ (data not shown), but is greatly inhibited by NH₄Cl.

Gyration Is Blocked by Free 5S DNA Sites

Free 5S DNA sites dissociate the TFIIIA-5S DNA complexes and block the gyration reaction in the oocyte S-150 (Kmiec et al., 1986a). As lane 3 of Fig. 4 shows, 8-min incubation of the TFIIIA-5S DNA complexes with 10% free 5S DNA sites completely blocks the gyration reaction. One might expect, a priori, that addition of 10% extra TFIIIA molecules would again occupy all the 5S DNA sites reestablishing concerted gyration. However, this is not so; only a small proportion of the total plasmid population gyrates after the addition of 20% extra TFIIIA (Fig. 3 b, lane 4), and
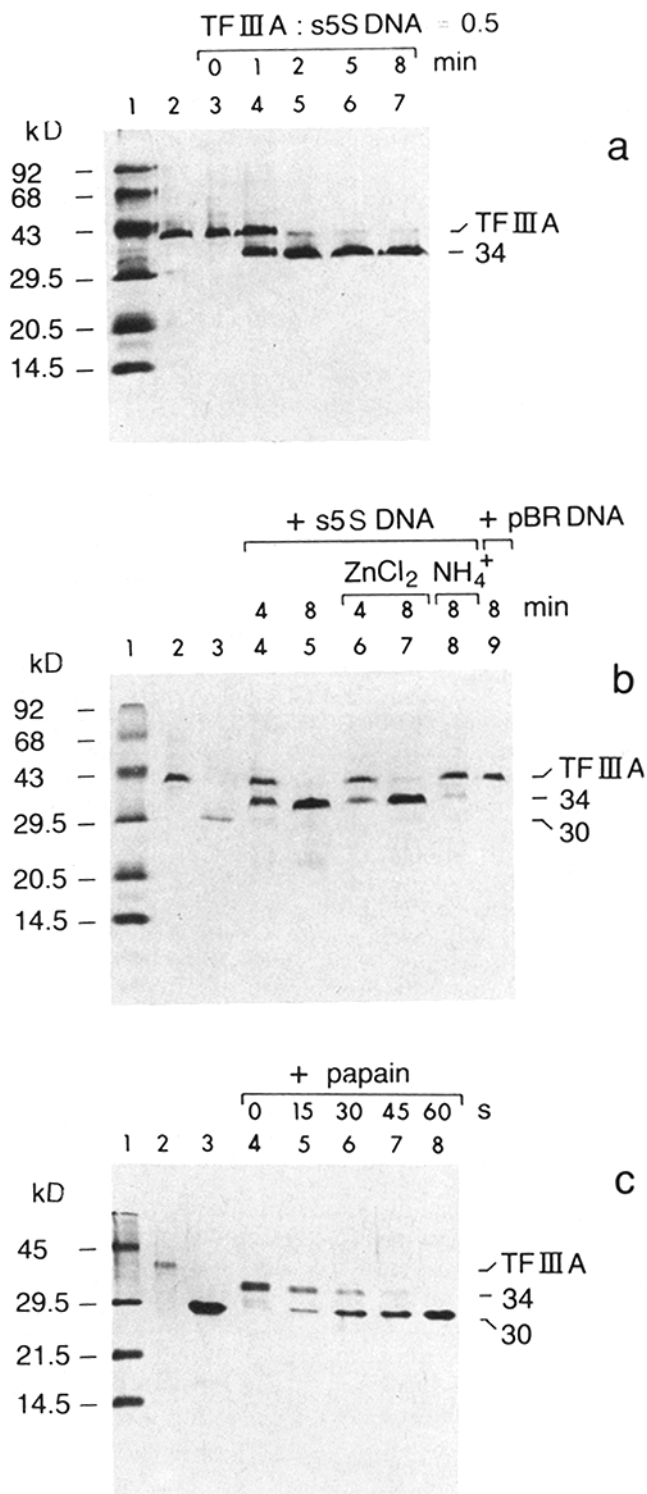
Figure 5. The 5S DNA exchange of factor leads to proteolysis at the C-terminal arm of TFIIIA. (*a*) 0.25 μg TFIIIA were incubated with 25 μg unlabeled relaxed pXbsF201 (TFIIIA:5S DNA ratio of 0.5) in 2 μl total volume for various times as indicated in lanes *3–7*. Lane *1*, molecular weight standards; lane *2*, TFIIIA. No TFIIIA breakdown was observed after an 8-min incubation at a TFIIIA:DNA ratio of 0.5 with pUC DNA or oocyte 5S DNA (data not shown). (*b*) 0.25 μg TFIIIA were incubated with 12.5 μg pXbsF201 (TFIIIA:5S DNA ratio of 1) in 2 μl volume for 15 min to form the TFIIIA–5S DNA complexes. After this time, the samples in lanes *4–8* received a 4% molar equivalent of the 249 bp somatic 5S DNA fragment (see Kmiec et al., 1986a), and were incubated for an addi-

this proportion does not increase after the addition of 200% extra TFIIIA molecules (Fig. 4, lane *5*).

It should be noted that not even this small fraction of plasmids gyrate if the additional TFIIIA molecules are added after the oocyte S-150 (Fig. 4, lane *6*). Under these conditions, the additional factor molecules are excluded because the plasmids assemble into static chromatin (Kmiec and Worcel, 1985; Ruberti and Worcel, 1986). It is not immediately apparent however, why the extra TFIIIA molecules cannot fully reverse, in the absence of the oocyte S-150, the inhibition brought about by the free 5S DNA sites. The fact that extra TFIIIA molecules at less than stoichiometric amounts are active (Fig. 4, lane *4*) indicates that most of the 5S DNA sites in the plasmids are probably not empty. In fact, this result resembles that obtained when most 5S DNA sites contain 30,000-mol-wt protein and only a small fraction of the 5S DNA sites contain TFIIIA (see Fig. 1 *b*).

The Direct Exchange of Factor between 5S DNA Sites Leads to Proteolysis at the C-terminal Arm of TFIIIA

The results displayed in Fig. 4 compelled us to examine the state of the protein after the TFIIIA–5S DNA exchange. To our surprise, the 40,000-mol-wt protein rapidly breaks down to a 34,000-mol-wt protein when the factor is incubated with 5S DNA at TFIIIA:5S DNA ratios below 1 (Fig. 5 *a*) or when a TFIIIA–5S DNA complex is challenged with protein-free 5S DNA (Fig. 5 *b*).

The rate of TFIIIA cleavage is a function of the concentration of protein-free 5S DNA sites. The factor breaks down promptly, within 2 min, at a TFIIIA:5S DNA ratio of 0.5 (Fig. 5 *a*) and very slowly, after 3 h, at a TFIIIA:5S DNA ratio of 1 (data not shown). The slow breakdown at a TFIIIA:5S DNA ratio of 1 is probably due to the presence of small amounts of free 5S DNA sites at equilibrium, because breakdown is not observed at a TFIIIA:5S DNA ratio of 1.2 (see Fig. 2).

The 249-bp fragment carrying the somatic 5S RNA gene is extremely efficient in catalyzing the TFIIIA exchange and breakdown, presumably because its excluded volume is much smaller than the excluded volume of the intact 5S DNA plasmid, and this results in a faster collision rate between the occupied and unoccupied 5S DNA sites. Thus, after the addition of only a 4% molar equivalent of the free 5S DNA fragment, about half of the factor molecules in the TFIIIA–5S DNA complexes break down within 4 min, and all of them break down within 8 min (lanes *4* and *5* in Fig. 5 *b*).

We have examined the effect of ZnCl₂ and NH₄Cl on the TFIIIA exchange and breakdown because those compounds appear to stabilize the TFIIIA–5S DNA interaction and enhance the DNase I footprint of the 5S DNA-bound TFIIIA (Smith et al., 1984). The addition of ZnCl₂ has no effect on the 5S DNA exchange reaction (data not shown), but slows

tional 4 or 8 min as indicated. The samples in lanes *6* and *7* also contained 25 μM ZnCl₂; the sample in lane *8* contained 70 mM NH₄Cl. The sample in lane *9* received a 4% molar equivalent of the 275 bp Bam HI-Sal I fragment of pBR322 (see Kmiec et al., 1986a). Lane *1*, molecular weight standards; lane *2*, TFIIIA; lane *3*, 30,000-mol-wt protein. (*c*) Purified 34,000-mol-wt protein was digested with papain as described in Materials and Methods for various times as indicated in lanes *4–8*. Lane *1*, molecular weight markers; lane *2*, TFIIIA, lane *3*, 30,000-mol-wt protein. Numbers at right, molecular weight ×10⁻³.

down slightly the rate of TFIIIA breakdown. On the other hand, NH₄Cl markedly inhibits both 5S DNA exchange (Fig. 3 d) and TFIIIA breakdown (lane 8 in Fig. 5 b). It should also be noted that this TFIIIA proteolysis is DNA sequence specific, because no breakdown occurs under these conditions with non-5S DNA (see lane 9 in Fig. 5 b).

To map the 5S DNA-induced break in TFIIIA, we cleaved the 34,000-mol-wt protein with trypsin and with papain. Trypsin cleavage of this protein generates a 20,000-mol-wt protein and a 14,000-mol-wt protein (data not shown), but these products cannot be oriented because trypsin cleaves TFIIIA symmetrically (Smith et al., 1984). Papain, on the other hand, gives an unambiguous answer because a 30,000-mol-wt protein is generated after the digestion of either TFIIIA or 34,000-mol-wt protein (see Fig. 5 c). We therefore conclude that the 5S DNA-mediated break occurs at the C-terminal arm of TFIIIA, about halfway between the C-terminal end and the papain-sensitive site.

Discussion

The interaction between TFIIIA and 5S DNA is not governed by a simple equilibrium because it proceeds via a number of steps that are probably accompanied by conformational changes in the protein and the DNA (Razvi F., E. B. Kmiec, and A. Worcel, manuscript in preparation). The kinetic analysis of this process is greatly complicated by the DNA-mediated transfer reaction. Transfer occurs when a factor transiently bound to a non-5S DNA site collides with another nonspecific DNA site, and this reaction is responsible for the vector effects on the rate of formation of the TFIIIA-5S DNA complexes. Direct transfer also occurs when the 5S DNA-bound factor collides with a free 5S DNA site, and this exchange reaction is responsible for the all-or-none nature of gyration in the cell-free extract.

The analysis is complicated further by the TFIIIA breakdown that follows the 5S DNA exchange. Thus, the standard saturation curve with increasing amounts of factor shown in Fig. 3 a does not reveal that TFIIIA has broken down to a 34,000-mol-wt protein in the samples incubated at TFIIIA: 5S DNA ratios below 1 (see Fig. 5 a). Because both TFIIIA and 34,000-mol-wt protein bind tightly to the 5S DNA site, the labeled 5S DNA is retained on the filter by either protein and a linear response to TFIIIA concentration is observed.

The gradual association curves displayed in Figs. 3, b and c are also somewhat misleading because they conceal the fact that TFIIIA-5S DNA complexes are stable only when all 5S DNA sites are occupied. In this case, filtering the solution through a nitrocellulose membrane has the same effect as the previously described DNA immobilization in agarose (Kmiec et al., 1986a): it prevents further collisions between a TFIIIA-5S DNA complex and a free 5S DNA site.

We feel that a thorough quantitative analysis of the TFIIIA-5S DNA interaction is premature until all the parameters of this complex process are better characterized. Therefore, we wish to comment here only on the qualitative aspects and possible biological relevance of these observations.

5S RNA Gene Activation Is Governed by the Rate of Association of TFIIIA with 5S DNA

As we point out in the analysis of the results displayed in

Figs. 2 and 3, the different association rates of TFIIIA with somatic and oocyte 5S DNA readily explain the differential 5S RNA gene activation previously observed under these experimental conditions (see Kmiec et al., 1986a). The irreversibility of the chromatin assembly process implies that association rates will probably play a crucial and general role in eucaryotic gene activation, because factors will be excluded by the nascent nucleosome unless they find their DNA sites within a fixed time after DNA replication (Ruberti and Worcel, 1986; Worcel, 1986). Clearly, the mechanism of factor-DNA search is not only intriguing physicochemically, but also important biologically.

Acceleration of the rate of formation of the TFIIIA-5S DNA complex by vector DNA indicates that the factor finds its DNA site by a DNA-mediated transfer mechanism. We have examined in detail the vector DNA effects using the gyration assay with excess 30,000-mol-wt protein as well as the filter binding assay (Fig. 3, b and c, and unpublished data), and we find that the effects are dependent on both DNA concentration and DNA sequence. Under our standard conditions, optimal acceleration of TFIIIA-5S DNA complex formation is observed with 7 molecules of pUC per molecule of somatic 5S DNA plasmid, a condition that shifts the association from 8 min to 30 s at a TFIIIA:5S DNA ratio of 1. Higher pUC levels inhibit TFIIIA-5S DNA association, probably by competing for TFIIIA. Similar vector effects are observed with the oocyte 5S DNA (see, e.g., Fig. 3 c), and they readily explain the pUC induced shifts in the TFIIIA dose-response of oocyte 5S DNA (see Figs. 3 and 4 in Kmiec et al., 1986a) and the pUC activation of the oocyte 5S RNA gene (see Fig. 1 in Gargiulo et al., 1984).

We have also examined the effect of different restriction fragments of pBR322 DNA on the TFIIIA-5S DNA association. Some DNA fragments accelerate, some have no effect, and some slow down the rate of formation of the TFIIIA-5S DNA complex. We surmise that the different effects reflect different TFIIIA affinities of those DNA molecules, and that inhibitory DNA fragments may carry a site of moderate TFIIIA affinity. We know that coincubated oocyte 5S DNA slows down the TFIIIA-somatic 5S DNA association (data not shown), and this kinetic effect may explain the minor shift in the TFIIIA dose-response of somatic 5S DNA by coincubated oocyte 5S DNA (see Fig. 2 in Kmiec et al., 1986a). Furthermore, similar DNA effects are also observed in cis. Thus, different 5S DNA constructs may display somewhat different rates of TFIIIA-5S DNA complex formation depending on what sequences are inserted next to the 5S DNA (Kmiec and Worcel, unpublished observations). We suspect these events may be the reason behind the "poison" effects of some vector sequences (Kressman and Birnstiel, 1979; Lusky and Botchan, 1981) and other peculiar effects of heterologous DNAs (Gargiulo et al., 1984, 1985).

To avoid these vector DNA effects, in all our comparative studies we have used similar pUC constructs of somatic and oocyte 5S DNA (see Material and Methods here and in Gargiulo et al., 1984 and Kmiec et al., 1986a). To explain the marked difference between the rate of TFIIIA association with somatic and oocyte 5S DNA in the homologous pUC vectors, we suggest that the factor adopts a number of different conformations (similar arguments have been advanced for the formation of the complexes between Escherichia coli RNA polymerase and DNA, see Chamberlin, 1974, and

Straney and Crothers, 1985). We suggest that soluble TFIIIA has a low DNA affinity while DNA-bound TFIIIA has a high DNA affinity, and that DNA-bound TFIIIA can adopt at least two conformations depending on whether the protein is transiently bound to a low affinity site or stably bound to its 5S DNA site.

The relatively slow rate of formation of the TFIIIA–5S DNA complexes, 8 min at 10^{-9} M TFIIIA and somatic 5S DNA, implies that the factor probably collides a number of times with the somatic 5S DNA site before a productive interaction occurs. The required conformational change that locks the protein onto its 5S DNA site may occur faster on the favorable environment of the somatic 5S DNA and slower on the less favorable nucleotide sequence of the oocyte 5S DNA. Thus, the transfer of TFIIIA after a collision with a low affinity site will abort the TFIIIA–oocyte 5S DNA interaction more frequently than the somatic 5S DNA interaction. In this view, more TFIIIA–5S DNA collisions will be required before a productive interaction occurs at the oocyte 5S DNA site, and this will result in a slower overall rate of association for oocyte 5S DNA.

A 5S DNA–induced TFIIIA Cleavage

The break of TFIIIA that follows direct transfer between 5S DNA sites underscores the major changes in conformation that must occur while the protein clamps onto its intragenic DNA site. Although we can not rule out the presence of small amounts of a contaminating protease in our preparations of purified TFIIIA, the absolute requirement for free 5S DNA and the rate dependence of the reaction on the concentration of free 5S DNA argues that the cleavage is probably self-inflicted during the process of exchange.

The behavior of the 34,000-mol-wt protein produced during the 5S DNA-mediated exchange of TFIIIA resembles that of the 30,000-mol-wt protein produced by TFIIIA digestion with papain. Both proteins bind to the 5S DNA site with the same affinity as TFIIIA, and neither protein can exchange to a free 5S DNA site nor induce DNA gyration in the oocyte S-150 (Razvi et al., manuscript in preparation). We may conclude, therefore, that the 6,000-mol-wt C-terminal arm of TFIIIA contains a protein domain(s) essential for interaction with both the incoming molecule of 5S DNA during the exchange reaction, and the additional required factor(s) in the S-150 during the gyration reaction.

The suggestion that all-or-none gyration is a consequence of TFIIIA exchange between 5S DNA sites (Kmiec et al., 1986a) is supported by the linear response to TFIIIA when the extra 5S DNA sites are occupied by the 30,000-mol-wt protein (Fig. 1). The TFIIIA cleavage that occurs when the factor is incubated with excess 5S DNA further ensures that 5S RNA gene activation is only triggered at a TFIIIA:5S DNA ratio of 1 or above. This novel and peculiar reaction also explains why preincubation with 5S DNA depletes the endogenous TFIIIA so efficiently (Kmiec and Worcel, 1985; Kmiec et al., 1986a,b). All of the free TFIIIA molecules probably break down to the 34,000-mol-wt protein after the 1-h preincubation of the S-150 with excess 5S DNA. In fact, our preincubation protocol is very similar to the protocol used in the experiment of Fig. 4, lane 6: the excess 5S DNA depletes TFIIIA by breaking down the factor, and the oocyte

S-150 inactivates the 5S DNA by forming static chromatin on the plasmid.

The cleavage of TFIIIA described here brings to mind the self-proteolysis of lexA and phage λ repressors of E. coli (Little, 1984). In the case of the two procaryotic repressors, the cleavage reaction is activated by rec A protein, whereas in the case of TFIIIA, it is activated by free 5S DNA. Further experiments are needed to determine whether a similar chemical mechanism is responsible for the proteolysis of these three sequence-specific DNA binding proteins.

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