Two TFIIIA activities regulate expression of the Xenopus 5S RNA gene families

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Immunoblotting experiments with polyclonal and monoclonal anti-transcription factor IIIA (TFIIfA) antibodies reveal different electrophoretic forms of TFIIfA in extracts from immature and mature oocytes of Xenopus laevis. The well-characterized 39-kD TFIIfA species is present in ~10^13 copies per cell in stage I–III previtellogenic oocytes and declines in abundance by 10- to 20-fold during oogenesis. An immunologically related protein of apparent molecular mass of 42 kD is present at 2–4% of the level of 39-kD TFIIfA in immature oocytes, and the level of this protein increases dramatically during oogenesis. Both the 39- and 42-kD proteins are complexed with 5S RNA in 7S ribonucleoprotein (RNP) particles. High-level transcription of the oocyte-type 5S genes in vitro requires 39-kD immature oocyte TFIIfA, whereas both 39-kD TFIIfA and the mature oocyte TFIIfA species of 42 kD support somatic-type 5S transcription. TFIIfA of 42 kD does not support oocyte-type 5S transcription in a fractionated transcription system derived from mature oocytes. Both proteins, however, bind the oocyte-type and somatic-type genes with comparable affinities and exhibit similar DNase footprints on both genes. These results suggest a model for the developmental regulation of 5S RNA gene transcription where 42-kD TFIIfA serves as an activator of somatic-type 5S transcription and as a repressor of oocyte-type transcription during early embryogenesis.

[Key Words: TFIIfA; transcription; Xenopus; oocyte]

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5S rRNA is encoded by repetitive gene families in the Xenopus genome. During oogenesis, a highly repeated oocyte-type gene family (with 20,000 copies per haploid genome) is actively transcribed, whereas in developing embryos and in the somatic cells of the adult organism a more moderately repetitive somatic-type gene family (400 copies per haploid genome) is preferentially transcribed. The somatic-type genes are also actively transcribed during oogenesis. In somatic cells, the somatic-type genes are at least 1000-fold more active than the oocyte-type genes on a per gene basis. Models to explain the differential transcription of the 5S gene families during development have been based on the availability of the 5S gene-specific positive transcription factor IIIA (TFIII A) (Gottesfeld and Bloomer 1982; Brown 1984; Brown and Schlissel 1985; Andrews and Brown 1987). The finding of declining levels of TFIIfA per cell during embryogenesis (Shastry et al. 1984; Andrews and Brown 1987) suggested that competition for this factor by the members of the two gene families may play a central role in the establishment of the preferential transcription of the somatic-type genes observed during blastulation (Wormington and Brown 1983; Wakefield and Gurdon 1983). In support of this view, elevation of TFIIfA levels in vivo by microinjection of embryos with synthetic TFIIfA mRNA results in the transient reactivation of the oocyte-type genes (Andrews and Brown 1987). However, inactivation of the oocyte-type genes occurs in the presence of high levels of TFIIfA [150 molecules per gene], suggesting mechanisms for the inactivation of the oocyte-type genes independent of TFIIfA.

Recently, we (Millstein et al. 1987; Peck et al. 1987) and others (McConkey and Bogenhagen 1987; Wolffe 1988; Xing and Worcel 1989) have been able to reproduce the preferential transcription of somatic-type genes in vitro with extracts prepared from mature oocytes or eggs (Glikin et al. 1984). These results were not dependent on transcription reaction conditions, DNA concentration effects, or differential RNA stability. The somatic-type gene preference observed in oocyte extracts does not depend on levels of TFIIfA either, because sufficient quantities of this protein are available to bind both active somatic-type and inactive oocyte-type genes (Millstein et al. 1987; Peck et al. 1987). We examined several different types of extracts prepared from Xenopus oocytes, eggs, and embryos for their relative ability to transcribe the oocyte- and somatic-type 5S genes.
stein et al. 1987; Peck et al. 1987]. We found two general classes of extracts: those that give approximately equivalent levels of transcripts from the two genes (in independent reactions), and those that preferentially support somatic-type transcription. The depressed levels of oocyte-type transcription observed with the latter class of extracts provide an in vitro model for study of the regulation of 5S gene transcription observed in developing embryos (Wakefield and Gurdon 1983; Wormington and Brown 1983).

Two explanations for preferential transcription of the somatic-type genes in the mature oocyte S-150 might be that these extracts are deficient in some component specifically required for oocyte-type transcription or that these extracts may contain a specific repressor of oocyte-type transcription (Peck et al. 1987). In this regard, Millstein et al. (1987) have shown that stable inactive complexes are formed on the oocyte-type genes in S-150 extracts and that these complexes are refractory to activation after microinjection into living oocyte nuclei; however, Wolfe (1988) has shown that the addition of a crude protein fraction containing TFIIC and other proteins to S-150 extracts can elevate oocyte-type transcription. Thus, evidence for both explanations is currently available. Wolfe and Brown (1987, 1988) proposed that the inactivation of oocyte-type transcription observed during early embryogenesis is due to destabilization of transcription complexes formed on oocyte-type, but not on somatic-type, genes. The biochemical mechanism responsible for this destabilization has yet to be established.

In this paper we show that antibodies raised against highly purified TFIIIA recognize different electrophoretic forms of this protein in extracts from immature oocytes and in extracts from mature oocytes, unfertilized eggs, and developing embryos. We find that high-level transcription of the oocyte-type genes in vitro correlates with the 39-kD form of TFIIIA that is present in great abundance in immature oocytes. This form of TFIIIA declines in abundance during oogenesis (Shastry et al. 1984) and is replaced by a mature oocyte form of TFIIIA with an apparent molecular mass of 42 kD. We purified both the immature and mature oocyte forms of TFIIIA and demonstrated with a fractionated transcription system derived from mature oocytes that immature oocyte TFIIIA is responsible for oocyte-type 5S gene transcription in vitro, whereas the mature oocyte form of TFIIIA does not serve as a positive transcription factor for the oocyte-type genes. Both forms of TFIIIA support the transcription of the somatic-type 5S genes. These results suggest a new model for the developmental regulation of 5S RNA gene transcription.

Results

Differential 5S gene expression in vitro

Because immature previtellogenic oocytes are the cells responsible for high-level oocyte-type gene transcription in vivo, we monitored the transcription of the oocyte- and somatic-type genes in mature oocyte S-150 extracts supplemented with extracts prepared from immature oocytes [Fig. 1]. In the absence of added immature oocyte extract, the somatic gene transcriptional advantage is 98:1 (measured at the 25:1 ratio of oocyte to somatic gene, lane 2). In the presence of the immature oocyte extract, however, the level of oocyte-type transcription is increased at least 10-fold and the transcriptional advantage of the somatic-type gene is reduced to 10:1 (at the 25:1 ratio, lane 6). This value is similar to that reported for the oocyte nuclear extract (Brown and Schlissel 1985; Peck et al. 1987). Similar results also have been obtained with oocyte nuclear extracts (Blanco et al. 1988) and with protein fractions isolated from oocyte and egg extracts (Wolfe 1988 and see below). These experiments suggest that factors specifically required for oocyte-type transcription are absent from or are present in insufficient concentrations to support oocyte-type transcription in S-150 extracts, but these factors may be found in the immature oocyte or nuclear extracts.

Figure 1. Effect of an immature oocyte extract on transcription of oocyte- and somatic-type genes in S-150 extracts. At the indicated molar ratios of oocyte- to somatic-type plasmids, 200 ng of 5S DNA was transcribed in S-150 extracts, as described previously (Millstein et al. 1987). The clones used were pXloA3' (oocyte) and pXls11 (somatic). [Lanes 1-3] Reactions were supplemented by the addition of 5 μl of H buffer; [lanes 4-8] reactions were supplemented with 5 μl of immature oocyte homogenate. The products of transcription were analyzed on a partially denaturing gel that separates the oocyte- and somatic-type transcripts (denoted o and s). The triplet of oocyte-type bands is due to inefficient termination (Bogenhagen and Brown 1981; Peck et al. 1987). The reason for two somatic-type bands is unknown at present.
Distribution of TFIIIA in extracts from oocytes, embryos, and somatic cells

Because it seemed likely that TFIIIA or some variant of TFIIIA would be required for oocyte-type transcription, we examined the distribution of this protein in extracts from immature and mature oocytes, unfertilized eggs, midblastula-stage embryos, and somatic cells in culture by immunoblotting with two polyclonal sera and one monoclonal antibody raised against TFIIIA purified from 7S particles isolated from immature oocytes [Fig. 2]. To our surprise, these antibodies showed strikingly different patterns of reactivity on Western blots of cellular proteins and revealed markedly different results with mature and immature oocyte extracts. Figure 2A shows the Coomassie blue stain of the total proteins in these extracts. In the immature oocyte extract, antiserum 470 [Fig. 2B] detects a major band with identical electrophoretic mobility as purified 7S particle-derived TFIIIA (39 kD, cf. lanes 1 and 9). This serum also detects a protein of lower electrophoretic mobility in the immature oocyte extract. The apparent molecular mass of this protein is 42 kD. This 42-kD protein is the major species detected with antiserum 470 in S-150 extracts from mature oocytes [lane 2], unfertilized eggs [lane 3], midblastula-stage embryos [lane 4], and whole-cell extracts from somatic cells in culture [lane 8]. On the basis of the results presented below, we designate the 42-kD immunoreactive species 42-kD TFIIIA and the 39-kD species 39-kD TFIIIA. Antiserum 470 also detects a polypeptide of ~100 kD; at present, we do not know the nature of this protein. Both polyclonal sera react artifactualy with species in the 60-kD range. This reactivity is present in gel lanes where no protein or molecular weight markers have been loaded. Antiserum 469 [Fig. 2C] shows much greater reactivity with 39-kD TFIIIA than with 42-kD TFIIIA. Antiserum 469 detects only small amounts of 39-kD TFIIIA in extracts from unfertilized eggs, midblastula-stage embryos, and somatic cells. Similar results have been obtained with a mouse monoclonal antibody generated with a similar preparation of 7S ribonucleoprotein [RNP] particles containing TFIIIA [Fig. 2D].

Figure 2. Western blot analysis of TFIIIA in extracts from oocytes, embryos, and somatic cells probed with polyclonal and monoclonal anti-TFIIIA antibodies. Soluble proteins from S-12 extracts from immature stage I and II oocytes [lane 1, 38 µg of total protein from 25 oocytes], S-150 extracts from stage V and VI oocytes [lane 2, 84 µg from 3 oocytes], unfertilized eggs [lane 3, 80 µg from 3 eggs], midblastula-stage embryos [lane 4, 75 µg], 7S RNP particles from immature oocytes [lane 5, 18 µg], 0.36 M KCl fraction from DEAE-cellulose chromatography of the immature oocyte extract [lane 6, 12 µg], 0.22 M KCl fraction from DEAE-cellulose [lane 7, 6 µg], tissue culture cell extract [lane 8, 17 µg], purified TFIIIA from 7S particles [lane 9, 3.5 µg], and molecular weight markers [lane M], were subjected to electrophoresis in a 10% polyacrylamide gel containing SDS and transferred to nitrocellulose. Blots were probed with rabbit anti-7S TFIIIA polyclonal sera 470 and 469 and monoclonal antibody O4E2, as indicated. Positive antibody reaction was detected with alkaline phosphatase-conjugated goat anti-rabbit antibody.
Furthermore, results similar to those obtained with S-150 extracts [Fig. 2] were obtained with sonicated whole oocytes.

Table 1 lists the amounts of 39- and 42-kD TFIIIA found in oocytes, eggs, and embryos. These data were obtained by comparing the antibody reaction in extracts from known numbers of cells with that obtained with known amounts of purified 39- and 42-kD proteins [see below]. For estimation of the amount of 39-kD TFIIIA in mature oocytes and embryos, Western blots of gels containing greater numbers of these cells were used (data not shown). For comparable amounts of protein, antisera 470 is more reactive with 42-kD TFIIIA than with the 39-kD protein; therefore, measurements of levels of these two proteins in different extracts must take this differential reactivity into account. The values that we obtained for 39-kD TFIIIA are similar to those obtained by other workers using different antibodies [Shastry et al. 1984; Andrews and Brown 1987]. In agreement with previous studies, 39-kD TFIIIA is the abundant species in immature oocytes, and the level of this protein declines 10- to 20-fold during oogenesis. Concomitant with the drop in 39-kD TFIIIA, the amount of the 42-kD species increases during oogenesis such that this protein is as abundant in mature oocytes as 39-kD TFIIIA is in immature oocytes. The amount of 42-kD TFIIIA doubles between oogenesis and ovulation and then remains fairly constant during early embryogenesis. We have not yet determined the amount of this protein at later stages of embryogenesis. At midblastula, the 42-kD protein is about 40-fold more abundant than 39-kD TFIIIA, thus providing 10^11 molecules of this protein per 5S RNA gene in each cell of the embryo.

It is important to stress that antisera 469 and 470 were raised against the same preparation of highly purified 39-kD immature oocyte TFIIIA [Fig. 2]. This raised two questions: whether 42-kD TFIIIA was an impurity in our original antigen preparation and whether the same antibodies in serum 470 are reacting with both TFIIIA species. We think cross contamination is unlikely because the immunogen was purified by two cycles of ion-exchange chromatography and preparative SDS-PAGE and was homogeneous by silver staining, but this possibility cannot be formally excluded. To answer the second question, the antibodies in antisera 470 reacting with either the 39- or 42-kD TFIIIA were affinity purified and reacted again with Western blots containing both protein species [Fig. 3]. Those antibodies present in serum 470 that first reacted with either TFIIIA species showed specificity for their respective target antigens on subsequent blots, suggesting that different classes of antibodies are reacting with the two TFIIIA.

Barrett and Sommerville [1987] described the 5S RNA and DNA-binding properties of proteins contained in the 42S RNP particles of immature oocytes that store both 5S and tRNA. Because the apparent molecular mass of 42-kD TFIIIA is similar to that of the smaller protein in the 42S particle [40-43 kD], we examined the distribution of the 39- and 42-kD TFIIIA by Western blotting of electrophoretically separated 42S and 7S RNP proteins [Fig. 4]. The 42S particle proteins have electrophoretic mobilities that are clearly different from those of the 7S particle proteins [cf. Fig. 4A, lane 3 with Fig. 4B and C, lanes 4]. Furthermore, neither polyclonal serum reacted with either the 40- or 48-kD proteins in the 42S RNP; rather, both TFIIIA are detected with the polyclonal sera in the 7S region of the gradient. Antiserum 469 detected a low level of 39-kD TFIIIA in the 42S particle fraction [Fig. 4B, lane 3]; however, we do not know whether this represents incorporation of 39-kD TFIIIA into a subset of 42S RNPs or spillover between the 7S and 42S fractions. In agreement with previous reports [Picard and Wwegnez 1979; Pelham and Brown 1980], we conclude that neither major protein of the 42S particle is TFIIIA.

On occasion, we have found mature oocyte S-150 extracts that are active in both somatic- and oocyte-type 5S transcription [Fig. 5B]. We used the two polyclonal sera to examine the distribution of 39- and 42-kD TFIIIA in these extracts and in extracts that transcribe the somatic-type gene preferentially. Figure 5 shows that there is a good correlation between the presence of 39-kD TFIIIA detected with antiserum 469 and oocyte-type transcription: extracts 4 and 5 transcribe the oocyte-type gene and contain markedly higher levels of 39-kD TFIIIA than extracts 1 and 3, which do not transcribe the oocyte-type gene. Extract 2 must be deficient in some factor other than TFIIIA. In each of these extracts, 42-kD TFIIIA is the major TFIIIA species detected with antiserum 470. TFIIIA of 39 kD is only detectable in these S-150 extracts with antiserum 469. Germinal

### Table 1. Amounts of 39- and 42-kD TFIIIA in oocytes, unfertilized eggs, and embryos

| Cell type                      | Nanograms/cell | Molecules/cell | Molecules/5S RNA gene |
|-------------------------------|----------------|---------------|-----------------------|
|                               | 39 kD          | 42 kD         | 39 kD                 | 42 kD                  | 39 kD | 42 kD |
| Immature oocytes [stage I–III] | 50–100         | 2             | 10^{12}               | 5 \times 10^{10}       | 10^7  | 5 \times 10^5 |
| Mature oocytes [stage V–VI]   | 5–10           | 150           | 10^{11}               | 2 \times 10^{12}       | 10^6  | 2 \times 10^7 |
| Unfertilized egg              | 5–10           | 250           | 10^{11}               | 4 \times 10^{12}       | 10^6  | 4 \times 10^7 |
| Midblastula-stage embryo [4000 cell stage] | 5–10           | 300           | 3 \times 10^7         | 1 \times 10^9          | 3 \times 10^2 | 1 \times 10^4 |

* Nanograms per embryo, molecules per cell.
Figure 3. Characterization of affinity-purified antibodies. A Western blot of S-150 proteins was either stained with amido black (lane 1) or probed with antiserum 470 (lane 2). Antibodies reacting with the 39- and 42-kD species were eluted separately from equivalent strips from the same blot (prior to reaction with secondary antibody and color reagents), as described in Materials and methods, and used to probe additional strips from the same blot of S-150 proteins. [Lane 3] Antibody reaction with 39-kD affinity purified antibodies; [lane 4] reaction with 42-kD affinity-purified antibodies.

vesicle extracts, which support both oocyte and somatic-type transcription (Brown and Schlissel 1985; Peck et al. 1987), contain both TFIIIA species [data not shown].

Oocyte components required for efficient oocyte-type 5S gene transcription

To determine whether the 42-kD immunoreactive protein is a 5S gene transcription factor, we separated the 39- and 42-kD proteins from one another by chromatography of oocyte extracts on DEAE-cellulose. Western blots of proteins contained in DEAE-cellulose fractions probed with anti-TFIIIA antibodies show that the 42-kD protein elutes from the resin at lower ionic strength than the 39-kD protein (Figure 2A–D, lanes 6 and 7). TFIIIA of 42 kD elutes at 0.22 M KCl, whereas 39-kD TFIIIA elutes at 0.36 M KCl. No detectable 39-kD TFIIIA is found in the fraction enriched in the 42-kD protein [lane 7]. Both proteins copurify with 5S RNA. To ensure that the 42-kD protein was free of contamination with 39-kD TFIIIA, we used as a source of starting material for transcription studies a mature oocyte S-150 extract that did not contain detectable amounts of 39-kD TFIIIA when probed with antiserum 469. The 42-kD protein was purified by chromatography on DEAE-cellulose, RNase digestion to liberate the protein from 5S RNA and, finally, phosphocellulose chromatography and is devoid of detectable 39-kD TFIIIA [Fig. 6A and B]. The approach we used to assay this fraction for transcription involves reconstitution with partially purified components (Shastry et al. 1982). In addition to RNA polymerase III and TFIIIA, 5S transcription in vitro requires two less well-characterized chromatographic fractions, TFIIIB and TFIIIC (Shastry et al. 1982; Setzer and Brown 1985). We tested the activity of both 5S genes with both TFIIIA species and a TFIIIB, TFIIIC, RNA polymerase III fraction (fraction BCP) derived from mature oocytes. Figure 6B (lane 3) shows that this fraction is devoid of either TFIIIA species. Transcription of the somatic-type gene is achieved with the BCP fraction and either TFIIIA fraction (Fig. 6C, lanes 9–14); however, oocyte-type transcription is obtained with only the 39-kD TFIIIA fraction and the BCP fraction (lanes 5–7). No transcription is observed with the BCP fraction alone [Fig. 6C, lanes 1 and 8]. Transcription of the somatic-type gene with the 42-kD TFIIIA fraction is not due to contaminating 39-kD TFIIIA in this fraction because positive transcription signals are obtained with both protein fractions at comparable TFIIIA protein to DNA ratios. Moreover, we do not detect 39-kD TFIIIA in the 42-kD TFIIIA fraction by Western blot analysis with either polyclonal antibody [Fig. 6B, lane 2]. Furthermore, renatured 42-kD protein recovered from an SDS–polyacrylamide gel is partially active in somatic-type transcription [data not shown]. Also, we find that prebinding of 42-kD TFIIIA to oocyte-type genes inactivates these
genes in extracts that support oocyte transcription [Fig. 7]. In this experiment, a large excess of 42- and 39-kD TFIIIA was used to ensure that all genes were complexed with the TFIIIA species being tested prior to addition of the extract. Neither TFIIIA fraction has any inhibitory effect on somatic-type transcription, and the 39-kD TFIIIA fraction has no inhibitory effect on oocyte-type transcription. These experiments indicate clearly that both the 42- and 39-kD TFIIIA act as positive transcription factors for the somatic-type genes, whereas only 39-kD TFIIIA supports transcription of the oocyte-type genes.

Characterization of two TFIIIA species

Electrophoretic bandshift experiments with labeled restriction fragments containing either the oocyte-type or somatic-type gene with both TFIIIA species were performed to assess the relative affinities of these proteins for the two genes [Fig. 8A and B]. Similar amounts of each protein are required to detect binding to either gene. Conversion of the labeled fragment from the protein-free to bound forms occurs at protein–DNA input ratios slightly greater than 1 : 1 [2 : 1 to 4 : 1, lanes 5 and 10], in agreement with previous reports [Wormington et al. 1981; Sakonju and Brown 1982; McConkey and Bogdenhagen 1987]. Because no other protein is present in sufficient quantity in the 42-kD TFIIIA fraction [Fig. 2, lane 7] to account for DNA binding at the protein–DNA ratios used in these experiments, these results demonstrate unequivocably that 42-kD TFIIIA is the protein species responsible for DNA binding. Models to explain the differential expression of the oocyte- and somatic-type gene families based on differences in factor affinity [Brown 1984] are not supported by the present data. Similar conclusions have been reached by McConkey and Bogdenhagen [1987] and Xing and Worcel [1989]. One difference in the binding properties of the two TFIIIA species observed is that the 39-kD TFIIIA fraction produces multiple bands [more than one protein molecule bound per DNA-binding site; Fried and Crothers 1981] at protein–DNA input ratios in excess of 7 : 1 [Fig. 8, lanes 11 and 12]. This is true for binding to both the somatic- [Fig. 8A] and oocyte-type genes [Fig. 8B]. We do not know the reason for this difference between 39- and 42-kD TFIIIA; however, these additional bands do not represent specific protein–DNA interactions as they can be competed with excess nonspecific DNA.

The gel mobility shift assay has also been used to determine which TFIIIA species is actually bound to 5S gene restriction fragments incubated in the S-150 extracts. A second-dimension SDS gel followed by Western blotting with the two polyclonal antibodies indicates that 42-kD TFIIIA is bound to the active somatic-type genes in mature oocyte S-150 extracts [data not shown]. Figure 8C shows the results of DNase footprint analysis of end-labeled DNA fragments containing either the oocyte- or somatic-type 5S genes with DEAE-cellulose fractions containing 39- and 42-kD TFIIIA, as well as with highly purified 39-kD TFIIIA. Each of the fractions protects the same intragenic region from DNase digestion. These footprints are due to the major TFIIIA species present in each fraction and not to traces of cross-contaminating TFIIIA species [or to some other protein in the DEAE-cellulose fractions], because as little as three molecules of TFIIIA per binding site will protect the labeled DNA in the presence of excess unlabeled 5S DNA [data not shown].

Discussion

Multiple forms of TFIIIA

It is important to consider why previous studies failed to recognize different forms to TFIIIA in immature and mature oocytes and early embryos by immunoblotting [Shastry et al. 1984; Andrews and Brown 1987]. A second form of TFIIIA [TFIIIA'], with an apparent molecular mass 2 kD larger than 39-kD TFIIIA, has been reported by these laboratories; however, this protein first appears late in embryogenesis and is the major TFIIIA species detected in somatic cells [Shastry et al. 1984; Andrews and Brown 1987, Darby et al. 1988], whereas the 42-kD species described here is present in oocytes and early embryos, as well as in somatic cells. TFIIIA' has been shown not to serve as a positive transcription factor for...
the somatic-type genes in a reconstituted system (Shastry et al. 1984); however, Pelham et al. [1981] concluded that somatic cell TFIIIA is a transcription factor for the somatic-type genes. This discrepancy has not been resolved. It remains to be determined whether the 42-kD species we find in oocytes corresponds to this somatic cell protein. A possible source of the difference between our present results and those reported earlier could come from the nature of the gel systems employed by each laboratory (using different extents of cross-linking and different buffer concentrations). We find that in different gel systems the two TFIIIA proteins described can migrate with different relative mobilities or little or no difference in mobility; however, equivalent quantitative results have been obtained with our polyclonal sera with each gel system. Thus, we conclude that our immunological reagents are quite different from those employed in the earlier studies.

What is the relationship between the two TFIIIA species? We considered the possibility of postsynthetic modification of 39-kD TFIIIA as the source of the difference in electrophoretic mobility between the two proteins; however, we failed to detect any of the common protein postsynthetic modifications [J. Blanco and J.M. Gottesfeld, unpubl.]. Preliminary comparative peptide mapping studies with both 39- and 42-kD TFIIIA suggest that these two proteins share some common trypsin and V8 protease-generated peptides, however, both proteins possess unique peptides. These data suggest that the two proteins may be related structurally, but they are distinct proteins. Thus, the two TFIIIA species may be products of two different genes or the result of alternative splicing of the TFIIIA primary transcript. At present, insufficient data are available to decide between these possibilities. Isolation of a cDNA clone encoding 42-kD TFIIIA should resolve this question.

Developmental regulation of 5S gene transcription

Many lines of evidence support the notion that TFIIIA levels control the differential expression of the oocyte- and somatic-type 5S gene families (Pelham et al. 1981; Brown and Schlissel 1985; Andrews and Brown 1987; see introductory section). Our present results are in agreement with this point of view but also suggest an alternative model for developmental 5S gene regulation. We have shown that the major TFIIIA species present in immature oocytes [39-kD TFIIIA] serves as a positive transcription factor for both gene families, whereas the abundant form of TFIIIA present in unfertilized eggs, developing embryos, and somatic cells recognized with our anti-TFIIIA antibodies [42-kD TFIIIA] fails to stimulate transcription of the oocyte-type genes in the in vitro system that we used. This TFIIIA species acts only as a
positive transcription factor for the somatic genes. Because 39-kD TFIIIA turns over markedly during oogenesis [Fig. 2, Table 1], we speculate that the positive transcription factor for the oocyte-type genes is no longer present in sufficient quantity in developing embryos to support high level transcription of the oocyte-type genes. Only those oocyte-type genes complexed with the remaining [maternal] 39-kD TFIIIA would support the low level of oocyte-type 5S transcription observed at the midblastula-stage transition (Wakefield and Gurdon 1983; Wormington and Brown 1983). We suggested previously that the abundant form of TFIIIA present in mature oocytes and embryos may even act as a repressor of oocyte-type transcription (Peck et al. 1987). This suggestion was based on the finding that both the active somatic-type and inactive oocyte-type 5S genes are complexed with TFIIIA in S-150 extracts in vitro. Our present data are consistent with this view. The finding of two distinct proteins with similar binding specificities [Fig. 8] may be surprising but is not without precedence. Recently, the class II transcription factors that bind the CCAAT-box element were isolated, and multiple cDNAs were obtained that encode different proteins (Landschulz et al. 1988; Santoro et al. 1988). Furthermore, multiple immunoglobulin gene enhancer binding proteins have been recognized in B cells and in non-B cells (Schreiber et al. 1988 and references therein).

In the case of 39- and 42-kD TFIIIA, we suggest that only 39-kD TFIIIA allows the subsequent productive binding of the other components of the transcription complex to the oocyte-type gene. Transcription complexes on the somatic gene can be formed with both TFIIIA species [Figs. 6 and 7]. Inhibition of oocyte-type transcription with 42-kD TFIIIA supports the notion that 42-kD TFIIIA could act as a repressor of oocyte-type transcription (Fig. 7). Although the somatic gene transcription preference observed with S-150 extracts is similar to that observed in vivo in developing embryos, it is important to note that this system does not mimic the in vivo regulation of the oocyte pseudogene or trace oocyte-type gene (Millstein et al. 1987; Peck et al. 1987; Xing and Worcel 1989). It will be crucial to establish whether the results obtained with the oocyte S-150 extract truly reflect the in vivo mechanism of regulation of the oocyte-type genes. To this end, currently we are examining the binding of TFIIIA to both classes of genes in vivo, using both genomic footprinting and cross-linking techniques.

Although different forms of TFIIIA present during oogenesis and early embryogenesis have not been observed previously, our data are not inconsistent with the conclusions of previous studies [Wolffe and Brown 1987; Wolffe 1988]. We believe that both the relative abundance of the two forms of TFIIIA and the absolute concentration of other transcription factors are critical for active expression of the oocyte-type genes. The developmental regulation of the oocyte-type genes must involve mechanisms responsible for both the initial inactivation of these genes and the subsequent maintenance of these genes in an inactive chromatin configuration. The latter mechanism appears to rely on packaging of the oocyte-type genes in nucleosomes containing histone H1 (Schlissel and Brown 1984) and possibly on the late replication of these genes during S phase (Guinata and Korn 1986; Guinata et al. 1986). The relative timing of replication could influence the availability of transcription factors to newly replicated 5S RNA genes [Gottesfeld and Bloomer 1982; Brown 1984]. The actual mechanism responsible for the initial repression of the oocyte-type genes observed during early embryogenesis has yet to be established. Repression occurs during gastrula stage when the ratio of 39-kD TFIIIA to 5S gene is ~100 : 1 [Andrews and Brown 1987]. Thus, some mechanism must limit the accessibility of this protein and/or the other components of the transcription complex to the oocyte-type, but not to the somatic-type, genes. Wolffe and Brown (1988) suggested that components present in embryos destabilize transcription complexes on the oocyte-type genes, thus allowing these genes to be packaged into chromatin structures containing histone H1. This relative instability of oocyte gene transcription complexes may explain the inactivation of oocyte-type 5S genes in normal and in TFIIIA-enhanced embryos [Andrews and Brown 1987]. Additionally, competition between 39-kD TFIIIA and the more abundant 42-kD TFIIIA for binding the oocyte-type genes could play a major role in the initial inactivation of these genes.

Materials and methods
Plasmids and restriction fragments
Plasmid pXloA3' + 176 contains one *Xenopus laevis* oocyte-type 5S gene, the AT-rich 5'-flanking sequence, and 60 bp of
Figure 8. DNA-binding activities of 39- and 42-kD TFIIAs. (A and B) Electrophoretic band-shift analysis of TFIIA-DNA complexes. (A) A 242-bp Mspl-Ddel fragment containing the somatic type 5S gene was analyzed as protein-free DNA (lanes 1 and 7) or with the amounts of TFIIA indicated below. (B) A 186-bp Fnu4HI–EcoRI fragment containing the oocyte-type gene was analyzed as protein-free DNA (lanes 1 and 7) or with 20 (lane 2), 50 (lane 3), 140 (lane 4), 430 (lane 5), or 1300 fmoles of 42-kD TFIIA (lane 6), or with 30 (lane 8), 90 (lane 9), 260 (lane 10), 760 (lane 11), or 2300 fmoles of 39-kD TFIIA (lane 12). The ratios of TFIIA to DNA-binding sites are indicated. (C) DNase footprint analysis. (Lanes 1–4) A 32P-end-labeled 474-bp Aval–HindIII restriction fragment containing the somatic-type gene with the label on the coding strand, 161 bases from the 3' end of the gene [pXls11; Peterson et al. 1980]. (Lanes 5–8) A 530-bp HindIII–EcoRI fragment with the end label 60 bases from the 3' end of the oocyte-type gene [pXloA3' + 176; Bogenhagen and Brown 1981]. (Lanes 1 and 5) Reactions contained no protein; (lanes 2 and 6), 100 ng of 42-kD TFIIA; (lanes 3 and 7) 100 ng of 39-kD TFIIA; (lanes 4 and 8) 80 ng of 39-kD TFIIA, purified to homogeneity by chromatography on Bio-Rex 70. Different electrophoresis times were employed for the oocyte and somatic fragments. Lanes M denote Hpal markers of pBR322, and arrows denote the location of the 5S-coding sequences and direction of transcription.

3'-flanking sequence but lacks the pseudogene sequence. This 530-bp HindIII–EcoRI insert was cloned in the polylinker of pUC19 after excision from the original pBR322 vector [Bogenhagen and Brown 1981]. Plasmid pXls11 contains the 883-bp HindIII fragment containing the somatic-type 5S repeating unit of X. laevis described by Peterson et al. [1980] cloned into the HindIII site of pUC19. For the preparation of singly end-labeled fragments, pXloA3' DNA was digested with EcoRI, treated with calf intestinal alkaline phosphatase, 5'-labeled with [γ-32P]ATP and polynucleotide kinase, and secondarily restricted with HindIII or Fnu4HI. Standard procedures were employed [Maniatis et al. 1982]. Restriction fragments of 530 bp [HindIII] or 186 bp [Fnu] were isolated from a nonadenaturing 6% polyacrylamide gel using the crush-soak method [Maniatis et al. 1982]. For pXls11, the 883-bp HindIII fragment was gel-purified after alkaline phosphatase treatment, 5'-end-labeled, and secondarily restricted with AvalII, and the 474-bp Aval–HindIII fragment was gel-purified. A 242-bp Mspl–Ddel fragment of pXls11 was obtained by digestion of the plasmid DNA [in pBR322], first with Mspl, isolation of a 521-bp fragment, end-labeling, and secondary restriction with Ddel.

Extracts and transcription reactions
S-150 extracts from mature [stage V–VI] oocytes were prepared by the method of Glikin et al. [1984], with modifications described by Millstein et al. [1987]. Similar extracts were prepared from unfertilized eggs and embryos [Millstein et al. 1987]. An extract from immature oocytes was prepared from the ovaries of small X. laevis females [6–9 months postmetamorphosis, 2.5–5 cm in length], containing exclusively nonpigmented stage I–III oocytes. These ovaries were washed in H buffer [Millstein et al. 1987] and homogenized in a minimum volume of H buffer with 20 strokes of a Dounce B homogenizer. The extract was centrifuged at 12,000g for 10 min at 4°C, and aliquots of the supernatant [termed S-12 extracts] were stored at –70°C. Transcription reactions with S-150 extracts were performed exactly as described [Millstein et al. 1987]. Tissue cul-
nature cell extract was prepared exactly as described by Pelham et al. (1981).

Extract fractionation

Aliquots (3 ml) of the immature oocyte S-12 extract (generally derived from the ovaries of 6–10 frogs) were chromatographed on DEAE–cellulose (DE-52, Whatman) on a column measuring 1.5 × 2.5 cm. The resin was equilibrated in buffer A [20 mM HEPES (pH 7.5), 0.5 mM DTT, 0.1 mM EDTA, 5 mM MgCl₂, 10% glycerol], containing 100 mM KCl. After application of the sample, the column was washed with five column volumes of buffer A containing 150 mM KCl, and proteins were eluted stepwise with buffer A containing 220 mM KCl and 360 mM KCl. Fractions of 1 ml were collected, and those fractions containing peaks of absorbance at 280 nm were pooled and aliquots stored at −70°C. The S-150 extracts from mature oocytes were used as the starting material for the preparation of 42-KD TFIIIA for transcription experiments. Generally, 30 ml of S-150, derived from four large ovaries, was chromatographed on DEAE–cellulose [20-ml bed volume], as described above, for the S-12 extracts. The 0.22 M KCl eluate was treated with RNase A at 50 µg/ml for 20 min at 22°C and subjected to chromatography on phosphocellulose [Whatman P-11] on a column of 15-ml bed volume equilibrated in buffer B containing 100 mM KCl (same as buffer A but with 0.5 mM spermine in place of MgCl₂) (Shastry et al. 1982). The chromatogram was developed with a 150-ml linear gradient of 100 mM–1 M KCl in buffer B. Aliquots of each fraction (6 ml) were subjected to SDS–gel electrophoresis, and those fractions containing the peak of 42-KD protein were pooled and dialyzed against buffer A containing 100 mM KCl. Aliquots were stored frozen at −70°C.

S-150 extracts also were used for isolation of a fraction containing TFIIIB, TFIIIC, and RNA polymerase III. Six milliliters of S-150, derived from the ovaries of two frogs, were chromatographed on phosphocellulose, as described above, except that the column was washed with 5 volumes of buffer B/100 mM KCl and step-eluted with 1 M KCl in buffer B. Fractions of 1 ml were collected, and those containing peak absorbance at 280 nm were pooled and dialyzed against 200 volumes of buffer A containing 100 mM KCl. This material was applied to a DEAE–cellulose column [as above], and proteins were step-eluted with buffer A containing 360 mM KCl. The peak fractions were pooled and dialyzed against buffer A/100 mM KCl. The DEAE column serves to remove all traces of TFIIIA. The TFIIIB, TFIIIC, RNA polymerase fraction is denoted fraction BCP. For transcription, 10 µl of BCP was used in a final reaction volume of 25 µl, supplemented with various amounts of TFIIIA. Reactions contained 500 ng of gene-containing plasmid, and the final ionic conditions were 8 mM MgCl₂, 64 mM KCl, 12 mM HEPES (pH 7.5), and 5.4% glycerol. Labeled and unlabeled nucleotides were as for S-150 (Millstein et al. 1987).

Analysis of transcription products

Labeled RNAs were analyzed either on denaturing 8% polyacrylamide gels containing 7.6% acrylamide, 0.4% bis-acrylamide, and 8 M urea in 1 × TBE [88 mM Tris-borate, 2 mM EDTA (pH 8.3)] or on partially denaturing gels, as modified from the original system described by Wakefield and Gurdon (1983). The latter gels were composed of 12% polyacrylamide (30 : 1.18 acrylamide to bis-acrylamide), 4 M urea in 1 × TBE (Peck et al. 1987). The gels measured 15 × 25 cm × 1.5 mm and were prerun for 4 hr at 400 V prior to electrophoresis, and then at 150 V for 16 hr. Xylene cyanol reaches the bottom of the gel after this period of electrophoresis. For both gel systems, RNA purified from the transcription reactions [Millstein et al. 1987] was dissolved in 95% formamide, 1 × TBE, 0.1% bromphenol blue, and 0.05% xylene cyanol and heated to 95–100°C for 5 min prior to electrophoresis.

Antibodies and immunodetection of TFIIIA

Polyclonal sera and monoclonal antibodies were generated against TFIIIA purified from 7S RNPs by glycerol gradient centrifugation, DEAE–cellulose chromatography, and Bio-Rex 70 chromatography (Millstein et al. 1987). For rabbit immunization, TFIIIA was subjected to SDS–PAGE, and the band containing TFIIIA was excised from the gel, lyophilized, crushed, rehydrated in water, and mixed with an equal volume of complete Freund’s adjuvant. Approximately 30–50 µg of TFIIIA was injected into each rabbit, and the rabbits were boosted with an additional injection of 30 µg at intervals of 2 weeks and 1 month. Serum was obtained 1 month after the final injection. Monoclonal antibodies were generated by the subcutaneous injection of 50 µg of TFIIIA as 7S particles with complete Freund’s adjuvant at monthly intervals into an F₃4 crossed mouse [BALB/c × B10BR]. After four inoculations, the mouse was rested for 2 months, and 50 µg of TFIIIA was injected into the tail vein. The mouse was sacrificed 3 days later, and the splenocytes were fused with myeloma SP-2/0-Ag14 and selected with hygromycin and azaserine. Positive clones were identified by ELISA screening and immunoblotting. Monoclonal antibodies were purified as described (Dillworth et al. 1987). Proteins from oocyte, egg, embryo, and somatic cell extracts were separated by polyacrylamide gel electrophoresis [Laemmli 1970] and transferred to nitrocellulose (Towbin et al. 1979). The Western blots were blocked with Carnation nonfat dry milk (Johnson et al. 1984). Both monoclonal antibodies were used at dilutions of 1 : 500, and positive antibody reaction was detected with either an alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega Protoblot system) or a peroxidase-conjugated anti-rabbit IgG (Tago). An additional intermediate rabbit anti-mouse antibody was used for the monoclonal antibody [Promega]. Antibodies present in the polyclonal sera were affinity-purified on Western blots of SDS–polyacrylamide gel separated proteins. Antibodies were eluted from gel strips with 200 µl of 6 M guanidine-HCl [30 min on a rotator] and renatured by stepwise dilution to 0.1 M with H buffer. Antibodies were used directly for reprobing filters.

Analysis of protein–DNA complexes

DE-52 column fractions containing the TFIIIA species were treated with RNase A [50 µg/ml, 10 min], and binding reactions were carried out in a volume of 20 µl of 20 mM HEPES (pH 7.5), 50 mM KCl, 25 mM ZnCl₂. In addition to 100 fmols of the labeled fragment, all reactions contained 200 ng of poly[dl-D]-C. After 20 min preincubation, the samples were made 10% in glycerol and were subjected to electrophoresis at 150 V for 2.5 hr in a 1.5-mm × 20-cm 5% nondenaturing polyacrylamide gel containing 44 mM Tris-borate [pH 8.3], and 0.1 mM EDTA. For DNA footprinting, end-labeled fragments were incubated as above, but with 200 ng of unlabeled carrier plasmid DNA, and digested with DNase I, as described (Peck et al. 1987). DNA purified from these digests was subjected to electrophoresis in 6% sequencing gels. Radioactive bands were detected by autoradiography with Kodak XRP or XAR-5 film and Dupont Cronex screens.

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