Presence of multiple species of polypeptides immunologically related to transcription factor TFIIIA in adult *Xenopus* tissues

Wataru Yasui and Masaru Ryoji*

Department of Molecular and Experimental Medicine, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

Received March 14, 1989; Revised and Accepted June 5, 1989

ABSTRACT

Transcription of 5S RNA gene in *Xenopus* oocytes requires a 38 kDa transcription factor TFIIIA, which interacts with the 50 bp internal control region of the gene. We looked for TFIIIA-like polypeptides in the extracts of adult *Xenopus* tissues on the basis of their antigenic cross-reactivity to anti-TFIIIA antibody. Several species of polypeptides ranging from 30 to 50 kDa were found in kidney, stomach, liver and testis. Although these polypeptides reacted specifically to anti-TFIIIA antibody, proteolytic peptide mapping of three representative ones did not reveal any mutual similarities. They also seemed to be distinct from TFIIIA. Possible functions of these proteins are discussed.

INTRODUCTION

Transcription of *Xenopus* 5S RNA gene requires at least three transcription factors TFIIIA, B and C in addition to RNA polymerase III (1–3). A single molecule of TFIIIA binds to the 50 bp internal control region of 5S RNA gene, and then attracts other factors to form a stable complex for the initiation of transcription (4–7). TFIIIA is also known as an RNA binding protein, which stores 5S RNA as 7S ribonucleoprotein complexes in the oocyte cytoplasm during early oogenesis (8,9). TFIIIA purified from premature oocytes is a 38 kDa, zinc-containing polypeptide (6,10,11). The DNA binding domain of this protein contains nine tandemly repeated units each consisting of about 30 amino acid residues (11). Every repeat carries two cysteines at one end and two histidines at the other which, by chelating one zinc ion, generate a finger-like structure (11). Recently, a number of other nucleic acid binding proteins have also been shown to contain similar structures, generally known as 'zinc fingers' (for reviews see refs. 12 and 13).

Xenopus laevis carries two types of 5S RNA genes and their transcription is differentially regulated in development (for review, see ref. 14). The oocyte-type genes (20,000 copies per haploid genome) are transcribed only in oocytes whereas the somatic-type genes (400 copies per haploid genome) are expressed both in oocytes and in somatic cells. A major question in this system has been focused on how the expression of the oocyte-type genes is selectively turned off in somatic cells despite the gene copy number 50-fold excess to that of the somatic-type genes. Several mechanisms have been proposed to account for such differential expression (for a review, see ref. 15). Less explored among them would be the possibility of the presence of altered TFIIIA specific for somatic-type genes. It is known that there is a peculiar protein present in the late stage of embryos and somatic cells. This protein, called TFIIIA', is antigenically related to authentic TFIIIA, but is about 2,000 dalton larger in molecular mass in SDS-PAGE (16–18). Cyanogen bromide (CNBr) peptide mapping of embryo- and cultured cell-derived TFIIIA' suggested a close similarity in the amino acid sequences to authentic TFIIIA (17). However, purified TFIIIA' from

cultured cells failed to exhibit a transcriptional activity (17). The genetic basis of such a variant of TFIIIA is not clear at present, since Southern blotting analysis indicated that there is only one gene for TFIIIA (19–21). Thus, it may have been generated by a post-translational modification of TFIIIA, which causes a slower mobility in SDS-PAGE. On the other hand, it is still possible that a decrease in the stringency of hybridization may allow detection of additional genes related to that for TFIIIA. Different splicing can also produce altered proteins from a single gene. Several minor bands of mRNA detected upon Northern analysis (19,21) may be an indication of the presence of proteins closely related to TFIIIA. It should be noted that various DNA-binding proteins have already been shown to be functionally or immunologically related to one another (22–26).

To learn more about the TFIIIA-like proteins in somatic cells of *Xenopus*, we took the following approach. Firstly, we used various organ tissues rather than cultured cells considering the possibility that partially dedifferentiated cultured cells might express oocyte TFIIIA. It was documented that a protein which comigrates with oocyte TFIIIA in electrophoresis was found only in cultured cells (16–18), and was barely detectable in adult liver (16–18). Secondly, based on the wide variation in size of the TFIIIA-equivalents purified from other species, e.g., 35 kDa in human and 48–51 kDa in yeast (27,28), we surveyed all the proteins separated in a gel. Previous studies by others put an emphasis on 38–40 kDa proteins, and proteins in other size ranges were not closely examined in regard to their cross-reactivities. In addition, we paid more attention to the specificity of the antibody reaction. As a result, we detected several novel polypeptides immunologically related to TFIIIA. We then compared their structures by proteolytic peptide mapping.

MATERIALS AND METHODS

Preparation of extracts from Xenopus tissues

For whole cell extracts, various tissues were removed surgically from *Xenopus laevis* (Nasco, Fort Atkinson, Wis.) under anesthesia by 0.3% *m*-aminobenzoic acid ethyl ester (Sigma), frozen immediately in liquid nitrogen and stored at -85°C . They were homogenized within three days. In the case of stomach, only mucosal and submucosal layers were taken using surgical blades. Frog tissues were ground in liquid nitrogen, washed with phosphate-buffered saline (PBS) and homogenized in a lysis buffer containing 1% Triton X100, 50 mM Tris HCl pH 7.5, 80 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml bacitracin (Sigma), 0.5 $\mu\text{g}/\text{ml}$ pepstatin A (Sigma), and 0.5 $\mu\text{g}/\text{ml}$ leupeptin (Sigma). After sonication and digestion with DNase I (Bohringer, about 1% of total protein) as well as RNase A (Sigma, about 1% of total protein) at 0°C for 60 min, sodium dodecylsulfate (SDS) was added to a final concentration of 6%. The samples were boiled and centrifuged at 15,000 g for 15 min, and the resulting supernatants were used as whole cell extracts.

For the preparation of high salt extracts, 2 M NaCl was used in the lysis buffer instead of 1% Triton X100. Nuclease treatment was omitted. After sonication and centrifugation, the supernatants were dialyzed against a low salt buffer containing 50 mM Tris HCl pH 7.5, 5 mM MgCl_2 , 0.5 mM dithiothreitol, 10 μM ZnCl_2 , 0.1 M KCl and 20% glycerol. Precipitates were removed by centrifugation, and the supernatants were used as high salt extracts. The precipitates did not contain significant amounts of cross-reactive proteins (data not shown). To analyze proteins in the high salt-insoluble fraction, the pellet of the first centrifugation was treated with 1% Triton X100, DNase I, and RNase A, and then with 6% SDS for solubilization.

Oocyte extracts were prepared from manually isolated stage VI oocytes (29) following the procedure for the whole cell extracts as above. However, in Figs. 1 and 4A, we used a low salt extract which was extracted directly with the low salt buffer described above. The protein concentrations were determined by the method of Lowry *et al.* (30) using bovine serum albumin (BSA) as a standard.

Preparation of polyclonal anti-TFIIIA antibody

TFIIIA was purified from the ovaries of immature *Xenopus laevis* following the method of Smith *et al.* (31). The purity of the preparation was greater than 95% in an overloaded SDS-PAGE stained with silver. Purified TFIIIA was finally separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the gel paste containing a single band of TFIIIA was injected into a rabbit along with complete Freund's adjuvant (GIBCO) as described previously (32). IgG fraction of the serum was used as immune IgG after dialysis against PBS.

Immunoblotting

Gel electrophoresis was carried out using 10% polyacrylamide as described (33). Proteins were electrotransferred onto nitrocellulose (Schleicher and Schuell) and immunoblotting was performed (34). Residual protein binding sites on the filter were then blocked by incubation with 2.5% BSA in PBS for 30 min. Immune IgG (10 μ g/ml) and 125 I-protein A (0.4 μ Ci/ml, ICN) were used in primary and secondary reactions, respectively. The filter was dried and exposed to X-ray film. Under these conditions, 2.5 ng of TFIIIA became an easily identifiable band after 12 hr exposure.

Absorption of TFIIIA-specific antibodies from immune IgG was carried out as follows. About 55 μ g of purified TFIIIA or BSA was subjected to SDS-PAGE and transferred onto nitrocellulose membrane. The bands corresponding to TFIIIA and BSA were cut out into small pieces. A portion of these membrane pieces containing 4 μ g of either protein were treated first with 2.5% BSA in PBS, and then gently mixed for 14 hrs in cold with 1.5 μ g of immune IgG in a final volume of 150 μ l unless it is indicated in the legends. The supernatant was then used as a TFIIIA- or BSA-absorbed antibody.

Electrophoretic elution of proteins from gels

Whole cell extracts or purified TFIIIA was separated by preparative SDS-PAGE with 0.1 mM sodium thioglycolate in the buffer to prevent the degradation of protein. To avoid acid hydrolysis of proteins, gels were stained for no more than 15 min and destained for less than 2 hr. The TFIIIA band or the cellular protein bands which we know migrate closely with each TFIIIA-like protein were cut out, and the proteins were eluted from the gel by electrophoresis as described (35).

Analysis of cyanogen bromide-cleaved peptides

Electroeluted proteins and purified TFIIIA were treated with 5 to 500 μ g of cyanogen bromide (CNBr) in 74 μ l of 70% formic acid for 24 hr at 25°C in the dark. The digests were lyophilized three times following rehydration in water. Finally, they were dissolved in the sample buffer (33) for separation by 15% SDS-PAGE.

One-dimensional peptide mapping

One-dimensional peptide mapping was performed as described by Cleveland *et al.* (36). Proteins eluted electrophoretically from gels were suspended in a buffer containing 0.125 M Tris HCl pH 6.8, 0.5% SDS, 10% glycerol and 0.001% bromophenol blue. Proteolytic digestions were carried out for 30 min at 37°C in the presence of various amounts of *Staph. aureus* V8 protease (Boehringer) or chymotrypsin A (Boehringer) as indicated in the figures. The reaction volumes were 7.5, 15, 22.5, and 22.5 μ l for TFIIIA, 35, 39,

and 50 kDa proteins, respectively. The digestion was terminated by boiling after addition of β -mercaptoethanol and SDS to final concentrations of 10% and 2%, respectively. The digests were separated by 17.5% SDS-PAGE, and analysed by immunoblotting.

RESULTS

Characterization of anti-TFIIIA antibody

We prepared a polyclonal anti-TFIIIA antibody from a rabbit immunized against TFIIIA. For this purpose, TFIIIA had been purified from the 7S ribonucleoprotein particles of immature oocytes by a standard procedure (31), and then isolated as a single band by SDS-PAGE in order to remove minor contaminants of different sizes (see Materials and Methods). To examine the specificity of the resulting immune IgG, we carried out an immunoblot analysis of a low salt extract (see Materials and Methods) of stage VI oocytes. As shown in Fig. 1, immune IgG detected the major 38 kDa band of TFIIIA together with three minor ones at 56, 40 and 35 kDa (lane with encircled +). While the 40 kDa band was detected even with preimmune control IgG (lane with encircled -) and thus considered to be nonspecific, the other two minor bands, i.e., 56 kDa and 35 kDa, seemed to be specific for anti-TFIIIA antibody. Further examination of whether these bands are really reacting to anti-TFIIIA antibody was carried out as follows: Immune IgG was absorbed first in a TFIIIA band blotted on nitrocellulose, and the remaining IgG in the supernatant was then used to see how the absorption affects the reactivity of the antibody

Figure 1. Specificity of anti-TFIIIA antibody. Soluble extract of stage VI oocytes was applied to 10% SDS-PAGE (25 μ g protein per lane) and subjected to immunoblotting as described in Materials and Methods. The blot was probed using immune IgG (lane with encircled +), preimmune IgG (lane with encircled -), immune IgG absorbed with BSA (lane +) and immune IgG absorbed with TFIIIA (lane -). The absorption reaction was carried out for only 2 hrs in this experiment.

to each protein band in the blot of the oocyte extract (Fig. 1, lane -; see Materials and Methods for detail). In another case, immune IgG was preabsorbed in a BSA band on nitrocellulose as a control (Fig. 1, lane +). We found that the intensities of TFIIIA and the 56 kDa band were greatly reduced upon preabsorption with TFIIIA, whereas 35 and 40 kDa bands were not affected (compare lanes + and -). The residual band seen at the position of TFIIIA after absorption (lane -) is due to an insufficient time for absorption (only 2 hrs in this experiment). This band was reduced to an almost background level if prolonged absorption (14 hrs) was carried out as seen in all the other experiments described below. The 56 kDa protein band, though specific, was very faint, and it seemed to be masked by the background smears in another preparation of the low salt extract (Fig. 4A, lane under O) despite the fact that we applied the same amount of protein in the gel. We concluded that authentic TFIIIA is the predominant protein reacting to the antibody in stage VI oocytes. These results also confirmed that the absorption test shown here can distinguish immunologically cross-reactive proteins from nonspecifically reacting ones although total immune IgG is used without affinity purification.

It has been reported that there are three CNBr cleavage fragments in TFIIIA (17,31). Smith *et al.* (31) designated these three as CB1, 2 and 3. CB2 and 3 represent the fragments from the C- and N-termini respectively, whereas CB1 corresponds to the central portion of TFIIIA molecule. Fig 2A depicts CNBr peptides of TFIIIA stained with silver. As expected, three fragments were seen at the appropriate positions. The lower molecular weight doublet corresponds to CB2 (11 kDa) and CB3 (9 kDa), and the intense band at about 19 kDa position is considered to be CB1. In addition, two faint bands closely migrating at around 30 kDa were observed. They are incomplete digests (CB1 + 2) and (CB1 +

Figure 2. Antibody reactivity to cyanogen bromide peptides of TFIIIA. Purified TFIIIA (5 µg) was digested with two different amounts of CNBr as indicated and portions of the reaction mixtures containing 1 µg (in A) and 0.5 µg (in B) of TFIIIA were applied to SDS-PAGE. The bands were then visualized by silver staining (in A) or by immunoblotting (in B). Control lanes contain 0.2 µg (A) and 0.1 µg (B) of purified TFIIIA without CNBr treatment.

Figure 3. Detection of immunologically related polypeptides to TFIIIA in female frog tissue extracts. Whole cell extracts of stage VI oocytes (O, 50 μ g protein), kidney (K, 100 μ g protein), stomach (S, 100 μ g protein) and liver (L, 100 μ g protein) were subjected to SDS-PAGE followed by immunoblotting. The blot was probed with the following antibodies: immune IgG absorbed with BSA (lanes +), immune IgG absorbed with TFIIIA (lanes -), immune IgG without absorption (lane with encircled +), and preimmune IgG (lane with encircled -). The numbers on the right indicate the positions of the bands described in the text.

3), which were also reported by Smith *et al.* (31). We then immunoblotted a gel of CNBr peptides using immune IgG as a probe (Fig. 2B). The antibody reacted to all CNBr fragments of TFIIIA, although the immunoreactivity was much stronger to the smaller fragments (CB2 and 3) than to the larger one (CB1). Therefore, our anti-TFIIIA antibody recognizes epitopes located in both the N-terminal portion including the first three units of the Zn-finger structure and the C-terminal non-finger tail. The reactivity to the bulk of the finger repeat (CB1) is considerably weaker.

Presence of polypeptides immunologically related to TFIIIA in frog tissues

With this anti-TFIIIA antibody in hand, we addressed the question of whether adult frog tissues contain TFIIIA-related proteins. Whole cell extracts soluble in an SDS-containing buffer (see Materials and Methods) were prepared from various tissue homogenates of an adult female frog, and examined by immunoblot analysis (Fig. 3). Several bands were detected in stage VI oocytes by the BSA-absorbed antibody (lane + under O), but only the TFIIIA band diminished in intensity upon absorption of immune IgG with TFIIIA (lane - under O). A minor protein (56 kDa) seen in the low salt extract (Fig. 1) was not detectable in this preparation. Detection of such a minor component would be difficult particularly in the whole oocyte extract, because a large amount of yolk and other proteins not present in the low salt extract would increase the background and, at the same time, reduce the contribution of the 56 kDa protein loaded in a gel. One of these extra proteins is evident in the 97 kDa region of this figure where a nonspecifically reacting smear is observed only in the whole oocyte extract.

To examine the kidney extract, we used four types of IgG as probes (lanes under K).

Figure 4. Solubilities of TFIIIA-related polypeptides of female frog tissues. High salt-soluble and -insoluble fractions of female frog tissue extracts were prepared as described in Materials and Methods. A, Soluble fractions of kidney (K, 100 μ g protein), stomach (S, 100 μ g protein) and liver (L, 100 μ g protein) were analyzed by immunoblotting. The control lane under O represents a low salt extract of stage VI oocytes (25 μ g protein) which displays the position of TFIIIA. The blot was probed with immune IgG absorbed with BSA (lanes +) and immune IgG absorbed with TFIIIA (lanes -). B, Insoluble fractions of tissue extracts (100 μ g protein) were subjected to immunoblotting using immune IgG as a probe. The numbers on the right indicate the positions of the bands described in the text.

Two control lanes, probed by total immune IgG (lane with encircled +) and by the one absorbed with BSA (lane +), were almost indistinguishable, indicating that BSA did not absorb antibodies nonspecifically. In contrast, preabsorption of immune IgG with TFIIIA (lane -) resulted in substantial reductions in the intensities of the bands at positions of 33, 35 and 50 kDa. Preimmune IgG (lane with encircled -) did not react to these bands, either. Thus these three proteins are considered to be immunologically cross-reactive to anti-TFIIIA antibody. In addition, as described below, we found a 39 kDa band specific for anti-TFIIIA antibody in other preparations of the kidney extract (Figs. 4 and 5). This band was barely visible in the extract shown in Fig. 3.

In stomach extract, four bands specific for anti-TFIIIA antibody were present (Fig. 3, lanes under S). The molecular masses of these proteins were 33, 35, 39, and 47 kDa. Examination of a few other blots indicated that the 39 kDa protein in fact migrated a little slower than 38 kDa TFIIIA (data not shown). Likewise, liver extract displayed two specific bands (35 and 50 kDa; see lanes under L) as well as a very faint band at about 47 kDa which also seemed to be specific for this antibody. This latter band was more clearly seen in another preparation of extract (Fig. 4B), and was referred to as the 46 kDa band, because it migrated slightly faster than the stomach 47 kDa band. A weak 39 kDa band was also detected in some preparations of the liver extract (Figs. 4B and 5). Thus, in each tissue, we detected several cross-reactive polypeptides ranging from 33 to 56 kDa in size by subjecting the same amount of total protein (100 μ g) to the analysis. Two of them may be tissue- or organ-specific, i.e., 47 kDa and 56 kDa proteins in stomach and ovary respectively. None of the cross-reactive polypeptides was found to comigrate with authentic TFIIIA. However, a protein (39 kDa) migrating a little behind authentic TFIIIA was present

Figure 5. Comparison of TFIIIA-related polypeptides in male and female frog tissues. Whole cell extracts (100 μ g protein) of kidney (K), stomach (S), liver (L), and testis (T) obtained from female (lanes F) and male (lanes M) frogs were examined by immunoblotting. Immune IgG absorbed with BSA (panel A) was used as a probe. The numbers on the right indicate the positions of the bands described in the text.

in stomach (Fig. 3) and less reproducibly in liver and kidney (Figs. 4 and 5). This protein may correspond to TFIIIA' described by others (see Discussion).

High salt extraction of the TFIIIA-related proteins

Since transcription factors are likely to be present in cells in high salt-extractable forms rather than in insoluble ones like the membrane or nuclear matrix proteins, we asked which cross-reactive polypeptides were extractable with 2 M NaCl but without the use of detergents. This study would also be important when one purifies these proteins for further characterization. Tissue homogenates were extracted with a 2 M NaCl buffer, and the remaining insoluble material was further extracted with an SDS-containing buffer (see Materials and Methods). These two fractions, i.e., 2 M NaCl-soluble and -insoluble fractions, were then subjected to immunoblot analysis (Fig 4). As seen in Fig. 4A, most of the TFIIIA-related proteins detected in the whole cell extracts were also present in the high salt extract. The distribution of the cross-reactive proteins into the two fractions was quantitatively assessed in Figs. 4A and B. It should be mentioned that the band intensities in these two figures cannot be compared directly, because the antibody reactions in these

Table 1. Immunologically related polypeptides to TFIIIA in frog tissue extracts.

Tissue	Molecular Mass (kDa) ^a		
	Group I	Group II	Group III
Kidney	50	(39) ^c	(35) ^c , 33
Stomach	47	39	35, 33
Liver	50, (46) ^{b,c}	(39) ^{b,c}	35
Testis	46/45	39	30
Ovary	(56) ^c	38	—

^a Molecular masses were estimated based on their mobilities on 10% SDS-PAGE.

^b These polypeptides were mainly found in the high salt-insoluble fraction (see text).

^c These proteins were barely visible or not detected in some extracts (see text).

two figures were carried out under slightly different conditions. In addition, the material applied in the gel was not proportional to the total amount of proteins recovered in each fraction. We estimated after corrections of such differences that about 50% of each protein was present in the high salt extract. Exceptions were found in the case of liver proteins: While the insoluble fraction contained a 46 kDa band (Fig. 4B, lane L), this band was barely visible in the soluble fraction (Figs. 4A, lane L). It was specific for anti-TFIIIA antibody based on the absorption test (data not shown). Similarly, a 39 kDa band, which was present in some preparations of the liver extract (Figs. 4B and 5), was seen only in the insoluble fraction (compare lanes under L in Fig. 4A and B). In this respect, the 39 kDa protein in liver differs from those present in kidney and stomach.

The quantitative variations from extract to extract, as seen with the 39 and 46 kDa proteins in liver, were also observed in kidney: Although the 35 kDa protein was a major band in the kidney extract shown in Fig. 3, only a very faint band was detected in the extract depicted in Figs. 4A. Instead, a 39 kDa protein, which was a minor one in Fig. 3, became a major protein in Figs. 4A and B (also see Fig. 5). Such variations could in part be a result of proteolytic degradation possibly occurring in the process of cell homogenization and extraction.

Male and female differences in TFIIIA-related proteins

Since authentic TFIIIA is present exclusively in the ovaries of female frogs, TFIIIA-related polypeptides may also vary in expression in male and female. We prepared whole cell extracts from male and female tissues, and compared them by immunoblotting (Fig. 5). The same amount of total protein (100 μ g) was used in each case. Most of the TFIIIA-related proteins found in the female tissues were also present in the male counterparts, although the relative amount of each polypeptide varied. However three of the proteins seemed to display sexual differences. A 46 kDa protein present in the female liver was not detectable in the male (see the bands indicated by the numbers on the right side of the figure). Instead, the liver of male contained an elevated level of a 39 kDa protein which was faint (Fig. 4B and 5) or even undetectable (Fig. 3) in the female liver. Testis appeared to have male-specific proteins; a band running slightly ahead of the liver 46 kDa protein and thus referred to as 45/46 in this figure was present only in this organ. In addition, a 30 kDa protein, which was the smallest polypeptide we found in the present studies, was also testis-specific. The absorption test confirmed that these proteins reacted specifically to anti-TFIIIA antibody (data not shown).

Table 1 summarizes the observations we have described so far. The TFIIIA-related polypeptides we found can be classified into three size groups; group I includes larger polypeptides (46–56 kDa), group II refers to authentic TFIIIA or the polypeptides slightly larger (39 kDa) than this, and group III corresponds to smaller polypeptides (30–35 kDa).

Structural analysis of TFIIIA-related polypeptides present in frog tissues

It is possible that the higher molecular weight group I polypeptides are produced from the TFIIIA gene by post-transcriptional events such as alternative splicing or protein modification. On the other hand, the lower molecular weight group III polypeptides could simply be the degradation products of authentic TFIIIA. To examine the possible relationships among the three groups of polypeptides and authentic TFIIIA, we characterized three representative polypeptides by proteolytic peptide mapping. To begin with, TFIIIA, which was purified and isolated as a single band by SDS-PAGE, was digested with various amounts of *Staph. aureus* protease V8 or chymotrypsin. Fig. 6 shows an immunoblot of the digests probed with immune IgG. Protease V8 generated four immunoreactive fragments

Figure 6. Proteolytic peptide mapping of TFIIIA. Purified TFIIIA was further separated by SDS-PAGE, and the protein was eluted from the TFIIIA band. A portion of the material containing 63 ng TFIIIA was digested with various amounts of chymotrypsin or *Staph.* protease V8 as described in Materials and Methods. The digests were subjected to immunoblotting using immune IgG as a probe.

about 22, 17.5, 16 and 10 kDa in size. An additional minor band of about 36 kDa is due to a nonspecific reaction to the large amount of protease V8 or its contaminants present in this lane. Chymotrypsin converted TFIIIA into four fragments (28, 15.5, 14 and 13 kDa).

As representatives of the three size groups, we chose the 50, 39, and 35 kDa proteins from kidney, stomach, and liver, respectively, and characterized them by the same proteolytic peptide mapping (Fig. 7). To compare the antigenicity of the resulting peptides with that of the TFIIIA-originated peptides, we adjusted the quantities of the crossreactive proteins applied in the gel so that they could give more or less equivalent intensities of bands upon Western blotting (see the lanes of 0 µg/ml proteases in Figs. 6 and 7). Increasing amounts of protease V8 almost completely digested the kidney 50 kDa protein, but only a very faint band of about 20 kDa was detected in an original autoradiograph of the immunoblot. Chymotrypsin converted the same protein into 43 and 33 kDa fragments. All these fragments differed in size from those of authentic TFIIIA. It is also evident that the immunoreactivities of the fragments from the 50 kDa protein were much reduced when compared to those of authentic TFIIIA, although the total reactivities of the starting materials were adjusted to be almost equivalent (see Discussion). These observations support the possibility that the 50 kDa protein and TFIIIA are products of two different genes despite their immunological cross-reactivities.

Proteolytic peptides of the stomach 39 kDa protein also displayed a distinct pattern from that of authentic TFIIIA (Fig. 7, lanes under 39 kDa). Two immunoreactive bands (25 and 22.5 kDa) were detected in the V8 digest, whereas three bands (29, 23 and 20.5 kDa) were seen in the chymotrypsin digest. Like the kidney 50 kDa protein described above, the immunoreactivities of the fragments were much weaker than those of the TFIIIA fragments. It should be pointed out that the stomach 39 kDa protein reported here is quite similar in size to TFIIIA' which is known to be present in various types of somatic cells (see Introduction). However, the ones isolated from *Xenopus* embryos and tissue culture cells have been reported to exhibit a very similar peptide mapping pattern to that of authentic

Figure 7. Proteolytic peptide mapping of TFIIIA-related proteins of frog tissues. TFIIIA-related polypeptides of 50 kDa (2.5 μ g) from kidney, 39 kDa (3 μ g) from stomach and 35 kDa (1 μ g) from liver were eluted from the corresponding area of the gel after electrophoresis, and were digested with indicated amounts of chymotrypsin or *Staph.* protease V8. The digests were analyzed by immunoblotting as Fig. 6.

TFIIIA (17). Therefore, they may be different from the protein reported here (see Discussion).

The liver 35 kDa protein (Fig. 7, lanes under 35 kd) generated no cross-reactive fragments by V8 digestion, while a faint band of about 24 kDa was produced by chymotrypsin. The 34 kDa band seen in both of the digests is a partial degradation product already present in the undigested material. In addition, we did not detect any cross-reactive fragments in the CNBr digest of this protein (data not shown). These digestion patterns do not bear a resemblance to those of TFIIIA, the kidney 50 kDa protein or the stomach 39 kDa protein. Thus the liver 35 kDa protein seems to be another distinct, cross-reactive polypeptide.

In summary, we have characterized three of the TFIIIA-related polypeptides. They neither share common proteolytic peptides with authentic TFIIIA nor seem to be mutually related. Thus, despite their antigenic cross-reactivities, these three proteins and TFIIIA are likely to be the products of four different genes.

DISCUSSION

In the present studies, we employed a polyclonal anti-TFIIIA antibody raised against purified TFIIIA. Although we did not use affinity-purified antibody, proteins reacting to other antibodies in the immune IgG were eliminated by an absorption test where antibodies were preabsorbed with purified TFIIIA. Only proteins which lost the reactivity after preabsorption were considered to be specific for anti-TFIIIA antibody. The validity of our assay depends upon the purity of the TFIIIA band used for immunization as well as for preabsorption. We have not carried out two-dimensional gel analysis in order to examine whether the

single band of TFIIIA in SDS-PAGE really represents a single spot in the two-dimensional gel. However, this preparation of TFIIIA was isolated through standard purification steps including ion exchange columns before being subjected to SDS-PAGE. Therefore, we presume that the band of TFIIIA did not contain significant quantities of other proteins differing in their isoelectric points. Furthermore, the fact that CNBr cleavage of purified TFIIIA produced only the expected fragments, which were detected both by silver staining and by anti-TFIIIA antibody, strongly indicated that the possible contamination in the TFIIIA band was physically as well as antigenically negligible (Figs. 2A and 2B). Further proof must await cDNA cloning and sequence analysis of these proteins. A puzzling characteristic of our antibody was a sudden loss of its cross-reactivity when proteins were partially digested (Fig. 7). Our hypothesis is that the majority, if not all, of the antibodies in our preparation might simultaneously recognize multiple domains within the protein rather than very local amino acid sequences. Separation of such domains by digestion would cause a loss of antigenicity.

Using such an antibody, we found that adult tissues contain several cross-reactive polypeptides of 30–56 kDa. Most of them differ in size from the one known as TFIIIA', and exist in high-salt extractable forms like many of the DNA binding proteins. Their behavior in proteolytic digestion indicated that at least three of these polypeptides are not only different from one another, but also distinct from authentic TFIIIA. We suggest that they are products of different genes. One of the crossreactive proteins was 39 kDa, which was a little slower in mobility than authentic TFIIIA. This might correspond to TFIIIA' observed in *Xenopus* adult liver by Darby *et al.* (18), although they did not present any data on peptide mapping. On the other hand, TFIIIA' reported by Shastry *et al.* (17) has a very similar peptide mapping pattern to that of authentic TFIIIA, and therefore seems to be distinct from the 39 kDa protein described here. This latter TFIIIA' was derived from embryos and tissue culture cells (17), which might express some proteins characteristic of dedifferentiated cells, but not seen in adult tissues.

What is the nature of these proteins antigenically related to TFIIIA? The antibody we used recognizes all three domains of TFIIIA defined by CNBr cleavage (Fig. 2B). However the reactivities of the smaller fragments originating from the N- and C- termini far exceed that of the larger fragment representing the central portion of TFIIIA. It has been proposed that the domains near either terminus are crucial for the activity of TFIIIA; the N-terminal fragment carrying the first three units of zinc-fingers is thought to be the tightest binding domain to the 5S RNA gene (37,38), whereas the C-terminal tail interacts with other factors and RNA polymerase III (31,37). It is conceivable that these two domains might well be conserved in the TFIIIA-like transcription factors if present, and thus our antibody would be able to detect them. Alternatively, a small population of our antibody could be directed to a local sequence such as the six amino acid element HTGEKP, which joins the first three fingers near the N-terminus of TFIIIA. Recently a family of zinc-finger proteins has been identified in *Xenopus* (39,40). It comprises at least 14 different members each carrying multiple fingers with a highly conserved HTGEKP sequence between two units (40). Hence, the polypeptides we have described may also belong to this family. However, these proteins seem to be distinct DNA binding proteins not related to the 5S RNA gene transcription since their amino acid sequences deduced from cDNA exhibited only 40–50% homology to TFIIIA (40). Despite these speculations, we do not even know at present whether the proteins we detected can bind to DNA, or more specifically to 5S RNA gene.

Such functional studies in combination with cDNA cloning and sequence analysis would provide definite information on the nature of these proteins reported here.

ACKNOWLEDGEMENT

We thank Joel Gottesfeld and Eiman Tominna for useful comments on this manuscript. This work was supported by National Institute of Health grant GM38075. This is publication number 5806-BCR from Scripps Clinic and Research Foundation.

*To whom correspondence should be addressed

REFERENCES

1. Segall, J., Matsui, T. and Roeder, R.G. (1980) *J. Biol. Chem.* **255**, 11986–11991.
2. Shastri, B.S., Ng, S.Y. and Roeder, R.G. (1982) *J. Biol. Chem.* **257**, 12979–12986.
3. Lassar, A.B., Martin, P.L. and Roeder, R.G. (1983) *Science* **222**, 740–748.
4. Bogenhagen, D.F., Sakonju, S. and Brown, D.D. (1980) *Cell* **19**, 27–35.
5. Sakonju, S., Bogenhagen, D.F. and Brown, D.D. (1980) *Cell* **19**, 13–25.
6. Engelke, D.R., Ng, S.Y., Shastri, B.S. and Roeder, R.G. (1980) *Cell* **19**, 717–728.
7. Bieker, J.J. and Roeder, R.G. (1984) *J. Biol. Chem.* **259**, 6158–6164.
8. Honda, B.M. and Roeder, R.G. (1980) *Cell* **22**, 119–126.
9. Pelham, H. and Brown, D.D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4170–4174.
10. Hanas, J.S., Hazuda, D.J., Bogenhagen, D.F., Wu, F.Y.H. and Wu, C.W. (1983) *J. Biol. Chem.* **258**, 14120–14125.
11. Miller, J., McLachlan, A.D. and Klug, A. (1985) *EMBO J.* **4**, 1609–1614.
12. Berg, J.M. (1986) *Science* **232**, 485–487.
13. Evans, R.M. and Hollenberg, S.M. (1988) *Cell* **52**, 1–3.
14. Brown, D.D. (1982) *Harvey Lect.* **76**, 27–39.
15. Folk, W. R. (1988) *Genes Dev.* **2**, 373–375.
16. Pelham, H.R.B., Wormington, W.M. and Brown, D.D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1760–1764.
17. Shastri, B.S., Honda, B.M. and Roeder, R.G., (1986) *J. Biol. Chem.* **259**, 11373–11382.
18. Darby, M.K., Andrews, M.T. and Brown, D.D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5516–5520.
19. Ginsberg, A.M., King, B.O. and Roeder, R.G. (1984) *Cell* **39**, 479–489.
20. Tso, J.Y., Van Den Berg, D.J. and Korn, L.J. (1986) *Nucleic Acids Res.* **14**, 2187–2200.
21. Taylor, W., Jackson, I.J., Siegel, N., Kumar, A. and Brown D.D. (1986) *Nucleic Acids Res.* **15**, 6185–6195.
22. Dorn, A., Bollekens, J., Staub, A., Benoist, C. and Mathis, D. (1987) *Cell* **50**, 863–872.
23. Chodosh, L.A., Baldwin, A.S., Carthew, R.W. and Sharp, P.A. (1988) *Cell* **53**, 11–24.
24. Santoro, C., Mermod, N., Andrews, P.C. and Tjian, R. (1988) *Nature* **334**, 218–224.
25. Landschulz, W.H., Johnson, P.F., Adashi, E.Y., Graves, B.J. and McKnight S.L. (1988) *Genes Dev.* **2**, 786–800.
26. Hai, T., Liu, F., Allegretto, E.A., Karin, M. and Green, M.R. (1988) *Genes Dev.* **2**, 1216–1226.
27. Wang, C.K. and Weil, P.A. (1989) *J. Biol. Chem.* **264**, 1092–1099.
28. Seifart, K.H., Wang, L., Waldschmidt, R., Jahn, D., and Wingerder, E. (1989) *J. Biol. Chem.* **264**, 1702–1709.
29. Dumont, J.N. (1972) *J. Morph.* **136**, 153–180.
30. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall R.J. (1951) *J. Biol. Chem.* **193**, 265–275.
31. Smith, D.R., Jackson, I.J. and Brown, D.D. (1984) *Cell* **37**, 645–652.
32. Ryoji, M., Karpen, J.W. and Kaji, A. (1981) *J. Biol. Chem.* **256**, 5798–5801.
33. Laemmli, U.K. (1970) *Nature* **227**, 680–685.
34. Tobin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
35. Hunkapiller, M.W., Lujan, E., Ostrander, F. and Hood, L.E. (1983) *Methods Enzymol.* **91**, 227–238.
36. Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) *J. Biol. Chem.* **252**, 1102–1106.
37. Vrana, K.E., Churchill, M.E.A., Tullius, T.D. and Brown, D.D. (1988) *Mol. Cell. Biol.* **8**, 1684–1696.

38. Fiser-Littell, R.M., Duke, A.L., Yanchick, J.S., and Hanas, J.S. (1988) *J. Biol. Chem.* **263**, 1607–1610.
39. Ruiz i Altaba, A., Perry-O'Keefe, H. and Melton, D.A. (1987) *EMBO J.* **6**, 3065–3070.
40. Köster, M., Pieler, T., Pötting, A., and Knöchel, W. (1988) *EMBO J.* **7**, 1735–1741.

**This article, submitted on disc, has been automatically
converted into this typeset format by the publisher.**