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# XrpFI, an Amphibian Transcription Factor Composed of Multiple Polypeptides Immunologically Related to the GA-Binding Protein α and β Subunits, Is Differentially Expressed during *Xenopus laevis* Development

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**XrpFI, first identified in the extract of *Xenopus laevis* oocyte nuclei, binds to a proximal sequence of the L14 ribosomal protein gene promoter. Its target sequence, 5′-TAACCGGAAGTTTGT-3′, is required to fully activate the promoter, and the two G's of the central motif are essential for factor binding and transcriptional activation; our data also suggest that XrpFI may play a role in cap site positioning. The binding site of XrpFI is homologous to the sequence recognized by the family of *ets* genes. Antibodies specific for Ets-1 and Ets-2 proteins did not react with XrpFI, but those raised against the rat α and β GA-binding proteins both supershifted the retarded bands formed by XrpFI. The *Xenopus* polypeptides related to GA-binding protein α interact with DNA both as monomers and as heterodimers associated with β-related proteins. Oocyte nuclei contain multiple forms of α- and β-related proteins: the α-like proteins remain throughout development, while the pattern of the β species changes in the embryonic stages examined. β-like proteins are undetectable in the cleavage period up to the neurula stage, but at later stages, when ribosomal protein genes are actively transcribed, two β-related polypeptides reappear.**

Control of eukaryotic mRNA transcription is governed by *cis*-acting elements and *trans*-acting factors (14, 27, 28, 30, 35). Initiation of transcription at defined points depends on the presence of the TATA box, bound by the factor TFIID (45). Multiple initiation sites usually characterize promoters lacking discernible TATA elements (13, 38). Promoters of ribosomal protein (rp) genes, which contain noncanonical TATA boxes, initiate transcription with precision; the major cap site is usually found within a run of consecutive pyrimidines (3). Additional elements, besides the TATA elements, might contribute to positioning and stabilization of preinitiation complexes (18). In rp genes, functional analysis demonstrated the clustering of activator elements within a few hundred base pairs around the cap site (10, 17).

Our previous results, obtained with 5′-deletion mutants of the L14 rp gene promoter, showed that efficiency of transcription depended on the presence of two upstream elements: a distal element at position −95 bound by an Sp1-like factor and a proximal sequence at position −53 (10). XrpFI is the factor which interacts with the proximal promoter element whose core is 5′-CTTCC-3′ (10). Site-specific mutation of the core sequence to -CTTAA- abolished binding of XrpFI and reduced expression of a linked reporter gene, CAT (chloramphenicol acetyltransferase [23]). Another DNA-binding activity, named XrpFII, binds to a sequence downstream of the TATA-like box. However, so far no specific role has been assigned to this activity because mutation of the XrpFII target site did not decrease the L14 promoter efficiency or affect the specificity of the transcription initiation sites.

The XrpFI DNA-binding specificity and functional role have been conserved during evolution; homologous factors in mouse (β factor) (17) and HeLa (HrpF) (23) cells have been identified. DNA sequences homologous to the XrpFI target site are present in the promoter of mouse (L30 and L32) and human (S14 and S17) rp genes (23). Again, deletion of these sequences decreased promoter efficiency (17). On the basis of these observations, we proposed a role for XrpFI, HrpF, and the mouse β factor as positive regulators of rp gene transcription (23).

Recently, a DNA-binding domain that recognizes a purine-rich core DNA sequence has been described (20, 33). This domain, named the *ets* domain, is common to all members of the *ets* proto-oncogene family and is highly conserved through evolution. Chicken *ets*-1 is the progenitor of a viral oncogene, v-*ets*, whose product is expressed as a fusion protein with v-Myb and Gag by the avian leukemia virus E26 (26, 32). Other members of the same family are *ets*-2 (5), *erg* (37), *elk*-1 (36), PEA3 (50), PU.1 (21), E74 (43), *fli*-1 (4), *elf*-1 (41), *yan* (24), and GA-binding protein α (GABPα) (25, 42). The products of *ets* genes have been implicated in regulation of transcription, cell transformation, and development (15, 24). Some *ets* genes show a very restricted pattern of expression (*ets*-1 [5], *erg* [37], *PU.1* [21], and *PEA3* [50]), and others are expressed in many cell types, such as *ets*-2 (5) and GABPα (25). Potential Ets binding sites are found in the promoters of genes involved in the early response to growth stimuli (16, 46) and genes that are highly expressed in transformed cells (47), but most of the target genes for the Ets regulators are still unknown. The tissue distribution of known *ets* domain-expressed proteins suggests that they could regulate the expression of tissue-specific genes (21, 36, 40, 41, 50). On the other hand, genes involved in basic cellular functions could be regulated by ubiquitous Ets proteins.

The DNA sequences bound by XrpFI and by members of

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the *ets* family are homologous; moreover, mutation of the same G residues in the 5′-GGAA-3′ core recognition sequence abolished binding and decreased efficiency of transcription in promoters regulated by XrpFI (23) or *ets*-expressed factors (33). These observations suggest that XrpFI belongs to the *ets* family. The members of the *ets* family *ets*-1 and *ets*-2, whose cDNAs were isolated from oocyte libraries (9, 40), were studied with *Xenopus laevis*. *ets*-1 and *ets*-2 gene transcripts were detected in the poly(A)$^+$ RNAs from the early stages of oogenesis to the late stages of embryogenesis, but only *ets*-2 transcripts were found in a variety of adult tissues (49). The *ets*-2 maternal gene appears to be required for the meiotic maturation of *Xenopus* oocytes (11).

For this paper, we extended the study of the DNA-binding activity of XrpFI to various stages of development. We also characterized the XrpFI protein components by biochemical and immunological assays. We found that the DNA-binding activity of oocyte XrpFI is composed of several polypeptides immunologically related to GABPα and -β subunits but not to Ets-1 or Ets-2. While the α polypeptides, which contact the DNA, are present in all developmental stages analyzed, we found significant differences in the patterns of the β polypeptides.

## MATERIALS AND METHODS

**Biological material.** Adult ovaries were manually dissected in Barth's solution and oocytes were staged by hand under the microscope according to the method of Dumont (12). Embryos, a generous gift from Paola Pierandrei-Amaldi of Consiglio Nazionale delle Richerche Cellular Biology Laboratory of Rome, were obtained as described by Brown and Littna (7) and were grown at 22°C in dechlorinated water and staged as described by Nieuwkoop and Faber (31). Embryos were collected at stages 2, 4, 6, 15, 27, and 40. Lung, heart, and whole ovaries were removed from anesthetized animals and washed several times with cold phosphate buffer containing heparin to eliminate blood. Nuclei were prepared from B3.2 cultured kidney cells, provided by Fabrizio Loreni, Department of Biology, University of Tor Vergata, Rome, Italy. A total of $3 \times 10^7$ cells were washed with phosphate-buffered saline and were resuspended in 1 ml of lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.4], 10 mM KCl, 1.5 mM Mg acetate, and 1% Nonidet P-40). After incubation on ice for 4 min, the lysate was centrifuged at 1,500 × *g* at 4°C for 10 min. The nuclear pellet was resuspended in 100 μl of J buffer (10). Germinal vesicles were manually isolated from oocytes of different stages in J buffer (10).

**Site-directed mutagenesis.** The −166 ptCAT clone (23) was mutated in the XrpFI binding site by changing the core CTTCC into CTTAA or CTTGG, with the double-stranded plasmid method of Inouye and Inouye (19). Mutations were controlled by sequencing with a U.S. Biochemicals Sequenase kit.

**Injection into oocytes and analysis of transcripts.** Wild-type and mutated −166 ptCAT clones were injected into *Xenopus* oocyte nuclei (20 nl per oocyte, 200 ng/μl) and were incubated for 5 h at 19°C; the CAT assay was performed with 30 μl (corresponding to 3 oocytes) of the supernatant of the homogenate of a pool of 30 to 50 oocytes, which had been centrifuged for 15 min at 13,000 rpm in a microcentrifuge at 4°C (10). The experiment was repeated with different oocyte batches, and a control plasmid was included to normalize quantitation (10). Primer extension was made with the total RNA corresponding to two oocytes exactly according to the protocol described in reference 1. The primer was a 33-mer oligonucleotide complementary to the CAT gene (10).

**Preparation of extracts.** Nuclear extract was prepared from oocyte germinal vesicles or cultured cell nuclei as previously described (10). Whole-cell S-100 extract was obtained from whole ovaries or from oocytes isolated by collagenase treatment according to the procedure described by Scotto et al. (39). S-100 extracts were obtained from staged embryos and from somatic tissues by the same procedure, with the exception that the homogenization buffer contained aprotinin and benzamidine from Boehringer in addition to phenylmethylsulfonyl fluoride. Protein concentration was determined with the Bio-Rad microassay (catalog no. 500-0006).

**DNA-protein binding and EMSA.** For DNA-protein binding assays and the electrophoretic mobility shift assay (EMSA), 5 to 10 μg of nuclear protein or 20 to 50 μg of whole-cell protein was preincubated with 0.5 to 2.0 μg of poly(dI-dC) (Boehringer) on ice in binding buffer (10). After 10 min, 0.2 to 1.0 ng of the double-stranded oligonucleotide B (5′-AGCTTACCACAAACTTCCGGTTATCAGGTGTTC CCA-3′) spanning the L14 rp gene promoter region from positions −61 to −32 (10), flanked by underlined *Hin*dIII ends, was added and the binding mixture was incubated for a further 20 min on ice. When used, antibodies were added to the protein extract and incubated for 30 min at room temperature before the addition of poly(dI-dC) and probe. After binding, the samples were assayed for complex formation by electrophoresis through a native 4.5 or 5% polyacrylamide gel in 0.25× TBE (Tris-borate-EDTA buffer). In the competition experiments, unlabelled specific competitors were included in the binding reaction. Double-stranded oligonucleotide competitors were 5′-TCGGGATAACCGGAAGTT TGTGC-3′ (oligonucleotide X), carrying the XrpFI binding site, and the nonspecific competitor 5′-AAGTTTATCATT TCACTGC-3′ (oligonucleotide A).

**Methylation interference analysis.** The L14 gene fragment (positions −63 to +12) containing the XrpFI target sequence and mutated in the XrpFII target site in order to prevent interference by another DNA-binding activity was end labeled with [α-$^{32}$P]dATP and Klenow enzyme. The radiolabeled DNA was partially methylated with dimethyl sulfate (29) before being added to the binding reaction mixtures. DNA ($3 \times 10^5$ cpm) was incubated in 40 μl of reaction mixture with 9 μg of poly(dI-dC) and 54 μg of oocyte nuclear protein as described for the EMSA. Bands corresponding to bound and free DNA were excised from the gel, and the DNA was eluted, purified on a prepacked ion-exchange resin minicolumn (NACS Prepac; Bethesda Research Laboratories), cleaved with piperidine, and electrophoresed through a 20% acrylamide–urea denaturing gel in TBE.

**Chromatographic procedures.** The chromatographic procedures were performed as described in reference 6. Briefly, the *Xenopus* oocyte extract diluted to 0.1 M KCl was applied to a column of heparin-agarose (Sigma [1 ml of resin for 10 mg of protein]) equilibrated with F buffer (20 mM HEPES-KOH [pH 7.9], 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride containing 0.1 M KCl). The column was washed with 5 column volumes of 0.1 M KCl in F buffer, and proteins were eluted sequentially with 2 column volumes of F buffer and 0.2, 0.35, and 1 M KCl. Individual fractions were assayed for XrpFI binding activity, and the active fractions were pooled, diluted to 0.1 M, and reapplied to the heparin-agarose column. Stepwise

elution was performed with 0.15, 0.2, 0.3, and 1 M KCl in F buffer.

**Molecular mass estimation of the XrpFI components.** One hundred to 250 μg of protein partially purified by heparin-agarose was precipitated by the addition of 4 volumes of acetone for 2 h at −20°C. Pellets were dissolved in 8 M urea–sodium dodecyl sulfate (SDS) sample buffer and were loaded onto an SDS-polyacrylamide gel (10% polyacrylamide) (22). After electrophoresis, the gel was sliced and the proteins were eluted and processed as described by Baeuerle (2). XrpFI DNA-binding activity was localized by EMSA.

**Antibodies.** Rabbit antibodies raised against GABPs were a generous gift from F. de la Brousse and S. L. McKnight. Affinity-purified c-Ets-1 and c-Ets-2 antibodies raised in sheep against synthetic 17-residue peptides from the central part of human c-Ets-1 and from the C terminus of human c-Ets-2, respectively, were purchased from Cambridge Research Biochemicals and AMS-Raggio-Italgene. A polyclonal antiserum (raised in mice) against a recombinant 30-kDa *Xenopus* Ets-1 protein was also used.

**Immunodepletion.** Nine micrograms of partially purified oocyte nuclear protein was incubated with antibodies for 30 min at room temperature. Protein A-Sepharose CL-4B (Pharmacia) swollen in IPP buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1% Nonidet P-40, 0.02% Na azide) was added to the protein-antibody reaction mixture to a final concentration of 0.25 mg/ml and was incubated for 2 h at 4°C. The resin was then pelleted in a microcentrifuge, and the supernatant proteins were recovered for further characterization.

**Immunoblotting.** Proteins from *Xenopus* oocytes or embryos were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE [10% polyacrylamide]) and electrotransferred to Schleicher and Schuell nitrocellulose membranes. The filters were blocked, incubated at room temperature for 2 h with rabbit antibodies (diluted to 1:1,000) under gentle agitation, and developed by the protocol supplied by the blotting alkaline phosphatase rabbit immunoglobulin C ABC detection kit (Vectastain).

**UV cross-linking.** Crude or partially purified oocyte nuclear protein was incubated with a uniformly bromodeoxyuridine (BrdU)-substituted double-stranded oligonucleotide, 5′-TTGATAACCGGAAGTTTG-3′, in the presence or absence of competitor DNAs. After incubation, binding reaction mixtures were irradiated for 30 s at a distance of 5 cm with UV light (254 nm) in a Spectrolinker X-1000 on ice. The UV cross-linked DNA-protein complexes were then subjected to SDS-PAGE or EMSA. After native gel electrophoresis, the DNA-protein complexes were excised from the gel, electroeluted, precipitated with 4 volumes of acetone, and analyzed by SDS-PAGE.

## RESULTS

**Role of XrpFI in positioning transcripts.** Previous analysis of the elements involved in the control of L14 rp gene transcription showed that about 40 to 50% of promoter efficiency depends on the presence of an element, bound by XrpFI, located −53 from the major transcription start site (10). In order to define the role of this factor, two different mutants of the XrpFI binding site were constructed: AA, in which CTTCC was changed into CTTAA, and GG, with CTTCC changed into CTTGG (Fig. 1a). The mutated and wild-type −166 to +12 L14 promoter regions, cloned upstream of a reporter CAT gene, were injected into the nuclei of stage VI *Xenopus* oocytes. The transcriptional activation

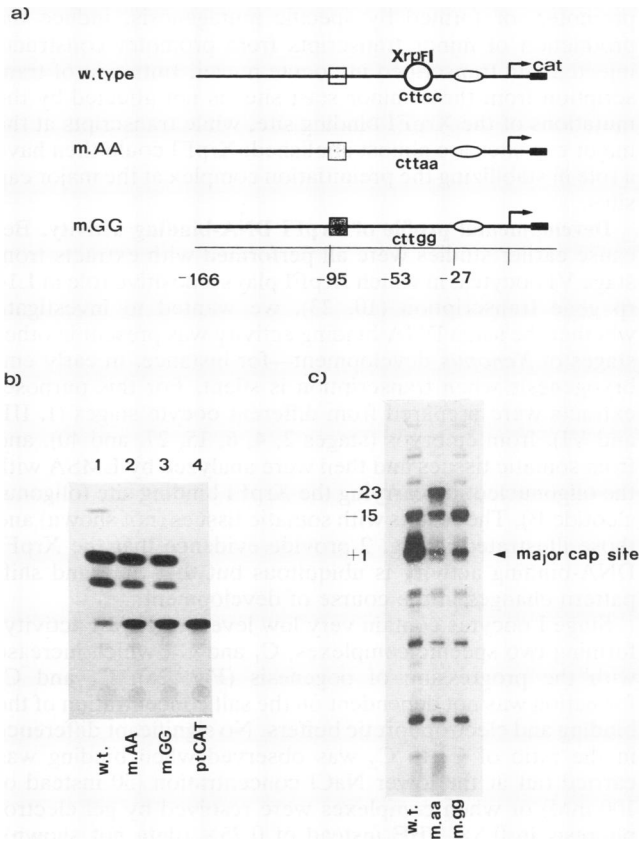


FIG. 1. Role of XrpFI in transcription. (a) The L14 promoter region from positions −166 to +12 with Sp1 (■), XrpFI (○), and XrpFII (◯) binding sites. CTTCC mutated into CTTAA (m.AA) or CTTGG (m.GG) is not recognized by XrpFI (data not shown). w. type, wild type. (b) CAT assay after injection of wild-type (w.t. [lane 1]) or mutated −166 ptCAT (m.AA and m.GG [lanes 2 and 3]) into oocyte nuclei. Lane 4, vector plasmid. A protein extract corresponding to three oocytes, incubated at 19°C for 5 h, acetylated 0.125 μCi of [$^{14}$C]chloramphenicol up to 97% (lane 1), 50% (lane 2), 42% (lane 3), and 1% (lane 4), according to Betascope 603 (Betagen) counting of the spots. (c) Initiation of RNA transcripts detected by primer extension of a CAT-specific primer hybridized to total RNA corresponding to two oocytes injected with wild-type or mutated −166 ptCAT.

of the reporter gene was monitored with the enzymatic CAT assay and analysis of CAT RNA transcripts. In both mutants, which did not bind XrpFI anymore, as seen with a band shift assay, CAT activity was reduced to about 50% (Fig. 1b) (23). Primer extension analysis of the transcripts directed by the mutants showed that the reduction in transcription efficiency was mainly affecting the initiation from the major cap sites (about 70% of the reduction [Fig. 1c]). The major start sites are at two consecutive cytosines in the run of uninterrupted pyrimidines peculiar to the ribosomal protein genes so far analyzed in vertebrates. In the injected CTTAA mutant, a new initiation was created at position −23; the transcripts start 26 bases downstream of the introduced mutation, which is probably recognized as a new TATA-like box. This did not happen with the CTTGG mutant. Other minor transcripts (−15 in Fig. 1c) in wild-type and mutated clones start 28 bases downstream of another TATA-like box, the TTAT sequence at position −46. The results show that TATA-like boxes naturally present in the

promoter, or formed by specific mutagenesis, induce the production of minor transcripts from promoter constructs injected and transcribed in oocyte nuclei. Initiation of transcription from these minor start sites is not affected by the mutations of the XrpFI binding site, while transcripts at the major cap sites are almost abolished: XrpFI could then have a role in stabilizing the preinitiation complex at the major cap sites.

**Developmental profile of XrpFI DNA-binding activity.** Because earlier studies were all performed with extracts from stage VI oocytes, in which XrpFI plays a positive role in L14 rp gene transcription (10, 23), we wanted to investigate whether the same DNA-binding activity was present in other stages of *Xenopus* development—for instance, in early embryogenesis when transcription is silent. For this purpose, extracts were prepared from different oocyte stages (I, III, and VI), from embryos (stages 2, 4, 6, 15, 27, and 40), and from somatic tissues and then were analyzed by EMSA with the oligonucleotide carrying the XrpFI binding site (oligonucleotide B). The results with somatic tissues (not shown) and those illustrated in Fig. 2 provide evidence that the XrpFI DNA-binding activity is ubiquitous but that the band shift pattern changes in the course of development.

Stage I oocytes contain very low levels of XrpFI activity, forming two specific complexes, $C_1$ and $C_2$, which increase with the progression of oogenesis (Fig. 2a). $C_1$ and $C_2$ formation was not dependent on the salt concentration of the binding and electrophoretic buffers. No significant difference in the ratio of $C_1$ to $C_2$ was observed when binding was carried out at the lower NaCl concentration (50 instead of 100 mM) or when complexes were resolved by gel electrophoresis in 0.5× TBE instead of 0.25× (data not shown). Some differences in the $C_1/C_2$ ratio occurred, depending on the batches of the nuclear extract used, because of the presence of contaminating cytoplasmic material. In fact, when EMSA was performed with oocyte whole-cell extract instead of the proteins from isolated nuclei, we observed an increase in the amount of $C_1$ complex and the appearance of two additional bands having electrophoretic mobilities different from those of $C_1$ and $C_2$ (data not shown). To further test this point, we analyzed nuclear and cytoplasmic extracts separately (Fig. 2b). Cytoplasmic extract, prepared from large oocytes (stage VI) after enucleation, produced only one specific complex migrating as $C_1$ and the two bands (indicated ns in panels b and c), which could be inhibited by increasing the amount of poly(dI-dC). The proteins forming the $C_2$ complex, which is composed of at least two retarded species, are then specifically located in the oocyte nucleus.

The $C_1$ complex is constantly present in the course of embryogenesis. It appears as a broad band, presumably composed of more than one component, both in the extract from the ovaries and in the extract from the embryos (Fig. 2c, lanes 1 to 6). Proteins responsible for the formation of the $C_1$ complex decrease up to stage 15, but they again increase from stage 27 to stage 40. The amount of total proteins from stage 15 embryos used in the band shift assay was increased 2.5-fold to obtain a comparable $C_1$ complex.

In the very early embryos (stage 2), the $C_2$ complex is barely visible and another specific complex ($C_3$) of electrophoretic mobility intermediate between $C_1$ and $C_2$ appears (Fig. 2c, lanes 1 and 2). In the subsequent stages (stages 6 and 15), both $C_2$ and $C_3$ disappear (Fig. 2c, lanes 3 and 4), but $C_2$ is again produced by extracts from late embryos at stages 27 and 40 (Fig. 2c, lanes 5 and 6) and from adult tissues (data not shown). The relative amount of the bands forming the $C_2$ complex also varies in the course of *Xenopus* development because the upper band is more abundant in embryos and tissues than in oocytes.

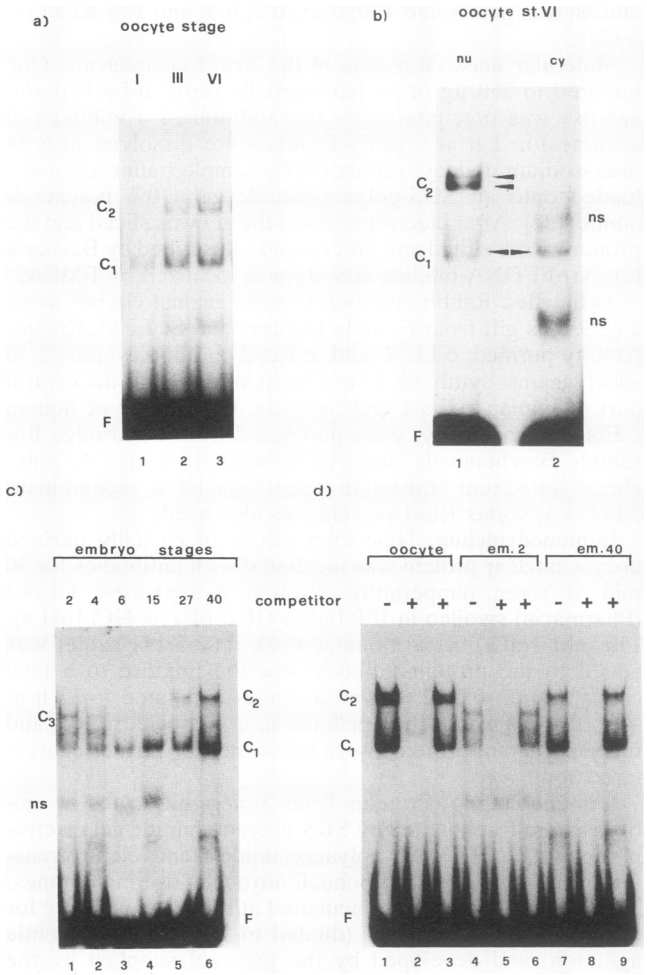


FIG. 2. Binding activity of XrpFI from different sources to the DNA probe (36-mer oligonucleotide B). Complexes were formed in the presence of poly(dI-dC) as nonspecific competitor and were run on a native 4.5% polyacrylamide gel. (a) Nuclear proteins corresponding to one (stages I and III) or a half (stage VI) oocyte. (b) DNA-protein complexes formed by nuclear (lane 1) and cytoplasmic (lane 2) proteins from stage VI oocytes. (c) Binding of whole-cell extract proteins of the indicated embryo stages to the DNA probe (20 μg for each stage and 50 μg for stage 15). (d) Binding by oocyte nuclear (lanes 1 to 3) and whole-cell embryo (em.) proteins (stages 2 and 40, lanes 4 to 6 and 7 to 9, respectively) was inhibited by the addition of poly(dI-dC) with a 500-fold molar excess of specific (lanes 2, 5, and 7) or nonspecific (lanes 3, 6, and 9) double-stranded oligonucleotide. Major and minor components of the $C_2$ complex and the $C_1$ complex are indicated by arrowheads in panel b. −, no competitor added; $C_1$, $C_2$, and $C_3$, specific XrpFI-DNA complexes; F, unbound probe; ns, nonspecific bands.

**Characterization of XrpFI.** The results presented above suggest that XrpFI is a heterogeneous activity constituted by multiple polypeptides. To define the size range of these polypeptides, we fractionated a preparation of the factor, partially purified by two heparin-agarose columns (6), by SDS-PAGE. Proteins were eluted from gel slices, allowed to renature, and then were assayed by EMSA with the DNA probe (Fig. 3a). Only proteins in the 44- to 68-kDa size range were able to bind. These renatured proteins yielded a major

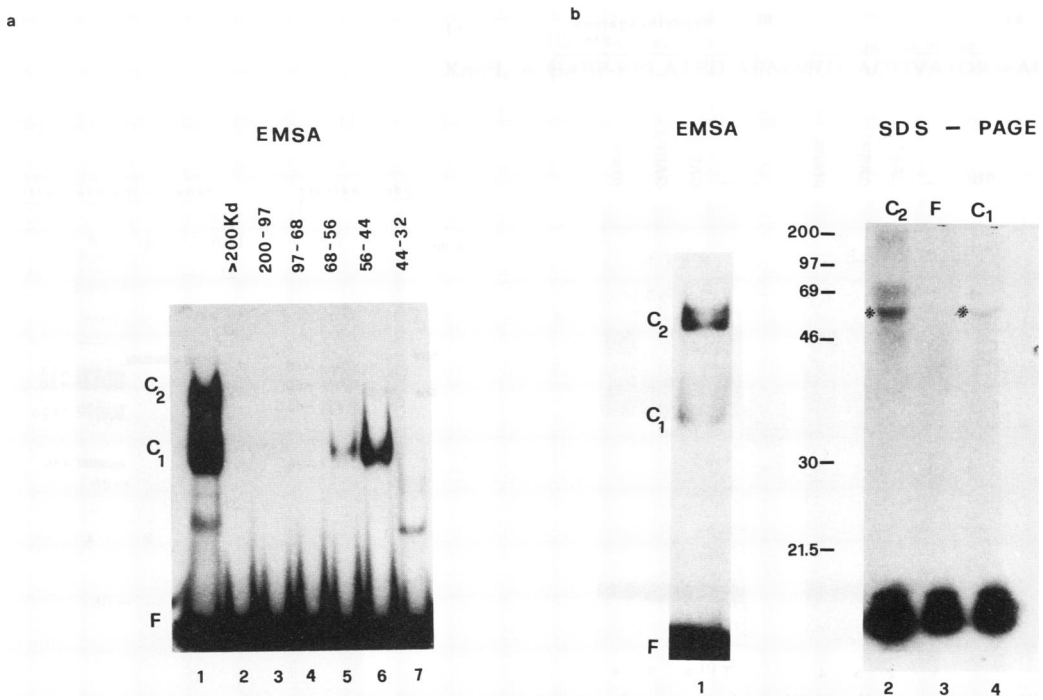


FIG. 3. XrpFI characterization. (a) SDS-PAGE fractionation and renaturation. Fifty micrograms of nuclear protein from heparin-agarose-purified XrpFI was fractionated by SDS-PAGE (10% polyacrylamide). Proteins of the indicated molecular mass range were eluted, renatured, and analyzed by EMSA in the presence of 250 ng of poly(dI-dC). Lane 1, complexes obtained by unfractionated starting protein. The exposure time was 3 days. (b) UV cross-linking. Partially purified XrpFI (9 μg) was bound to the labeled BrdU-substituted probe, exposed to UV light for 30 s on ice, and then run on a 4% native polyacrylamide gel (lane 1). Molecular species present in the gel slices corresponding to $C_2$, F (free probe), and $C_1$ complexes were electroeluted and then analyzed by SDS-PAGE (lanes 2 to 4). Size markers (in kilodaltons) are shown on the left. Asterisks indicate the 60-kDa species common to $C_1$ and $C_2$.

band corresponding to the $C_1$ complex (Fig. 3a, lanes 5 and 6), while $C_2$ was visible only after a very prolonged exposure. The failure to completely reconstitute the slower $C_2$ complex could be due to incomplete renaturation of some polypeptides which lose the ability to make protein-protein interactions. Another possibility is that these polypeptides are not included in the 44- to 68-kDa range; this seems unlikely, because no improvement was obtained by complementing the proteins of the 44- to 68-kDa fractions with those of the other fractions of the same gel (data not shown).

Further analysis was performed by UV cross-linking. A BrdU-substituted oligonucleotide containing the XrpFI target site was incubated with partially purified nuclear oocyte protein. After UV cross-linking, the binding mixture was run on a native gel (Fig. 3b, lane 1). $C_1$ and $C_2$ complexes were excised out of the gel, electroeluted, and rerun by SDS-PAGE (Fig. 3b, lanes 2 to 4). Both yielded a product approximately 60 kDa in size, with the addition, in the sample derived from the $C_2$ complex, of two broad bands with high molecular masses. The results show that, in $C_2$, the same polypeptide as in $C_1$ is cross-linked to DNA but is combined with additional proteins.

**GABP-related polypeptides constitute XrpFI.** mRNAs coding for two highly related *ets* family members, Ets-1 and Ets-2, were identified in *X. laevis* oocytes (9, 11, 40, 49). The consensus Ets binding site (33) is homologous to the sequence contacted by XrpFI, and close contact is established within the same central core GGAAG (10, 33). Taken together, these observations suggest that XrpFI belongs to the *ets* family. To test this hypothesis, we first used Ets-1- and Ets-2-specific antibodies in band shift experiments. Neither of these antibodies had a specific effect on the mobility of the $C_1$ and $C_2$ complexes formed by XrpFI (data not shown).

In a second set of experiments, we used antibodies against rat GABPα and -β subunits, known to be active on the β factor, the murine homolog of XrpFI (50). The effect of anti-GABP sera on XrpFI is shown in Fig. 4a and b. Preincubation of crude oocyte nuclear protein with anti-GABPα serum supershifted both $C_1$ and $C_2$ complexes (panel a, lane 2) while only $C_2$ was supershifted by anti-GABPβ serum. The anti-α serum appears to be weaker than the anti-β serum; in fact, reaction with anti-β serum entirely abolished the $C_2$ complex and caused a slight increase in the amount of $C_1$ (lane 3). On the other hand, the anti-α serum, used under the same experimental conditions as the anti-β serum (1 μl of undiluted serum), was unable to completely supershift the two complexes. Similar results were obtained with the same sera for purified XrpFI (panel b, lanes 2 and 3).

The effects produced by the sera are specific because no inhibition or supershifting of XrpFI complexes occurred with rabbit preimmune serum (Fig. 4a and b, lanes 4). These data show that XrpFI contains polypeptides immunologically related to GABP subunits and that GABPα-like components contact the DNA alone ($C_1$) or are associated with GABPβ-like species ($C_2$). The data from the band shift and cross-linking analyses reported above indicate that XrpFI is composed of several polypeptides. To further investigate this point, we used anti-GABP antibodies on Western blots (immunoblots) of crude and purified proteins from oocyte nuclei. Two α-related species, the major one 46 kDa and the

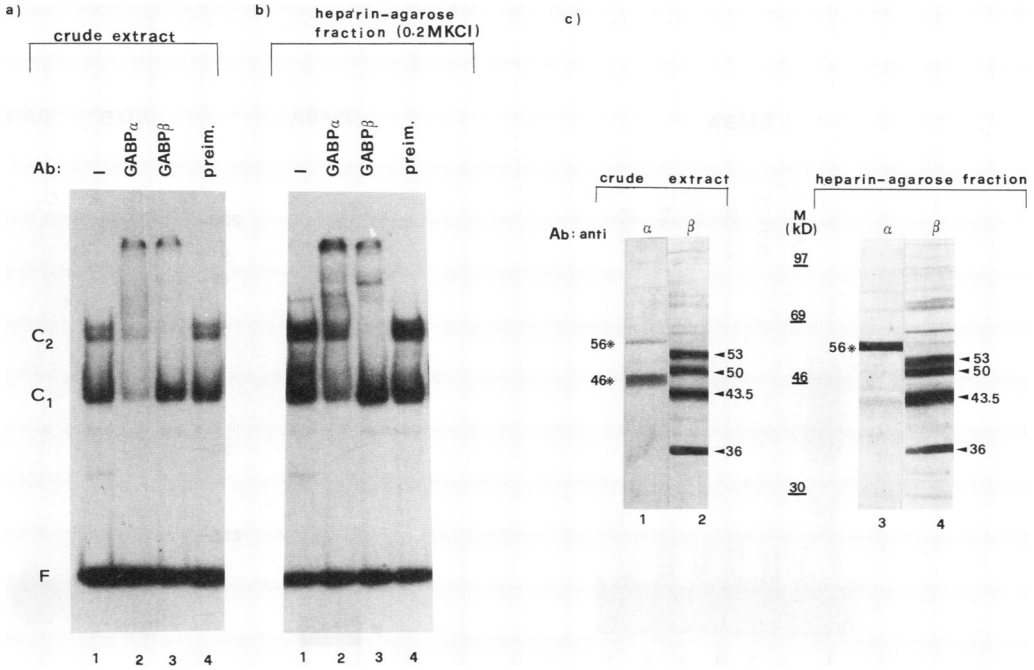


FIG. 4. Effect of antisera on XrpFI DNA-binding activity. Undiluted sera (1 μl each) were reacted with *Xenopus* oocyte nuclear extract for 30 min at room temperature before binding to the DNA probe. Complexes were electrophoresed on native gels. (a) Anti-rat GABPα, GABPβ, and preimmune (preim.) sera on crude extract (5 μg). –, no serum addition; Ab, antibody. (b) The same as panel a but on partially purified extract (4.5 μg). (c) Immunoblots of crude (100 μg) and heparin-agarose-purified (25 μg) XrpFI reacted with anti-GABPα (lanes 1 and 3) and anti-GABPβ (lanes 2 and 4) sera. M, molecular size markers (in kilodaltons). Asterisks indicate the 56- and 46-kDa α species. Arrowheads point to the β species.

minor one 56 kDa, and four major β-related polypeptides of 53, 50, 43.5 (probably a doublet), and 36 kDa were detected in the crude oocyte nuclear extract (Fig. 4c, lanes 1 and 2, respectively): the 56-kDa species became the major α-related component in the blot of the partially purified XrpFI preparation, while the α-like 46-kDa species was undetectable (Fig. 4c, lane 3). The purified preparation of XrpFI used in the immunoblot, band shift, and cross-linking experiments corresponds to the 0.2 M KCl–heparin-agarose fraction. The XrpFI DNA-binding activity elutes from the heparin-agarose column between 0.18 and 0.3 M KCl. The 0.2 M KCl fraction is composed of proteins which form the $C_1$ and $C_2$ complexes but is enriched with proteins which produce the $C_2$ complex; at higher salt concentrations (0.25 to 0.3 M KCl), proteins containing the 46-kDa α species and forming only the $C_1$ complex are eluted (data not shown).

The supershifting experiments suggest that the $C_2$ complex is formed by the association of α- with β-related polypeptides; the question is whether all of the β-related proteins, detected by immunoblotting, associate with the α counterpart. Another question is whether the associated β-like polypeptides contact the DNA probe, as reported for the rat GABPβ (42). UV cross-linking and methylation interference assays were performed to investigate this point.

In order to simplify the patterns of UV cross-linked species, we used the partially purified XrpFI fraction in which only the 56-kDa α-related and β-related forms are present. The extract was incubated with anti-GABPβ serum or with preimmune serum, and the antigen-antibody complexes were adsorbed to protein A-Sepharose. Untreated and depleted samples were then bound to the BrdU-substituted probe, exposed to UV light, and run on SDS-PAGE gels (Fig. 5). The sample treated with the preimmune serum (panel a, lane 1) and the untreated XrpFI fraction (panel b, lanes 1 to 5) produced two strong (85- and 95-kDa) and two faint (60- and 115-kDa) cross-linked species. Other bands with lower molecular masses (37 and 25 kDa) are likely due to degradation after UV cross-linking because they increase if the exposure time to UV light is extended over 30 s. All of these cross-linked species were susceptible only to specific competition (panel b, lanes 2 and 3). Addition of 1,000-fold molar excess of the specific double-stranded oligonucleotide X inhibited the minor bands and caused a 50% reduction in the prominent species. The inability to completely inhibit binding is due to a lower affinity for XrpFI of the oligonucleotide X compared with the BudR probe. Depletion of β-like polypeptides caused a strong reduction in the amounts of the cross-linked species with high molecular masses (115, 95, and 85 kDa) and a net increase in the amount of the 60-kDa product (panel a, lane 2). This result confirms that the α-related component of XrpFI binds to DNA as a monomer in the 60-kDa band; the high-molecular-mass bands are, instead, dependent on the presence of the β-related components, which, when associated with α, are in contact with the DNA probe and can be cross-linked by UV light. In agreement with the data presented above, we observed that the $C_2$ complex resulting from the association of α and β polypeptides produces a more extended methylation interference pattern than that of $C_1$ (Fig. 6, panel a). In addition, the $C_2$ complex seems to be more stable in specific competition because, on increasing the amount of unlabeled specific oligonucleotide (oligonucleotide X), the $C_1$ complex dissociates before the $C_2$ complex (Fig. 6b, lanes 3 and 4).

**Immunoblot analysis of embryonic XrpFI.** Band shift ex-

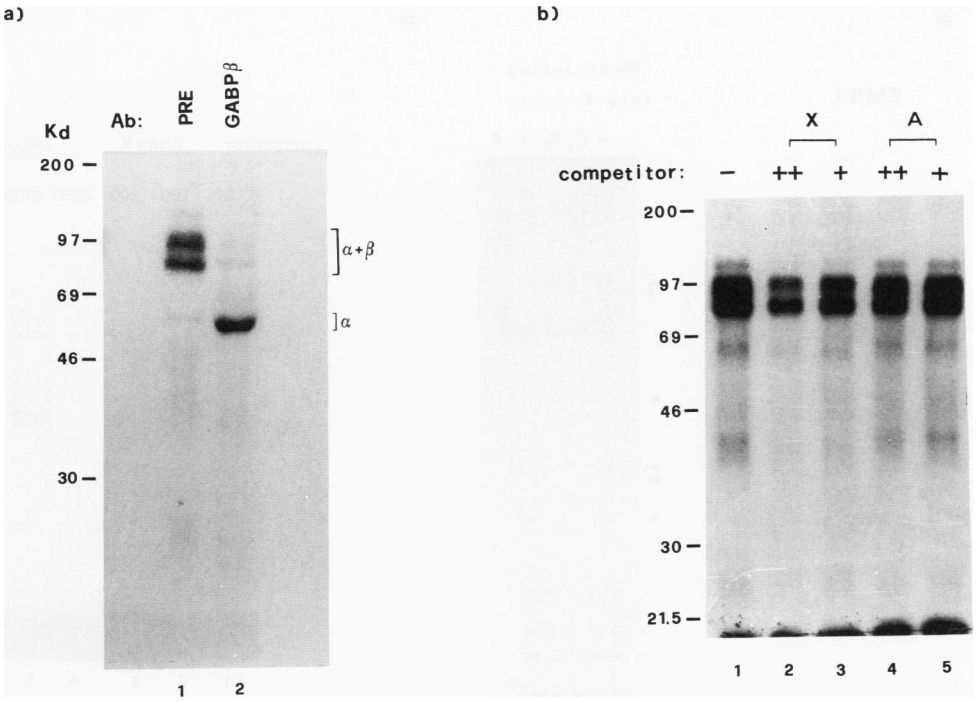


FIG. 5. Analysis of α- and β-related components of XrpFI by UV cross-linking to DNA. (a) Depletion of β-related polypeptides. Nine micrograms of heparin-agarose-purified oocyte nuclear protein was preincubated for 30 min at room temperature with 1.5 μl of undiluted anti-GABPβ serum (lane 2) or with 1.5 μl of rabbit preimmune (PRE) serum (lane 1). Depletion was carried out with protein A-Sepharose as described in Materials and Methods. Residual proteins were bound to the BrdU-substituted probe, UV cross-linked, and analyzed by SDS-PAGE. Ab, antibody. (b) Competition. Heparin-agarose-purified XrpFI (9 μg) was incubated with 1 ng of labelled BrdU-substituted probe in the presence of specific (X) or nonspecific (A) oligonucleotide competitors. Molar excesses were 1,000-fold (++) and 500-fold (+). –, no competitor added. Size markers (in kilodaltons) are indicated on the left.

periments employing embryonic extracts show that proteins from early embryos produce the $C_1$ complex but little or no $C_2$ complex. A decrease in the amount or the absence of β-related polypeptides able to associate with α in the early embryogenesis might account for this observation. To test this hypothesis, we analyzed embryonic proteins by immunoblotting with anti-GABP antibodies and comparing the results with those from nuclear and cytoplasmic oocyte proteins. The two major polypeptides 56 and 46 kDa in size detected in the blots of oocyte nuclear proteins by the anti-GABPα serum (Fig. 4 and Fig. 7, panel a, lane 2) are also present as very faint bands in the blots of oocyte cytoplasm and early and late embryo proteins (Fig. 7, lanes 1 and 3 to 5). However, the major α-related component present in the embryo extracts appears to be a polypeptide which migrates as a 50-kDa band. Moreover, other bands with sizes greater and smaller than 50 kDa appear in the blot of proteins from two-blastomere embryos. All of the α species, although present, are underrepresented in the blot of proteins from embryo stage 15.

More pronounced differences in β-like proteins emerged from the comparative analysis of oocyte and embryonic proteins. None of the β-related protein species found in the oocyte nucleus were evidenced by anti-β serum in the oocyte cytoplasm or in the extracts from embryos of stages 2 to 15 (Fig. 7b, lanes 1, 4, and 5); extract from embryos of stage 6 (not shown) produced results identical to those of stage 15. However, the anti-β serum revealed one 45-kDa species in the blot of stage 2 proteins; this polypeptide, probably of maternal origin, is absent at later stages. In contrast, β-related polypeptides comigrating with the oocyte 53- and 50-kDa species again appeared in the Western blots of proteins from stage 40 embryos (shown in lanes 3 and 5). The immunoblotting data correlate well with those obtained by EMSA reported in Fig. 2 and with those in Fig. 8 which show the effects produced by anti-GABP antibodies on the DNA-protein complexes of stage 2 (Fig. 8, lanes 1 to 4), stage 15 (lanes 5 to 8), and stage 40 (lanes 9 to 12) embryos. A complex smaller than $C_2$, called $C_3$, is present only in stage 2 embryo extracts and is also supershifted by the anti-β serum (Fig. 8, lane 4). At stage 15, binding of proteins to the labelled DNA probe is not affected by the anti-β serum treatment nor can we detect polypeptides able to associate with the α-related counterparts to form $C_2$ complexes.

## DISCUSSION

For this report we studied the role of XrpFI in L14 gene transcription and its DNA-binding activity during oogenesis and development; we also sought to determine the correlation between XrpFI and some members of the *ets* family.

XrpFI, in terms of DNA-binding activity, is found in every oocyte stage, in embryos, and in all adult tissues (not shown) analyzed. XrpFI is composed of polypeptides immunologically related to the α and β subunits of rat GABP (25, 42). Under our experimental conditions, the α-related components are able to bind as monomers to a sequence identical to the Ets consensus, while β-like polypeptides are never found in these small complexes. The α-like components also form heteromeric complexes with the β-related proteins; these

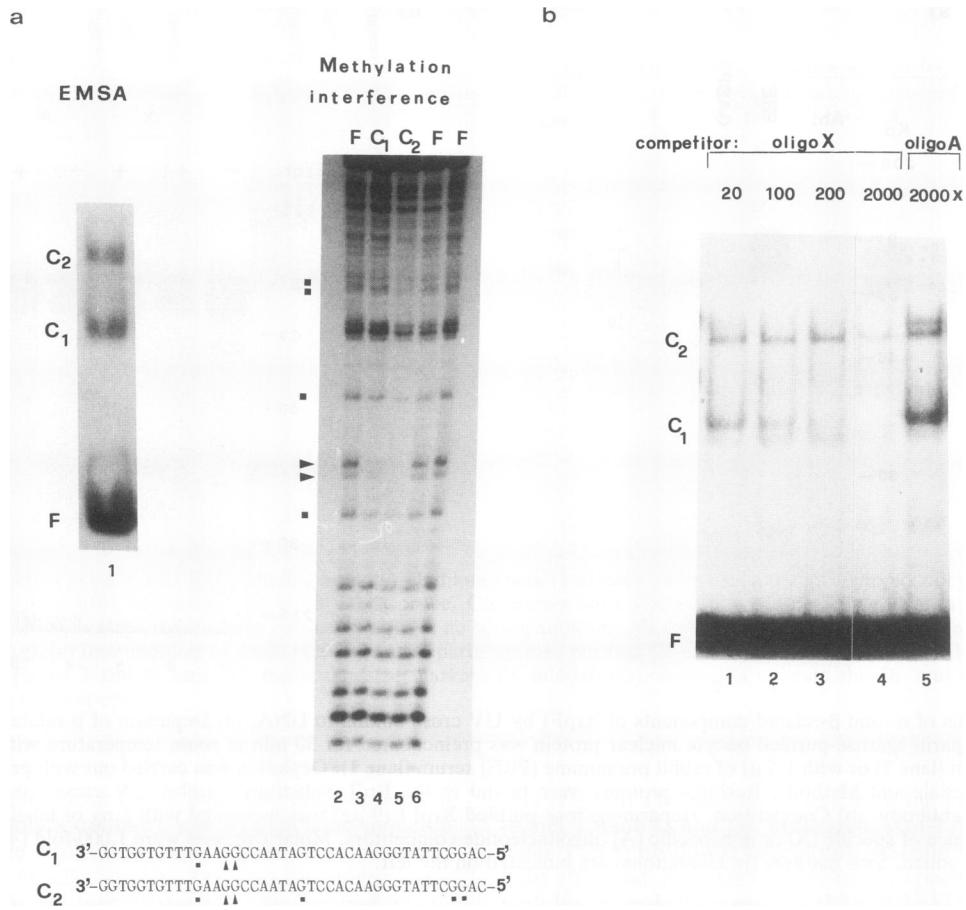


FIG. 6. Analysis of the XrpFI $C_1$ and $C_2$ complexes by dimethyl sulfate methylation interference and competition assays. (a) The partially methylated radiolabeled (500,000 cpm) XrpFI probe was bound to oocyte nuclear protein. Bound ($C_1$ and $C_2$) and free (F) probes were eluted from the native gel (lane 1), cleaved with piperidine, and analyzed on a 20% sequence gel (lanes 2 to 6). The data are summarized at the bottom of the figure; arrowheads show the G residues of the probe denoting complete interference. Partial interference, more extended in the pattern produced by the $C_2$ complex, is indicated by solid squares. (b) Relative stability of the $C_1$ and $C_2$ complexes versus specific competition. Molar excess of the unlabeled specific DNA competitor (oligonucleotide X) is indicated on the top. Control competition with a 2,000-fold molar excess of nonspecific oligonucleotide (oligonucleotide A) is shown in lane 5.

β-like proteins, when associated with α, contact DNA and can be cross-linked to the probe. The αβ complex produces a more extended methylation interference pattern and shows an increased stability during specific DNA competition, in agreement with the properties of the GABP factor (25). Some new findings emerged from our study, including the presence of multiple α- and β-related polypeptides and the differential expression of the β-related components in embryogenesis.

Preparations of GABP from rat liver contain three polypeptides: one α and two β (β1 and β2) (25). In *Xenopus* oocytes, we find two α- and four β-like peptides, but in differentiated tissues (tadpole stage) we find one major α polypeptide and two β-related species, such as those found in rat liver. At the moment, we cannot explain the occurrence in oocytes of multiple α- and β-related protein species. Even though unspecific partial proteolysis cannot be ruled out, other molecular cellular events, such as specific proteolytic cleavage, alternative splicing, and posttranslational modifications, might account for multiple gene products. Another possibility is that *Xenopus* oocytes express genes specific for germinal cells.

HrpF, the human homolog of XrpFI, which forms a complex with the XrpFI target sequence (23) also reacts with anti-GABP serum and appears to be composed of multiple α- and β-related species (unpublished results). HrpF might correspond to NRF-2, the factor recently described as composed of several β-like subunits (44).

A peculiar feature of the early stages of embryogenesis is the absence of polypeptides related to GABPβ. Both immunoblotting and EMSA failed to detect β species or heteromeric complexes ($C_2$) in stage 6 and 15 embryos, while at stage 2, a β-like species, probably of maternal origin, was evident. We presume that a correlation between transcription and the appearance of β-like proteins does exist.

XrpFI is composed of α monomers ($C_1$) and αβ dimers ($C_2$), whose function, at the moment, is only a matter of speculation. Both monomers and dimers could act as activators of transcription of several genes, but the β proteins could positively modulate this activity. There are some indications in favor of this hypothesis: the β-like proteins, able to interact with the α polypeptides, are exclusively located in the nucleus and are absent when transcription in the embryo is absent or very low but again appear at stages

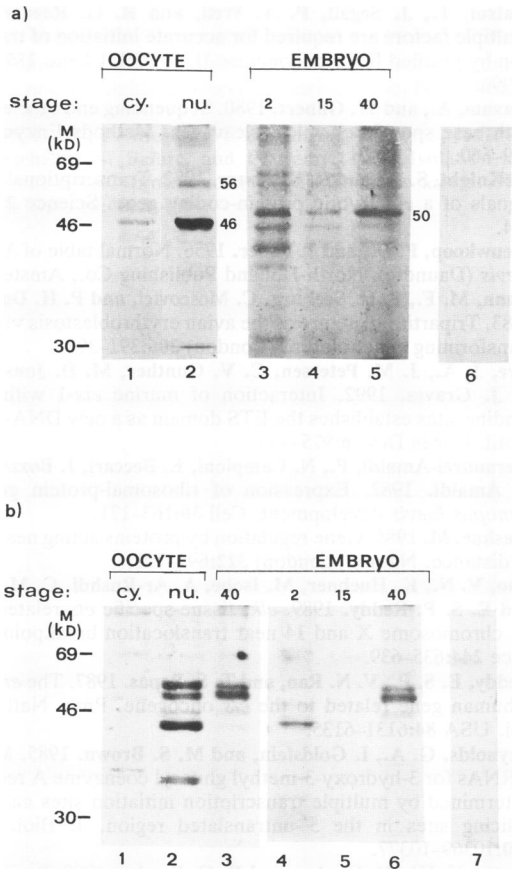


FIG. 7. Identification of GABPα- and β-related polypeptides in oocyte and embryonic extracts by immunoblotting. (a) α-related polypeptides detected by anti-GABPα serum on a Western blot employing 140 μg of oocyte cytoplasmic (cy.) extract protein (lane 1), 60 μg of crude oocyte nuclear (nu.) extract (lane 2), or 125 μg of whole-cell extract protein from embryos of the indicated stages (lanes 3 to 5). Proteins from oocyte nuclei in lane 6 were treated with preimmune serum as a control. (b) β-related polypeptides in oocyte cytoplasmic proteins (lane 1), oocyte nuclear proteins (lane 2), and embryos at the indicated stages (lanes 3 to 6). As a control, the first antibody treatment was omitted in the oocyte nuclear protein blot (lane 7). Numbers on the left of the figure indicate protein size markers (in kilodaltons).

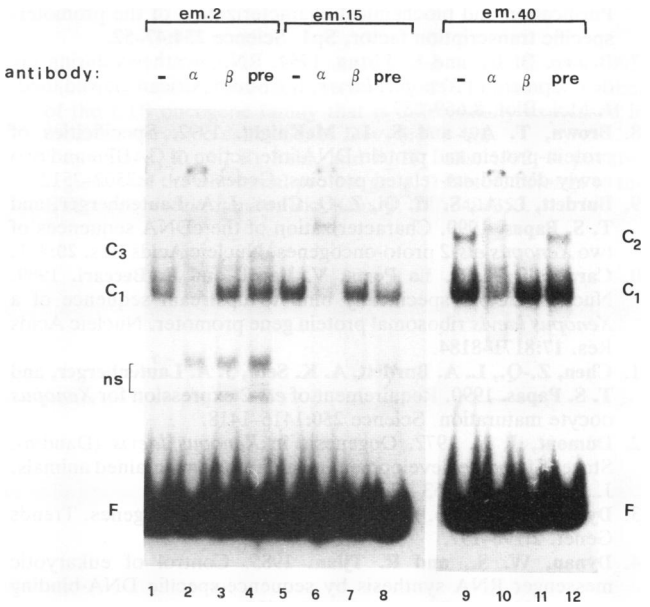


FIG. 8. Effect of anti-GABPα and -β subunit sera on the DNA-binding activity of embryonic proteins. Extract proteins from embryo (em.) stages 2 (20 μg, lanes 1 to 4), 15 (50 μg, lanes 5 to 8), and 40 (20 μg, lanes 9 to 12) were treated with 1 μl of anti-α or anti-β undiluted serum or with 1 μl of preimmune (pre) rabbit serum for 30 min before binding to the labelled DNA probe, and the resulting complexes were analyzed by EMSA on a native 5% polyacrylamide gel. Embryo stages and antibodies are indicated on the top of the figure. −, no serum addition; $C_1$, $C_2$, and $C_3$, specific DNA-protein complexes; F, free probe; ns, nonspecific bands.

characterized by enhanced transcription (34). They are also important in increasing stability of the monomer bound to DNA. Mutation of the XrpFI binding site specifically affects transcripts starting from the major cap sites; thus, stabilization of preinitiation complexes assembled at suitable distance could be mediated by direct interaction of the β-like polypeptides with some component of the basal transcription machinery. It is interesting to note that in the mouse and human rp genes regulated by factors homologous to XrpFI, the distance of the target sequence from the cap site is conserved. In the rp gene promoters so far analyzed, the XrpFI binding site is present in a single copy, while the herpes simplex virus type 1 *cis*-regulatory element consists of three imperfect GA-rich repeats (25) and the rat cytochrome *c* oxidase subunit IV (44) consists of two tandem repeats. Duplication of the target site for αβ dimers might occur in the genes with a housekeeping function, which need to be expressed at high rates in some tissues. Different combinations of αβ forms create a versatile factor that is able to discriminate among target promoters and to establish specific protein-protein interactions.

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