Volume 313, number 3, 205–209 FEBS 11787 November 1992

Identification of nuclear factors which interact with the 5′ flanking region of the EF-1αO gene in *Xenopus laevis*

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Received 21 July 1992; revised version received 7 October 1992

The EF-1αO gene of *Xenopus laevis* is a stage-specific gene, being transcribed in oogonia and oocytes, but not in postmeiotic germ cells and terminally differentiated cells. We found that two *trans*-acting factors from oocyte nuclear extract are able to interact with a DNA sequence in the 5′-upstream region of the EF-1αO gene. Methylation interference experiments suggested that the two factors recognised the same DNA element. Gel retardation assays indicated that part of the protein binding site could be confined to a 21 bp sequence, located between −51 and −72, relative to the cap site. Interestingly, this region shares great homology to a negative regulatory segment in the promoter of the TFIIIA gene, another developmentally regulated gene.

Stage-specific transcription; DNA binding protein; EF-1αO; *Xenopus laevis* oocytes

1. INTRODUCTION

Protein-coding genes in eukaryotes have promoters and enhancers containing specific sequences, *cis*-acting elements, which are able to bind to general and promoter-specific transcription factors [1–3]. Through protein–DNA and protein–protein interactions, the transcription factors and the *cis*-acting elements collaborate in controlling the rate of transcription initiation, known to be a major gene regulatory step in eukaryotic cells [1,4].

The EF-1αO gene of *Xenopus laevis* is an interesting model for studying the gene expression in developmentally regulated genes. The EF-1αO gene encodes a stage-specific elongation factor, EF-1αO, consisting of 461 amino acids, and sharing 97% homology with the normal and well-known eukaryotic elongation factor, EF-1α. EF-1αO mRNA is present in oogonia and oocytes in considerable amounts, but postmeiotic germ cells and terminally differentiated cells (such as liver cells) contain no detectable EF-1αO mRNA [5–7].

We have previously sequenced and characterized the EF-1αO gene together with its promoter. The promoter sequence of the EF-1αO gene contains neither a TATA-box, typical of genes expressed in terminally differentiated cells, nor a pyrimidine tract immediately upstream of the capsite, as is seen in many 'housekeeping' genes, nor a 17 bp initiator motif 5′-GCCTCATTCTGGA-GAC-3′ around the site of transcription initiation as seen in some developmentally regulated genes [8].

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Another *Xenopus* gene, transcription factor IIIA (TFIIIA), has an expression profile comparable to that of EF-1αO [9]. The promoter of the TFIIIA gene contains several well-defined regulatory elements [10,11]. Recently, we reported that the region from −83 to −22, relative to the capsite in the EF-1αO gene, is partly homologous to a negative regulatory element in the TFIIIA promoter [8].

In the present study we focused our attention on the first 180 bp of the EF-1αO promoter. Gel-retardation assay and methylation-interference experiments allowed us to identify two protein factors capable of interacting with the investigated promoter element.

2. MATERIALS AND METHODS

2.1. *Plasmids and DNA probes*

A 687 bp DNA fragment containing the 5′ flanking region as well as the first exon and part of the first intron of the *Xenopus laevis* EF-1αO gene was cloned in the *Eco*RI site of the vector pUC18. The construct was controlled by direct plasmid sequencing. To prepare labelled DNA fragments, the plasmid was digested either with *Hin*dIII or *Eco*RI, and the 5′ overhang was filled in using Klenow polymerase and [α-^{32}P]dATP [12]. Subsequent digestion with *Rsa*I or *Dra*I and polyacrylamide gel electrophoresis allowed the purification of end-labelled probes.

2.2. *Preparation of germinal vesicle extracts from oocytes*

To obtain oocytes, adult female *Xenopus laevis* were anaesthetized in tap water containing 0.01% ethyl *p*-aminobenzoate. Ovaries were removed, rinsed in Barth's solution and germinal vesicles were prepared either manually as described by Carnevali et al. [13] or by the large-scale method [14]. Shortly before use the germinal vesicles were disrupted by pipetting 5–10 times in the presence of 0.4 M NaCl. Nuclear debris could then be removed from the lysate by centrifugation for 10 min, 500 × *g* at 4°C. Protein concentration in extracts was determined by the method of Bradford [15].

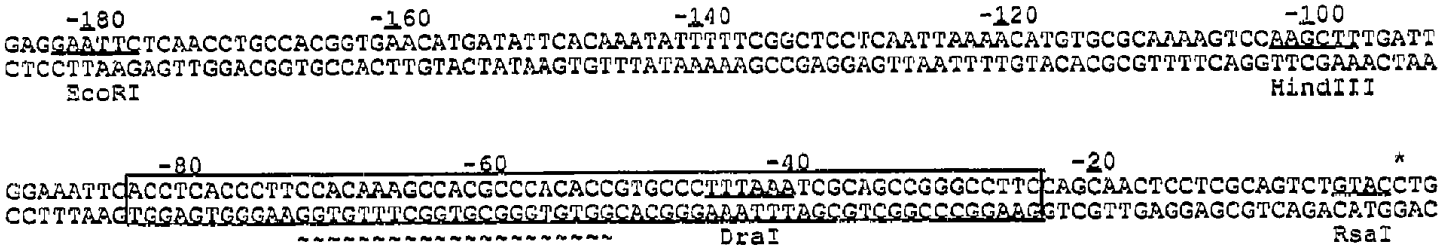

Fig. 1. The proximal promoter region of the Ef-1αO gene. The start site of transcription is marked by an asterisk. Relevant restriction enzyme sites are underlined, and the putative TFIIIA regulatory region is boxed. Wavy line is the oligonucleotide used for the gel-retardation experiments in Fig. 5.

2.3. *Gel retardation experiments*

5–8 μg of crude nuclear extract were preincubated on ice in a total volume of 20 μl containing 15 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM NaCl, 0.5 mM DTT, 5% glycerol and 1–3 μg non-specific competitor DNA, either sonicated herring sperm DNA or double-stranded poly(dI-dT). After 10 min a few nanograms of end-labelled DNA (5,000 cpm) were added, and the incubation continued for 20 min. The binding reactions were loaded onto a 6% polyacrylamide gel and electrophoresed at 10 V/cm in the cold room. After migration, the gel was dried and subjected to autoradiography. For the competition experiments, cold-specific competitor DNA was added together with the labelled DNA following the preincubation step. The unspecific competitor DNA used in competition experiments was either an *Alu*I digest of the plasmid pUC19 (New England Biolab) which yields 16 restriction fragments ranging in length from 19 to 679 nucleotides or sonicated herring sperm DNA. Oligonucleotides for competition experiments: 5′-CCACAAAGCCACGCCCACACC-3′ and 5′-GGTGTGGGCGTGGCTTTGTGG-3′ were chemically synthesized and annealed by heating to 75°C for 5 min followed by slow cooling.

2.4. *Methylation interference*

For methylation interference experiments we used end-labelled DNA, which had been partially methylated by DMS, according to standard procedures [16]. The modified DNA was precipitated and used in binding reactions. Binding reactions contained 60–75 μg of nuclear protein, 4 μg of sonicated herring sperm DNA and were carried out in the same environment as described above but in a total volume of 50 μl. After 10 min of preincubation the partially methylated DNA (500,000 cpm) was added and the incubation continued for 20 min. The reaction mixtures were then run on a native gel in the cold room. After electrophoresis the gel was exposed for 3 h and the bands corresponding to free and retarded DNA were cut out and eluted. The eluates were purified on Sephadex 50 and ethanol precipitated. The pellets were resuspended in 100 ml 1 M piperidine, cleaved at methylated G residues [16], and analyzed on a 10% sequencing gel.

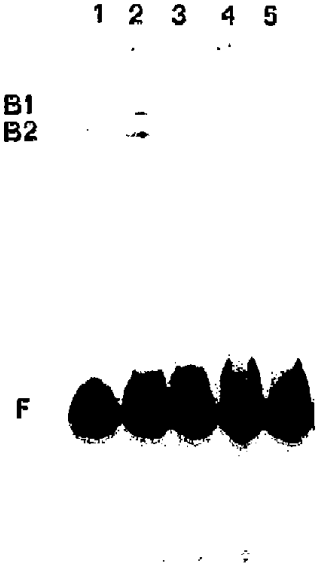

Fig. 2. Gel-retardation experiment using the 179 bp *Eco*RI–*Rsa*I probe and nuclear extracts from various tissues. Lane 1, no extract; lane 2, *Xenopus* oocyte; lanes 3–5, *Xenopus* liver, *Xenopus* brain and human HeLa cells, respectively.

3. RESULTS

3.1. *The 5′-upstream region of the EF-1αO gene*

The fact that the EF-1αO gene is developmentally expressed, made us believe that specific *trans*-acting proteins participate in the regulation of the gene. To test

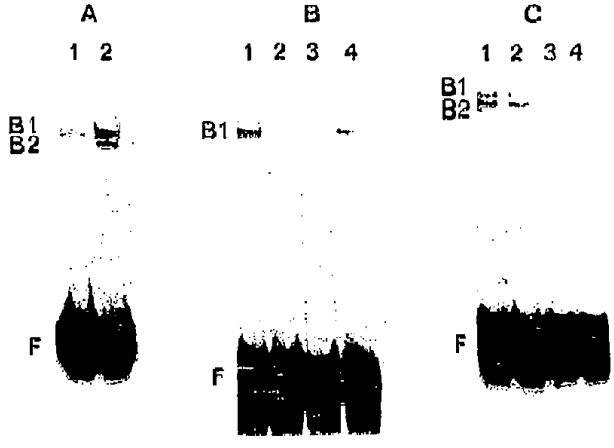

Fig. 3. Binding specificity of crude nuclear extract to the EF-1αO promoter. (A) Binding of the nuclear factors to the −180 to −1 promoter region. (1) Probe, 2 μg carrier DNA and stage I nuclear extract (6 μg proteins). (2) Same as (1) but with stage V nuclear extract. (B) Stage I nuclear extract. (1) *Hin*dIII–*Rsa*I probe, 8 μg of previtellogenic extract and 3 μg of sonicated carrier DNA. (2) As in (1), plus a 10-fold molar excess of the same unlabelled *Hin*dIII–*Rsa*I fragment. (3) As in (1), competed by a 100-fold molar excess of unlabelled fragment. (4) As in (1), competed by a 100-fold molar excess of unspecific DNA fragments. (C) Stage V nuclear extract. (1) *Hin*dIII–*Rsa*I probe, 6 μg crude stage V nuclear extract and 3 μg of sonicated carrier DNA. (2) As in (1) plus a 100-fold molar excess of herring sperm DNA fragments. (3) As in (1) plus a 100-fold molar excess of unlabelled probe. (4) As in (1) plus a 10-fold molar excess of unlabelled probe.

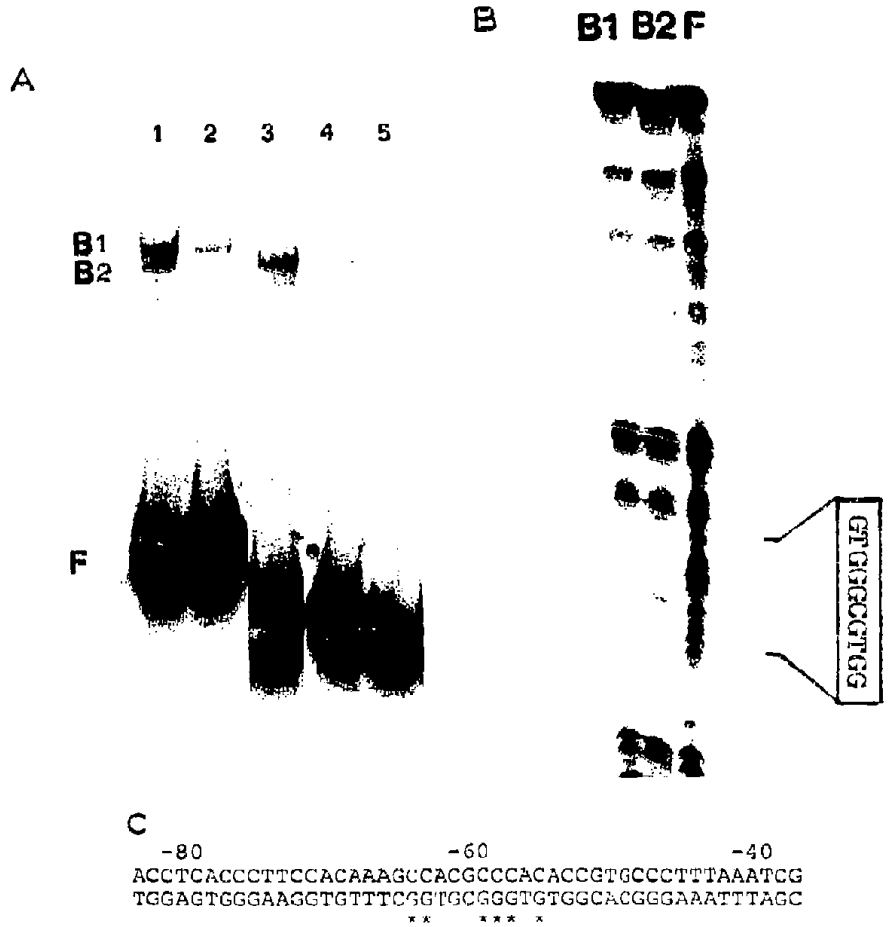

Fig. 4. One factor of the crude nuclear extract bind to the proximal promoter region. (A) Gel retardation experiment using different DNA probes. Lanes 1 and 2, the *Hin*dIII–*Rsa*I probe; lanes 3–5, the *Eco*RI–*Dra*I probe. DNA probe (5,000 cpm) was incubated with 2 μg sonicated herring sperm DNA and 5 μg nuclear proteins, either from stage V oocytes (lanes 1 and 3) or previtellogenic oocytes (lanes 2 and 4). Lane 5, no nuclear proteins added. (B) Methylation interference assay on the *Hin*dIII–*Rsa*I probe, labelled at the bottom strand. The guanines whose methylation interferes with binding of nuclear proteins are included in the box. F = free DNA. B1 and B2 = DNA purified from the retarded B1 and B2 complexes shown in Fig. 2. (C) The nucleotide sequence between −40 and −80 relative to the cap site. Asterisks indicate the G residues involved in protein binding in the methylation interference assay.

our hypothesis we concentrated on the 181 bp DNA segment immediately flanking the initiation codon of the *Xenopus laevis* EF-1αO gene (Fig. 1). This segment contained a 54 bp sequence sharing homology to an established negative regulatory region in the promoter of the TFIIIA gene [8]. We prepared crude nuclear extracts from oocytes and other *Xenopus* tissues and incubated them with labelled DNA. Complexes, formed between nuclear proteins and the DNA fragment, were detected by the electrophoretic mobility shift assay. A nuclear extract from HeLa cells was kindly provided by Dr. Handa (Tokyo University). The nuclear extract from *Xenopus* oocytes resulted in two retarded complexes (Fig. 2, lane 2), whereas proteins from *Xenopus* liver or brain and from human HeLa cells did not form compiexes (Fig. 2, lanes 3–5).

3.2. *Stage-specific binding of nuclear proteins to the upstream region of the EF-1αO gene*

We wanted to see if nuclear proteins from different oocyte stages resulted in different protein–DNA complexes in the analyzed region. To do this, we separately prepared crude nuclear extracts from stage I and stage V *Xenopus* oocytes. The two oocyte extracts were then incubated with labelled DNA and examined by the electrophoretic mobility shift assay. Incubation with stage V oocyte nuclear extract gave two equally intense retarded bands (B1 and B2), whereas one of the bands (B1) appeared significantly more intense than the other when stage I nuclear extract was used (Fig. 3A).

To determine the specificity of the observed protein–DNA interactions we performed competition experiments. Labelled DNA fragments were incubated with the two nuclear extracts in the presence of specific unlabelled DNA fragment or in the presence of an equal amount of non-specific competitor DNA. As seen in Fig. 3B,C, the shifted stage V extract bands (B1 and B2) and the shifted stage I band (B1) could be specifically prevented by the addition of a 100-fold molar excess of unlabelled versus labelled DNA fragment, whereas a

Fig. 5. (A) Competition experiment with the oligonucleotide shown in Fig. 1 (1) Probe, crude nuclear extract plus 2 μg of sonicated carrier DNA. (2) As in (1), competed by 200-fold molar excess of unlabelled oligonucleotide. (3) As in (1), competed by a 200-fold molar excess of unspecific DNA fragments.

100-fold molar excess of unspecific DNA fragments only competed very weakly for complex formation.

3.3. *DNA region for protein binding*

To determine the sequence motif able to interact with oocyte nuclear extract more precisely, we used alternative restriction enzyme sites when preparing the labelled DNA probe. This provided us with two smaller probes, a *Hin*dIII–*Rsa*I fragment spanning from −101 to −3 and an *Eco*RI–*Dra*I fragment spanning from −181 to −43 (see Fig. 1). As shown in Fig. 4A both restriction fragments produced two retarded bands (B1 and B2) when incubated with stage V oocyte nuclear extract. It is noteworthy that the same gel-retardation pattern was seen for both probes, and so we concluded, that the sequences involved in protein binding are located in the overlapping region, that is between −101 and −43.

The methylation interference assay was used to identify the exact contact points on DNA of complexes B1 and B2. The *Hin*dIII–*Rsa*I fragment was end-labelled at the bottom strand and partially methylated by DMS prior to incubation with nuclear extract. After separation on a native gel, B1 and B2 complexes as well as free DNA were cleaved by piperidine and the cleavage products were analyzed on a sequencing gel (Fig. 4B). Six guanines of the bottom strand, indicated by asterisks in Fig. 4C, clearly participated in the binding. Precise mapping of the contact points revealed that the sequence involved was: 5′-CCACGCCCAC-3′, located between −64 and −55 from the capsite. Both retarded bands produced the same interference pattern, indicating the involvement of the same G residues in the protein–DNA complexes seen in B1 and B2. This suggests that the DNA sequences involved in the complexes B1 and B2 might be identical.

3.4. *Specificity for protein–DNA binding*

To confirm our conclusion that B1 and B2 are related to the found DNA region, we synthesized a 21 bp double-stranded oligonucleotide and tested this as competitor in a binding assay with mixed oocyte nuclear extract. The oligonucleotide covered a central part of the protein-binding segment and included the region sharing greatest homology to the TFIIIA promoter (Fig. 1). It can be seen in Fig. 5 that a 200-fold molar excess of cold oligonucleotide makes both the retarded bands disappear, whereas a similar molar excess of unspecific competitor has no detectable effect. This result suggests that the 21 bp oligonucleotide sequence is involved in the binding of the oocyte nuclear proteins.

4. DISCUSSION

Gel-retardation assays demonstrated, that crude nuclear extracts from oocytes bind specifically to the upstream region of the EF-1αO gene, resulting in two slow migrating complexes, B1 and B2. Apparently, the *trans*-acting factor giving rise to complex B1 dominates in previtellogenic oocytes (stage I), whereas nuclear extracts from stage V oocytes contain equal amounts of the factors responsible for complexes B1 and B2. Nuclear extracts from *Xenopus* liver cells seemingly did not contain any factors able to bind to the EF-1αO promoter.

A methylation interference assay revealed that the contact points on DNA of complexes B1 and B2 were identical. This may be due to a common DNA binding protein in the two complexes, whereas their dissimilarities in electrophoretic mobility could be explained by association with auxiliary non-DNA-binding proteins. Another hypothesis is that the two retarded bands are caused by the same DNA-binding protein in different phosphorylated states. Phosphorylation– dephosphorylation of proteins is known to be an important regulatory step in many cellular processes.

Using alternative DNA probes, we could restrict the protein-binding element of the DNA to −101 to −43 relative to the cap-site. Methylation interference and competition experiments with a chemically synthesized oligonucleotide suggested that part of the protein binding took place in the 21 bp segment between −52 and −73. It is noteworthy that this segment partly encom-

passes a region sharing homology with a negative regulatory element in the promoter of the TFIIIA gene [10].

Acknowledgements: We thank Dr. Francesca Carnevali, University of Rome, for excellent suggestions and helpful discussions. Professor Brian F.C. Clark provided impetus to the initial experiments in this project, and his input and advice is warmly appreciated.

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