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Nuclear factors specifically bind to upstream sequences of a *Xenopus laevis* ribosomal protein gene promoter

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ABSTRACT

The upstream region of the *Xenopus laevis* L14 ribosomal protein gene was deleted starting from the 5′ extremity in order to define the promoter length necessary to express a linked reporter CAT gene. The functional analysis indicated that a sequence located between −63 and −49 from the capsite is important for an efficient promoter activity. Band shift and ExoIII protection assays evidenced the binding to this region of a factor, called XrpFI, present in the crude nuclear extract from *X.laevis* oocytes. Methylation interference analysis localized the contacts in the G residues belonging to a short box, 5′ CTTCC 3′, positioned between −53 and −49 from the capsite. An additional factor, XrpFII, makes contacts with the sequence 5′GCCTGTTCGCC 3′ located between −27 and −17 from the capsite. The deletion mutant still containing this sequence is poorly transcribed, but resumes activity when a short fragment containing the binding site for factor XrpFI is cloned in an upstream position.

INTRODUCTION

Increasing interest has recently been devoted to the study of the promoters of the 'housekeeping' genes which seem to differ from those of genes expressed in terminally differentiated cells in that often an AT rich region substitutes for a canonical TATA box and a GC rich stretch is present in the region immediately upstream of the transcription initiation site (1,2).

The genes coding for the proteins of the ribosome so far analyzed in *Xenopus laevis* (3,4) and mouse (5) share the features of the housekeeping genes in their promoter regions. In mouse functional analysis has shown that maximal expression of the r-protein genes does require upstream and downstream sequences relative to the capsite (6,7). In yeast full expression of most r-protein genes depends on the presence of an upstream activating sequence, the so-called UAS RPG, located in a region 200−400 bp upstream of the capsite (8). Recent data demonstrate that a nuclear factor binds specifically to these sequences; it has been suggested that the coordinated expression of the genes coding for the components of the translational apparatus could be mediated by this common factor(s)(9−11).

In this paper we report a functional characterization of the region extending some hundred bases 5′ to the capsite of the *Xenopus laevis* L14 r-protein gene. Interaction of factors present in the *X.laevis* nuclear extract was investigated. Gel retardation, ExoIII protection and methylation interference assays enabled us to identify two sequences, located at −53 and −27 from the capsite, which bind two distinct *X.laevis* nuclear factors. The deletion mutant analysis demonstrated that the distal sequence is essential for the transcriptional activity of the promoter.

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MATERIALS AND METHODS

Plasmids and recombinant DNA constructions

The L14 gene region analyzed extends from the AvaII site at −890 to the AvaI site at +12 (12). To obtain −890pTCAT, this fragment was filled in and cloned in the filled XhoI site of the vector pTCAT (13), carrying the bacterial chloramphenicol acetyltransferase gene (CAT) and the SV40 late polyadenylation signal located at the end of the CAT gene.

The L14 deletion mutants shown were obtained by cloning into the polylinker region of the pTCAT vector restriction fragments of the −890 to +12 region: −166pTCAT extends from the Hinfl site at −166 to AvaI (+12); −63pTCAT from the −63 HaeIII site to AvaI (+12); −49pTCAT from the HpaII site (−49) to AvaI (+12).

The −29pTCAT construct derived from a L14 cDNA clone that naturally contained 29 bp upstream of the major capsite (4). The EcoRI-EcoRV cDNA fragment inserted in pTCAT is made by 29 bp preceding the capsite, the first exon of the L14 gene (42 bp) and 9 bp of the second exon. Two copies of the B oligo (see below) were inserted into the HindIII site of the polylinker region of −29pTCAT to form 29BBpTCAT : in this construct both the B oligos were cloned in the reverse orientation 40 bp upstream of the L14 sequence. Every construct was controlled by sequencing according to Maxam and Gilbert (14).

CAT assay in injected oocytes

All recombinant CAT plasmids were CsCl purified prior to injection. DNA was dissolved in injection buffer (15) at 200 μg/ml and 20 nl were injected into the nucleus of stage VI *X.laevis* oocytes: in each experiment a minimum of 50 oocytes were injected for each template. Incubation was in Barth solution (16) for 3 hr at 18°C. For the CAT assay, oocytes were homogenized (10 μl/ oocyte) in 0.25 M Tris-HCl pH 7.5 and spun for 10 min at 12.000 rpm in the cold room with a microfuge. 20μl from the super were added to 130 μl of a solution made by 0.25 M Tris-HCl pH 7.5, 4mM AcCoA, 0.05 μCi (14C)-chloramphenicol. After 1 hr incubation at 37°C, chloramphenicol and derivatives were extracted with ethyl-acetate, dried and resuspended in 25 μl of the same solvent. Samples were spotted on silica gel and chromatographed with chloroform-methanol (95:5). An aliquot of the homogenates was used to quantitate the amount of plasmid DNA injected: 10 μl were mixed with SDS to 1% final concentration, heated breafly and run on a 1% agarose gel. The DNA was blotted and hybridized with a nick-translated isolated CAT DNA sequence.

Quantitation of the CAT transcripts

RNA was prepared from 30−50 injected oocytes by proteinase K/ SDS treatment and the amount corresponding to 4 oocytes run on MOPS-formaldehyde agarose and blotted as described (15). CAT transcripts were detected by hybridization with the nick-translated 783 bp CAT DNA isolated from pTCAT by BamHI digestion. The amount of RNA loaded on the filter was quantitated by a second hybridization with a nick-translated 5S DNA from *X.borealis* (17) and the CAT transcripts normalized to the 5S RNA value and to the amount of injected CAT plasmids which also became visible in the Northern blot after hybridization with the CAT DNA. In one set of injections CAT transcripts were normalized to 5S RNA transcribed after coinjecting the different L14 constructs with 200 ng/μl of a *X.borealis* 5S DNA plasmid (17) and with 0.8 μCi/μl of 32P −UTP. Oocytes were incubated for 3 hr at 18 C, RNA extracted, run and blotted as described and the filter exposed to X-ray film to detect labeled RNA prior to hybridizing with the CAT probe. All quantitatations were made on X-ray films with a LKB Ultroscan XL laser densitometer.

Preparation of the germinal vesicle extract

Germinal vesicles (GV) were prepared from large *Xenopus laevis* oocytes essentially as described by Birkenmeier et al (17). GVs were stored intact at −70°C in J-buffer (18), 1 μl for each GV. Extract was prepared disrupting GVs by pipetting (8−10 times) in the presence of 0.4 M NaCl and incubating the extract on ice for 20 min. The resulting lysate was centrifuged for 10 min at 500×g at 4°C to pellet nuclear debris and the supernatant was used or quickly frozen in dry ice and stored at −70°C.

Gel retardation assay

DNA fragments were end-labeled with T4 polynucleotide kinase and isolated by polyacrylamide gel electrophoresis. The purified DNA fragments were incubated with the *Xenopus* nuclear extract and assayed for protein binding by mobility shift analysis. Binding reactions contained 0.5−2 ng (5000 cpm) of labeled fragment and 5−10μg nuclear extract proteins in 20 μl of binding buffer (15 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 5 mM $MgCl_2$, 0.1 M NaCl, 0.5 mM DTT, 5% glycerol) in the presence of 0.75−1μg double stranded poly (dI-dC) or 2−4μg sonicated *E. coli* chromosomal DNA as non specific competitors. After 30 min incubation on ice, the binding reactions were added with 3% glycerol and run on a 4% acrylamide gel in 0.25×TBE (22.5 mM Tris-base, 22.5 mM boric acid and 0.25 mM EDTA). Pre-electrophoresis (30 min) and electrophoresis were carried out in the cold room at 10 V/cm. Gels were dried and autoradiographed.

ExoIII protection

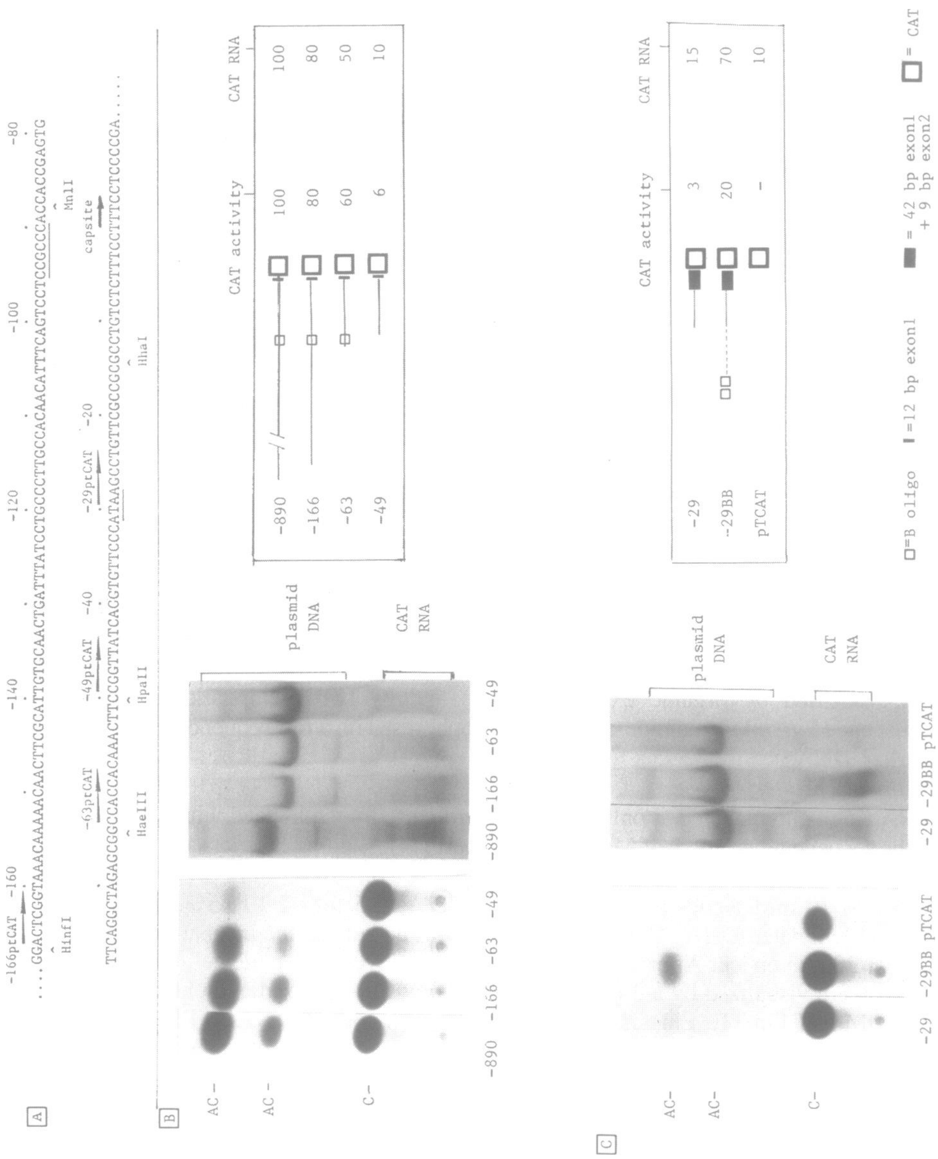
ExoIII protection assay was performed following the protocol described by Wu (19). A DNA fragment, 5′ end-labeled at one terminus (20−40.000 cpm), was incubated for 30 min on ice with 15μg nuclear extract proteins and 1.0μg ds poly (dI-dC). 3−5 μl Exonuclease III (Boehringer-Mannheim, 225 U/ μl) were added to the binding reactions and incubation was carried out at 30°C for 13 min. Control reactions, without extract in the binding mix, were digested with 1 or 2 μl ExoIII. Digestions were stopped with one volume of stop mix (20mM Na2EDTA, 1% SDS), diluted with one volume TE, and DNA purified by organic extraction twice and by two precipitations. Pellets were resuspended in 5μl formamide-dye and electrophoresed on a 6% sequencing gel along with marker DNAs.

Methylation interference

DNA fragments, labeled at only one end, were partially methylated with DMS according to the sequencing protocol of Maxam and Gilbert (14). Binding reactions (60 μl), containing 60μg nuclear extract proteins and 6μg sonicated *E. coli* DNA, were preincubated 10 min on ice in 10 mM Hepes pH 7.9, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 0.1 M NaCl. Methylated DNA (450−500.000 cpm) was added and the incubation continued for further 20 min on ice. After separation on a 4.5% native acrylamide gel, free and retarded bands were visualized by a 3 hr exposure to X-ray film, excised and eluted o.n. at 50°C in 0.2 M NaCl in TE. Eluates were purified on mini-columns (NACS Prepac from BRL) and ethanol precipitated. Pellets resuspended in 40 μl 1 M piperidine, were processed according to Maxam and Gilbert (14). An equal number of cpm of free and bound DNA were loaded into each slot of a 10% sequencing gel.

Preparation and cloning of the B oligonucleotide

The 36mer 5′AGCTTACCACAAACTTCCGGTTATCAGGTGTTCCCA 3′ oligonucleotide and its complementary 5′ AGCTTGGGAACACCTGATAACCGG-AAGTTTGTGGTA 3′ strand were synthesized with a Dupont Coder 300 authomatic DNA synthesizer. Purification of the oligonucleotides and reannealing was according to the


A
-166ptCAT -160 -140 -120 -100 -80
....GGACTCGGTAAACAAAAACAACTTCGCATTGTGCAACTGATTTATCCTGCCCTTGCCACAACATTTCAGTCCTCCGCCCACCACCGAGTG
HinfI
MnlI
-63ptCAT -49ptCAT -40 -29ptCAT -20 capsite
TTCAGGCTAGAGCGGCCACCACAAACTTCCGGTTATCAGGTGTTCCCATAAGCCTGTTCGCCGCGCCTGTCTCTTCCTTTCCTCCCCGA.....
HaeIII
HpaII
HhaI
B
AC -
AC -
C -
-890 -166 -63 -49
-890 -166 -63 -49
plasmid DNA
CAT RNA
CAT activity
CAT RNA
-890 100 100
-166 80 80
-63 60 50
-49 6 10
C
AC-
AC-
C-
-29 -29BB pTCAT
-29 -29BB pTCAT
plasmid DNA
CAT RNA
CAT activity
CAT RNA
-29 3 15
-29BB 20 70
pTCAT - 10
□=B oligo
▮=12 bp exon1
■ = 42 bp exon1 + 9 bp exon2
□= CAT

protocol developed by Kadonaga and Tjian (20). The double stranded oligo is the copy of the −61 to −30 L14 promoter sequence, flanked by HindIII cohesive ends.

RESULTS

Analysis of the upstream region of the L14 gene

The gene coding for the ribosomal protein L14 had previously been characterized and sequenced (4,12). The initiation of transcription was shown not to be unique but to be localized for the large majority of transcripts about 30 bp downstream from a non-canonical TATA box, in a tract of consecutive pyrimidines typical of the ribosomal protein genes capsite.

The region of the L14 r-protein gene initially analyzed for promoter activity extended, with respect to the capsite, from −890 to +12. This DNA fragment, cloned in the plasmid pTCAT (13) efficiently directed expression of the reporter CAT gene (fig.1B).

In order to identify the sequences relevant for the expression of the L14 promoter, we performed a deletion mutant analysis by shortening the region from the 5′ end. The deletion mutants were also cloned in the ptCAT vector, which contains the SV40 late polyadenylation signal. Due to the poly(A) tail the CAT trancripts were very stable in the oocyte and the CAT assay could be performed 3 hr after injection. Incubations for 4 or 5 hr gave similar results. No CAT activity after injection of pTCAT alone was detected in these short incubation times (fig.1C). When transcription was allowed to continue overnight, however, the unspecific transcripts from the vector accumulated to give a positive signal in the assay (not shown). A minimum of 50 oocytes were injected with the same amount of the different constructs (4 nanograms / oocyte). This is almost a saturating amount for the oocyte: by doubling the amount of plasmid we obtained less than a doubling of CAT activity; four times as much was inhibitory (not shown).

The CAT assay gave a fast answer while testing the various deletion constructs, but due to the multiple sources of variability in the injection of the oocytes, transcription of the various constructs was analyzed by Northern blots and the specific CAT transcripts normalized to oocyte endogenous 5S RNA and to the amount of CAT plasmid present in the same slot. CAT DNA and CAT RNA were distinguished by RNAse treatment of the samples (not shown). In fig. 1B we show one of the CAT assays made with some deletion mutants and the values of CAT activity indicated in the inset are the average of 3 different experiments. The −166pTCAT construct directed CAT transcription at a level

Figure 1. Expression of deletion mutants of the L14 promoter region. A) sequence of the −168 to +12 L14 region. The arrows indicate the 5′ end of the fragments cloned into pTCAT. A putative Sp1 binding site at −95 and the TATA-like box at −31 are underlined. B) CAT assay (left) and Northern blot (right) after injection of 4 ng/ oocyte of the recombinant pTCAT plasmids. Proteins corresponding to 2 oocytes were used in the CAT assay. Purified total nucleic acids corresponding to 4 oocytes were used for the Northern blot which was hybridized to isolated 780 bp CAT DNA. The values given in the inset are the average of 3 experiments and are expressed as a percent of the positive control used in each series of injections (−890pTCAT). C) Cat assay and Northern blot made in the same conditions as in B) after insertion of 2 copies of the B oligo (see fig.3) upstream of −29pTCAT. This plasmid contains 29 bp of L14 5′ flanking sequences, the entire first exon (42 bp) and 9 bp of the second exon cloned in front of the CAT sequence in pTCAT. The position of the oligo B in the plasmid is shown in the drawing. The values in the inset are expressed as percent of the positive control (−890pTCAT).

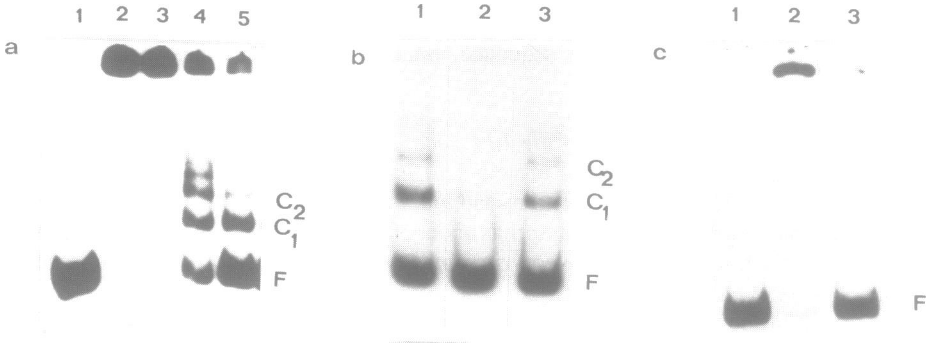


Figure 2. Binding specificity of the GV extract to the L14 upstream region. a) binding of the nuclear factors to 1.5 ng of the −568 to +12 L14 promoter region. 1) free probe 2) plus nuclear extract (5μg proteins) and no competitor DNA 3−5) as in 2 plus 0.5, 1, 2 μg *E. coli* DNA. b) 1) 1.5 ng of the same probe incubated with 5μg of nuclear proteins and 4μg of *E. coli* sonicated DNA. 2) as in 1), competed by a 30-fold molar excess of the same L14 unlabeled fragment. 3) as in 1), competed by a 30-fold molar excess of a prokaryotic 550 bp DNA fragment. c) Binding to a 279 bp pSP64 DNA fragment containing the SP6 promoter. 1) free probe 2) incubated with 5μg of nuclear proteins and no competitor 3) as in 2) but competed by 4μg *E. coli* DNA.

very similar to the −890 region. −63pTCAT showed 75−60% activity with respect to the longer constructs. A sharp drop of CAT activity and CAT transcription was observed with the −49pTCAT plasmid: these results indicate that a sequence located between position −63 and −49 is important for the efficient expression of the reporter gene.

To confirm the relevance of the sequence with a positive control, we synthesized a 36 bp double stranded oligonucleotide (B oligo) containing the sequence whose deletion negatively affected CAT transcription. One or two copies of the B oligo were cloned in upstream position in the HindIII site of the −29pTCAT mutant (described in Methods), which produced low levels of transcripts and undetectable CAT activity. Two copies of the B oligo enhanced transcription of the CAT gene and partially restored CAT activity (fig.1c). The effect was less pronounced when one copy of the B oligo was inserted (not shown). The failure of −29pTCAT and −29BBpTCAT to produce a CAT activity proportional to transcription could be explained by the presence in the mRNAs of two ATGs upstream to the one of the CAT gene: the first, located at the end of the L14 first exon, was in frame with the CAT ATG, but the second, created by the cloning procedure, was out of frame.

X. laevis crude nuclear extracts contain factors that specifically interact with upstream L14 sequences

Having established that the cloned L14 gene was efficiently transcribed after injection in the nucleus of *Xenopus laevis* oocytes (15) and that the 5′ non-coding region promoted expression of a linked reporter gene, we assayed crude *Xenopus* nuclear extracts for the presence of trans-acting factors capable to interact with the promoter region. Specific binding of nuclear proteins to the 5′ flanking region of the L14 gene was detected by gel retardation assay as shown in fig.2. Binding activity was monitored by incubating the end-labeled DNA fragment with the extract in the absence and in the presence of a non specific competitor DNA. In the absence of competitor DNA all of the probe was retained at the top of the gel (fig 2a, lane 2). With the addition of increasing amounts (0.5 to 2 μg) of *E. coli* sonicated DNA as competitor, a major retarded band was detected (fig 2a, lane

5, complex C1). Further increase of competitor DNA up to 8μg did not reduce the amount of the shifted species (not shown). Comparable results were obtained using *E. coli* sonicated DNA or double stranded poly(dI-dC) as competitors. We tested the optimal conditions for binding: 10 minutes were the minimum requirement and very little increase was observed beyond 30 minutes. The presence of Mg^{++} in the binding mixture was not required: Mg^{++} concentrations exceeding 5 mM had destabilizing effects. Optimal NaCl concentration was between 100 and 150 mM (results not shown).

In equivalent binding conditions relative to the amount of GV extract and of competitor DNA, a prokaryotic DNA fragment produced a barely detectable band shift, indicating that the factors responsible for binding to L14 do not recognize the heterologous DNA (fig.2c, lane 3).

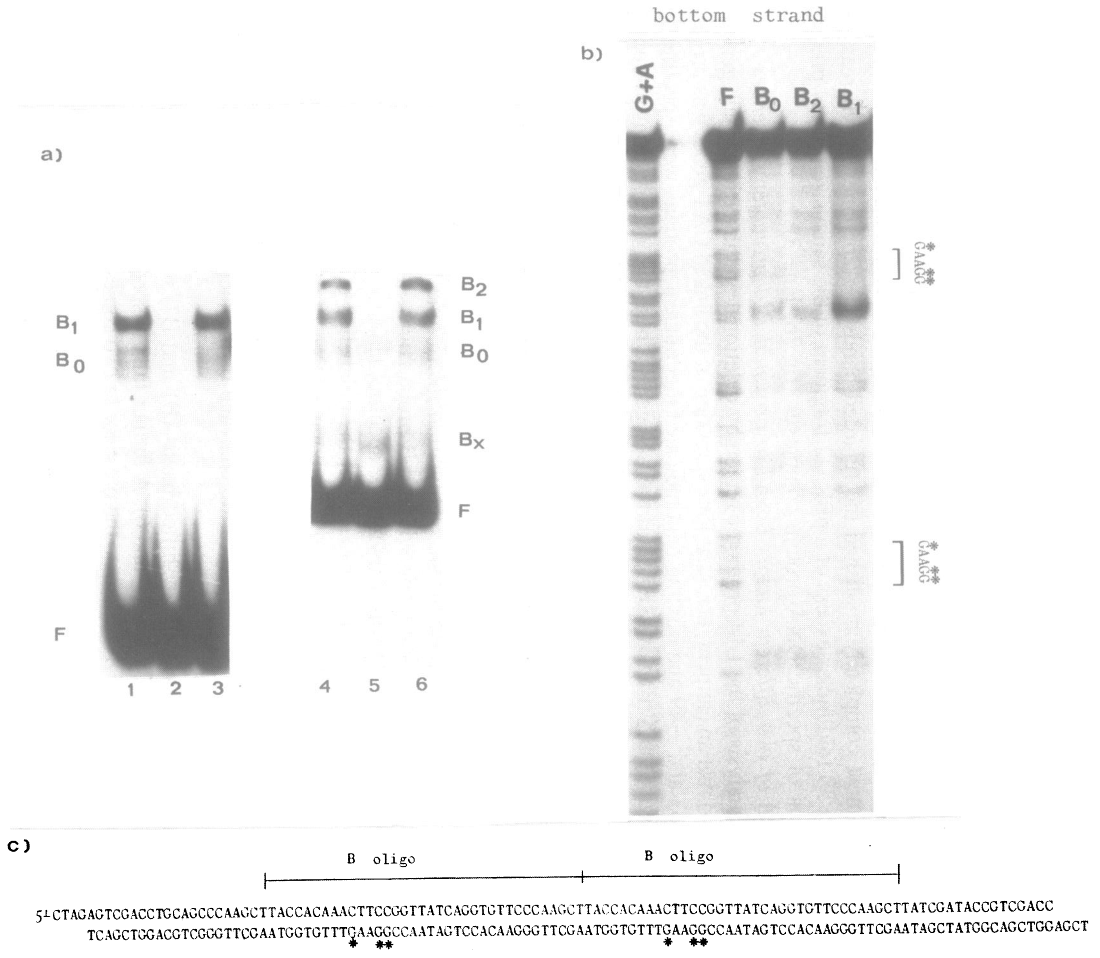
To test if the observed interactions were specific we performed the competition experiment shown in fig.2b. The addition of a 30-fold molar excess of the cold versus the labeled fragment almost completely abolished the retarded bands (fig.2b, lane 2), whereas the addition of a 30 fold molar excess of a pBR322 fragment of the same size did not prevent the formation of specific DNA-protein complexes (fig.2b, lane 3).

Identification of two different factors bound to proximal L14 promoter sequences

Functional analysis of the L14 promoter region by deletional and insertional mutagenesis demonstrated that an element, essential for expression of the linked CAT gene, was present between −63 and −49 and binding data indicated that nuclear proteins were specifically interacting with the 5′ flanking region of the L14 gene. These two observations prompted us to search for the factor(s) interacting with the −63 and −49 region of the L14 gene and to define the sequence element involved in the binding. For this purpose we first used a double stranded DNA probe containing one or two tandemly linked copies of the 36 bp B oligonucleotide encompassing the transcriptionally relevant promoter element (B probe and BB probe respectively). DNA-protein complexes formed between the probes and the GV extract were seen by the gel retardation assay (fig.3a) and the G residues involved in the binding were identified by the methylation interference analysis (fig.3b).

In the gel retardation assay made with the B probe (fig 3, lanes 1−3) one prominent DNA-protein complex (B1) was shown to be specific, since it was completely competed out by addition of a 100-fold molar excess of the unlabelled oligonucleotide, but not by addition of the same molar excess of an heterologous DNA of the same size (fig.3a, lanes 2 and 3). A broad faster migrating band (Bo), specific on the basis of competition experiments, constantly appeared in the gels, but its relative amount varied from batch to batch of the extract; a partial proteolitic cleavage of the factor might account for the presence of these species.

Using the BB probe, two major shifted bands (B1 and B2) were observed in addition to the Bo component (fig.3a,lanes 4 and 6). A further faint unspecific band (Bx) sometimes migrated close to the free DNA. Due to the presence of two copies of the relevant sequence, two different band shifts were expected when one or both sites were occupied by the factor(s). The appearance of the slow migrating B2 band when the free DNA was still present and the fact that B1 and B2 abundance is the same, suggest cooperativity in the binding of this factor. To identify the contact points of the protein on DNA, we made the methylation interference assay shown in fig.3b. The BB probe, labeled at the top or the bottom strand, was partially methylated and used in a binding reaction as described in Methods. Piperidine cleavage products, relative to free DNA and B0,B1 and B2 complexes, were analyzed on sequencing gels. All the retarded bands produced the same


a)
B1
B0
F
1 2 3
4 5 6
B2
B1
B0
Bx
F
b)
bottom strand
G+A
F
B0
B2
B1
GAAGG
GAAGG
c)
B oligo
B oligo
5'-CTAGAGTCGACCTGCAGCCCAAGCTTACCACAAACTTCCGGTTATCAGGTGTTCCCAAGCTTACCACAAACTTCCGGTTATCAGGTGTTCCCAAGCTTATCGATACCGTCGACC
TCAGCTGGACGTCGGGTTCGAATGGTGTTTGAAGGCCAATAGTCCACAAGGGTTCGAATGGTGTTTGAAGGCCAATAGTCCACAAGGGTTCGAATAGCTATGGCAGCTGGAGCT

interference pattern, suggesting the involvement of the same factor, that was termed XrpFI (*Xenopus* ribosomal protein factor I). Three guanines of the bottom strand were clearly involved in the binding. These guanine residues, indicated by asterisks in the sequence reported in the figure, belong to a short sequence motif CTTCC that in the fragment used is repeated twice. Hypermethylation sites, mapping very close to the binding sites, were present. No methylation interference was observed on the G residues of the top strand.

In order to confirm these observations, gel retardation, methylation interference and ExoIII protection assays were repeated on a DNA fragment containing the −63 to +12 L14 promoter region (L14 probe). The size of this probe (105 bp), flanked by polylinker sequences, is the same as the previously used BB probe. The L14 probe is shown in fig.4e. In the mobility shift assay, two shifted bands were obtained incubating the L14 probe with the GV extract: a fast non resolved doublet (A), preceded by a faint smear, and a slower single band (B). All of these bands were shown to be specific by competition with a 30-fold molar excess of the unlabeled probe fragment (fig.4a, lane 4). The band shift pattern obtained with the L14 probe appeared to be different from that produced by the B probe, which shares with the former the −63 to −30 L14 promoter region.

To identify the complexes formed by the same factor, the BB probe and the L14 probe, bound to the GV extract, were run on the same gel (fig.4 b, lanes 1 and 2). The B oligo was used to compete the binding on the L14 probe (lane 3). The A complex in these conditions is not competed, indicating that it is formed by a factor different from XrpFI, while the B complex disappears.

A further evidence for the binding of two different factors comes from an ExoIII protection assay (fig.4c). Digestion of the L14 probe, 5′ labeled in the top strand, resulted in a prominent resistant fragment which mapped at position −38, just bordering the right side of the CTTCC box. The ExoIII digestion on the fragment labeled in the bottom strand produced two major resistant fragments not present in the control digest, mapping at −63 and −36 from the capsite. The −63 stop borders the left side of the CTTCC element, consistent with the binding of a factor on that motif. The other stop indicates that an additional protein is bound closer to the capsite. Precise mapping of the contact points of this second factor, that we called XrpFII, was obtained by methylation interference (fig.4d): the sequence involved was 5′GCCTGTTCGCC 3′, located between −27 and −17 from the capsite. A scheme of the localization of the two factors on the promoter and of the guanines that make contacts is shown in fig.4e.

Figure 3. Gel retardation and methylation interference analysis on the B and BB probes. a) the B probe (HindIII-HindIII monomer of the B oligo), (left) and the BB probe, shown in c), (right), were bound to 7.5μg of nuclear proteins in the presence of 0.75μg of sonicated chromosomal *E.coli* DNA (lane 1 and 4). A 100-fold molar excess of the unlabeled B probe (2 and 5) or of a pBr322 fragment of the same size (3 and 6) were added as competitors. F=unbound probe. B(n)= DNA/protein complexes. b) Methylation interference pattern of the nuclear proteins on the BB bottom strand. F= unbound DNA; B(n)=bound DNAs; G+A= Maxam and Gilbert sequencing reactions on the same probe. Brackets indicate the guanines whose methylation prevents binding. c) sequence of the BB probe, made by two linked copies of the B oligo and flanking polylinker regions. Astrerisks indicate theμg residues involved in binding.

DISCUSSION

Sequence data of the region preceding the capsite of the *Xenopus laevis* L14 gene indicated that this housekeeping gene lacks both TATA and CAAT boxes: primer extension and S1 mapping showed that the majority of the L14 transcripts in the oocyte starts 30 bp downstream from the ATAA sequence reminiscent of the TATA box, in a stretch of consecutive pyrimidines (4).

We were interested in the identification of the essential promoter elements in a gene with housekeeping functions and used the CAT assay to monitor promoter activity in the natural environment of the r-protein genes, i.e. the frog oocyte and also assayed the oocyte nuclear extract for the presence of specific DNA binding factors.

The functional analysis showed that the 900 bp region 5′ to the L14 gene efficiently promoted the expression of the CAT gene in the oocytes and that this activity was not significantly reduced in the mutants up to −166 (data not shown). A reduction was obtained when the upstream sequences were deleted to −63 from the capsite. In particular the −63 mutant retained about 60−70% of the whole fragment activity. The −49 deletion mutant, on the contrary, showed a dramatic drop of CAT activity and of the relative mRNA. A sequence element located between −63 and −49 is then essential for L14 promoter function. A confirmation was obtained by cloning the B oligo, which is a copy of the −61 to −30 L14 region, in front of the CAT gene in the −29ptCAT deletion mutant, which showed CAT activity close to the background. Two copies of the B oligo, cloned in reverse orientation 40 bp upstream of the L14 sequence, very efficiently enhanced transcription from this construct.

The analysis of nuclear protein binding, restricted to the −63 to +12 region, showed the interaction of two distinct factors: XrpFI which makes contacts with the upstream box, 5′ CTTCC 3′, and XrpFII, interacting with the proximal element 5′GCCTGTTCGCC 3′, located between the TATA-like box and the capsite. No binding was observed on the capsite.

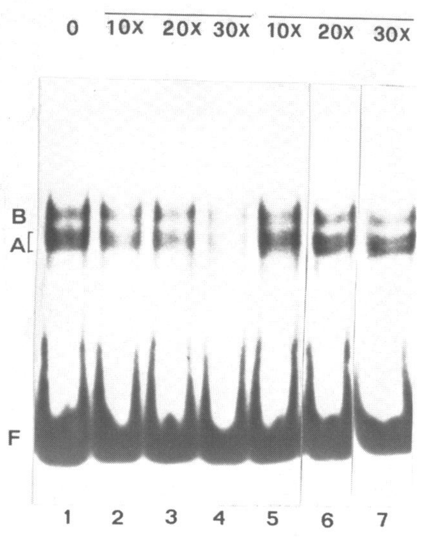
The two factors seem to bind to their recognition sequences independently, since deletion of the XrpFI binding site (data not shown), or competition with excess B oligo DNA (fig.3b), did not prevent the interaction of the XrpFII to the proximal sequence element.

The efficiency of L14 promoter transcription in *X. laevis* oocytes depends on the presence of the distal CTTCC element and, presumably, XrpFI represents the corresponding transcriptional activator.

A drop of activity is observed in the deletion from the −166 to the −63 mutant. The presence of an Sp1 binding site at −95 from the capsite could represent an additional element contributing to the promoter expression. A *Xenopus* factor, homologous to human Sp1, has been identified in nuclear extracts by Tebb and Mattaj (21).

At the moment we have no data to assign a function to the proximal element or to the XrpFII factor, since their presence is not per se enough to stimulate transcription. However we cannot exclude their contribution to the basal level of transcription or their participation in the formation of a stable preinitiation complex.

The involvement of downstream promoter elements in the control of ribosomal protein transcription is well documented in mouse (6,7). From the results presented here, it emerges a)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10X
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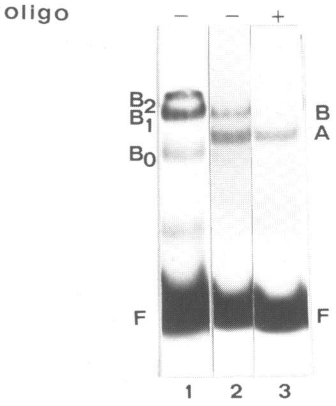
b)


oligo
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B_2
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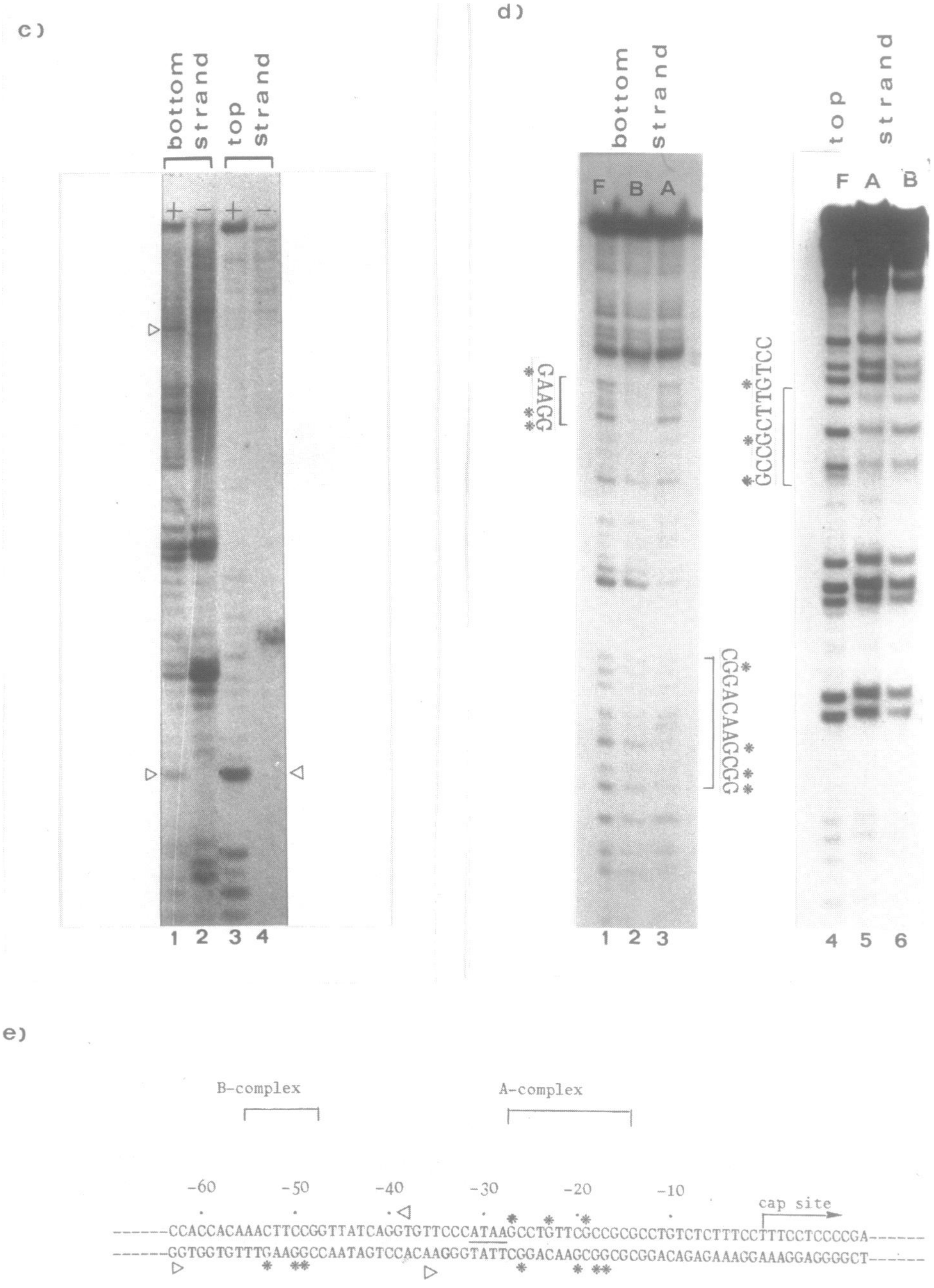
c)
bottom strand
top strand
+
−
+
−
1
2
3
4
d)
bottom strand
F B A
GAAGG
CGGACAAGCGG
1
2
3
top strand
F A B
GCCGCTTGTCC
4
5
6
e)
B-complex
A-complex
-60
-50
-40
-30
-20
-10
cap site
------CCACCACAAACTTCCGGTTATCAGGTGTTCCCATAAGCCTGTTCGCCGCGCCTGTCTCTTTCCTTTCCTCCCCGA------
------GGTGGTGTTTGAAGGCCAATAGTCCACAAGGGTATTCGGACAAGCGGCGCGGACAGAGAAAGGAAAGGAGGGGCT------

that the promoter activity of the L14 gene strongly depends on 5′ flanking sequences. However the contribution of downstream elements cannot be excluded since most of the deletion mutants, here analysed, retain only a few bases beyond the capsite. The only exception concerns the −29pTCAT constructs in which the whole first exon and 9 bases of the second are present. If the CAT transcripts directed by sequences present in −29pTCAT are compared with those of the −49pTCAT mutant, which maintains only 12 bases of the first exon, it appears that the construct with the whole first exon produces 5% more transcripts (fig.1). Functional analysis of the L14 downstream sequences and binding of nuclear factors to these regions are under investigation in order to identify additional promoter elements.

It has been suggested (1,22) that the promoters of the genes expressed in a constitutive way would show less complexity than that of the inducible ones and that strong or very complex enhancers or tissue specific elements would not be required.

In yeast, most of the available data are consistent with a coordination of the synthesis of the various components of the ribosomal protein family operating at the level of transcription, with a regulatory mechanism which acts through the interaction of cis-acting elements (UAS-rpg and HOMOL1) with trans-acting factors (10).

In higher eukaryotes, sequence data of the rp-genes from organisms as different as human, *Drosophila* and *Xenopus* show a striking similarity in the architecture of the rp-promoters. The upstream or downstream regulative elements which control in a coordinate way the rp-gene expression are under investigation. In mouse, elements necessary for full activity of the rp-gene promoters have been identified in the region preceding the capsite, in the first exon and in the proximal end of the first intron (6−7).

In *Xenopus laevis* we found a transcriptionally relevant sequence in the region preceding the capsite of the L14 gene. It is worth to notice that in the L14 gene of the closely related species *Xenopus tropicalis* the region up to −100 from the capsite is 90% conserved (4).

We have searched for the presence of sequences homologous to the L14 CTTCC box in the upstream region of two other *Xenopus laevis* rp-genes: two similar sequences are found at −50 and −170 in the S19 rp-gene (P.Mariottini, personal communication) and

Figure 4−Distinct factors of the X.laevis GV extract bind to the −63 to +12 L14 promoter region. a) Mobility shift of the −63 to +12 L14 region (L14 probe) shown in e). Binding was performed with 7.5μg of GV proteins, 1.25μg sonicated *E. coli* DNA and the indicated molar excess of unlabeled L14 probe (1−4) or a prokaryotic DNA fragment of the same length (5−7). F=unbound DNA; A and B= DNA/ protein complexes which are competed only by the homologous fragment. b) Band shift assay with the BB probe (1), with the L14 probe (2) and with the L14 probe competed by a 100 fold molar excess of the BB probe (3). Band A, remaining after competition, is due to a factor interacting with the proximal half of the region. c) Exonuclease III protection analysis. The L14 probe, labeled in the top or bottom strand, was digested with Exonuclease III in the presence (+) or in the absence (−) of GV extract. Open arrowheads indicate the main ExoIII stops. d) Methylation interference assay on the same probe. The guanines whose methylation interferes with binding of nuclear proteins are included into brackets. F= free DNA. A and B = DNA purified from the retarded A and B complexes shown in panel a) of this figure. e) Schematic representation of the interactions between *X.laevis* nuclear factors and the −63 to +12 L14 region. Open arrowheads indicate the ExoIII stops. Asterisks mark guanines whose methylation interferes with protein binding. The TATA like box at −29 is underlined.

at −69 and on the capsite in the L1 rp-gene. The involvement of these sequences in transcription is under investigation.

Interestingly we have noted that sequence elements homologous to the XrpFI binding site are present in the transcriptionally relevant upstream sequences of human (23) and mouse (6) rp-genes. Moreover, a good homology seems to exist between the yeast UAS-rpg consensus sequence ACACCCATACATTT and the L14 region from −53 and −35.

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