Nucleic Acids Research, Vol. 19, No. 23 6633–6637

Identification of a sequence-specific protein binding the 5′-transcribed spacer of rat ribosomal genes

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Received April 4, 1991; Revised and Accepted October 18, 1991

ABSTRACT

A novel 85-kD protein factor which interacts specifically with the 5′-transcribed spacer of rat ribosomal genes was identified using the gel mobility shift, DNase I protection and UV-crosslinking techniques. The binding site of the factor is located inside the 36 bp AluI-HindIII fragment of transcribed spacer, most probably in the region +94 to +115 with respect to the transcription initiation site. Factors giving very similar gel mobility shift patterns were also found in mouse and human cell extracts. Sequences resembling the binding site of this factor were revealed in corresponding regions of mouse and human ribosomal genes. The biological function of FTS remains to be elucidated.

INTRODUCTION

Upstream regions located 5′ to transcription initiation sites of ribosomal genes have some structural features which are typical for higher eukaryotes. In particular, the RNA-polymerase I main promoter consists of two functional regions, the core promoter and the upstream control element (UCE), which are highly homologous among higher eukaryotes. This type of organization was shown for *Xenopus laevis* (1), mouse (2), rat (3) and human (4, 5) ribosomal genes. Besides the main promoter, 5′-upstream regions of rat and mouse ribosomal transcription units also contain spacer promoters (6, 7) and spacer terminators (8–10).

It is believed that the mechanisms of transcription initiation by RNA polymerase I are similar for higher eukaryotes and include interaction of various protein factors with functional promoter elements (reviewed in (11)). Transcription factors UBF and SL 1 were shown to interact with the core element and UCE of the human, rat and mouse main promoters as well as rat spacer promoter. UBF, SL 1 and RNA-polymerase binding factor TIF-1C (12) together are sufficient for initiation of transcription by RNA polymerase I (3, 13–21). UBF is not species-specific and can be substituted by its analogues from other mammals in an *in vitro* transcription system (16, 19, 20). This factor is represented by two polypeptides with molecular mass values of 82 and 87 kD for *Xenopus* (21), and 94 and 97 kD for human, rat and mouse cells (13, 16, 17, 21). In contrast to UBF, SL 1 is a species-specific protein which when bound to DNA determines the specificity of transcription initiation (15, 16, 18, 20). Mouse SL 1 can bind ribosomal promoter sequences independently of UBF and its binding site is identical to that of another 44-kD mouse transcription factor TIF 1B (16, 22, 23).

In addition to the above mentioned transcription factors there is a 16-kD rat protein factor belonging to the HMG family (24). The 16 kD protein increases the efficiency of transcription initiation from main and spacer promoters and specifically binds to two 200-bp regions located downstream from the spacer promoter and within the transcribed spacer, respectively.

Previously (25) we have identified several protein factors which bind specifically to different regions of rat ribosomal genes. These factors bind to a putative enhancer, spacer promoter, a fragment containing the main promoter and a part of the transcribed spacer. In this work we characterize a protein factor that interacts specifically with the 5′-transcribed spacer of the rat ribosomal unit. We have localized the binding site of this factor, determined a molecular mass value and studied some of its binding properties.

MATERIALS AND METHODS

Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, Klenow fragment of *E.coli* DNA polymerase I were obtained from 'Ferment' (USSR). DNAse I, HEPES, deoxyribonucleoside triphosphates and *E.coli* tRNA (Sigma), poly d(AT) (Boehringer) were used. [alpha-^{32}P]dATP and [alpha-^{32}P]dCTP with a specific activity of 3000 Ci/mM were obtained from 'Isotop' (USSR).

The 17-nt M13 sequencing primer was prepared by Dr. B.Chernov. 5′-Bromo-2′-deoxyuridine triphosphate (BrdU) was obtained by bromination of 2′-deoxyuridine triphosphate (sodium salt, Sigma) and purified by ion-exchange chromatography on DEAE cellulose.

Plasmids, phages and DNA fragments

The pRr 56 plasmid was characterized earlier (25). Its subclone P3 harboring the SalI-HindIII fragment containing rat ribosomal promoter (P_o) in corresponding sites of pUC19 polylinker was obtained using standard cloning techniques (26). The 297 bp fragment P_o, representing the sequence from −176 to +122 of the spacer and including the UBF and SL 1 binding sites, was excised from P3 with restriction endonucleases EcoRI and HindIII, end-labelled using the Klenow fragment of *E.coli* DNA polymerase I, and electrophoretically purified. The fragment P_oR (136 bp, −14 to +122) was cut out of the plasmid P3 with RsaI and HindIII, end-labelled and purified similar to P_o. This

fragment does not contain the UBF binding site and contains less then a half of the core promoter element. The 36 bp fragment P_0A (3'-end of the P_0R fragment, +87 to +122) was cut out of the plasmid P3 by digestion with AluI and HindIII and subcloned in two orientations in phage vectors M13mp18 and M13mp19 (26).

Presented above are the lengths of the coding strands of the fragments. Figure 1 illustrates the location of the fragments within the ribosomal unit.

Cell lines and nuclear extracts

Nuclear extracts from the cell lines NIH 3T3 (mouse fibroblasts), OAB2A (rat fibroblasts) and HeLa were obtained as described previously (25).

Gel mobility shift assay (27)

For DNA-protein binding, one of the end-labelled fragments P_0, P_0R or P_0A (0.2–1 ng) was mixed with 2 μg of poly d(AT) and 5–10 μg of the nuclear extract protein in a binding buffer (12 mM Hepes-KOH pH 7.9, 12% glycerol, 60 mM KCl, 0.3 mM EDTA, 0.6 mM dithiothreitol, 5 mM $MgCl_2$), resulting in a final volume of 15 μl (25). The binding reaction was carried out for 30 min at room temperature and the resulting mixture was loaded on a 5% (P_0, P_0R) or 7% (P_0A) polyacrylamide gel with an acrylamide/bisacrylamide ratio of 30:1 and electrophoresed at 4°C for 2.5 hours at 200 V in 0.5×TBE buffer. After separation the gels were dried and autoradiographed.

Fractionation of OAB2A nuclear extract

The OAB2A nuclear extract protein in the binding buffer (see above) was loaded on a heparin-agarose column and the column was washed extensively with the same buffer. After this the column was stepwise eluted with the same buffer, additionally containing 0.4 M and 0.6 M KCl (final concentrations of 0.46 and 0.66 M KCl, respectively), and binding activities of eluted fractions and of a flow-through fraction with respect to P_0A were measured by the gel mobility shift assay. About 10–15% of the extract protein was eluted in the 0.66 M KCl fraction.

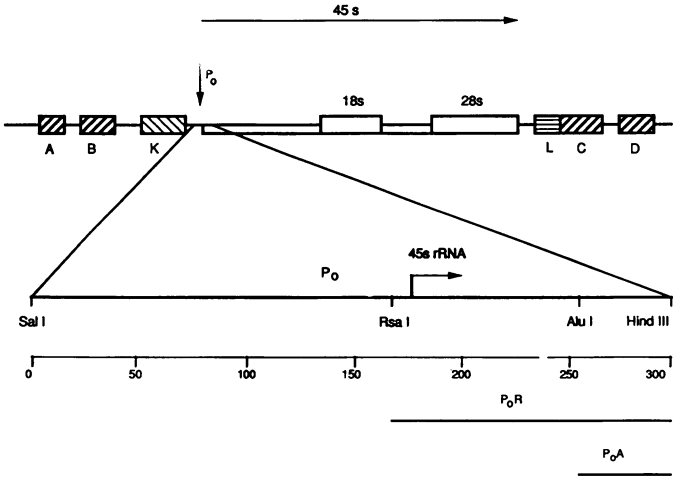

Figure 1. The structure of P_0 region containing the main promoter and a part of the external transcribed spacer of rat ribosomal unit. P_0R and P_0A denote DNA fragments used for the determination of the protein factor binding site and its molecular mass value, respectively.

DNAse I footprinting (28)

In order to form DNA-protein complexes 0.5 ng of P_0R (3'-labelled on coding strand using the Klenow enzyme) was mixed with 15–100 μg of the OAB2A nuclear extract protein fraction which had been eluted from the column with 0.66 M KCl and 5 μg of poly d(AT). The mixture was incubated for 30 min at room temperature in binding buffer (see above). $MgCl_2$ and $CaCl_2$ were then added up to concentrations of 5 mM and 2.5 mM, respectively. P_0R bound to proteins was digested with 10 ng of DNAse I in a final volume of 50 μl for 1 min at 20°C. The reaction was stopped by addition of EDTA up to a final concentration of 20 mM. The mixture was then digested with proteinase K and extracted with phenol-chloroform. The DNA was precipitated with ethanol, washed and dissolved in 20 mM NaOH, 60% formamide, 0.0025% bromophenol blue and 0.0025% xylene cyanol buffer. The sample was then denatured for 2 min in boiling water and applied to a 10% sequencing gel (29).

Preparation of labelled and BrdU-substituted P_0A

The second strand of either M13mp18 or M13mp19 P_0A-containing templates (annealed with the 17-nt sequencing primer) was synthesized using 25 μCi [alpha-^{32}P]-dATP or [alpha-^{32}P]-dCTP, 25 μM of the lacking NTPs (BrdU triphosphate instead of TTP) as described (30). In either case the resulting 'uniformly' labelled fragment, P_0A, was cut out from the double-stranded DNA with EcoRI and HindIII and its single-stranded ends were filled in with Klenow enzyme. Finally, the fragment was electrophoretically purified in a 6% non-denaturing polyacrylamide gel. As a result, double-stranded P_0A DNA was obtained in which either coding or noncoding strand was labelled with [^{32}P]dA or [^{32}P]dC and contained BrdU residues substituted for thymidine. Substitution of thymidine residues by BrdU can increase the yield of UV-induced DNA-protein crosslinks by an order of magnitude (30).

Preparation of UV-crosslinked DNA-protein complexes

0.1 pmole P_0A DNA with either coding or noncoding labelled and BrdU-substituted strand was mixed with 4 μg of poly d(AT) and 15 μg of the OAB2A nuclear extract protein, incubated for 30 min at room temperature in a binding buffer, and UV-crosslinked as described earlier (30). The crosslinked DNA-protein complexes were separated from unbound P_0A on a

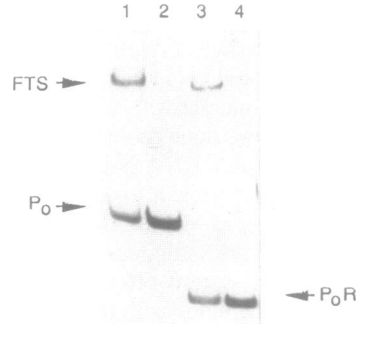

Figure 2. Gel mobility shift assay for P_0 and P_0R. The reaction mixture contained 2 μg of poly d(AT), 10 μg of OAB2A extract protein, 1 ng of end-labelled P_0 (lanes 1, 2), 0.5 ng of end-labelled P_0R (lanes 3, 4), 15 ng of unlabelled P_0 (lanes 2, 4).

mobility shift gel. Autoradiographically localized DNA-protein complexes were excised from the gel and eluted as described (29) in the presence of 1 mM PMSF and 30 μM leupeptin. The eluted complexes were precipitated with ethanol, washed and then dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) with 0.05% SDS.

The label transfer (30)

The isolated UV-crosslinked DNA-protein complexes were digested with 10 μg of DNAse I in the presence of 5 mM $MgCl_2$, 1 mM PMSF and 30 μM leupeptin for 20 min at 37°C. The reaction was terminated by addition of SDS, glycerol and bromophenol blue to final concentrations of 2, 20 and 0.0025%, respectively. The reaction mixture was heated for 5 min at 100°C and loaded on an SDS–polyacrylamide gel (31).

Two-dimensional PAGE

The P_oA fragment was mixed with poly d(AT) and nuclear extract and then incubated and UV-crosslinked as described above. The resulting mixture was loaded onto the mobility shift gel (first dimension). After the separation, the gel strip containing the labelled P_oA and P_oA-protein complexes was cut out, incubated in 50 mM Tris-HCl, pH 6.8, 1% SDS, 2% 2-mercaptoethanol for 30 min at 37°C. The gel strip was then put on top of a 5% stacking gel and electrophoresed in the second dimension in a 15% SDS-containing polyacrylamide gel as described (31). The gel was dried and autoradiographed.

RESULTS AND DISCUSSION

A protein which specifically interacts with the P_oR sequence and its binding site

We have shown previously by means of mobility shift and competition that SalI-HindIII fragment (P_o, see Figure 1), spanning the main RNA-polymerase I promoter and part of the transcribed spacer of rat ribosomal unit, specifically binds to a protein factor (25). A more detailed study of the fragment P_o interaction with the protein factor (Figure 2), allowed us to isolate a 136 bp RsaI-HindIII fragment containing its binding site. The fragment was designated as P_oR (Figure 1). The residual SalI-RsaI fragment of P_o does not bind any protein factors under the conditions used, as judged by the results of the mobility shift assay (data not shown). The fragment P_oR was further used for identification of the protein factor binding site in DNAse I protection experiments. Rat nuclear extract was fractionated on a heparin-agarose column prior to the footprinting reaction. It is noteworthy that the protein factor in question is tightly bound to heparin-agarose and can be eluted only at 0.66 M KCl (Figure 3).

A P_oR coding strand DNA protection pattern obtained by the footprinting is presented in Figure 4. The protected region is about 22 nucleotides long and is located in the transcribed spacer close to a HindIII site. It spans nucleotides +94 to +115 relative to the transcription initiation site of 45 S RNA. Based on these data the 36 bp AluI-HindIII fragment P_oA (Figure 1), which is a part of P_oR and contains the whole protected region, was cut

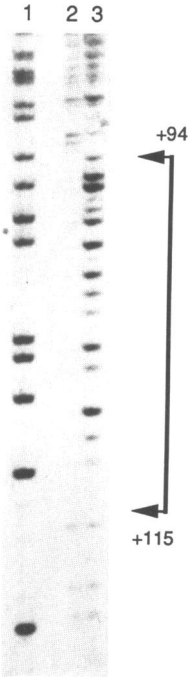

Figure 4. DNAse I protection. The reaction mixture contained 0.5 ng of end-labelled P_oR (lanes 2, 3), 5 μg of poly d(AT) (lanes 2, 3), 50 μg of protein from 0.66 M KCl eluted fraction (lane 2). The fragment was C-sequenced in a lane 1. FTS protects the region of P_oR from +94 to +115.

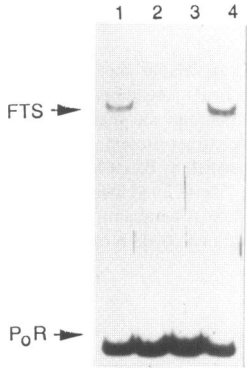

Figure 3. Fractionation of the OAB2A nuclear extract on heparin-agarose column. The reaction mixture contained 1 ng of end-labelled P_oR, 2 μg of poly d(AT), 10 μg of unpurified OAB2A extract protein (lane 1), 10 μg of flow-through fraction protein (lane 2), 3.6 μg of protein from a 0.44 M KCl eluted fraction (lane 3), 3.6 μg of protein from a 0.66 M KCl eluted fraction (lane 4).

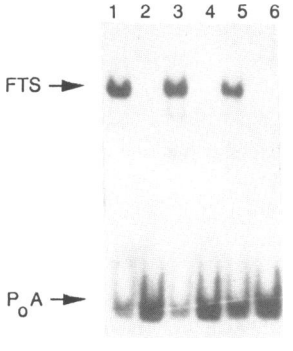

Figure 5. Gel mobility shift assay for P_oA. The reaction mixture contained 0.2 ng of end-labelled P_oA, 1 μg of poly d(AT), 5 μg of rat OAB2A extract protein (lanes 1, 2), 5 μg of HeLa extract protein (lanes 3, 4), 5 μg of mouse NIH 3T3 cells extract protein (lanes 5, 6) and 30 ng of unlabelled P_o (lanes 2, 4, 6).

out, subcloned and used in mobility shift experiments. The fragment specifically interacts with protein factors from rat, human and mouse extracts (Figure 5), the mobility of a single specific DNA-protein band being the same for all three extracts. P_oA competes for the protein factor in a similar way to P_o (25).

The protein component of the DNA-protein complexes

In order to characterize the protein recognizing the P_oA DNA, P_oA with radioactively labelled and BrdU substituted either coding or noncoding strands was complexed with OAB2A nuclear extract protein and the DNA-protein complexes were analyzed by two-dimensional gel electrophoresis. After the DNA-protein complexes were formed in mixtures with nuclear extracts, the complexes were crosslinked by UV-light and electrophoretically separated in the first dimension under conditions of mobility shift assay (Figure 6A). Only one dominant band corresponding to a specific DNA-protein complex was visible after this separation. An appropriate gel strip was electrophoresed in a second dimension SDS−polyacrylamide gel (Figure 6B, C).

Only one polypeptide can be crosslinked by UV to the DNA in the specific protein-DNA complex, because the main band of the first dimension gave only a single spot of cross-linked DNA-protein material in the second dimension (Figure 6). Nevertheless, other non-crosslinkable proteins can participate in the formation of the complex. The protein, specifically binding and crosslinkable to the P_oA DNA was designated as FTS (the transcribed spacer DNA-binding factor).

Determination of the molecular mass of the FTS

To determine the molecular mass of FTS the label transfer technique (30) was used. DNA-protein complexes of a UV-irradiated mixture of uniformly labelled 36 bp P_oA and the rat nuclear extract were electrophoresed in a non-denaturing mobility shift gel, and the band of the specific complex was cut out and its content was eluted from the gel. The DNA of the eluted covalently linked complex was further digested with DNase I and the digestion products were electrophoretically separated in an SDS−polyacrylamide gel as shown in Figure 7. FTS labelled with a residual short single-stranded oligonucleotide migrated as a homogeneous protein with a molecular mass value of 85 kD irrespective of the source of the label (either coding or noncoding strand). An average chain length of the residual oligonucleotides

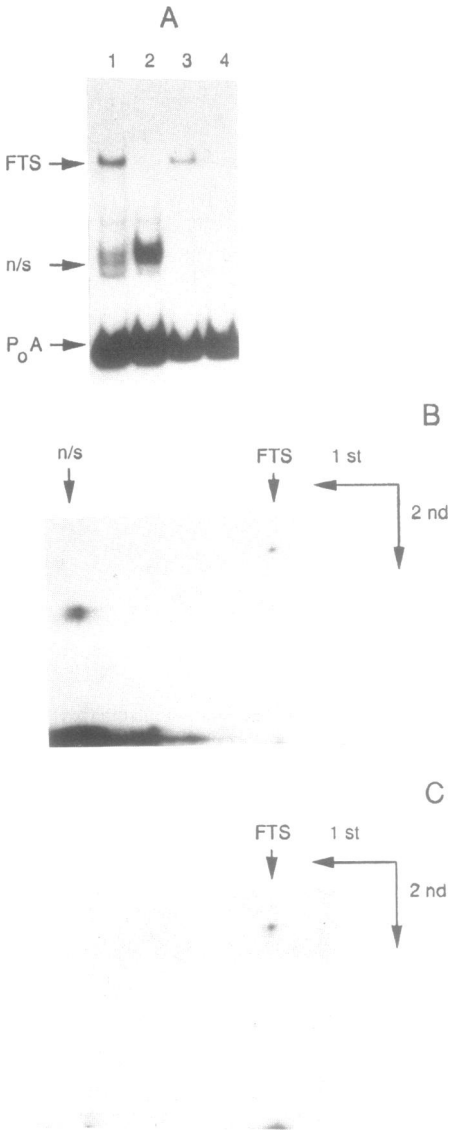

Figure 6. Two-dimensional PAGE 'mobility shift — UV cross-linked DNA-protein complex'. A. 0.8 ng of P_oA with BrdU-substituted and [^{32}P]-adenosine labelled noncoding (lanes 1, 2) or coding (lanes 3, 4) strands were mixed with 4 μg of poly d(AT), 20 μg of OAB2A extract protein, 45 ng of unlabelled P_o (lanes 2, 4), UV-crosslinked and electrophoresed in a band motility shift gel (first dimension). 'N/S' stands for non-specific bands. B. Second dimension. The lane analogous to 1 but after longer separation in the first dimension was put on a 12% SDS−polyacrylamide gel. C. The same as B, but with lane 3.

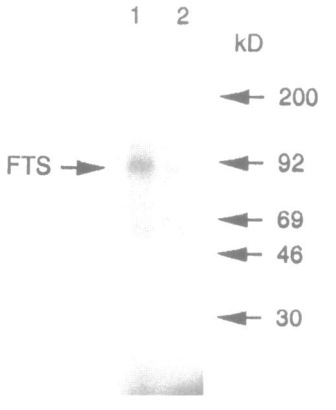

Figure 7. Determination of a molecular mass of the protein factor FTS by 'label transfer'. 2 ng of P_oA with BrdU-substituted and [^{32}P]cytidine labelled coding (lane 1) or noncoding (lane 2) strand was mixed and incubated with 8 μg of poly d(AT) and 50 μg of OAB2A extract protein. After UV-crosslinking the specific DNA-protein complex was purified by band-shift gel electrophoresis (see Materials and Methods), digested with DNase I and loaded on a 12% SDS−polyacrylamide gel. Molecular weight markers (Amersham): myosin — 200 kD, phosphorylase — 92 kD, bovine serum albumin — 69 kD, ovalbumin — 49 kD, carboanhydrase — 30 kD.

Table 1. Sequence motifs of rat, human and mouse ribosomal genes homologous to the FTS binding site.

Species	Sequence	Position	Ref.
Rat	CTTC...TGCGCATGGGCCTCT.TCG	+94, +115	[35]
Mouse	tTaC...TG.GCtTGGGtCTgTcgCG	+92, +113	[36,37]
Man	CTcCggaTGCGCgcGGGgCTCT.ggc	+46, +70	[38,39]
Consensus	TGnGCnnGGGnCTnT		

determined from the normalized content of the radioactive label before and after the DNAse treatment was about 5–6 nucleotides. Oligonucleotides of that size are known to cause only minor changes in mobility on SDS–PAGE of proteins which have been labelled in this way (30).

CONCLUSIONS

We have identified a new 85-kD rat protein factor (FTS) which specifically interacts with a transcribed region (TR) of rat ribosomal genes adjacent to the transcription initiation site. Protein factors binding P_0A and probably analogous to FTS seem to be also present in human and mouse nuclear extracts (Figure 5). External transcribed spacers of human and mouse ribosomal genes contain sequence motifs with up to 68% homology to the FTS binding site (Table 1). This suggests the existence of human and mouse FTS analogues interacting with these motifs. The region 3′ to the rat spacer promoter has no clear-cut homologies to the FTS binding site and apparently no factors bind there. A comparison of the sequence recognized by FTS with those recognized by polymerase II and III transcription factors (cited in (32)) did not reveal any striking homology.

As far as we know, the role of TR in transcription initiation was not revealed. TR seems not to be essential for *in vitro* transcription. Indeed, 3′-deletion mutants of the human ribosomal gene promoter have equal activity in transcription assays *in vitro* irrespective of the presence of TR (5). Moreover, constructs for *in vitro* transcription from rat ribosomal promoter do not contain this region at all (3) and the deletion of the corresponding mouse sequence does not affect the binding of mouse transcription factors by the promoter (33). TR region of mouse ribosomal genes also seems dispensable for transcription in transfected CHO cells (34). However, the role of TR (and consequently FTS) in transcription regulation can not be excluded. Except for FTS, an HMG-like protein, P16, which interacts with AT-rich sequence elements is known to bind the transcribed spacer. The interaction of P16 with either this or another binding site located between the main and spacer promoters stimulates transcription from both promoters *in vitro* (24).

In a previous paper (25) we described a specific DNA-protein complex formation in the rat spacer promoter region. On analysis of these complexes in two-dimensional polyacrylamide gels, we have found out that two closely migrating bands in mobility shift gels (25) contained at least four different proteins binding the spacer promoter region. These proteins have close molecular mass values not identical to that of FTS. Preliminary data suggest that not all of these proteins or perhaps none belong to the UBF and SL 1 groups. Therefore, it can be expected that new proteins interacting with the spacer region will be identified.

ACKNOWLEDGEMENTS

We are indebted to Dr E.Braga for kindly donating the clones of rat ribosomal genes and to Dr E.Zalmanson for the OAB2A rat cell line. We are also grateful to Drs B.Glotov, A.Itkes and M.Poznansky for critical reading of the manuscript.

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