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The nucleolar transcription activator UBF relieves Ku antigen-mediated repression of mouse ribosomal gene transcription

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ABSTRACT

Previously we have shown that the RNA polymerase I (Pol I)-specific transcription factor UBF stimulates transcription by both facilitating transcription complex formation and by relieving repression exerted by a negative-acting factor which competes for binding of the murine factor TIF-IB to the ribosomal gene promoter (1). We have purified and functionally characterized this repressor protein from Ehrlich ascites cells. The final preparation contained two polypeptides with molecular masses of 75 and 90 kDa, respectively. Both polypeptides interact with the rDNA promoter as revealed by UV-crosslinking experiments. The specificity of binding to the ribosomal gene promoter was demonstrated in an electrophoretic mobility shift assay and by DNase footprinting. The biochemical properties of this negative-acting factor closely resemble those of the Ku antigen, a human nuclear DNA-binding heterodimer which is the target of autoantibodies in several autoimmune diseases. Anti-Ku antibodies precipitate the repressor activity and overcome transcription inhibition. The data demonstrate that regulation of Pol I gene transcription may involve an antirepression mechanism as already documented for Pol II genes and suggest that Ku protein may be causally involved in repressor-mediated down regulation of rRNA synthesis.

INTRODUCTION

Regulation of gene expression is a complex process that can be achieved in multiple steps. Numerous studies have demonstrated that gene expression is primarily regulated at the level of transcription initiation. A great deal of effort has been directed toward the identification and isolation of positive-acting proteins that stimulate transcription. However, regulation of transcription also involves inhibitory processes, and multiple factors which specifically repress transcription have been described. These negative-acting proteins inhibit transcription either by steric hindrance or by direct competition with transcription activators.

In view of the growing number of specific factors which antagonize stimulatory proteins and therefore may play an important regulatory role, it is not surprising that the stringent control of rDNA transcription also involves the balanced action of both positive and negative factors. Similar to genes transcribed by RNA polymerase II and III, transcription initiation from the ribosomal gene promoter by RNA polymerase I (Pol I) is a multistage process requiring the action of at least four basal initiation factors which assemble at the promoter together with Pol I in an ordered fashion to form active preinitiation complexes (2). Promoter recognition is brought about by TIF-IB, a multisubunit factor which contains the TATA-box binding protein (TBP) and which is responsible for the observed species-specificity of rDNA transcription (3–5). In different groups and different systems this factor has been given different names, like SL1 (6, 7), PC-D, TFID or factor D (8–10), and Rib1 (11). This selectivity factor forms a strong cooperative complex at the rDNA promoter together with another DNA-binding protein, designated UBF for 'upstream binding factor' (12–14). Once TIF-IB and UBF are tethered to the DNA, Pol I together with two associated factors (TIF-IA and TIF-IC) can bind.

In previous studies on the mechanism of UBF-mediated transcription activation we found that the degree of UBF-directed transcription stimulation was highly variable depending on both the purity of transcription factors used and on the concentration of template DNA (1). In a reconstituted system containing highly purified Pol I and the initiation factors TIF-IA, TIF-IB, and TIF-IC, UBF only moderately augments transcription (ca. 3-fold). In a cruder system containing partially purified transcription factors and Pol I, UBF stimulates basal transcription at least 50-fold. This activation was not observed at high template concentrations or after preincubating the template DNA with TIF-IB. These results suggested that UBF activates transcription by relieving inhibition exerted by a negative-acting factor(s) present in crude Pol I and TIF-IA/TIF-IC fractions which prevents the formation of functional preinitiation complexes. The repressing activity depends on binding to the rDNA promoter and is therefore not due to 'squelching' of positively acting factors. By analogy to what is understood of the mechanism of transcriptional

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activation, the repressor(s) appears to destabilize or inhibit the formation of preinitiation complexes, probably by interfering with TIF-IB/promoter interactions. UBF, on the other hand, relieves the repression in the presence of TIF-IB, probably by stabilizing binding of TIF-IB to the rDNA promoter which in turn results in the formation of a stable preinitiation complex that can promote multiple rounds of initiation by Pol I.

As an initial step toward understanding the functional role of this transcriptional repressor in vitro, we have purified this activity and show that the biochemical properties of the purified factor closely resemble those of the Ku protein, a human nuclear autoantigen found in sera from patients with several autoimmune diseases (15–17). The data suggest that Ku protein may repress Pol I transcription and that UBF relieves this inhibition.

MATERIALS AND METHODS

Purification of transcription factors and RNA polymerase I

Cultivation of Ehrlich ascites cells, extract preparation and fractionation of individual transcription factors has been described previously (2). The fractionation scheme is diagrammed in Figure 1A. Extracts (200 ml of a mixture of nuclear and cytoplasmic extracts) were chromatographed on DEAE-Sepharose CL-6B, followed by fractionation on Heparin-Ultrogel A4-R by step-elution with buffer AM (20 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 20% glycerol, 5 mM $MgCl_2$) containing different salt concentrations. TIF-IA and TIF-IC eluted at 200 mM KCl, RNA polymerase I at 400 mM KCl, and TIF-IB at 600 mM KCl. The crude Pol I fraction (H-400) was further fractionated on a S-Sepharose FPLC and on a Mono Q FPLC column. To reconstitute transcriptional activity, Pol I was supplemented with TIF-IA and TIF-IC which were present in the H-200 fraction and were further purified on Q-Sepharose and on Mono Q as described before (2). TIF-IB was obtained by chromatography of the H-600 fraction on CM-Sepharose and step-elution with 400 mM KCl. UBF was purified from the fractions eluting at 1M KCl from the Mono Q column, followed by a sequence-specific DNA affinity column containing two complementary oligonucleotides derived from the human UCE region 5′-CAGGTGTCCGTGTCGCGCGTCGCCTGGGCC-GGCGGCG-3′ (22).

In vitro transcription assays

The template pMrWT (from nucleotides −170 to +155) was linearized with Nde I or Nar I to generate 371 nt or 319 nt transcripts, respectively. 25 μl assays contained 5–10 ng of template DNA, partially purified Pol I (3 μl of Mono Q fraction), 2 μl of TIF-IB (CM-400 fraction), 3 μl of TIF-IA/TIF-IC (Mono Q fraction) and 5 ng of UBF. To assay the negatively-acting factor in the reconstituted transcription system, the first template (pMrWT/Nde I) was preincubated for 15 min at 30°C with 3–6 μl of the inhibitor-containing fractions before the second template (pMrWT/Nar I), the transcription factors and the nucleotides were added. Transcription was stopped after 60 min and transcripts were analyzed by gel electrophoresis and autoradiography.

Purification of the repressor

The Ku-related repressor was purified from cultured Ehrlich ascites cells according to the fractionation scheme displayed in Figure 4A. Cell extracts were fractionated on DEAE-Sepharose and Heparin Ultrogel as described before (2). The H-200 fraction was then chromatographed on Q-Sepharose (Q-300).followed by purification on a polyethyleneimine-HPLC (PEI) column using a salt gradient from 250 to 1000 mM KCl. Fractions eluting at 700 mM KCl were applied to a Mono Q-FPLC column. Bound proteins were recovered with a linear gradient ranging from 200–300 mM KCl with the peak of repressor activity at 280 mM KCl. The repressor-containing Mono Q fractions were dialyzed against buffer AM containing 80 mM KCl (AM-80) before loading onto a BioRex-70 (200–400 mesh) column and step elution with 150 mM KCl. The last step in the purification procedure included chromatography on a DNA affinity column which contained the human UCE (22). After washing with buffer AM-200 containing 0.05% NP40, the repressor was recovered with buffer AM-600/0.05% NP40.

UV-crosslinking

Body-labelled rDNA promoter probe was prepared by annealing a single stranded oligonucleotide (5′-GATCTTTTCTATCTG-TTCCTATTGGACCTGGAGATAGGTACTAGTTTTTTTGG-T-3′) with a 14-base complementary primer (5′-ACCAAAA-AAACTAG-3′) and filling the coding strand with Klenow DNA polymerase in the presence of 75 μM dGTP, 150 μM 5′Br-dUTP and 25 μCi each of (α-^{32}P)dATP and (α-^{32}P)dCTP. The double-stranded labelled DNA probe was purified by chromatography on a Sephadex-G50 column. Purified protein fractions (3 μl) were incubated with 400,000 cpm (30 fmoles) of labelled DNA under gel shift conditions for 30 min at 30°C. After binding, samples were irradiated under a UV lamp for 10 min. The mixtures were then treated with 2 μg of DNase I and 20 units of micrococcus nuclease for 30 minutes at 37°C in the presence of 3.5 mM $CaCl_2$. Reaction products were separated by electrophoresis, stained with silver nitrate, and analyzed by autoradiography.

Western immunoblot analysis

Proteins of nuclear extracts were separated by electrophoresis on a 7% SDS-polyacrylamide gel, the proteins were transferred to nitrocellulose membranes and immunoreacted with either anti-UBF antiserum (K8; 1:1,000 dilution) or anti-Ku antiserum (1:4,000 dilution) followed by incubation with an anti-rabbit or anti-human horseradish-peroxidase-conjugated secondary antibody as previously described (25). Protein-antibody complexes were visualized by an enhanced chemiluminescence Western blotting detection system (Amersham).

DNase I footprinting

Footprinting was performed essentially as described (12) with minor modifications. The incubation mixtures (50 μl) contained 5 mM HEPES (pH 7.9), 25 mM KCl, 5 mM $MgCl_2$, 2.5 mM KF, 2% polyvinyl alcohol, 5% glycerol and 1–2 ng of a 5′-labeled DNA fragment which contains rDNA sequences from −168 to +56. The labeled DNA was incubated for 15 min at 30°C with 12.5 μl of the repressor-containing fraction. Then 1–2 ng of DNase I as well as 2.3 mM $CaCl_2$ were added and digestion was carried out for 1 min at room temperature. The reaction was stopped by addition of 350 mM ammonium acetate, 20 mM EDTA and 0.2 mg/ml yeast tRNA. After phenol extraction and ethanol precipitation, the samples were analyzed on a 6% polyacrylamide-8 M urea sequencing gel.

Electrophoretic mobility shift assays

The oligonucleotide (5′-GATCTTTTCTATCTGTTCCTATT-GGACCTGGAGATAGGTACTG-3′) containing the core region

of the murine rDNA promoter (−39 to +4) was 3′-end labeled with (α-^{32}P)dATP and (α-^{32}P)dCTP. 20,000−30,000 cpm of labeled oligonucleotide (10−15 fmoles) were incubated for 30 min at 30°C with 1−5 μl of the protein fractions in a 25 μl reaction mixture containing 40 mM HEPES (pH 8.4), 10 mM Tris−HCl (pH 7.9), 4.5 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM dithioerythritol and 2.5 μg bovine serum albumin. After binding, the samples were analyzed on a 4% native polyacrylamide gel.

Immunodepletion of repressor-containing fractions

50 μl fractions were incubated with 1.5 μl of control or anti-Ku serum for 15 min at room temperature with gentle mixing. Immunodepletion was performed by adding 15 μl of protein A-agarose beads (Boehringer) which have been pretreated with buffer AM-100 containing 10 mg/ml of bovine serum albumin. After incubation for 30 min at room temperature, beads were pelleted by brief centrifugation and supernatants were assayed for DNA binding and transcriptional activity.

RESULTS

A negative-acting factor stably associates with the rDNA promoter

Initial studies on the chromatographic separation of individual Pol I transcription factors from mouse cells have shown that the fraction which eluted from the heparin column at 400 mM KCl (H-400 fraction) contained the bulk of cellular RNA polymerase I (Pol I) activity and a yet to be characterized factor whose presence was required for maximal transcriptional activation by UBF (1). This putative factor has been suggested to be a negatively acting protein which binds to the murine rDNA promoter and represses transcription. Subsequent studies have indicated that this or a similar activity was also found in the flow-through fraction of the heparin column (H-200).

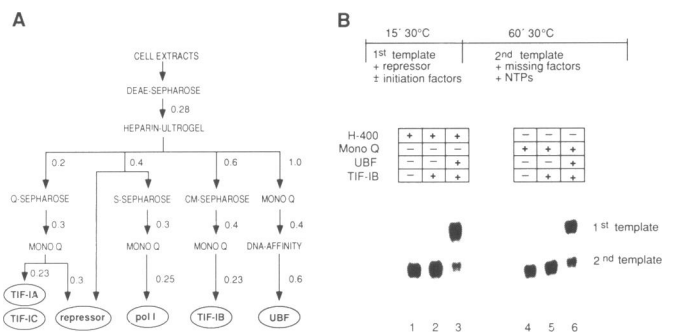

Figure 1. Transcriptional repression of the mouse rDNA promoter by (a) protein(s) present in both the H-400 and the Mono Q fraction. **(A)** Diagram of the first fractionation steps used to separate individual transcription factors. **(B)** A negative-acting factor is present in both the H-200 and H-400 fraction. Transcription reactions contained 5 ng each of pMrWT/Nde I (template 1) and pMrWT/Nar I (template 2). The first template was preincubated for 15 min at 30°C in the presence of the individual transcription factors indicated above the lanes. Then the second template, the nucleotides and the missing factors were added and transcription was proceeded for 60 minutes. The inhibitory activity was present either in the 400 mM KCl step from the Heparin column (H-400; lanes 1−3) or in the H-200 fraction which has been concentrated by chromatography on a Mono Q FPLC column (lanes 4−6).

To determine whether both activities are functionally identical, the H-200 fraction was concentrated on Q-Sepharose and further purified by chromatography on a Mono Q column. The inhibitory activity present in either the H-400 or the Mono Q fraction was tested in a partially purified reconstituted transcription system using a preincubation protocol which is depicted in Figure 1B. Two identical rDNA promoter templates were employed which were cleaved with either Nde I or Nar I to yield 371 and 319 nt run-off transcripts, respectively. The first template (pMrWT/Nde I) was preincubated with the H-400 or Mono Q fraction before the second template (pMrWT/Nar I), the missing factors and the nucleoside triphosphates were added. Both templates were transcribed with the same efficiency when the preincubation period was omitted (data not shown). However, preincubation of the first template with either the H-400 or the Mono Q fraction prior to addition of the second template and the missing components prevented transcription from the first template indicating that in the absence of UBF the first template was repressed by a DNA binding protein present both in the H-400 and the Mono Q fraction (lanes 1 and 4). The presence of moderate amounts of TIF-IB did not prevent this inhibition (lanes 2 and 5). If, however, UBF was present during the preincubation reaction, the first template showed a preferential transcriptional commitment (lanes 3 and 6), a finding which supports our conclusions that (i) an inhibitory factor represses transcription by stably binding to the rDNA promoter and preventing transcription complex formation, and (ii) UBF stimulates ribosomal gene transcription by counteracting this inhibitory factor and thus functions as transcriptional antirepressor.

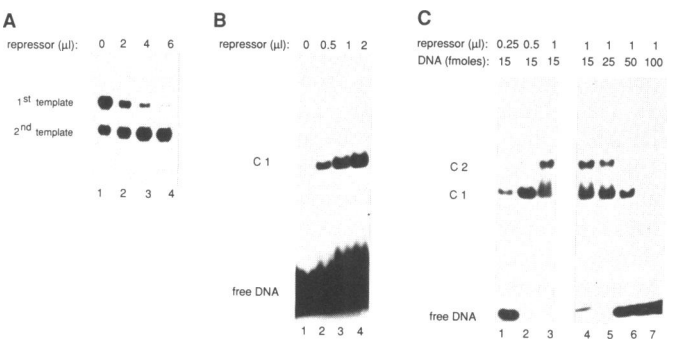

Figure 2. Binding of the repressor to the rDNA promoter. **(A)** Transcription assay. The first template (pMrWT/Nde I) was preincubated for 15 min at 30°C either in the absence (lane 1) or in the presence of increasing amounts of the repressor-containing fraction (lanes 2−4) before the second template (pMrWT/Nar I), 3 μl of Pol I, 3 μl of TIF-IA/TIF-IC, 2 μl of TIF-IB, 5 ng of UBF and the nucleoside triphosphates were added and incubation was continued for 60 min. Individual reactions contained different amounts of repressor. Lane 2: 2 μl; lane 3: 4 μl; lane 4: 6 μl). **(B)** Electrophoretic mobility shift assay. 135 fmoles (20,000 cpm) of 32P-labeled, double stranded oligonucleotide encompassing the mouse rDNA core promoter were incubated in the absence of the repressor (lane 1) or with 0.5 μl (lane 2), 1 μl (lane 3) and 2 μl (lane 4) of the repressor-containing fraction (Mono Q). **(C)** Two protein-DNA complexes are formed at high protein/DNA ratios. Lanes 1−3: 15 fmoles of the labelled oligonucleotide were incubated with 0.25 μl (lane 1), 0.5 μl (lane 2), and 1 μl (lane 3) of the repressor-containing fraction (Mono Q). Lanes 4−7: 15 fmoles of labelled core promoter oligonucleotide (lane 4), plus increasing amounts of the same oligonucleotide to yield a final concentration of 25 fmoles (lane 5), 50 fmoles (lane 6), or 100 fmoles (lane 7), respectively, were incubated with 1 μl of the Mono Q fraction and complexes formed were analyzed by gel electrophoresis. Free probe and the two DNA-protein complexes (C1 and C2) are labelled.

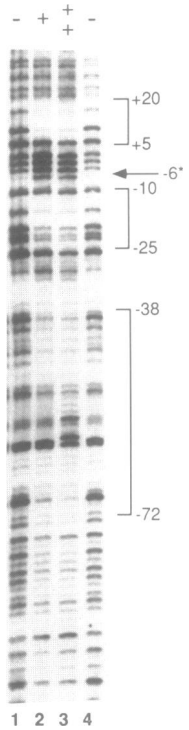

Figure 3. DNAse I footprinting of the repressor on the mouse rDNA promoter. The 5'-end labelled rDNA probe was incubated in the absence (lanes 1 and 4) or presence of 5 μl (lane 2) and 10 μl (lane 3) of a repressor- containing Mono Q-fraction, digested with DNase and the products were analyzed on a sequencing gel. Enhanced cleavage is marked by an asterix, protected regions are bracketed.

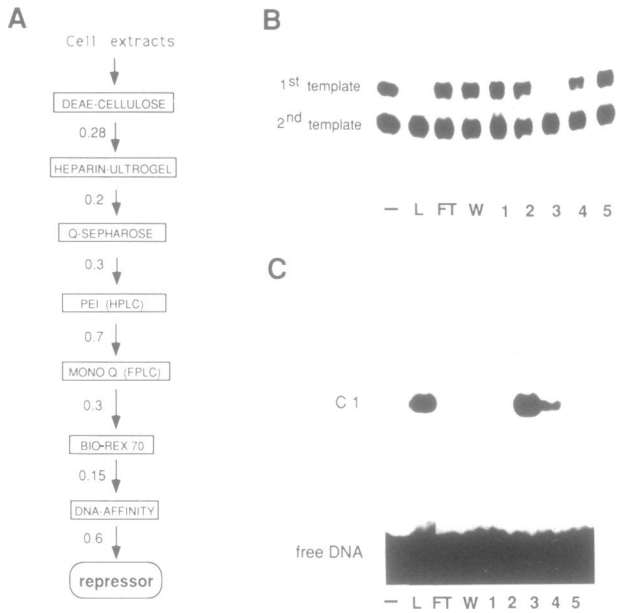

Figure 4. Purification of the repressor. **(A)** Scheme for the purification of the repressor from Ehrlich ascites cell extracts. **(B)** Inhibition of transcription by the fractions eluted from the DNA-affinity column. 4.5 μl of fractions were assayed in the two template assay as described in the legend to Fig. 1B. The first lane (–) shows transcripts synthesized in the absence of the repressor during the preincubation period. L shows the activity of pooled Bio-Rex 70 fractions applied to the affinity column. FT represents the flow-through and W the 100 mM salt fraction used to wash the column. The numbers indicate individual fractions that were eluted with 200 mM KCl (fractions 1 and 2) and with 600 mM KCl (fractions 3–5) from the DNA-affinity column. **(C)** DNA binding of affinity purified repressor. Electrophoretic mobility shift assay of the fraction applied to the affinity column (L), the flow-through (FT), the 100 mM KCl wash (W), and individual fractions that were eluted from the DNA-affinity column.

DNA binding of the repressor

As a first step to analyze the properties of the repressor, we partially purified the activity from nuclear extracts and studied its DNA-binding specificity. Extracts were fractionated on DEAE-Sepharose followed by chromatography on Heparin-Ultrogel, Q-Sepharose, PEI-HPLC and Mono-Q (see Materials and methods). Activity was monitored both in the reconstituted transcription system and in an electrophoretic mobility assay. As shown in Figure 2A, the addition of increasing amounts of the Mono Q fraction to the template occlusion assay described above inhibited transcription. The extent of inhibition was proportional to the amount of fraction present during the preincubation period. Even at the highest concentrations added, transcription from the second template was not affected indicating that the repressor was stably bound to the first template.

To demonstrate binding of this inhibitory factor to the rDNA promoter, the fraction was incubated with a synthetic oligonucleotide containing sequences from −39 to +4 relative to the transcription initiation site and analyzed by native gel electrophoresis. A specific DNA-protein complex (C1) was formed depending on the amount of fraction added (Fig. 2B, lanes 2–4). At high concentrations the association of the repressor with the promoter fragment yielded a second, slower migrating complex (C2). Formation of this second complex depended on the ratio of repressor to DNA present in the assay. In the experiment shown in Fig. 2C, at low oligonucleotide concentrations (15 fmoles) 1 μl of repressor-containing fraction shifted the probe to approximately equal amounts of complexes C1 and C2 (lane 3). When the concentration of the probe was increased by adding unlabelled core oligonucleotide, complex C2 disappeared before complex C1 (lanes 4–7). This concentration-dependent formation of two DNA-protein complexes indicates that at high protein to DNA ratios two factor molecules bind to the DNA probe.

To address the specificity of binding, a series of competition experiments was performed using different oligonucleotides or DNAs (data not shown). We found that complex formation was not only suppressed by addition of a 50- to 100-fold molar excess of unlabelled core-oligonucleotide but also—although to a lower degree—by an excess of other synthetic DNA fragments, such as the 'Sal-box' rDNA terminator sequence (Grummt et al., 1986), the human upstream control element (UCE) or by poly (dG-dC). On the other hand, supercoiled plasmid DNA, single stranded DNA or poly d(AT) were no effective competitors.

Interaction of the repressor with the rDNA promoter

Having established that the purified factor binds to the core promoter probe, we tried to further characterize the specificity of the DNA-protein interaction by DNase footprinting experiments. The footprinting characteristics of a highly enriched repressor-containing fraction is shown in Figure 3. Clearly, the repressor produced a distinctive footprint on the murine rDNA promoter. Characteristic features of the footprint are the decreased DNase accessibility downstream of the transcriptional start site (from +5 to +20), in the core promoter (from −10 to −25) and in the upstream region of the rDNA promoter (from

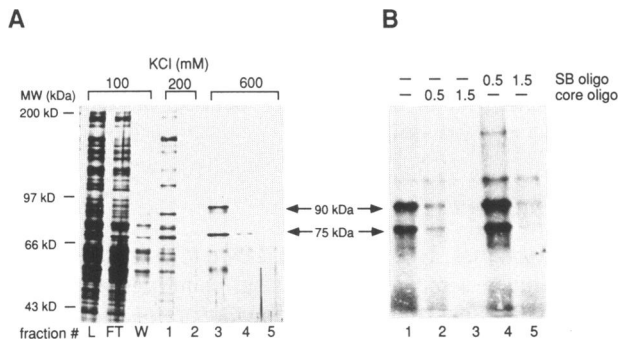

Figure 5. The repressor consists of two subunits. **(A)** SDS-gel electrophoresis of the repressor. Proteins that were applied to the DNA-affinity column (L), the flow-through (FT), the 100 mM wash fraction (W), the 200 mM eluate (fractions 1 and 2), and the 600 mM eluate (fractions 3–5) were separated on SDS-polyacrylamide gels and visualized by silver staining. **(B)** UV-crosslinking of the purified repressor. A ^{32}P-labelled rDNA fragment was cross-linked to partially purified repressor as described in Materials and methods. Reactions were performed in the absence (lane 1) or in the presence of 0.5 pmoles (lanes 2 and 4) or 1.5 pmoles (lanes 3 and 5) of an oligonucleotide containing either the core promoter sequence (core-oligo; lanes 2 and 3) or the 'Sal-box' termination signal (SB-oligo; lanes 4 and 5).

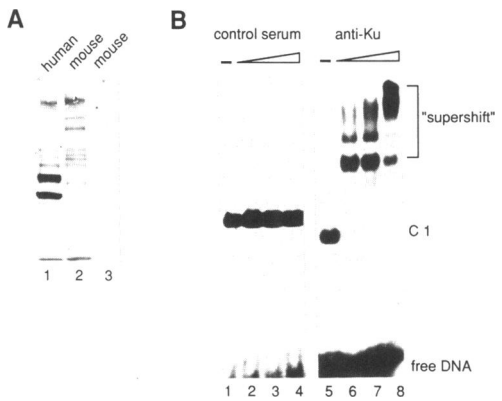

Figure 6. The repressor is related to Ku antigen. **(A)** Immunoblot of Ku polypeptides. 20 μg of nuclear extract proteins from HeLa cells (lane 1), nuclear extract proteins from Ehrlich ascites cells (lane 2) or 20 ng of purified repressor (lane 3) were subjected to Western blot analysis using anti-Ku serum. **(B)** Effect of anti-Ku serum on DNA binding. 3 μl of purified repressor (Bio-Rex 70 fraction) were preincubated for 15 min at room temperature with increasing amounts (0, 0.05, 0.1 or 0.3 μl) of control serum (lanes 1–4) or anti-Ku antibodies (lanes 5–8). Then the ^{32}P-labelled DNA probe was added and a mobility shift analysis was performed.

–38 to –72). In addition, protein binding caused increased cleavage at nucleotide –6. Thus the repressor binding overlaps with functionally important promoter domains. A similar protection pattern and similar biochemical properties was observed with highly purified repressor preparations (see below) indicating that DNA binding and inhibition of transcription is caused by the same protein moiety.

Purification of the repressor

Further purification of the repressor activity was achieved by chromatography of the Mono Q fraction on BioRex 70 followed by oligonucleotide affinity chromatography as shown in Figure 4A (for details, see Materials and methods). The activity of the affinity-purified fractions was monitored by in vitro transcription (Fig. 4B) and by the electrophoretic mobility shift assay (Fig. 4C). Once again, both the DNA-binding and the transcriptional repression activity coincided. The peak of activity was found in fraction # 3 eluting at 600 mM KCl from the DNA affinity column. The protein composition of these fractions is shown in Figure 5A. The affinity-purified fractions contained two major polypeptides of 75 and 90 kDa (Fig. 5A, fractions 3 and 4) which are present in stoichiometric amounts both in different factor preparations and at different purification stages and, therefore, very likely represent two subunits of this factor.

To confirm directly that these two polypeptides represent the DNA binding activity and to investigate whether both subunits are involved in DNA binding, the proteins were UV-crosslinked to a body-labelled mouse core promoter probe. An aliquot from a partially purified repressor-containing fraction (BioRex 70) was incubated with the labelled DNA, the reaction was exposed to UV-light and then electrophoresed on an SDS-polyacrylamide gel. Two predominant polypeptides were crosslinked to the core promoter (Fig. 5B, lane 1) whose apparent molecular weights of 75 and 90 kDa, respectively, correspond to the silver-stained protein bands observed in the purified factor fractions. The preferential interaction of these proteins with the core promoter was confirmed by competition with specific and nonspecific oligonucleotides. The presence of 0.5 pmoles of unlabelled core

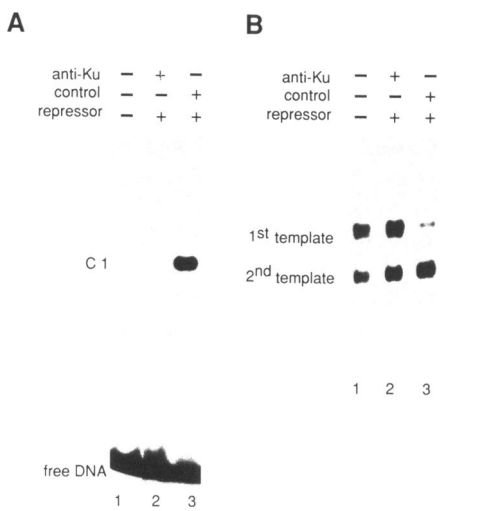

Figure 7. Immunoprecipitation of the repressor by anti-Ku serum. The repressor-containing fraction (Bio-Rex 70) was incubated for 15 min at room temperature with either control serum or anti-Ku serum. The immunocomplexes were precipitated by protein A bound to agarose beads and 3 μl or 6.5 μl, respectively, of the supernatants were analyzed in the electrophoretic mobility shift assay **(A)** or in the reconstituted transcription system **(B)** using the template occlusion assay described in Fig. 1B. Lanes 1 show control reactions where the repressor-containing fraction was omitted.

promoter oligonucleotide decreased labelling about 3-fold (lane 2). The same amount of an unrelated oligonucleotide containing the 'Sal-box' terminator sequence (18) did not compete for binding (lane 4). However, at higher concentrations significant competition by the nonspecific oligonucleotide was also observed (lane 5). This finding is in agreement with the gel shift competition data indicating that the factor has a low DNA binding specificity and that its binding is not restricted to the rDNA promoter.

The repressor is identical or related to the Ku protein

The molecular weight of the two mouse proteins and their binding pattern to DNA resembles those previously described for human Ku protein (15–17). The human Ku protein is a DNA-binding nuclear protein complex containing two proteins, p70 and p86, which are recognized by autoantibodies in sera of certain patients with systemic lupus erythematosus and other rheumatic diseases. To examine the possibility whether the repressor of rDNA transcription is identical or related to the Ku protein, a patient serum containing Ku-specific antibodies was tested in immunoblots and in immunoprecipitation assays.

On immunoblots of nuclear extract proteins, the serum reacted strongly with human p70 and p86 (Fig. 6A, lane 1). No cross-reactivity was observed with nuclear extract proteins from mouse cells (lane 2) or with the purified rDNA repressor (lane 3). The poor reactivity of human autoantibodies with murine Ku protein on Western blots has been observed with all patient sera tested and with monoclonal antibodies directed against human Ku proteins (19, 20). This failure of the antibodies to recognize the mouse protein has been attributed to specific amino acid substitutions in an immunodominant conformational epitope located in the carboxyl-terminal part of human p70 (20). However, most of the anti-Ku patient sera which were negative in Western blots with the rodent Ku proteins have been shown to react with the native protein (19–21). Thus, if our assumption was correct and the repressor was identical or related to Ku protein, then it might recognize the native protein and, consequently, affect DNA-binding and/or transcription.

In a first set of experiments anti-Ku serum was tested in the mobility shift assay. For this, purified repressor was preincubated with increasing amounts of anti-Ku serum before adding the labelled DNA probe and analyzing the DNA-protein complexes by gel electrophoresis. As shown in Fig. 6B, addition of a serum from scleroderma patients containing anti-Ku antibodies shifted the prominent repressor-DNA complex C1 (lane 5) to slower migrating complexes (lanes 6–8), demonstrating that both Ku protein and the antibody have bound to the DNA fragment leading to the formation of a higher-order complex. Four different anti-Ku patient sera were tested (not shown) and found to cause this specific supershift whereas control human sera did not affect the formation or mobility of the complex (lanes 1–4). This result demonstrates that the repressor is Ku antigen or a Ku-related protein and that antibodies bound to the complex do not prevent DNA binding.

Precipitation of the repressor activity by Ku-antibodies

The demonstration that the protein responsible for binding to the rDNA promoter reacts with Ku-antibodies raised the question of whether the same protein is causally involved in inhibition of rDNA transcription. For this, samples of the repressor-containing fraction were incubated with anti-Ku serum, then antigen-antibody complexes were removed by adsorption on protein A-agarose beads, and the supernatant was tested both in the electrophoretic mobility shift assay and in the reconstituted transcription system. As shown in Fig. 7A, the Ku-antiserum completely depleted the protein(s) responsible for specific DNA-protein complex formation (lane 2). The reaction was specific for the anti-Ku serum because a control human serum did not affect complex formation (lane.3). Similarly, transcription inhibition activity as tested in the two template assay was removed from the supernatant when immunoprecipitations were performed with the Ku-antiserum (Fig. 7B, lane 2). In contrast, in the presence of control serum the transcriptional inhibition of the first template remained unchanged (lane 3). These data provide additional experimental support for the conclusion that the negative-acting factor is very likely identical or related to Ku-antigen.

DISCUSSION

In previous studies, UBF1 has been shown to drastically enhance transcription in a reconstituted system using partially purified RNA polymerase I and transcription factors (1, 11, 22–25). These studies have revealed that the main function of UBF1 is to assist in the assembly of transcription initiation complexes. This function is brought about by two ways. First, UBF1 stabilizes binding of TIF-IB to the gene promoter, and second, it overcomes the inhibitory action of a DNA binding protein which interacts with the rDNA promoter and represses transcription (1). Thus, both positive and negative factors interact with the core element of the rDNA promoter in order to regulate rRNA synthesis.

In an attempt to biochemically characterize this negative-acting factor and to study its function in rDNA transcription, we have isolated this activity by a series of chromatographic steps. Interestingly, the molecular weights of the two subunits and the biochemical properties of the factor closely resemble those of Ku protein. Ku is a complex of 70 and 86 kDa proteins, which was originally detected as an autoantigen reacting with antibodies from patients with rheumatic disorders (26). This protein, variously termed Ku (15), anti-Ki (19), TREF (27), PSE1 (28), or NFIV (29), is an abundant nuclear protein which binds DNA in a non-sequence specific manner. Despite its relaxed DNA binding specificity, some selective interaction with defined promoter elements has been demonstrated. Examples for Ku binding to regulatory gene sequences are the human transferrin receptor promoter (27) and the proximal and distal promoter elements of the U1 snRNA gene (30). Moreover, a Ku-related protein (NFIV) has been purified from HeLa cells with apparent sequence specificity to molecular ends of adenovirus type 2 DNA (29). In view of these findings, Ku has been suggested to serve several important cellular functions and to be involved in active DNA processes, such as transcription, replication, recombination, or repair. Indeed, recent in vitro studies support the idea that Ku may be a transcriptional activator. Immunodepletion and addback experiments have demonstrated that transcription from both the U1 snRNA and the human transferrin receptor gene promoter requires PSE1, a factor identical or related to Ku, and that this transcriptional activation is dependent on specific binding of this factor to the promoter (28, 30).

Interestingly, the results presented in this study demonstrate that besides its positive effect on U1 snRNA and human transferrin receptor transcription, Ku exerts a negative effect on Pol I-dependent transcription initiation. Based on DNase footprinting and specific transcription experiments, we have found that the Ku protein interacts with the rDNA promoter. Interestingly, the regions of interaction with the promoter overlap with the sequences characterized by cooperative binding of both UBF and TIF-IB (13). This specific interaction has been observed with homogenous Ku preparations and, therefore, is not due to TIF-IB and/or UBF binding to the promoter. We propose that binding of Ku protein to the Pol I promoter prevents TIF-IB binding to its target sequence and thus blocks the formation of transcription initiation complexes. Apparently UBF counteracts

Ku antigen by forming a cooperative complex with TIF-IB which in turn significantly increases its affinity to the rDNA promoter. As a consequence, the repressor is displaced from the DNA and transcription complexes may assemble by the stepwise association of Pol I, TIF-IC and TIF-IA (2). Alternatively, UBF could interact directly with the Ku protein and displace it from the DNA. We consider this possibility as rather unlikely because we have never observed an effect of UBF on Ku binding in gel shift and DNase footprinting experiments. However, we cannot exclude a third possibility, namely, that the direct target of antirepression by UBF is not Ku itself but another factor which—by analogy to the Pol II system—may be referred to as an 'intermediary protein', 'adaptor', or 'coactivator'. Such a putative protein may associate with Ku and bridge the interaction with UBF. In view of recent findings demonstrating that Ku autoantigen is the regulatory component of a template-associated protein kinase (31, 32) such a mediator could either be the catalytic subunit of the DNA-dependent kinase or another polypeptide which is recruited to the template by Ku.

Numerous studies have shown that proteins which are involved in chromatin structure such as nucleosomes, high mobility group (HMG) proteins, histone H1 and others can inhibit transcription (for review, see 33). This transcriptional repression is counteracted by various specific transcriptional activators, having different activating and/or DNA binding domains. Therefore, Ku-mediated inhibition of ribosomal gene transcription which is overcome by UBF may represent a novel example of a general repression-antirepression mechanism and may not be restricted to the Pol I promoter.

The expression of the 70 and 86 kDa subunits of human Ku protein has been studied during cell proliferation (34). These studies have demonstrated that Ku gene expression correlates with the proliferative state of the cells and that nucleolar localization of the Ku protein is cell-cycle-dependent. Interestingly, nucleolar localization of the Ku protein negatively correlated with cell proliferation. In quiescent lymphocytes Ku was highly enriched within the nucleolus. After growth stimulation by phytohaemagglutinin the nucleolar staining was significantly decreased (34). These and our own studies (unpublished observations) indicate that Ku protein is present in the nucleolus during early G1-phase. During late G1/early S-phase, when rRNA synthesis was most active, the protein was exclusively located in the nucleoplasm. Therefore, cell-cycle and growth-dependent variations in the nucleolar localization of Ku may contribute to fluctuations of rDNA transcriptional activity in response to extracellular signals. A critical balance of antagonizing factors would allow cells to have more flexibility in regulating their level of ribosomal RNA according to physiological conditions. Detailed dissection of this mechanism will be of considerable importance to our understanding of how activators in general and UBF in particular act to set up a functional transcription complex.

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