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E_1BF/Ku Interacts Physically and Functionally With the Core Promoter Binding Factor CPBF and Promotes the Basal Transcription of Rat and Human Ribosomal RNA Genes

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We have previously characterized an RNA polymerase (pol) I transcription factor, E_1BF, from rat cells. This protein is immunologically related to Ku autoantigen and is required in pol-I directed transcription of rodent ribosomal RNA gene (rDNA). Glycerol density gradient fractionation and in situ UV cross-linking analysis of the purified factor showed directly that it consists of a heterodimer of 85 and 72 kDa polypeptides. E_1BF also interacted with the human core promoter and augmented transcription of human rDNA as much as fivefold in HeLa nuclear extract, whereas transcription from adenovirus major late promoter, CMV or SV40 early promoters by pol II and of U6 and 5S RNA genes by pol III were either unaffected or minimally inhibited by the antibodies. Purified rat E_1BF partially restored the suppression of human rDNA transcription by anti-Ku antibodies. Immunoprecipitation of rat cell extract with the anti-Ku antibodies followed by SDS-PAGE of the precipitated proteins and Southwestern analysis showed that E_1BF interacts with CPBF, a core promoter binding factor. When the majority of CPBF and E_1BF was removed from the reaction mixture by preincubation with a core promoter oligo nucleotide fragment, rDNA transcription was severely impaired. Addition of exogenous CPBF or E_1BF to such a reaction resulted in significant restoration of the transcription, whereas inclusion of both factors caused further enhancement of rDNA transcription. These data demonstrate that E_1BF is a basal pol I transcription factor that interacts with a core promoter binding factor both physically and functionally, and that is not a general pol II or pol III transcription factor.

rDNA transcription Pol II transcription Pol III transcription Anti-Ku antibodies Protein-protein interaction

INITIATION of transcription in eukaryotic cells is a highly regulated process involving specific interaction between *cis*-acting elements and *trans*-acting factors. Significant advances have been made in the identification of the *cis*-acting sequences required for basal and maximal transcription of ribosomal RNA gene (rDNA) by RNA polymerase I (pol I) in a variety of eukaryotic organisms (Paule, 1993; Moss, 1994; Jacob, 1994). These elements include the core promoter sequence and enhancer sequences in the spacer region. There has also been significant progress in the characterization of essential *trans*-acting factors required for pol I transcription. UBF (up-

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stream binding factor), which interacts with both upstream control element (UCE) and the core promoter sequence, is the first well-characterized pol I *trans*-factor purified initially from HeLa cells (Bell et al., 1988) and subsequently from rat (Smith et al., 1990), mouse (Bell et al., 1990), and frog (Pikaard et al., 1989). This factor is essential for human pol I transcription (Bell et al., 1988, 1990), whereas it is involved in the enhancement of pol I transcription in rodents (Voit et al., 1992; Smith et al., 1993). Studies with the cloned UBF demonstrated the existence of two species designated UBF1 and UBF2, the latter species being produced by alternative splicing of the primary transcript (O'Mahony and Rothblum, 1991). Recent study (Smith et al., 1993; Kuhn et al., 1994) has shown that UBF1 is the functional form of the factor.

A regulatory factor, designated factor C (Brun et al., 1994), TFIC (Mahajan and Thompson, 1990), or TIF-IA (Schnapp et al., 1993) by different laboratories, has been purified essentially to homogeneity. Factor C is closely associated with the functional RNA polymerase I, but can be separated by extensive purification. Factor C or TIF-IA can restore mouse rDNA transcription in inactive extracts prepared from stationary-phase or protein synthesis-inhibited cells. Similarly, glucocorticoid treatment can downregulate rDNA transcription in the mouse lymphosarcoma cell line P1798, which can be restored by the addition of the purified factor TFIC (Mahajan and Thompson, 1990). A comparison of the properties of these three factors suggests that they are not structurally identical (Brun et al., 1994). Further study is needed to elucidate the functional and structural relationships among these factors. Another factor, SL-1, which directs species-specific pol I transcription, was purified from human cells (Learned et al., 1985). Immunoprecipitation of HeLa nuclear extract with antibodies raised against human TATA box binding protein (TBP) revealed that human SL-1 consists of TBP complex with three additional polypeptides of 110, 68, and 48 kDa (Comai et al., 1992). Subsequently, the polypeptides associated with SL-1 complex were also characterized in mouse (Eberhard et al., 1993) and *Acanthamoeba castellani* (Radebaugh et al., 1994). Recently, we characterized a factor, CPBF, from the rat mammary adenocarcinoma ascites (Liu and Jacob, 1994) and HeLa cells (Z. Liu and S. Jacob, unpublished data) that specifically interacts with the rDNA core promoter sequence, as demonstrated by Southwestern, electrophoretic mobility shift, and UV cross-linking assays. Using a reconstitution assay, we showed that ribosomal gene transcription requires this protein (Liu and Jacob, 1994). Another protein, E_1BF, purified from rat mammary adenocarcinoma cells (Zhang and Jacob, 1990), consists of two polypeptides with molecular masses of 72 and 85 kDa, which interacts with the nonrepetitive (Zhang and Jacob, 1990; Hoff and Jacob, 1993) and repetitive (Ghosh et al., 1993) enhancer sequences, and the core promoter sequence (Zhang and Jacob, 1990; Hoff and Jacob, 1993) of rat rDNA. Subsequent study (Hoff and Jacob, 1993) showed that the size and immunological characteristics of this protein resemble those of the human Ku autoantigen. Using specific antibodies against the smaller subunit of the Ku protein or those against a peptide corresponding to the same subunit, we demonstrated (Hoff et al., 1993) that rat rDNA transcription requires E_1BF/Ku, which acts primarily in the preinitiation complex formation and that dissociation of the two polypeptides comprising E_1BF/Ku results in inhibition of transcription. Recent study in our laboratory demonstrated that a modified form of E_1BF (E_1BFs), produced during serum starvation of cells, prevents initiation of rDNA transcription and thus functions as a transcription repressor (Niu and Jacob, 1994). This factor does not resemble factor C or TFIC or TIF-IA structurally or functionally. We undertook the present study to show directly heterodimerization of E_1BF in the native state, the relative pol I specificity of the factor, and its potential interaction with other pol I transcription factor(s).

MATERIALS AND METHODS

Preparation of Rat Enhancer 1-Binding Factor (E_1BF)

Whole cell extract was prepared from the rat mammary adenocarcinoma ascites cells as described (Zhang and Jacob, 1990). E_1BF was purified from the whole cell extract by a series of fractionations that consisted of chromatography on DEAE-Sephadex, Heparin-Sepharose, CM Sepharose, and oligo affinity column constructed of a 37 bp enhancer sequence (Zhang and Jacob, 1990).

Preparation of HeLa Nuclear Extract

HeLa cells were cultured in Eagle's-MEM medium with 5% fetal calf serum and harvested at a density of 5×10^5 cells/per ml by centrifuging for 10 min at $3000 \times g$. Nuclear extracts were prepared as described (Dignam et al., 1983).

Glycerol Gradient Sedimentation Analysis

Purified E_1BF (200 μl) was loaded onto 11-ml glycerol density gradient in 0.2 M NaCl, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.01% NP-40, and 1 mM DTT, and was sedimented at 39,000 rpm for 52 h at 4°C in a Beckman SW41 rotor. Fractions of 300 μl were collected from the tube top. Alcohol dehydrogenase and β-amylase (Sigma) with molecular masses of 150 and 200 kDa, respectively, were used as markers and were centrifuged in parallel. The fractions were assayed by the electrophoretic mobility shift and SDS-PAGE analysis followed by silver staining.

Electrophoretic Mobility Shift and UV Cross-Linking Assays

Two synthetic oligonucleotides that contain the rat and human rDNA promoter (−36 to +18) sequences (Rothblum et al., 1982; Firancsek et al., 1982) were labeled separately at the 3′ ends with [α-^{32}P]dATP (3000 Ci/mmol) and purified by electroelution from the gel as described (Zhang and Jacob, 1990). The electrophoretic mobility shift assays using the labeled probes were performed as described previously (Garg et al., 1989). For in situ DNA-protein cross-linking assays, polyacrylamide gels from the electrophoretic mobility shift assays were placed 2 in. below the 254 nm UV light source and irradiated for 25 min at 4°C; DNA-protein complexes were excised and gel slices were analyzed by SDS/7.5% PAGE (Moliter et al., 1990).

In Vitro Transcription Assays

The transcription reaction contained 3–5 μl of HeLa nuclear extract (10 mg/ml) in 25 μl reaction volume for 45 min at 30°C essentially as described (Garg et al., 1989). The plasmid (pBEs) that contains the flanking sequence (−513 to +697) of human rDNA was linearized with Sal I and used as template in the pol I transcription assays. The plasmid pML(C_2AT) containing adenovirus major late gene promoter (−404 to +10) linked to a G-less cassette was used for pol II-directed transcription assays in the presence of RNase T1 and the chain terminator 3′-*O*-methyl-GTP as described (Sawadogo and Roeder, 1985). The plasmid pSV-2 cat, which contains SV40 promoter and CAT gene (Gorman, et al., 1992) linearized with EcoR I, and the plasmid pcDNAI/Amp-CAT, which contains CMV promoter and CAT gene (Invitrogen Corporation) linearized with BamH I, were used for pol II-directed transcription assays. The correct transcription of the plasmids containing adenovirus, SV40 and CMV promoters from the +1 site must yield 390, 350 (the predominant transcript out of the possible two), and 875 nucleotides-long transcripts, respectively. Transcription of the mouse U6 RNA and 5S RNA genes by pol III was carried out as described (Yuan and Reddy, 1991). After incubation for 45 min at 30°C, the reactions were stopped and the RNAs were extracted twice with phenol/chloroform, precipitated with ethanol, and analyzed by 4% or 10% polyacrylamide gel electrophoresis and autoradiography.

Immunoprecipitation Analysis

Whole cell extract from rat hepatoma (N1-S1) cells was incubated with monoclonal antibodies against Ku-p70 for 1 h at 4°C. Protein A-Sepharose beads (50 μl of 50% Slurry) were added to the mixture and incubated for another hour. After brief centrifugation, the pellets were gently washed twice with buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM Vanadate, 1 mM NaF, protease inhibitor cocktail (1% aprotinin, 10 mM pepstein, 0.1% leupeptin, 0.5 mM phenylmethylsulfonylfluoride), and 15% glycerol. The immunoprecipitated protein was resolved by SDS-PAGE (10%), electroblotted to nitrocellulose membrane, and subsequently subjected to Western or Southwestern blotting analysis.

Southwestern Analysis of DNA Binding Proteins

After the proteins were resolved on SDS-PAGE and transferred to nitrocellulose membrane, the filter was dried and soaked in 6 M guanidine HCl in buffer A (20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 5 mM $MgCl_2$ 1 mM DTT, 0.04% NP-40) for 20 min at 4°C with gentle shaking. Subsequently, the filter was successively washed with 3, 1.5, 0.75, 0.38, and 0.19 M guanidine HCl in buffer A for 5 min. The filter was soaked for 5 min, and was given a final wash in buffer A alone, and was then blocked with 5% fat-free milk in the buffer to prevent nonspecific binding. After washing, the filter was preincubated with poly (dIdC) in buffer A (1 μg/ml) for 20 min at room temperature, and incubated with the labeled core promoter probe (at least 0.25×10^6 cpm/ml) for 2 h at room temperature with gentle shaking. Finally, the filter was washed with buffer A two to three times and then analyzed by autoradiography.

Antibodies

Antibodies against the peptide fragment (322–358) of 70 kDA subunit and the 86 kDa polypeptide of the human Ku protein were generously provided by Dr. Bellur S. Prabhakar, University of Texas Medical School, Galveston, TX.

RESULTS

Direct Proof for the Heterodimeric Structure of Rat Pol I Transcription Factor E_1BF

To prove direct heterodimerization of the two E_1BF polypeptides, purified E_1BF was subjected to glycerol density gradient fractionation along with the molecular size markers to measure the native molecular weight of this factor. Following centrifugation, the fractions were collected and assayed for the rat rDNA promoter binding activity of E_1BF by electrophoretic mobility shift assay (EMSA) using a ^{32}P-labeled promoter probe. Relative to the position of the marker, the peak DNA binding activity of E_1BF was located between 150 and 200 kDa (Fig. 1). Only one major DNA–protein complex was formed in the EMSA except in the presence of relatively high concentrations of E_1BF, which gave an additional slower band (also see Zhang and Jacob, 1990). This result suggests that E_1BF forms a heterodimer or the homodimer of the two polypeptides. To distinguish between these two possibilities, the density gradient fractions were assayed for proteins by silver staining. Both 85 and 72 kDa polypeptides were seen in all the fractions that exhibited the promoter binding activity (Fig. 1, lanes 16–20). These data indicate that E_1BF is a heterodimer of the two polypeptides. We have shown previously that dissociation of the two polypeptides by anti-Ku antibodies leads to inhibition of rDNA transcription. These observations, taken together, indicate that the structural and functional integrity of E_1BF/Ku requires heterodimerization of the two polypeptides.

E_1BF Is Not a Species-Specific Transcription Factor

Because pol I transcription is generally species specific, it was of interest to investigate whether E_1BF purified from rat cells could interact with human pol I promoter and modulate human pol I transcription. To test this possibility, we initially studied its interaction with human rDNA promoter element in an EMSA using the ^{32}P-labeled oligo probe corresponding to rat rDNA promoter. A competition EMSA was performed by first forming the DNA–protein complex with ^{32}P-labeled rat promoter probe in the presence of varying amounts of unlabeled human promoter fragment (Fig. 2). The binding of E_1BF to rat promoter was competed out by incubating with excessive unlabeled human promoter fragment (Fig. 2, compare lane 4 with lane 1). Rat E_1BF could also bind human core promoter directly (Fig. 2, lane 5). As observed with the rat promoter, binding to ^{32}P-labeled human promoter probe was also competed out with excess of unlabeled human promoter fragment (Fig. 2, compare lane 6 with lane 5). An excess (100 ng) of a nonspecific competitor [poly(dIdC)] used in the assay (lane 7) achieved only a minimal competition. These data suggest that E_1BF is not a species-specific factor for rDNA transcription.

To determine further whether rat E_1BF interacts with human and rat promoter elements in the same manner, in situ UV cross-linking analysis was performed using ^{32}P-labeled rat and human promoter probes. The affinity-purified E_1BF formed DNA–protein complexes with each of the two probes (Fig. 3B). It was resolved under nondenaturing conditions and then subjected to UV irradiation in situ (Fig. 3A). Subsequent SDS/PAGE of the cross-linked DNA–protein adducts excised from these irradiated gels (Fig. 3B) revealed the presence of two labeled bands (114 kd, 95 kd) that correspond to the purified polypeptides of E_1BF. Although the upper band was relatively weak, it was reproducible. The apparent higher molecular weights of the protein bands were due to the oligo probe cross-linked to the two polypeptides. When the cross-linked DNA–protein adducts in solution were treated with DNase I, the molecular weights of the bands were reduced in size (data not shown). Because silver staining of the affinity-purified E_1BF showed only two polypeptides, it is unlikely that the UV cross-linking involved any minor contaminants. This result further indicates that the two polypeptides of E_1BF form a unique heterodimeric complex that binds to both rat and human ribosomal gene promoter sequences.

To establish the role of E_1BF in human rDNA transcription, a suboptimal amount of HeLa whole cell extract was used to transcribe human rDNA, a strategy used in our earlier studies with rat cell extract (Zhang and Jacob et al., 1990). Transcription of the Sal I-linearized human rDNA in a recombinant plasmid (pBEs) must yield a 700 nucleotides-long run-off transcript if transcription starts at +1 site (Firancsek et al., 1982). The expected transcript (700 nt) was produced under these conditions (Fig. 4). Increasing amounts of purified E_1BF were added to the reaction. A five-fold increase in the amount of the run-off tran-

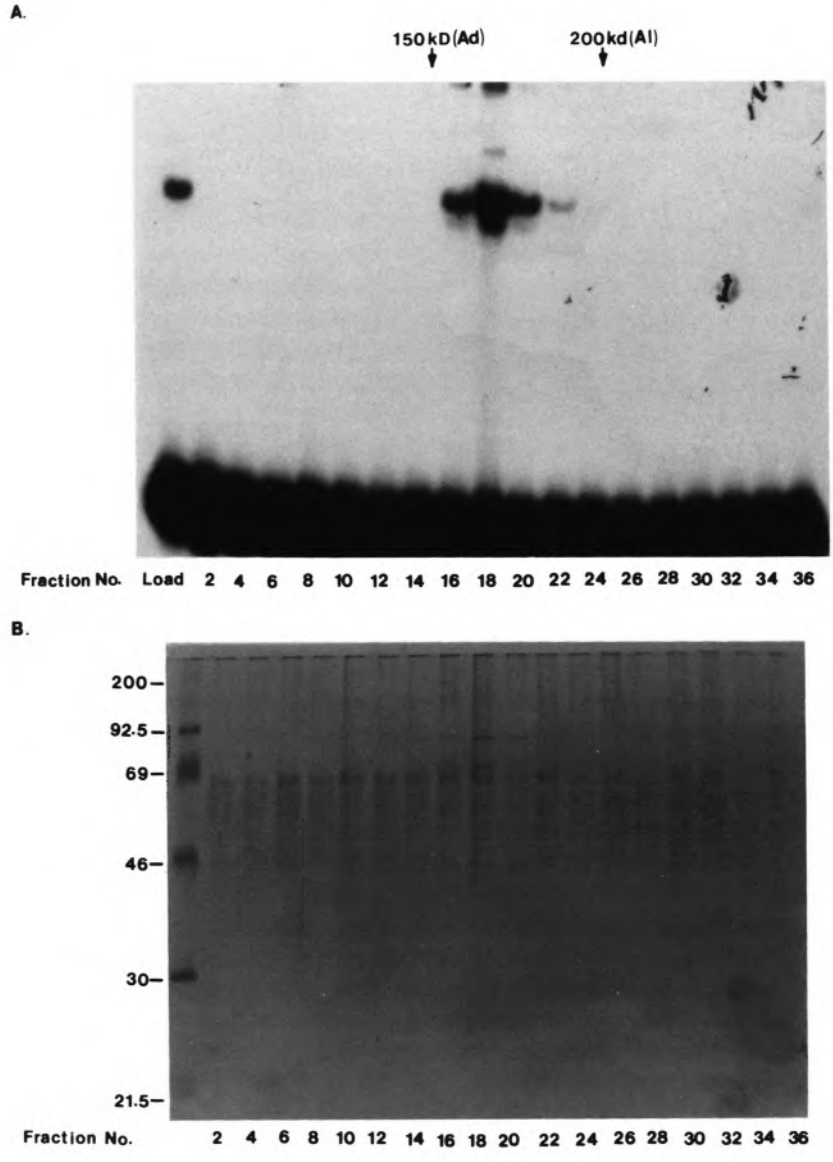

FIG. 1. Glycerol density gradient fractionation of E_1BF. Purified E_1BF was sedimented through a 15–35% glycerol gradient. (A) Gradient fractions (3 μl) were assayed for promoter binding activity of E_1BF by electrophoretic mobility shift analysis using ^{32}P-labeled rat core promoter fragment as the probe. The sedimentation position of the marker proteins, 150 kDa alcohol dehydrogenase (Ad) and 200 kDa β-amylase (A1), are indicated. The numbers at the bottom indicate the fractions used in the assay. Load: represents the purified E_1BF fraction loaded on the glycerol density gradient. (B) Polypeptide composition of glycerol gradient fractions. The same gradient fractions described in (A) were analyzed by SDS-PAGE (10%) followed by silver staining. The molecular size (in kDa) is denoted on the left.

script was observed in response to exogenous rat E_1BF as measured by densitometric scanning of the autoradiogram.

Antibodies Against Human Ku Protein Inhibit Human rDNA Transcription In Vitro

Recent study in our laboratory (Hoff et al., 1994) has demonstrated that anti-Ku antibodies can inhibit rat rDNA transcription in vitro. The inhibition of transcription was observed with antibodies raised against either a short peptide (pep 7) corresponding to amino acids 344–358 of the human Ku p70 or the entire p70 polypeptide. To investigate whether these antibodies produce similar effects on the pol I-directed transcription of human rDNA, HeLa nuclear extract was preincubated with the anti-pep 7 antibodies prior to addition of the template and nucleotides to start the reaction. The run-off transcription of human

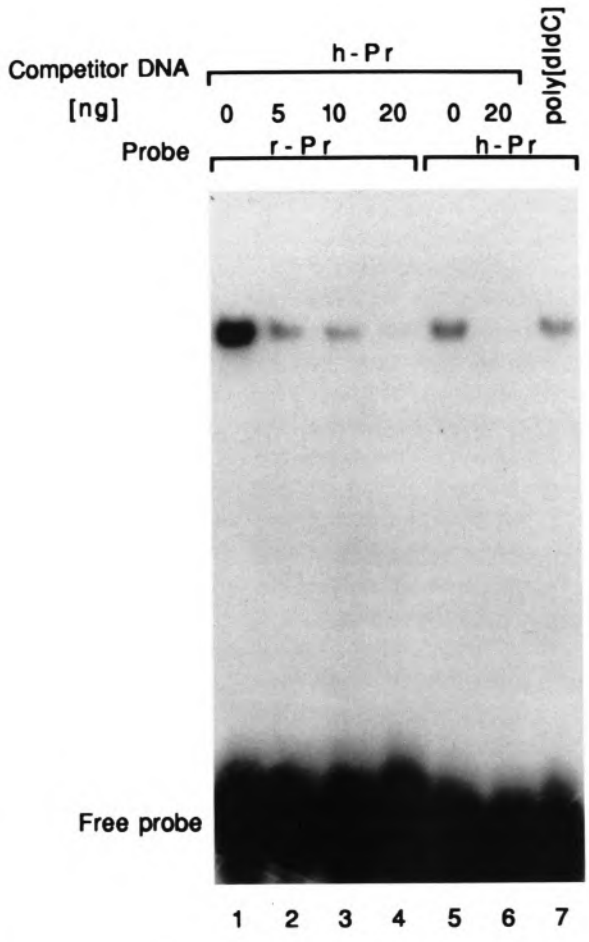

FIG. 2. Interaction of rat E_1BF with the promoter elements of human ribosomal DNA. Electrophoretic mobility shift assay was performed using purified E_1BF and human or rat rDNA promoter probe (see Materials and Methods for details). The mobility shift assay was performed using different amounts of unlabeled human promoter probe as competitor. Lane 1: labeled rat promoter probe in the absence of competitor DNA. Lanes 2, 3, and 4: 5, 10, and 20 ng unlabeled human core promoter element, respectively, were included as a competitor. Lane 5: labeled human core promoter probe in the absence of competitor DNA. Lanes 6 and 7: unlabeled human (20 ng) core promoter element and nonspecific poly(dIdC) (100 ng), respectively, were used as competitors.

rDNA was inhibited by increasing concentrations of anti-pep 7 antibodies (Fig. 5, compare lanes 2–4 with lane 1). As observed for rat rDNA transcription (Hoff et al., 1994), transcription was not affected by preincubation with anti-p86 antibodies or mouse IgG (Fig. 5, lanes 5–7). The unabated transcription in the presence of anti-p86 antibodies was probably due to retention of the heterodimeric structure, as opposed to dissociation of the heterodimer with anti-p70 antibodies (Hoff et al., 1994). These data suggest that a Ku-related protein is also essential for rDNA transcription in human system and that E_1BF/Ku protein is involved in the basal ribosomal DNA transcription in vitro in at least higher eukaryotes.

Rat E_1BF Can Restore Human rDNA Transcription Inhibited by Anti-Ku Antibodies

Next, we investigated whether inhibition of human rDNA transcription by anti-Ku antibodies can be overcome by purified rat E_1BF. To test this possibility, HeLa nuclear extract was preincubated with anti-pep 7 antibodies (0.3 μg), which resulted in more than 80% decrease of human rDNA transcription relative to transcription in the absence of antibodies (Fig. 6, compare lanes 1 and 2). Increasing amounts of purified rat E_1BF that had been preincubated with the template for 10 min were than added along with the nucleotides to the reaction mixture. The transcription was restored to approximately 50% of the control level, as determined by densitometric scanning of the autoradiogram. The ability of rat E_1BF to restore human rDNA transcription inhibited by anti-Ku antibodies strongly suggests that this factor is not entirely species specific and that it plays an important role in basal rDNA transcription. The lesser extent of recovery of human rDNA transcription by rat E_1BF compared to rat rDNA transcription suggests that rat E_1BF and the human Ku protein may not be identical. It should be noted that inhibition/restoration experiments are technically quite complex, which might explain the absence of a strictly concentration-dependent increase in transcription by exogenous E_1BF (Fig. 6; Hoff et al., 1994). The recovery of the antibody-inhibited human rDNA transcription was reduced if exogenous purified E_1BF was not preincubated with the template prior to addition to the HeLa nuclear extract (data not shown). This observation supports the notion that E_1BF is primarily involved in the formation of preinitiation complex, which is consistent with its effect on the rat rDNA transcription (Hoff et al., 1994).

Anti-Ku Antibodies Do Not Inhibit or Minimally Affect Pol II-Directed Transcription From Adenovirus, SV40 and CMV Promoters, or Pol III-Directed Transcription of U6 RNA and 5S RNA Genes

To investigate whether E_1BF affects pol II-directed transcription, the effect of anti-Ku antibodies on the transcription of hybrid plasmid containing the adenovirus major late promoter (AdML) linked to a synthetic 390 bp DNA frag-

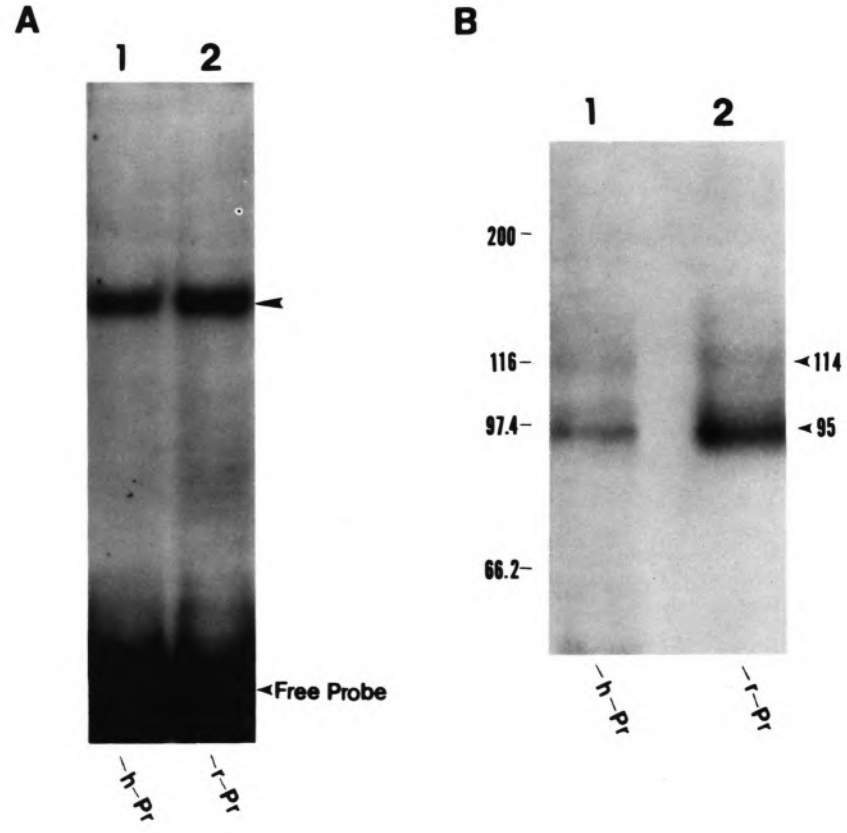

FIG. 3. In situ UV cross-linking analysis of DNA–protein complexes formed between rat E_1BF and human or rat rDNA promoter elements. E_1BF (10 ng) was incubated with human or rat rDNA probes (see Materials and Methods for details). Protein–DNA complexes were resolved by electrophoretic mobility shift assay (A). The DNA–protein complexes were then subjected to UV irradiation in situ and subjected to SDS-PAGE (B).

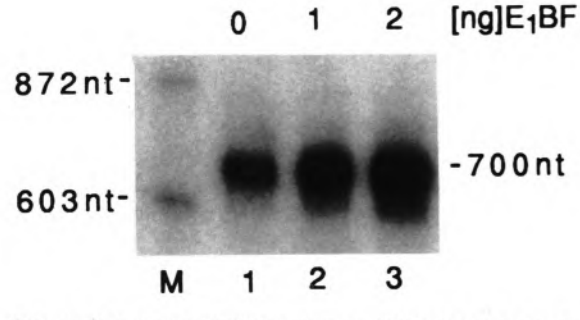

FIG. 4. Effect of rat E_1BF on human rDNA transcription in vitro. The plasmid pBEs (200 ng), which contains the human rDNA promoter element, was linearized with Sal I and used as the template for pol I transcription in HeLa nuclear extract. The transcripts were analyzed by denaturing polyacrylamide (4%) gel electrophoresis. Lane M: molecular size markers. Lanes 1–3: transcription reactions in the presence of 0, 1, and 2 ng of rat E_1BF, respectively. The position of specific transcript (700 nt) is indicated on the right.

ment was studied. This fragment lacks cytidine residues on the transcribed strand and generates a transcript with no guanosine residues. In vitro transcription was performed in the presence of RNase T1 and the chain terminator 3′-*O*-methyl-GTP (see Materials and Methods for details). Under these conditions, a 390 nucleotides-long RNase T1-resistant transcript resulting from correct initiation at the major late adenovirus promoter was produced (Fig. 7, lane 1). When HeLa nuclear extract was preincubated with the anti-Ku antibodies before the addition of templates and nucleotides, transcription from the adenovirus gene promoter was not inhibited by the anti-pep 7 or anti-p86 antibodies (Fig. 7A, lanes 2–7). Similarly, pretreatment of the HeLa nuclear extract with the anti-Ku antibodies did not inhibit pol II-directed transcription from the SV40 promoter (Fig. 7B) or CMV promoter (Fig. 7C). In fact, a notable stimulation of transcription from SV40 promoter has been consistently observed following incubation of the extract with the anti-pep 7

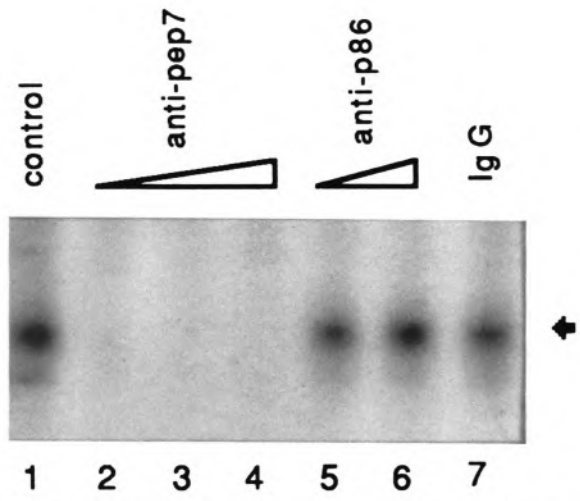

FIG. 5. Inhibition of pol I-directed transcription in HeLa nuclear extract by anti-Ku antibodies. HeLa nuclear extract (30 μg) was incubated with antibodies for 15 min at room temperature. The template pBEs (200 ng) and nucleotides were then added to the extract and the reactions were carried out as described in Materials and Methods. Lane 1: control reaction, no antibodies added. Lanes 2–4: incubation with 0.4, 0.8, and 1.6 μg of anti-pep 7 antibodies, respectively. Lanes 5 and 6: incubation with 1.25 and 2.5 μg of anti-p86 antibodies, respectively. Lane 7: incubation with 5.0 μg of mouse IgG. The transcript (700 nt) is indicated by arrow on the right.

antibodies (Fig. 7B, compare lanes 2 and 3 with lane 1). The significance of this observation is not known. We also studied the effect of anti-Ku antibodies on pol III-directed transcription of U6 RNA gene and 5S RNA gene. In contrast to the complete inhibition of rDNA transcription, the same amount of anti-Ku antibodies did not affect U6 RNA gene transcription or minimally inhibited (<25%) 5S RNA gene transcription (Fig. 8A, B). These data indicate that, unlike pol I transcription, the basal pol II and pol III transcription do not require E_1BF/Ku.

Interaction of E_1BF With CPBF, a Core Promoter Binding Factor

Recently, we have characterized a core promoter binding factor (CPBF) that binds specifically to the core promoter sequence in rat (Liu and Jacob, 1994) and human rDNA (Z. Liu, unpublished data). Reconstitution experiments showed that CPBF is required for the basal rDNA transcription in rat system (Liu and Jacob, 1994). Both CPBF and E_1BF copurified through several column chromatographic fractionations until the final DNA affinity column, which suggested a close functional interaction between these two proteins. The physical interaction between CPBF and E_1BF was investigated by immunoprecipitation of whole cell extract from the rat hepatoma (N1-S1) cells with anti-Ku p70 antibodies that are known to interact with rat E_1BF/Ku (Hoff and Jacob, 1993). The precipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and renatured. Next, the core promoter binding proteins in the renatured product were identified in a typical Southwestern assay using ^{32}P-labeled rat core promoter probe (Fig. 9). Only one protein corresponding to a molecular mass of 44 kDa was obtained by this analysis (Fig. 9, lane 3). Under this condition, control mouse IgG did not precipitate any core promoter binding protein (Fig. 9, lane 2). Western blot analysis showed that the two polypeptides of E_1BF were immunoprecipitated by anti-Ku antibodies (Fig. 10, lane 2) whereas control antibodies did not immunoprecipitate this factor (Fig. 10, lane 1). Similar results were obtained when fraction DE-B was used instead of whole cell extract in immunoprecipitation. Because only three core promoter binding polypeptides of 116, 44, and 39 kDa were detected in the Southwestern analysis (see Liu and Jacob, 1994), a single promoter binding polypeptide in the immunoprecipitate is particularly striking. Although rat CPBF consists of two polypeptides with molecular masses of 44 and 39 kDa, and both polypeptides interact with the core promoter probe (Liu and Jacob, 1994), interaction between CPBF and E_1BF may be mediated through the 44

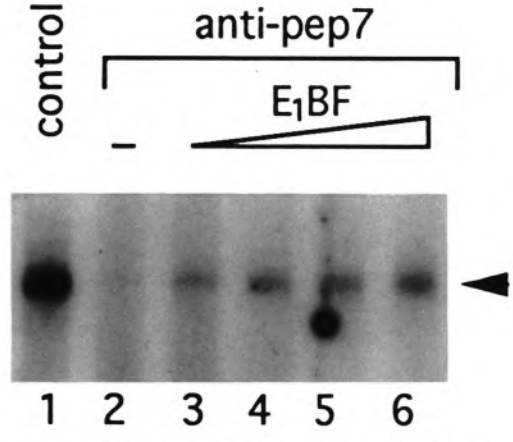

FIG. 6. Restoration of anti-pep 7 antibodies-inhibited human rDNA transcription by purified rat E_1BF. HeLa nuclear extract was incubated with 0.3 μg of anti-pep 7 antibodies for 15 min at room temperature. After preincubation of the plasmid pBEs with 0, 2, 4, 6, and 8 ng of purified rat E_1BF (lanes 3–6) for 10 min at room temperature, the mixture was added to the extract preincubated with the antibodies. The reactions were carried out as described in Materials and Methods. Lane 1: transcription in the absence of antibodies. Lane 2: transcription in the presence of antibodies. The specific transcript (700 nt) is indicated by arrow on the right.

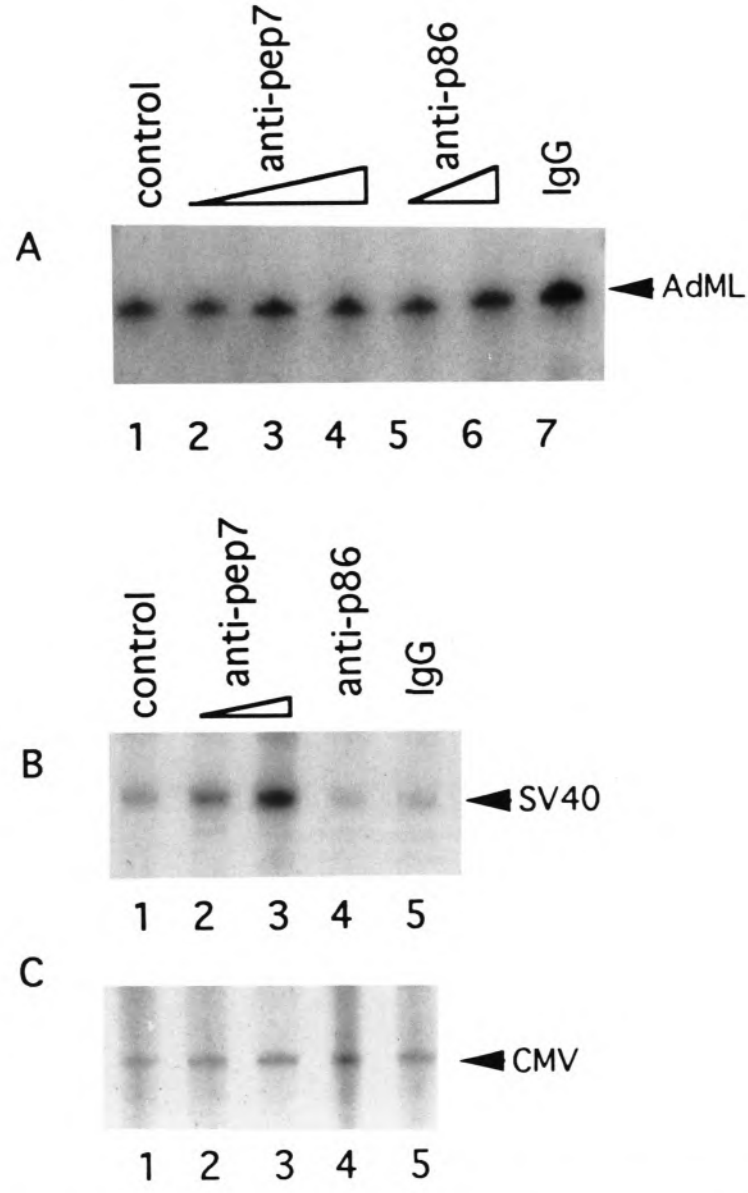

FIG. 7. Effect of anti-Ku antibodies on pol II-directed transcription from the adenovirus, SV40 and CMV promoters. HeLa nuclear extract (30 μg) was incubated with anti-Ku antibodies as described in the legend to Fig. 5. The plasmid pML (C_2AT) (1 μg), which contains the adenovirus major late promoter and G-less cassette (A), the plasmid pSV2-cat (1 μg), which contains SV40 early promoter linearized by EcoRI (B), or the plasmid pcDNA I/Amp-CAT (0.5 μg), which contains CMV promoter linearized by BamH I (C), were used as templates to perform the transcription reaction in the antibody-treated extract. Transcription of these plasmids yielded products of the anticipated size (see Materials and Methods for details). The transcripts were analyzed by denatured polyacrylamide gel (4%) electrophoresis. (A) Lane 1: control reaction, no antibodies added. Lanes 2–4: incubation with 0.4, 0.8, and 1.6 μg of anti-pep 7 antibodies, respectively. Lanes 5 and 6: incubation with 1.25 and 2.5 μg of anti-p86 antibodies. Lane 7: incubation with 5.0 μg of mouse IgG. (B) and (C) represent transcription from SV40 and CMV promoters, respectively. Lane 1: control reaction, no antibodies added. Lanes 2 and 3: incubation with 0.8 and 1.6 μg of anti-pep 7 antibodies, respectively. Lane 4: incubation with 2.5 μg of anti-p86 antibodies. Lane 5: incubation with 5.0 μg of mouse IgG. The specific transcript is indicated by arrow on the right.

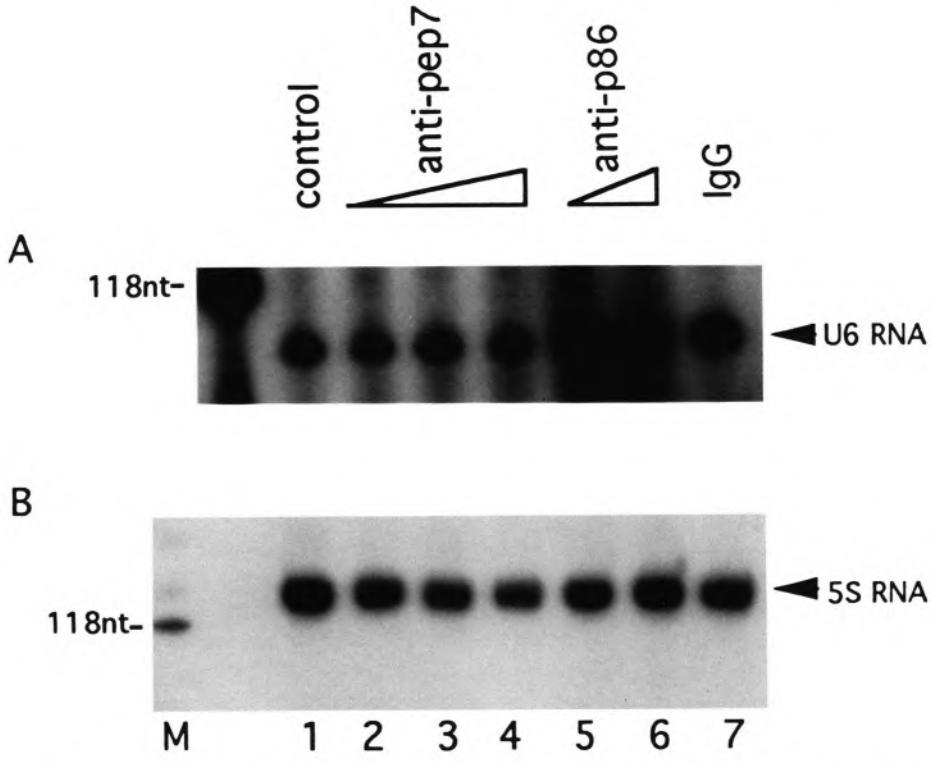

FIG. 8. Effect of anti-Ku antibodies on the pol III-directed transcription of U6 RNA and 5S RNA genes. HeLa nuclear extract (30 μg) was incubated with antibodies as described in the legend to Fig. 5. The supercoiled plasmid (1 μg), which contains the mouse U6 RNA gene (A) or 5S RNA gene (B), was added to the extract and the reactions were carried out as described in Materials and Methods. The transcripts were analyzed by denatured polyacrylamide (10%) gel electrophoresis. Lane M: molecular size marker. Lane 1: control reaction, no antibodies added. Lanes 2–4: incubation with 0.4, 0.8, and 1.6 μg of anti-pep 7 antibodies, respectively. Lanes 5 and 6: incubation with 1.25 and 2.5 μg of anti-p86 antibodies, respectively. Lane 7: incubation with 5.0 μg of mouse IgG. The specific transcript is indicated by arrow on the right.

kDa polypeptide of CPBF. Immunoprecipitation of this complex with the antibodies probably results in dissociation of 39 kDa polypeptide from the complex, which might explain absence of the latter polypeptide in the immunoprecipitate.

To demonstrate the functional significance of the physical interaction between E_1BF and CPBF, we took advantage of the known affinity of the two factors for the core promoter (Zhang and Jacob, 1990; Liu and Jacob, 1994). Fraction DE-B that contains pol I and all the pol I accessory factors required for basal transcription (Garg et al., 1989) was preincubated with the core promoter oligonucleotides to deplete these two factors. To determine the optimal condition for depleting the factors from the extract, fraction DE-B was first preincubated with various amounts of the oligonucleotide before the initiation of the transcription reactions. As shown in Fig. 11A, the specific transcript was gradually abolished when increased amounts (50, 100, and 200 ng) of the oligonucleotides were added to the reactions. A concentration of 150 ng of 58 bp core promoter oligo per reaction was selected for depleting the factors from fractionated extract. Transcription of rat rDNA was significantly reduced, but not eliminated, following preincubation with the oligonucleotide when used at the above concentration (Fig. 11B, lane 2). Under this condition, limiting amounts of both E_1BF and CPBF should be available for transcription. When 4 ng of CPBF or E_1BF was added separately to the treated fraction DE-B, a significant recovery of the transcript (4.4-fold with CPBF and 2-fold with E_1BF) was obtained (lanes 3 and 4). When 2 ng of each factor (CPBF and E_1BF) was added together to the factor-depleted extract, an even more dramatic stimulation (9.7-fold) of transcription was observed, as determined by densitometric scanning of the transcripts (lane 5). We used only 2 ng of either factor for combined addition (lane 5) to maintain the total protein concentration identical to that in the

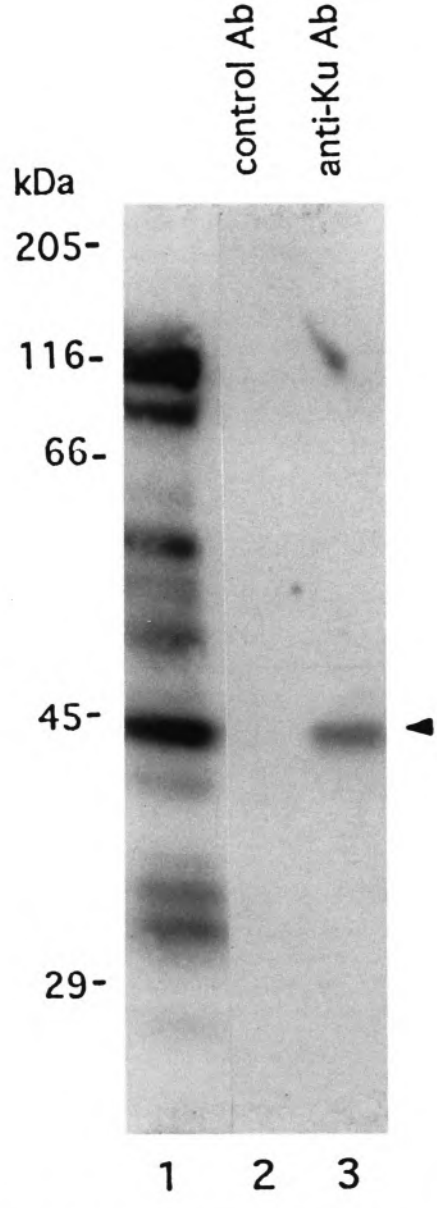

FIG. 9. Interaction of CPBF with E_1BF. Whole cell extract from rat hepatoma (N1-S1) cells was immunoprecipitated by control antibodies or anti-Ku-p70 antibodies as described in Materials and Methods. The immunoprecipitated pellet was resolved on SDS-PAGE (10%), transferred to nitrocellulose membrane, and subjected to Southwestern blotting analysis (see Materials and Methods) using rat rDNA core promoter probe. Lane 1: whole cell extract (80 μg) prior to immunoprecipitation. Lane 2: whole cell extract (240 μg) immunoprecipitated by control mouse IgG (2 μg). Lane 3: whole cell extract (240 μg) immunoprecipitated by anti-Ku-p70 antibodies (2 μg).

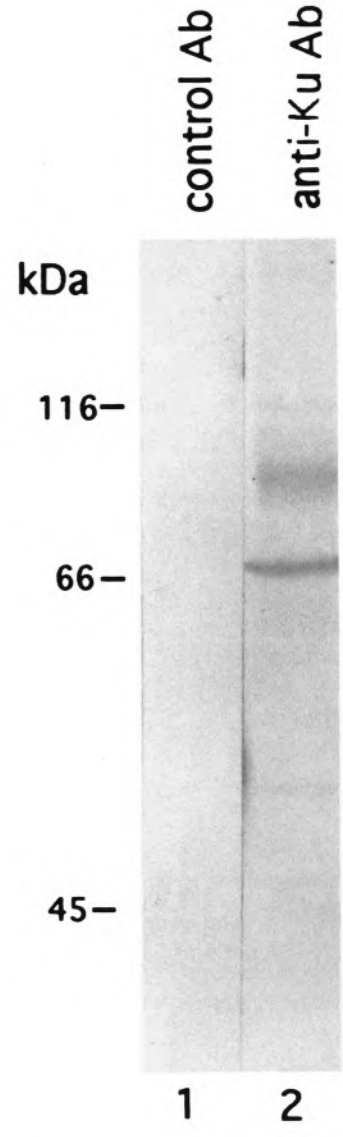

FIG. 10. Immunoprecipitation of E_1BF by anti-Ku-p70 antibodies. Whole cell extract from rat hepatoma (N1-S1) cells was immunoprecipitated by control antibodies or anti-Ku-p70 antibodies as described in Materials and Methods. The immunoprecipitated pellet was resolved by SDS-PAGE (8%) and subjected to Western blotting analysis using anti-Ku antibodies. Lane 1: whole cell extract (200 μg) immunoprecipitated by control mouse IgG (2 μg). Lane 2: whole cell extract (200 μg) immunoprecipitated by anti-Ku-p70 antibodies (2 μg).

reaction containing either CPBF or E_1BF (lanes 3 and 4). The noteworthy observation is that CPBF and E_1BF function synergistically to promote pol I transcription. These data clearly show that CPBF interacts with E_1BF both physically and functionally.

DISCUSSION

Past studies on rDNA transcription using a reconstituted system failed to identify the Ku protein or a Ku-related protein as an essential *trans*-acting factor, as none of the reconstituted transcription systems utilized the most highly purified components. The fraction containing the SL1 complex was used in reconstituted transcription.

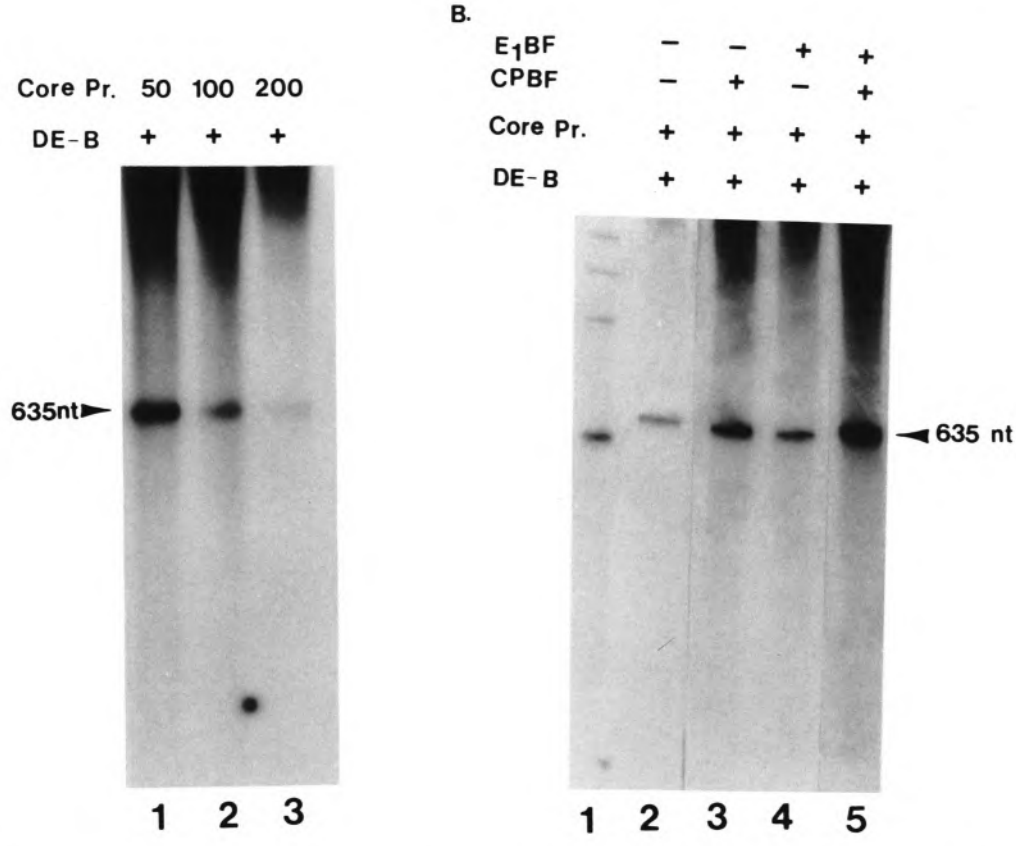

FIG. 11. Enhanced stimulation of rat rDNA transcription in response to the combined addition of E_1BF and CPBF. (A) Depletion of CPBF and E_1BF from fraction DE-B. Fraction DE-B (35 μg) was preincubated with 58 bp core promoter DNA fragment for 10 min at room temperature before starting the transcription reactions to determine the conditions for depletion of the factors. Lanes 1 to 3: in the presence of 50, 100, and 200 ng of 58 bp oligonucleotide corresponding to the rat rDNA core promoter, respectively. (B) Effect of exogenous E_1BF and CPBF on the rDNA transcription inhibited by preincubation with the oligonucleotide. The transcription assay was performed as described in Materials and Methods. After preincubation of fraction DE-B with core promoter oligo (150 ng), CPBF and E_1BF were added to the transcription reactions. Lane 1: molecular size markers. Lane 2: control transcription reaction in the absence of either factor. rDNA transcription activity of the depleted extract supplemented with 4 ng of CPBF (Lane 3), 4 ng of E_1BF (Lane 4), 2 ng each of E_1BF and CPBF (Lane 5).

This fraction invariably contains a trace amount of E_1BF, sufficient to direct basal rDNA transcription (Liu and Jacob, 1994). In addition, unless pol I is purified by glycerol density gradient fractionation or by a selective DNA affinity chromatography, it is associated with E_1BF (Liu and Jacob, 1994; Zhang and Jacob, 1990). The role of E_1BF/Ku in pol I transcription cannot, therefore, be assessed unless it can be inactivated by specific antibodies or depleted from the reaction by preincubation of the extract with an oligonucleotide fragment corresponding to the core promoter sequence. The latter strategies are largely responsible for our success in elucidating the functional role of this unique protein in rDNA transcription (Hoff et al., 1994, and the present data). One may argue that minimal level of ribosomal RNA synthesis could occur in vivo in the absence of E_1BF/Ku and that this protein acts more like a regulatory factor in vivo. Preliminary study does not, however, support this contention (A. Ghosh, unpublished data).

The similar extent of interaction between E_1BF and human or rat pol I promoter, and the stimulation of rat rDNA transcription in rat and human cell extracts in response to rat E_1BF indicate that E_1BF is not a species-specific factor. The present data also suggest that E_1BF/Ku is required specifically for pol I transcription. Anti-Ku antibodies inhibited human rDNA transcription completely, whereas similar concentration of the antibodies did not either inhibit or minimally block pol II-directed transcription from the adenovirus major late promoter, SV40 or CMV promoter (Fig. 7), or pol III-directed transcription of U6 RNA, and 5S RNA genes (Fig. 8).

A Ku-like transcription activator, PSE1, has been shown to bind the proximal sequence element of the human U1 promoter and share amino acid sequence with a protein called TREF, which binds to the human transferrin receptor (HTFR) promoter (Knuth et al., 1990). Immunodepletion with anti-PSE1 antibodies decreased U1snRNA gene transcription in vitro and addition of purified PSE1 reversed this effect (Knuth et al., 1990; Gunderson et al., 1990). Unlike the complete inhibition of pol I transcription by anti-Ku antibodies (Hoff et al., 1994), pol II transcription of U1snRNA gene was only partially inhibited. It appears from these reports that PSE1/Ku protein is not essential for the basal pol II transcription. Interestingly, transcription from either adenovirus major late promoter or the histone H2B promoter was independent of PSE1 (Knuth et al., 1990; Gunderson et al., 1990). This study, coupled with our observations, suggests that proteins of the Ku multigene family might transactivate selected pol II promoters. Alternatively, E_1BF characterized in our laboratory may be a distinct member of the Ku family and may have unique *trans*-activating function in pol I transcription. Unlike the classical Ku protein (Griffith et al., 1992), E_1BF can also bind to internal sequences in rDNA (Ghosh et al.,1993), which is consistent with the interaction of PSE1 with internal sequences in U1RNA gene (Knuth et al.,1990). Although members of the Ku-related proteins show similarities in peptide sequence, they are not structurally identical and may have functionally distinct roles. Attempts to obtain a satisfactory peptide sequence analysis of E_1BF have so far failed. It appears that unlike larger polypeptides of Ku, Ku-2, and PSE1, the p86 of E_1BF is blocked at N-terminus (K. Ghoshal and A. Ghosh, unpublished data). These observations suggest that E_1BF may be a unique Ku-related protein. Further study is required to address this issue and to determine whether PSE1, Ku-2, or any other Ku-related polypeptides are functionally related to E_1BF in regard to their role in pol I transcription.

Ku protein is one of the components of DNA-dependent protein kinase that phosphorylates the carboxyl-terminal domain (CTD) of RNA polymerase II (Gottilieb and Jackson, 1993). CTD phosphorylation is important at a postinitiation phase of the transcription reaction, which facilitates entry of transcription into the elongation stage (Payne et al., 1989). It is not known why E_1BF and another Ku-related protein, PSE_1, are incapable of *trans*-activating some pol II or pol III promoters. Because these proteins can bind to elements other than promoter sequence (Ghosh et al., 1993), the presence of other *cis*-acting elements in the plasmid construct may be required for detecting the effect of the Ku proteins in transcription of certain genes. Alternatively, transcription of the latter genes may be dependent upon other members of the Ku multigene family that have not been fully characterized.

Because specific pol I transcription requires TATA box binding protein (TBP) and its associated factors (TAFs) (Comai et al., 1992), one may raise the possibility that E_1BF or one of its constituent polypeptides functions as a rat TAF. This is particularly relevant, as E_1BF interacts with the promoter element and is involved in the preinitiation/initiation reaction (Hoff et al., 1994). Immunoprecipitation of the rat cell extract with anti-TBP antibodies did not, however, immunoprecipitate the E_1BF polypeptides (unpublished data). It is possible that one or both polypeptides comprising E_1BF may interact with TBP/TAFs complex following the initial interaction of this complex with the promoter. Further study is needed to address this issue.

Finally, interaction of E_1BF/Ku with CPBF, the core promoter binding factor, deserves comment. The latter factor binds to the core promoter element specifically and is essential for rat rDNA transcription (Liu and Jacob, 1994). Interestingly, no other core promoter binding proteins, including the well-characterized UBF, were immunoprecipitated with the anti-Ku antibodies. It should be noted that dissociation of the polypeptides comprising E_1BF causes significant reduction in its interaction with the core promoter elements. This may explain why E_1BF was not detected by Southwestern analysis after immunoprecipitation with anti-Ku-antibodies (Fig. 10) or direct Southwestern analysis of the cell extracts (Liu and Jacob, 1994). This experiment demonstrates the specificity of the interaction between E_1BF/Ku and CPBF. Mixing E_1BF and CPBF also resulted in enhanced DNase I footprinting on the core promoter sequence between -22 bp and $+3$ bp, which illustrates further the protein-protein interaction between E_1BF and CPBF (data not presented). We have not established at what stage during the initiation E_1BF interacts with CPBF. Studies along these lines are now in progress.

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