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Purification and Characterization of TTFI, a Factor That Mediates Termination of Mouse Ribosomal DNA Transcription

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Termination of rRNA gene transcription is dependent on an 18-base-pair sequence motif, AGGTCGAC CAG$^{AT}_{TA}$NTCCG (the Sal box), which is present several times in the spacer region downstream of the 3′ end of the pre-rRNA coding region. We report here the purification to molecular homogeneity of a nuclear factor which specifically interacts with the Sal box element. Addition of the isolated protein to S-100 extracts which contain low levels of the Sal box-binding protein and are therefore termination incompetent restores terminating activity, indicating that this protein is a polymerase I-specific transcription termination factor. The purified protein (termed TTFI) has a molecular weight of approximately 105,000 on sodium dodecyl sulfate-polyacrylamide gels. Mild proteolysis generates a relatively protease-resistant core which still specifically recognizes its target sequence. However, the termination activity has been lost, suggesting that the interaction with the DNA and the interaction with the transcription apparatus reside in different protein domains.

The control of gene expression in eucaryotes often results from the specific interaction of defined DNA-binding proteins with regulatory gene sequences. Attempts to understand transcription of eucaryotic genes have so far focused on the identification of sequences that are required for accurate and specific transcription initiation and on the purification and characterization of the protein factors that recognize these sequences, thus mediating selective gene expression. For a number of genes, specific transcription factors have been identified which recognize defined signal sequences of the gene promoter and impart selectivity to the RNA polymerases (for a review, see references 13, 16, and 20).

Much less is known about the sequences and protein factors responsible for the process of transcription termination in eucaryotes. Transcription termination is a complex process which not only causes the cessation of RNA chain growth but also leads to the release of the nascent RNA molecules. Furthermore, the RNA polymerase has either to be liberated from the template or to be modified in order to move along the DNA until it associates with another transcription initiation complex. To dissect the individual steps of the termination reaction, studies in cell-free systems which will allow the identification and functional characterization of the *cis*-acting elements and *trans*-acting cellular factors mediating this process are required.

We have shown previously that mouse RNA polymerase I terminates transcription 565 base pairs (bp) downstream of the 3′ end of the 28S rRNA-coding region in front of a conserved 18-bp sequence element, AGGTCGACCAG $^{AT}_{TA}$NTCCG (the Sal box). This nucleotide sequence is repeated eight times in the 3′-terminal spacer region between nucleotides +587 and +1178 relative to the 28S rRNA terminus (6). The Sal box is recognized by a nuclear protein which functions as a termination factor. Deletions, insertions, or point mutations which disturb the integrity of the Sal box consensus sequence affect the interaction of the nuclear factor with the DNA and inhibit or abolish transcription termination (7).

In this study, we describe the purification of a 105-kilodalton (kDa) nuclear protein that binds specifically to the Sal box element. This purification was achieved by a combination of conventional fractionation procedures and specific DNA affinity chromatography. The purified Sal box-binding protein retained the ability to mediate transcription termination in a reconstituted in vitro system and thus represents a bona fide transcription termination factor. Furthermore, we demonstrate that this factor exhibits a pronounced susceptibility to digestion by endogenous and exogenous proteases. A limited proteolytic treatment yields a protease-resistant core which is still able to specifically interact with the 18-bp termination signal. However, the ability of the factor to direct termination of mouse pre-rRNA synthesis was lost after protease treatment. This finding suggests that the factor consists of two functionally distinct domains which can be physically separated, one which interacts with the DNA and one which interacts with the transcription machinery.

MATERIALS AND METHODS

Recombinant plasmids. The recombinant plasmids used have been described previously. pMr600 contains a mouse ribosomal DNA (rDNA) promoter region extending from nucleotides −328 to +292 (21). pPTBH is a minigene construct which contains 324 bp from the 5′ region of the rDNA repeat (from −169 to +155 with respect to the initiation site) fused to a 381-bp *Bam*HI-*Hin*fI fragment extending from nucleotide +335 to +715 relative to the 3′ end of 28S rRNA (6). pUCT$_2$ contains an 83-bp *Nci*I fragment from the 3′-terminal spacer (from +604 to +686) cloned into the *Sma*I site of pUC9 (7).

Cell extract preparation. Ehrlich ascites cells grown in the peritoneal cavity of the mouse were cultured for 40 h in RPMI 1640 medium containing 5% newborn calf serum. They were harvested at a density of 1 to 1.2 × 10^6/ml. S-100 extracts were prepared as described by Weil et al. (22), and nuclear extracts were prepared as described by Dignam et al.

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(3). The proteins still bound to DNA after extraction of the nuclei were solubilized by the procedure described by Manley et al. (14). The nuclei were lysed by the addition of 0.5 M ammonium sulfate, the DNA was pelleted by centrifugation for 3 h at 4°C, and the soluble proteins were precipitated with 3 M ammonium sulfate. The proteins were dissolved at a concentration of 2 mg/ml and dialyzed against buffer A (see below) containing 100 mM KCl.

Transcription assays. Cell-free transcriptions were performed in 50-μl assays containing 30 ng of template DNA (pPTBH-*Eco*RI), 70 ng of pUC9 carrier DNA, and 30 μl of cell extract in a reaction mixture containing 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.9]); 85 mM KCl; 0.12 mM EDTA; 5 mM $MgCl_2$; 10 mM creatine phosphate; 0.6 mM each ATP, CTP, and UTP; 12.5 μM GTP; and 1 μCi of [α-^{32}P]GTP. The mixture was incubated for 60 min at 30°C and was processed for gel analysis as previously described (5).

Purification of TTFI. For protein purification, 100 ml of nuclear extracts (1.85 g of total protein) derived from about 80 liters of cultured Ehrlich ascites cells was diluted to 50 mM KCl with buffer A (20 mM Tris hydrochloride [pH 7.9], 0.2 mM EDTA, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) and loaded on a DEAE-Sepharose column (1 ml of resin for each ml of extract) equilibrated with buffer A containing 50 mM KCl (buffer A50). The column was washed with 2 volumes of buffer A50, and the factor was step-eluted with 280 mM KCl. After dialysis against buffer A100, the active fractions were applied to a heparin Ultrogel column (8-ml column volume). Bound proteins were eluted with two salt steps containing 280 and 600 mM KCl. Factor TTFI activity which was monitored by exonuclease III (exo III) protection and mobility shift assays was eluted in the 600 mM KCl fraction. The active fractions were pooled, dialyzed, and loaded onto a 4-ml CM Sepharose column equilibrated with buffer A50 and eluted with 200 mM KCl. The last step in the purification procedure involved chromatography on a specific DNA affinity column. The oligonucleotide used (CCCGGGATCC TTCGGAGGTCGACCAGTACTCCGGGCGAC) contained 30 bp of duplex DNA covering the sequence of the first Sal box plus a 9-nucleotide single-strand extension on each 5' end and was covalently coupled to Sepharose Cl 2B according to a protocol provided to us by Carl Wu. The dialyzed CM column eluate was adsorbed to 1.2 ml of the affinity resin by incubation at 4°C for 1 h with slow rotation. The material was then placed in a small column, and the bound proteins were eluted with buffer A containing 0.1% Nonidet P-40 (NP-40) and 0.35, 1.2, and 2 M KCl. The fractions eluting at 1.2 M KCl were pooled and subjected to a second round of affinity chromatography. By this procedure, TTFI was more than 80% pure as indicated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12) and silver staining of the proteins. The protein concentration of the fractions was measured by the method of Heil and Zillig (8).

Electrophoretic mobility shift assay. The gel mobility assay was performed essentially as described previously (1). The double-stranded 39-bp oligonucleotide that had been used for affinity chromatography was labeled at the 3' ends, and approximately 20,000 cpm (ca. 2 fmol of DNA) was incubated in a 25-μl assay containing 12 mM HEPES (pH 8.0), 120 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 8% glycerol, 2 μg of poly(dI-dC) heteropolymer, and 10 μg of tRNA in the presence of 25 μg of extract protein. After incubation for 20 min at room temperature, the samples were loaded onto low-ionic-strength (30 mM Tris hydrochloride [pH 8.0], 30 mM boric acid, 1 mM EDTA) 8% polyacrylamide gels and electrophoresed at 10 V/cm for 4 h at room temperature. The gel was then dried and analyzed by autoradiography.

For identification of the protein(s) bound to the specific probe, the amount of the oligonucleotide was scaled up to 10 pmol, and 5 μl of partially purified TTFI (heparin 600 mM KCl eluate) was incubated under standard binding conditions. After electrophoresis, the specific complexes were visualized by autoradiography, and the gel slices were transferred into the slots of a denaturing SDS-polyacrylamide gel (12) and electrophoresed along with protein size markers. The proteins were silver stained as described by Blum et al. (2).

Exo III protection assay. A 114-bp *Eco*RI-*Hin*dIII fragment was prepared from $pUCT_2$ by cutting with *Eco*RI followed by phosphatase treatment and 5'-end labeling with polynucleotide kinase. The labeled DNA was digested with *Hin*dIII, and 0.5 ng of DNA (ca. 5,000 cpm) was incubated for 15 min at room temperature in a 25-μl reaction mixture containing 12 mM HEPES, pH 8.0; 75 mM KCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 0.5 mM dithiothreitol; 4 mM NaF; 12% glycerol; 1 μg of poly(dI-dC); and 10 μg of yeast tRNA as well as various amounts of extract protein or column fractions. After the binding reaction, 6 U of exo III was added and incubation was continued for another 8 min. The reaction was terminated by the addition of 25 μl of 350 mM ammonium acetate and 10 mM EDTA. The DNA was purified by phenol-chloroform extraction and analyzed on 6% sequencing gels.

RESULTS

Complementation assay to study termination of rDNA minigene transcription. Previously we have used artificial ribosomal minigenes, i.e., plasmid constructs that represent fusions of the mouse rDNA promoter region with 3'-terminal spacer fragments, to study termination of polymerase I transcription in nuclear extracts derived from cultured Ehrlich ascites cells. In the recombinant plasmid pPTBH (6), 3'-terminal sequences (from +335 to +715 relative to the 3' end of 28S rRNA) were inserted downstream of the rDNA promoter. In the presence of nuclear extracts, 385- and 440-nucleotide transcripts (Fig. 1A, T_1 and T_2) whose 3' termini mapped 21 bp upstream of the first and second Sal box elements, respectively, were synthesized. In addition, various amounts of 540-nucleotide readthrough transcripts were generated depending on the concentration of template DNA and the amount of termination factor present in individual extract preparations. The most drastic differences were observed when cytoplasmic (S-100) and nuclear extracts were compared. Both types of extracts possess a similar capacity to generate faithfully initiated runoff transcripts from pMr600-*Eco*RI, indicating that the initiation factors are present in comparable quantities (Fig. 1B, lanes 1 and 2). When the same extracts were assayed with the minigene construct pPTBH-*Eco*RI, remarkable differences in the ratio of terminated versus readthrough transcripts were observed. The major RNA products synthesized in the presence of S-100 extracts were readthrough transcripts (Fig. 1B, lane 3). However, in nuclear extracts, most RNA chains were terminated at site T_1 or T_2, indicating that the amount or activity of the termination factor is significantly higher in nuclear than in S-100 extracts.

The failure of S-100 extracts to direct efficient termination provides a means of establishing a complementation assay

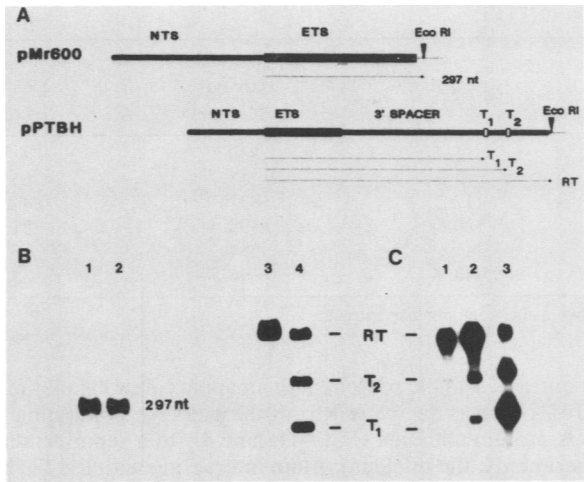

FIG. 1. Transcription of rDNA templates by different types of cell extracts. (A) Schematic diagram of pMr600, pPTBH, and the transcripts derived from these templates. nt, Nucleotide. (B) Autoradiogram of transcripts generated from pMr600-*Eco*RI (lanes 1 and 2) and pPTBH-*Eco*RI (lanes 3 and 4) in the presence of S-100 (lanes 1 and 3) and nuclear extracts (lanes 2 and 4). (C) Complementation of S-100 extracts by nuclear lysates. The transcription reaction was performed in a 25-μl assay in the presence of 7.5 μl of S-100 extract without nuclear lysate (lane 1) or with 2.5 μl (lane 2) or 5 μl (lane 3) of nuclear lysate. RT, Readthrough transcripts; T_1 and T_2, transcripts which terminated at the first or second Sal box present in pPTBH.

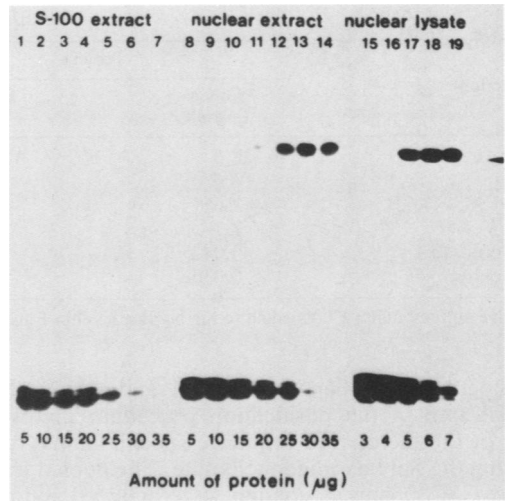

FIG. 2. Exo III protection assay with S-100, nuclear extract, or chromosomal proteins. Each reaction contained about 0.5 ng (ca. 5,000 cpm) of the 114-bp *Eco*RI-*Hin*dIII fragment from $pUCT_2$ and increasing amounts of extract protein derived from S-100 extracts (lanes 1 through 7), nuclear extracts (lanes 8 through 14), or the chromosomal protein fraction (lanes 15 through 19). The amount of protein present in the assays is indicated. The decrease of the labeled fragments observed at higher protein concentrations is due to phosphatase activity of the extracts.

for the functional identification of the termination factor. When nuclei after extraction of the soluble proteins were lysed by the procedure of Manley et al. (14), a chromosomal protein fraction was obtained which alone was incompetent to direct rDNA transcription because of the lack of initiation factor TIF-IB activity (unpublished data). Addition of these soluble chromosomal proteins to S-100 extracts (Fig. 1C) resulted in a shift from readthrough transcripts to terminated RNA molecules, indicating that this fraction contains significant amounts of factor activity which reconstitutes transcription termination.

To quantify the termination factor in the different types of cell extracts and to demonstrate a correlation between the terminating activity and the protein interacting with the Sal box sequence element, exo III protection experiments were performed. A 5′-end-labeled fragment containing the termination site T_2 was incubated with increasing amounts of S-100 or nuclear extract or with the solubilized chromosomal protein fraction (nuclear lysate) and subjected to exo III digestion. Both in the absence of protein and in the presence of S-100 extract, the double-stranded DNA was progressively degraded by exo III until the substrate was single-stranded and resistant to further digestion (Fig. 2, lanes 1 through 7). In the presence of nuclear extract, a 54-bp exo III-resistant fragment was generated (Fig. 2, lanes 8 through 14). The size of this fragment maps the 3′ boundary of the protected region to position +638 on the coding strand, i.e., 4 bp upstream of the Sal box sequence. Maximal protection was achieved with about one-fifth of the protein concentration of the nuclear lysate (Fig. 3, lanes 15 through 19) compared with the nuclear extract, a finding that suggests a close association of the factor with the chromatin.

Purification of the termination factor. To characterize the transcription termination factor, we purified the protein according to the fractionation scheme diagrammed in Fig. 3. Factor activity was monitored by both the exo III protection and the mobility shift assays (4) and by the ability to direct termination in the reconstituted transcription system. In a typical large-scale purification, 100 ml of nuclear extract was fractionated on DEAE-Sepharose, which was followed by

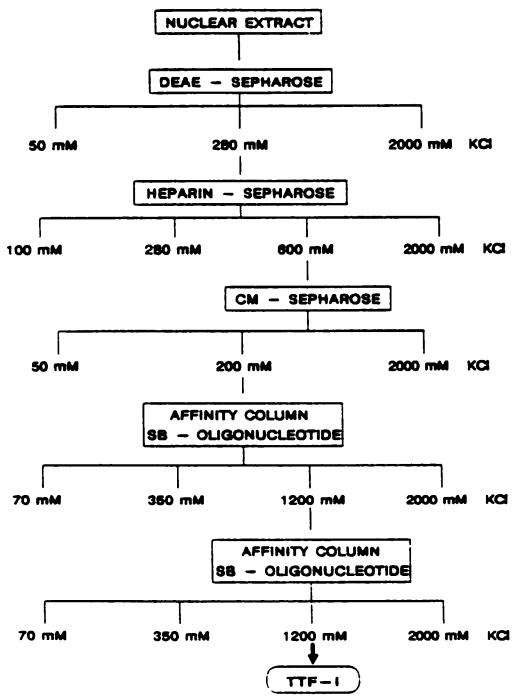

FIG. 3. Purification scheme for the transcription termination factor. SB, Sal box.

TABLE 1. Purification of TTFI

Fraction	Protein		Sp act (U/mg)[a]	Total activity (U)[a]	Yield (%)
	Concn (mg/ml)	Total amt (mg)			
Nuclear extract	18.5	1,850	1	1,850	
DEAE	4.6	555	2.2	1,215	65.7
Heparin	2.65	47.7	17.2	821	44.4
CM	2.1	19.1	20.8	398	21.3
Affinity column 1	0.0425	0.085	3,341	284	15.4
Affinity column 2	0.030	0.012	8,040	96.5	5.2

[a] Relative activity units; 1 U is equal to the binding level in 1 mg of protein of Ehrlich ascites cell nuclear extract.

chromatography on heparin-Ultrogel and CM-Sepharose. The final step in the purification procedure involved the binding of the factor to a sequence-specific affinity column containing the Sal box oligonucleotide. The pooled fractions from the CM-Sepharose column were dialyzed and applied to the oligonucleotide column at 70 mM KCl. After the column was washed with 0.35 M KCl to remove nonspecific DNA-binding proteins, the factor activity eluted at 1.2 M KCl. After two subsequent cycles of affinity chromatography, one predominant protein was obtained (see below). The entire procedure resulted in a roughly 8,000-fold purification of the Sal box-binding protein with a total yield of about 5% (Table 1). Activity yields were determined via quantitative exo III protection assays performed in the presence of increasing protein concentrations. The amount of protein required for saturation of a constant amount of DNA is a measure for the specific activity of the Sal box-binding protein.

Identification of the Sal box-binding protein as a 105-kDa polypeptide. To functionally identify the isolated protein as a termination factor, the high salt fractions eluting from the oligonucleotide column were assayed for specific DNA binding and termination activity. Figure 4 shows the result of such an analysis. Figure 4A shows an exo III protection experiment, which indicates the presence of the Sal box-binding activity in fractions 11 and 12. Figure 4B shows a transcription reconstitution assay, which also reveals the terminating activity within these two fractions. Figure 4C shows a silver-stained SDS gel in which proteins present in the same fractions were analyzed. There is only one 105-kDa polypeptide visible which peaks in fractions 11 and 12. Application of this fraction 5 times more to the gel reveals a faint larger protein of about 130 kDa and a few hardly detectable bands (less than 2% each) in the range between 80 and 200 kDa (I. Grummt, J. Clos, I. Bartsch, and M. Hannappel, UCLA Symp. Mol. Cell. Biol., in press). Quantitative exo III protection-competition experiments using a constant amount of purified protein and increasing concentrations of the Sal box oligonucleotide (not shown) indicate that the 105-kDa band is the only protein present in a quantity sufficient to account for stoichiometric binding to the Sal box probe. The correlation of the presence of this protein with both the specific DNA binding and the termination activity strongly suggests that the 105-kDa Sal box-binding protein functions as a transcription terminator.

To demonstrate that the Sal box-binding activity resides within the 105-kDa polypeptide, the factor-DNA complex was isolated on a preparative scale by electrophoresis (Fig. 4D, lane 1), and the eluted protein was analyzed by SDS-polyacrylamide gel electrophoresis. The partially purified factor preparation used for this experiment (heparin 600 mM KCl eluate) contained many polypeptides (Fig. 4D, lane 3). In contrast, a single protein with an apparent molecular mass of 105 kDa was recovered from the gel slice containing the DNA-protein complex (Fig. 4D, lane 4). In a separate set of experiments, the nuclear protein interacting with the Sal box motif has been identified by both UV cross-linking and renaturation of binding activity after electrophoresis in denaturing gels (Grummt et al., in press). Thus, it has been shown by several independent methods that the Sal box-binding protein is a polypeptide with an apparent molecular weight of about 100,000.

Murine and human termination factors share a common protease-resistant protein domain responsible for DNA binding. We consistently observed an extreme lability of the termination factor in crude extracts and during early purification steps. This decrease in terminating activity was accompanied by an increased heterogeneity of DNA-protein complexes in the mobility shift assay (Grummt et al., in press), suggesting that the factor molecule is very sensitive to proteolysis. To test this hypothesis, nuclear extracts were incubated with the labeled Sal box oligonucleotide probe in the presence of increasing concentrations of proteinase K. The murine DNA-protein complex was converted into a faster-migrating complex (Fig. 5B, lanes 1 through 6). This transition into the protease-resistant DNA-binding domain often occurred via defined intermediates (Grummt et al., in press). Both the slow- and fast-migrating complexes are specific, as shown by competition with $pUCT_1$ (Fig. 5B, lane 5), a plasmid containing the murine termination signal and 5′ flanking sequences. The analogous mutant plasmid $pUCT_1$ 589/590, which is incompetent for factor binding because of two nucleotide exchanges within the Sal box (7), did not compete for complex formation (Fig. 5B, lane 6). Obviously the protease had clipped off a considerable part of the factor molecule without greatly affecting its DNA-binding capacity. The same alteration in the mobility shift was also obtained after digestion with other proteases, such as elastase and chymotrypsin (data not shown), indicating that the DNA-binding domain of the factor has a compact protease-resistant structure.

Previously we have shown that the signal sequence mediating termination of human rDNA transcription is similar to the murine terminator sequence (1). The human sequence (GGGTCGACCPuPuC) is almost identical to the proximal part of the murine Sal box sequence motif. It is recognized by a protein which is functionally equivalent to the murine termination factor but exerts a different sequence specificity and different electrophoretic mobility in gel retardation assays. The different mobilities of the protein-DNA complexes of both species (Fig. 5A, lanes 1 through 6) indicate different physicochemical properties of the murine and human factors. Interestingly, these differences in the complex mobility of both species disappeared after protease treat-

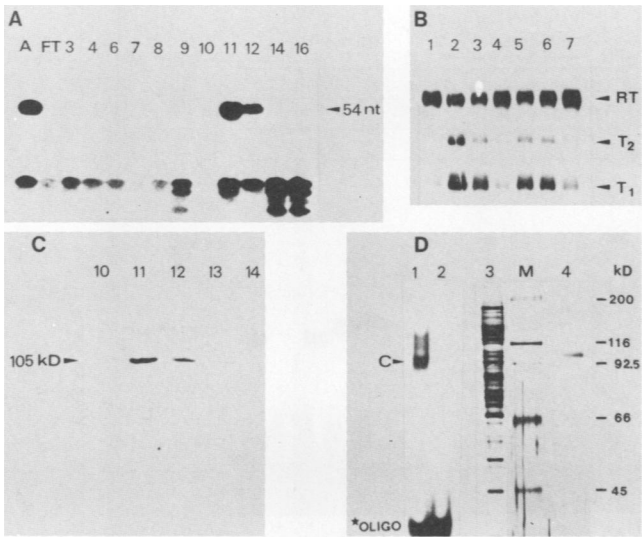

FIG. 4. Analysis of fractions from the specific oligonucleotide affinity column. (A) Exo III protection assay. A 114-bp DNA fragment from plasmid $pUCT_2$ was 5'-end labeled at the coding strand and incubated under standard conditions with 5 μl of column fractions. Lane A, Control reaction with the material applied to the column; lane FT, flowthrough fraction. The numbers above the lanes indicate the column fractions assayed. Fractions 3 through 9 were eluted by 350 mM KCl; fractions 10 through 16 were eluted by 1.2 M KCl. Specific protein binding resulted in the appearance of a 54-nucleotide exo III-resistant DNA fragment. (B) In vitro transcription assay. Each 25-μl assay contained 15 ng of pPTBH-*Eco*RI, 35 ng of pUC9 DNA, 7.5 μl of S-100 extract, and 7.5 μl of either buffer A100 (lane 1), nuclear extract (lane 2), the fraction applied to the affinity column (lane 3), or fractions 10 through 14 (lanes 4 through 7) eluting from the column at high salt. T_1 and T_2, Transcripts which terminated at the first or second Sal box present in pPTBH; RT, readthrough transcripts. The amount of terminated transcripts in lane 5 does not quantitatively reflect the termination activity of this fraction because of some losses during sample processing. (C) SDS-polyacrylamide gel electrophoresis of the purified factor. Individual column fractions (20 μl) were precipitated with 4 volumes of acetone in the presence of 10 μg of acrylamide. The proteins were electrophoresed on a 10 to 15% SDS-polyacrylamide gel and stained with silver. The molecular weight of the factor was determined by comparing its mobility with defined marker proteins. (D) Identification of TTFI protein in specific DNA-protein complexes. Labeled Sal box oligonucleotide (5×10^5 cpm) was mixed with 10 pmol of an unspecific oligonucleotide (lane 1) or the Sal box oligonucleotide (lane 2) and incubated with 5 μl of a protein fraction eluting from the heparin column at 600 mM KCl. The specific DNA-protein complex was separated from unbound DNA by electrophoresis, and the complex (C) from lane 2 was cut out as indicated. The gel slice was transferred into the slot of an SDS-polyacrylamide gel, and the proteins were analyzed by electrophoresis and silver staining (lane 4). For comparison, the protein pattern of the heparin fraction is shown (lane 3). Lane M shows marker proteins whose sizes are indicated.

ment (Fig. 5B). The murine and human protease-resistant complexes exhibited the same electrophoretic mobility, suggesting that the DNA-binding domains of the human and mouse termination factors are very similar.

DNA binding and termination activity reside in different protein domains. Next, we investigated whether the DNA-binding protein core obtained after mild proteolysis of the factor is functionally active in directing transcription termination in the cell-free system. For this investigation, the complementation assay described above was used. Partially purified termination factor was used to reconstitute S-100 extracts devoid of terminating activity. After addition of this protein fraction, which alone is incompetent to promote rDNA transcription (Fig. 6, lane 2) to S-100 extracts, a shift from readthrough to terminated transcripts was observed (lane 3). In the reactions depicted in lanes 4 and 5 of Fig. 6, the factor was treated with proteinase K in the presence and absence of phenylmethylsulfonyl fluoride. In parallel reactions, the conversion of the factor into its protease-resistant core was controlled by the gel retardation assay (data not shown). The protease-treated nuclear factor, which still efficiently and specifically binds to its target sequence (Grummt et al., in press), clearly does not support transcription termination. The ratio of readthrough to terminated RNA chains is virtually the same as in S-100 extracts (Fig. 6, lane 5). The decrease in the basal transcriptional activity observed in the protease-treated samples (Fig. 6, lanes 4 and 5) is probably due to an incomplete inhibition of proteinase K by phenylmethylsulfonyl fluoride. Nevertheless, the failure of the protease-resistant core to terminate rDNA transcription indicates that the protein domain that is essential for transcription termination is outside the DNA-binding domain and that both regions can be physically separated.

DISCUSSION

Previously we have identified in nuclear extracts an activity which mediates termination of *pol* I transcription on ribosomal minigene constructs. Termination is dependent on an 18-bp sequence element in the 3'-terminal spacer (the Sal box) which is recognized by a nuclear protein(s) directing transcription termination. In this paper we describe the purification of this sequence-specific DNA-binding protein and demonstrate that it functions as a termination factor. The fractionation procedure which used three conventional chromatographic steps combined with a sequence-specific DNA affinity column yielded a single 105-kDa polypeptide. As estimated from stained SDS gels, the factor preparations were regularly more than 80% pure. The purified protein exhibited the same binding specificity and exo III protection properties as the Sal box-binding protein in the crude material, indicating no gross structural changes during purification.

The 105-kDa polypeptide has been functionally identified as a termination factor in a transcription-complementation assay. S-100 extracts obtained after homogenization of cells in hypotonic buffer contain sufficient amounts of transcription factors and *pol* I to support specific rDNA transcription initiation but contain very low levels of termination factor. This fact enabled us to devise a reconstitution assay to measure termination factor activity. Addition of partly or highly purified preparations of the Sal box-binding protein to S-100 extracts resulted in the generation of correctly terminated RNA chains. The terminating activity copurified with the specific DNA-binding activity during all fractionation steps. This quantitative correlation between (i) the amount of the nuclear factor which interacts with the Sal box motif, (ii) the activity of the termination factor, and (iii) the appearance of the 105-kDa protein suggests that both site-specific DNA binding and transcriptional function reside within the same polypeptide. However, the present studies do not unequivocally prove that the 105-kDa protein alone is responsible for *pol* I transcription termination. Although the transcription complementation assay used showed a clear dependence of the termination reaction on the Sal box-binding protein, we cannot exclude that its main or sole

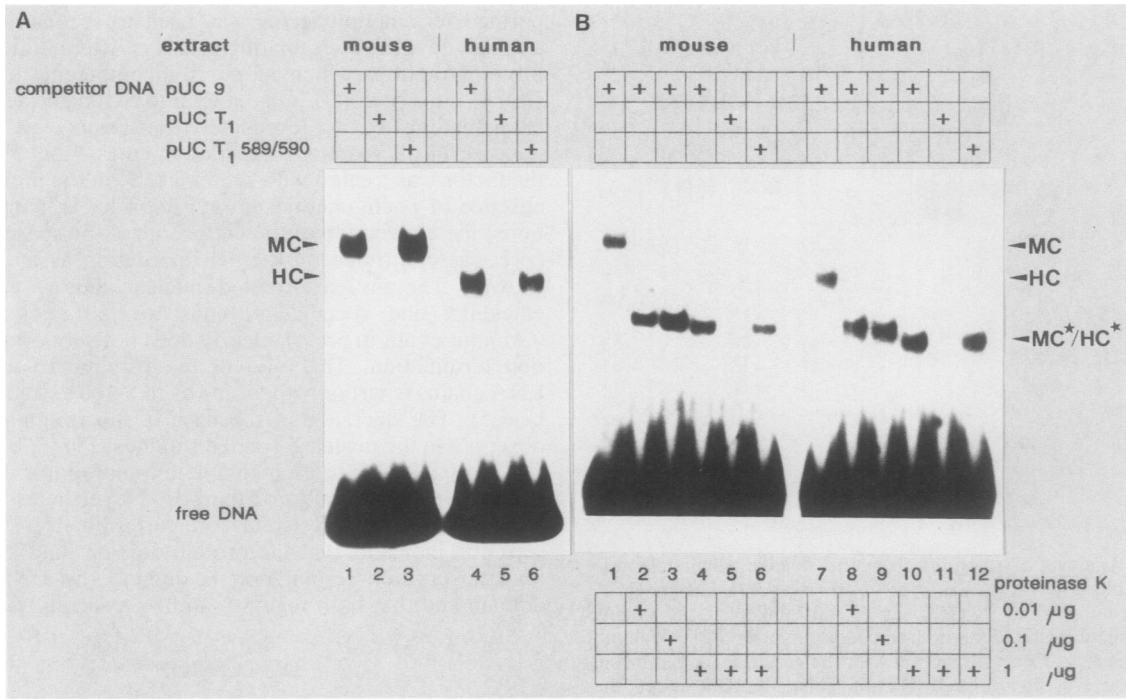

FIG. 5. Murine and human Sal box-binding proteins with similar protease-resistant protein domains. (A) Mobility shift assay with 2 fmol of labeled Sal box oligonucleotide and 50 µg of mouse (lanes 1 through 3) or human (lanes 4 through 6) nuclear extract proteins. Complex formation was performed in the presence of nonspecific competitor DNA pUC9, specific competitor DNA $pUCT_1$, and mutant DNA $pUCT_1589/590$ (7) as indicated above the lanes. (B) Mouse (lanes 1 through 6) or human (lanes 7 through 12) extracts were incubated at room temperature with the labeled Sal box oligonucleotide in the presence of different competitor DNAs. After 15 min, increasing amounts of proteinase K were added, and incubation was continued for 5 min at 37°C. Complex formation was monitored by the mobility shift assay. MC and HC, Intact murine and human complexes, respectively; MC* and HC*, fast-migrating murine and human complexes, respectively, observed after protease treatment.

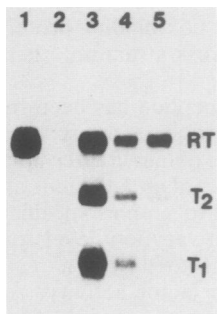

FIG. 6. Inability of the protease-resistant core of TTFI to direct transcription termination. The termination complementation assay contained 30 ng of template DNA pPTBH cut with *Eco*RI, 70 ng of pUC9, and a total of 30 µl of protein fractions. Lanes: 1, 30 µl of S-100 extract; 2, 30 µl of partially purified Sal box-binding protein (heparin 600 mM KCl fraction); 3 through 5, 15 µl of S-100 extract plus 15 µl of the heparin fraction. To assay the activity of the protease-resistant core, the heparin fraction was incubated for 15 min at 30°C with the template DNA before treatment for 3 min at 30°C with 250 ng of proteinase K. The protease was inhibited by addition of 2 mM phenylmethylsulfonyl fluoride, and the reaction was started by addition of the salt mixture and the S-100 extract (lane 5). As a control, phenylmethylsulfonyl fluoride was added before proteinase K treatment (lane 4). RT, Readthrough transcripts; T_1 and T_2, transcripts which terminated at the first or second Sal box present in pPTBH.

function is to mediate pausing of the RNA polymerase at a defined point in the spacer and that the formation of correct 3′ ends and the release of the nascent RNA chains are brought about by a specific nuclease or an RNA-associated polypeptide that interacts with the Sal box-binding protein.

The termination factor TTFI shows a remarkable sensitivity to endogenous and exogenous proteases. A conversion of the 105-kDa polypeptide into smaller proteins is often observed after storage of the extracts or after repeated freezing and thawing. Concomitant with this appearance of heterogenous DNA-protein complexes a loss of termination activity occurred, suggesting that the factor is very susceptible to proteolysis. This view is supported by the finding that the DNA-protein complexes formed in crude extracts can be converted into a unique faster-migrating complex by mild protease digestion. The proteolytic fragment obtained exhibits identical boundaries of exo III protection and the same binding specificity as the intact factor (Grummt et al., in press). UV cross-linking experiments indicate that the protease treatment generates a 49-kDa DNA-binding protein core (Grummt et al., in press). Interestingly, this protease-resistant protein domain appears to be very similar in both the murine and the human factors, which in their native form exhibit different electrophoretic mobilities in gel retardation assays (1). We suggest that the DNA-binding domain of the *pol* I-specific termination factor of both species has been conserved, although a molecular coevolution has taken place between the termination signal sequences and the genes coding for the termination factors.

The proteolytic core has lost the ability to direct transcription termination. This result indicates that the protein domains responsible for specific DNA binding and for interaction with the transcription machinery do not overlap. Such a physical separation of DNA binding and transcriptional function has also been observed in other eucaryotic regulatory proteins as yeast GAL4 (11), GCN4 (10), and the tRNA-specific transcription factor τ (15). Most of the amino acid sequences of these factors can be removed without affecting DNA binding. It has also been reported that the 5S gene-specific transcription factor TFIIIA contains a C-terminal protein domain which is not involved in DNA binding but which enables the protein to direct initiation, probably through interaction with RNA polymerase (19). On the other hand, a colocalization of both DNA binding and *trans*-acting functions to a small core region of a regulatory protein has been described for both the human and the rat glucocorticoid receptors (9, 17). There, a central 88-amino-acid region contains all the information required for DNA binding and transcriptional activation.

At present we are still ignorant of the molecular mechanism by which the termination factor causes the stop of the elongation reaction and the release of the nascent RNA chains. There are definitely basic differences in the mechanism of transcription termination in procaryotes compared with eucaryotic *pol* I-specific transcription. The crucial procaryotic signal sequences have been shown to reside in the RNA, and the significant participation of DNA-protein interactions in the termination process has been ruled out (for a review, see reference 18). Termination of mammalian *pol* I transcription, however, is mediated by a protein factor that specifically binds to the termination signal sequence located downstream of the site of 3'-end formation. It is not yet known how the factor interacts with the transcription machinery and thus exerts its biological function. Furthermore, it remains to be investigated whether the heterogeneity of the Sal box-binding protein which we observe in partially purified factor preparations (Grummt et al., in press) is solely due to the extreme protease sensitivity of TTFI or has to be attributed to related proteins with similar sequence specificity. The final proof of whether the DNA-binding domain of TTFI is encoded by a unique gene or whether it is a member of a gene family with still unknown functions will hinge on genetic analysis. Cloning of the gene coding for TTFI and genetic engineering of the protein will be required to answer this question and to define the functional domains involved in the interaction with the template, with *pol* I, and with the nascent RNA.

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