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Species-Specific rDNA Transcription Is Due to Promoter-Specific Binding Factors

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RNA polymerase I transcription factors were purified from HeLa and mouse L cell extracts by phosphocellulose chromatography. Three fractions from each species were found to be required for transcription. One of these fractions, virtually devoid of RNA polymerase I activity, was found to form a stable preinitiation complex with small DNA fragments containing promoter sequences from the homologous but not the heterologous species. These species-specific DNA-binding factors can explain nucleolar dominance in vivo in mouse-human hybrid somatic cells and species specificity in cell-free, RNA polymerase I-dependent transcription systems. The evolution of species-specific transcriptional control signals may be the natural outcome of a special relationship that exists between the RNA polymerase I transcription machinery and the multigene family coding for rRNA.

Interspecific cell hybrids between rodent species express the rRNA genes (rDNA) of both parental cell lines (8, 18, 29). However, rodent-human cell hybrids exhibit nucleolar dominance (4, 9, 23, 27, 43), resulting in the preferential transcription of the rDNA of one species. This suggests that rDNA transcription may be species specific.

Species-specific RNA polymerase I transcription has in fact been demonstrated in vitro in cell-free transcription systems. A *Drosophila melanogaster* Kc cell extract is capable of transcribing a *D. melanogaster* but not a *Drosophila virilis* rDNA template in vitro (16). *Physarum*, *Drosophila*, and *Acanthamoeba* spp. rDNAs are not transcribed in mouse cell extracts (11). In addition, human cell extracts do not transcribe mouse or rat rDNA templates, nor do mouse cell extracts initiate transcription from human rDNA (12, 20, 30) (see Fig. 1C).

The results from observations of hybrid cells in vivo and in vitro studies on rDNA transcription stand in stark contrast to those for most genes transcribed by RNA polymerase II. For example, protein-coding genes are usually expressed in mouse-human hybrid cells regardless of their species origin, and this fact has been utilized extensively in the construction of human genetic maps (34). The transcription of 5S RNA genes and tRNA genes by RNA polymerase III factors has also been shown to be independent of species origin (38, 48). In addition, nucleotide sequence comparisons of eucaryotic genes transcribed by RNA polymerases II and III have identified sequences at defined positions surrounding the initiation site which are evolutionarily conserved and appear to be involved in the accurate initiation of transcription (39). However, this is not true for eucaryotic rDNA. Comparisons of nucleotide sequences for a considerable distance around the origins of transcription of human (10, 20, 26), mouse (28), *Xenopus* (41), *Drosophila* (21), and yeast (42) genes show that no apparent sequence conservation for all these species exists. Therefore, species-independent RNA polymerase II and III transcription reflects sequence conservation of proposed regulatory signals, whereas rDNA control sequences (see references 11 and 19) seem to have evolved under less severe constraints, resulting in species-specific transcription (1).

We report in this paper the partial purification and characterization of factors required for in vitro RNA polymerase I-dependent transcription of human and mouse rDNAs. We demonstrate that a factor(s) from each species binds to rDNA segments required for in vitro transcription (promotor sequences) in a species-specific fashion. This result is discussed in terms of the evolutionary mechanisms which may have led to the species specificity in rDNA transcription by coevolution of rDNA-binding factors and their homologous rDNA-binding domains.

MATERIALS AND METHODS

Fractionation of whole-cell extracts. HeLa or L cells (20 liters) were grown as suspension cultures, and extracts were prepared essentially as described by Manley et al. (22). Briefly, logarithmically growing cells were harvested, and cell pellets were frozen at −20°C before homogenization. Proteins in the subsequent high-speed supernatant were precipitated with 60% ammonium sulfate. This protein pellet was dialyzed against DB buffer, consisting of 100 mM KCl, 2 mM dithiothreitol, 0.1 mM EDTA, 17% glycerol, and 40 mM Tris (pH 7.9 at 25°C). The ratio of the optical densities measured at 260 and 280 nm (OD_{260}:OD_{280}) was usually 1.3, with the final protein concentration estimated at 10 to 20 mg/ml. Extracts were applied to a phosphocellulose column (Whatman P-11) and equilibrated with DB buffer at a concentration of 10 mg of protein per ml of bed volume. Step elutions were performed with KCl by the method of Matsui et al. (24). Peak OD_{280} fractions were pooled, dialyzed against DB buffer containing 12.5 mM $MgCl_2$, and stored as aliquots at −80°C.

RNA polymerase I assay. Using saturating levels of UTP, we assayed dilutions of the pooled fractions in a 25-μl reaction mixture containing 50% DB buffer with 12.5 mM $MgCl_2$, 500 μM each ATP, CTP, and GTP, 50 μM UTP, 10 μCi of [α-^{32}P]UTP (740 Ci/mmol; New England Nuclear Corp.), 200 μg of α-amanitin per ml, and 5 μg of native calf thymus DNA. After 20 min at 30°C, the solution was spotted

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onto DE81 (Whatman) paper disks, washed with 5% Na_2HPO_4 as described by Hammond and Holland (13), and counted in liquid scintillation fluid.

rDNA templates. Construction of mouse and human rDNA clones has been previously described (26). The human rDNA template, T1, was linearized 750 base pairs (bp) 3′ of the initiation site, and T2 was linearized at the *Sst*I site, which is 200 bp further downstream. The mouse template was a *Pvu*II digest of the mouse rDNA clone, which results in a 300-bp runoff transcript (28).

In vitro transcription. The standard 25-μl reaction mixture contained 250 ng of template DNA, 500 μm each ATP, CTP, and UTP, 50 μM GTP, 10 μCi of [α-^{32}P]GTP (710 Ci/mmol; New England Nuclear), 200 μg of α-amanitin per ml, and 13 μl of unfractionated extract or 5 μl each of fractions A, C, and D (see Fig. 1A). Under these conditions the concentration of the transcription factors in the reconstituted system was the same as in the unfractionated extract. The reaction was stopped after 1 h at 30°C and run on 5% acrylamide–8 M urea gels as previously described (26).

Competition assays. DNA fragments to be used as competitors were generated by the appropriate restriction enzyme digestions and made blunt-ended by repair with DNA polymerase I (Klenow fragment). These were isolated by electroelution from preparative acrylamide gels after visualization by UV shadowing (25). All samples recovered (3 to 7 μg) were adjusted to a standard DNA concentration of 50 μg/ml, based on the equation: 1 OD_{260} = 50 μg/ml. This final concentration was confirmed by high-resolution acrylamide gels, using DNA standards. The preincubation procedure was essentially that described by Davison et al. (5). At room temperature we mixed 5 μl of fraction D with 120 ng of polyinosinic acid · polycytidylic acid [poly(I) · poly(C)] (Miles Laboratories) for 5 min, followed by a 10-min preincubation with 10 to 200 ng of the competitor DNA fragment. To this we added 250 ng of template DNA, and after another 10 min of incubation, we added 5 μl each of fractions A and C and the remaining components of the transcription reaction mixture described above. After 45 min at 30°C the reaction was terminated, and transcription was analyzed by gel electrophoresis. Since these gels were run so that the 300-bp mouse runoff transcript was near the bottom, we were unable to detect any shorter transcripts which may have been produced by the origin-containing rDNA fragments H2 and M1 used as competitors.

RESULTS

Fractionation and reconstitution of a HeLa cell extract. Human cell extracts (22) were fractionated by phosphocellulose chromatography (Fig. 1A). We determined the minimum number of fractions needed to reconstitute the in vitro

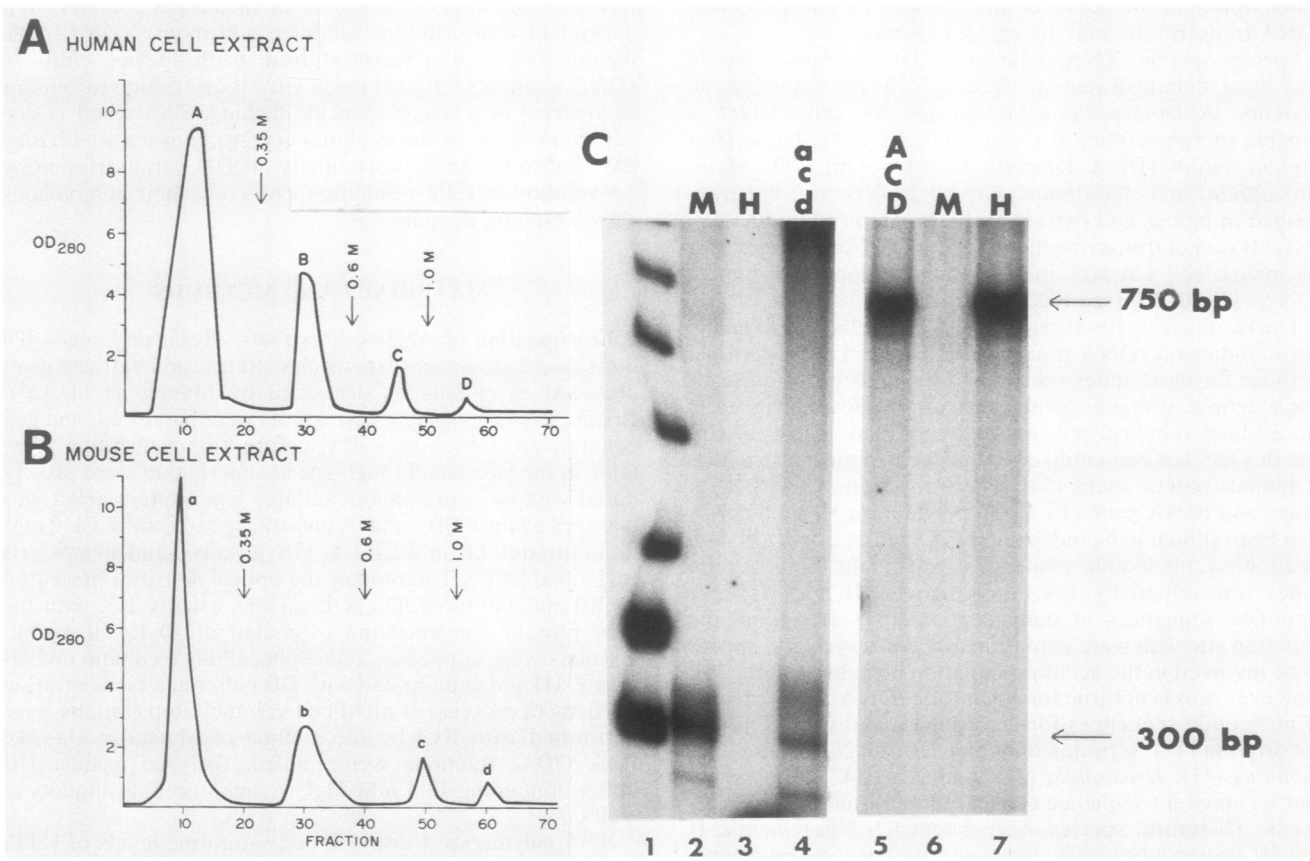

FIG. 1. Phosphocellulose chromatography of human (A) and mouse (B) whole cell extracts. Peak OD_{280} fractions were pooled and used in the reconstituted transcription systems. A, C, and D, Minimum required human (H) fractions; a, c, and d analogous mouse (M) fractions. (C) Autoradiograph of the reconstituted in vitro transcription systems. Lanes 2 through 4, Mouse rDNA template producing a 300-bp runoff transcript added to mouse (M), human (H), or reconstituted mouse (a, c, d) extracts. Lanes 5 through 7, Human rDNA template producing a 750-bp runoff transcript added to a reconstituted human (A, C, D), mouse (M), or human (H) extracts. Lane 1, End-labeled DNA fragments from a *Hin*cII digest of ϕX174.

TABLE 1. RNA polymerase I activity in phosphocellulose fractions

Source	Fraction	RNA polymerase I activity	
		U/ml[a]	% of total
HeLa cell extract	A	23.8	23.5
	C	75.2	74.3
	D	2.4	2.2
L cell extract	a	11.7	9.4
	c	110.7	88.7
	d	2.4	1.9

[a] 1 U = 1 pmol of UTP incorporated in 20 min at 30°C in the presence of 200 μg of α-amanitin per ml.

transcription system by assaying for the ability of the fractions to accurately transcribe an rDNA template in the presence of 200 μg of α-amanitin per ml. It was found that only three (A, C, and D) of the four phosphocellulose fractions were required to give the same specific runoff transcript as the unfractionated extract (Fig. 1C). Elimination of any one of these three fractions resulted in no transcription (data not shown). Table 1 shows the distribution of RNA polymerase I activity in fractions A, C, and D. The majority of RNA polymerase I activity was in fraction C, with the remainder being in fraction A; fraction D contained negligible amounts.

A factor in fraction D forms a stable preinitiation complex. We tested each required fraction for factors with DNA-binding activity, using one template (T1) which gave a 750-bp runoff transcript and another (T2) which gave a 950-bp runoff transcript (26) (Fig. 2). T2 was identical to T1 except that it contained an additional 200 bp of downstream sequences. When fractions A, C, and D were incubated for 15 min with an equal amount of T1 and T2, followed by the addition of the other components required to initiate transcription, the amount of runoff transcript from each template was found to be equal (Fig. 2, lane 1). However, if T1 or T2 was first incubated alone with A, C, and D, then virtually all of the transcript originated from this template, even though the second template was present for the 1-h reaction period during which transcription occurred (Fig. 2, lanes 2 and 3). This shows that one or more of the three fractions contains a factor(s) which appears to form a stable preinitiation complex similar to that reported recently in an RNA polymerase II system (5).

To identify which of the three fractions contains the factor(s) responsible for this preferential transcription, we repeated the competition experiments but used only one fraction at a time in the incubation. The concentration of each of the three fractions (see above) was in the linear range for transcriptional activity determined by titration experiments (data not shown). The results (Fig. 2, lanes 4 to 6) showed that the differential transcription seen in lanes 2 and 3 could not be due to a DNA-binding factor in fraction A since incubation of a template with A did not result in its preferential transcription. Incubation with fraction C resulted in an altered ratio of transcription of T1 (lane 8) or T2 (lane 9), depending on which was added first. This suggests that some factor(s) in fraction C can form a complex with the template.

However, an even more dramatic difference was observed when fraction D was incubated with T1 (lane 11) or T2 (lane 12). Nearly all of the transcription occurred with the incubated template, mimicking the results seen when all three fractions were used. Incubation with fraction D alone also resulted in a much greater degree of transcription. Since incubation with fraction C did show some preferential transcription, we tested the possibility that C and D together may increase transcription even more. There was no detectable enhancement from adding fraction C along with D during the incubation period (Fig. 2, lanes 13 to 15). One possible explanation for the ability of fraction C alone to form a complex (lanes 8 and 9) is that C might be contaminated with small amounts of D. Regardless of this, however, our data clearly identify the component(s) in fraction D as the major contributing factor(s) to the formation of stable preinitiation complexes, and further characterization of this reaction was carried out.

Localization of the binding domain. To determine where, relative to the origin of transcription, the factor(s) in fraction D was binding, we carried out competition experiments analogous to those of Davison et al. (5), using small DNA segments surrounding the origin of transcription. As did their experiments, ours included mixing a predetermined amount of poly(I) · poly(C) with fraction D before preincubation. We found that this step was required for significant levels of specific binding by this partially purified transcription factor(s) (data not shown). After this poly(I) · poly(C) step, fraction D was preincubated with a test DNA segment for 10 min. A full-length transcription template was then added to the mixture, and the mixture was incubated for an additional 10 min. Finally, transcription was initiated by adding the remaining transcription factors (A and C) and other components. Three human rDNA fragments (H1, H2, and H3) were tested (Fig. 3A). In the first experiment (Fig. 3B), it appeared that H2 (lane 2) was having an inhibitory effect relative to the sequences further upstream (H1) or downstream (H3). Additional experiments (Fig. 3D, cf. lanes 1 and 2 and lanes 6 and 7) fully confirmed this observation (see also Fig. 5). The H2 fragment contained the sequences from −170 to +80 relative to the human rDNA origin of transcription (10, 20, 26), including those which are absolutely required for in vitro transcription (19).

The interaction of the required transcription factor(s) in

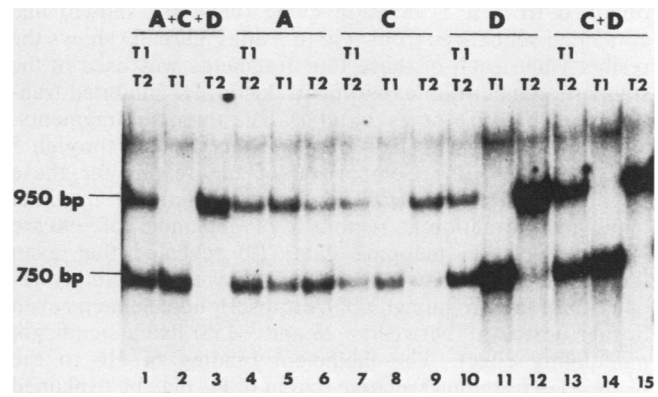

FIG. 2. Phosphocellulose fractions from HeLa cells were incubated either simultaneously or sequentially with 250 ng each of two human rDNA tempates, T1 or T2. The components of each 15-min incubation are shown above the lanes. After this incubation, all the remaining reactants were added and incubated for 1 h at 30°C. This final reaction mixture contained fractions A, C, and D, T1 and T2, nucleotide triphosphates, and α-amanitin. The upper band (>1,200 bp) is an artifact which was occasionally but not always observed and was independent of the source of template DNA added (data not shown).

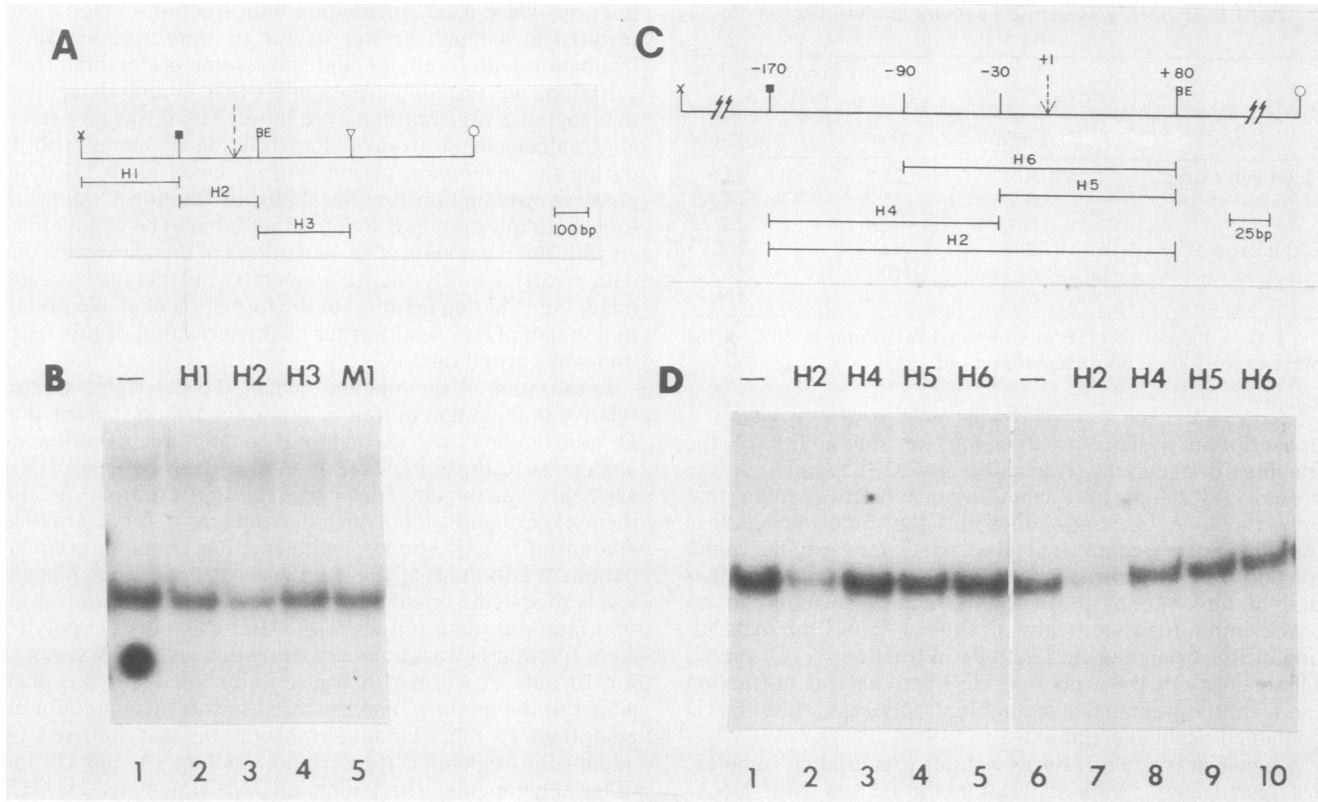

FIG. 3. (A) Map of the fragments used as competitors for the binding factor(s) present in human fraction D. (B) Competition experiment in which fraction D was preincubated with 100 ng of each of three human rDNA fragments (H1, H2, and H3) or a mouse rDNA fragment (M1; see Fig. 5A) before the addition of template T1. (C) Additional human rDNA fragments used to map the binding domain (see the text for details). (D) Results of competition experiments with the human rDNA competitor fragments shown in panel C. The preincubation step contained 100 (lanes 2 through 5) or 200 (lanes 7 through 10) ng of competitor DNA. Symbols (A and C): X, EcoRI; ■, *Hin*cII, BE, *Bst*EII, ▽, *Xor*II; ○, *Sal*I; ↓, origin of transcription.

fraction D with H2 was dissected further by using subfragments (Fig. 3C). H4 and H5 were obtained by cleaving H2 with *Hin*fI, which resulted in 140-bp and 110-bp fragments upstream and downstream, respectively, of −30. H6 was obtained from a 5′ deletion clone (data not shown) and contained sequences from −90 to +80. Figure 3D shows the results when each of these four fragments was used in the standard competition experiment. Again, H2 inhibited transcription (Fig. 3D, lanes 2 and 7). The three subfragments, however, had no effect on transcription (lanes 3 through 5 and 8 through 10). Comparison of the results for these fragments with those for H2 suggests that for maximal binding with fraction D, regions 3′ of −30 and 5′ of −90 are needed. Deletion mapping data (19) revealed that even though elements between −26 to +7 were absolutely required, for in vitro human rDNA transcription elements even further upstream (between −26 and −158) had a significant modulating effect. The inefficient binding of H6 to the factor(s) in fraction D relative to that of H2 may be explained by the absence of these upstream elements. Although H4 contained sequences 5′ of −30, they were not by themselves sufficient for maximal binding, and therefore sequences 3′ of −30 must also be required.

We carried out DNase I footprinting studies with fraction D, which we estimate contains 0.4% of the protein originally present in the transcription extract, but we were unable to map the binding site. An additional 20-fold purification of fraction D by Biorex 70 chromatography (see the legend to Fig. 4) was still inadequate. Further purification of the transcription factor(s) in fraction D, perhaps with the approaches developed by Samuels et al. (36) and Dynan and Tjian (7), appears to be necessary.

Mouse cells also contain an rDNA-binding factor. To determine whether mouse cells contain a factor which forms a preinitiation complex with mouse promoter sequences, we fractionated a mouse L cell extract (M) by phosphocellulose chromatography, using the same elution conditions as for the HeLa extract (Fig. 1B). The relative protein contents and RNA polymerase I activities of the fractions were very similar in the two species (cf. Fig. 1A and 1B; Table 1). We found that, as with the HeLa extract, mouse fractions a, c, and d were all required for transcription (Fig. 1C, lane 4). We carried out a competition experiment in which mouse fraction d was preincubated with mouse rDNA promoter (M1) and downstream (M2 or M3) sequences, followed by the addition of a full-length mouse template. M1 was the only fragment which inhibited transcription (Fig. 4B, lanes 2 and 7). In vitro transcription (28) and deletion mapping studies (11) have shown that all the sequences required for in vitro transcription are within the M1 fragment. Our results, therefore, support the idea that, like human cells, mouse cells also contain a transcription factor which interacts specifically with rDNA promoter sequences.

Human and mouse rDNA-binding factors are species specific. The results discussed above show that fraction D from human cells and fraction d from mouse cells each contain a

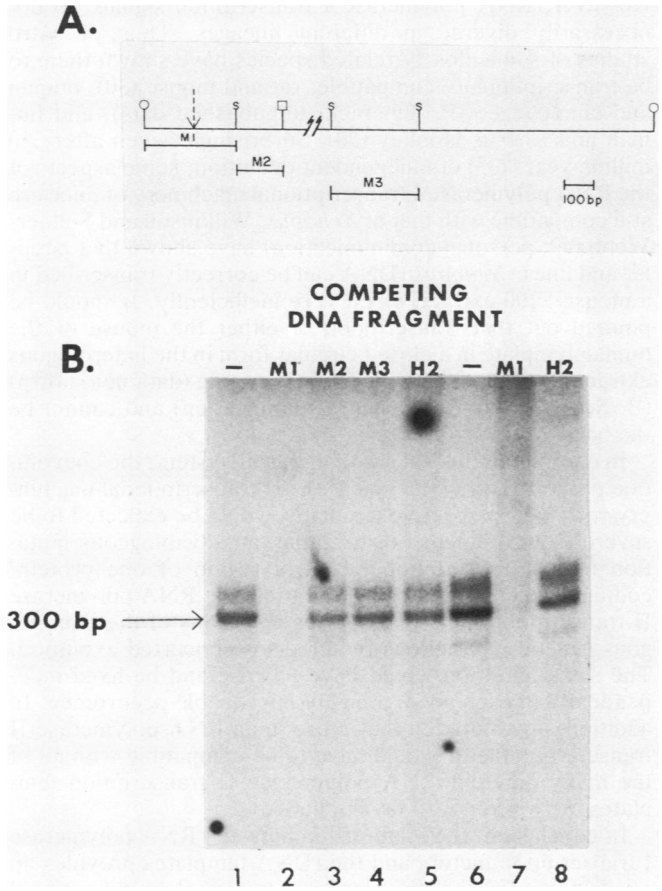

FIG. 4. Competition experiments in which 50 ng of the mouse fragments M1, M2, and M3 (panel A) or the human rDNA fragment H2 was preincubated with mouse fraction d, followed by the addition of the mouse rDNA full-length template and the additional mouse components (fractions a and c, nucleotide triphosphates, etc.). Lanes 6 through 8, 5 μl of a Biorex 70 column fraction which contained the mouse d transcription factor(s), enriched 20-fold relative to the phosphocellulose fraction d used in the lanes 1 through 5. The d activity was eluted from the Biorex 70 column by a 0.5 to 1.5 M KCl gradient. Symbols: (○, *Sal*I; S, *Sma*I; □, *Pvu*II; ↓, origin of transcription.

factor(s) which binds fragments containing the homologous rDNA origin of transcription (H2 and M1, respectively). We next tested the ability of these binding factors to form preinitiation complexes with the heterologous rDNA fragments. In preincubation experiments, the mouse fragment M1 had no effect on human rDNA transcription (Fig. 3B, lane 5). Conversely, the human origin of transcription containing fragment H2 did not inhibit mouse transcription (Fig. 4B, lanes 5 and 8). We confirmed this observation by repeating the competition experiments in triplicate (Fig. 5). Even over a range of concentrations, both M1 (Fig. 5A) and H2 (Fig. 5B) specifically decreased transcription only in the homologous reaction. In these particular experiments, unlike those shown in Fig. 3B and 4B, there was a nonspecific decrease in transcription positively correlated with the total amount of competitor DNA added. At all concentrations, however, the homologous DNA segment interfered with transcription to a far greater extent than did the heterologous one. Nonspecific effects were observed by Davison et al. (5) in their analysis of RNA polymerase II-mediated transcription. It is important to realize, however, that since M1 had no specific effect on human rDNA transcription in the human system (Fig. 3B and 5B), the inhibition of mouse rDNA transcription by M1 could not be due to contamination by a nonspecific inhibitory substance. Similarly, H2 must act as a specific inhibitor in the human system, since it did not reduce transcription in the mouse system (Fig. 4B and 5A). The extent of specific inhibition seen (Fig. 3 and 4) was very reproducible and was observed for two independent competitor DNA preparations with mouse and human fractions obtained from more than one phosphocellulose column (data not shown). The basis for the 10-fold difference in amount of competing DNA fragments needed to inhibit the human and mouse systems (100 and 10 ng, respectively) is not known.

DISCUSSION

We have identified a function of two related transcription factors which are required for in vitro RNA polymerase I-dependent transcription of homologous rDNA templates. The finding that they each form preinitiation complexes in the absence of RNA polymerase I with DNA fragments containing sequences found to be important for transcription suggests that they may play a role in the regulation of the transcriptional initiation event. A HeLa cell factor(s) which binds DNA has recently been identified as an important component of RNA polymerase II in vitro transcription systems (5). Sequences upstream from both the adenovirus type 2 major late promoter and the conalbumin gene were both able to serve as binding domains only if their respective TATA boxes remained intact. The promoter-binding factors of RNA polymerase III systems have been even more extensively studied. The regulation of *Xenopus* 5S RNA gene expression by a positively acting DNA-binding transcription factor has been well characterized (17). In addition, preliminary studies with fractionated *Drosophila* cell extracts have been described which suggest that a factor(s)

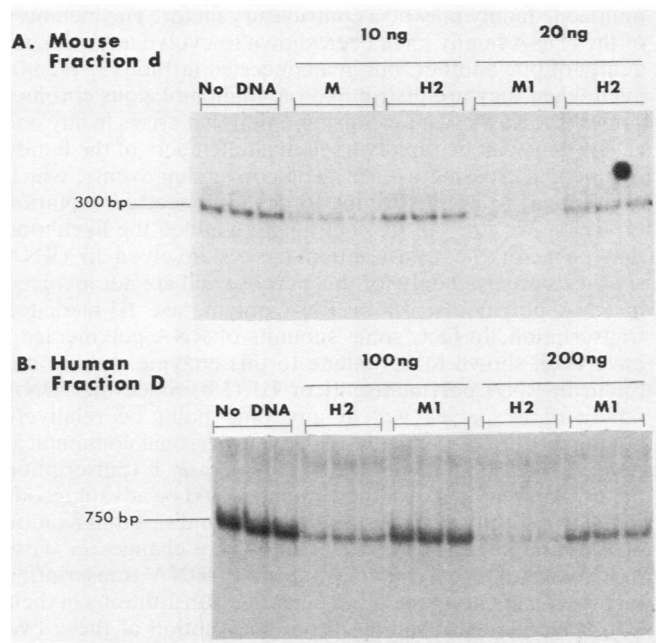

FIG. 5. Competition experiments performed in triplicate with the human (H2) or mouse (M1) binding domain-containing fragments. (A) Mouse fraction d in the mouse system. (B) Human fraction D in the human system.

forms a stable binding complex with the internal T-control region of a *Drosophila* tRNA gene (37).

Genes transcribed by RNA polymerases II and III have conserved promoter sequences which have been shown to be required for in vitro transcription (39). This conservation allows RNA polymerase II and RNA polymerase III transcription templates to be transcribed in a wide variety of heterologous in vitro systems (38, 45–48). As reviewed above, in vitro studies on the RNA polymerase I system are very different and show species specificity. Our results demonstrate that the inability of the required transcription factor(s) in fractions d and D to form stable preinitiation complexes with heterologous templates is the basis for the observation that mouse and human rDNA transcription is species specific in vitro (12, 20, 30) (Fig. 1C). If our binding factors were the only species-specific component, we would expect that if a human template was added to a transcription mixture containing all the human factors, but with mouse d substituted for human D, then no transcription would occur, although a mouse rDNA template added to this same mixture would be transcribed. Recently, Mishima et al. (30) reported just this result. It is interesting that although their species-specific factors were also eluted by a 1 M KCl wash from a phosphocellulose column, they reported that 30% of the RNA polymerase I activity was contained in this fraction. However, we found that fractions d and D contained only about 2% of the RNA polymerase I activity in our reconstituted system (see Table 1). Finally, the phenomenon of nucleolar dominance in mouse-human hybrid cells can be explained by our results if both binding factors are not present in each particular line. In fact, it has recently been observed that mouse-human hybrid cells not expressing human rDNA lack the human factor(s) in fraction D (R. Miesfeld, B. Sollner-Webb, C. Croce, and N. Arnheim, manuscript submitted for publication).

How is it that transcription factors and rDNA promoter sequences from different species can become incompatible during evolution? The special genetic behavior of the rDNA multigene family may be a contributory factor. The members of the rDNA family have been shown to evolve not independently of one another, but in a concerted fashion (3, 14, 44), even when they are distributed on nonhomologous chromosomes (2). Any advantageous mutation that arises in any one rDNA gene can be rapidly fixed in all members of the family by unequal crossing-over or gene conversion events, which are thought to be the driving forces of concerted evolution (1, 6, 31, 32, 40). To this fact must be added the likelihood that some of the transcription factors involved in rRNA synthesis are used only for this purpose and are not involved in RNA polymerase II- or RNA polymerase III-mediated transcription. In fact, some subunits of RNA polymerase I have been shown to be unique to this enzyme and are not found in RNA polymerase II or III (15). Since the rDNA family of a species will at any time point be relatively homogeneous (T. Ohta and G. Dover, personal communication), a mutation in an RNA polymerase I transcription factor that interacts with the template could be advantageous to the transcription of most of the genes and result in fixation of the altered factor. Thus, evolutionary changes in these particular RNA polymerase I-specific rDNA transcription factors can occur along with nucleotide substitutions in their rDNA templates, resulting in the coevolution of these two components of transcription (1).

If the direction of coevolutionary change is different in independent evolutionary lineages, then transcriptional incompatibility can arise, as is the case in humans and rodents. However, RNA polymerase I transcription signals do not necessarily diverge in different lineages. Thus, in vitro studies of some closely related species have shown them to be transcriptionally compatible: rat and mouse (30), human and chimpanzee (P. Seperack, unpublished data), and human and Rhesus monkey (20). Surprisingly, even after 350 million years (33) of independent evolution, some aspects of the RNA polymerase I transcriptional machinery of mice are still compatible with that of *Xenopus*. Wilkinson and Sollner-Webb (49; personal communication) have shown that circular and linear *Xenopus* rDNA can be correctly transcribed in a mouse S100 extract, albeit very inefficiently. It should be pointed out that transcription of either the mouse or the human template in a closed circular form in the heterologous extract is still undetectable by S1 mapping (data not shown) (B. Sollner-Webb, personal communication) and cannot be the basis for in vitro species specificity.

In contrast to the RNA polymerase I system, the coevolution of the RNA polymerase II or III transcriptional machinery with their respective templates would be expected to be severely constrained. For example, an advantageous mutation in the transcriptional control region of one protein-coding gene could not be spread to all the RNA polymerase II transcription units in a species by means of the homologous genetic interactions required for concerted evolution. The same mutation would have to arise and be fixed independently in each gene, a highly improbable occurrence. In addition, any mutation that arose in an RNA polymerase II transcription factor would have to be compatible with all of the many different RNA polymerase II transcription templates if it were not to be eliminated.

In conclusion, the relationship between RNA polymerase I transcription factors and the rDNA templates provides an opportunity for the divergence of essential transcriptional control signals during evolution. For the other two transcription systems, however, fundamental control signals required by all the genes would by necessity be expected to be highly evolutionarily conserved. We also wish to point out that the coevolutionary principle under which the RNA polymerase I system may evolve could also be applied to a subset of RNA polymerase II or III transcription units which undergo concerted evolution and interact with a DNA-binding factor required for the expression of such a subset of genes.

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