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Transcription from the rat 45S ribosomal DNA promoter does not require the factor UBF

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For efficient transcription from the rat ribosomal DNA (rDNA) promoter by RNA polymerase I in vitro, at least two transcription factors, rat UBF and rat SL-1, are required. Transcription cannot take place in vitro in the absence of SL-1. On the other hand, there is considerable difference of opinion concerning the necessity for UBF in in vitro transcription mediated by RNA polymerase 1, and the requirement for UBF is not clear. Mammalian cells code for UBF1 and UBF2, two forms of UBF that differ in HMG box-2, one of four HMG boxes or DNA-binding domains. We have used a monospecific antibody raised to recombinant rat UBF to determine whether UBF1 and UBF2 are required for RNA polymerase I-mediated transcription. This antibody can detect as little as 1.35×10^{-15} moles of UBF1 or UBF2 in an immunoblot. Fractionated extracts that were competent for transcription had no detectable UBF1 or UBF2 when assayed in immunoblots with this antiserum. This evidence supports the hypothesis that UBF is not required for transcription of the rat rDNA promoter in vitro and most likely functions as an auxiliary transcription factor. In addition, we have fractionated rat UBF1 from UBF2 and tested each of them in in vitro transcription assays in which the 45S or spacer rDNA promoter template is limiting. UBF1 can activate transcription from either the 45S or spacer promoter under these conditions, whereas UBF2 cannot. This implies that there is a functional difference in the transactivation of RNA polymerase I by UBF1 and UBF2 in vitro. Furthermore, we show that COS cells, which are transfected with and overexpress $p21^{H\text{-}ras}$, express only UBF1 and not UBF2, as detected by Western blotting. This suggests that UBF2 is also dispensable in vivo for the transcription of the ribosomal RNA genes.

Analysis of the molecular anatomy of the vertebrate ribosomal RNA (rRNA) gene promoter has revealed two separable domains: the core promoter element (CPE), which extends from nucleotides −31 to +6 and is required for transcription in vitro, and the upstream promoter element (UPE) or upstream control element (for review see Sollner-Webb and Tower, 1986). Truncated templates that terminate just upstream of the CPE may be transcribed weakly in vitro but are not inactive (Cassidy et al., 1987; Smith et al., 1990). On the other hand, the CPE is necessary but insufficient to direct transcription in vivo (Henderson and Sollner-Webb, 1990; Xie and Rothblum, 1992). The UPE, although less well defined than the CPE, is required for transcription in vivo (Henderson and Sollner-Webb, 1990) and for the formation of a stable preinitiation complex in vitro (Cassidy et al., 1987).

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At least two RNA polymerase I transcription factors interact with the UPE: UBF, of which there are two forms, UBF1 and UBF2 (O'Mahony and Rothblum, 1991); and SL-1 (Smith et al., 1990; Learned et al., 1986). UBF contains four HMG boxes, which represent the DNA-binding domains. UBF1 and UBF2 differ in that UBF2 has a deletion of 37 amino acids in HMG box-2 (O'Mahony and Rothblum, 1991). UBF1 and UBF2 also differ in their DNA-binding and dimerization properties (O'Mahony et al., 1992b). Both are phosphoproteins (O'Mahony et al., 1992a).

A third factor, E_1BF, purified from rat adenocarcinoma ascites cells, has been shown to interact with both the 174 bp enhancer element (located between nucleotides −2,183 and −2,219 of the spacer region) and the CPE (Zhang and Jacob, 1990) and is related to the human autoantigen Ku (Hoff and Jacob, 1993). Recent data suggest that the regulation of rDNA gene transcription may involve an antirepressor mechanism, which may be mediated through Ku (Kuhn et al., 1993), and that UBF activates transcription by relieving the inhibition exerted by negatively acting factor(s).

The UPE extends from nucleotides −60 to about −140. DNase I footprints of rat UBF extend from nucleotides −60 to −120 in the UPE of the rDNA promoter (Smith et al., 1990; Pikaard et al., 1990). Linker-scanning mutagenesis demonstrated that at least two distinct regions within the UPE of the rat rDNA promoter are required for maximal transcription (Smith et al., 1990; Xie et al., 1992). The region between nucleotides −101 and −106 is required for both the UBF footprint and UBF-mediated activation of transcription in vitro (Smith et al., 1990; Xie et al., 1992). Furthermore, the region between nucleotides −129 and −124 is also required for UBF-mediated activation of in vitro transcription; it lies within the region protected by SL-1 (Smith et al., 1990; Xie et al., 1992).

There are considerable data to suggest that SL-1 and RNA polymerase I are sufficient for transcription in vitro, and that UBF is not required. We have reported that UBF could not be detected by DNase I footprinting assays of DE-175 fractions, which are sufficient to direct transcription from the rat rDNA promoter (Xie et al., 1991). Furthermore, we could not detect the presence of UBF in DE-175 fractions by a number of other assays, including UV cross-linking assays with a radiolabeled rDNA promoter probe (Xie et al., 1991) and in vitro phosphorylation of the DE-175 fractions with casein kinase II, which effectively phosphorylates UBF in vitro (D. J. O'Mahony and L. I. Rothblum, unpublished data). However, the sensitivity of those assays was never established.

The purpose of the present experiments is to determine whether UBF is required for transcription from a mammalian rDNA promoter. Using a monospecific, polyclonal antiserum raised to recombinant UBF—which can recognize as little as 1.35×10^{-15} moles of UBF—we failed to detect UBF in fractions fully competent for transcription. Furthermore, in transcription assays in which the rDNA promoter template is limiting, we demonstrate that UBF1—but not UBF2—stimulates transcription from either the 45S or spacer promoter, suggesting a functional difference between UBF1 and UBF2 in the in vitro transactivation of RNA polymerase I. In addition, we show that COS cells, transfected with a cDNA coding for $p21^{H\text{-}ras}$, express UBF1 but not UBF2, suggesting that UBF2 is also dispensable in vivo under certain conditions.

Materials and methods

In vitro transcription

The conditions used for in vitro transcription were described previously (Cassidy et al., 1986; Haglund and Rothblum, 1987). A standard 50 μl reaction contained 0.1 μg of the truncated template and 1.0 μg of nonspecific DNA, either pBR322 or pUC18. The efficiency of transcription was quantitated as described previously (Cassidy et al., 1986). For assays demonstrating that UBF is not essential for transcription, the pooled RNA polymerase I-containing fractions from the first DEAE-Sephadex column (described below) were used. This fraction contains RNA polymerase I and SL-1 and is referred to as the "UBF-depleted extract."

Fractionation of nuclear extracts

Nuclear extracts were prepared from Novikoff hepatoma ascites cells, as described previously (Haglund and Rothblum, 1987; Kurl et al., 1984). The extracts were fractionated by DEAE-Sephadex column chromatography, as described (Smith et al., 1990). The column was washed

with DE-50 and eluted stepwise with solutions containing 175 and 500 mM $(NH_4)_2SO_4$, generating the fractions referred to as DE-175 and DE-500, respectively. The DE-175 fraction is the same as the DE-B fraction used by other investigators (Kurl et al., 1984). The subsequent treatment of DE-175 and DE-500 has been described previously (Smith et al., 1990). DE-175 contained RNA polymerase I and SL-1 but was depleted of UBF, as determined by DNase I footprinting of the rat 45S rDNA promoter. DE-500 was enriched for UBF. The chromatographic separation of UBF1 from UBF2 from Novikoff hepatoma cells was performed by CM-Sephadex chromatography in which UBF1 was eluted at 500 mM KCl, and UBF2 was eluted at 200 mM KCl (Smith et al., 1990).

Immunoblots and purification of recombinant UBF

The monospecific antiserum to UBF was described previously, as was the protocol for Western blots (O'Mahony et al., 1992a). To determine the lower limit of detection in an immunoblot, serial dilutions of purified UBF were spotted onto Immobilon-P membrane (Millipore) or subjected to Western blotting using the same batch of Immobilon-P. To control for nonspecific binding in the "spotting" assay, the final concentration of protein was kept at 1 mg/ml by adding bovine serum albumin. The same preparation of antiserum (C21) at a 1:500 dilution was used in both types of immunoblotting experiments. The antigen-antibody complexes were visualized with the same lot of ^{125}I-labeled goat anti-rabbit IgG (FAB_2 fragment from DuPont NEN). The blots were exposed for autoradiography for 24 hours at −70°C.

UBF1-486, the recombinant UBF containing the N-terminal domain and the four HMG boxes of UBF1, was expressed from the plasmid pIH902 in Escherichia coli, as described previously (O'Mahony et al., 1992b), and was purified by CM-Sephadex column chromatography, essentially as described for the purification of UBF from Novikoff cells (Smith et al., 1990). After a single column, the material was homogenous when fractionated by SDS-PAGE and stained with either Coomassie blue or silver staining (data not shown). The purified recombinant UBF1-486 was subsequently used to standardize the immunoblots.

Analysis of UBF expression in COS cells transfected with a cDNA coding for $p21^{H\text{-}ras}$

COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS) as described (Kinsella et al., 1991). The cultured cells were transfected with the plasmid pCMV5 or with pCMV5ras, a plasmid that overexpresses $p21^{H\text{-}ras}$ in COS cells, essentially as described (Kinsella et al., 1991). Forty-eight hours after transfection, unfractionated cell lysates (100 μg) from control COS cells or COS cells transfected with pCMV5 or pCMV5ras were fractionated by SDS-PAGE, transblotted onto nitrocellulose, and screened with the antibody to $p21^{H\text{-}ras}$ or with the UBF antiserum, essentially as described (Kinsella et al., 1991; O'Mahony et al., 1992a, respectively).

Results

In vitro transcription assays (Fig. 1) demonstrate that DE-175 is capable of transcription without

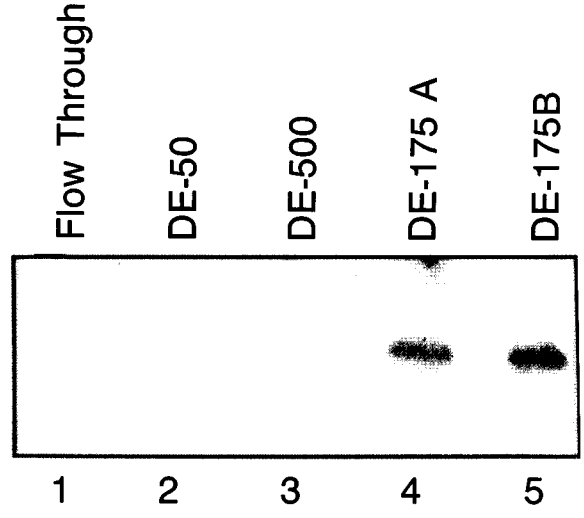

Figure 1. In vitro transcription by DEAE-Sephadex fractions of a nuclear extract. The fractions indicated (5 μl each) were assayed for their ability to support in vitro transcription. One-third of the transcription reaction was analyzed by PAGE and autoradiography. The transcript demonstrated was 628 nt according to the position of molecular weight size markers (not shown). The autoradiograph was quantitated as described previously (Cassidy et al., 1987). DE-175A and DE-175B were DEAE-175 fractions from two different column runs of two different starting nuclear extracts. The two fractions contained 0.4 mg/ml and 1.2 mg/ml protein, respectively.

supplementation with fraction DE-500. This reproduces our previous results. We reported previously that the DE-175 fraction was depleted of UBF, as determined by DNase I footprinting assays (Smith et al., 1990), UV cross-linking assays (Xie et al., 1991), and in vitro phosphorylation assays with casein kinase II (unpublished data). UBF contains nine consensus phosphorylation sites for casein kinase II and is phosphorylated in vitro by this kinase on both the NH_2-terminal domain and the acidic C-terminal domain (O'Mahony et al., 1992b). Since the DE-175 fraction is transcription-competent, it is of interest to establish how UBF-depleted it is.

The pooled DEAE-Sephadex column fractions were analyzed by Western blotting for the presence of UBF. UBF was detected in the initial nuclear extract (Fig. 2, lane 1), and in the material eluted at 500 mM $(NH_4)_2SO_4$ (DE-500; Fig. 2, lane 3). No UBF was detected in the 175 mM $(NH_4)_2SO_4$ eluates of four different columns (Fig. 2, lanes 2, 4, 5, and 6). Further, after exposure of this Western blot for 48 hours, no UBF was detected in any of the 175 mM $(NH_4)_2SO_4$ fractions. It should be noted that the Western blots of the DE-175 fractions used four times the amount of extract protein utilized in the transcription assays.

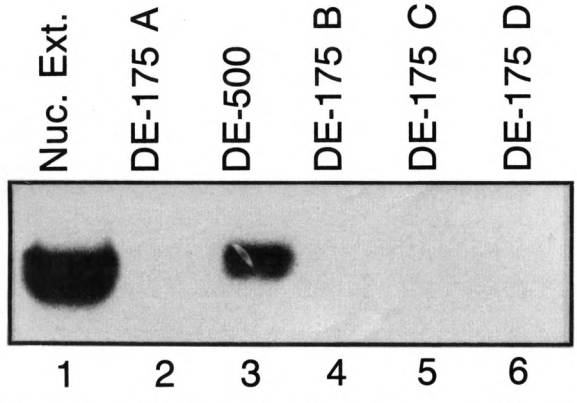

Figure 2. UBF is found in DE-500 but not in DE-175. Unfractionated nuclear extract (12 μg) and the indicated DEAE fractions of one or more extracts were fractionated by SDS-PAGE, blotted to Immobilon-P, and assayed for the presence of UBF with the UBF antiserum C21 at a 1:500 dilution, as described previously (O'Mahony et al., 1992b). Two of the DE-175 fractions, DE-175A and DE-175B, were those used in Figure 1. Two other DE-175 fractions were also assayed. The following amounts of protein were applied: lane 1, 12 μg; lane 2, 8 μg; lane 3, 1.8 μg; lane 4, 24 μg; lane 5, 20 μg; and lane 6, 22 μg. The blot was exposed for autoradiography for 24 hours at −70°C.

The sensitivity of the Western blot was estimated by standardizing the blot protocol, using an Immobilon-P filter containing dot blots or Western blots of known amounts of recombinant UBF (UBF1-486; Fig. 3, panels A and B, respectively). For these assays, UBF was purified to homogeneity from the plasmid construct pIH902, which overexpresses recombinant UBF1-486 (O'Mahony et al., 1992b) in the soluble fraction of the induced culture (D. J. O'Mahony and L. I. Rothblum, unpublished data). The limit of detection in these assays was estimated to be 1.35×10^{-15} moles of UBF.

To determine whether there is a functional difference between UBF1 and UBF2 in the in vitro transactivation of RNA polymerase I, UBF1 and UBF2 were separated by fractionation on CM-Sephadex chromatography. The separation of UBF1 and UBF2 was confirmed by Western blotting, using the same dilution of the UBF antiserum C21 (Fig. 4A). The UBF1 fraction consisted entirely of UBF1, whereas the UBF2 fraction contained trace amounts of UBF1 (Fig. 4A). Limiting quantities of the 45S or spacer rDNA promoters were assayed with the DE-175 fraction alone or the DE-175 fraction supplemented with purified UBF1 or UBF2. As shown in Figure 4B, neither DE-175 nor DE-175 supplemented with UBF2 efficiently promotes RNA polymerase I-mediated transcription from the 45S or spacer promoter when either template is limiting. In contrast, UBF1 stimulated transcription from both promoters under these conditions (Fig. 4B). Taken together, these results indicate that UBF1 and UBF2 differ in their abilities to transactivate RNA polymerase I in vitro.

To investigate whether UBF expression is necessary in growing cells, we analyzed the expression of UBF1 and UBF2 in COS cells transfected with a pCMV5-based plasmid, pCMV5ras, which overexpresses $p21^{H\text{-}ras}$ in vivo (Kinsella et al., 1991). Both control COS cells and COS cells transfected with pCMV5 express UBF1 and UBF2, as determined by Western blotting (Fig. 5). In contrast, in the case of COS cells transfected with pCMV5ras, Western blotting revealed only UBF1 expression, with no discernible expression of authentic UBF2, suggesting that UBF2 is dispensable in vivo. The cross-reacting band of low molecular weight, which

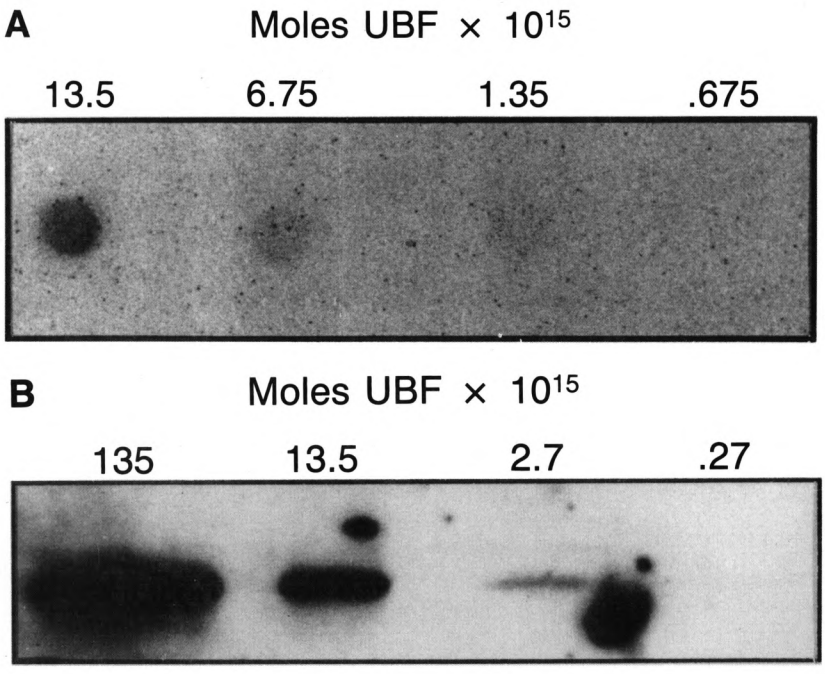

Figure 3. Immunoblots of recombinant UBF. **A.** Serial dilutions in 1 mg/ml bovine serum albumin of purified recombinant UBF (UBF1-486) were applied to an Immobilon-P membrane and subjected to immunodetection by the UBF antiserum C21 at a 1:500 dilution, as described in Materials and Methods. **B.** Western blots of recombinant UBF following SDS-PAGE and electroblotting to Immobilon-P. The blots were probed with the UBF antiserum C21 at a 1:500 dilution and exposed for autoradiography for 24 hours at −70°C.

migrates at 84 kDa in the pCMV5ras-transfected COS cells, may represent either unphosphorylated or processed forms of UBF.

Discussion

We have examined the hypothesis that transcription in vitro of the rat rDNA promoter does not require the presence of UBF. Previously we reported the inability to detect UBF in the fractions competent for transcription, as determined by DNase I footprinting (Smith et al., 1990) and UV cross-linking assays (Xie et al., 1991). In this manuscript, we determined our lower limit of detection to be 1.35×10^{-15} moles of UBF, and we present evidence consistent with a model of transcription by RNA polymerase I in which UBF is not essential. It should be noted that longer exposure of the autoradiograms presented in Figure 3 lowered the limit of detection to $<1.35 \times 10^{-15}$ moles of UBF. On the other hand, longer exposures of the Western blot shown in Figure 2 failed to demonstrate UBF in the DE-175 fraction. Nonetheless, this level of

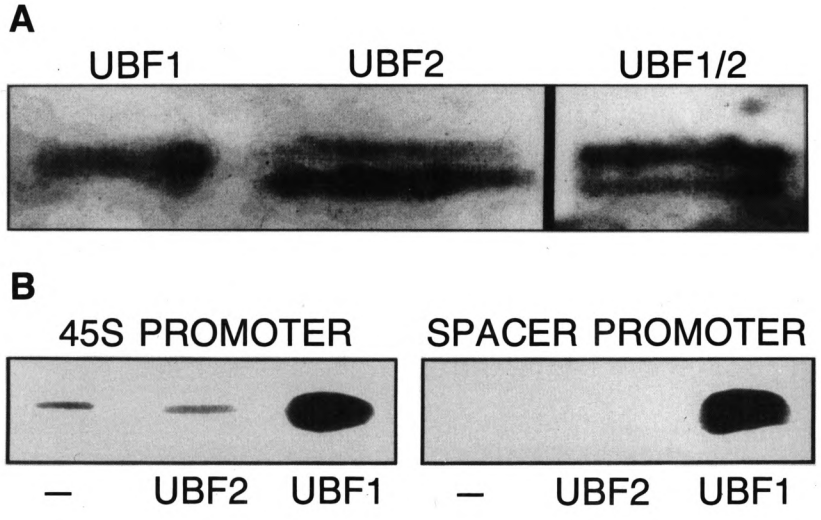

Figure 4. UBF1 and UBF2 differ in the transactivation of RNA polymerase I. **A.** UBF1 was separated from UBF2 by CM-Sephadex chromatography. Western blots of samples containing the UBF antiserum and UBF1, UBF2, or a mixture of UBF1 and UBF2 are shown. **B.** In vitro transcription. Transcription from limiting quantities of the 45S or spacer rDNA promoter was performed with the DE-175 fraction alone (— lane), the DE-175 fraction supplemented with UBF2 (UBF2 lane), or UBF1 (UBF1 lane).

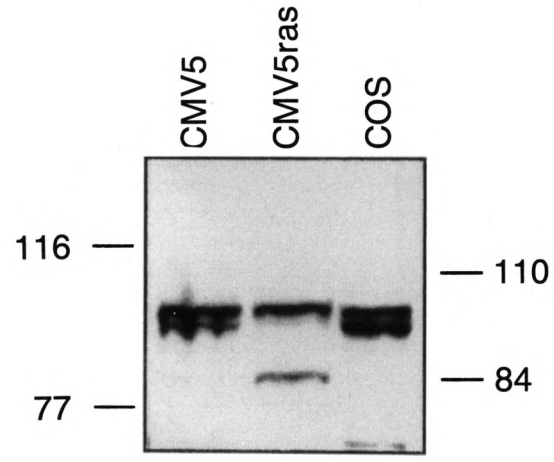

Figure 5. UBF2 is dispensable in vivo. A Western blot using the UBF antiserum shows control COS cells (COS lane), COS cells transfected with plasmids pCMV5 (CMV5 lane), or pCMV5ras (CMV5ras lane). The positions of the molecular weight size standards (in kDa) are shown.

detection is not necessary for the results presented here to be consistent with our hypothesis.

A typical transcription reaction driven by DE-175 contains 0.1×10^{-12} moles of template and 4 μg protein and yields 0.28×10^{-13} moles of transcript. Thus, we observe approximately 1 mole of transcript per 4 moles of template. The immunoblots indicate that there can be no more than 0.34×10^{-15} moles of UBF per assay (one-fourth the lower limit of detection). This translates to 3.4×10^{-3} moles of UBF/mole of template, i.e.,

$$\frac{0.34 \times 10^{-15} \text{ moles UBF, assay}^{-1}}{0.1 \times 10^{-12} \text{ moles template, assay}^{-1}}.$$

Both Smith et al. (1990) and Schnapp and Grummt (1991) have reported that UBF commits to the initiation complex. Thus, if UBF were required for transcription in vitro, only templates to which it was bound would be transcribed (i.e., 0.34×10^{-15} moles of UBF:template complex). In this situation, a typical reaction would produce 0.28×10^{-13} moles transcript/0.34×10^{-15} moles UBF:template complex/30 minutes or 100 transcripts/complex in 30 minutes. As the complete transcripts are 640 nucleotides (nt) long, the elongation rate for this reaction would have to be greater than 35 nt/second (100 transcripts × 640 nt transcript^{-1} × [1800 seconds]$^{-1}$), and 99 rounds of termination and initiation, including promoter escape, would have to be instantaneous. Promoter escape has been estimated to required 15–30 seconds (see Table IC and Figure 7 in Gokal et al., 1990). Thus, the minimum elongation rate would have to be 201 nt/second. As it is highly unlikely that these conditions could exist, our data are consistent with the hypothesis that UBF is not required for transcription in vitro in the rat system.

We reported previously that UBF-depleted extracts were capable of forming a stable preinitiation complex and initiating transcription (Smith et al., 1990). This result appears to agree with those reported by Schnapp and Grummt (1991) in their studies of the formation of the transcription complex on the mouse rDNA promoter. On the other hand, our results contrast with those reported by McStay et al. (1991), who demonstrated an apparently absolute requirement for UBF during the transcription of the Xenopus laevis rDNA promoter in vitro.

Prior to this report, there was the possibility that our previous observations and those of Schnapp and Grummt (1991) were due to the presence of UBF in our preparations of RNA polymerase I and SL-1. However, based on the Western blotting data reported here, this must be considered highly unlikely. Thus, we conclude that UBF is dispensable for in vitro transcription mediated by RNA polymerase I. This in turn raises the question whether UBF is essential for in vivo transcription of the ribosomal RNA genes.

In normal growing COS cells, transfected with a plasmid that codes for $p21^{H\text{-}ras}$, we can detect the expression of UBF1—but not UBF2—by Western blotting, using the UBF antiserum. In contrast, both control COS cells and COS cells transfected with pCMV5 express both UBF1 and UBF2. Thus, it appears that UBF2 is dispensable in vivo, and that UBF1 alone may be sufficient for transcription of the rDNA genes in *ras*-transfected cells. Interestingly, direct microinjection of activated ras protein into primary neonatal rat ventricular cardiac myocytes is able to activate several features of the hypertrophic phenotype in these cells (Thornburn et al., 1993).

We showed previously that UBF increased in mass in hypertrophic cardiomyocytes grown in tissue culture, relative to noncontracting myocytes (O'Mahony et al., 1991). Furthermore, serum stimulation of contracting cardiomyo-

cytes elevates the amount of UBF protein in these cells compared to contracting control cells (Xie and Rothblum, 1993), with a significant increase in the expression of UBF1 compared to UBF2.

In the study reported here, we show that, under specific conditions, UBF1 alone will stimulate transcription by RNA polymerase I from either promoter, whereas UBF2 will not. This in turn suggests that UBF1 and UBF2 are functionally different in the in vitro transactivation of RNA polymerase I. Preliminary experiments indicated that when UBF2 was added after an initial preincubation of the spacer promoter with the UBF1 fraction, there was transcription from the spacer promoter. When the initial preincubation contained UBF2, and UBF1 was added subsequently, there was no transcription from the spacer promoter (data not shown). This suggests that UBF2 may block the stimulatory effect of UBF1. Furthermore, we found previously that both UBF1 and UBF2 differ in their DNA-binding and dimerization characteristics (O'Mahony et al., 1992b). Our results suggested that UBF2 had a "poorer" DNA-binding ability than UBF1 (O'Mahony et al., 1992b).

Thus, we have considered three lines of evidence: (1) expression of UBF1 and not UBF2 in *ras*-transfected COS cells; (2) the more efficient binding of the rDNA promoter in vitro by UBF1 than by UBF2; and (3) the inability of UBF2 alone, but not UBF1, to stimulate transcription from the rDNA promoter in vitro. From this evidence, together with our inability to detect UBF1 or UBF2 by Western blotting in transcription-competent fractionated extracts (i.e., DE-175 fractions), we conclude that transcription from the rat 45S ribosomal RNA gene promoter does not require the transcription factor UBF, and that UBF1 and UBF2 differ in their transactivation of RNA polymerase I. Since it is dispensable for basal transcription, rat UBF should be considered an auxiliary transcription factor or co-activator.

How then does one rationalize apparently conflicting models for transcription by RNA polymerase I? It is more likely that there are species-specific variations in the affinity of each transcription factor for its respective promoter(s). In such a model, if SL-1 has a very low affinity for the core and upstream promoter elements, then the requirement for additional factors that would stabilize or facilitate formation of that complex becomes more apparent. Conversely, if SL-1 has a high affinity for the promoter, then UBF—or another factor—would have less effect on transcription and a lower affinity for that promoter.

There are experimental data that fit this model. Human SL-1 does not yield a detectable footprint over the human rDNA promoter. In fact, in the presence of UBF, the putative binding of SL-1 to the promoter was said to "extend" the UBF footprint (Learned et al., 1986). On the other extreme, mouse SL-1 recognizes the mouse promoter, but mouse UBF interacts weakly with the mouse promoter (Bell et al., 1990). However, UBF homologues isolated from species as disparate as Xenopus laevis and rat recognize one another's promoters equally (Pikaard et al., 1990).

Thus, as suggested earlier by Miesfeld and Arnheim (1984), we hypothesize that the rDNA promoter represents a unique example of molecular coevolution. Not only has there been a variation in the core promoters necessitating changes in the SL-1 homologues, but there have also been corresponding changes in the interactions of UBF with the UPE to balance the interactions of SL-1 with the promoter.

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