

Cellular regulation of ribosomal DNA transcription: both rat and *Xenopus* UBF1 stimulate rDNA transcription in 3T3 fibroblasts

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ABSTRACT

A novel RNA polymerase I (RPI) driven reporter gene has been used to investigate the *in vivo* role of the architectural ribosomal transcription factor UBF in gene activation and species specificity. It is shown that the level of UBF overexpression in NIH3T3 cells leads to a proportionate increase in the activities of both reporter and endogenous ribosomal genes. Further, co-expression of UBF antisense RNA suppresses reporter gene expression. Thus, UBF is limiting for ribosomal transcription *in vivo* and represents a potential endogenous ribosomal gene regulator. In contrast to some *in vitro* studies, *in vivo*, the mammalian and *Xenopus* forms of UBF1 show an equal ability to activate a mouse RPI promoter. This activity is severely impaired in mutants compromised for either dimerization or DNA binding. Similarly, the natural UBF2 splice variant shows a severely impaired capacity to activate RPI transcription. The data strongly suggest that UBF predominantly regulates ribosomal transcription by binding to and activating the ribosomal genes, but does not eliminate a possible secondary role in titrating ribosomal gene repressors such as Rb. Consistent with the DNA folding ability and cellular abundance of the UBF, we suggest that the protein may regulate a structural transition between the potentially active and active chromatin states.

INTRODUCTION

The ribosomal genes encoding the 18S, 5.8S and 28S ribosomal RNAs (rRNA) are exclusively transcribed by a dedicated polymerase, RNA polymerase I (RPI). These genes are typically responsible for 35% of total cellular RNA production while their products constitute 80% of the total mass of cellular RNA. It is known that regulation of rRNA transcription is the key factor in regulating ribosome biogenesis (reviewed in 1–3). Thus, ribosomal transcription is a major factor in determining cell growth rate. The ribosomal genes are typically present in animal genomes in several hundreds of copies and, with certain exceptions, are

organized in simple tandem arrays. Although as early as the 1970s, regulation of the ribosomal genes had been shown to occur at two distinct levels (4,5): (i) the number of transcripts per gene and (ii) the number of active genes, the mechanisms underlying this regulation still remain a question. The initiation rate per ribosomal gene is probably regulated at the level of RPI (6–10; reviewed in 1,2). Various experiments have identified the levels of initiation competent RPI to be subject to regulation, and it has been shown that this is probably due to changes in certain RPI associated factors. However, the molecular nature of these factors has to date remained unidentified. As for the mechanism which determines active gene number, we are still lacking a good candidate to mediate this level of regulation.

In vitro, RPI initiation requires the polymerase and a specific TBP-complex, SL-1 (1–3,10,11). Initiation is, however, strongly enhanced by the HMG-box factor UBF (12,13).

Indeed, *in vitro* footprinting on the human rDNA promoter has shown that UBF plays an essential role in facilitating the interaction of SL-1 with the RPI promoter (14). In other mammalian systems it has been shown that UBF is essential *in vitro* at low template concentration and that it allows the activation of a histone bound template (15). Thus, binding of UBF to the ribosomal genes could represent a key step in gene activation *in vivo*. However, despite extensive *in vitro* studies of the role of UBF in ribosomal transcription, almost nothing has been done to date to relate these findings to the situation *in vivo*.

RPI transcription is, to a great extent, species-specific. Rodent SL-1 cannot replace human SL-1 for *in vitro* transcription of the corresponding ribosomal promoters (1–3,12 and references therein). UBF has also been demonstrated to contribute to the species specificity of ribosomal transcription. While human and rodent UBF are completely interchangeable (16), *Xenopus* UBF can only to some degree replace the mammalian UBF in mammalian *in vitro* transcription assays, and mammalian UBF was inactive in the *Xenopus in vitro* transcription system (16–18). This species specificity has been ascribed to the absence in xUBF of the mammalian HMG-box 4 DNA binding domain (19–21). In both mammals and *Xenopus*, UBF has been found to be subject to differential splicing. In mammals the UBF2 isoform is missing the HMG-box2 DNA binding domain. It has been suggested from *in vitro* studies that this isoform is significantly compromised in

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its ability to bind DNA and activate transcription (22,23). However, the significance of these findings to the *in vivo* situation are not known.

Here we have compared the capacities of various UBF isoforms including rodent UBF, *Xenopus* UBF and UBF mutants compromised for dimerization, to activate ribosomal transcription in mouse cells. We demonstrate that overexpression of UBF1 is sufficient to significantly increase accurate transcription from both a cotransfected rodent rDNA promoter construct and also the endogenous ribosomal genes in NIH3T3 cells. rUBF2 was one third as active as rUBF1 in transactivating the same promoter. A construct directing expression of antisense UBF inhibited basal pSMECAT activity. Interestingly, overexpression of *Xenopus* UBF (xUBF) but not sxUBF, a deletion mutant of xUBF lacking the dimerization domain, was just as efficient as rUBF in stimulating rodent rDNA transcription. At the same time, a *Xenopus* rDNA promoter construct was inactive in NIH3T3 cells and was not stimulated by the overexpression of either xUBF or rUBF. These results argue that *in vivo* the activity of UBF is not species-specific and that the appropriate UBF cannot abrogate the need for the homologous form of SL-1. Together these data suggest that (i) altering the cellular content of UBF is sufficient to modulate rRNA synthesis and (ii) the observed increase in rDNA transcription following overexpression of UBF results from a direct action of UBF at the level of RPI transcription that is dependent on its ability to dimerize.

MATERIALS AND METHODS

Cell culture

Monolayer cultures of NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. For transfections, the cells were plated on 60 mm dishes at 0.3×10^6 cells/dish 24 h before transfection.

Construction of rDNA transcription reporter constructs (Fig. 1A)

To construct pSMECAT and pSMECAT-7, the murine ribosomal promoter fragment (24) containing nucleotides -152 to +60, relative to the site of transcription initiation (+1), was subcloned by PCR using the primers 5'-GAATTCTGAGGTCCGGTCTTTTCG-3' and 5'-GAATTCCTTAAATCGAAAGGGTCTC-3'. Amplification of this subclone with the primers 5'-GAATTCTGAGGTCCGGTCTTTTCG-3' and 5'-CCTTCCAGGTATTCTCTG-3' resulted in a 150 bp fragment containing a G to A mutation of base -7 within the core promoter. The mutated fragment was used in combination with the primer 5'-GAATTCCTTAAATCGAAAGGGTCTC-3' to generate a complete mutant promoter, i.e. -150 to +64. Both the mutant and the wild-type mouse promoters were then inserted into the *EcoRI* site of pBSECAT (25), which contains the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) upstream of the *Escherichia coli* chloramphenicol acetyl transferase gene. pXMECAT and piXMECAT were similarly constructed using the primers 5'-GAATTCTCCCGCGGAGGC-CCCGAT-3' and 5'-GGAATTCGAGGCGGAAACGCCCCG-GTC-3' to amplify sequences -152 to +60 of the *Xenopus laevis* ribosomal promoter (26).

Construction of UBF expression constructs

A number of full-length and mutant rat and *Xenopus* UBF expression constructs were used in this study (Fig. 1B). pCMV-rUBF1 was generated by PCR from the rat UBF1 cDNA (27) using a primer containing a *Bam*HI linker followed by the first 20 nucleotides of the 5' end of the UBF1 coding region, and a second primer complementary to the last 19 nucleotides of the UBF1 cDNA followed by an *Eco*RI linker. pCMV-FLAG-rUBF1 and pCMV-FLU-rUBF1 were constructed using the same 3' primer as used for pCMV-rUBF1, however the 5' primers contained an additional 24 and 28 nucleotides coding for the FLAG (IBI) or FLU (HA) epitopes, respectively. The tags were inserted in frame, between the start ATG and second codon of UBF1 cDNA. After PCR amplification, the products were cloned into the mammalian expression vector pCDNA3 (Invitrogen). This vector drives expression of UBF1 under the control of the cytomegalovirus (CMV) promoter. The orientations of the inserts were confirmed by sequencing and restriction endonuclease mapping. pGAL4-rUBF1 and pGAL4-rUBF2 were constructed by inserting the DNA-binding domain of Gal4 (amino acids 1-92; 28) in frame upstream, in place of the initiation codons of UBF1 and UBF2. PCMV-r/xUBF1 encodes a chimeric rUBF in which the mammalian-specific HMG-box 4 was replaced with the equivalent region from xUBF1. This plasmid was constructed from pCMV-FLAG-rUBF1 by precisely replacing the rUBF1 coding region for amino acids 363-479 with the equivalent, but shorter, region from xUBF1. Silent mutations were first introduced into the rUBF1 coding region to create *Xho*I and *Hind*III sites bracketing the region to be replaced. The intervening rUBF1 fragment was then replaced by a PCR product amplified from the xUBF1 cDNA (21,29) using primers 5'-CTCGAGAATCTCCCA-GAAGAG-3' and 5'-GGGTCTCTGGAAGCTTAGCCCTC-3'. pCMV-FLU-XUBF1 was constructed in a similar way starting from the xUBF1 cDNA (21,29) incorporating an ATG followed by a FLU (HA) epitope coding segment in place of the natural ATG (pCMV-xUBF1) or of the codon for arginine 97 (pCMV-sxUBF1).

Transfection and CAT/ β -galactosidase assays

NIH3T3 cells were cotransfected in the presence of OptiMEM (Gibco BRL) with the indicated constructs, (0.5-2 μ g/60 mm dish) and pSV40- β Gal (0.5 μ g/60 mm dish) (Promega Corp.) using Lipofectamine (Gibco BRL). An appropriate amount of the basic vector, pCDNA3, was included in the transfection media so that all cells were exposed to the same total amount of DNA. Five hours after transfection, the culture media was replaced with fresh DMEM/10% FBS. Twenty-four hours after transfection the cells were harvested, lysates prepared as described (30) and frozen at -80°C until assayed for either chloramphenicol acetyl transferase (CAT) or β -galactosidase activity (β Gal) (30). The results of the CAT assays were normalized by the results of the β -galactosidase assays to correct for variations in the efficiency of transfection. Typical transfection efficiency, as assayed by an *in situ* β -galactosidase assay (Promega Corp.), was ~40%.

S1 analysis

The transcript from pSMECAT was mapped using a double stranded probe extending from -150, the upstream boundary of the mouse RPI promoter, to +207, within the EMCV IRES sequence (31). The probe was 5'-³²P-labeled at +207 using T4

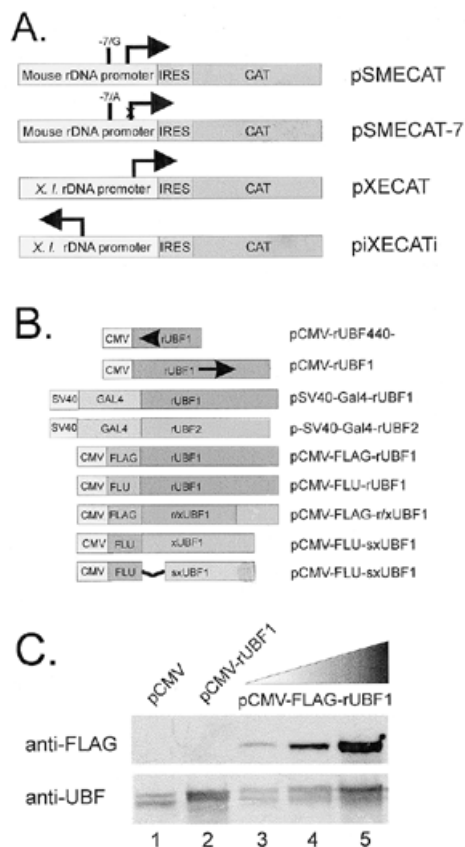


Figure 1. Reporter constructs for 45S rDNA transcription and characterization of UBF1 expression plasmids. (A) Schematic representation of the 45S rDNA reporter constructs: pSMECAT, mouse rDNA promoter reporter construct; pSMECAT-7; mouse pXECAT; and piXECAT. (B) Schematic representation of the UBF1 and UBF 2 expression constructs: pCMV-rUBF1, full-length rat UBF1; pCMV-rUBF440-, nucleotides 1–440 of rat UBF1 in the antisense orientation; pCMV-FLAG-rUBF1, full-length rat UBF1 tagged with the FLAG epitope; pCMV-FLU-rUBF1, full-length rat UBF1 tagged with the FLU epitope; pSV40-GAL4-rUBF1, full-length rat UBF1 tagged with the GAL4 DNA-binding domain; pSV40-GAL4-rUBF2, full-length rat UBF2 tagged with the GAL4 DNA-binding domain; pCMV-FLAG-r/xUBF1, rat-*Xenopus* chimeric construct tagged with the FLAG epitope; pCMV-FLU-xUBF1, full-length *Xenopus* UBF1 tagged with the FLU epitope; pCMV-FLU-sxUBF1, *Xenopus* UBF1 containing a deletion in the NH₂-terminal dimerization domain tagged with the FLU epitope. The relevant segments of each of the vectors are indicated and a complete description of each construct is given in Materials and Methods. CMV, cytomegalovirus promoter. SV40, simian virus promoter. (C) Twelve hours after transfection with pCMV (lane 1, 2 µg), pCMV-rUBF1 (lane 2, 2 µg) or pCMV-FLAG-rUBF1 (lanes 3–5: 0.5, 1 and 2 µg), nuclear proteins were isolated from NIH3T3 fibroblasts, fractionated by SDS–PAGE, and blotted to nylon membranes. Wild-type, recombinant authentic and recombinant FLAG-Tagged UBF was detected with the polyclonal anti-UBF antibody (upper panel) and a monoclonal antibody to the FLAG epitope (lower panel) as described in Materials and Methods.

kinase, denatured and hybridized at 48°C in 80% formamide with 32 µg of total RNA isolated from control NIH3T3 cells or cells cotransfected with pSMECAT and pCMV-rUBF1. The hybrid was characterized on 8% denaturing acrylamide gels in 90 mM Tris-borate, 2.5 mM EDTA, 8 M urea. The size of the protected fragment was determined by comparison with the mobility of the *RsaI* restricted S1 probe and with *HpaII* restricted pBR322.

Western analysis

NIH3T3 cells were released from the plates by treatment with 0.25% trypsin–EDTA. The cell pellets were washed once with ice-cold PBS, resuspended in 1 ml of EBC buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 100 µl/ml aprotinin, 100 µM benzamide, 10 µM leupeptin), and incubated on ice for 20 min. The cells were broken by vortexing, clarified by centrifugation (10 000 g for 15 min) and the supernatant stored at –70°C. Protein and DNA determinations were performed as described (32). Western blots with a polyclonal anti-UBF, a monoclonal anti-flag M2 antibody (cat no. IB13026/6D1311, IBI) and a monoclonal anti-Flu antibody (Boehringer Mannheim) were carried out as described previously (11–13)

Nuclear run-on transcription

Transcription from the rDNA promoter in isolated nuclei was measured by the hybridization of *in vitro* synthesized, ³²P-labeled run-on transcripts to a 45S rDNA clone as described previously (32).

Coimmunoprecipitation

Coimmunoprecipitation was performed at 4°C as described previously (33). Whole cell extracts were pre-cleared by incubating for 30 min with protein A-agarose beads (pre-washed with PBS). The beads were removed by gentle centrifugation for 5 s, and the pre-cleared extracts were incubated with 2 µg of anti-UBF antibody and tumbled for 2 h. Subsequently, 25 µl of washed protein A agarose beads were added and the sample tumbled for an additional 2 h. The beads were washed three times in PBS containing 0.5% NP-40, resuspended in 50 µl of Laemmli SDS–PAGE sample buffer and incubated at 95°C for 10 min. The immunoprecipitated proteins were resolved on SDS–PAGE and transferred to Immobilon-P for western blot analysis. For FLAG immunoprecipitation, whole cell extracts were incubated with 25 µl of anti-FLAG resin (IBI) and tumbled for 2 h. The beads were washed three times in PBS containing 0.05% NP-40, 200 mM NaCl and resuspended in 50 µl of PBS containing FLAG peptide (0.5 mg/ml). After incubation for 10 min on ice, the samples were centrifuged for 10 s and the supernatant removed and heated at 95°C for 10 min with 10 µl of 5× Laemmli SDS–PAGE sample buffer. The immunoprecipitated proteins were resolved on SDS–PAGE and transferred to Immobilon-P for western blot analysis.

Overlay assays

Overlay assays were performed as previously described (34). Partially purified full-length rat UBF1 protein (200 ng) expressed in Sf9 cells was separated by SDS–PAGE and transferred to nitrocellulose membranes. The protein blots were then denatured in 6 M guanidine–HCl in renaturation buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 6 mM MgCl₂, 0.6 mM EDTA, 2 mM DTT and 20% glycerol) for 30 min and renatured in 100 mM guanidine–HCl in renaturation buffer supplemented with 0.02% polyvinyl pyrrolidone twice for 2 h at room temperature. The blots were then blocked with 3% skim milk in renaturation buffer for 1 h at room temperature. The ³⁵S-labeled rUBF1, xUBF1 and sxUBF1 were prepared with cDNA templates pCMV-FLAG-rUBF1, pCMV-FLU-rUBF1 and pCMV-FLU-sxUBF1, respectively, using the Coupled T7 Rabbit Reticulocyte *in vitro* Transcription Translation Kit (Promega) supplemented with [³⁵S]methionine. Equal amounts (~50 µl) of the ³⁵S-labeled *in vitro* transcribed

translated lysates were incubated in 2 ml of renaturation buffer with the renatured protein blots containing baculovirus UBF, for 16 h at room temperature with gentle shaking. The blots were washed three times in renaturation buffer containing 200 mM KCl for 10 min at room temperature, lightly blotted dry and analyzed on a PhosphorImager.

RESULTS

Overexpression of rat UBF1 drives rDNA transcription

To study the parameters governing ribosomal transcription *in vivo* we have constructed several novel polymerase I driven CAT reporter gene constructs. These contain either the mouse or *Xenopus* rDNA promoters and as controls, either a transcriptionally inactive mouse promoter (-7 G to A) or an inverted *Xenopus* promoter (Fig. 1 and Materials and Methods).

As a first step in investigating whether the overexpression of UBF would be sufficient to drive elevated levels of rDNA transcription in an immortalized cell line, we examined whether UBF itself could be overexpressed. Several constructs capable of driving the expression of rUBF1, rUBF2 and xUBF1 were assembled (Fig. 1B). Western blotting was carried out to ensure that the various forms of UBF were expressed equally. NIH3T3 fibroblasts were transfected with either pCMV-rUBF1 (Fig. 1C, lane 2), or with increasing amounts of pCMV-FLAG-rUBF1 (Fig. 1C, lanes 3–5). After 24 h, whole cell lysates were prepared from the transfected cells, fractionated by SDS-PAGE, transferred to Immobilon-P and probed with either an anti-FLAG antibody (upper panel) or an anti-UBF antibody (lower panel). Transfection with pCMV-rUBF1 resulted in the overexpression of rUBF1 as demonstrated by western blotting with anti-UBF antibody (lane 2, lower panel). As the rUBF1 was not FLAG-tagged it did not react with the anti-FLAG antibody (lane 2, upper panel). Transfection with pCMV-FLAG-rUBF1 resulted in the overexpression of FLAG-tagged rUBF1, as demonstrated by blotting with both anti-UBF or anti-FLAG antibodies. Similar levels of expression were detected when the other UBF expression vectors were tested (e.g. see Fig. 7A).

As the level of expression of UBF1 was proportional to the amount of DNA used in the transfection, we then examined whether the level of rDNA transcription would correlate with the altered cellular content of UBF. In these experiments we first used pSMECAT, a reporter for rDNA transcription. pSMECAT uses the mouse rDNA promoter upstream of an IRES to drive the expression of CAT. Previous efforts to use such reporters have met with varied degrees of success, as quite often the RPI promoters, or the constructs themselves, contain cryptic RPII promoters (35–37). To control for this possibility, we constructed pSMECAT-7 (Fig. 1). This construct is identical to pSMECAT except that the G at -7 of the rDNA promoter, essential for transcription by RPI (1–3), is mutated to an A. Thus, pSMECAT-7 should not be transcribed by RPI, and serves as a control for fortuitous expression from cryptic RPII or even RPIII sites. As shown in Figure 2A, the level of CAT activity from pSMECAT-7 was <10% of the level of CAT activity supported by the non-activated pSMECAT (compare the levels of the acetylated chloramphenicol products in lanes 1 and 5). The residual level of CAT activity from pSMECAT-7 was also completely unaffected by the overexpression of UBF (Fig. 2A, lanes 2–4). In contrast,

cotransfection of 3T3 cells with increasing levels of pCMV-FLAG-rUBF1 resulted in proportionately increasing levels of CAT activity (Fig. 2A, lanes 5–8). Compilation of the results from a series of such experiments (Fig. 2B) demonstrated that the overexpression of UBF1 increased transcription from pSMECAT by up to 6.5-fold.

The control experiments with pSMECAT-7 provided considerable assurance that pSMECAT was in fact being transcribed by RPI and not by RPII. However, this question was further assessed by determining the site of transcription initiation. RNA was isolated from cells cotransfected with pSMECAT and pCMV-FLAG-rUBF1 and the transcription initiation site mapped. As shown in Figure 2C, S1 nuclease analysis of the RNA obtained from the transfected cells demonstrated that the site of transcription initiation was that predicted for transcripts synthesized by RPI.

UBF-dependent activation of transcription is specific to RPI

In combination, the above experiments provided complementary evidence that pSMECAT is transcribed specifically and predominantly by RPI. However, we were interested to know if UBF might also have the potential to activate RPII transcription. Cells were cotransfected with the rDNA reporters and a reporter for RPII, pSV β -Gal and both CAT and β Gal levels determined. As shown in Table 1, transcription from pSMECAT, but not transcription from pSMECAT-7, was stimulated 6-fold when the 3T3 cells were cotransfected with pCMV-FLAG-rUBF1. However, the absolute β -galactosidase levels measured in each of the transfections fell within 10% of one another, regardless of the level of expression of exogenous rUBF. UBF clearly activated RPI transcription, but did not activate RPII transcription, at least from the model late SV40 promoter. Thus, UBF appears to be an RPI specific transcription factor *in vivo* and maintains this specificity even when overexpressed. This is consistent with the exclusive nucleolar localization of the UBF (38,39).

The overexpression of UBF1 can drive an elevated level of transcription from the endogenous rRNA genes

The above experiments demonstrated that the overexpression of UBF can significantly activate transcription from a cotransfected RPI reporter in NIH3T3 cells. As such, they are consistent with the hypothesis that cells regulate rDNA transcription by modulating the activity of UBF. However, it was possible that overexpression of UBF could affect transcription from the reporter but have no effect on transcription from the endogenous genes.

To examine this question, nuclei isolated from cells cotransfected with the indicated amounts of pCMV-rUBF1 and pSV β -Gal were used in nuclear run-on assays and the levels of rDNA transcription measured. As shown in Figure 3, the nuclear run-on assay demonstrated that transfection with pCMV-rUBF1 resulted in a 2-fold stimulation of endogenous rRNA gene transcription. A parallel set of plates were stained *in situ* for β -galactosidase activity. We found that ~40% of the cells were transfected. Thus, overexpression of UBF1 activated transcription from the endogenous genes by ~5-fold ($2 \times 1/0.4$). This was very similar to the effect of overexpressing UBF on pSMECAT activity. Taken together, the two sets of results strongly support the hypothesis that the rate of rDNA transcription is regulated *in vivo* by the level of UBF1 within the cell.

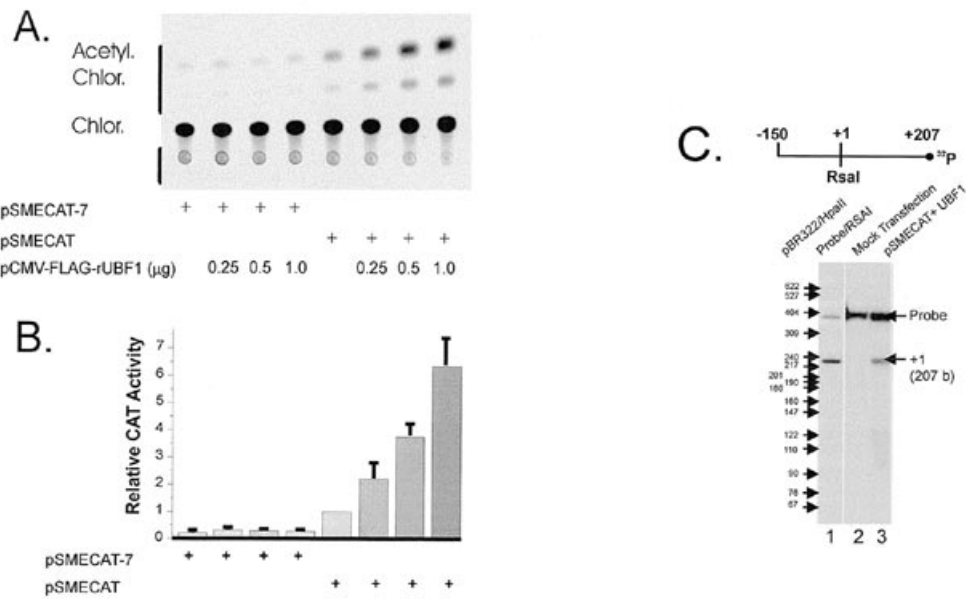


Figure 2. Overexpression of UBF increases transcription from pSMECAT and is initiated at +1. (A) NIH3T3 cells were transfected with the indicated constructs and the control vector, pSV40βGal, using lipofectamine as described in Materials and Methods. After 24 h, cell lysates were prepared and assayed for CAT activity and β-galactosidase as described in Materials and Methods. (B) The results from 5–7 separate experiments similar to those in Figure 2A were averaged and adjusted for the efficiency of transfection. The results are presented as the average fold increase (± SD) in pSMECAT activity in cells cotransfected with increasing amounts of rUBF expression vector as compared with cells transfected with pSMECAT alone. (C) Total RNAs from control NIH3T3 cells and NIH3T3 cells transfected with pSMECAT (1 μg) and pCMV-rUBF1 (3 μg), were used in S1 nuclease assays using an end-labeled probe (–150 to +207). After S1 nuclease digestion, the reaction products from the control (lane 2) and pSMECAT/UBF transfected cells (lane 3) were fractionated by denaturing gel electrophoresis and detected by autoradiography. Parallel lanes contained the the S1 probe digested with *RsaI* (cleavage between –1 and +1) and end-labeled fragments of pBR322. The S1 protected fragment showed an identical mobility with that of the *RsaI* cleaved probe, while both fragments showed an anomalous migration relative to the *HpaII*/pBR322 molecular weight marker.

Table 1. UBF activates transcription by RPI, but not by RPII

3T3 Cells	CAT % Conversion	β-Gal mUnits	Ratio [(CAT/β-Gal)x100]	Average
pSMECAT-7	0.09	108.7	0.08	0.07
pSMECAT-7 + UBF	0.07	111.6	0.06	
pSMECAT-7 + UBF	0.21	123.8	0.17	0.11
pSMECAT-7 + UBF	0.08	120	0.06	
pSMECAT	0.82	114.2	0.72	0.68
pSMECAT + UBF	0.68	104.1	0.65	
pSMECAT + UBF	5.12	115.9	4.47	4.42
pSMECAT + UBF	4.97	113.7	4.37	

The overexpression of UBF2 can result in elevated levels of rDNA transcription

Two previous *in vitro* investigations have reported that the natural splice variant UBF2 cannot activate rDNA transcription (22,39). In our investigation of this problem, we concluded that the low level of activation by UBF2 seen in *in vitro* transcription reactions was probably due to cross contamination of the UBF2 with UBF1 (22). We investigated this question using the reporter system described above and vectors, pGAL4-rUBF1 and pGAL4-rUBF2, that expressed full-length UBF1 and UBF2 tagged with the DNA-binding domain of Gal4. As shown in Figure 4, cotransfection of 3T3 cells with pSMECAT and increasing amounts of pGAL4-rUBF1 resulted in dose-dependent activation of transcription from pSMECAT that was very similar in level to that seen with the other rUBF expression vectors. Interestingly, cotransfection with pSME-

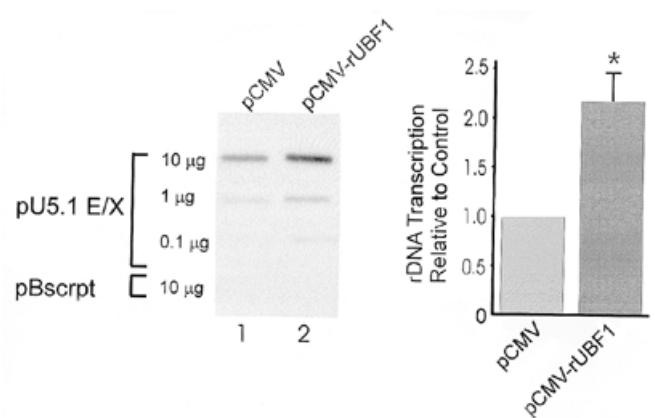


Figure 3. Overexpression of UBF1 stimulates transcription of the endogenous ribosomal genes in NIH3T3 cells. NIH3T3 cells were transfected with pCMV-rUBF1 (1 μg/60 mm dish) as described in Materials and Methods, nuclei were isolated, and ribosomal DNA transcription was measured as described in Materials and Methods. Radiolabeled rRNA transcripts were purified from the reaction mix, and hybridized to 45S rDNA (clone pU5.1E/X) or control pUC 19 DNA. After stringent washes, the hybrids were visualized by autoradiography (see autoradiogram). The radioactivity of 45S run-on transcripts obtained from three separate experiments were quantified by laser densitometry and are presented as the average percentage increase ± SD in rDNA transcription in response to contraction over the transcription observed in contraction-arrested cells. *, denotes significance from control cells (P < 0.01)

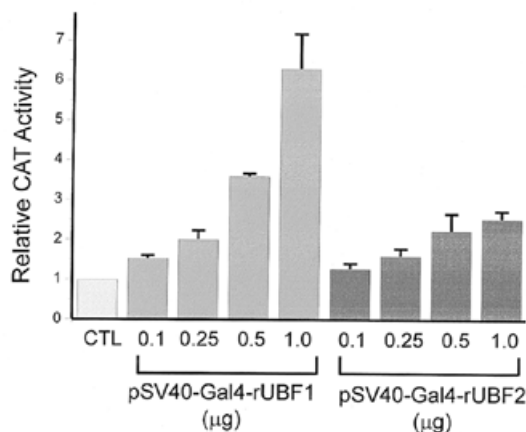


Figure 4. UBF2 is only one third as active as UBF1 at transactivating the rDNA promoter *in vivo*. NIH3T3 cells were transfected with increasing amounts of pSV40-rUBF1 or pSV40-rUBF2 (0.5–1 μg/60 mm dish) and pSMECAT (1 μg/60 mm dish). After 24 h, cell lysates were prepared and assayed for CAT activity and β-galactosidase as described in Materials and Methods. The results from 3–4 separate experiments were averaged and adjusted for the efficiency of transfection. The results are presented as the average fold increase (±SD) in pSMECAT activity in cells cotransfected with increasing amounts of rUBF1 or rUBF2 expression vector as compared with cells transfected with pSMECAT alone.

CAT and increasing amounts of pSV40-GAL4-rUBF2 also resulted in a dose-dependent activation of transcription from pSMECAT (Fig. 4). However, the maximum level of activation achieved using pGAL4-rUBF2 was only 30% of that observed using pGAL4-rUBF1 (compare the relative CAT activity levels when cells are cotransfected with pSMECAT and 1 μg of either pGAL4-rUBF1 or pGAL4-rUBF2). These results suggest that UBF2 is not as effective as UBF1 in activating rDNA transcription. Nonetheless, UBF2 does exhibit some activation potential. This result in fact agrees with our previous *in vitro* observation showing that UBF2 was 30% as active as UBF1 and suggests that the level of *in vitro* activation observed in those experiments reflected the true activity of UBF2.

UBF anti-sense RNA inhibits transcription from pSMECAT

The above studies demonstrated that artificially raising the cellular content of UBF results in a stimulation of rDNA transcription. We were also interested in determining if a reduction in the cellular content of UBF would result in a decrease in the level of basal rDNA transcription. To test this, a plasmid was constructed to express the antisense strand of the first 440 bp of rUBF, pCMV5-rUBF440(-). Cotransfection of NIH3T3 cells with pSMECAT (1 μg) and increasing amounts of pCMV-rUBF440- (0.5–2 μg) resulted in decreasing levels of CAT activity (Fig. 5, upper panel). Compilation of the results from a series of such experiments (Fig. 5, lower panel) demonstrated that the antisense UBF construct (2 μg) decreased basal transcription from pSMECAT by up to 75%.

The overexpression of *Xenopus* UBF1 results in elevated levels of transcription from the mouse rDNA reporter in murine cells

Transcription of vertebrate ribosomal RNA genes is species-specific (1–3). One clear demonstration of this property is the inability of active extracts of human cells to transcribe the rat and mouse rDNAs

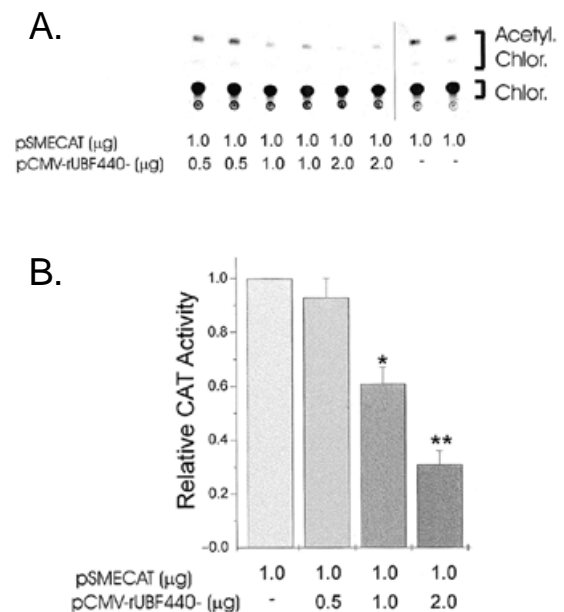


Figure 5. Overexpression of antisense UBF inhibits rDNA transcription. (A) NIH3T3 cells were transfected with increasing amounts of a construct that expresses antisense rUBF, pCMV-rUBF440- and pSMECAT. After 24 h, cell lysates were prepared and assayed for CAT activity and β-galactosidase as described in Materials and Methods (upper panel). (B) The results from 3–4 separate experiments similar to that presented in Figure 7A were averaged and adjusted for the efficiency of transfection and presented graphically. The results are the average-fold increase (±SD) in pSMECAT activity in cells cotransfected with increasing amounts of pCMV-rUBF440- expression vector as compared with cells transfected with pSMECAT alone. (* and **, denote statistically significant differences from control values of $P < 0.05$ and $P < 0.01$, respectively.)

in vitro (e.g. 12). This species specificity has predominantly been ascribed to the TBP-containing rDNA transcription factor referred to as SL-1 (12). It has also been shown that the structural differences between the mammalian and amphibian UBFs may represent a further level of species specificity. However, species-specific UBF activity has been noted only under certain precise conditions of protein and template concentration *in vitro*. Under other conditions the mammalian and *Xenopus* forms of UBF appear equivalent (18,40).

We investigated the role of UBF in species specificity in an *in vivo* context. We first determined if xUBF and rUBF could heterodimerize, and then determined the effect of xUBF on rDNA transcription *in vivo*. Vectors were constructed that would direct the expression of FLU-tagged forms of either rUBF1 or xUBF1, (pCMV-FLU-rUBF1 and pCMV-FLU-xUBF1). A third vector was created to express a rat–*Xenopus* UBF chimera, r/xUBF1, in which the sequences of rUBF1 HMG-box 4 had been replaced with the equivalent HMG-box-less region of xUBF1. In addition, as a control for the expression of xUBF, a vector that would express a mutant of xUBF lacking the NH₂-terminal dimerization domain was constructed, pCMV-FLU-sxUBF1. It has been reported that UBF lacking the NH₂-terminal domain is unable to dimerize and cannot activate rDNA transcription *in vitro* (1,2,41,42).

As shown in Figure 6A, overlay assays demonstrated that rUBF1 and xUBF1 can both homo- and heterodimerize and confirmed that sxUBF1 was unable to heterodimerize with rUBF1 (and by inference cannot therefore homodimerize). The

ability of xUBF to heterodimerize with rUBF was also examined *in vivo* (Fig. 6B). NIH3T3 cells were transiently transfected with either the 'empty' expression vector pCDNA3 (lane 1), pCMV-Flu-xUBF (lane 2) or a combination of pCMV-Flu-xUBF1 and pCMV-FLAG-rUBF (lane 3). Following transfection, whole cell lysates were prepared and UBF complexes were immunoprecipitated using anti-UBF antibodies or anti-FLAG antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nylon membranes and blotted with either anti-FLU, anti-FLAG or anti-UBF antibodies. Antibodies to rat UBF immunoprecipitated both wild-type rUBF and also FLAG-tagged rUBF (Fig. 6B, lanes 4 and 5). FLU-tagged xUBF was also immunoprecipitated (lane 6). Control experiments demonstrated that our antibodies to rat UBF do not immunoprecipitate *Xenopus* UBF. Thus, the Flu-tagged xUBF can only have been coimmunoprecipitated if it was heterodimerized with rUBF. Similarly, anti-FLAG antibodies immunoprecipitated both FLAG-tagged rUBF and Flu-tagged xUBF. In this case, the FLU-tagged xUBF could only have been coprecipitated with the FLAG tagged rUBF if it was in the form of a Flu-xUBF/rUBF-FLAG heterodimer. These studies demonstrate that xUBF and rUBF can heterodimerize both *in vitro* and *in vivo* and confirm that deletion of the NH₂-terminal domain of xUBF prevents this dimerization.

We then compared the effects of overexpressing FLU-xUBF and FLU-rUBF on transcription from pSMECAT. Western blots of extracts from cells transfected with 0.5 µg of either pCMV-FLU-rUBF1, pCMV-FLU-xUBF1 or pCMV-FLU-sxUBF1 demonstrated that the three vectors supported approximately equal levels of expression of the three FLU-tagged proteins (Fig. 7A). Similar expression levels were also observed for the mammalian-*Xenopus* UBF chimera, r/xUBF1 (results not shown). Cotransfection of 3T3 cells with pSMECAT and pCMV-FLU-rUBF1 resulted in a maximum level of activation of 6.3-fold (Fig. 7B). Interestingly, when cells were cotransfected with pSMECAT and pCMV-FLU-xUBF1, the maximum level of activation of 6.1-fold was very similar. Furthermore, a very similar level of activation was also seen when the r/xUBF chimera was over expressed with pSMECAT, (Fig. 7B). Transfection of NIH3T3 cells with pSMECAT and pCMV-Flu-sxUBF1 resulted in a weak, but not statistically significant, activation (Fig. 7B). These results demonstrate that *Xenopus* UBF1 can activate transcription from a mouse rDNA promoter in a mouse cell line. Interestingly, when the *Xenopus* rDNA reporter was used in the same experiments, we did not observe transcription from the *Xenopus* promoter either in the absence or in the presence of exogenous rUBF1 or xUBF1 (Fig. 7C). pXECAT has been shown to direct CAT expression in *X.laevis* cells (data not presented). These results argue that *in vivo*, the activity of UBF is not species-specific and further that the appropriate UBF cannot abrogate the need for the homologous form of SL-1.

DISCUSSION

The transcription of the ribosomal RNA genes is a process essential for cell growth. Not only is it known to be regulated in response to growth rate changes but it may also represent a means of regulating long term growth. Despite this, little or nothing is known of the manner in which the ribosomal RNA genes are regulated. Here we present the first demonstration that the ribosomal transcription factor UBF is functionally limiting for RPI transcription in NIH3T3 fibroblasts. We show that the

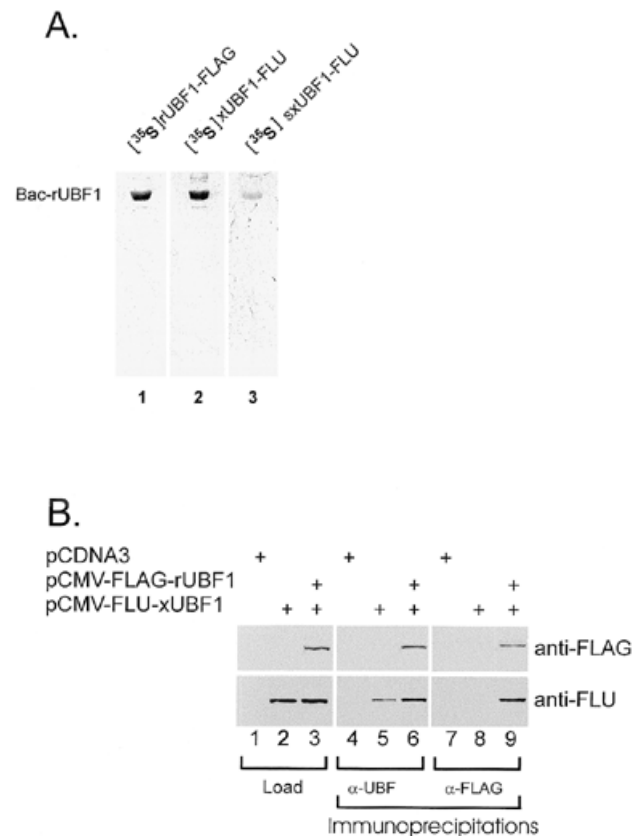


Figure 6. *Xenopus* UBF1 dimerizes with rat UBF *in vitro* and *in vivo*. (A) Full-length xUBF1 but not xUBF1 missing the NH₂ terminal domain dimerizes with rat UBF. Full-length rat UBF1 protein (500 ng) purified from Baculovirus (pVL1393) was separated by SDS-PAGE and transferred to nitrocellulose membranes. The protein blots were then denatured and renatured as described in Materials and Methods. After blocking with 3% skim milk in renaturation buffer for 1 h at room temperature, the membranes were incubated in 2 ml of renaturation buffer containing ³⁵S-labeled *in vitro* transcribed translated rUBF1, xUBF1 or sxUBF1 for 16 h at room temperature with gentle shaking. The blots were washed three times in renaturation buffer containing 200 mM KCl for 10 min at room temperature, lightly blotted dry and analyzed on a PhosphorImager. The experiments were repeated three times and typical images are shown. (B) xUBF1 dimerizes with rUBF *in vivo*. NIH3T3 cells were transfected with either pCMV, pCMV-FLU xUBF1 or a combination of pCMV-FLU-xUBF1 and pCMV-FLAG-rUBF. After 24 h, cell lysates were prepared and the UBF proteins immunoprecipitated with anti-rUBF antibodies or anti-FLAG antibodies. The immunoprecipitated proteins were fractionated by SDS-PAGE, transferred to Immobilon-P and blotted with the described antibodies.

activity of a novel CAT reporter construct, pSMECAT, whose expression is driven by the mouse RPI promoter, depends directly on the cellular level of UBF. We demonstrate that overexpression of UBF leads to a dose dependent activation, up to 6.5-fold, of the reporter gene and that cotransfection of an antisense UBF construct suppresses reporter activity. This is the first observation of the dependence of mammalian ribosomal transcription on a known transcription factor *in vivo*. The observation is all the more significant since we also show that the endogenous ribosomal genes can be activated by increasing the cellular concentration of UBF. Thus, the reporter construct pSMECAT appears to provide a model system that closely reflects endogenous ribosomal transcription regulation. We further show that activation of transcription by UBF is specific to RPI transcription. It is

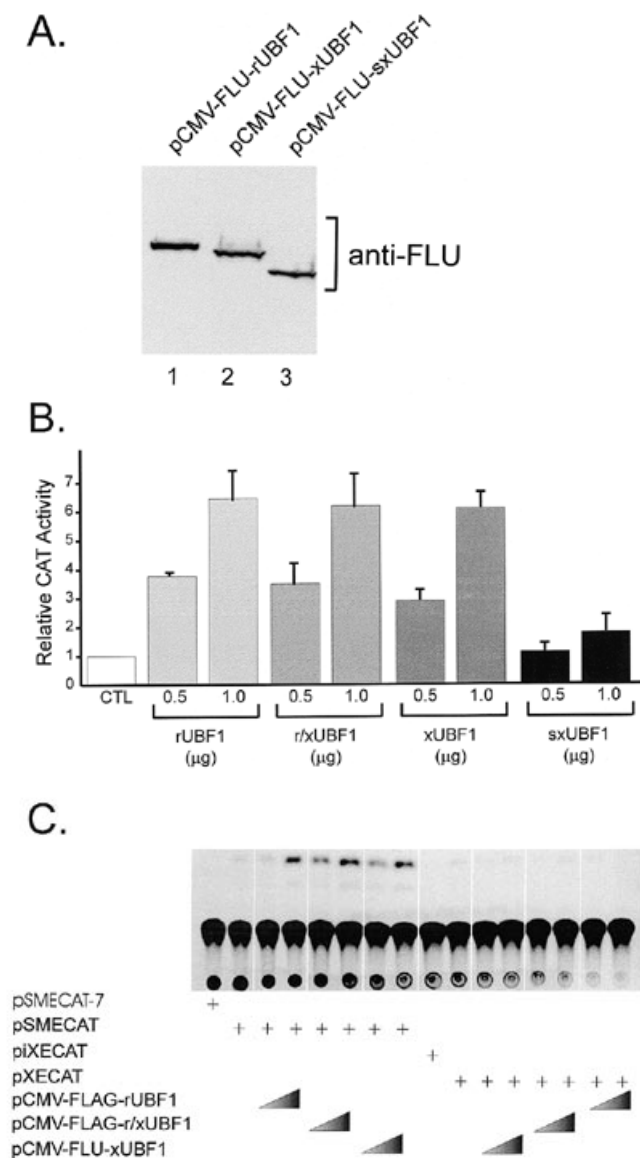


Figure 7. Overexpression of *Xenopus* UBF1 results in elevated levels of transcription from the mouse rDNA reporter in NIH3T3 cells. (A) pCMV-Flu-rUBF1, pCMV-FLU-xUBF1 and pCMV-sxUBF1 are expressed at similar levels in NIH3T3 cells. Twelve hours after transfection with pCMV-Flu-UBF1 (lane 1, 2 µg), pCMV-Flu-xUBF1 (lane 2, 2 µg) or pCMV-Flu-sxUBF1 (lane 3, 2 µg), nuclear proteins were isolated, fractionated by SDS-PAGE and blotted to nylon membranes. Recombinant Flu-Tagged UBF was detected with a monoclonal anti-Flu as described in Materials and Methods. (B) r/xUBF1 and xUBF1 but not sxUBF drive transcription from pSMECAT. NIH3T3 cells were transfected with the indicated amounts of the described constructs and pCMVβ. After 24 h, cell lysates were prepared and assayed for CAT activity and β-galactosidase as described in Materials and Methods. The results from 3–4 separate experiments were averaged and adjusted for the efficiency of transfection and presented graphically. The results are the average-fold increase (± SD) in pSMECAT activity in cells cotransfected with increasing amounts of the indicated UBF expression constructs compared with cells transfected with pSMECAT alone. (C) Overexpression of *Xenopus* UBF1 does not drive transcription from the *Xenopus* rDNA promoter in murine cells. NIH3T3 cells were transfected with the indicated UBF expression constructs (1 and 2 mg/60 mm dish) and either pSMECAT-7, pSMECAT, pXECAT or piXECAT (1 µg/60 mm dish) as described in Materials and Methods. Twenty-four hours following transfection cell lysates were prepared and assayed for CAT activity as described in Materials and Methods.

concluded that the cellular level of UBF is a potential regulator of ribosomal gene transcription, a view consistent with the several observations of growth regulated UBF expression (reviewed in 43).

Ribosomal transcription is species-specific, the human promoter does not function in mouse and neither the human nor the mouse promoters function in *Xenopus* (1–3). Though it is clear that this specificity resides predominantly with the TBP-complex SL-1, *in vitro* experiments have also suggested that some degree of species specificity exists at the level of UBF (16). We clearly show, however, that *in vivo* there exists no significant difference in the capacity of rat or *Xenopus* UBF to activate RPI transcription. On the other hand, in confirmation of *in vitro* data, we show that the xUBF cannot abrogate the need for the cognate SL-1, since the *Xenopus* rDNA promoter remains silent in NIH3T3 cells even in the presence of excess xUBF1. Furthermore, we show that the activity of xUBF depends on its ability to dimerize, strongly suggesting that the predominant mode of activation by the UBFs *in vivo* is mediated by their ability to bind DNA (19,41). In support of this conclusion, we have more recently shown that single point mutations that effect DNA binding by xUBF also lead to a similar loss of activation (V.Stefanovsky and R.Hannan, unpublished data). It is possible that a secondary role of UBF could be to indirectly activate transcription by squelching repressors (15) of ribosomal transcription such as Rb (44,45). Indeed, sxUBF does show a residual level of activation that could be explained by such an effect and this conclusion is consistent with the observed activity of rUBF2, discussed below.

Though natural splice variants of UBF occur in all systems studied to date, no function for these variants has yet been described. In mammals the second HMG-box DNA binding domain is missing in the UBF2 splice variant. *In vitro* studies have suggested that UBF2 is non-functional in transcription activation and that its affinity for DNA is greatly reduced as compared with UBF1 (22,39). Using the RPI reporter we have tested the *in vivo* role of UBF2 in ribosomal transcription and find that in fact it does activate transcription, but only ~30% as well as UBF1. This level of activation is in fact similar to the residual levels of activation seen with the NH₂-terminal deletion mutant sxUBF and point mutants of xUBF1, (data not shown), all of which are also compromised in DNA binding. This suggests that part of the function through which UBF acts may squelch the action of a repressor such as Rb (46,47) and that this is the only part of the mechanism through which UBF2 may act.

How then might UBF function in regulating the ribosomal genes? UBF is able to form an alternative chromatin based on the ribosomal enhancosome. The enhancosome resembles a nucleosome in terms of total DNA and protein content, but differs in that a dimer of UBF replaces the histones and the DNA component is wound into a single not into a double loop as it would be in the nucleosome (48,49). *In vitro* studies show that the interaction of UBF with SL-1 and the rDNA promoter facilitates, but may not be necessary for, the formation of the RPI initiation complex (12,50). Hence, *in vivo* the availability of UBF may regulate the number of genes actively engaged in transcription via a direct gene interaction. In any given cell the number of potentially active ribosomal genes generally represents only a fraction of the total gene number. In yeast, this fraction is regulated in response to growth rate changes as demonstrated by psoralen crosslinking (51). Interestingly, the fraction of potentially active/active ribosomal genes (versus inactive genes) does not appear to change in a given mammalian cell as measured by the same

technique (52). If the chromatin structure is not different between active and potentially active ribosomal genes, perhaps it is the association of UBF with a gene which determines that it may be active. Interestingly, the number of visible nucleoli (i.e. the number of active ribosomal gene loci or active nucleolar organizers) has also long been recognized as a measure of tumor cell growth rate (e.g. 53,54).

Psoralen probes the chromatin state of the ribosomal genes, inactive and active (or potentially active) loci showing significantly different accessibilities. This difference could represent a switch in chromatin structure between the repressed nucleosomal state and the enhancer structure (UBF-DNA). Estimates of UBF concentration are quite variable, but suggest that sufficient UBF exists within the cell to engage not only the promoter but also a large segment of the intergenic spacer including the enhancer rDNA elements of the active ribosomal genes (recent measurements demonstrate 200 000 copies/3T3 cell; L.Rothblum and A.H.Cavanaugh, unpublished observation). The observation that the DNA-binding-mediated activity of UBF is limiting for ribosomal gene activity is consistent with its potential to induce a structural change in ribosomal gene chromatin and hence its ability to recruit genes to the active pool.

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