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Functional differences between the two splice variants of the nucleolar transcription factor UBF: the second HMG box determines specificity of DNA binding and transcriptional activity

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The nucleolar transcription factor UBF consists of two proteins, UBF1 and UBF2, which originate by alternative splicing. Here we show that deletion of 37 amino acids within the second of five HMG box motifs in UBF2 is important for the dual role of UBF as transcriptional activator and antirepressor. UBF1 is a potent antirepressor and transcriptional activator, whereas the ability of UBF2 to counteract histone H1-mediated repression and to stimulate ribosomal gene transcription both *in vivo* and *in vitro* is at least one order of magnitude lower. The difference in transcriptional activity between UBF1 and UBF2 is due to their different binding to the ribosomal gene promoter and enhancer. Apparently, the presence of an intact HMG box2 modulates the sequence-specific binding of UBF to rDNA control elements. However, the interaction of UBF with rDNA does not entirely depend on sequence recognition. Both UBF isoforms bind efficiently to four-way junction DNA, indicating that they recognize defined DNA structures rather than specific sequences. The results demonstrate that the HMG boxes are functionally diverse and that HMG box2 plays an important role in specific binding of UBF to rDNA.

Key words: four-way junction DNA/HMG box proteins/ribosomal genes/RNA polymerase I/transcription factors

Introduction

Regulation of ribosomal RNA gene (rDNA) transcription has been shown to play a central role in adapting ribosome synthesis to environmental conditions. A cascade of events is involved in the transduction of extracellular signals into the nucleolus. We have previously shown that growth-dependent fluctuations of rDNA transcriptional activity is mediated by an essential RNA polymerase I (Pol I) specific transcription initiation factor, called TIF-IA, whose level or activity is modulated according to cell proliferation (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1990a, 1993). TIF-IA is a monomeric 75 kDa protein that associates with Pol I, thus converting it into an initiation-competent enzyme. Promoter selectivity is conferred by TIF-IB, the murine rDNA-specific factor which contains the TATA binding protein and three tightly associated polypeptides known as TAFs (Clos *et al.*, 1986; Schnapp *et al.*, 1990b; Eberhard *et al.*, 1993). The functionally analogous factors in human and *Xenopus laevis* have been termed SL1 (Learned *et al.*, 1986) and Rib1 (McStay *et al.*, 1991a) respectively. TIF-IB is believed to be the fundamental transcription factor for Pol I, i.e. it appears to serve a role akin to that of TFIID for RNA polymerase II or TFIIIB for RNA polymerase III promoters. Stable binding of TIF-IB to the mouse rDNA promoter is augmented by another protein, the upstream binding factor (UBF). UBF is a sequence-tolerant DNA binding protein that interacts with both the core, the upstream control element (UCE) and the enhancer repeats of rDNA, and therefore functions both as an enhancer binding protein and as a general transcription factor (Bell *et al.*, 1990; Pikaard *et al.*, 1990).

UBF appears to serve multiple functions. It is involved in recruiting or stabilizing TIF-IB binding which in turn stimulates transcription. In addition to its positive effect on the formation and stability of preinitiation complexes, UBF functions as a transcriptional antirepressor. UBF has been demonstrated to overcome transcription inhibition caused by a repressor protein which competes with TIF-IB for DNA binding and thus prevents the formation of functional preinitiation complexes (Kuhn and Grummt, 1992). This repressor protein has been isolated and shown to be identical or related to the Ku autoantigen (Kuhn *et al.*, 1993). Thus UBF appears to exert part or most of its transactivating function via an antirepression mechanism.

UBF has been purified to homogeneity from human, mouse, rat and frog cells (Bell *et al.*, 1988; Pikaard *et al.*, 1989; Smith *et al.*, 1990; Voit *et al.*, 1992). The molecular cloning of cDNA encoding UBF has led to the identification of multiple domains of the protein which are functionally relevant. UBF is a member of a family of DNA binding proteins whose DNA binding domains are homologous to the non-specific DNA binding domains of high mobility group (HMG) proteins 1 and 2, and therefore have been termed HMG boxes (Jantzen *et al.*, 1990; Hisatake *et al.*, 1991; McStay *et al.*, 1991; O'Mahony and Rothblum, 1991). In contrast to other members of the HMG box protein family, UBF is unique in having more than two HMG boxes which are required for specific DNA binding. Despite the lack of apparent sequence homology of the different target sites, UBF is structurally conserved in vertebrates and exhibits similar DNA binding properties. It interacts with both the core elements and the UCE of rDNA promoters as well as with the enhancer repeats present in the rDNA spacer (Bell *et al.*, 1990; Pikaard *et al.*, 1990). As with HMG 1 and 2, UBF has a very acidic carboxyl-terminus which is essential for transactivation (Jantzen *et al.*, 1992; Voit *et al.*, 1992). Purified UBF derived from mammalian cells is made up of a doublet of 97 and 94 kDa proteins, referred to as UBF1 and UBF2 respectively (Bell *et al.*, 1988; Smith *et al.*, 1990; Chan *et al.*, 1991). In cultured cells, the two polypeptides are present in approximately equimolar amounts. UBF2 differs from UBF1 in that it lacks 37 amino acids within the second HMG box; this difference

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has been shown to result from alternative splicing (Hisatake *et al.*, 1991; O'Mahony and Rothblum, 1991). Previous studies have demonstrated that UBF1 expressed from cloned cDNA either using a vaccinia virus or an *in vitro* translation system displays all of the functional properties of cellular UBF (Jantzen *et al.*, 1990, 1992; McStay *et al.*, 1991; Voit *et al.*, 1992).

The essential role of UBF in rDNA transcription suggests that the two isoforms may serve different functions. The deletion within the second HMG box could result in conformational changes of UBF which in turn may affect the interaction with other components of the initiation complex. To resolve this question, we have isolated cellular UBF1 and UBF2 and compared their biochemical properties. The results demonstrate that the internal deletion within the second HMG box has profound functional consequences. UBF1 efficiently activates rDNA transcription both *in vitro* and *in vivo*, whereas UBF2 is virtually inactive. The two forms differ markedly in their activity to counteract histone H1-mediated transcriptional repression and exhibit significant differences in their ability to bind to rDNA. Interestingly, both UBF1 and UBF2 efficiently bind to synthetic cruciform DNA in a sequence non-specific manner. The results suggest that the two splice variants may serve distinct functions. We propose that UBF1 plays a role in transcription initiation that requires sequence-specific DNA binding, whereas UBF2 may be involved in formation of loops between the enhancer and the gene promoter.

Results

Chromatographic separation of UBF1 and UBF2

UBF purified from mammalian cells yields a homogenous preparation containing two proteins of 97 kDa and 94 kDa, referred to as UBF1 and UBF2. Since the two forms of UBF differ only by 37 amino acids and, therefore, have very similar physico-chemical properties, it is difficult to separate them by chromatography. On most chromatographic systems tested, UBF1 and UBF2 copurify. However, separation of UBF1 and UBF2 can be achieved by gradient elution on a Bio-Rex 70 column (Figure 1B). The Western blot displayed in Figure 1C demonstrates that the peak fractions of UBF which elute at ~450 mM KCl (fractions 10–12) contain equal amounts of the 97 and the 94 kDa polypeptides. In the lower salt fractions preceding the main peak the smaller form (i.e. UBF2) predominates, whereas the fractions eluting at higher salt contain mainly UBF1. Figure 1D shows a silver-stained gel of fractions containing UBF1 and UBF2, which were used for most of the experiments described below. Both silver-staining and Western blotting revealed that each form of UBF was highly enriched and contained at most 5–10% of the other form.

Transcriptional properties of UBF1 and UBF2

To examine whether the two isoforms of UBF serve different functions, their activity was assayed in a reconstituted transcription system which contains partially purified protein fractions (Figure 2A), namely a crude Pol I fraction (H-400 fraction), TIF-IA, TIF-IB and TIF-IC (Schnapp and Grummt, 1991; Kuhn and Grummt, 1992). At the template concentrations and amounts of TIF-IB used, transcription was dependent on addition of UBF. Figure 2B illustrates the effect of increasing amounts of UBF1 and UBF2 on rDNA

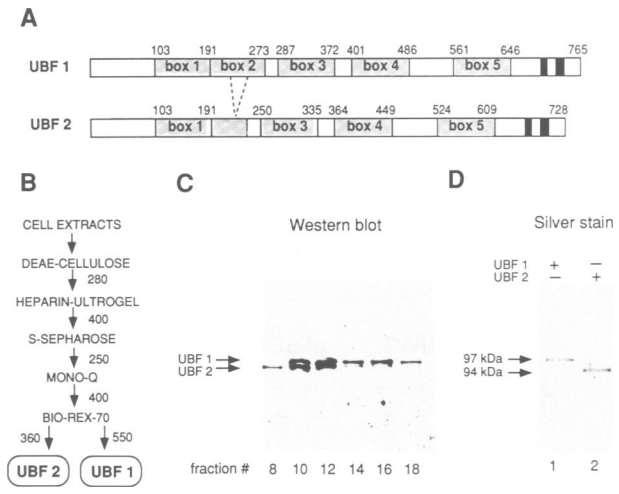

Fig. 1. Separation of UBF1 and UBF2 by chromatography on Bio-Rex 70. (**A**) Schematic diagram of the structure of UBF1 and UBF2. The position of HMG boxes and the acidic tail in the amino acid sequence are indicated by the numbers above the diagram. (**B**) Fractionation scheme of UBF1 and UBF2. (**C**) Western blot analysis of fractions eluting from Bio-Rex 70. Cellular UBF fractionated by chromatography on DEAE–Sepharose, Heparin-Ultrogel, S-Sepharose and Mono Q was applied to a Bio-Rex 70 column and eluted with a linear salt gradient from 0.3 to 1 M KCl. Individual fractions were analyzed on immunoblots using anti-mUBF antibodies. (**D**) Silver-staining of 20 ng of UBF1 and UBF2.

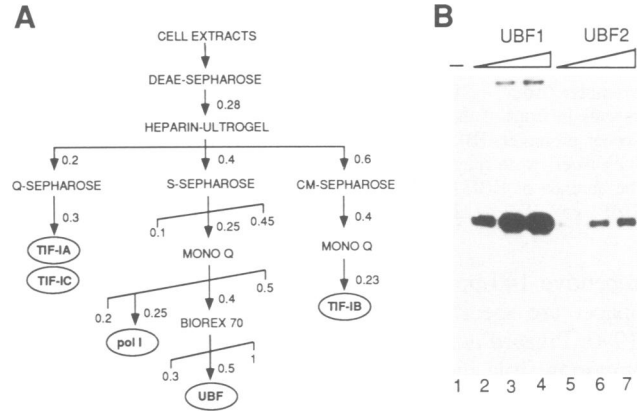

Fig. 2. Transcriptional properties of UBF1 and UBF2. (**A**) Diagram of the first fractionation steps used to purify and separate individual transcription factors. (**B**) Transcriptional activity of UBF1 and UBF2. Transcriptions were performed in the reconstituted transcription system containing partially purified Pol I, TIF-IA, TIF-IB and TIF-IC (see A) and 7 ng of the template pMrWT/*Nde*I. Transcriptions were performed in the absence of UBF (lane 1) or in the presence of 1 ng (lanes 2 and 5), 4 ng (lanes 3 and 6) or 8 ng (lanes 4 and 7) of UBF1 and UBF2, respectively.

transcription. In the absence of UBF, transcription from the rDNA promoter was hardly detectable (lane 1). Addition of UBF1 resulted in ~50-fold stimulation of transcriptional activity (lanes 2–4). UBF2, on the other hand, was practically inactive (lanes 5–7). The low level of transcriptional activation could be due to traces of UBF1 present in the UBF2 fraction.

Having identified functional differences in the activity of the two isoforms of UBF, we wondered whether the same result would be obtained if different rDNA templates were used. Previously, we and others have shown that the

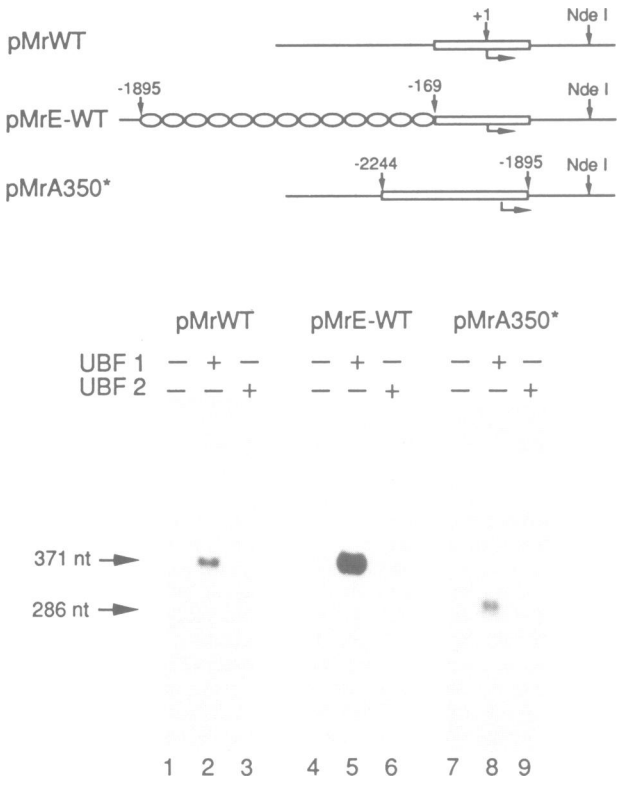

Fig. 3. Transcriptional activity of the two forms of UBF on different rDNA templates. (**A**) Schematic representation of the recombinant plasmids used. The pMrWT construct contains the murine rDNA promoter (from −170 to +155), pMrE-WT contains 13 enhancer repeats in front of the rDNA promoter and pMrA350* contains the spacer promoter. (**B**) 2.5 fmol of the individual templates (truncated with *Nde*I) were transcribed in the reconstituted transcription system in the absence of UBF (lanes 1, 4 and 7), or in the presence of 2 ng of UBF1 or UBF2 as indicated above the lanes.

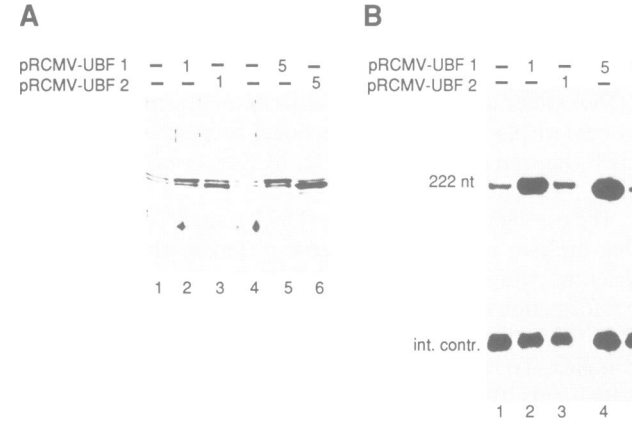

Fig. 4. Transactivation of a ribosomal minigene construct by cotransfection with expression vectors encoding UBF. (**A**) Western blot analysis of UBF overexpressed in NIH3T3 cells. 12.5 μg of pMrE-CAT were co-transfected either with the vector pRCMV (lanes 1 and 4) or with 1 μg (lanes 2 and 3) or 5 μg (lanes 5 and 6) of pRCMV-UBF1 (lanes 2 and 5) and pRCMV-UBF2 (lanes 3 and 6). Expression of UBF was analyzed by SDS−PAGE of 5 μg of extract proteins followed by immunoblotting. (**B**) Primer extension of RNA synthesized from the reporter gene pMrE-CAT. Transfection was performed as described in (A) and Pol I-specific transcripts were quantified by primer extension. Specific transcripts yield a 222 nt cDNA. For quantification and to account for losses during sample processing, an internal CAT-specific control RNA which generates a 108 nt cDNA product was added to the individual reactions.

repetitive 140 bp enhancer elements in the murine rDNA spacer are specifically recognized by UBF (Kuhn *et al.*, 1990; Pikaard *et al.*, 1990). Thus UBF appears to play an important role in enhancer function. To find out whether the two splice variants of UBF may serve a different activity in conjunction with the enhancer repeats, a construct containing 13 enhancer elements fused upstream to the rDNA promoter (pMrE-WT) was tested in the reconstituted system. In this experiment a different TIF-IB preparation and lower DNA concentrations were used, conditions which enhanced the stimulatory effect of UBF1 and augmented the functional differences between UBF1 and UBF2. As with the enhancer-less construct pMrWT (Figure 3, lanes 1−3), transcription from the enhancer-containing template was strongly stimulated by UBF1 but not by UBF2 (Figure 3, lanes 4−6), indicating that UBF1 stimulated both basal and enhanced transcription. The template having the 140 bp repeats attached to the promoter was transcribed about three times better than the construct containing only the promoter fragment (compare lanes 2 and 5).

Next we examined whether UBF1 and UBF2 exert different effects on transcription from the spacer promoter (Kuhn and Grummt, 1987; Tower *et al.*, 1989; Smith *et al.*, 1990). The spacer promoter and the major gene promoter have only limited homology, which may explain the reduced stability of the preinitiation complex and the lower activity of the spacer promoter. As transcription from the spacer promoter has been shown to require UBF, we studied whether or not each form of UBF would similarly affect transcription from the spacer and the gene promoter. For this, a template which contains the murine spacer promoter (pMrA350*) was used in the reconstituted transcription system and assayed in the absence of UBF (Figure 3, lane 7) or in the presence of UBF1 (lane 8) or UBF2 (lane 9). Again, only UBF1 activated transcription. Thus, despite the fact that the gene promoter and the spacer promoter share only limited sequence homology within the proximal part of the core promoter (Kuhn and Grummt, 1987), the molecular interactions of UBF with basal components of the transcription apparatus appear to be similar at the two promoters.

UBF1—but not UBF2—stimulates rDNA transcription in vivo

To assess the functional effects of UBF1 and UBF2 *in vivo*, we carried out DNA-mediated gene transfer experiments. Transfection of NIH3T3 cells with two plasmids expressing UBF1 or UBF2 under the control of the CMV promoter (pRCMV-UBF1 and pRCMV-UBF2) resulted in expression of both forms of UBF in a concentration-dependent manner (Figure 4A). With this transfection system, we asked whether overexpression of UBF would affect Pol I transcription. Plasmid DNA containing the mouse Pol I promoter fused to part of the chloramphenicol acetyltransferase (CAT) gene (pMrE-CAT) was cotransfected with pRCMV-UBF1 or pRCMV-UBF2, and the transcripts derived from the rDNA promoter were monitored by primer extension using a CAT-specific oligonucleotide (Kuhn *et al.*, 1990). Cotransfection of the pMrE-CAT reporter gene with 1 or 5 μg of pRCMV-UBF1 resulted in a 2- to 5-fold increase in Pol I-specific transcription (Figure 4B, lanes 2 and 4). On the

other hand, pRCMV-UBF2 or a control vector that contained no UBF cDNA insert did not affect rDNA expression (lanes 1, 3 and 5). This result qualitatively corresponds to the *in vitro* transcription data which demonstrated that under the experimental conditions employed, only UBF1 stimulated transcription, whereas UBF2 was more or less inactive.

Antirepression activity of UBF1 and UBF2

Recently we have shown that the biochemical mechanism by which UBF activates Pol I transcription involves relief of transcription inhibition brought about by a negatively-acting factor which binds to the rDNA promoter (Kuhn and Grummt, 1992). This negatively-acting factor turned out to be identical or closely related to Ku autoantigen (Kuhn *et al.*, 1993). These studies demonstrated that Pol I transcription regulation may involve antirepression as already documented for Pol II genes (Croston *et al.*, 1991, 1992).

We have established a convenient assay system in which the antirepression effect of UBF can be studied (Kuhn and Grummt, 1992). At elevated template and TIF-IB concentrations, UBF-independent transcription occurs (Figure 5, lanes 1–3). In the presence of histone H1, rDNA transcription is strongly repressed (lane 4). To investigate the ability of UBF to relieve histone H1-mediated transcriptional repression, recombinant UBF was used that had been overexpressed in the vaccinia virus system. In the first experiment we have compared the properties of full-length UBF1 with those of the carboxy-terminal deletion mutant ΔC552. This truncated protein binds efficiently to DNA but does not stimulate transcription (Jantzen *et al.*, 1992; Voit *et al.*, 1992), suggesting that the carboxyl-terminal acidic tail plays an essential role in transcription activation. Histone H1-mediated transcriptional repression was effectively overcome by full-length UBF (lane 5). However, the ability of the deletion mutant ΔC552 to relieve H1-mediated transcriptional repression was severely impaired (lane 6). This result suggests that the acidic tail is required to disrupt local histone H1–DNA interactions and demonstrates that this region is required not only for true activation but also for efficient antirepression.

If the acidic tail of UBF was sufficient to counteract transcriptional repression, both UBF1 and UBF2 should be able to prevent H1-mediated transcription inhibition. However, this was definitely not the case. Again, UBF1 counteracts H1-mediated repression at least one order of magnitude more efficiently than UBF2 (lanes 7–9). Thus, antirepression correlates with transcriptional activity, implying that transcriptional antirepression by UBF requires both the DNA binding domain(s) and the acidic tail.

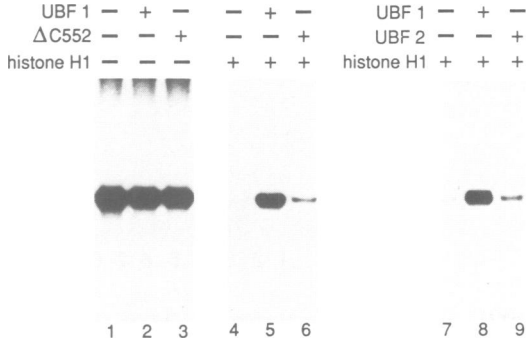

Fig. 5. Different antirepressor activity of UBF1, ΔC552 and UBF2. 40 ng of pMrWT/*Nde*I were transcribed in the reconstituted system containing 6 μl of Pol I, 2 μl of TIF-IB and 3 μl of TIF-IA/C in the absence (lanes 1–3) or presence of 100 ng of histone H1 (lanes 4–9). The assays contained 7.5 ng of recombinant UBF which has been expressed in vaccinia virus and purified to apparent homogeneity.

Both forms of UBF dimerize

UBF1 has been shown to form dimers in solution and dimerization appears to be essential for transcriptional activity. The amino-terminal 102 amino acids play a most important role in dimerization (McStay *et al.*, 1991), but other domains, presumably the HMG box2, may contribute to dimer formation (O'Mahony *et al.*, 1992). We therefore wondered whether the differences in transcriptional activity of the two isoforms of UBF could be due to different dimerization properties. ^{35}S-labelled UBF1 and UBF2, translated in a reticulocyte lysate system, were purified on a sequence-specific DNA affinity column and then assayed for homodimer formation. For this, each form of UBF was incubated in the absence or presence of glutaraldehyde, and analyzed on an SDS–polyacrylamide gel. In the presence of glutaraldehyde both forms of UBF were completely converted into a dimer species migrating at ~200 kDa (Figure 6A). Thus, both UBF1 and UBF2 form homodimers in solution.

Next, we asked whether the two UBF polypeptides differ in their potential to form heterodimers. For this, a Far-Western blot experiment was performed. A fraction of cellular UBF containing both forms of UBF was separated by SDS–PAGE and transferred to nitrocellulose, and individual strips were incubated with radiolabelled UBF1 or UBF2 to monitor dimer formation with membrane-bound UBF. In parallel, the two forms of UBF were visualized by immunoblotting. As shown in Figure 6B, the 97 and 94 kDa polypeptides were labelled at a ratio corresponding to the relative amounts of UBF1 and UBF2 present in this fraction as estimated by immunoblotting. This result indicates that the two UBF isoforms have identical dimerization properties.

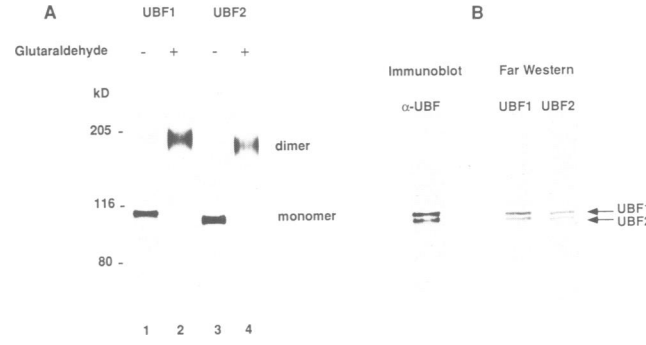

Fig. 6. Both UBF isoforms form homo- and heterodimers. (**A**) Glutaraldehyde cross-linking of UBF1 and UBF2. *In vitro*-translated UBF was purified by DNA affinity chromatography and equal amounts of protein were treated with 0.005% glutaraldehyde for 40 min. Samples were resolved on 6% SDS–polyacrylamide gels. The molecular weight standards are shown at the left. (**B**) Far-Western blotting of UBF1 and UBF2. A fraction containing cellular UBF1 and UBF2 was electrophoresed on an 8% SDS–polyacrylamide gel, blotted onto nitrocellulose, and the filter was incubated with ^{35}S-labelled UBF1 or UBF2 as described in Materials and methods. To quantify the relative amounts of the two UBF isoforms, one strip of the blot was incubated with anti-UBF antibodies and UBF was visualized by the ECL Western blotting system.

DNA binding of UBF1 and UBF2

Despite the extensive sequence divergence of Pol I promoters, UBF derived from *X.laevis*, rat, mouse or human produced identical footprints on distinct rDNA promoter elements of different species (Bell *et al.*, 1989, 1990; Pikaard *et al.*, 1989, 1990). The consensus recognition sequence is highly degenerate and the relative affinity of UBF for different promoter elements varies considerably. UBF from frog, human or mouse binds well to the human rDNA promoter, but only weak interactions are observed at the mouse promoter (Bell *et al.*, 1990). However, UBF binds efficiently to the murine 140 bp enhancer repeats (Kuhn *et al.*, 1990). To compare the binding affinity of the two UBF isoforms, the interaction of UBF1 and UBF2 with a fragment containing three enhancer repeats was assayed (Figure 7A). As has been reported before, the most remarkable feature of UBF footprints at the enhancer is the appearance of hypersensitive sites within the repeats which are flanked by protected regions (Pikaard *et al.*, 1990; Voit *et al.*, 1992). This characteristic footprint pattern was easily observed in the presence of UBF1 (lanes 2–4) but not with UBF2 (lanes 6–8). In several independent experiments using different preparations of UBF1 and UBF2, it was observed that binding of UBF2 was ~10-fold weaker than that of UBF1. The actual differences in binding could be considerably higher given that UBF2 is contaminated by low amounts of UBF1 (see Figure 1C). Thus the differences in transcriptional activity of the two forms of UBF could be due mainly to differences in their DNA binding affinity.

To verify this finding, footprinting was also performed on the mouse rDNA promoter. Consistent with previous studies, a weak but significant interaction of UBF was detected both at the upstream region and at the core yielding a protected region between positions −102 and −106 and hypersensitive sites at nucleotides −12/−13, −89 and −107/−108 (Figure 7B, lanes 1–3). Surprisingly, a qualitatively similar but much more pronounced pattern was observed with the pMrLS109-75 linker-scanning mutant. This mutant contains 38 instead of the natural 35 bases between nucleotides −109 and −75 and is transcriptionally as active as the wild-type (Schnapp *et al.*, 1990b). As on the wild-type promoter, UBF binding to this mutant template causes hypersensitive sites within the core promoter, a protected region in the upstream region (−102 to −106) and a strong enhanced cleavage site within the foreign sequence downstream of the UCE (Figure 7B, lanes 4–6).

The availability of a promoter mutant with increased affinity for UBF enabled us to investigate whether the two

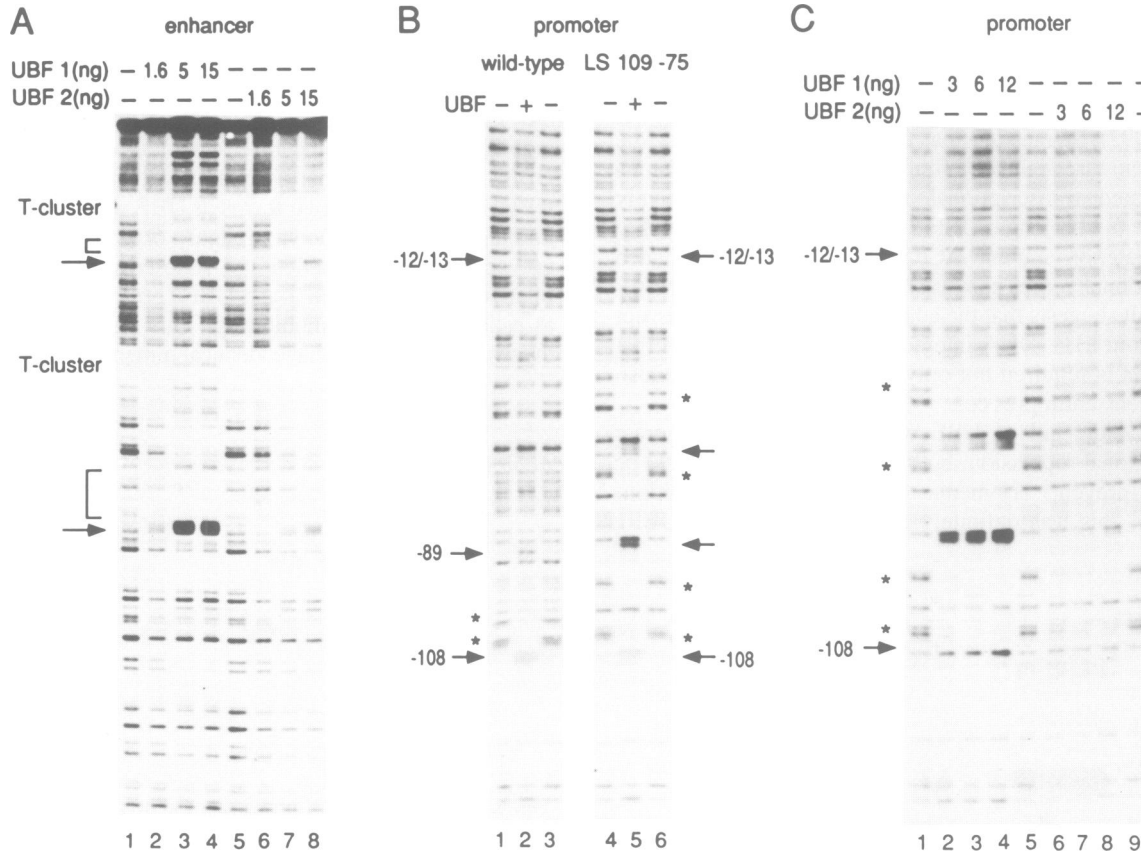

Fig. 7. UBF1 and UBF2 have different DNA binding affinities. (A) Footprinting of UBF1 and UBF2 on the mouse 140 bp enhancer repeats. The binding of increasing amounts of UBF1 (lanes 2–4) and UBF2 (lanes 6–8) is shown together with the digestion pattern of the naked DNA (lanes 1 and 5). The amount of UBF present in the individual reactions is indicated above the lanes. Each 140 bp element is bounded by clusters of T residues (marked as T-cluster). Protected regions are bracketed; hypersensitive sites are indicated by arrows. **(B)** DNase I footprinting of UBF on the rDNA promoter. The non-coding strand of pMrWT (lanes 1–3) or the linker-scanning mutant pMrLS109-75 (lanes 4–6) were footprinted with a Bio-Rex 70 fraction containing equal amounts of UBF1 and UBF2 (lanes 2 and 5). Lanes 1, 3, 4 and 6 contained no protein. Asterisks mark the protected regions and arrows the locations of enhanced DNase I cleavage sites. **(C)** Binding of UBF1 and UBF2 to the rDNA promoter. The non-coding strand of pMrLS109-75 was used for footprinting. Reactions contained increasing amounts of UBF1 (lanes 2–4) and UBF2 (lanes 6–8) as indicated. Lanes 1, 5 and 9 show control reactions without protein. The asterisks mark protected regions.

UBF isoforms exert different binding affinities to the promoter (Figure 7C). Once again, binding of UBF2 was significantly impaired (lanes 6–8). The highest amount of UBF2 tested (12 ng, lane 8) showed a weaker signal than the lowest amount of UBF1 assayed (3 ng, lane 2). This result suggests that an intact HMG box2 is required for efficient and specific binding to rDNA control elements.

UBF binds to four-way junction DNA

Since the initial characterization of the HMG box motifs of UBF, a superfamily of HMG box proteins has emerged all of which bind DNA. The relaxed DNA binding specificity of UBF suggests that, like HMG1, the structure of the DNA may be as important or even more important than sequence requirements. To test this hypothesis, we examined binding of UBF to synthetic four-way junction DNA in a gel mobility-shift assay. As a control, a bacterially expressed 'minimal HMG box' peptide, containing the first HMG box of rat HMG1, was used. This peptide, HMG1bA, efficiently binds to four-way junction DNA in a concentration-dependent manner (Figure 8A, lanes 1–6). No shift was observed with a linear DNA probe with an identical base composition as one half of the four-way junction probe (lanes 7–12). The more slowly moving complex formed in the presence of increasing concentrations of HMG1 probably contains multiple copies of the protein bound per DNA molecule.

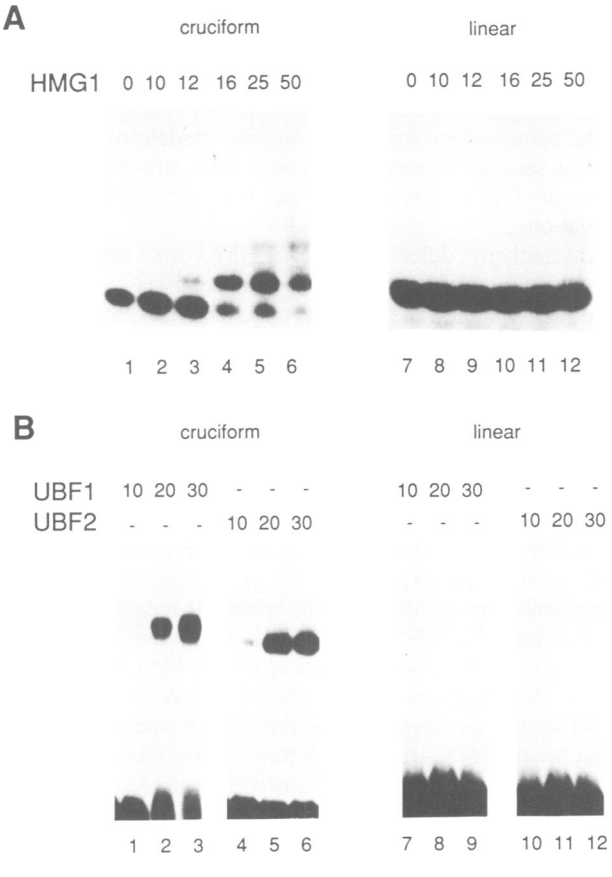

Fig. 8. UBF recognizes four-way junction DNA. (**A**) Mobility shift with HMG1 peptide. Labelled cruciform DNA (lanes 1–6) or linear duplex DNA (lanes 7–12) were incubated with the indicated amounts (in ng) of HMG1 peptide and assayed by gel electrophoresis. (**B**) Mobility shift with UBF1 and UBF2. Increasing amounts of recombinant UBF were incubated with cruciform DNA (lanes 1–6) or linear duplex DNA (lanes 7–12) and assayed by gel electrophoresis. The amounts of UBF (in ng) are indicated above the lanes.

To investigate binding of UBF to cruciform DNA, we used the same synthetic probes and the same mobility shift assay as for HMG1. In initial experiments we used cellular UBF and observed a well-defined, slowly moving complex with the four-way junction probe (data not shown). To compare binding of UBF1 and UBF2 to the cruciform DNA, we used recombinant UBF which had been expressed in bacteria (Figure 8B). Interestingly, although the two UBF fractions differed by at least 10-fold in their ability to interact with the rDNA promoter and enhancer, they bound with similar affinity to the four-way junction probe (lanes 1–6). As with HMG1, binding to linear DNA of similar base composition was hardly visible and required high protein: DNA ratios (lanes 7–12). A series of controls, such as specific competitions and co-purification of shift-activity with UBF protein, verified that the mobility shift of the cruciform DNA was in fact due to UBF binding (data not shown). The result demonstrates that (i) the selectivity of UBF is towards DNA structure rather than towards defined sequences, and (ii) the integrity of HMG box2 is not required for sequence-independent DNA binding.

Discussion

Mammalian UBF consists of two proteins with apparent molecular weights of 97 and 94 kDa which are encoded by one gene; their mRNAs are produced by alternative splicing. The role of the two isoforms of UBF in Pol I transcription is not yet known. Recently Hisatake *et al.* (1991) have determined the relative amounts of mRNAs encoding murine UBF1 and UBF2 in different tissues as well as in growing and quiescent NIH3T3 cells. They found that both mRNAs were present in eight tissues examined and in cultured mouse cells. In most cases, UBF2 mRNA was slightly more abundant than UBF1. Similarly, UBF2 mRNA was found at higher concentrations in stationary phase cells, whereas the two mRNAs were expressed at almost the same level in exponentially growing mouse fibroblasts. These data, as well as our own results demonstrating that in differentiating F9 cells and during mouse embryogenesis both the ratio of mRNA encoding the two forms of UBF and the ratio of UBF1 to UBF2 protein decrease (R.Evers, unpublished data), imply that there are functional differences between the two alternatively spliced forms of this transcription factor. Furthermore, both the ubiquitous coexpression of UBF1 and UBF2, and the existence of the same two forms of UBF mRNA containing the same deletion of 37 amino acids in rat, mouse, human and hamster (O'Mahony and Rothblum, 1991) underscores the functional relevance of the two forms of UBF.

In previous studies UBF has been structurally and functionally characterized, and different domains of UBF have been shown to carry out distinct functions such as DNA binding, dimerization, transactivation and nucleolar localization (Jantzen *et al.*, 1990, 1992; McStay *et al.*, 1991; Maeda *et al.*, 1992; Voit *et al.*, 1992). In this communication we have investigated whether the two UBF isoforms are functionally equivalent or not. Since cellular UBF preparations usually contain equimolar amounts of the 97 and 94 kDa polypeptides, it was necessary to separate the two proteins or to express the two cDNAs *in vivo* and purify the recombinant proteins. We have applied both approaches,

separating the two forms of cellular UBF by chromatography and also purifying recombinant UBF produced either in bacteria or in vaccinia virus. Our data demonstrate that UBF1 efficiently activates rDNA transcription from templates containing the spacer promoter and the major gene promoter and from enhancer-containing constructs. On the other hand, the transcriptional activity of UBF2 was at least one order of magnitude lower than that of UBF1. As pointed out above, this difference may be much more pronounced because we cannot exclude the possibility that the 5–10% cross-contamination of UBF2 with UBF1 is responsible for most of the transcriptional activity and sequence-specific binding of UBF2. In agreement with this presumption, activation of a Pol I-driven reporter gene in transient transfection assays was observed only with UBF1, whereas UBF2 had virtually no effect.

Like other promoter and enhancer binding proteins, an important function of UBF is to overcome chromatin-mediated transcriptional repression (Kuhn and Grummt, 1992; Kuhn *et al.*, 1993). Using purified histone H1 bound to DNA as a model system for the repressed state of the rDNA promoter, we have shown that cellular UBF counteracts histone H1-directed repression of basal transcription. In this experimental system, the carboxy-terminally truncated protein, ΔC552, which is transcriptionally inactive, did not alleviate repression. This finding suggested that it is the acidic tail which mediates antirepression and transcriptional activation. Therefore, the positive effect of UBF could be due simply to non-specific removal of histones from the template DNA by ionic interactions between the acid tail and the basic histones. This, however, is definitely not the case. Only UBF1, and not UBF2, was an efficient antirepressor. We found that UBF1 and UBF2, both of which contain the acidic tail, differ markedly in their ability to relieve repression, UBF1 being a much better antirepressor. Therefore, in addition to the carboxyl-terminus, efficient DNA binding appears to be a prerequisite for antirepression. Comparable results were recently reported by Croston *et al.* (1992). These authors showed that for antirepression by a GAL4–VP16 fusion protein in a system comparable to that we have used, the DNA binding domain of GAL4 alone was sufficient to disrupt local histone H1–DNA interactions, but the transcriptional activation region of VP16 was additionally necessary for antirepression. These data suggest that an activation region is not only required for true activation but also for efficient antirepression.

Somewhat surprisingly, we found that UBF2 binds much more weakly than UBF1 both to the murine rDNA promoter and to the repeated enhancer elements. Thus the differences in transcriptional activity of the two UBF isoforms correlate with their respective DNA binding activity. This result apparently contradicts recent data from Jantzen *et al.* (1992), who investigated the contribution of individual HMG boxes to specific UBF interactions. They inferred that deletion of HMG box2 from human UBF does not affect binding to the human rDNA promoter. Since UBF is known to bind with much higher efficiency to the human rDNA promoter than to the mouse rDNA promoter, we assumed that the apparent discrepancy between our results and those of Jantzen *et al.* could have been due to the difference between the systems used. To exclude this possibility, we also tested the interaction of mUBF with the human rDNA promoter and found a similar difference in binding of UBF1 and UBF2 (A.Kuhn, unpublished data). However, on the human promoter the different binding activities of UBF1 and UBF2 are much more pronounced at the core promoter than in the UCE. The decreased binding efficiency of UBF2 (~5-fold) in the core region is also clearly visible in the footprint of Jantzen *et al.* (Figure 1). Therefore, in both the human and the mouse systems, UBF2 has a lower affinity for the promoter, which in turn results in a strong reduction of transcriptional activity.

The contribution of the multiple HMG boxes to specific DNA interactions has been defined by assaying internal UBF deletion mutants by DNase I footprinting (Jantzen *et al.*, 1992). These data demonstrate that HMG box1 is necessary and sufficient for specific binding of UBF to the human rDNA promoter. However, both the amino-terminus and the other HMG boxes that lack detectable specific DNA binding by themselves appear to enhance binding by HMG box1. A similar study with *Xenopus* UBF (xUBF) revealed that HMG boxes 2 and 3 as well as the amino-terminal domain play a significant role in determining the affinity of xUBF for DNA (Leblanc *et al.*, 1993). Therefore, the presence or absence of HMG box2 appears to have pronounced functional implications. It is conceivable that the presence of box2 determines a defined structure or a critical spacing of UBF domains that play a role in sequence-specific DNA binding. It has recently been suggested that, by binding as a dimer, the carboxyl-terminal segment of xUBF directly or indirectly interacts with HMG box1 by extensive folding of the core promoter within the DNA–xUBF complex (Leblanc *et al.*, 1993). Thus one intriguing possibility is that a protein–protein interaction between UBF and a component of the transcription initiation complex, which involves the correct spacing between the amino- and carboxy-terminal parts of UBF, plays a crucial role in transcriptional activation.

Alternatively, deletion of part of HMG box2 may change the structure of UBF. All HMG boxes identified to date have a similar structure. They contain three α-helices, which form an L-shaped structure as determined by two-dimensional ^{1}H-NMR spectroscopy (Weir *et al.*, 1993). The deleted region in UBF2 comprises helix 2 and part of helix 3, thereby changing the three-dimensional structure of the molecule. The shape of the HMG box motif, which is distinct from that of hitherto characterized DNA binding motifs, may be significant in relation to its DNA binding properties. An interesting feature of both HMG1 and HMG box-containing transcription factors, such as the mammalian testis determining factor, SRY, and the lymphoid-specific enhancer binding proteins, LEF-1 and TCF-1, is that they bind to the minor groove of DNA (Ferrari *et al.*, 1992; Lilley, 1992; van de Wetering and Clevers, 1992). Moreover, a number of observations suggest that the HMG domain has an influence on DNA structure. From comparison of the binding sites of UBF and other HMG proteins, it seems that the structure of the DNA may be as important or even more important than sequence requirements. In this communication we show for the first time that UBF, like HMG1 and SRY, binds to double-stranded crossover sequences. UBF binds to four-way junction DNA of unrelated sequence with much higher affinity than it does to linear duplex DNA of the same sequence or to its authentic target site. Although the significance of this finding has yet to be established, we assume that binding of UBF to cruciform DNA may reflect UBF binding to double helical crossovers formed within

individual enhancer repeats, the UCE and the core, or even within single transcription units. Consistent with this assumption we have observed UBF-induced looping of individual enhancer repeats by electron microscopy (A.Kuhn and H.Zentgraf, unpublished data). Moreover, interaction of UBF with DNA may induce local contortions within the DNA such as bending. Recent studies by Giese *et al.* (1992) have demonstrated that the HMG domain of the lymphoid-specific transcriptional regulator LEF-1 can bend DNA almost back on itself. Bending was also found to accompany DNA binding by the SRY HMG domain. Although not yet proven, it is likely that UBF also induces a flexure in the DNA. This is an attractive hypothesis considering that not only the two essential sequence elements of the rDNA promoter but also the repetitive enhancer elements interact with UBF. UBF-mediated DNA bending would augment the interaction between proteins bound to the distant promoter elements and facilitate assembly of transcription initiation complexes.

Although the functional significance of UBF2 is not yet understood, this factor may serve a potentially important but thus far unknown regulatory function, such as maintenance of a certain structural organization of rDNA which may include wrapping or looping of the DNA. The presence of an intact HMG box2, on the other hand, appears to facilitate the specific interaction with rDNA control elements. These results suggest that the different HMG boxes are functionally diverse and that HMG box2 contributes to sequence-specific binding of UBF. Furthermore, the ability of UBF to form homo- and heterodimers suggests that a combination of different dimeric proteins may exert different functions. Thus the potential to obtain related factors with different DNA binding properties and different functions increases the level of complexity and versatility in the regulation of gene expression.

Materials and methods

Plasmid constructs

The plasmid pMrWT (Skinner *et al.*, 1984) contains a 324 bp 5′-terminal *Hin*dII−*Sma*I fragment from the murine ribosomal transcription unit including sequences from −169 to +155. To yield the enhancer-containing construct pMrE-WT, a 1762 bp *Sal*I fragment (−1930 to −169) encompassing 13 repeats of the 140 bp enhancer elements was inserted upstream of the rDNA promoter (Kuhn *et al.*, 1990). In transfection experiments the construct pMrE-CAT (Kuhn *et al.*, 1990) was used, which contains the 1632 bp *Bam*HI−*Hin*dIII fragment from pSV2CAT cloned into the *Sma*I site of pMrE-WT. The template pMrA350* contains a 349 bp *Ava*I−*Sal*I fragment of the rDNA spacer (from −2244 to −1895) encompassing the spacer promoter. In this construct the spacer fragment was present in the opposite orientation than in pMrA350 (Kuhn and Grummt, 1987). The linker-scanning mutant pMrLS109-75 contains 38 nucleotides of foreign sequence instead of the natural 35 bases between nucleotides −109 and −75 (Schnapp *et al.*, 1990b).

To clone UBF2 cDNA, 1 μg of RNA from Ehrlich ascites cells was reverse transcribed with oligo(dT) primers and the cDNA was subjected to 25 cycles of PCR with two primers, 5′-GGAGGTGGCTGGACAGCTGGCATA-TGAACGGAGAAGCGGACTG-3′ (sense oligo, complementary to nucleotides −23 to +20 of mUBF1 cDNA) and 5′-CTTCATGTT-GGCCATGAGCTCC-3 (antisense oligo, complementary to nucleotides +930 to +951). The amplified 871 bp fragment containing the mUBF2 splice junction was purified by gel electrophoresis and digested with *Nde*I and *Msc*I, and the fragment was used to replace the corresponding fragment of pBAT-UBF1 (Annweiler *et al.*, 1991) to generate pBAT-UBF2.

Before cloning into eukaryotic expression vectors, unique restriction sites (*Hin*dIII and *Xba*I) were introduced into UBF cDNA upstream and downstream of the coding region by PCR-mediated mutagenesis to remove the 5′ and 3′ untranslated regions. The oligonucleotides 5′-TGGTAACGT-TGCTGGAGAATGAACGGAGAAGCG-3′ and 5′-AGGTCTAGACTG-AGCTCAGTTGGAGTCAGAATC-3′ were used as forward and backward primers respectively for amplification of the mUBF1 and mUBF2 coding sequence. The resulting 2318 bp (UBF1) and 2207 bp (UBF2) *Hin*dIII−*Xba*I fragments were cloned into Bluescript SK^+ plasmid vectors, and the integrity of the sequences was verified by DNA sequencing. For expression in mammalian cells, the UBF1 and UBF2 cDNA constructs were inserted between the *Hin*dIII and *Xba*I sites of the eukaryotic expression vector pRCMV (Invitrogen) resulting in pRCMV-UBF1 and pRCMV-UBF2.

For expression in *Escherichia coli*, the UBF1 and UBF2 cDNAs were inserted between the *Nde*I and *Bam*HI sites of the expression vector pET6His, resulting in pETUBF1 and pETUBF2, respectively.

Purification of UBF1 and UBF2

The partial purification of TIF-IA, TIF-IB, TIF-IC and Pol I has been described (Schnapp and Grummt, 1991). UBF was purified from the fractions eluting at 0.4 or 1 M KCl from Heparin-Ultrogel (Voit *et al.*, 1992) and fractionated on a Mono S column by gradient elution from 100 to 450 mM KCl. Active fractions (300 mM KCl) were chromatographed on a Mono Q column using a linear salt gradient from 200 to 500 mM KCl. UBF1 and UBF2 were partially separated on Bio-Rex 70 (200−400 mesh) with a linear gradient from 0.3 to 1 M KCl. Fractions which contained predominantly one of the two polypeptides were finally purified to apparent homogeneity by chromatography on a sequence-specific DNA affinity column as described before (Bell *et al.*, 1988).

Bacterially expressed UBF1 and UBF2 were obtained by sonication of *E.coli* BL21(DE3)pLysS transformed with pETUBF1 and pETUBF2, respectively, in buffer B (20 mM HEPES, pH 7.9, 0.1 mM EDTA, 5 mM $MgCl_2$, 300 mM KCl, 20% glycerol, 0.05% Nonidet-P40, 1 mM DTE, 1 mM PMSF). After precipitation with 50% NH_4SO_4, proteins were resuspended in buffer B containing 100 mM KCl and 5 mM imidazole and chromatographed on a nickel chelate agarose column. UBF was eluted with 100 mM imidazole and subsequently purified to near homogeneity by chromatography on a Mono Q column.

In vitro transcription assays

The template DNAs, pMrWT, pMrE-WT and pMrA350*, were linearized with *Nde*I to generate 371 nt and 286 nt transcripts, respectively. The UBF-responsive transcription system was essentially as described by Kuhn and Grummt (1992). 25 μl assays contained 2.5−3.5 fmol of template DNA, 6 μl of partially purified Pol I (H-400 fraction), 2 μl of TIF-IB (CM-400 fraction), 2 or 4 μl of TIF-IA/TIF-IC (Q-Sepharose fraction) and 2 ng of UBF.

Western blot analysis

The two forms of UBF were resolved on an 8% SDS−polyacrylamide gel, transferred to nitrocellulose and probed with the anti-mUBF polyclonal antiserum K8 which has been described before (Voit *et al.*, 1992). The membranes were blocked for 1 h at room temperature with PBS, pH 7.5, 2.5% milk powder and 0.2% Tween 20. The filters were incubated with an anti-UBF antiserum (1:1000 dilution) followed by incubation with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Promega). Protein−antibody complexes were visualized using an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham).

In vitro translation and cross-linking of UBF

The pBAT-UBF1 expression vector has been described before (Voit *et al.*, 1992). This plasmid or a similar construct encoding UBF2 was transcribed with T3 RNA polymerase and translated in a rabbit reticulocyte lysate system (Promega). ^{35}S-labelled UBF was purified on a sequence-specific DNA affinity column (Jantzen *et al.*, 1992). For chemical cross-linking, equal amounts of labelled UBF1 or UBF2 were incubated for 40 min at 30°C in buffer AM-120 (20 mM HEPES, pH 7.9, 120 mM KCl, 0.1 mM EDTA, 20% glycerol, 0.5 mM DTE, 5 mM $MgCl_2$) containing 0.005% glutaraldehyde. The reactions were stopped by addition of lysine to a final concentration of 30 mM, and the products were analyzed by SDS−PAGE and fluorography.

DNase I footprinting

Footprinting was performed essentially as described by Bell *et al.* (1990) with minor modifications. The incubation mixtures (50 μl) contained 5 mM HEPES (pH 7.9), 25 mM KCl, 5 mM $MgCl_2$, 2.5 mM KF, 2% polyvinyl alcohol, 5% glycerol and 1−2 ng of a 5′-labelled DNA fragment. The enhancer probe contains the *Stu*I−*Sal*I (−640 to −168) fragment labelled at the *Stu*I site. The promoter fragment extends from −169 to +155. The labelled DNA was incubated for 15 min at 30°C with UBF before 1−20 ng of DNase I and 2.5 mM $CaCl_2$ were added and digestion was carried out for 1 min at room temperature. The reaction was stopped by the addition

of 350 mM ammonium acetate, 20 mM EDTA and 10 μg/ml yeast tRNA. After phenol extraction and ethanol precipitation, the samples were analyzed on a 6% polyacrylamide−8 M urea sequencing gel.

Far-Western blot

The two forms of UBF were separated by electrophoresis on 8% SDS−polyacrylamide gels and electrotransferred to nitrocellulose filters. The filters were incubated for 30 min at room temperature with 6 M guanidinium−HCl in buffer AM-100 without glycerol. Bound proteins were renatured by washing the membrane for 90 min in buffer AM-100 and for 1 h in buffer AM-100 containing 5% milk powder. After three washes with buffer AM-100, the membrane was incubated with ^{35}S-labelled UBF (10^5 c.p.m./ml) in buffer AM-100 containing 0.1% NP-40 for 4 h at 4°C. The filters were washed several times with buffer AM-100 + 0.1% NP-40 before radiolabelled proteins were visualized by autoradiography.

Electrophoretic mobility assay

Four-way junction DNA was obtained by annealing appropriate oligonucleotides which are partially complementary to each other and, when annealed, assemble into a cruciform molecule. Details of the construction and the sequences of the oligonucleotides are given in Bianchi (1988). As controls for structure-specific binding, a linear duplex DNA was used which is composed of one strand of the four-way junction annealed to its antiparallel complement (Bianchi, 1988). For mobility shift assays, one of the oligonucleotides was labelled with T4 polynucleotide kinase before annealing to the other oligonucleotide(s). 10 μl binding assays contained 8% Ficoll, 200 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.9), 5 mM KCl, 1 mM EDTA, 1 mM spermidine, 0.5 mM DTE, ~1 nM of labelled oligonucleotides and varying amounts of either bacterially expressed recombinant UBF1 and UBF2 or a bacterially expressed HMG peptide (HMG1bA, Bianchi *et al.*, 1992). After incubation for 10 min at room temperature, the samples were applied to 6.5% polyacrylamide gels in 0.5 × TBE and electrophoresed at 11 V/cm for 3−4 h at room temperature.

DNA transfection and transient expression assays

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and were transfected by the calcium phosphate technique. Cells were plated at a density of 5 × 10^5 cells per 10 cm plate and transfected with 17.5 μg of supercoiled plasmid DNA. After 44−48 h, RNA was isolated and 50 μg of total cellular RNA was hybridized for 3 h at 37°C to 5 × 10^5 c.p.m. of a 5′-labelled CAT-specific oligonucleotide and subjected to primer extension analysis as described before (Kuhn *et al.*, 1990).

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