Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3180–3184, April 1991
Biochemistry

Identification of two forms of the RNA polymerase I transcription factor UBF

(rRNA-encoding DNA/transcription)

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Communicated by Robert P. Perry, January 25, 1991 (received for review December 10, 1990)

ABSTRACT **The structure of the rat homologue of the RNA polymerase I transcription factor UBF was investigated. The sequence of the protein was deduced from the sequence of overlapping cDNAs isolated from a cDNA library and from clones of the products generated by the polymerase chain reaction from random-primed, first-strand cDNA. The sequences of these clones indicated that there were two mRNAs for UBF and that the encoded proteins were similar but not identical. One form of rat UBF was essentially identical to human UBF. The second class of UBF mRNA contained an in-frame "deletion" in the coding region that results in the deletion of 37 amino acids from the predicted protein sequence. This deletion reduces the predicted molecular size of the encoded form of UBF by ≈4400 from 89.4 kDa to 85 kDa and significantly alters the structure of one of the four HMG-1 homology regions (HMG box-2) in that form of UBF. Evidence for the existence of two mRNAs in rat cells was confirmed by a probe protection assay, and we provide evidence that other vertebrate cells contain these same two forms of UBF mRNA. These results are consistent with the observation that UBF purified from four different vertebrates migrates as two bands upon SDS/PAGE. It has been hypothesized that the HMG motifs are the DNA-binding domains of UBF. Altering one of these "boxes," as in the second form of UBF, may alter the functional characteristics of the transcription factor. Thus, the existence of different forms of UBF may have important ramifications for transcription by RNA polymerase I.**

Despite significant sequence differences (1) the promoters of the vertebrate rRNA genes consist of a core promoter element, CPE (2–4), and an upstream promoter element, UPE (5–9). Accurate and efficient transcription initiation by vertebrate RNA polymerase I requires at least two DNA-binding proteins. One of these factors, referred to as SL-1 (7, 9–11) or PC-D (12), appears to be sufficient for transcription *in vitro* (9, 11), apparently recognizes the CPE and the UPE (9, 11), and is required for species-specific transcription (9, 11, 12). A second transcription factor, UBF (13), has been isolated from several vertebrates.

UBF binds to the UPE of the vertebrate 45S RNA promoter (9, 11, 13, 14) and to the UPE of the promoter located in the intragenic spacers of these genes (15). UBF can also bind to the CPEs of these genes (11, 13) and to the repetitive spacer elements capable of enhancer activity (16). Furthermore, UBF isolated from one species can recognize the UBF-binding site on a heterologous promoter (16–18). Despite this abundance of binding sites, no consensus DNA-binding sequence has been demonstrated for UBF.

UBF is a member of the family of DNA-binding proteins that contain DNA-binding domains homologous to HMG-1 (refs. 19 and 20 and references therein). SDS/PAGE analysis of the UBF homologues isolated from four different vertebrate species demonstrated that each preparation consisted of two bands (9, 11, 16). The mammalian UBF homologues consist of two bands with apparent molecular sizes of ≈97 and ≈94 kDa (9, 11, 17). In the case of rat UBF (rUBF) both polypeptides have been shown to be DNA-binding proteins (21). Recombinant human UBF (hUBF), with a predicted molecular size of 89.4 kDa, comigrates with the 97-kDa band upon SDS/PAGE (19). The two UBF polypeptides might be related through some form of posttranslational processing or they may be independent translation products. Our results suggest that the two polypeptides are the translation products of two different mRNAs. The higher molecular size form of UBF, referred to as UBF1, contains four HMG-1 homology regions as reported for hUBF. The second form of UBF, referred to as UBF2, contains a partial deletion of HMG box-2, significantly altering the structure of that DNA-binding domain and the size of the protein.*

MATERIALS AND METHODS

Tissue Culture Growth of Mammalian Cells. Rat N1-S1 cells, murine SP2/0-Ag14 myeloma cells, Chinese hamster ovary (CHO) cells, and HeLa cells were obtained from the American Type Culture Collection and cultured as recommended.

Cloning of Target cDNAs. All DNA and RNA manipulations were performed as described (22–24). Total cellular RNA was isolated from N1-S1, HeLa, CHO, and murine myeloma cells (≈10^8 cells in each case) essentially as described (25). First-strand cDNA synthesis was performed in 25-μl reaction mixtures using 1.4 μg of total RNA and 100 pmol of random hexamers (26). After 60 min at 37°C, the reaction mixtures were incubated at 85°C for 10 min and then diluted to a final volume of 100 μl in polymerase chain reaction (PCR) buffer (Perkin–Elmer/Cetus) containing 100 pmol each of amplification primers and 2.5 units of Taq DNA polymerase. PCR amplification of target cDNAs was performed as follows: denaturation at 94°C, 1 min; primer hybridization at 46°C, 1.5 min; elongation at 72°C, 3 min; 40 cycles. For cloning purposes the PCR products were extracted with phenol, precipitated with ethanol, resuspended in 10 mM Tris/1 mM EDTA, pH 7.0, fractionated by electrophoresis on low-melting-temperature agarose, purified, and cloned into *Sma* I-digested plasmid pUC19.

Isolation of a 5′ Coding cDNA Clone for rUBF. A partial cDNA clone coding for nucleotides 144–438 of hUBF (19) was isolated following PCR amplification with sequence-specific primers from first-strand cDNA synthesized from HeLa cell RNA and cloned into pUC19, generating plasmid pUChUBF1. A ^{32}P-radiolabeled probe of the DNA insert in

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Abbreviations: CPE, core promoter element; hUBF and rUBF, human and rat homologues of UBF; PCR, polymerase chain reaction; UPE, upstream promoter element; CHO, Chinese hamster ovary.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M61724, M61725, and M61726).

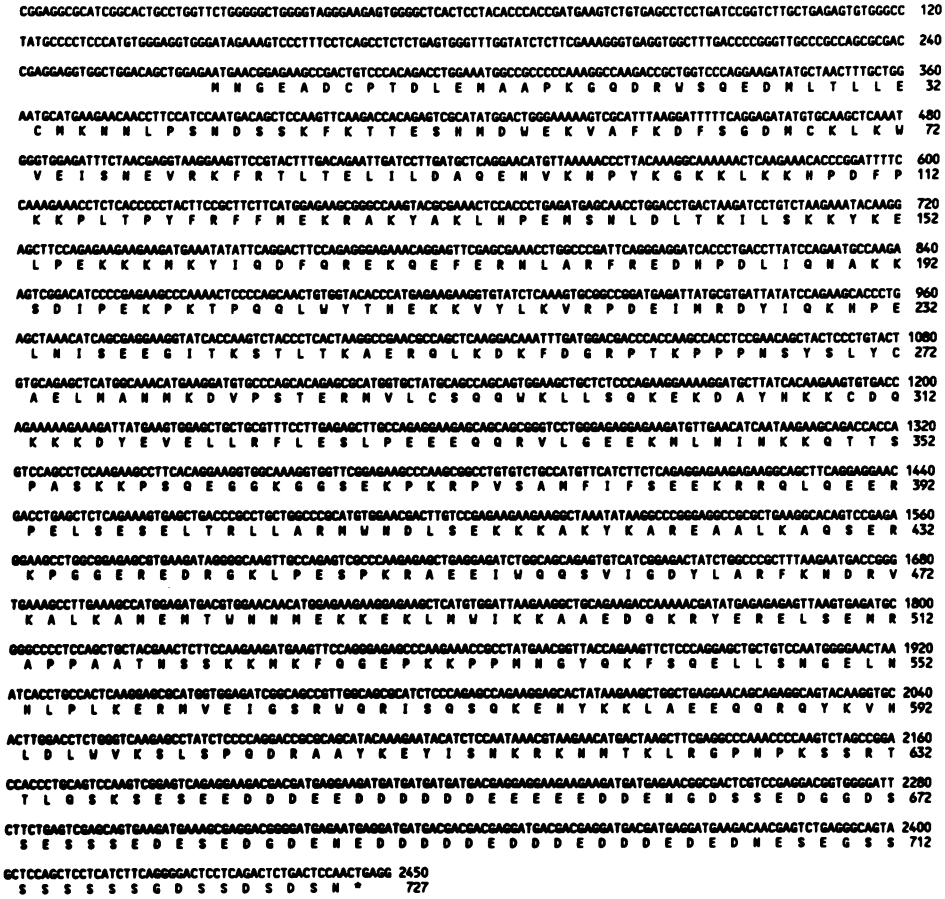

FIG. 1. Nucleotide sequence of the full-length cDNA coding for rUBF2 and the deduced amino acid sequence of rUBF2 extending from the methionine codon at position 267 to the stop codon at position 2446.

pUChUBF1 was used to screen a Reuber hepatoma cell cDNA library (Stratagene). Plating, screening of the cDNA library in *Escherichia coli* XL blue, and *in vivo* excision of the positive, recombinant plasmid p405rUBF from the bacteriophage ZAP405rUBF were performed essentially as outlined by the supplier (Stratagene).

Cloning of the 3′ Coding Region for rUBF. The oligonucleotides Dom50 (corresponding to nucleotides 1453–1470 of the rUBF cDNA coding strand on p405rUBF) and Dom15 (complementary to nucleotides 2424–2444 of the hUBF cDNA sequence) were used for PCR amplification of randomly primed first-strand cDNA derived from N1-S1 RNA. A

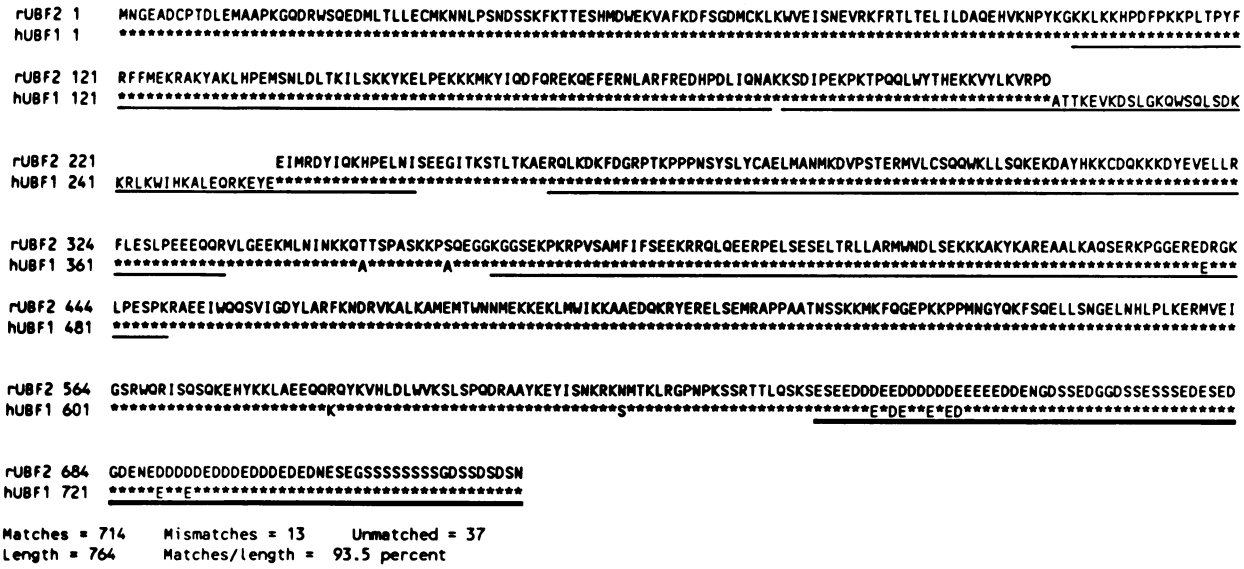

FIG. 2. Comparison of the deduced amino acid sequences of rUBF2 and hUBF1 (19). An asterisk denotes identical amino acids. Positions of the four HMG boxes, residues 103–190 (box-1), 191–236 (box-2), 250–335 (box-3), and 364–449 (box-4), of rUBF2 are underlined. The position of the acidic tail (residues 639–725 of rUBF2) is heavily underlined. Amino acids 221–257 of hUBF1 are deleted in the corresponding region of rUBF2.

1-kilobase (kb) fragment that hybridized to a probe based on the hUBF cDNA sequence (bases 2221–2244) was cloned into plasmid pUC19, generating p406rUBF.

Northern Blot Analysis. Five micrograms of poly(A)$^+$ mRNA isolated from N1-S1 cells was resolved by electrophoresis in a formaldehyde/formamide agarose gel, transferred to a Hybond N nylon membrane, and probed using a ^{32}P-radiolabeled riboprobe (27) complementary to the insert in plasmid p405rUBF.

Analysis of the UBF Coding mRNAs by PCR and Southern Hybridization. First-strand cDNAs to N1-S1, HeLa, CHO, and murine myeloma cell RNAs were amplified for UBF cDNA sequences using the primer pairs indicated in the legend to Fig. 3. The PCR products were analyzed by agarose gel electrophoresis, transferred to Hybond N membranes, and probed using radiolabeled "internal" oligonucleotides complementary to the rUBF cDNA sequence. Following amplification of the N1-S1 cDNA with primers that correspond to bases 145–164 of the hUBF sequence (Dom14) and 1054–1071 of the rUBF2 sequence (Dom69), respectively, two DNA fragments that hybridized to an internal probe were purified, cloned into pUC19, generating p32rUBF1 and p43rUBF2, and sequenced.

S1 Nuclease Assay. S1 nuclease assays were carried out as described (28). The 5′ end-labeled probe was a synthetic hexadecamer complementary to nucleotides 662–712 of the rUBF cDNA coding strand sequence on plasmid p32rUBF1 (numbering relative to the initiation codon of the rUBF2 sequence in Fig. 1), which also contained a 10-base extension at the 3′ end. The

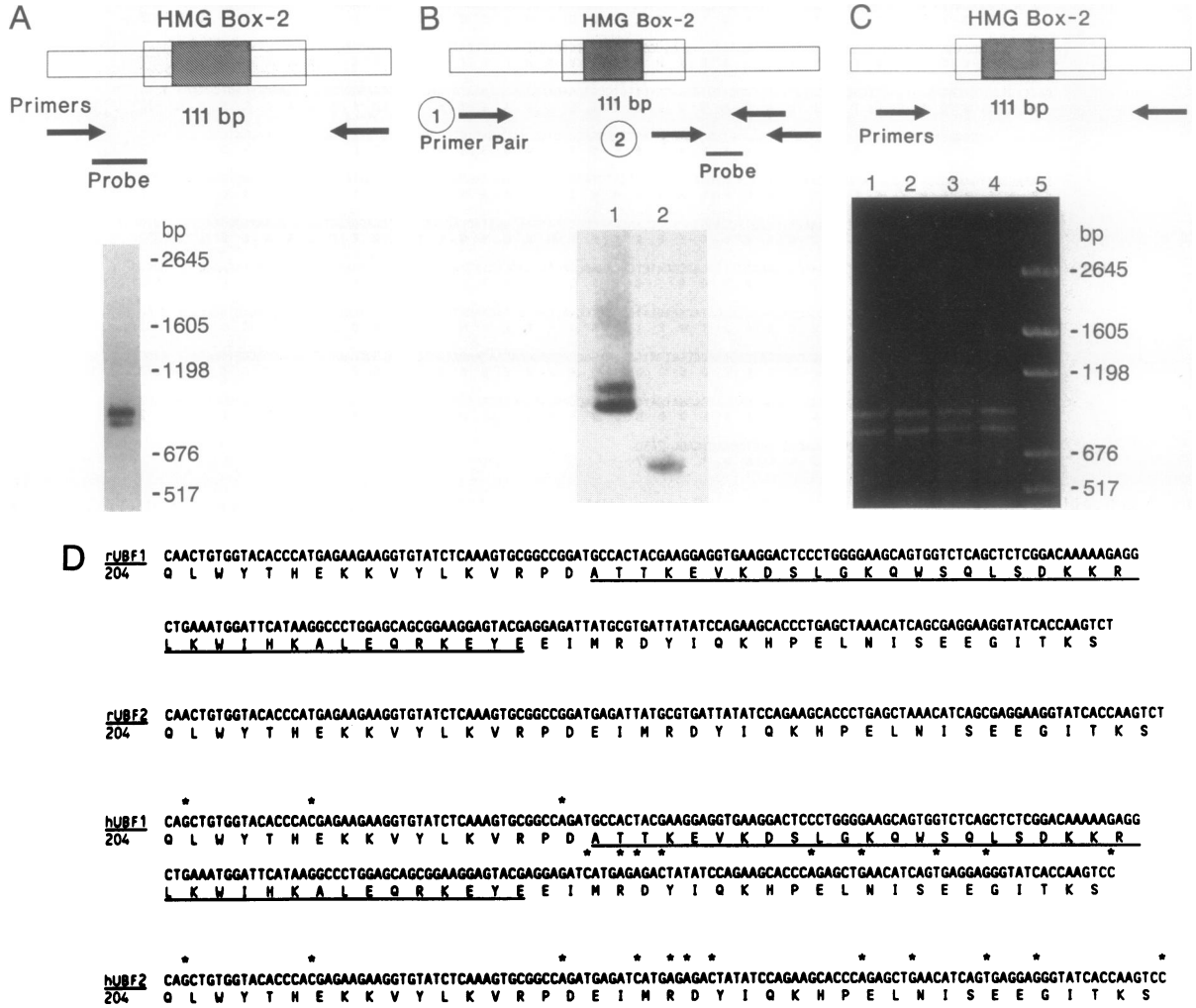

FIG. 3. Identification of two forms of UBF in rat, human, hamster, and mouse cells. (*A*) Randomly primed first-strand cDNA from rat N1-S1 cells was amplified by PCR using the primer pair Dom14/Dom69, which flanks the HMG box-2 coding region. The amplified DNA was resolved by agarose gel electrophoresis, blotted, and probed with an internal probe (Dom54; bases 520–537 of rUBF2). Two DNA fragments of 807 base pairs (bp) (band 2) and 918 bp (band 1) hybridized to the probe. Positions and sizes of the pGEM DNA size standards are shown. (*B*) Randomly primed first-strand cDNA from rat N1-S1 cells was amplified by PCR using the primer pairs (873–890)/(2046–2030) (primer pair 1) and (1416–1440)/(2178–2162) (primer pair 2). Following agarose gel electrophoresis, the amplified DNA was blotted and probed with the primer Dom50. Primer pair 1 generated two PCR products of 1173 and 1284 bp, which hybridized to the radiolabeled probe (lane 1). Primer pair 2 generated a single PCR product of 630 bp, which hybridized to the labeled probe (lane 2). (*C*) Randomly primed first-strand cDNA from human (lane 1), rat (lane 2), CHO (lane 3), and mouse (lane 4) cells was amplified by PCR using the primer pair Dom14/Dom34 (complementary to bases 997–1013 of hUBF cDNA sequence) and analyzed by agarose gel electrophoresis. In each case two PCR products of 752 and 863 bp were synthesized. Each DNA fragment hybridized to a mixture of internal end-labeled primers (data not shown). The smaller PCR product synthesized from the HeLa cDNA was gel purified and cloned into pUC19, generating pHB2hUBF2. Positions and sizes of the pGEM DNA size standards (lane 5, markers) are shown. (*D*) DNA sequences and corresponding amino acid sequences for rUBF1, rUBF2, and hUBF2 in the vicinity of HMG box-2 present on plasmids p32rUBF1, p43rUBF2, and pHB2hUBF2, respectively. The corresponding DNA and amino acid sequences of hUBF1 have been published (19). The sequences of rUBF1 and hUBF1 contain an additional 37 amino acids (underlined) within HMG box-2 in comparison to rUBF2 and hUBF2, respectively. An asterisk denotes differences in the nucleic acid sequence between the corresponding rat and human cDNA sequences.

protected products were analyzed by electrophoresis on a 10% denaturing polyacrylamide gel and autoradiography.

RESULTS

Molecular Cloning and Sequence Analysis of rUBF. Plasmid p405rUBF contained a 1.47-kb insert corresponding to the 5′ coding region of rUBF, whereas p406rUBF, coding for the 3′ region of the rUBF, was obtained by PCR amplification of first-strand cDNA of N1-S1 cell total RNA. The nucleotide sequence of these overlapping cDNA clones together with the deduced amino acid sequence for rat UBF are presented in Fig. 1. The nucleic acid sequence coding for rUBF demonstrated an 86.5% sequence identity with that reported for hUBF. However, there was a "deletion" of 111 nucleotides at position 927 of the rat cDNA sequence when compared to the hUBF cDNA sequence. This deletion was in frame, resulting in the deletion of 37 amino acids, and was located within one of the HMG boxes, HMG box-2 of the rUBF sequence (Fig. 2).

Analysis of the Heterogeneity of the HMG Box-2 Coding Region of rUBF mRNA. PCR amplification of the HMG box-2 coding domains of N1-S1 cell first-strand cDNA generated two DNA fragments that hybridized to an internal end-labeled oligonucleotide (Fig. 3*A*). These two bands were cloned, generating plasmids p43rUBF2 (band 2) and p32rUBF1 (band 1), and sequenced (Fig. 3*D*). The DNA sequence of the smaller PCR product, band 2, was the same as the corresponding region of the rUBF sequence shown in Fig. 1. However, the DNA sequence of the larger PCR product, band 1, was longer than that of the corresponding region of the rUBF sequence by 111 bp and the deduced peptide sequence was identical to hUBF across HMG box-2 (discussed below).

To examine these results further, we carried out additional PCR reactions on first-strand cDNA in which we amplified UBF coding sequences with primer pairs that either flanked the deleted region or that would amplify the sequence 3′ to the deletion. Following PCR amplification, the products were resolved by agarose gel electrophoresis, blotted, and hybridized to an internal UBF-specific probe (Dom50). The primers that flanked the deleted region generated two distinct PCR products that differed in size by 111 bp and that hybridized to the internal probe (Fig. 3*B*, lane 1). In contrast, the reaction using the primers for the region 3′ to the deletion in the HMG box-2 coding sequence generated a single product that hybridized to the internal probe (Fig. 3*B*, lane 2).

Analysis of the Heterogeneity of the HMG Box-2 Coding Region of mRNAs from Human, Hamster, and Murine Cell Lines. In view of our finding that rat cells contained two mRNAs coding for distinct forms of UBF we chose to investigate whether other vertebrates might also contain analogous UBF mRNAs. Randomly primed cDNA templates synthesized from RNA preparations isolated from human, hamster, and murine cells were subjected to PCR using primers that flank the HMG box-2 coding region of rUBF. As shown in Fig. 3*C*, each reaction produced two PCR products, which hybridized to UBF-specific probes based on either the rat or human sequence (data not shown).

The smaller of the PCR products from HeLa first-strand cDNA (Fig. 3*C*) was cloned and sequenced. The nucleic acid sequence (Fig. 3*D*) is identical to the corresponding region reported for the hUBF cDNA, except for the deletion of 111 bp that maps to the same position found in the rUBF2 sequence. The deduced amino acid sequence of the human fragment is identical to that of the smaller form of rUBF in this region. Thus, it would appear that two forms of UBF mRNA with the potential to code for distinct forms of UBF are present in each species tested.

Analysis of N1-S1 UBF mRNAs by Northern Blot and S1 Nuclease Assays. Analysis of poly(A) mRNA isolated from N1-S1 cells by Northern blot using a rUBF riboprobe demonstrated a single transcript of 3.2 kb (Fig. 4*A*). This is similar to the size reported for the mRNA for hUBF (19). A smaller transcript that hybridized weakly comigrated with 18S rRNA and likely represents background hybridization. To establish whether rat may indeed contain two distinct transcripts differing in the HMG box-2 coding domain an S1 nuclease digestion analysis was performed. Two distinct protection products are visible (Fig. 4*B*). The smaller product comigrated with a 30-base oligonucleotide size standard, whereas the larger protected fragment has an estimated size of 50 bases. The sizes of these protection products were those predicted from the DNA sequence analyses of the cloned UBF cDNAs.

DISCUSSION

We have isolated cDNAs encoding rUBF from a cDNA library from rat Reuber hepatoma cells and by PCR ampli-

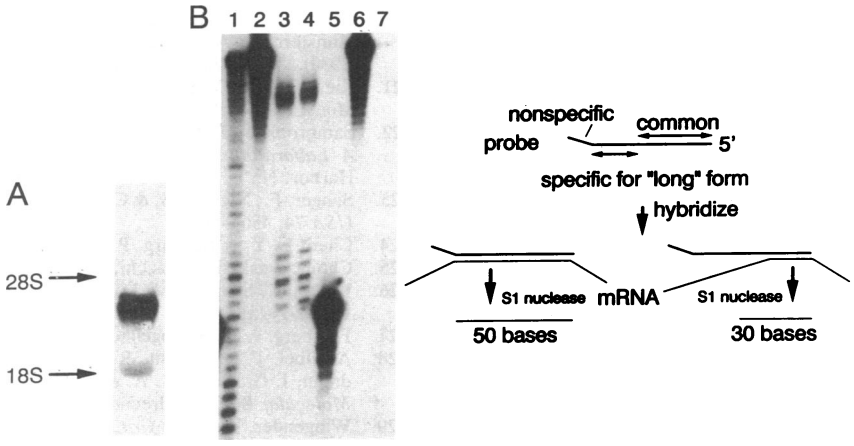

FIG. 4. Analysis of the mRNA coding for rUBF by Northern blot and probe protection assay. (*A*) mRNA isolated from rat N1-S1 cells was resolved by formaldehyde/formamide agarose gel electrophoresis, blotted, and hybridized to a riboprobe complementary to the rUBF mRNA coding sequence on plasmid p405rUBF. Positions of 18S and 28S rRNA are indicated. (*B*) The synthetic oligonucleotide Dom81 was end-labeled and hybridized to total N1-S1 cell RNA; hybridization products were digested with S1 nuclease and analyzed by polyacrylamide gel electrophoresis and autoradiography. Dom81 was designed so that after S1 nuclease digestion the mRNA for rUBF1 would yield a 50-base S1 product and the mRNA for rUBF2 would yield a 30-base product. The results of two experiments in which the probe was hybridized to total N1-S1 cell RNA are presented (lanes 3 and 4), along with control lanes containing partially digested probe (lane 1), undigested probe (lanes 2 and 6), a radiolabeled 30-mer oligonucleotide (lane 5), and the Dom81 digested with S1 nuclease in the absence of rat N1-S1 RNA (lane 7).

fication of first-strand cDNA from the rat N1-S1 cell line. We have found that rat cells contain two different mRNAs that in turn have the capacities to code for two distinct forms of UBF that differ in one of their putative DNA-binding domains. S1 nuclease experiments confirmed the existence of the two forms of UBF mRNA predicted from the molecular cloning experiments. The deduced amino acid sequence for one form of rUBF, rUBF2, was very nearly identical to that reported for hUBF1 except for the deletion of 37 amino acids. The deleted region, which is α-helical in nature, resulted from an in-frame deletion of 111 bp within the HMG box-2 coding region of the mRNA coding for UBF2. Apart from the deletion of 37 amino acids in rUBF2 there are only 13 differences in 764 residues between hUBF and rUBF that arise primarily by single base substitutions. Ten of these differences are the substitution of aspartic and glutamic acid for glutamic and aspartic residues in the highly acidic carboxyl tail of the protein. Considering the strong sequence conservation between rUBF and hUBF it is not surprising that these transcription factors are interchangeable in *in vitro* transcription assays and in DNase I footprinting experiments (S. D. Smith and S. Bell, personal communication).

We subsequently obtained evidence for the existence of the same two forms of UBF mRNA in three other species: mouse, human, and hamster. Furthermore, PCR products coding for the HMG box-2 coding domain derived from HeLa cell mRNA were cloned and sequenced. The amino acid sequence deduced from our partial cDNA clone for hUBF2 contains the same deletion of 37 amino acids in this domain as found in rUBF2. Consequently, we can conclude that rats and humans synthesize the same two distinct forms of UBF. Similarly, the identification of equivalent PCR products derived from murine and CHO cell mRNA suggests that these species also code for the same two forms of UBF. The predicted sizes of the two forms of UBF differed by 4.47 kDA, similar to the observed size difference of the two bands present in purified UBF isolated from rat, mouse, and human cells.

There are numerous examples of variants of the same protein with the potential to recognize the same DNA sequence (29), including TF-IIIA (30), *Jun* (31–33), and NF-E1 (34). The two forms of UBF mRNA may be the products of two genes or they might result from alternative splicing.

Although the existence of two isoforms for UBF appears to add another level of complexity to what is presently understood about transcription by RNA polymerase I, our results may help to eliminate one of the conundrums in this field.

The various vertebrate homologues of UBF bind to two parts of the 45S promoters (UPE and CPE), to the spacer promoters, and to the variable repeat elements in the nontranscribed spacers of the rRNA-encoding DNA (rDNA) repeats. However, despite this abundance of DNA-binding sites there is no clear consensus UBF recognition sequence. Jantzen *et al.* (19) proposed that the HMG homology boxes were the DNA-binding domains of UBF1 and demonstrated that a truncated UBF containing the amino-terminus and HMG box-1 (residues 1–204) could bind to the human rDNA promoter. It is possible that specific DNA binding by UBF requires only HMG box-1. Alternatively, the specific recognition of DNA by UBF1 may result from the cooperative interactions of the four HMG boxes. In one model the specific recognition of DNA by UBF1 results from the cooperative interactions of four independent DNA-binding domains. Computer analysis predicts that the HMG boxes are essentially α-helical in nature, bounded by regions that are either turns or random coils. In a second model the boxes could function in pairs to form the two-helix motif found in the DNA-binding domains of other proteins, such as the large fragment of *E. coli* DNA polymerase I (35). Thus, deleting a significant portion of one of these boxes, as in UBF2, might change or eliminate one of the components of the DNA-binding reaction. Furthermore, the deletion of a portion of the protein might change its structure and the topological relationships between the DNA-binding domains and between the DNA-binding domains and activating domains of the protein. The result would be to alter the DNA sequences recognized and/or the ability of UBF—i.e., UBF2—to activate transcription in specific instances. Therefore, it is formally possible that most preparations of UBF may actually contain two isoforms of UBF with dissimilar DNA-binding properties.

We wish to express our thanks to the other members of the laboratory for their helpful comments and suggestions. We thank Drs. David Carey, Theresa Kinsella, and Howard Morgan for helpful comments and suggestions concerning the manuscript. We also thank Brian Shoop for his expert assistance in the production of the final form of the figures. This work was supported by the Geisinger Foundation.

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