Differential Phosphorylation and Localization of the Transcription Factor UBF in Vivo in Response to Serum Deprivation

IN VITRO DEPHOSPHORYLATION OF UBF REDUCES ITS TRANSCRIPTIVE PROPERTIES*

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Daniel J. O'Mahony, WenQin Xie, S. David Smith, Harold A. Singer, and Lawrence I. Rothblum

From the Sigfried and Janet Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania 17822-3618

We have analyzed the expression, phosphorylation, and localization of the ribosomal DNA transcription factors UBF1 and UBF2 in Chinese hamster ovary cells in response to serum deprivation. In vivo labeling experiments demonstrate that UBF1 and UBF2 are phosphoproteins. Phosphoamino acid analysis of the in vivo labeled proteins demonstrate that UBF is phosphorylated on serine residues. Following serum deprivation there is no alteration in the cellular levels of UBF1 and UBF2 as determined by Western blotting, but there is an 80% reduction in the level of phosphorylation of UBF compared with logarithmically growing cells. Following serum deprivation there is a redistribution of UBF between the nucleolus, the nucleus, and the cytoplasm. Phosphatase-treated UBF demonstrated a reduced ability to rescue transcription by RNA polymerase I from the rDNA spacer promoter in vitro. These findings suggest that phosphorylation of UBF is a prerequisite for transactivation of RNA polymerase I.

The promoters of vertebrate ribosomal RNA genes consist of elements with similar functions: a core promoter element (CPE),1 an upstream promoter element (UPE), and a promoter-proximal terminator site (T.) (Ref. 1, and references therein). Transcription of ribosomal RNA genes requires at least two defined factors referred to as SL-I and UBF (1–3). Although the CPE is sufficient for transcription in vitro, the UPE is required for transcription in vivo (4). In vitro experiments indicate that the transcription factor UBF footprints over the UPE of the 45 S and spacer promoters (1–6). Whereas it has been established that SL-I interacts with both the CPE and the 5’ boundary of the UPE (1, 3) the factor has not been characterized in detail. UBF has been isolated from a number of species, including human, rat, mouse, and Xenopus laevis. In the cases of human, rat, and mouse the purified factors consist of two distinct polypeptides of 94 and 97 kDa in size (1, 3). The cDNAs coding for human, rat, Xenopus, and mouse UBF1 and UBF2 have been cloned and sequenced (7–11).

Previous studies on the regulation of ribosomal RNA synthesis in response to serum deprivation in eukaryotes have led to the model that transcription by RNA polymerase is regulated by the post-translational modification of either RNA polymerase 1 (12) and/or a factor which is tightly associated with the polymerase, such as TFIC (13). In this respect we have analyzed the expression, localization, and phosphorylation of UBF in cultured Chinese hamster ovary (CHO) cells in response to serum deprivation. Cells deprived of serum, and the growth factors contained therein, cease to progress through the cell cycle and shut off ribosomal RNA synthesis (Ref. 12, and references therein). Our evidence demonstrates that both UBF1 and UBF2 are phosphoproteins and that the degree of phosphorylation of UBF is significantly reduced when CHO cells are serum-deprived. Immunolocalization studies show loss of UBF from the nucleolus following serum deprivation. In vitro transcription experiments demonstrated that treatment of UBF with alkaline phosphatase reduces the transcriptional activation properties of UBF.

EXPERIMENTAL PROCEDURES

Materials—All DNA and RNA modifying enzymes and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Promega. Taq DNA polymerase was from Perkin-Elmer/Cetus.123-Labeled goat anti-rabbit IgG, F(ab)2 fragment, [32P]orthophosphate, [α-32P]UTP, and [γ-32P]ATP were purchased from Du Pont-New England Nuclear. Nitrocellulose and Immobilon-P were purchased from Millipore, respectively. RIBI adjuvant was from ImmunoChem Research, Inc. RPMI-1640 media was from MediaTech; protein G-agarose, newborn calf serum, and dialyzed newborn calf serum were from GIBCO/BRL. Prestained protein size standards were purchased from Bio-Rad. Bacterial alkaline phosphatase was obtained from Pharmacia LKB Biotechnology Inc. Rhodamine-conjugated, affinity-purified, goat anti-rabbit IgG was obtained from Vector Laboratories. Protein G-agarose, newborn calf serum, and dialyzed newborn calf serum were from Sigma. TLC cellulose plates were obtained from Kodak.

Expression of Recombinant UBF2 in Escherichia coli and Antibody Production—A cDNA fragment coding for amino acids 53–390 of rUBF2 was amplified by PCR from the plasmid template p405rUBF (8) and cloned into the vector pET3a (14), generating pETrUBF53–390. IPTG-induced expression of rUBF53–390 in the host E. coli BL21 (DE3) was performed essentially as described (14, 15). The recombinant protein rUBF53–390 was purified from induced cultures by preparative SDS-PAGE (16). Rabbits were immunized with 100 µg of rUBF53–390 protein in RIBI adjuvant following an immunization scheme recommended by the manufacturer. The antiserum cross-reacted with both recombinant UBF1 and UBF2 synthesized in E. coli (data not shown).

Tissue Culture of CHO Cells and in Vivo Labeling with [32P]Orthophosphate—CHO cells were cultured in RPMI-1640 plus 10% newborn calf serum (NBS). For serum deprivation studies, cells were initially plated at a density of 3.5 × 10^5 cells/60-mm dish. After 24 h in complete media, the cells were washed with RPMI-1640 and serum-starved for either 24, 48, or 68 h by incubation in RPMI, 0.05% NBS. Cell viability, determined by the trypan blue dye exclusion assay, was greater than 90% after 24 or 48 h of serum starvation.

For in vitro phosphorylation studies, lognometrically growing and serum-starved cells were plated at a density of 3.5 × 10^5 and 5.5 × 10^5 cells, respectively, per 60-mm dish in RPMI, 10% NBS (3 ml) and grown for 24 h. Cells were then washed in phosphate-free RPMI media. Log growing cells were labeled for 16 h in phosphate-free RPMI, 20% dialyzed NBS containing 1 mCi/ml [32P]orthophosphate. Where specified, separate cultures were either serum-starved (RPMI, 0.05% NBS), or cultures were serum-starved and then labeled in phosphate-free RPMI, 20% dialyzed NBS containing 1 mCi/ml [32P]orthophosphate.

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The abbreviations used are: CPE, core promoter element; UPE, upstream promoter element; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NBS, newborn calf serum.

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precipitated with protein G-agarose as described by the supplier, were fractionated by SDS-PAGE, blotted onto Immobilon-P membrane, and subjected to autoradiography (17). For quantitation, the immunoprecipitated bands were cut from the membranes and subjected to liquid scintillation spectrometry. Phosphoamino acid analysis was carried out as described (18).

Western Blotting and Immunofluorescence Studies—Whole cell lysates (100 μg of protein) from CHO cells cultured as described were fractionated by SDS-PAGE, blotted onto nitrocellulose, and probed with the anti-UBF serum (1:500 dilution) (17). For immunofluorescence studies, CHO cells were grown on coverslips and were fixed and stained with either anti-UBF serum (1:80 dilution) or antiserum to fibrillarin essentially as described (19). The second antisera were used as recommended by the suppliers.

Analysis of the mRNAs Coding for UBF1 and UBF2 in CHO—First strand cDNA was synthesized using total RNA (20), random hexamer nucleotide primers, and reverse transcriptase followed by amplification of the UBF coding sequences by PCR and analysis by Southern blotting using a mixture of 5' end-labeled, internal oligonucleotide probes as described (8).

In Vitro Transcription Studies—UBF1 and UBF2 were purified through the CM-Sepharose chromatography step (1). In vitro transcription of the rat rDNA spacer promoter (6) was carried out as previously described (1, 6) including the addition of a radiolabeled internal standard to monitor the recovery of nucleic acids (1, 6). Treatment of the purified UBF1/UBF2 with bacterial alkaline phosphatase in buffer C20 (1) was performed essentially as described (12).

RESULTS

Analysis of the Expression of UBF1 and UBF2 in Different Species and in Response to Serum Deprivation—Western blot analysis of cell lysates prepared from Novikoff (rat), HeLa (human), COS (monkey), CHO (hamster), and BFE (bovine) cells showed that in each case the anti-UBF serum recognized two bands at 97 and 94 kDa (Fig. 1A), confirming that both UBF1 and UBF2 are highly conserved in the different eukaryotic species tested. Western blot analysis (Fig. 1B) of cell lysates from CHO cells which were either grown in the presence of serum, serum-starved for 24, 48, or 68 h, or were serum-starved and refed for the specified time periods showed that, with the exception of cells which were serum-starved for 68 h (Fig. 1B, lane 7), there was no significant change in the levels of UBF1 and UBF2 in the CHO cells grown under these different conditions. Analysis of the UBF coding sequences in RNA isolated from logarithmically growing CHO cells, serum-starved CHO cells, and starved and refed CHO cells showed that there was no substantial change in the steady-state levels of the UBF coding mRNAs (Fig. 1C). Taken together, these results indicate that the level of expression of UBF1 and UBF2 does not change in response to serum deprivation.

Analysis of the Immunolocalization of UBF in Response to Serum Deprivation—Immunolocalization studies using the anti-UBF antibody confirmed that UBF in CHO cells is a nucleolar protein (Fig. 2, panels A and B) as indicated by the specific staining of the nucleolus in logarithmically growing cells. However, following serum starvation for 24 h, the distribution of UBF was altered. The nucleolar fluorescence decreased, and a diffuse nuclear and cytoplasmic staining was evident (Fig. 2, panel C). When cells serum-starved for 43 h were refed for 5 h, UBF was again located in the nucleolus (Fig. 2, panel D). In post-confluent cells UBF was again distributed between the nucleolus, the nucleus, and the cytoplasm (Fig. 2, panel E). In contrast, the nucleolar localization of fibrillarin was not altered under these growth conditions although the level of nucleolar fluorescence decreased upon serum starvation and when the cells were confluent (Fig. 2, panels A–E, respectively).

The Phosphorylation of UBF Is Altered in Response to Serum Deprivation—In order to test whether UBF1 and UBF2 are phosphorylated, CHO cells were labeled with [32P]orthophosphate, and UBF1 and UBF2 were immunoprecipitated, fractionated by SDS-PAGE, and blotted onto Immobilon. A typical precipitate formed in the presence of the anti-UBF antiserum but not preimmune serum contained two radioactive bands at 94 and 97 kDa (Fig. 3A, lane 2).

When cells were serum-starved for 24 or 48 h there was an 80% reduction in the degree of UBF1/UBF2 phosphorylation (Fig. 3A, lanes 3 and 4) in comparison with cells grown in the presence of serum (Fig. 3A, lane 2). When cells were serum-starved for 24 or 48 h and refed with complete medium for 8 h and labeled with [32P]orthophosphate for 16 h, the level of phosphorylation of UBF1 and UBF2 recovered to that observed in logarithmically growing cells (Fig. 3A, lanes 6 and 7). In contrast, cells which were serum-starved for 72 h contained no detectable levels of phosphorylated UBF (Fig. 3A, lane 5) even upon refeeding (Fig. 3A, lane 8). Parallel analyses of the level of phosphorylation of whole cell extracts from serum-starved (24 and 48 h) and starved/refed cells...
Fig. 2. Immunocytochemical analysis of the distribution of UBF in response to altered growth conditions. Panels A and B show phase and fluorescence photographs, respectively, of logarithmically growing CHO cells stained with the UBF antiserum (α-UBF, upper panel) or the fibrillarin antiserum (α-fibrillarin, lower panel). Panels C, D, and E show serum-starved (24 h), serum-starved/refed (43 h/5 h), and confluent CHO cells, respectively, stained with α-UBF (upper panels) or α-fibrillarin (lower panels). The arrowheads in panels C and D indicate areas where cytoplasmic staining with the α-UBF antiserum was readily apparent.

Fig. 3. Analysis of the phosphorylation of UBF in response to altered growth conditions. A, UBF1/UBF2 were immunoprecipitated from 32P metabolically labeled CHO cells using the anti-UBF antiserum, fractionated by SDS-PAGE, blotted onto nitrocellulose, and subjected to autoradiography. Lanes 1 and 2, immunoprecipitates from logarithmically growing cells using the preimmune and anti-UBF antiserum, respectively. Lanes 3-5 are immunoprecipitates from cells serum-starved for 24, 48, and 72 h, respectively. Lanes 6-8 are immunoprecipitates from 24-, 48-, and 72-h serum-starved cells which were reared for 8 h in complete medium and labeled for 16 h, respectively. Equivalent numbers of cells were used for each analysis. The position and size (kDa) of the molecular weight size standards are indicated. B, phosphoamino acid analysis of 32P metabolically labeled, immunoprecipitated UBF1/UBF2 was performed as described under "Experimental Procedures." Lane 1, no URF; lane 2, UBF; lane 3, UBF preincubated at 4 °C for 30 min; lane 4, UBF and bacterial alkaline phosphatase (0.28 unit) added at the start of the transcription reaction; lane 5, UBF preincubated with bacterial alkaline phosphatase (0.28 unit) at 4 °C for 30 min. Trans. and Int. Std. denote the transcript and internal standard, respectively.

Fig. 4. Analysis of the in vitro trans-activation properties of phosphatase-treated UBF. In vitro transcription from the spacer promoter using bacterial alkaline phosphatase-treated UBF was performed as described under "Experimental Procedures." Additions: lane 1, no UBF; lane 2, UBF; lane 3, UBF preincubated at 4 °C for 30 min; lane 4, UBF and bacterial alkaline phosphatase (0.28 unit) added at the start of the transcription reaction; lane 5, UBF preincubated with bacterial alkaline phosphatase (0.28 unit) at 4 °C for 30 min. Trans. and Int. Std. denote the transcript and internal standard, respectively.

level of phosphorylation of UBF1 and UBF2 increases dramatically.

Phosphoamino acid analysis of radiolabeled, immunoprecipitated UBF1 and UBF2 from serum-starved, serum-starved/refed, and serum-fed CHO cells demonstrated that the phosphorylated amino acid(s) in UBF1/UBF2 is serine in each case (Fig. 3B, lanes 1, 4, 2, 5, and 3, 6, respectively).

In Vitro Transcription Studies Using Bacterial Alkaline Phosphatase-treated UBF1/UBF2—To establish whether the phosphorylation of UBF plays a direct role in the ability of UBF to activate transcription, the spacer promoter (which is transcriptionally inactive in the absence of UBF) was used as a template in in vitro transcription assays. Transcription from this promoter is dependent upon the presence of UBF in this assay (6, 21). UBF rescued transcription from this promoter (Fig. 4, lanes 2 and 3). Bacterial alkaline phosphatase-treated UBF (Fig. 4, lane 5) demonstrated a reduced ability (67% reduced) to activate transcription from the spacer promoter. Addition of equivalent amounts of bacterial alkaline phosphatase directly to the transcription reaction did not significantly affect UBF-dependent transcription (Fig. 4, lane 4).
DISCUSSION

The results presented here demonstrate that: 1) the RNA polymerase I transcription factor UBF is a phosphoprotein, 2) the modified amino acid is serine, 3) the phosphorylation of UBF is reduced approximately 80% in serum-starved CHO cells relative to the level of phosphorylation of UBF in log-arithmically growing cells, 4) the subcellular distribution of UBF shifts from being predominantly nucleolar to a dispersed nucleoplasmic/cytoplasmic distribution following serum starvation, and 5) treatment of purified UBF with bacterial alkaline phosphatase reduced the transactivation properties of UBF in an in vitro RNA polymerase I transcription system.

The phosphorylation state of certain transcription factors is important in modulating their transcriptional properties (Refs. 22 and 23, and references therein). The results presented here strongly suggest that the phosphorylation state of UBF may affect its transactivation properties. The reduced transactivation properties of phosphatase-treated UBF in our in vitro transcription system suggest that the phosphorylation of UBF may be a prerequisite for transcriptional activation. The finding that authentic UBF can be phosphorylated in vitro by casein kinase II (data not shown) and the observation that the level or extent of phosphorylation of UBF actually increases when serum-starved cells are re-fed suggests that the degree of phosphorylation of UBF is regulated.

There is also the possibility that the phosphorylation of UBF is necessary for its nucleolar localization. The events controlling protein translocation in response to various conditions, including serum starvation, have recently been addressed (Ref. 24, and references therein). It appears that the nuclear localization of several proteins, e.g. c-Fos (24), NFB (25), and SV-40 T-antigen (26), is regulated by phosphorylation. We have seen that concomitant with a reduction in the level of UBF phosphorylation, UBF redistributes between the nucleolus, the nucleus, and the cytoplasm. Such an event would effectively reduce nucleolar, UBF-dependent RNA polymerase I transcription in serum-starved cells. Relevant to this hypothesis is the observation that serum starvation is associated with the translocation of another nucleolar phosphoprotein, B23 or N038, from the nucleolus to the nucleoplasm (27).

Several laboratories have reported that when cell growth is shut off in response to stimuli such as starvation (12, 28), glucocorticoids (13), and serum deprivation (29) that the ability of RNA polymerase I extracted from those cells to correctly initiate transcription in vitro is reduced. This has been ascribed to a modification of RNA polymerase I itself (12, 28, 29) or to the modification of a factor such as TFIIIC (13) that is tightly associated with the polymerase. Our results suggest that cells respond to serum starvation by altering the phosphorylation state of UBF as well. Although the above mentioned studies on the regulation of eukaryotic rDNA transcription reported no changes in the activities of the other transcription components in the whole cell extracts of the down-regulated cells one must remain cognizant of the facts that 1) standard in vitro transcription reactions of the mouse and rat 45 S RNA promoter do not appear to require UBF (1, 3) and 2) those studies used whole cell extracts which would contain a small amount of phosphorylated UBF. Our results indicate that the amount of UBF present in down-regulated cells is unchanged and suggest that the phosphorylation of UBF affects its role in transcription and in its subcellular localization, thus regulating its activity in transcription in two ways. Therefore, our observations may complement earlier results, as it is formally possible that the same enzyme or signal transduction cascade responsible for regulating the modification of RNA polymerase I, TIF-1A, TIF-1B, or TIF-1C may also be involved in regulating the phosphorylation of UBF. We are currently attempting to identify the kinase(s) and/or phosphatase(s) which modify UBF in vivo in response to serum and to ascertain the possible roles of such systems in coordinately regulating the activity of the individual components required for efficient rRNA transcription. UBF, which has potential phosphorylation sites for casein kinase II in its carboxyl-terminal acidic tail is phosphorylated by casein kinase II in vitro (data not shown). The identification and systematic mutagenesis of the phosphorylated amino acid(s) in UBF1 and UBF2 should allow us to define the mechanism(s) by which the phosphorylation of UBF modulates its role in transactivation of RNA polymerase I.

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