

Competitive Recruitment of CBP and Rb-HDAC Regulates UBF Acetylation and Ribosomal Transcription

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Summary

RNA polymerase I (Poll) transcription is activated by the HMG box architectural factor UBF, which loops approximately 140 bp of DNA into the enhancosome, necessitating major chromatin remodeling. Here we show that the acetyltransferase CBP is recruited to and acetylates UBF both in vitro and in vivo. CBP activates Poll transcription in vivo through its acetyltransferase domain and acetylation of UBF facilitates transcription derepression and activation in vitro. CBP activation and Rb suppression of ribosomal transcription by recruitment to UBF are mutually exclusive, regulating in vivo Poll transcription through an acetylation-deacetylation “flip-flop.” Thus, Poll transcription is regulated by protein acetylation, and the competitive recruitment of CBP and Rb.

Introduction

The level of ribosomal gene transcription has been shown to be finely regulated in response to changes in cell growth rate and the state of differentiation. This regulation is believed, at least in part, to be due to a change in the number of actively transcribed genes (Moss and Stefanovsky, 1995; Paule, 1998). Transcription of the ribosomal genes by RNA polymerase I (Poll) is activated both in vitro and in vivo by the architectural upstream binding factor (UBF) (Grummt, 1998; Hannan et al., 1998b; Zomerdijk and Tjian, 1998). The recruitment of this protein to the Poll promoter is in fact the first step in ribosomal gene activation, permitting the subsequent association of the TATA-binding protein (TBP)-containing complex SL-1, and hence of the polymerase (Moss and Stefanovsky, 1995; Paule, 1998). UBF con-

tains multiple tandem homologies to the DNA binding domain of high mobility group 1 (HMG-1), the HMG box (Figure 1C), and loops approximately 140 bp of ribosomal DNA into a single turn, a structure we have called the ribosomal *enhancosome* (Bazett-Jones et al., 1994; Stefanovsky et al., 1996). Data on the promotion of Poll transcription in vertebrates are compatible with the formation of two precisely juxtaposed enhancosomes on the Poll promoter as a prerequisite to promoter recognition by SL-1 (Moss et al., 1998). Mammalian and *Xenopus* UBFs have recently been shown to be functionally interchangeable for this task in vivo (Hannan et al., 1999). However, enhancosome formation is clearly incompatible with the nucleosomal chromatin structure of the inactive genes (Lucchini and Sogo, 1998; Moss et al., 1998). The transition from the inactive to active ribosomal gene state, therefore, requires the replacement of one or more nucleosomes with enhancosomes. Chromatin remodeling has been shown to be facilitated by the recruitment of co-activators with acetyltransferase activity (Shikama et al., 1997; Wolffe and Hayes, 1999; Kouzarides, 2000). Further, the HMG box of *Drosophila* TCF/LEF was recently shown to functionally recruit the histone acetyltransferase (HAT) CREB-binding protein (CBP) (Waltzer and Bienz, 1998). We therefore studied the potential of this molecule to activate ribosomal transcription by Poll.

Results and Discussion

CBP and UBF Interact

Glutathione S-transferase (GST) pulldown assays (see Experimental Procedures) showed that an interaction occurred in vitro between bacterially expressed *Xenopus* UBF (xUBF) and the GST-fused interaction domain 2 of mouse CBP (CBP2); while no interaction was detected with interaction domain 1 (CBP1) (Figures 1A and 1B). Similarly, xUBF was found to bind to *Drosophila* CBP2 (data not shown). The site of interaction between CBP2 and xUBF mapped to HMG-1 boxes 1 and 2 (Figures 1C and 1D). CBP2 showing no detectable affinity for either HMG box 3 or for the whole C-terminal half of xUBF (box345+tail). Neither the dimerization of HMG box 1 via the N-terminal domain of xUBF (Nbox1) nor the combination of HMG boxes 1 and 2 (Nbox12) notably enhanced CBP2 binding, suggesting that these HMG boxes constituted two independent binding targets. Full-length xUBF, however, was observed to be a significantly better binding substrate than any of its subdomains (cf. Figures 1B and 1D), suggesting a certain degree of cooperativity in the interaction between CBP2 and UBF. When HA-tagged CBP was expressed in NIH3T3 cells and subsequently immunoprecipitated (see Experimental Procedures) from nuclear protein extracts (Figure 1E), both splice forms of the endogenous mouse UBF were co-immunoprecipitated. This interaction was completely specific; when HA-tagged extracellular signal-regulated kinase 1 was expressed in these cells, UBF was not co-immunoprecipitated (data not

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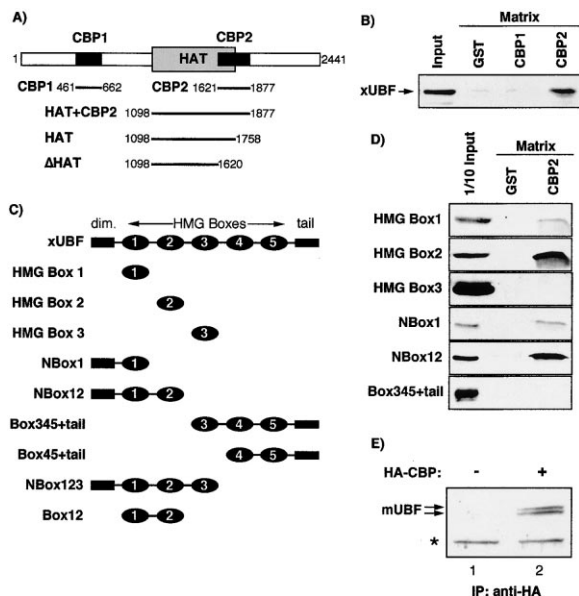


Figure 1. CBP Interacts with UBF In Vitro and In Vivo

(A) Schematic representation of mouse CBP and the mutant constructions used in this study. CBP1 (aa 461–662) and CBP2 (aa 1621–1877) have been described previously (Bannister and Kouzarides, 1995), as have ΔHAT (aa 1098–1620), HAT (aa 1098–1758) and HAT-CBP2 (aa 1098–1877) (Bannister and Kouzarides, 1996).

(B) GST pull-down assays with full-length xUBF. Bacterially produced 6HISxUBF retained on GST-CBP1 (CBP1; aa 461–661), GST-CBP2 (CBP2; aa 1620–1877), or GST was visualized on Western blot using an anti-xUBF antibody.

(C) Schematic representation of xUBF and the mutants constructs used in this study (Guimond and Moss, 1992; Leblanc et al., 1993; Stefanovsky et al., 1996; see Experimental Procedures).

(D) Mapping of the xUBF-CBP interaction by GST pull-down. xUBF subdomains retained on GST-CBP2 (CBP2) were visualized by Western blot using the anti-xUBF antibody. In each case, approximately the same amount of each xUBF mutant was applied to the binding matrix. Hence, the variation in band intensities reflects the differential affinity of the polyclonal antibody for the various subdomains, rather than the actual protein input.

(E) Endogenous mouse UBF (mUBF) co-immunoprecipitates with CBP. Nuclear extracts from NIH3T3 cells transfected (see Experimental Procedures) with pCDNA3 (–, lane 1) or pCMV-2N3T-CBP (Ramirez et al., 1997) (HA-CBP) (+, lane 2) were immunoprecipitated with anti-HA antibody. mUBF was visualized on Western blot using an anti-rat UBF antibody (Hannan et al., 1996). The doublet band is due to the two splice forms of mUBF, which are also known to heterodimerize (Hannan et al., 1999). The band identified by an asterisk represents crossreaction with a mouse protein present in the nuclear extracts and acts as a convenient control for protein loading.

shown). Thus, the endogenous UBF was available to interact with CBP in vivo. Along with the CBP regulation of *Drosophila* TCF (Waltzer and Bienz, 1998), our data suggest that CBP recruitment could be a common property of HMG box DNA binding domains.

UBF Is a Substrate for the CBP-HAT Domain and Is Acetylated In Vivo

The N-terminal half of UBF (Nbox123) containing the CBP2 binding site was initially tested as substrate for acetylation by CBP (Grant et al., 1997; see Experimental Procedures). The HAT domain of CBP, with or without

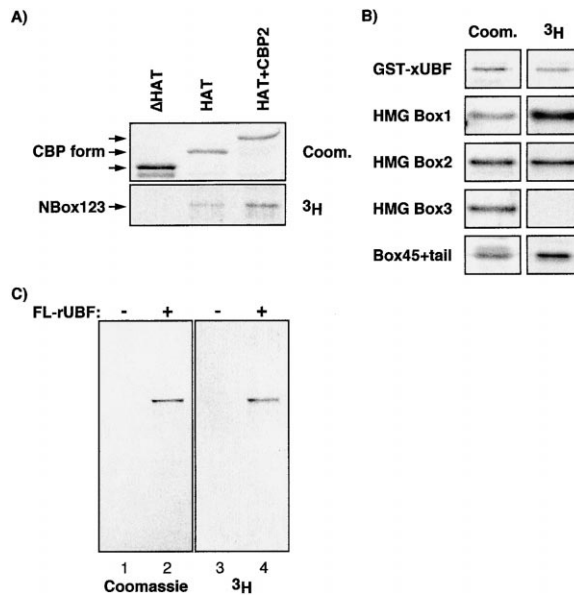


Figure 2. UBF Is Acetylated Both In Vitro and In Vivo

(A) The N-terminal half of xUBF (Nbox123; Figure 1C) was used as substrate for the CBP truncation mutants ΔHAT, HAT, and HAT+CBP2 (Figure 1A). Acetylation was monitored by ³H fluorography (³H). The Coomassie (Coom.) gel analysis of the recombinant CBP proteins is also shown.

(B) Mapping of the CBP acetylation sites in xUBF. Full-length xUBF and its subdomains were acetylated with HAT in vitro. Both the Coomassie-stained gel and the ³H of the same gel are shown.

(C) UBF is acetylated in vivo. NIH3T3 cells were transfected with pCDNA3 (–, lane 1 and 3) or pCMV-FLAG-rUBF1 (Hannan et al., 1996) (+, lane 2 and 4) and labeled with [³H] Na acetate (see Experimental Procedures). FLAG-rUBF1 was recovered from nuclear extracts by affinity chromatography on anti-FLAG M2-agarose (Sigma), and analyzed by SDS-PAGE (Coomassie) and by fluorography (³H). The single band seen on the Coomassie-stained and the fluorographed gel (lanes 2 and 4) was further shown to correspond to rUBF1 on Western blot using an rUBF1 specific antibody and by MALDI-ToF analysis (data not shown).

the adjacent CBP2 interaction domain (see Figure 1A), efficiently acetylated Nbox123 in vitro (Figure 2A). As expected, the noncatalytic deletion mutant ΔHAT was unable to acetylate the same substrate. Full-length xUBF was also an in vitro substrate for acetylation by the HAT domain of CBP (Figure 2B). In vitro acetylation of the individual HMG boxes paralleled the interaction assays. HMG box 1 and HMG box 2, both of which interacted with CBP2, were acetylated, but HMG box 3 was not. The C-terminal region of xUBF (box45+tail) was also acetylated at one or more sites. Since the last 62 aa of the acidic tail did not contain a lysine residue, it could be concluded that the C-terminal acetylation sites also lay within HMG boxes or their immediate flanking sequences. Rat UBF (rUBF) was found to be acetylated in NIH3T3 cells to a similar degree as that observed in vitro for xUBF (Figure 2C). Using matrix-assisted laser desorption/ionization–time of flight (MALDI-ToF) analysis of tryptic peptides on a PerSeptives Biosystems Voyager Elite (Borealis Biosciences, Inc.), eight potential internal acetylations have been identified on in vivo acetylated rUBF (G. Pelletier, unpublished data).

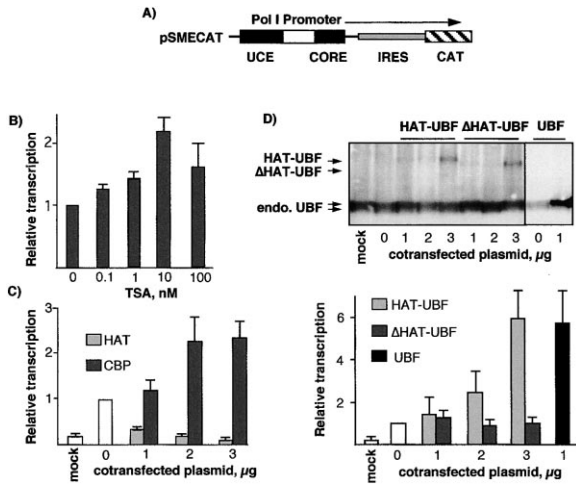


Figure 3. A Targeted Acetylation Activates Ribosomal Transcription
(A) Schematic representation of the internal ribosome entry site-CAT reporter plasmid pSMECAT, which is driven by the mouse RNA polymerase I promoter (Hannan et al., 1996; Hannan et al., 1999; see Experimental Procedures).
(B) NIH3T3 cells were transfected with pSMECAT and then treated for 16 hr with the indicated concentrations of TSA. Levels of conversion of chloramphenicol were normalized to those in the absence of TSA.
(C) NIH3T3 were cotransfected with pSMECAT and increasing amounts of pCMV-CBP-HA (CBP) or pCDNA3-HAT (HAT; Figure 1A; Lundblad et al., 1995).
(D) NIH3T3 cells were also cotransfected with pSMECAT and increasing amounts of the HAT- or ΔHAT-UBF (Figure 1A) fusion plasmids pCDNA3-HAT-rUBF1 and pCDNA3-ΔHAT-rUBF1. The upper panel shows the expression of the rUBF fusion proteins and the endogenous mUBF detected on a Western blot of nuclear protein using the rUBF antibody. This panel also shows a shorter exposure of the same Western blot indicating the relative level of exogenous rUBF expression required to activate transcription. The lower panel shows transcription of the pSMECAT reporter. The results from the CAT assays were also verified by selective S1 mapping of the Poll transcripts from pSMECAT (Hannan et al., 1999; data not shown). pCDNA3-HAT-rUBF1 and pCDNA3-ΔHAT-rUBF1 were constructed by fusing the coding region for aa 1098–1758 or aa 1098–1620, respectively, of mouse CBP upstream of the full-length rUBF1 open reading frame contained in pCDNA3-FLAG-rUBF1 (Hannan et al., 1996). The data in (B), (C), and (D) represent the means and SDs of four or more independent experiments.

Targeted Acetylation Activates Poll Transcription In Vivo

Inhibition of protein deacetylation with Trichostatin A (TSA) induced approximately 2.5 times activation of transcription from the Poll-driven reporter construct (pSMECAT [Hannan et al., 1996; Hannan et al., 1999]) in NIH3T3 cells (Figures 3A and 3B). This level of activation is typical of Poll transcription regulation. It is similar to that induced by the overexpression of UBF (two to seven times) (Hannan et al., 1996; Hannan et al., 1999) and to effects of amino acid starvation and growth factors on endogenous Poll transcription (2- to 4-fold), (see, e.g., Grummt et al., 1976; Larson et al., 1991; Hannan et al., 1998a). Maximal activation of Poll transcription occurred by 10 nM TSA, a concentration about 30 times lower than that typically required to activate RNA polymerase II genes (Brehm et al., 1998). Poll transcription was therefore particularly sensitive to the level of endog-

enous protein acetylation. In vivo acetylation of FLAG-tagged rUBF was only slightly increased (12%) by treatment of cells with TSA (data not shown), suggesting that if UBF acetylation was implicated in transcription activation, it was the result of the modification of specific sites rather than of a bulk change in the level of UBF acetylation. Expression of full-length CBP activated Poll transcription to a similar degree as TSA (2.5 times) (Figure 3C). On the other hand, expression of the HAT domain of CBP alone did not activate Poll transcription, and actually repressed it somewhat (Figure 3C). Together these data suggested that protein acetylation is necessary, but insufficient, for activation and that the CBP acetyltransferase activity needs to be correctly targeted. If, as we suspected, this targeting was to UBF, covalent tethering of the CBP-HAT domain to UBF should also activate Poll transcription. The coding regions for the catalytically active HAT domain and an inactive truncation mutant (ΔHAT) (Figure 1A) were separately fused with the full-length rat UBF (rUBF) coding region. Expression of the HAT-rUBF chimera was observed to activate Poll transcription up to six times, which is better than that achieved with CBP alone; while the ΔHAT-rUBF had no effect at all on transcription (Figure 3D, lower panel). Overexpression of rUBF has been shown to activate Poll transcription, but this effect requires a severalfold increase in the concentration of nuclear UBF (Hannan et al., 1999; see Figure 3D). Neither expression of the HAT-rUBF nor of the ΔHAT-rUBF chimera significantly affected the nuclear concentration of UBF (Figure 3D, upper panel), clearly attesting to the potency of targeting a limited level of HAT activity exclusively to UBF. It was concluded that recruitment of the HAT domain of CBP to UBF was sufficient for Poll transcription activation in vivo, and this activation was dependent on the acetyltransferase activity of CBP.

CBP Competes with Rb for the Same Site on UBF

The retinoblastoma protein (Rb) has been reported to suppress ribosomal transcription in vitro via a direct interaction with UBF (Cavanaugh et al., 1995; Voit et al., 1997). An interaction has been identified in vitro between Rb aa 379–928 and the HMG boxes 1 and/or 2 of mUBF (Voit et al., 1997). Other data further suggest that the Rb “pocket” (aa 379–792) is sufficient for transcriptional suppression in vitro and for the Rb-UBF interaction in vivo (Cavanaugh et al., 1995; Hannan et al., 2000). Since HMG boxes 1 and 2 also bound CBP, it was possible that Rb and CBP binding to UBF were exclusive events. If this were the case, suppression by Rb and activation by CBP would also be exclusive. However, the significance of an Rb-UBF interaction has not yet been demonstrated in vivo.

We first showed that Rb suppressed ribosomal transcription in vivo (Figure 4A). Consistent with a role for the Rb pocket in this suppression, we also found that the Rb-related pocket protein p107 was equally effective in this suppression (data not presented). We next determined if the pocket domain of Rb (aa 379–792) could bind HMG boxes 1 and/or 2 of xUBF. As demonstrated in Figure 4B, Rb(379–792) bound a polypeptide containing HMG boxes 1 and 2, but did not bind to sequences C-terminal of HMG box 2. Thus, in agreement with Cavanaugh

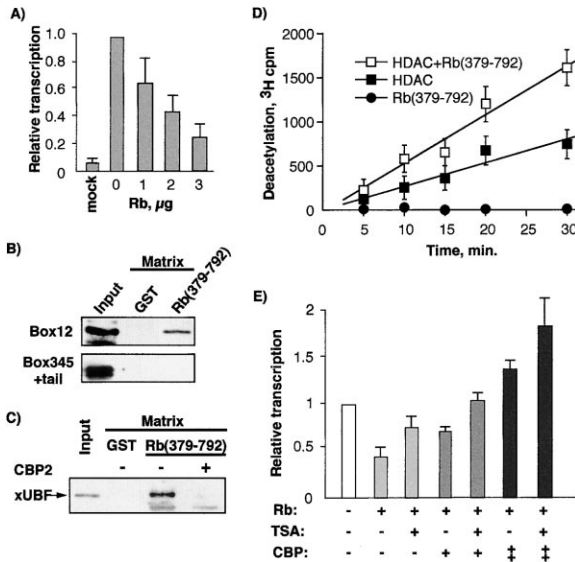


Figure 4. Rb and CBP Competitively Regulate Ribosomal Transcription

(A) Rb inhibits ribosomal transcription in vivo. NIH3T3 cells were cotransfected with the Poll reporter pSMCAT (Figure 3A) and increasing amounts of pCMV-Rb [Rb(aa 1-928); Qin et al., 1992]. (B) GST pulldown assay of xUBF subdomains (Figure 1C) retained on GST-Rb(379-792) (Kaelin et al., 1991). Visualization was by Western blot using an anti-xUBF antibody. (C) Binding of CBP and Rb to xUBF are mutually exclusive. 6HIS-xUBF was preincubated with recombinant CBP2 (Figure 1A) or binding buffer only before being applied to immobilized GST-Rb(379-792) (see Experimental Procedures). A 5-fold M excess of CBP2 over Rb(379-792) was used. Detection was as in (B). (D) Rb increases the rate of deacetylation of xUBF by HDAC1. Immobilized GST-xUBF was acetylated in vitro with the CBP-HAT domain (Figure 1A) and then used as substrate for deacetylation by baculovirus-expressed HDAC1 (Hassig et al., 1997; see Experimental Procedures) in the presence or absence of bacterially expressed Rb (aa 379-792). Aliquots of the reaction supernatants were taken at the times indicated and the released [³H]-acetate determined. The data are the average of three independent assays. (E) Addition of TSA in combination with CBP rescues Poll transcription from suppression by Rb. NIH3T3 cells were cotransfected as in (A) with or without 1.5 μg of pCMV-Rb and with the addition of 1 μg (+) or 2 μg (++) of pCMV-CBP-HA. Where indicated, (+) cells were treated for 16 hr with 10 nM TSA. The data in (A) and (E) represent the means and SDs of four or more independent experiments.

et al. (1995), the pocket region of Rb was sufficient to bind xUBF. We also found that, consistent with Voit et al. (1997), the individual HMG boxes 1 and 2 bound the Rb pocket region less efficiently than did the box12 combination (data not shown). Rb and CBP were then placed in competition for xUBF. Preincubation of full-length xUBF with the interaction domain CBP2 was sufficient to inhibit subsequent binding to Rb(379-792) (Figure 4C). Conversely, pre-incubation of xUBF with Rb(379-792) inhibited its subsequent binding to CBP2 (data not shown). These data strongly suggest that suppression of Poll transcription by Rb results, at least in part, from its capacity to interfere with the recruitment of CBP to UBF (see below). However, in other systems, Rb has also been shown to suppress transcription by the recruitment of histone deacetylase 1 (HDAC1) (Brehm et

al., 1998; Magnaghi-Jaulin et al., 1998). Hence, Rb could also potentially reverse the catalytic effects of recruiting CBP to UBF. We therefore asked if HDAC1 could deacetylate xUBF acetylated with the CBP-HAT domain, and if so, whether the presence of Rb would enhance this deacetylation. The rate of deacetylation of xUBF by HDAC1 in the presence of Rb(379-792) was nearly two times more rapid than in its absence (Figure 4D). Suppression of Poll transcription in vivo by Rb (and p107; data not shown) was also found to be at least partly relieved by inhibiting deacetylation with TSA (Figure 4E). The fact that we were unable to completely reverse Rb suppression with TSA alone was consistent with Rb also preventing CBP recruitment to UBF. In fact, we found that the Rb-induced suppression of Poll transcription could be completely relieved, and indeed reversed, by the coexpression of CBP in combination with TSA treatment.

UBF Acetylation Facilitates Poll Transcription In Vitro

The recruitment of CBP to UBF could activate transcription (1) by the acetylation of UBF, (2) by the acetylation of local chromatin, (3) by displacing Rb, or (4) by a combination of these effects. The fact that Rb could cooperate in the deacetylation of UBF (Figure 4D) suggested that acetylated UBF could effectively bind Rb and this was confirmed in pulldown experiments similar to those in Figures 4B and 4C (data not shown). We also investigated the role of CBP, Rb, and acetylation in DNA binding by UBF. Neither an excess of CBP2 nor saturation acetylation of UBF with the HAT domain of CBP had any detectable effect on its capacity to bind the ribosomal promoter DNA as determined by footprinting (Figure 5B). We also found that Rb(379-928), [for the pocket domain Rb(379-792) or GST-Rb(379-928); data not shown], had no effect on DNA binding by UBF (Figure 5B). This latter finding contrasts with the data of Voit et al. (1997) but is fully consistent with the data of Cavanaugh et al. (1995) and with the more recent footprinting data of Hannan et al. (2000). The reasons for this inconsistency in the published data are still unclear, but could possibly result from the use of an unnatural UBF binding site, that is, cruciform DNA, in the experiments of Voit et al. (1997).

Since the DNA binding of UBF was unaffected by CBP or Rb binding or by acetylation, we next asked if acetylated UBF was necessary for transcription activation in vitro. Bacterially produced UBF, which is necessarily unacetylated, has been found in many laboratories to be refractory for in vitro transcription, (see, e.g., Voit et al., 1995). However, UBF produced in mammalian and insect cells or by in vitro translation has been found to be functional (see, e.g., McStay et al., 1991; Jantzen et al., 1992; Voit et al. 1999). This suggests that post-transcriptional modification of UBF may be important, and indeed this has been shown to be the case for UBF phosphorylation (O'Mahony et al., 1992; Voit et al., 1992; Voit et al., 1995; Kihm et al., 1998; Tuan et al., 1999; Voit et al., 1999). Rat and mouse nuclear extracts were therefore depleted of endogenous UBF and used to study the capacity of bacterially expressed (i.e., unacetylated) UBF to activate transcription from the rat or mouse Poll promoters (Grummt, 1982; Cassidy et al.,

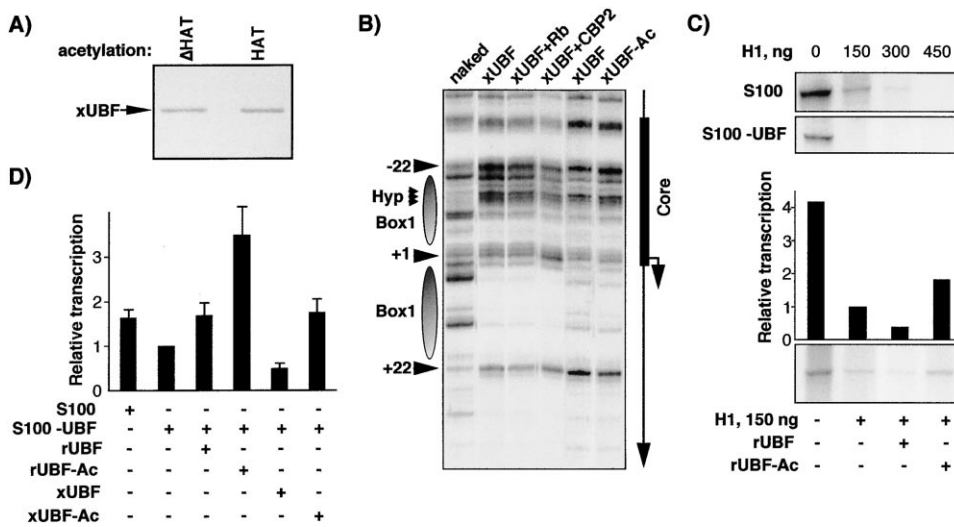


Figure 5. Acetylation of UBF Does not Change Its DNA Binding Properties but Facilitates Transcription Activation and Derepression In Vitro (A) Typical example of the PAGE analysis of UBF after acetylation and mock acetylation reactions. (B) Footprint analysis of xUBF bound to the *Xenopus* core promoter region. The previously mapped positions of the HMG box 1 domains of a xUBF dimer are indicated (Box1), as are the characteristic sites of hypersensitivity (Hyp) due to foldback of the C-terminal domain of full-length UBF (Leblanc et al., 1993; Bazett-Jones et al., 1994). Rb(379–928) and CBP2 were added, where indicated, in a 2.5-times M excess over xUBF. (C) The upper panel shows titrations of in vitro transcription from the rat Poll promoter with the repressor histone H1 in rat S100 undepleted or UBF depleted extracts (S100-UBF). The histogram and lower panel shows the quantitation of transcription before and after additions of H1 and of bacterially expressed rUBF, either acetylated (rUBF-Ac) or unacetylated. One hundred and fifty nanograms of H1 was added to the depleted extract and 100 ng of the modified or unmodified rUBF. (D) Quantitation of in vitro transcription from the mouse Poll promoter in mouse undepleted S100 or depleted S100 (S100-UBF) extracts. Where indicated, reactions contained 75 ng of acetylated (rUBF-Ac) or unacetylated bacterial rUBF, or 150 ng of acetylated (xUBF-Ac) or unacetylated bacterial xUBF. The data are the means of three independent assays.

1986). Bacterially produced rUBF and xUBF were either acetylated with matrix-immobilized active CBP HAT domain or mock acetylated (unacetylated) with the immobilized inactive ΔHAT domain and then the HAT protein removed by centrifugation (Figure 5A). UBF has been shown both to derepress Poll transcription in vitro as well as to activate it (Grummt, 1998; Zomerdijk and Tjian, 1998). We first investigated the derepression properties of rUBF in the rat extract in competition with added histone H1 (Figure 5C). As expected, addition of H1 to the rat extract repressed transcription of the rat promoter (Kuhn and Grummt, 1992), and this repression was even more pronounced after UBF depletion. Addition of unacetylated rUBF did not relieve H1 repression, and even increased it somewhat. On the other hand the acetylated rUBF relieved H1 repression and gave about a 2-fold increase in transcription (or more than 4-fold the level observed in the presence of the same amount of unacetylated UBF). The capacity of UBF to activate transcription from the mouse promoter was also tested in a UBF-depleted mouse nuclear extract (Figure 5D). Here the unacetylated rUBF gave a small degree of transcription activation (1.7 times), but the acetylated rUBF activated much more effectively (3.5 times). *Xenopus* UBF has been shown to activate the mouse promoter in vivo, although it has also been shown to be ineffective in activating the rat or human promoters in vitro (Bell et al., 1989; Pikaard et al., 1990). Figure 5D shows that bacterial unacetylated xUBF has a clear repressive (0.5 times) effect on the mouse promoter in vitro, but after

acetylation, this repression was completely relieved and transcription was somewhat activated (1.8 times).

Our data strongly support a “flip-flop” model for the regulation of ribosomal transcription by CBP and Rb-HDAC1 (Figure 6). (The term “flip-flop” is used to describe a system with two alternative semistable states, here CBP-bound or Rb-bound UBF.) The formation of a UBF-CBP complex activates transcription by acetylation of UBF itself, and perhaps also by opening up the adjacent ribosomal chromatin, allowing further UBF ingress and gene activation. Excess Rb prevents formation of a UBF-CBP complex and, by recruiting HDAC1, catalyses UBF deacetylation and hence its inactivation. We show that acetylation of UBF significantly enhances its ability to activate Poll transcription in vitro. Although we have excluded changes in DNA binding and the ability of UBF to bind Rb, the mechanism by which UBF acetylation functions remains unknown. One

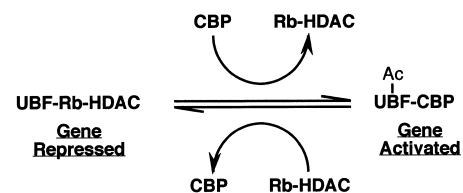


Figure 6. A Schematic Diagram of the “Flip-Flop” Regulation of Ribosomal Genes by CBP and Rb. Ac, acetylation. See text for discussion.

possible explanation that we are actively pursuing is that acetylation induces a structural change in UBF. Quite possibly Rb recruitment to UBF has roles other than just to promote UBF deacetylation. It has recently been shown that Rb can inhibit SL-1 recruitment to UBF (Hannan et al. 2000). Rb may also cooperate in deacetylation of adjacent histones. As we have noted previously (Moss et al., 1998), enhancer structure, with its single 140 bp loop of DNA, could accommodate the core histones in a weak association with the DNA. On the other hand, xUBF can also associate stably with nucleosomes (Moss et al., 1998). Thus, the CBP/Rb flip-flop could catalyze the transition between a predominantly nucleosomal and a predominantly enhancer gene state, the transition not necessarily requiring complete displacement of either core histones or UBF. It has in fact been observed that the core histones remain associated with the active ribosomal genes, but only via their N-terminal domains (Dimitrov et al., 1990). Whether a nucleosome-enhancer transition is facilitated by UBF acetylation, histone acetylation, or a combination of the two must now be determined.

Recent data suggest that both acetylation and phosphorylation can cooperate to activate transcription *in vivo* (Dumaz and Meek, 1999; Cheung et al., 2000; Lo et al., 2000). UBF is known to be multiply phosphorylated, mainly within the C-terminal acidic domain but also in HMG box 5 (O'Mahony et al., 1992; Voit et al., 1992; Voit et al., 1995; Kihm et al., 1998; Tuan et al., 1999; Voit et al., 1999). Each of these modifications has been shown to activate transcription *in vitro* and, in the case of the acidic domain, phosphorylation was shown to enhance recruitment of SL-1 (Kihm et al., 1998; Tuan et al., 1999). Here we have shown that acetylation is also important for UBF function. In future work we will attempt to test whether a functional link exists between the phosphorylation and the acetylation of UBF.

Experimental Procedures

UBF Constructions

6HISxUBF was produced by subcloning aa 16–677 of xUBF2b into pET-15b (Novagen). HMG box 1 (aa 110–188), HMG box 2 (aa 194–266), HMG box 3 (aa 292–364), and box12 (aa 110–266) were subcloned from xUBF2b into pGEX2T (Amersham Pharmacia Biotech), while box345+tail (aa 292–677) and box45+tail (aa 392–677) were subcloned into pGEX4T3 (Amersham Pharmacia Biotech).

GST Pulldown Assays

GST pulldown assays were performed in binding buffer (50 mM Tris-HCl [pH. 7.9], 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid [EDTA], 20% glycerol). Fifty to one hundred nanograms of xUBF or the relevant subdomain was applied to 0.5 μ g of immobilized protein partner. Bound protein was analyzed by Western blot using a polyclonal anti-xUBF antibody. Competitive binding experiments were performed in the same way, except that UBF was preincubated for 15 min at 4°C with thrombin-released CBP2 [or Rb(379–792)] prior to loading onto immobilized GST-Rb(379–792) (or GST-CBP2). A 5-fold M excess of competitor over immobilized binding protein was used in these experiments. GST-fusion proteins were expressed and purified on G-Sepharose 4B (Amersham-Pharmacia Biotech) according to the manufacturer's protocol, and were eluted after digestion with thrombin (Sigma) where necessary. Bacterial extracts containing 6HISxUBF were applied to diethylaminoethyl (DEAE)-Sephacel (Amersham-Pharmacia Biotech) and washed in 0.07 M TM buffer (50 mM Tris-HCl [pH. 7.9], 1.5 mM MgCl₂, 1 mM EDTA, 20% glycerol, 0.07 M KCl) before elution in 0.22 M TM Buffer. The DEAE

eluate was then affinity purified on Ni-NTA (Qiagen) according to the manufacturer's nondenaturing protocol. All buffers were supplemented with 1 mM dithiothreitol (DTT), 0.1 mM phenyl-methyl-sulfonyl fluoride (PMSF), 1 mg/ml leupeptin, and 1 mg/ml pepstatin (Roche Biochemicals).

In Vitro Acetylation Assays

Acetylation assays (Grant et al., 1997) were performed, unless otherwise stated, using the G-Sepharose-bound HAT domain of CBP (aa 1098–1758) and 100–200 ng of substrate protein. Reactions were fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie brilliant blue R250, treated with En³Hance (Dupont), dried, and visualized by fluorography. GST-Nbox123, used in the deacetylation assays, was acetylated using the thrombin-released HAT domain from bacterially produced GST-HAT. For transcription and footprinting reactions, acetylation of UBFs was performed in the absence of [³H]acetyl-coenzyme A (CoA) and the concentration of unlabeled acetyl-CoA was increased from 2 to 20 μ M.

Coimmunoprecipitation

Twenty-four hours post-transfection, nuclei were prepared as follows: All buffers were supplemented with 1 mM DTT, 0.1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml pepstatin (Roche Molecular Biochemicals). Cells were washed in phosphate-buffered saline and then disrupted in homogenization buffer (10 mM Tris.HCl [pH 7.9], 0.3 M sucrose, 3 mM CaCl₂, 2 mM Mg Acet.₂, 0.1% NP-40, 0.1% Triton X-100) by repeated micropipetting. Nuclei were recovered by microcentrifugation at 14,000 \times g, for 30 s at room temperature, resuspended in ice-cold 0.42 M TM, and sonicated for 30 s using the microtip at 70% maximal intensity on a Branson cell disruptor. The nuclear extract was centrifuged at 30,000 rpm for 30 min at 4°C in an SW-50.1 rotor (Beckman). The concentration of KCl in the supernatant was reduced from 420 mM to 75 mM by serial dilution. The extract was precleared for 2 hr at 4°C with 50 μ l of protein A-Sepharose (Amersham Pharmacia Biotech) under agitation and then incubated with 10 μ l of anti-HA ascites (12CA5) for 1 hr. 15 μ l of protein A-Sepharose was added and incubation allowed for another hr. The resin was then extensively washed with ice-cold NETN +10% glycerol (20 mM Tris-HCl [pH 7.9], 100 mM NaCl, 1 mM NaEDTA, 0.5% NP-40, 10% glycerol). An equal volume of 2X SDS-PAGE loading buffer was added to the resin and the samples were resolved by 8% SDS-PAGE. Western blotting was performed using Hybond-C (Amersham-Pharmacia Biotech); mUBF and rUBF fusion proteins were detected using a polyclonal anti-rUBF antibody and revealed by HRP chemiluminescence (ECL).

Cell Transfection

Mouse NIH3T3 cells were grown in Dulbecco's Modified Eagle Medium high glucose (Gibco-BRL) complemented with 10% fetal bovine serum (Wisent) and antibiotics-antimycotics (Gibco-BRL) in a humidified incubator at 37°C and 5% CO₂. Cells were transfected using Lipofectamine (Gibco BRL) according to the manufacturer's protocol. DNA concentrations were maintained constant in cotransfection experiments by addition of the empty pCDNA3 vector.

In Vivo Labeling

Forty-eight hour post-transfection, cells were incubated for 2 hr in 1 mCi/ml of [³H] sodium acetate, specific activity 8 Ci/mmol (Amersham Pharmacia Biotech). Nuclear extracts were made as described for the co-immunoprecipitations, except that all buffers contained 5 mM sodium butyrate. The nuclear extract was incubated with 10 μ l M2-anti-FLAG affinity resin (Sigma) and eluted with FLAG peptide (Sigma), according to the manufacturer's protocol. Eluates were analyzed by SDS-PAGE as described for the acetylation assays.

Deacetylase Assays

Immobilized acetylated GST-Nbox123 was extensively washed with binding buffer + 0.5% NP-40, before equilibration in the deacetylation buffer (35 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol). Homogeneous affinity-purified baculovirus-expressed HDAC1 was added to a total reaction volume of 120 μ l in deacetylase buffer and the reaction incubated at 37°C. One eighth of the initial supernatant

volume was removed at the indicated times, and the liberated [³H] acetate was quantified by liquid scintillation counting. The total released cpm were then estimated at each time point. Deacetylation assays were also performed in the same manner after addition of either 200 ng of thrombin-released Rb(379–792) and HDAC1 or just 200 ng of Rb(379–792). Linear regressions were calculated by the least squares method.

Footprinting

Footprinting of homogeneous GST-xUBF on the *Xenopus laevis* ribosomal promoter was performed as previously described (Read et al., 1992).

In Vitro Transcription Assays

S100 nuclear extracts were prepared from rat N1S1 cells and mouse L1210 cells as previously described (Miller and Sollner-Webb, 1981). The extracts were partially depleted of UBF, between 80% and 90% being removed, using a polyclonal anti-rUBF antibody. Extracts were incubated at 4°C for 1 hr with the antibody followed by 30 min incubation with protein A-Sepharose (Amersham Pharmacia Biotech). The mouse Poll promoter fragment (–165 to +292; Sall to PvuII) cloned in pUC9 was linearized at +292 while the rat promoter (pU5.1; –286 to +638) (Cassidy et al., 1986) was linearized at +638. Transcription reactions were performed essentially as described in (Miller and Sollner-Webb (1981) in a final volume of 25 μl but containing, where indicated, 75 to 100 ng of recombinant GST-rUBF or 150 ng of recombinant GST-xUBF, either acetylated or mock acetylated, 7 μl of S100 or depleted S100 extract, and 100 ng of the rat or mouse template.

Acknowledgments

The authors wish to thank A. J. Bannister, D. Trouche, M. V. Govindan, J. K. Tong, and W. G. Kaelin for supplying the various expression vectors and for providing advice on their use. This work was supported in Canada by an operating grant from the Medical Research Council of Canada, an FCAR-FRSQ Santé scholarship to G. P., and an MRC-Canada Scientist award to T. M.; and in France by the Centre National de la Recherche Scientifique (CNRS) with grants from the Fondation pour la Recherche Médicale (FRM) and the Association pour la Recherche sur le Cancer (ARC). L. J. R. acknowledges support from the NIH, grant GM46991.

Received May 31, 2000; revised August 14, 2000.

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