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Xenopus NF-Y pre-sets chromatin to potentiate p300 and acetylation-responsive transcription from the *Xenopus hsp70* promoter *in vivo*

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We identify *Xenopus* NF-Y as a key regulator of acetylation responsiveness for the *Xenopus hsp70* promoter within chromatin assembled in *Xenopus* oocyte nuclei. Y-box sequences are required for the assembly of DNase I-hypersensitive sites in the *hsp70* promoter, and for transcriptional activation both by inhibitors of histone deacetylase and by the p300 acetyltransferase. The viral oncoprotein E1A interferes with both of these activation steps. We clone *Xenopus* NF-YA, NF-YB and NF-YC and establish that NF-Y is the predominant Y-box-binding protein in *Xenopus* oocyte nuclei. NF-Y interacts with p300 *in vivo* and is itself a target for acetylation by p300. Transcription from the *hsp70* promoter in chromatin can be enhanced further by heat shock factor. We suggest two steps in chromatin modification at the *Xenopus hsp70* promoter: first the binding of NF-Y to the Y-boxes to pre-set chromatin and second the recruitment of p300 to modulate transcriptional activity.

Keywords: acetylation/chromatin/*hsp70* promoter/NF-Y/p300/Y-box

Introduction

Eukaryotic transcription is regulated within a potentially repressive chromatin environment. Both architectural features of chromatin and the capacity to target the remodeling of nucleosomes allow the transcriptional machinery to function effectively. Many promoters are pre-set by their assembly into specific nucleoprotein complexes prior to transcriptional activation (Becker, 1994; Wallrath *et al*., 1994; Svaren and Horz, 1997). Transcription is then controlled by the subsequent recruitment or modification of specific transcription factors and coactivators at these regulatory structures (Fascher *et al*., 1990; P.B.Becker *et al*., 1991; Chrivia *et al*., 1993; Marcus *et al*., 1994; Kamei *et al*., 1996). Several transcriptional coactivators are histone acetyltransferases whose enzymatic activity may contribute to chromatin disruption and transcription (Brownell *et al*., 1996; Ogrzyko *et al*., 1996; Yang *et al*., 1996; Chen *et al*., 1997; Spencer *et al*., 1997). Many vertebrate transcription factors interact with the structurally related coactivator/acetyltransferases p300 and CBP (Chrivia *et al*., 1993; Chakravarti *et al*., 1996; Hanstein *et al*., 1996; Kamei *et al*., 1996; Smith *et al*., 1996; Chen *et al*., 1997; Nakajima *et al*., 1997; Puri *et al*., 1997a,b; Shikama *et al*., 1997; Yao *et al*., 1998). The acetyltransferase activity of CBP stimulates transcription in a model system (Martinez-Balbas *et al*., 1998). The association of targeted chromatin disruption with transcriptional activation is well documented in *Saccharomyces cerevisiae* (Almer and Horz, 1986; Almer *et al*., 1986; Straka and Horz, 1991; Svaren and Horz, 1997). The yeast coactivator GCN5p requires histone acetyltransferase activity to regulate transcription, and is known both to modify histones locally in the vicinity of the regulated promoter and to facilitate chromatin disruption (Candau *et al*., 1997; Gregory *et al*., 1998; Kuo *et al*., 1998; Wang *et al*., 1998). These observations suggest that the assembly and subsequent modification of chromatin on particular promoters has an essential role in transcriptional control.

In metazoans, the heat shock promoters of *Drosophila* have provided particularly useful paradigms both for the assembly of pre-set promoters and for investigating the role of chromatin structure in transcriptional control (Rougvie and Lis, 1988; Thomas and Elgin, 1988; Becker and Wu, 1992; Giardina *et al*., 1992; Lu *et al*., 1993; Tsukiyama *et al*., 1994; Varga-Weisz *et al*., 1995; Wall *et al*., 1995). These promoters exist in DNase I-hypersensitive sites prior to transcriptional activation (Wu *et al*., 1979). These sites contain bound transcription factors such as the GAGA factor, components of the basal transcriptional machinery and RNA polymerase (reviewed by Lis and Wu, 1993, 1994). Regulation of the *hsp26* promoter within chromatin requires the regulated activation and association of heat shock factor (HSF) at a pre-set chromatin structure which then increases the reinitiation rate of RNA polymerase (Sandaltzopoulos and Becker, 1998). The reinitiation of RNA polymerase might be facilitated by modification of the basal transcriptional machinery (O'Brien and Lis, 1991) or by modification of chromatin by acetylation (Nightingale *et al*., 1998).

We have suggested that a regulatory role for chromatin exists for the *Xenopus hsp70* promoter (Landsberger and Wolffe, 1995, 1997; Landsberger *et al*., 1995). Constitutive transcriptional activity of the *Xenopus hsp70* promoter in oocytes is dependent on a specialized CAAT box, subsequently defined as a Y-box (CTGATTGGC/TC/TAA) (Bienz, 1984a,b, 1986; Mantovani, 1998). Transcription factors such as NF-Y that selectively recognize the Y-box (Dorn *et al*., 1987; Ronchi *et al*., 1995) facilitate the formation of tissue-specific transcription complexes (Milos and Zaret, 1992; Wright *et al*., 1994). In the *Xenopus hsp70* promoter, the Y-box elements are important for disrupting local chromatin structure and for facilitating

the association of HSF and transcriptional activation in chromatin (Landsberger and Wolffe, 1995). In this work, we have examined the role of NF-Y in the control of *hsp70* promoter activity in chromatin. Our experiments focus on the chromatin organization of the promoter prior to transcriptional activation and on the role of acetyltransferases and deacetylases in the regulation of transcription.

Histone deacetylase functions to repress transcription in chromatin (Kadosh and Struhl, 1998; Wong *et al*., 1998). Inhibition of histone deacetylase using trichostatin A (TSA; Yoshida *et al*., 1995) activates transcription from diverse promoters (Almouzni *et al*., 1994; Wong *et al*., 1998). Remarkably, we find that the transcriptional activation of the *Xenopus hsp70* promoter in chromatin by TSA is dependent uniquely on Y-box elements. In addition, the Y-boxes are also essential for the assembly of a DNase I-hypersensitive site on the *hsp70* promoter. These observations led us to define NF-Y as the regulatory factor that binds to the Y-box in oocyte nuclei and the role of NF-Y-associated acetyltransferases and chromatin assembly in transcription. We find that NF-Y interacts with p300 *in vivo* and is itself an acetylation substrate. Our experiments indicate that NF-Y establishes a pre-set promoter architecture that can facilitate transcription within chromatin by recruiting the p300 acetyltransferase.

Results

The Y-boxes determine DNase I hypersensitivity and the acetylation response of the Xenopus hsp70 promoter

In earlier studies, we and others have defined the two Y-box elements in the *Xenopus hsp70* promoter (Figure 1A) as essential for both oocyte-specific and heat shock-regulated transcription within chromatin (Bienz, 1986; Landsberger *et al*., 1995). The presence of the Y-box elements prevents the assembly of a canonical nucleosomal array over the *Xenopus hsp70* promoter (Landsberger and Wolffe, 1995). We examined the DNase I cleavage of *hsp70* promoters assembled into chromatin following the microinjection of a small mass (0.75 ng) of *hsp70* promoter into oocyte nuclei followed by an overnight incubation at 18°C (Figure 1). The injection of this small amount of DNA facilitates the efficient assembly of chromatin (Almouzni and Wolffe, 1993; Landsberger and Wolffe, 1995). We used templates with both Y-boxes intact (Figure 1A, WT), with both Y-boxes mutagenized (Δ Y1/Y2; Landsberger *et al*., 1995), and with all the heat shock response elements (HSEs) and the distal Y-box deleted (Δ HSE; Landsberger *et al*., 1995). We find that while the WT construct is assembled into a DNase I-hypersensitive site (Figure 1B, lanes 1–11), deletion of both Y-boxes eliminates DNase I hypersensitivity (Figure 1B, lanes 12–19). Retention of the single proximal Y-box Y1 significantly reduces DNase I hypersensitivity over the promoter; however, some selectivity of DNase I cleavage around the proximal Y-box and the start site of transcription remains (Figure 1B, lanes 20–27). Incubation of *Xenopus* oocytes in concentrations of TSA sufficient to induce transcriptional activation (30 nM; Almouzni *et al*., 1994; Wong *et al*., 1998, see Figure 1C) and to inhibit *Xenopus* oocyte histone deacetylase (Jones *et al*., 1998; Wade *et al*., 1998) had no influence on DNase I cleavage patterns. Inhibition of deacetylase does not lead to a major reconfiguration of the nucleoprotein complexes assembled on the *hsp70* promoter (see also Wong *et al*., 1998). We conclude that the presence of Y-box elements is important to generate a DNase I-hypersensitive site on the *Xenopus hsp70* promoter in chromatin. Our next experiments examined the influence of TSA on transcription of the *hsp70* promoter in chromatin.

The presence of both Y-boxes in the *hsp70* promoter is important for basal transcriptional activity at room temperature (Figure 1C, compare lanes 1, 5 and 9). The presence of HSEs is important for transcriptional activation in response to heat shock (34°C) (Figure 1C, compare lanes 1 and 3 with lanes 9 and 11). The addition of TSA activates transcription to levels equivalent to heat shock itself on the wild-type *hsp70* promoter (Figure 1C, compare lanes 2 and 4). TSA has no significant effect on the transcriptional activity of the *hsp70* promoter lacking Y-boxes (Figure 1C, compare lanes 5–8). The presence of the single proximal Y-box in the absence of HSEs is sufficient to confer TSA responsiveness (Figure 1C, compare lane 9 with 10, and lane 11 with 12). We conclude that the Y-boxes are essential for conferring the responsiveness of the *Xenopus hsp70* promoter to the deacetylase inhibitor TSA. This requirement for a specific regulatory element is surprising due to the generally repressive actions of histone deacetylase, and the capacity of TSA to activate transcription of diverse promoters in oocytes (Almouzni *et al*., 1994; Jones *et al*., 1998; Wong *et al*., 1998). A potential explanation is that proteins bound to the Y-boxes have an essential and focused function in recruiting coactivators that have acetyltransferase activity to the *hsp70* promoter and that this recruitment leads to the same net consequence for the acetylation status of the chromatin assembled on the *hsp70* promoter as inhibiting deacetylase activity (see Figure 2H and I, and Discussion).

In earlier work, we found that transcriptional coactivators appear to be deficient in early *Xenopus* development (Almouzni and Wolffe, 1995). In preliminary experiments to address the nature of this deficiency, we screened oocyte mRNA by Northern analysis using probes for PCAF (Yang *et al*., 1996), SRC-1 (Onate *et al*., 1996), CBP and p300 (Ogrzyko *et al*., 1996). We detected abundant p300 mRNA in the oocyte as confirmed by partial sequencing, but did not easily detect the mRNAs of any other known coactivators (A.Imhof, Q.Li and A.P.Wolffe, in preparation). We detected low levels of endogenous p300 protein using specific monoclonal antibodies (Dallas *et al*., 1997) (Figure 1D, lane 1). The abundance of p300 could be significantly increased, typically by 4- to 5-fold, by the microinjection of synthetic mRNA encoding human p300 (Materials and methods; Figure 1D, lanes 1 and 2). We subsequently have confirmed the identity of the endogenous p300 and that derived from the translation of exogenous mRNA by biochemical fractionation and enrichment of endogenous oocyte p300 by chromatography using Mono Q and heparin agarose. The properties of this complex will be described elsewhere (Q.Li, P.L.Jones and A.P.Wolffe, in preparation). Our subsequent coactivator studies focus on the role of p300 in transcriptional control of the *hsp70* promoter.

Using the various *Xenopus hsp70* promoter constructs,

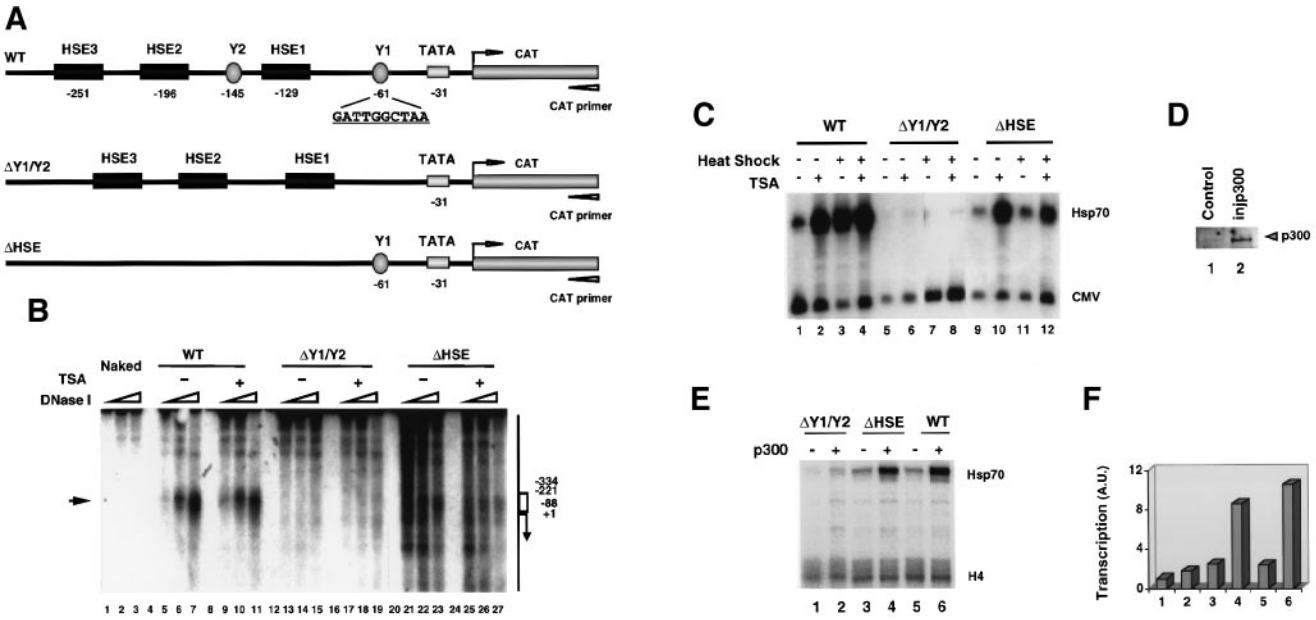


Fig. 1. The Y-box elements in the *Xenopus hsp70* promoter are essential for the assembly of a DNase I-hypersensitive site and for the activity of TSA and p300. (**A**) Schematic presentation of the wild-type (WT) and mutant types of the *Xenopus hsp70* promoter (ΔY1/Y2 and ΔHSE) used in the study. The CAT primer utilized for transcription analysis is shown. This was a 30mer, 3′-TACCTCTTTTTTTAGTGACCTATATGGTGG-5′, complementary to the CAT gene. A 167 nucleotide extension product is obtained from the *hsp70* promoter. The CMV promoter gives rise to an extension product that is 137 nucleotides long. (**B**) The DNase I-hypersensitive sites on the different promoters during TSA induction. Oocytes were microinjected with 0.75 ng of double-stranded DNA (see Materials and methods) and they were then incubated for 12 h in the absence or presence of 30 nM TSA. DNase I digestions were as described (Materials and methods). Lanes 1–3, DNase I digestions of naked promoter DNA are shown as a control; in lanes 4–11, the wild-type promoter is shown after chromatin assembly (WT); lanes 12–19, the Y-box elements are deleted (ΔY1/Y2); and in lanes 20–27, the HSE elements are deleted (ΔHSE). The solid hooked arrow denotes the transcription start site, defined as +1. The open box indicates the location of the *hsp70* regulatory elements and the TATA box. The solid horizontal arrowhead indicates the DNase I-hypersensitive site. On the right are the distances in base pairs relative to the start site of transcription (+1). (**C**) The response of different promoters to TSA treatment and heat shock induction. Oocytes were microinjected with 0.75 ng of double-stranded *hsp70* DNA and 0.25 ng of CMV CAT as a control. They were then incubated for 12 h in the presence or absence of 30 nM TSA. At this time, the incubation was continued either at 18°C or at the heat shock temperature of 34°C for a further 2 h as indicated. RNA transcription was analyzed by primer extension (Materials and methods). Lanes 1–4 show the wild-type promoter (WT), lanes 5–8 the Y-box element deletion promoter (ΔY1/Y2), and lanes 9–12 the HSE element deletion promoter (ΔHSE). The positions of *hsp70* and CMV transcripts are indicated. The experiment was repeated four times with a degree of reproducibility for relative transcription levels of ±5%. (**D**) p300 antibody detection of endogenous levels of p300 in the oocyte. Detection was using the monoclonal antibody NM9 (Dallas *et al*., 1997; the kind gift of Dr E.Moran, Fels Institute, Philadelphia). Lane 1, protein extract from non-injected oocytes, i.e. the endogenous p300 level; lane 2, protein extract from oocytes injected with human p300-WT mRNA (Ogryzko *et al*., 1996). After injection, oocytes were incubated overnight (16 h) before homogenization (Materials and methods). One oocyte equivalent was loaded per gel lane. (**E**) p300 activity on different *hsp70* promoters by primer extension. Lanes 1 and 2, the Y-box deletion promoter (ΔY1/Y2); lanes 3 and 4, the HSE element deletion promoter (ΔHSE); lanes 5 and 6, the wild-type promoter (WT). Double-stranded DNA (0.75 ng) as indicated was injected in each group of oocytes. The amount of p300-WT mRNA injected for the + group of oocytes was 2.5 fmol. The experiment was repeated four times with a degree of reproducibility for relative transcription levels of ±5%. (**F**) Quantitation of the experiment in (E) by PhosporImage analysis. The endogenous H4 signal is used as a loading control. The *hsp70* transcription signals from different promoters are plotted as fold induction relative to each control reaction [lanes 1, 3 and 5 in (E)].

we found that expression of exogenous p300 would potentiate transcription from the wild-type promoter (Figure 1E and F, lanes 5 and 6) and also from the promoter containing a single Y-box (Figure 1E and F, lanes 3 and 4). The *hsp70* promoter lacking both Y-boxes showed a much reduced response to p300 (Figure 1E and F, lanes 1 and 2). There is a strong correlation between the requirement for the Y-box elements to activate transcription in response to TSA and in the presence of p300 (Figure 1C and E). To understand the mechanistic basis of this phenomenon further, we examined the role of acetyltransferase activity in p300 function at the *hsp70* promoter. Functional domains of p300 that interact with transcription factors and other coactivators have been well documented (Figure 2A). We deleted a segment of the histone acetyltransferase domain from amino acid 1472 to 1522 to create p300-hm (for histone acetyltransferase mutant). This peptide segment contains amino acids conserved between all histone acetyltransferases (Neuwald and Landsman, 1997) and is essential for acetyltransferase activity (Martinez-Balbas *et al*., 1998; data not shown). We expressed both wild-type and p300-hm in *Xenopus* oocytes (Figure 2B, lanes 3 and 4). While the wild-type p300 stimulated transcription (Figure 2C and D, lanes 1, 2 and 3), the p300-hm was much reduced in activity (Figure 2C and D, lanes 1, 4 and 5). These results suggest that the acetyltransferase domain of p300 is important for transcriptional activation. A feature of p300- and CBP-regulated transcription is the inhibition by the viral oncoprotein E1A of transcriptional activation mediated by a variety of DNA-bound transcription factors (Eckner *et al*., 1994, 1996a,b; Arany *et al*., 1994; Lundblad *et al*., 1995; Chakravati *et al*., 1996; Gerritsen *et al*., 1997; Aarnisalo *et al*., 1998; Blobel *et al*., 1998). We find that expression of increasing amounts of E1A together with p300 eliminates the stimulatory effect of p300 on *hsp70* transcription (Figure 2E, F and G, lanes 1–3) and then represses *hsp70* transcription to levels below those obtained in the absence

of exogenous p300 (Figure 2E, F and G, compare lanes 1 and 4). Our results suggest that the acetyltransferase p300 can activate transcription from the *hsp70* promoter and that the targeting of p300 to the *hsp70* promoter requires the Y-box elements.

We wished to test further the hypothesis that the dependence of the histone deacetylase inhibitor TSA and p300 on the Y-boxes of the *hsp70* promoter for transcriptional induction (Figure 1C and E) had a common mechanistic basis. Since E1A inhibits transcriptional activation by both exogenous p300 and endogenous co-activators/acetyltransferases (Figure 2F and G), we established conditions such that E1A inhibited transcription (Figure 2H and I, lane 4) and examined the effect of TSA on transcription in the presence or absence of E1A. We find that the presence of E1A severely compromises the capacity of TSA to activate transcription (Figure 2H and J, compare lanes 2 and 3 with 5 and 6). Moreover, the repressive effect of E1A on TSA-induced transcription was more severe under conditions of more efficient chromatin assembly, i.e. at 12 versus 5 h after intranuclear injection of template DNA [Figure 2I, compare lane 6 (12 h) with lane 5 (5 h), respectively]. We suggest that the effect of E1A on both p300- and TSA-induced transcription is probably directed on the overall acetylation status of proteins in the vicinity of the *hsp70* promoter. We next investigated the transcription factor that might recruit p300 to the *hsp70* promoter in *Xenopus* oocytes.

Cloning and characterization of Xenopus NF-Y

Earlier experiments have led to some confusion as to the major transcriptional regulator that binds to the Y-box in *Xenopus* oocyte nuclei. Possible candidates have included C/EBP (Graves *et al*., 1986), NF-1 (Roulet *et al*., 1995; Puzianowska-Kuznicka and Shi, 1996), FRGY2 (Tafuri and Wolffe, 1990), CBTF (Brewer *et al*., 1995) and NF-Y (this study). The Y-box consists of an inverted CCAAT sequence flanked by other base pairs. Several studies demonstrate that these flanking sequences have a major effect on transcriptional activity in *Xenopus* oocytes

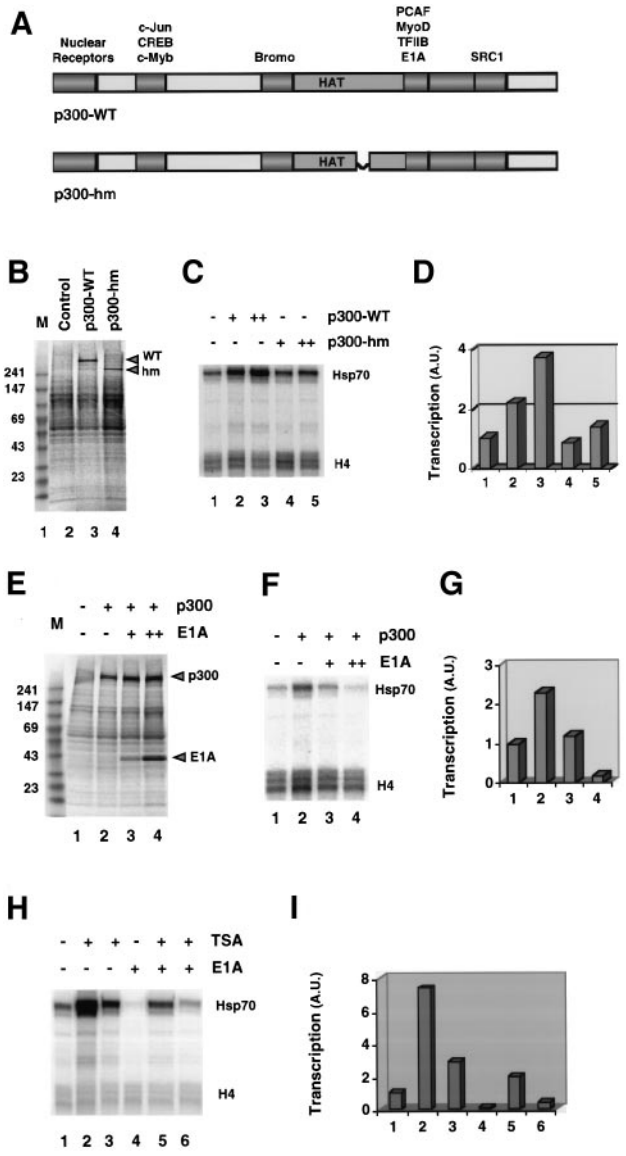
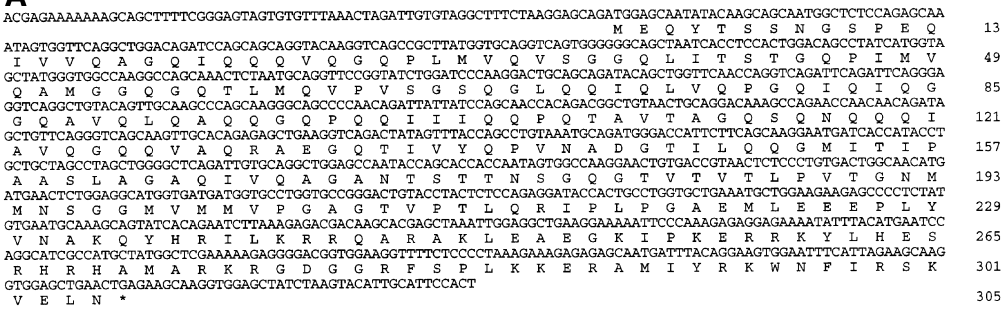


Fig. 2. p300 activates the *Xenopus hsp70* promoter in *Xenopus* oocytes in an acetyltransferase-dependent manner, and E1A inhibits the p300- and TSA-mediated activation of the *hsp70* promoter. (**A**) The schematic presentation of human wild-type p300 (p300-WT) and a histone acetyltransferase activity-deficient mutant (p300-hm, with deletion of amino acids 1472–1522). (**B**) The [^{35}S]methionine labeling of newly synthesized human p300 following mRNA injection of the oocytes. Lane 1, the pre-stained size marker (M); lane 2, a control reaction without any injected mRNA; lane 3, wild-type p300 (p300-WT) mRNA is injected; and in lane 4, the mRNA encoding the histone acetyltransferase mutant of p300 (p300-hm) is injected. The location of the p300 in each lane is denoted by an arrow on the right side of the panel. (**C**) Transcriptional activation of the *hsp70* promoter by p300. A primer extension analysis of transcription is shown (see Materials and methods). Lane 1, a control reaction without any exogenous p300; lanes 2 and 3, oocytes were injected with wild-type p300 (p300-WT) mRNA, 1.25 and 2.5 fmol, respectively; lanes 4 and 5, oocytes were injected with the acetyltransferase mutant p300 (p300-hm) mRNA, 2.5 and 5.0 fmol respectively. Double-stranded hsp-CAT template (0.75 ng) was injected in 9.2 nl for each group of oocytes. The experiment was repeated four times with a degree of reproducibility for relative transcription levels of ±5%. (**D**) Quantitation of the experiment in (C) by PhosporImage analysis. The endogenous H4 signal is used as an internal control. The *hsp70* transcription signal from the injected promoter DNA is plotted as the fold-induction relative to the control reaction (lane 1 in C). (**E**) The [^{35}S]methionine labeling of newly synthesized p300 and E1A following mRNA injection of the oocytes. Lane 1, mock-injected oocytes; lane 2, oocytes injected with 1.25 fmol of wild-type p300 mRNA; lane 3, oocytes injected with 1.25 fmol of p300 and 7.5 fmol of E1A mRNA; lane 4, oocytes injected with 1.25 fmol of p300 and 25 fmol of E1A mRNA. (**F**) Transcriptional regulation by p300 and E1A. Primer extension from the oocytes in lanes 1–4 from (E). Double-stranded hsp-CAT (0.75 ng) was injected in 9.2 nl for each group of oocytes. The experiment was repeated four times with a degree of reproducibility for relative transcription levels of ±5%. (**G**) Quantitation of the experiment in (F). The *hsp70* signal is plotted as fold induction relative to the control reaction (lane 1 in F). (**H**) Effects of E1A on TSA induction of the *hsp70* promoter by primer extension. First, ~20 oocytes were either mock injected (lanes 1–3) or injected (lanes 4–6) with 10 fmol of E1A mRNA and incubated at 18°C for 3 h to allow E1A protein synthesis. Then each group of oocytes was nuclear injected with 0.75 ng of double-stranded hsp-CAT template in 9.2 nl and incubated at 18°C for either 5 h (lanes 2 and 5) or 12 h (lanes 3 and 6) to allow chromatin assembly before the addition of 30 nM TSA. The oocytes were harvested 17 h post-nuclear injection to perform the primer extension. The experiment was repeated three times with a reproducibility for relative transcription values of ±5%. (**I**) Quantitation of the experiment in (H). The *hsp70* signal is plotted as fold induction relative to the control reaction (lane 1 in H).

NF-YA

A



B

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xNF-YA    MEQYT.SSNG SPEQIVVQAG QIQQQ..... .......... ...... ... ....VQGQPL MVQVSGGQLI TSTGQPIMVQ AM.GGQGQTL   59
hNF-YA    -----AN--S -T-------- -----QQGGV TAVQLQTEAQ VASASGQQVQ TLQV------ ---------- ---------- -VP------I   90
rCBF-B    -----AN--S -T-------- -----QQGGV TAVQLQTEAQ VASASGQQVQ TLQV------ ---------- ---------- -VP------I   90
yHAP-2

xNF-YA    MQVPVSGSQG LQQIQLVQPG QIQIQGGQAV QLQAQQGQPQ QIIIQQPQTA VTAGQSQNQQ QIAVQGQQVA QRAEGQTIVY QPVNADGTIL  149
hNF-YA    -------T-- ---------- ---------- -V-G----T- ---------- -----T-T-- ---------- -T-------- ----------  180
rCBF-B    -------T-- ---------- ---------- -V-G----T- ---------- -----T-T-- ---------- -T-------- ----------  180
yHAP-2              MSADETDAK FHPLETDLQS DTAAATSTAA ASRSPSLQEK PIEMPLDMGK APSPRGEDQR VTNEEDLFLF NRLRASQNRV   79

xNF-YA    Q.....QGMI TIPAASLAGA QIVQAGANTS TTNSGQGTVT VTLPVTGNMM NSGGMVMMVP GAGTVPAIQR IPLPGAEMLE EEPLYVNAKQ  234
hNF-YA    -VTVPVS--- ---------- ----T----N --S------- -----A--VV ---------- ---S------ ---------- ----------  270
rCBF-B    -.....---- ---------- ----T----N --S------- -----A--VV ---------- ---S------ ---------- ----------  265
yHAP-2    MDSLEPQQQS QYTSSSVSTM EPSADFTSFS AVTTLPPPPH QQQQQQQQQQ QQQQLVVQAQ YTQNQPNLQS DVLGT-..IA -Q-F------  167

xNF-YA    YHRILKRRQA RAKLEAEGKI PKERRK.YLH ESRHRHAMAR KRGDGGRFSP LKKERAMIYR KWNFIRSKVE LN                    305
hNF-YA    ---------- ---------- ------.--- ---------- ---E----F- -KE-DSPHMQ DPNQADEEAM TQIIRVS                 346
rCBF-B    ---------- ---------- ------.--- ---------- ---E----F- -KE-DSPHMQ DPNQADEEAM TQIIRV                  340
yHAP-2    -Y------Y- -----EKLR- SR--.-P--- ----K---R- P--E----LT AAEIKAMKSK KSGASDDPDD SHEDKKITTK IIQEQPHATS
          TAAAADKKT                                                                                          265
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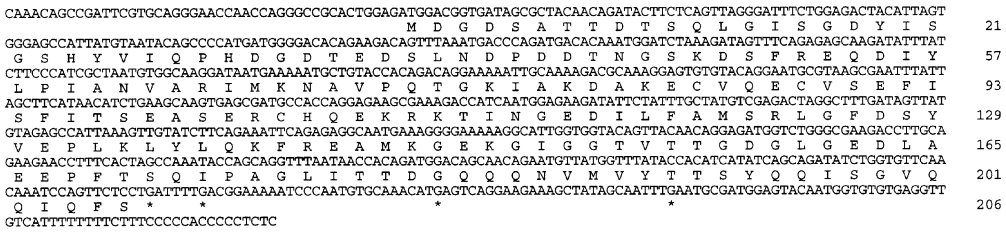
NF-YB

C



D

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xNF-YB      MDGDSATT DTSQLGISGD YISGSHYVIQ PHDGDTEDSL N..DPDDTNG SKDSFREQDI YLPIANVARI MKNAVPQTGK IAKDAKECVQ   86
hNF-YB    MT-----S-- -A------A- --G------- ---.-----M -..-HE---- --E------- ---------- ----I----- ----------   87
rCBF-A    -------S-- -A------A- --G------- ---.-----M -..-HE---- --E------- ---------- ----I----- ----------   87
yHAP-3                         MNTNESEHVS TSPE--QENG GNASSSGSLQ QTSTL----R W---N----L ---TL-PSA- VS--------   70

xNF-YB    ECVSEFISFI TSEASERCHQ EKRKTINGED ILFAMSTLGF DSYVEPLKLY LQKFREAMKG EKGIGGTV.T TGDGLGEDLA EEPFTSQIPA  175
hNF-YB    ---------- ---------- ---------- ---------- ---------- ---------- ------A-.- AT---S-E-T --A--N-L--  176
rCBF-A    ---------- ---------- ---------- ---------- ---------- ---------- -----A-.S AT---S-E-T --A--N-L--  176
yHAP-3    ---------- -----D--AA D--------- --ISLHA--- EN-A-V--I- -A-Y-QQQAL KNQLMYEQDD EEVP                    144

xNF-YB    GLITTDGQQQ NVMVYTTSYQ QISGVQQIQF S                                                                 206
hNF-YB    ---------- ---------- ---------- -                                                                  207
rCBF-A    ----A----- ---------- ---------- -                                                                  207
yHAP-3
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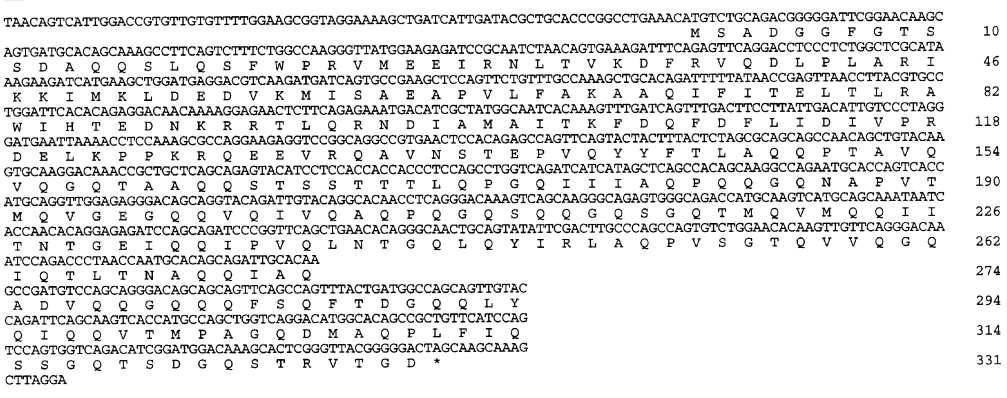
E



F

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xNF-YC                                                                                                  MSADG      5
rCBF-C                                                                                                  --TE-      5
yHAP-5     MIQREEMNMP RQYSEQQQLQ ENEGEGENTR LPVSEEEFRM VQELQAIQAG HDQANLPPSG RGSLEGEDNG NSDGADGEMD EDDEEYDVFR     90
hH1TF2A

xNF-YC     GFG.TSS.DA QQSLQSFWPR VMEEIRNLT. .....VKDFR VQDLPLARIK KIMKLDEDVK MISAEAPVLF AKAAQIFITE LTLRAWIHTE     87
rCBF-C     ---ST--S-- ---------- ---------. .....----- --E------- ---------- ---------- ---------- ----------   89
yHAP-5     NVGQGLVGHY KEIMIRYWQE LINEIESTNE PGSEHQDDFK SHSLPFARIR KV--T----- -------II- ---CE----- --M---CVA-   180
hH1TF2A                                    MELTG RNSIDNIHRL TEARPSNYHE TEAYKRCFDW AGIIDTLLIH HFFYKAYVKR     55

xNF-YC     DNKRRTLQRN DIAMAITKFD QFDFLIDIVP RDELKPPKRQ EEVRQTVNST EPVQYYFTLA QQPTAVQVQG QTAAQQSTSS TTTLQPGQII   177
rCBF-C     ---------- ---------- ---------- ---------- -----S-TPA ---------- ---------- -QQ---T--- ---I------   179
yHAP-5     R-------KA ---E-LQ-S- M------V-- -RPLPQ                                                          216
hH1TF2A    VLLKVEGQLP GSHELFHYLI RDLLAPGL-L C--------- -----S-TPA ---------- ---------- -QQG--T--- ---I------   145

xNF-YC     IAQPQQGQNA PVTMQVGEGQ QVQIVQAQPQ GQSQQGQSG. .QTMQVMQQI ITNTGEIQQI PVQLNTGQLQ YIRLAQPVSG TQVVQGQIQT   265
rCBF-C     --------TT ---------- ---------- --A--T---T G--------- ---------- -----A---- ---------- ----------   269
yHAP-5
hH1TF2A    --------TT ---------- ---------- --A--T---T G--------- ---------- ---------- ---------- ----------   235

xNF-YC     L.TNAQQIAQ ADVQQGQQQF SQFTDGQQLY QIQQVTMPAG QDMAQPLFIQ SSGQTSDGQS TRVTGD                            330
rCBF-C     -A------T- TE-------- --------.-- ---------- --L---M--- -AN-P----T PQ----                            334
yHAP-5
hH1TF2A    ---------- ---------- -------Q-- -------LRA RTSPS-...C --S--TSPP- AGPP--RRLR A                       303
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Fig. 3. Cloning of *Xenopus* NF-Y. (**A**, **C** and **E**) The cDNA and the deduced amino acid sequences of NF-YA, NF-YB and NF-YC are shown (DDBJ/EMBL/GenBank accession Nos AF041203, AF041204 and AF041205). (**B**) Sequence alignment between *Xenopus* NF-YA, human NF-YA (D.M.Becker *et al*., 1991; Li *et al*., 1992a; accession No. P23511), rat NF-YA (Maity and Crombrugghe, 1990, P18576) and yeast HAP2 (Pinkham *et al*., 1987; Xing *et al*., 1993; accession No. P06774). (**D**) Amino acid sequence comparison between *Xenopus* NF-YB, human NF-YB (Li *et al*., 1992a; accession No. P25208), rat NF-YB (Vuorio *et al*., 1990; Hooft von Huijsduijnen *et al*., 1992; Li *et al*., 1992a; accession No. P22569) and yeast HAP3 (Hahn *et al*., 1988; Xing *et al*., 1993; accession No. P13434). (**F**) Sequence homology between *Xenopus* NF-YC, human NF-YC (Nakshtri *et al*., 1996; Bellovini *et al*., 1997; accession No. U62296), rat CBF-C (Sinha *et al*., 1995; accession No. U17607), yeast HAP5 (McNabb *et al*., 1995; accession No. Q02516) and human H1TFIIA (Martinelli and Heintz, 1994; accession No. S74703). Dashes indicate identical amino acids.

(Graves *et al*., 1986; Landsberger *et al*., 1995). Mutations that deviate from the exact consensus for NF-Y binding (Dorn *et al*., 1987; Mantovani, 1998) reduce transcription (Graves *et al*., 1986; Landsberger *et al*., 1995). These results, coupled with our failure to regulate the *Xenopus hsp70* promoter positively in oocytes or embryos following expression of C/EBP, NF-1 or FRGY2 (Ranjan *et al*., 1993; M.Ranjan and A.P.Wolffe, unpublished observations), led us to clone and characterize *Xenopus* NF-Y.

NF-Y is a three-subunit protein containing NF-YA, NF-YB and NF-YC (Hooft van Huijsduijnen *et al*., 1990; Vuorio *et al*., 1990; D.M.Becker *et al*., 1991; Bellovini *et al*., 1997). The cDNA of each subunit was cloned from a *Xenopus laevis* ovary library using PCR (see Materials and methods). The deduced amino acid sequences have a high degree of sequence identity with human, rat and yeast transcription factor NF-Y (Figure 3). The sequence homology between *Xenopus* NF-YA (Figure 3B) and human (D.M.Becker *et al*., 1991; Li *et al*., 1992a) or rat (Maity and de Crombugghe, 1992) is ~75%. Yeast HAP2 (Pinkham *et al*., 1987; Xing *et al*., 1993) shows high homology only in a 60 amino acid stretch at the C-terminus of the protein (74%). The protein is extremely glutamine rich (Hatamochi *et al*., 1988; Maity *et al*., 1990). The calculated molecular mass of the 305 amino acid deduced polypeptide is 33 kDa. For *Xenopus* NF-YB, the calculated molecular mass of the 205 amino acid protein is 22.5 kDa (Figure 3C). *Xenopus* NF-YB shows a 90% amino acid identity with the human protein (Li *et al*., 1992a) and 89% identity with the rat protein (Vuorio *et al*., 1990; Hooft von Huijsduijnen *et al*., 1992; Li *et al*., 1992a). The homology to the yeast transcription factor HAP3 (Hahn *et al*., 1988; Xing *et al*., 1993) is still ~50%, though the C-terminus shows a much higher homology (73% in a 75 amino acid stretch) (Figure 3D). For *Xenopus* NF-YC, the calculated molecular mass of the 330 amino acid deduced protein is 36.5 kDa (Figure 3E). The sequence identity between *Xenopus* and human NF-YC (Nakshtri *et al*., 1996; Bellovini *et al*., 1997) is ~90% (Figure 3F); the same percentage of homology is shown for rat CBF-C (Sinha *et al*., 1995). In the case of yeast HAP5 (McNaab *et al*., 1995), the alignment shows an amino acid identity of 47% for the full-length protein. However, the C-terminus, as in the case of HAP2 and HAP3, shows an homology of 74%. Homology also exists between *Xenopus* NF-YC and the human CCAAT-binding factor H1TF2A (Martinelli and Heintz, 1994), which does not belong to the NF-Y transcription factor family. H1TF2A binds to the histone H1 subtype-specific consensus sequence which previously has been shown to be necessary for temporal regulation of histone H1 transcription during the cell cycle (LaBella *et al*., 1989). For the entire peptide sequence, H1TF2A shows 60% identity to *Xenopus* NF-YC. However, a 190 amino acid stretch at the C-terminus shows 89% identity.

To test whether a functional NF-Y complex could be synthesized *in vitro*, we transcribed and translated directly from circular plasmid DNA templates all three subunits of *Xenopus* NF-Y (Figure 4A). DNA binding assays were carried out as shown in Figure 4B using either oocyte nuclear extract (germinal vesicle extract, GV; Birkenmeier *et al*., 1978) or reticulocyte extract containing NF-YA, NF-YB and NF-YC. A 224 bp fragment of the *Xenopus hsp70* promoter encompassing both Y-boxes and HSE 1 and 2 (Figure 1A) was used initially to bind the heterotrimeric NF-Y complex *in vitro* (Figure 4B). All three subunits of NF-Y need to be co-translated in order to generate a protein able to bind to DNA (Figure 4B, lanes 1–5). The mobility shifts obtained with the NF-Y translated *in vitro* are identical to those observed in oocyte nuclear extract (Figure 4B, compare lanes 6–11 with 12–18). Specificity of binding was demonstrated using competition assays with unrelated oligonucleotides, oligonucleotides encompassing the Y-boxes of the *hsp70* promoter or oligonucleotides with point-mutated Y-boxes. Similar results in DNA binding assays with double-stranded oligonucleotides support the presence of a functional NF-Y complex (data not

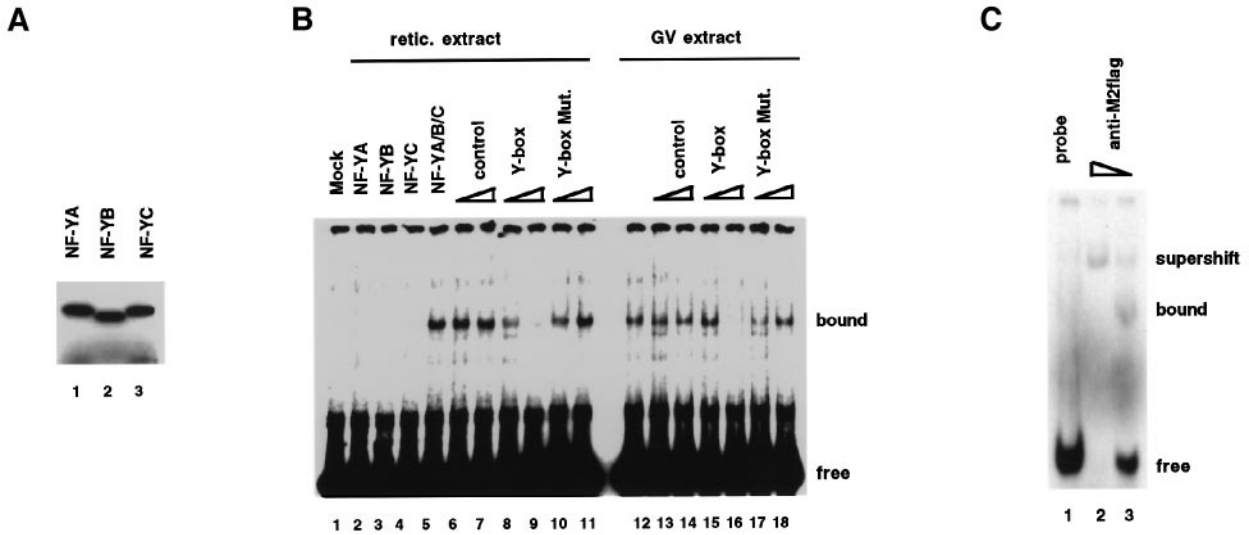

Fig. 4. *In vitro* binding of NF-Y to the Y-boxes of the *hsp70* promoter region. (**A**) *In vitro* translation of all three *Xenopus* NF-Y subunits in reticulocyte extract. (**B**) Competition gel shift analysis of the *hsp70* Y-box promoter. Germinal vesicle (GV) or reticulocyte extract were incubated with the radioactively labeled *hsp70* promoter fragment (see Materials and methods). Control bandshifts in the absence of competitor DNA and various combinations of NF-Y subunits are shown in lanes 1–5 and 12. Competition assays with increasing amounts of the following oligonucleotides are shown: an unrelated control oligonucleotide (lanes 6, 7, 13 and 14), oligonucleotides encompassing the Y-boxes of the *hsp70* gene upstream region (lanes 8, 9, 15, 16) and oligonucleotides with point-mutated Y-boxes (lanes 10, 11, 17 and 18). The sequences of the oligonucleotides are shown in Materials and methods. The positions of free oligonucleotides and bound complex are indicated on the right margin. (**C**) Supershift analysis: M2 flag-tagged NY-B was *in vitro*-translated with NY-A and NY-C. After nucleoprotein complex assembly with the *hsp70* promoter fragment (lane 1) (as in B), 1 (lane 3) or 5 μl (lane 2) of M2-flag serum was added to the binding reaction before resolution on non-denaturing gels. Residual nuclease activity in the serum accounts for the reduced radioactivity in lane 2.

shown). Failure to bind NF-Y correlates with the failure of the *hsp70* promoter containing identical mutations in the Y-boxes to assemble a DNase I-hypersensitive site (Figure 1B) and the failure to activate transcription in response to either TSA (Figure 1C) or expression of p300 (Figure 1E and F). Antibodies against NF-YB (kind gift of R.Currie, Picower Institute) and those against M2 flag-tagged NY-B were used to confirm the identity of the nucleoprotein complexes (Figure 4C). These same antibodies also indicate that endogenous NF-Y is abundant in *Xenopus* oocytes, but declined during early embryogenesis. Finally, we microinjected mRNA encoding all three subunits into *Xenopus* embryos and found that they activated *hsp70* transcription (M.Herrler and A.P.Wolffe, in preparation). We conclude that *Xenopus* NF-Y is the predominant Y-box-binding transcription factor in *Xenopus* oocyte nuclei.

NF-Y interacts with p300 and is an in vivo substrate for acetylation

As the Y-box elements are essential for acetylation-responsive transcription (Figure 1) and NF-Y is the major Y-box-binding protein in oocyte nuclei (Figure 4), we next examined whether NF-Y interacted with p300 *in vitro* and *in vivo*. In these experiments, we make use of a variety of deletion mutants of p300 (Figure 5A).

The p300 coactivator is a histone acetyltransferase (Ogryzko *et al*., 1996). p300 will also acetylate p53 and the basal transcription factors TFIIE and TFIIF (Gu and Roeder, 1997; Imhof *et al*., 1997). The acetylation of a protein by p300 indicates that there is at least a transient interaction between enzyme and substrate (see, for example, Gu and Roeder, 1997; Gu *et al*., 1997). We examined whether purified p300 or deletion mutants would acetylate NF-YB. We find that both p300-WT and p300-C acetylate NF-YB (Figure 5A and B, lanes 1 and 3). These proteins also autoacetylate (Figure 5A and B, lanes 1 and 3) and acetylate core histones (Figure 5A and B, lanes 2 and 4). Two more severely truncated proteins that retain the histone acetyltransferase and PCAF interaction domains, p300-BD and p300-BD1, do not acetylate NF-YB efficiently but do acetylate the core histones as well as autoacetylating (Figure 5C, compare lanes 1–3 with 4–6). p300-BD retains the capacity to interact with E1A, whereas in p300-BD1 the E1A interaction domain has been deleted. This indicates that the C-terminal portion of p300 beyond the PCAF interaction domain (Figure 5A) is required to target p300-mediated acetylation of NF-YB. These *in vitro* results suggest that p300 interacts with NF-YB. Earlier work by Currie (1998) indicated that NF-Y interacts with human GCN5 and PCAF. We next examined whether PCAF would also acetylate NF-YB. Purified recombinant PCAF will acetylate core histones but will not acetylate NF-YB, while p300 will acetylate both histones and NF-YB (data not shown). We did not explore further the functional interactions of PCAF with NF-Y. We suggest that the p300 and PCAF acetyltransferases have considerable specificity in their choice of non-histone substrates (see also Imhof *et al*., 1997). We have not yet investigated the acetylation of other components of NF-Y *in vitro* because of difficulties in expressing large amounts of recombinant protein. Our *in vitro* results with NF-YB encouraged the further investigation of interactions with p300 *in vivo*.

In vitro acetylation of proteins does not establish that they are either substrates for modification *in vivo* or indeed capable of stable interaction with the acetyltransferase *in vivo*. Therefore, we made use of antibodies against

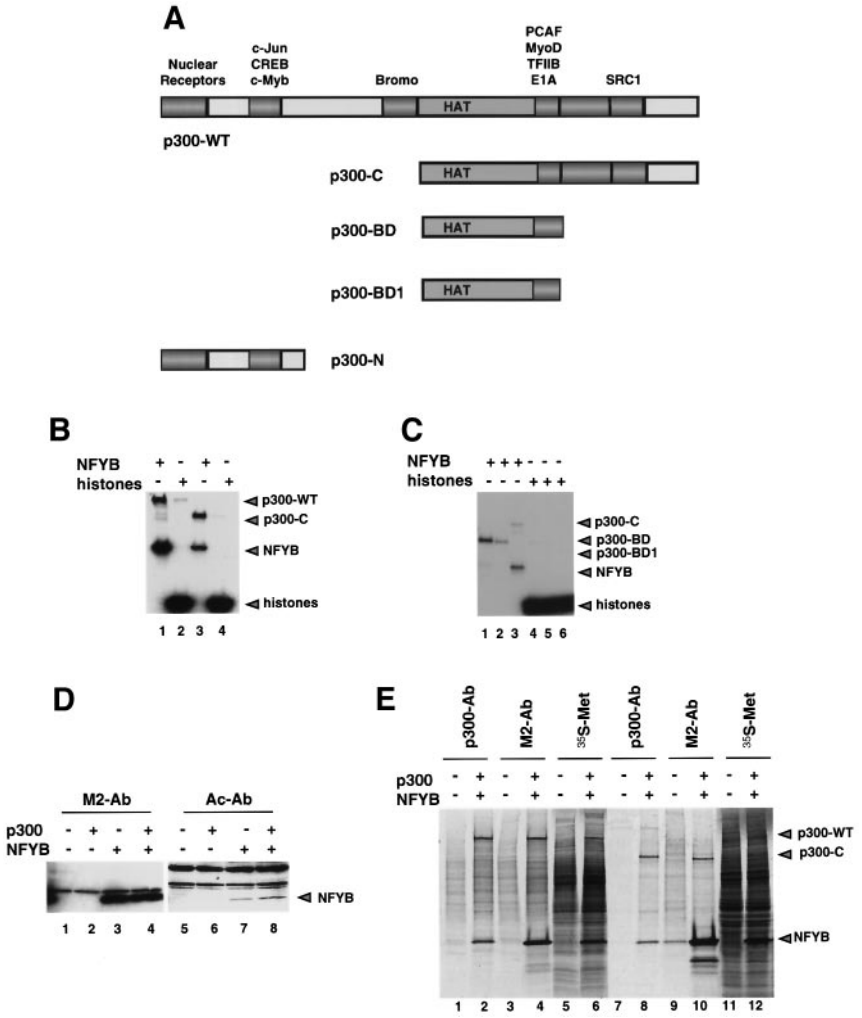

Fig. 5. A component of the Y-box-binding protein, NF-YB, presents a target for acetylation by p300 both *in vitro* and *in vivo*, and interacts physically with p300. (**A**) Schematic presentation of the different forms of human p300 used in the experiments. P300-WT, amino acids 1–2414; p300-N, amino acids 1–670; p300-C, amino acids 1135–2414; p300-BD, amino acids 1185–1810; and p300-BD1, amino acids 1195–1760 (Ogryzko *et al*., 1996). (**B**) The acetylation of NF-YB by p300. Purified recombinant p300 from the baculouvirus system and NF-YB from the *E.coli* sytem were used for the HAT assay (Materials and methods). Lanes 1 and 3, acetylation of NF-YB by wild-type (p300-WT) and C-terminus of p300 (p300-C), respectively; lanes 3 and 4, the acetylation of histones by p300-WT and p300-C, which are used as a positive control. (**C**) Definition of the domain of p300 that possesses the acetyltransferase activity for NF-YB. p300-BD and p300-BD1 cannot acetylate NF-YB efficiently even though they can acetylate histones. Lanes 1–3, acetylation of NF-YB by p300-BD, p300-BD1 and p300-C, respectively; lanes 4–6 acetylation of histones by p300-BD, p300-BD1 and p300-C respectively. (**D**) The *in vivo* acetylation of exogenous M2-tagged NF-YB by p300-WT. The p300-WT and/or M2-tagged NF-YB were synthesized in the oocyte and analyzed by Western blot analysis. Lanes 1–4, the Western blot shows the newly synthesized NF-YB protein level by M2 antibody (M2-Ab); lanes 5–8, the same blot as in lanes 1–4 detected using an antibody against the specific acetylated lysines (Ac-Ab) in the NF-YB. (**E**) Immunoprecipitation of p300 and NF-YB. P300-WT and NF-YB mRNA were co-injected into the oocytes and followed by 16 h incubation. The protein extract prepared then was used immediately for the pull-down assays. Lanes 1–6 illustrate the interaction of wild-type p300 (p300-WT) with NF-YB; lanes 7–12 show that the C-terminus of p300 (p300-C) contains the binding ability for NF-YB. Lanes 1, 2, 7 and 8, the pull-downs by p300 antibody (p300-Ab); lanes 3, 4, 9 and 10, the pull-downs using M2 antibody (M2-Ab); lanes 5, 6, 11 and 12, the input protein profile from oocytes labeled by [^{35}S]methionine (^{35}S-Met).

acetylated lysine (Hebbes *et al*., 1994) to examine whether NF-YB was acetylated *in vivo*. Immunoprecipitation of NF-YB synthesized *in vivo* in the presence (Figure 5D, lane 4) or absence of p300 (lane 3) followed by immunoblotting against acetylated lysine (Figure 5D, lanes 7 and 8) reveals that NF-YB is acetylated *in vivo*. We next examined whether p300 and NF-Y might interact stably *in vivo*. Full-length human p300 and M2-tagged NF-YB were expressed in *Xenopus* oocytes following microinjection of synthetic mRNAs into the cytoplasm (Figure 5E, lane 6). Monoclonal antibodies against p300 immunoprecipitated NF-YB (Figure 5E, lane 2) and antibodies against the M2 epitope immunoprecipitate both NF-YB and p300 (Figure 5E, lane 4). In order to begin to delimit the domain of p300 that interacts with NF-YB and to provide an additional control, we also co-expressed the C-terminal portion of p300 (p300-C) with NF-YB (Figure 5E, lanes 7–12). The C-terminal portion of p300 retains the capacity to interact specifically with NF-YB (Figure 5E, lanes 8 and 10). We conclude that p300 interacts with NF-Y *in vivo*.

Domain requirement of p300 for transcriptional activation of Xenopus hsp70 in chromatin

We have established that p300 stimulates transcription from the *Xenopus hsp70* promoter *in vivo* and that deletion of a segment of the histone acetyltransferase domain

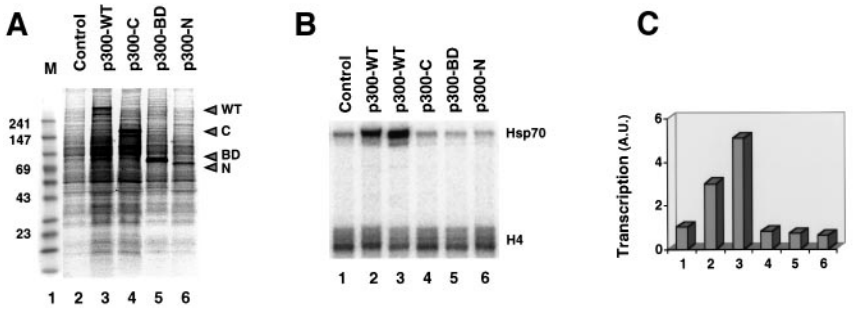

Fig. 6. The requirement for wild-type p300 in the induction of the *hsp70* promoter in *Xenopus* oocytes. (**A**) [^{35}S]methionine labeling of newly synthesized p300 following mRNA injection of oocytes. Lane 1, the pre-stained size marker (M); lane 2, a control reaction without any injected mRNA; lane 3, oocytes were injected with wild-type p300 (p300-WT) mRNA; lane 4, oocytes were injected with the C-terminus of p300 mRNA (p300-C); lane 5, oocytes were injected with p300-BD mRNA; and lane 6, oocytes were injected with the N-terminus of p300 (p300-N) mRNA. (**B**) Transcriptional activation of the *hsp70* promoter by mutants of p300 analyzed by primer extension. Lane 1, a control reaction without any exogenous p300; lanes 2 and 3, oocytes were injected with wild-type p300 mRNA, 2.5 and 5.0 fmol, respectively; lanes 4, 5 and 6, with 2.5 fmol of mRNA encoding p300-C, p300-BD and p300-N, respectively. Single-stranded hsp-CAT template (0.75 ng) was injected in 9.2 nl for each group of oocytes. The experiment was repeated three times with a degree of reproducibility for relative transcription levels of ±5%. (**C**) Quantitation of the experiment in (B). The endogenous H4 signal is used as an internal control. The *hsp70* transcription signal from the injected promoter DNA is plotted as fold induction relative to the control reaction [lane 1 in (B)].

essential for activity severely reduces the capacity of p300 to stimulate transcription (Figures 1 and 2). We next investigated whether the capacity to acetylate free histones and NF-YB was sufficient for transcriptional stimulation of the *hsp70* promoter. The C-terminal portion of p300 retains the capacity to interact with NF-YB and to acetylate both NF-YB and the core histones (Figure 5). The p300-BD protein can acetylate histones, but does not acetylate NF-YB efficiently (Figure 5). All of the deletion mutants and wild-type p300 (Figure 5A) are synthesized and appear stable in *Xenopus* oocytes (Figure 6A). The p300-WT activates transcription of the *hsp70* promoter; however, the deletion mutants do not activate *hsp70* transcription in chromatin (Figure 6B and C). This result is consistent with p300 exerting important functional roles in the transcription process in addition to those associated with acetylation of either NF-YB or histones. These may include interactions with other components of the basal transcriptional machinery (Imhof *et al*., 1997) or other sequence-specific transcription factors, or the RNA polymerase holoenzyme (Nakajima *et al*., 1997; Torchia *et al*., 1998).

The influence of p300 on transcriptional control dependent on heat shock factor

The *Xenopus hsp70* promoter is regulated through both developmentally controlled and heat-inducible pathways (Bienz, 1985). The Y-boxes and their association with NF-Y have a major controlling influence in oocyte-selective developmental control (Bienz, 1986; Landsberger and Wolffe, 1995). The Y-boxes pre-set the *hsp70* promoter for subsequent activation by HSF (Figure 1; Landsberger and Wolffe, 1995). In earlier work, we have shown that the range of transcriptional regulation of the *hsp70* promoter in response to heat shock is dependent on the pathway of chromatin assembly (Landsberger *et al*., 1995). Chromatin assembly on a replicating single-stranded template is more rapid and more repressive to basal transcription than on non-replicating double-stranded templates microinjected in oocyte nuclei (Almouzni and Wolffe, 1993; Wong *et al*., 1995). We find that expression of HSF in the presence of more limiting amounts of exogenous p300 than generally used earlier (1.25 fmol compared with 2.5 fmol of mRNA injected) augments transcription under heat shock conditions effectively when chromatin is assembled using a replicative template (Figure 7A and B).

Optimal transcription from the *hsp70* promoter within chromatin assembled under replicative conditions only occurs under heat shock conditions in the presence of exogenous HSF (Figure 7A and B; Landsberger and Wolffe, 1995, 1997; Stump *et al.*, 1995). We next asked to what extent the addition of TSA to inhibit deacetylase might substitute for the combined action of p300 and activated HSF on chromatin assembled by the replicative pathway. We find that exogenous HSF activated under heat shock conditions in the presence of exogenous p300 augments transcription to only slightly greater levels than that achieved by TSA alone or by TSA in combination with p300 (Figure 7C and D, compare lane 2 with 3 and 4). These results indicate that acetylation responsiveness is a major regulatory component of transcriptional control on the *hsp70* promoter within chromatin in the oocyte nucleus. We suggest that the inhibition of histone deacetylation compensates for the positive recruitment of acetyltransferases to particular promoters (Figure 7C and D, compare lanes 2–6; see Discussion). Other regulatory pathways may contribute to stimulating transcription above that achieved by modulation of net acetyltransferase and deacetylase activity, these might include the targeted modification of the basal transcriptional machinery through phosphorylation (Lis and Wu, 1994) and facilitated recycling of RNA polymerase (Kraus and Kadonaga, 1998; Sandaltzopoulos and Becker, 1998).

Discussion

The major conclusion of this work is that a single regulatory protein–DNA interaction defines acetylation responsiveness for the *Xenopus hsp70* promoter in chromatin. *Xenopus* NF-Y interacts with the Y-boxes in the *hsp70* promoter, pre-setting chromatin architecture (Figures 1, 3 and 4). NF-Y interacts with p300 (Figure 5). p300 stimulates *hsp70* transcription and requires Y-boxes

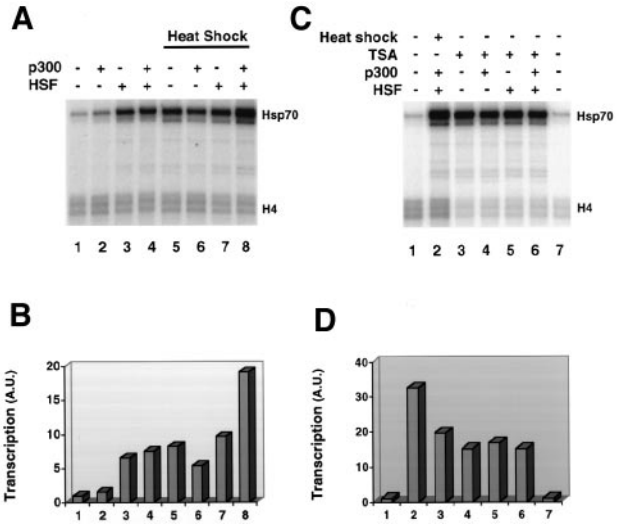

Fig. 7. The role of p300 in the regulation of the *hsp70* promoter in response to heat shock induction. (**A**) Primer extension analysis of the heat shock response from the injection of single-stranded templates containing the *hsp70* promoter. Lanes 1–4, the primer extension from the *hsp70* promoter following heat shock at 34°C for 2 h (Heat Shock). Lanes 5–8, the regulation of the *hsp* promoter at 18°C. Single-stranded hsp-CAT template (0.37 ng) was injected in each group of oocytes. The amount of p300-WT and/or HSF injected for the + group of oocytes was 1.25 fmol. This experiment was repeated three times with a degree of reproducibility for relative transcription levels of ±5%. (**B**) Quantitation of the experiment in (A). The endogenous H4 signal is used as an internal control. The promoter activity (*hsp70*) is plotted as fold induction relative to the control reaction [lane 1 in (A)]. (**C**) The regulation of *hsp70* promoter by TSA using single-stranded templates. Lanes 3–7, the activation of the *hsp70* promoter by TSA (30 nM) in the presence and absence of exogenous p300 and HSF. Lanes 1 and 2, the p300 acitivity on the promoter during heat shock induction used as a comparison. Single-stranded hsp-CAT template (0.75 ng) was injected in each group of oocytes. The amount of p300-WT and or HSF injected for the + group of oocytes was 2.5 fmol. This experiment was repeated three times with a degree of reproducibility for relative transcription levels of ±5%.
(**D**) Quantitation of the experiment in (C). The endogenous H4 signal is used as an internal control. The *hsp70* promoter activity is plotted as fold induction relative to the control reaction [as lanes 1 and 7 in (C), respectively].

for this effect (Figure 1). Histone deacetylase inhibitors also require the Y-boxes to stimulate *hsp70* promoter activity. We propose that NF-Y recruits p300 to the *hsp70* promoter in oocyte nuclei and that the acetyltransferase activity of p300 is important for transcription of the *hsp70* promoter in chromatin (Figure 2). The Y-box elements mediate acetylation responsiveness (Figure 1). The requirement for NF-Y and p300 can be largely eliminated by the addition of the deacetylase inhibitor TSA (Figures 1 and 2). E1A inhibits p300 activity and transcription; moreover, E1A also substantially reduces transcriptional activation in response to TSA (Figure 2). This indicates that a major function of NF-Y is to recruit the acetyltransferase function of p300. Thus there are two steps in the modulation of chromatin structure and function at the *Xenopus hsp70* promoter: the establishment of a DNase I-hypersensitive site and the capacitation of basal transcription, followed by the subsequent recruitment of p300 to modulate transcription.

NF-Y, acetylation and pre-set chromatin

NF-Y contains two subunits that are histone fold proteins resembling H2A and H2B (Baxevanis *et al*., 1995; Sinha *et al*., 1995). Metazoan NF-Y and *S.cerevisiae* HAP-2, -3 and -5 are highly related trimeric proteins. The histone fold proteins NF-YB (related to H2B) and NF-YC (resembles H2A) need to associate with DNA together with the third subunit NF-YA that confers sequence specificity (Sinha *et al*., 1995). Our results confirm the need for all three subunits for association with DNA (Figures 3 and 4). The sequence specificity of NF-Y binding to the Y-box (Figure 4) exactly parallels that of mammalian NF-Y (Dorn *et al*., 1987; Hatamochi *et al*., 1988; Nakshtri *et al*., 1996; Mantovani, 1998) and the sequence requirements for transcriptional activation by the Y-box in oocyte nuclei (Graves *et al*., 1986; Wolffe *et al*., 1992; Landsberger *et al*., 1995). We find that NF-Y is the most abundant Y-box-binding protein in *Xenopus* oocyte nuclei (Figure 4; data not shown). The Y-box is a common feature of many promoter sequences that are transcribed preferentially in oocytes (Bienz, 1986; Toyoda and Wolffe, 1992; Wolffe *et al*., 1992; Cho and Wolffe, 1994); thus, understanding the functions of the Y-box/NF-Y complex may underlie oocyte-selective transcription.

The Y-box is essential for the assembly of a DNase I-hypersensitive site in the chromatin of the *Xenopus hsp70* promoter (Figure 1). Both Y-boxes assemble a stronger site than the single proximal Y-box. Distal and proximal Y-boxes are separated by exactly eight helical turns of DNA (84 bp), and it is possible that adjacent NF-Y complexes may interact. We suggest that NF-Y may have an architectural role in establishing an accessible chromatin structure on the *Xenopus hsp70* promoter comparable with that of the GAGA transcription factor on the *Drosophila* heat shock promoters (Lu *et al*., 1993; Tsukiyama *et al*., 1994; Wall *et al*., 1995). The Y-boxes are important for basal transcription from the *Xenopus hsp70* promoter (Bienz, 1986; Landsberger and Wolffe, 1995). They are known to facilitate pre-initiation complex assembly (Milos and Zaret, 1992; Wright *et al*., 1994) and to facilitate the subsequent association of *Xenopus* HSF and transcriptional activation from the *hsp70* promoter (Landsberger and Wolffe, 1995).

Insight into the functional role of the Y-boxes is provided by the remarkable dependence of the *hsp70* promoter on the Y-box elements for transcriptional activation in response to deacetylase inhibitors and the p300 transcriptional coactivator (Figure 1). Earlier work on promoters such as *Xenopus* H1° which is activated by the deacetylase inhibitors sodium butyrate and TSA had failed to delineate any single element that accounted for the acetylation response (Khochbin and Wolffe, 1993; Almouzni *et al*., 1994). Addition of deacetylase inhibitors does not promote transcription or the assembly of DNase I hypersensitivity on the *hsp70* promoter lacking Y-boxes (Figure 1). We suggest that alterations in protein acetylation status do not influence the association of other components of the basal transcriptional machinery with the *hsp70* promoter in the absence of NF-Y. However, a single Y-box is sufficient to generate DNase I sensitivity and to promote transcription in the presence of TSA (Figure 1). The requirement for a Y-box in order to generate acetylation responsiveness is consistent with the hypothesis that proteins bound to it will recruit acetyltransferases, and that acetyltransferase activity will

be important for transcriptional regulation. Consistent with this hypothesis is the capacity of E1A expression to inhibit severely the TSA responsiveness of the *hsp70* promoter (Figure 2). E1A is known to interact with p300 and inhibit its transcriptional activation functions (Rochette-Egly *et al*., 1990; Stein *et al*., 1990; Wang *et al*., 1993).

NF-Y, HSF and p300

NF-Y interacts with p300 *in vivo* (Figure 5). Endogenous p300 is present in *Xenopus* oocyte nuclei (Figure 1), and expression of exogenous p300 facilitates transcription from the *hsp70* promoter in chromatin (Figures 1, 2 and 6). The physiological relevance of this interaction is indicated by the capacity of E1A expression to interfere with transcription of the *hsp70* promoter (Figure 2). Expression of E1A also effectively inhibits the expression of the *hsp70* promoter driven by endogenous acetyltransferases and leads to a 70% reduction in the capacity of TSA to activate the *hsp70* promoter (Figure 2H and I). Taken together, these results generate a strong argument for an involvement of p300 in *hsp70* promoter regulation through targeting by NF-Y. NF-Y is a transcriptional activator (Li *et al*., 1992b). The yeast homologs HAP2, -3 and -5 regulate transcription through interaction with the ADA2/ADA3/GCN5 complex (Pinkham *et al*., 1987; Berger *et al*., 1992; Georgakopoulos and Thireos, 1992; Marcus *et al*., 1994). GCN5p is a histone acetyltransferase (Brownell *et al*., 1996) whose acetyltransferase activity is required to regulate transcription (Kuo *et al*., 1998; Wang *et al*., 1998) and disrupt chromatin (Gregory *et al*., 1998). Mammalian NF-Y interacts with PCAF *in vitro* (Currie, 1998); however, PCAF does not acetylate NF-Y, nor does it potentiate *hsp70* transcription *in vivo* when expressed from exogenous mRNA (data not shown). In contrast, p300 interacts with NF-Y *in vivo* and potentiates transcription (Figures 1, 2, 5 and 6). Thus, in the oocyte system, we propose that p300 is a functional coactivator for *hsp70* transcription. We suggest that p300 functions in an analogous manner to that defined for GCN5p in *S.cerevisiae*.

We find that the acetyltransferase activity of p300 is important for transcriptional activation of the *hsp70* promoter. Histones represent one substrate for p300 (Ogrzyko *et al*., 1996), and we find that NF-Y itself is acetylated *in vivo* (Figure 5). Thus, NF-Y joins the growing list of transcription factors that are acetylation substrates for p300 (Gu and Roeder, 1997; Imhof *et al*., 1997; Wong *et al*., 1998). The functional consequences of NF-Y acetylation are being tested. It should be noted that all of our interaction assays have been performed with NF-YB. It is possible that the formation of a complex with NF-YA and NF-YC will alter the nature of any interaction with p300. These possibilities are also under experimental investigation. Deletion analysis of p300 indicates that binding to NF-Y is within the C-terminal domain that includes the histone acetyltransferase domain. This p300-C domain will also acetylate NF-Y and the histones, but is insufficient to activate transcription. Thus, p300 must have other functions at the *hsp70* promoter in chromatin in addition to NF-Y-targeted acetyltransferase activity. These most probably include recruitment of components of the basal transcriptional machinery and RNA polymerase (Nakajima *et al*., 1997). Modification of the chromatin environment is also likely to have a role in transcriptional regulation.

Acetylation of the histones helps promote transcription factor access to DNA in chromatin (Lee *et al*., 1993; Vetesse-Dadey *et al*., 1996), will weaken internucleosomal interactions (Garcia-Ramirez *et al*., 1995; Tse *et al*., 1998) and will promote the processivity of RNA polymerase through nucleosomal arrays (Ura *et al*., 1997; Nightingale *et al*., 1998). A clear example of where acetyltransferases and deacetylases have a regulatory role in transcription is provided by the activation of HIV-1 gene expression in latently infected cell lines (Van Lint *et al*., 1996a,b; Sheridan *et al*., 1997). Whereas most genes (332 out of 340 examined) do not show detectable changes in activity in response to histone deacetylase inhibitors, the HIV-1 promoter is induced >10-fold. This activation response implies that a high degree of selectivity will exist for acetylation-responsive transcription *in vivo*. Mechanistic studies indicate that chromatin remodeling is a necessary component of efficient transcriptional activation and reinitiation on the HIV-1 promoter (Verdin *et al*., 1993; Van Lint *et al*., 1996b; Sheridan *et al*., 1997). Our results with the deacetylase inhibitor TSA suggest that acetylation *per se* is not sufficient to influence association of other DNA-binding proteins with the *hsp70* promoter or transcription itself in the absence of the Y-box elements (Figure 1). The Y-box dependence shows that selectivity also exists within *Xenopus* oocytes for acetylation-responsive transcription. Y-boxes are also required for chromatin remodeling on the *Xenopus hsp70* promoter (Figure 1; Landsberger and Wolffe, 1995). How then might the interaction of NF-Y with p300 influence the transcription process? The acetyltransferase activity of p300 will modify NF-Y, histones and, potentially, TFIIE and TFIIF (Figure 5; Imhof *et al*., 1997). Acetylation might enable NF-Y to make more productive contacts with other transcription factors. Acetylation of TFIIE and TFIIF might also facilitate their function within chromatin. These possibilities remain to be tested rigorously. Moreover, the basal transcription factors might still require chromatin modifications to reveal the functional consequences of transcription factor acetylation. Alternatively, NF-Y might represent such a key component of the transcriptional machinery on the *hsp70* promoter that without its presence the basal transcriptional machinery cannot function. If this is the case, then NF-Y would have the dual role of recruiting both the basal machinery and p300 to create a chromatin environment competent for basal levels of transcription and for potentiating transcription in the presence of HSF.

Materials and methods

Plasmid DNA

The *Xenopus hsp70* promoter cloned into the *Xba*I–*Hin*dIII sites of pCAT Basic (Promega) to generate pHSP-CAT has been described previously by Ranjan *et al*. (1993). Deletion of the heat shock element 3 (HSE 3) resulted in the plasmid pΔ224. The 224 bp *Xba*I–*Hin*dIII fragment was used for electrophoretic mobility shift assays with oocyte nuclear extract (germinal vesicle, GV) or reticulocyte (RE) extract containing *in vitro*-translated NF-Y. Mutation of both CCAAT-boxes (CCAAT1 and CCAAT2 were changed to CGGCC and TATCTC, respectively, Landsberger and Wolffe, 1995) generated the plasmid pHSP-CAT (ΔY1/Y2). The M13Hsp70CAT vector and derivatives were constructed by removing the 2002 bp *Hin*dIII–*Bam*HI fragment containing the *Xenopus hsp70* promoter and the CAT gene from the WT343

construct (hsp-CAT basic; Ranjan *et al*., 1993) and inserting it into the linker region of M13mp18DNA (Gibco-BRL). Single-stranded DNA from bacteriophage M13 mp18 is prepared from phage purified by CsCl buoyant density centrifugation. Double-stranded supercoiled form I M13 derivatives are isolated and prepared as described (Maniatis *et al*., 1982). The human cytomegalovirus (CMV) clone 101 (kindly provided by Lother Hennighausen, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) contains the promoter from the immediate early gene 1 from human CMV. This promoter was used to drive transcription of the CAT gene transcription unit (CMV CAT) (Almouzni and Wolffe, 1993). XHSF1 cDNA was subcloned into pSP64pA previously described by Landsberger and Wolffe (1995). Plasmid constructs were cloned by PCR for plasmids pNF-YA, pNF-YB and pNF-YC (see below) into the vector pSP64pA (Promega) for *in vitro* transcription of *Eco*RI-linearized plasmid with SP6 RNA polymerase (Bouvet *et al*., 1994). All 5′ primers were designed with a *Hin*dIII site preceded by a sequence to enhance restriction digestion for cloning, an initiating AUG codon preceded by the sequence AAAG to enhance translation efficiency, and a 24 nucleotides sequence coding for an eight amino acid FLAG eptitope tag (Kodak). NF-Y sequences followed this designed sequence. 3′ Primers were designed with an *Xba*I site, followed by a stop codon and NF-Y sequence. After PCR, fragments were cloned into the *Hin*dIII and *Xba*I sites of pSP64pA. pET30a(+) (Novagen) was used for overexpression of NF-YB. NF-YB cDNA was subcloned into the *Eco*RI–*Xho*I sites for N-terminal fusion to cleavable His·Tag and S·Tag sequences. Recombinant NF-YB protein was overexpressed and purified according to the protocol from Novagen. All the constructs were confirmed by DNA sequencing.

The different human p300 expression constructs (DDBJ/EMBL/GenBank accession No. U01877, Eckner *et al*., 1994) were engineered by blunt end cloning of an *Xho*I–*Not*I fragment from different baculoviral constructs (Ogryzko *et al*., 1996) into the *Eco*RV site of T7TS vector (Zorn and Krieg, 1997) to generate T7TSp300. The E1A expression plasmid was constructed by *Bam*HI–*Hin*dIII digestion of PCDNA1 plasmid and blunt end ligation into the *Eco*RV site of pT7TS vector, an RNA expression vector constructed by Dr P.A.Krieg and colleagues (Zorn and Krieg, 1997).

Cloning and sequencing of NF-YA, NF-YB and NF-YC cDNA

Total RNA was isolated from *Xenopus* oocytes. Collected oocytes were homogenized with 20 mM Tris–HCl (pH 8.0) and extracted by the RNAzol™ method (Tel-Test, Friendswood). RT–PCR was performed with different concentrations of total RNA (0.25–1.5 μg) and either an oligo(dT) primer or degenerated oligonucleotide primers (A2), (B4) and (C6), derived from conserved NF-YA, NF-YB and NF-YC peptide sequences:

(A1) 5′-GAA(G)CCA(T)CTGTAC(T)GTG(T)AAC(T)GCA(T)AAA-(G)CAA(G)TAC(T)C-3′;
(A2) 5′-GCCTTGAAGCTTG(AT)GCCATG(AT)GCA(G)TGG(ATC)-CG(T)G(A)TG-3′;
(B3) 5′-GCCTTGAAGCTTGCGAGTGAGCGATGCCACC-3′;
(B4) 5′-GCCTTGAAGCTTGCTGAACACCAGATATCTGCTGATA-3′;
(C5) 5′-ATGAAA(G)ACA(CT)GAC(T)GTG(CT)AAA(G)ATGAT-3′;
(C6) 5′-GGC(A)ACC(A)AT(C)G(A)TCG(A)ATCAGG(A)AAG(A)TC-G(A)AA-3′.

Primers (A2), (B3) and (B4) were designed with a *Hin*dIII site preceded by a six nucleotide sequence to enhance restriction digestion for cloning. The oligonucleotides were obtained from Bioserve Biotechnologies, Laurel and Gibco-BRL, Gaithersburg. Samples were denatured for 10 min at 65°C and incubated for 1 h at 37°C in 10 μl of PCR buffer (10 mM Tris–HCl pH 8.2, 1.2 mM EDTA, 25 mM NaCl, 0.1% Tween-20, 0.1% gelatine) supplemented with 5 U of RNasin/ml, 0.1 M dithiothreitol (DTT), 10 mM dNTPs and 1 U of reverse transcriptase. After heat inactivation at 94°C for 5 min, the transcription reactions were subjected to PCR amplification after the addition of one of the forward primers (A1), (B3) or (C5), and *Taq* polymerase. Thirty cycle PCR amplifications with 2 min elongation time were performed using different combinations of primers and annealing temperatures. For NF-YA, a single 141 bp PCR product obtained with the N-terminal primer (A1) and primer (A2) derived from one of the internal peptides was inserted into pCR2.3™ (Invitrogen, San Diego, CA), cloned into *Escherichia coli* XL-1-Blue cells and sequenced with Sequenase 2.0 (US Biochemicals, Amersham Life Sciences, Arlington Heights). In the case of NF-YB, a 308 bp product was obtained with the primers (B3) and (B4), and for NF-YC the (C5) and (C6) generated a specific 203 bp product. Both PCR products were subcloned into pCR2.3™ and sequenced subsequently.

The randomly primed (Rediprime™, Amersham Life Sciences, Arlington Heights) [^{32}P]dCTP-labeled *Hin*dIII inserts from pCR141, pCR308 and pCR203 were used as probes for screening an *X.laevis* stage VI oocyte cDNA library in pBluescript, kindly provided by D.Patterton (Patterton *et al*., 1995). Library screening was done by standard techniques (Sambrook *et al*., 1989). Hybridizations were performed according to the standard protocols (Sambrook *et al*., 1989). Positive phages were plaque purified, and the inserts were subcloned into pBluescript or pCR2.3™ and sequenced. Overlapping cDNA clones were mapped and sequenced.

Microinjection of Xenopus oocytes

The defolliculated *Xenopus* stage VI oocytes were prepared as previously described (Almouzni and Wolffe, 1993). The indicated amounts of mRNA for p300s and/or HSF were injected into oocyte cytoplasm in 27.6 nl volume. Protein synthesis was allowed for 16 h by incubating the oocytes at 18°C. For transcription assays, the nuclear injection of either double strands or a single strand of hsp-CAT reporter DNA was done routinely 2 h after mRNA injection into cytoplasm with 0.75 ng of DNA in 9.2 nl. The injected reporter DNA was incorporated into chromatin and transcribed during 14 h of incubation of the oocytes at 18°C. Heat shock treatment was carried out by incubating the oocytes at 34°C for 2 h following the 14 h incubation at 18°C (Wolffe *et al*., 1984). TSA treatment (30 nM) was concurrent with DNA injection.

In vitro transcription and translation

Full-length capped and poly(A)$^+$ adenylated NF-YA, NF-YB, NF-YC and XHSF1 RNA was produced from *Eco*RI-linearized template DNA with the mMessage™ kit (Ambion) by SP6 RNA polymerase. *In vitro*-translated protein was made either from RNA template in a rabbit reticulocyte lysate system (mMachine™ kit, Ambion) or directly from circular plasmid DNA template with the TNT® lysate-coupled transcription/translation kit (Promega). Usually the translation reactions were assembled in a final volume of 50 μl (25 μl of TNT® lysate, 2 μl of TNT® buffer, 1 μl of TNT® SP6 RNA polymerase, 1 mM amino acid mixture minus methionine, 40 μCi of [^{35}S]methionine, 40 U of RNasin® ribonuclease inhibitor and 1 μg of DNA template), but translation reactions could also be performed on a small scale by reducing the reaction volumes proportionally. The reaction mixture was incubated at 30°C for 2 h.

Gel shift assay

Xenopus oocyte nuclear extract (GV extract; Birkenmeier *et al*., 1978) was kindly provided by Takashi Sera, NICHD, Bethesda, MD. The following oligonucleotides were used:

Y2-sense 5′-CTGGTTGCT GATTGGCTAACGAA-3′;
Y2-antisense 5′-TTTCGTTAGCCAATCAGCAA CCA-3′;
Y1-sense 5′-CTGTGCCTTGATTGGCTAATCTG-3′;
Y1-antisense 5′-ACAGATTAG CCAATCAAGGCA CA-3′;
(-Y2) sense 5′-CTGGTTGCTTCTGGCTACACGAA-3′;
(-Y2) antisense 5′-TTTCGT GTAGCCAGAAGCAACCA-3′;
(-Y1) sense 5′-CTGTGCCTTTCTGGC TACATCTG-3′;
(-Y1) antisense 5′-ACAGATGTAGCCAGAAAGGCACA-3′.

Consensus sense and antisense oligonucleotides were annealed and 5′-end labeled for 45 min at 37°C with T4 polynucleotide kinase. The 224 bp *Xba*I–*Hin*dIII fragment derived from plasmid pΔ224 was also 5′-end labeled. For a 50 μl reaction, 1 μl of double-stranded oligonucleotide (at 100 ng/μl) and 5 μl of [γ-^{32}P]dATP (3000 Ci/mmol at 10 mCi/ml) was used. Unincorporated nucleotides were removed through either gel filtration (Ausubel *et al*., 1996) or purification by gel electrophoresis. Poly(dI–dC) and/or salmon sperm DNA were added as non-specific competitor. The DNA-binding reactions were assembled in a final volume of 10 μl (10× binding buffer: 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 5% glycerol; 2 μg of poly(dI–dC)/salmon sperm DNA; 4–6 μg of GV or reticulocyte lysate extract) and incubated at room temperature for 5–10 min. Then 1 μl (50 000–200 000 c.p.m.) of ^{32}P-labeled *Hin*dIII–*Xba*I fragment or consensus oligonucleotide were added to each reaction and incubated for 20 min at room temperature. For the supershift experiments using antibodies, the indicated volume of antibody was added for the final 10 min of incubation. DNA binding reactions were analyzed on a 4 or 8% non-denaturing polyacrylamide gel. Gels were run at room temperature in 0.5-fold Tris-borate/EDTA buffer at 350 V for ~2 h, then dried and exposed to X-ray film overnight at –70°C with an intensifying screen.

Protein extraction and Western blot analysis

The protein from oocytes was prepared by homogenizing the oocytes in 20 mM HEPES buffer containing 70 mM KCl, 5% sucrose and 1 mM DTT. The homogenate was centrifuged for 10 min at 10 000 r.p.m. and 4°C, and separated on 4–20% Tris–glycine gradient gel (Novex). For Western blot, the proteins were transferred to Hybon ECL filter (Amersham) in 1× Tris–glycine buffer/20% methanol at 5 V/cm for 2 h at room temperature. The dilution of p300 and M2 antibody for the Western blots was 1:1000. The Western procedure was carried out in PBST and detected by the ECL system (Pierce).

'Histone' acetyltransferase (HAT) assay

For the HAT assay, 50–100 ng of either purified p300, deletion mutants or PCAF was incubated with ~1 μg of substrate protein, NF-YB or histone, in the presence of [^{3}H]acetyl-CoA in 1× HAT buffer [50 mM Tris pH 8.0, 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 30 min at 37°C in 20 μl volume. The reaction was stopped by an additional 20 μl of 2× SDS gel loading buffer and subjected to 4–20% SDS–PAGE analysis. The gel was then stained by Coomassie Blue R for 10 min and destained overnight. The signal of transferred [^{3}H]acetyl-CoA was enhanced by Amplify (Amersham), the gel was vacuum dried and exposed to MR film (Kodak).

Immunoprecipitation assay

A 10 oocyte equivalent of protein was used for each pull-down assay. Monoclonal antibody against p300 (a kind gift from Dr Betty Moran) was coupled to protein G–Sepharose (Phamacia Biotech). The M2 antibody was beaded to agarose (Kodak). The [^{35}S]methionine-labeled protein extract was incubated with either the beaded p300 or M2 antibody in 150 μl of 1× binding buffer (100 mM NaCl, 20 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 1 mM DTT and 1 mM PMSF) for 1 h at 4°C. The reaction was washed three times in 1 ml of washing buffer (250 mM NaCl, 20 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF and 0.05% NP-40). The pellet from the last wash was dissolved in 20 μl of SDS sample buffer and analyzed on 4–20% SDS–PAGE.

mRNA preparation and primer extension

The total RNA from oocytes was prepared by using RNA STAT-60 as instructed (Tel-Test, Inc). Primer extension was performed on two oocyte equivalents of RNA. CAT primer, 5′-GGTGGTATATCCAGTGATTTTT-TTCTCCAT-3′ was used to detect the specific *hsp70* transcript. H4 primer, 5′-GGCTTGGTGATGCCCTGGATGTTATCC-3′, was employed to probe the endogenous H4 mRNA as an internal control for correcting sample loading. Annealing was carried out in a 10 μl volume in 1× first strand buffer (Gibco-BRL) at 65°C for 10 min, followed by 55°C for 25 min, and finally 42°C for 10 min. The extension was performed in a 20 μl volume at 42°C for 1 h with Superscript II with conditions as recommended (Gibco-BRL). Then the reaction was stopped by 15 μl of denaturing loading buffer, and 5 μl of the sample was analyzed by 6% denaturing sequencing gel.

DNase I hypersensitivity analysis

Stage VI oocytes were injected and incubated as previously described. Groups of 45 healthy oocytes were collected and homogenized with 725 μl of the following buffer: 10 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol and 5 mM $MgCl_2$. The lysate was divided into three tubes, and 50, 60 and 70 U of DNase I, respectively, were added. The reaction mixture was incubated for 5 min at room temperature. The digestion was stopped by adding an equal volume of stop solution containing 0.5% SDS and 20 mM EDTA.

The samples were RNase A-treated for 1 h at 37°C. The DNA was purified by proteinase K treatment, phenol–chloroform extractions and isopropanol precipitations. The purified DNA was linearized by restriction enzyme digestion. The DNA was incubated for 10 min at 95°C in the presence of 0.1 M NaOH and then was neutralized with HCl. After purification by phenol–chloroform extraction and ethanol precipitation, the DNA was resuspended in 50 μl of a mixture containing 250 μM deoxynucleoside triphosphates (each), 1× *Taq* buffer, 5 mM $MgCl_2$, 0.8 pmol of a ^{32}P-labeled primer and 2 U of *Taq* polymerase (Promega). A linear PCR was performed. DNA thermal cycles were 95°C for 2 min, 52°C for 2 min and 70°C for 3 min. This process was repeated 15 times.

SDS was then added to a final concentration of 0.2%, and the DNA was ethanol precipitated. The primer utilized to footprint the HSEs was a 25mer, 5′-CCTTCATCGTCTTAGATTCGACTGT-3′, complementary to the hsp–CAT construct. The primer for the TATA box footprint was the same 30mer utilized in the primer extension assay.

For indirect end-labeling, the purified DNA was restricted with *Nco*I before resolution on a non-denaturing 1.5% agarose gel, transfer to a filter and hybridization with an *Eco*RI–*Nco*I DNA fragment that had been radiolabeled by random priming.

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Transcriptional regulation of the *MDR1* gene by histone acetyltransferase and deacetylase was recently shown to be mediated by NF-Y (Jin and Scotto, 1998).