Adaptor-mediated Recruitment of RNA Polymerase II to a Signal-dependent Activator*

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Communications

The second messenger cAMP stimulates the expression of a number of target genes via the protein kinase A-mediated phosphorylation of CREB at Ser-133 (Gonzalez, G. A., and Montminy, M. R. (1989) Cell 59, 675–680). Ser-133 phosphorylation enhances CREB activity by promoting interaction with a 265-kDa CREB binding protein referred to as CBP (Arias, J., Alberts, A., Brindle, P., Claret, F., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. (1994) Nature 370, 226–228; Chrvia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) Nature 365, 855–859). The mechanism by which CBP in turn mediates the interaction with specific transcription factors is unknown but is thought to involve recruitment of basal transcription factors to the promoter. Here we demonstrate that CBP associates specifically with RNA polymerase II in HeLa nuclear extracts. This association in turn permits RNA polymerase II to be recruited to CREB in a phospho-(Ser-133)-dependent manner. Anti-CBP antiserum, which inhibits recruitment of CBP and RNA polymerase II to phospho-(Ser-133) CREB, attenuates transcriptional induction by protein kinase A in vitro, our results demonstrate that the CBP–RNA polymerase II complex is critical for expression of cAMP-responsive genes.

A number of hormones and growth factors stimulate the expression of target genes by inducing the reversible phosphorylation of specific transcription factors (4). Although phosphorylation has been shown to regulate a number of nuclear factors by inducing their nuclear targeting or DNA binding activities, the cAMP-responsive transcription factor CREB belongs to a group whose transactivation potential is affected (1, 5). In this regard, Chrvia et al. (3) have characterized a nuclear CREB binding protein, termed CBP, which binds to CREB in a phospho-(Ser-133)-dependent manner. The requirement for CBP in mediating CREB-dependent transcription has been demonstrated by cellular microinjection experiments in which CBP antisera blocked transcriptional induction by cAMP (2) and by transient transfection experiments in which overexpression of CBP could potentiate CREB activity in response to agonist (2, 6). Here we examine the mechanism by which CBP interacts with the transcriptional apparatus to induce target gene expression in response to hormonal stimulation. Our results suggest that CBP is constitutively associated with specific components of the transcriptional apparatus and that this association in turn permits recruitment of certain basal factors to promoters of cAMP-responsive genes.

EXPERIMENTAL PROCEDURES

Preparation of Nuclear Extracts and in Vitro Transcription Assays—Nuclear extract preparations and in vitro transcription assays were carried out as described previously (5). To evaluate the effect of PKA on in vitro transcription reactions, purified recombinant PKA catalytic subunit (1 μg) (kindly provided by S. Taylor) was added to HeLa nuclear extracts during transcription reactions. CREB activity was monitored with an adenovirus major late promoter template containing three cAMP-responsive elements (CREs) from the rat somatostatin promoter (~56 to ~32). Affinity-purified antisera were added to in vitro transcription assays as reported (5).

Immuno precipitation Assays—For immunoprecipitation assays, HeLa nuclear extract (100 μg) was precleared with protein A-Sepharose for 30 min at 4 °C. Precleared extract was incubated with primary antibody for 1 h at 4 °C. To detect RNA polymerase II, a monoclonal antibody, raised against a C-terminal domain polypeptide of the large subunit (Promega), was used. Antibody complexes were recovered by incubation with protein A-Sepharose for 1 h at 4 °C. Beads were washed three times with buffer (B100) containing 100 mM KCl, 10 μg/ml Tris, 1% Nonidet P-40, resuspended in 2 × SDS loading buffer, and analyzed by SDS-polyacrylamide gel electrophoresis.

Glutathione-Sepharose Chromatography—A CREB cDNA fragment encoding the kinase-inducible domain (aa 88–160) was fused in-frame to the glutathione S-transferase (GST) cDNA in the pGEX-2T plasmid (Promega). GST-KID fusion protein was expressed, and purification from BL21 Escherichia coli was carried out as described previously (3). GST-KID fusion protein was phosphorylated at Ser-133 using purified PKA catalytic subunit as described previously (7). For GST affinity chromatography, HeLa extracts (100 μg) were added to glutathione-Sepharose beads (50 μl) containing GST-KID or GST-phospho-(Ser-133)KID polypeptides and co-incubated for 1 h at 4 °C. Beads were washed three times with B100 buffer (see above) and then evaluated by Western blot analysis.

RESULTS AND DISCUSSION

Preliminary evidence suggesting that CBP migrates as a high molecular mass complex of 2000 kDa during gel filtration chromatography (not shown) prompted us to examine whether CBP might stimulate cAMP-responsive genes by virtue of its association with specific basal transcription factors. When purified from HeLa nuclear extracts by phosphocellulose chromatography (Fig. 1, top), CBP was detected predominantly in the 0.3 M KCl “B” fraction, which also contained RNA polymerase II. Following subsequent fractionation on a Mono-S ion exchange resin (Fig. 1, bottom), CBP again eluted with peak fractions of RNA polymerase II. In contrast to the relatively short elution profile for CBP, however, RNA polymerase II appeared to be more broadly distributed, indicating that only a fraction of RNA polymerase II may be associated with CBP. By contrast with RNA polymerase II, CBP did not co-elute from the Mono-S column with TFII B, a basal factor that has been reported to interact with CBP in GST pull-down assays (6).

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FIG. 1. The CREB co-activator CBP fractionates with RNA polymerase II during purification from HeLa nuclear extracts. Top, Western blot analysis of HeLa nuclear extract following fractionation over P11 phosphocellulose resin. A–D correspond to HeLa protein fractions eluted from the P11 column with 100 mM, 300 mM, 500 mM, and 1.0 M KCl, respectively. Analysis of CBP, RNA polymerase II (RNA Pol II), RNA polymerase I (RNA Pol I), and TFIIH in individual fractions as indicated on left. Bottom, Western blot analysis of the 300 mM KCl HeLa "B" fraction (top) following fractionation over Mono-S ion exchange resin. Elution profile for CBP and RNA polymerase II as indicated on left. FT, flow-through.

To test whether CBP in fact associates with RNA polymerase II, we performed co-immunoprecipitation studies with two distinct anti-CBP antiseras directed against aa 1–100 (5729) and aa 455–679 (5614) of the protein (Fig. 2A). Although neither antiserum was capable of recognizing purified RNA polymerase II directly by Western blot or immunoprecipitation assay (not shown), the large subunit of RNA polymerase II was detected in immunoprecipitates of HeLa extracts with both antisera under non-denaturing conditions. In agreement with the broad elution profile of RNA polymerase II following Mono-S chromatography, only a limited fraction (10–20%) of the RNA polymerase II large subunit appeared to be associated with CBP in HeLa extracts. By contrast with RNA polymerase II, other basal transcription factors such as TBP and TFIIB did not elute in immunoprecipitates of HeLa extracts with both antisera.

But PKA treatment had no effect on an internal control ade-

Addition of PKA to crude HeLa nuclear extracts induced transcription from a cAMP-responsive template containing three consensus cAMP-responsive elements (3 × CRE) approximately 4-fold. As predicted by GST affinity chromatography experiments, we performed immunoprecipitation assays on crude HeLa nuclear extracts (Fig. 3). Using a CREB antiserum (253) that can recognize the CREB-CBP complex (8), we detected CBP in immunoprecipitates from PKA-treated but not untreated HeLa nuclear extracts. Similarly, the large subunit of RNA polymerase II was recovered specifically from immunoprecipitates of PKA-treated HeLa extracts, demonstrating that PKA induces formation of a phospho(Ser-133) CREB-CBP-RNA polymerase II complex.

In order to determine whether recruitment of the CBP-polymerase II complex to phospho-(Ser-133) CREB is critical for transcriptional induction by PKA, we performed in vitro transcription assays on crude HeLa nuclear extracts (Fig. 4). Addition of PKA to nuclear extracts induced transcription from a cAMP-responsive template containing three consensus cAMP-responsive elements (3 × CRE) approximately 4-fold. But PKA treatment had no effect on an internal control ade-

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Fig. 3. PKA stimulates formation of a phospho-(Ser-133) CREB-CBP-RNA polymerase II (POL II) heteromeric complex in HeLa nuclear extracts. Western blot analysis (αCBP, αRNA POL II) of immunoprecipitates (IP) prepared from control (−) or PKA (+) treated HeLa nuclear extract with various antisera under non-denaturing conditions. ONPUT, crude HeLa nuclear extract prior to immunoprecipitation; 253, polyclonal anti-CREB antiserum raised against the full-length recombinant CREB protein (aa 1–341); 5729, CBP antiserum raised against a recombinant CBP polypeptide extending from aa 1–100; Pl, preimmune serum.

Fig. 4. The CBP-RNA polymerase II complex is required for transcriptional induction by PKA in vitro. Primer extension analysis of in vitro transcription reactions performed with HeLa nuclear extracts in the presence or absence of purified PKA catalytic subunit, as indicated above each lane (−, +). 3XCRE, transcription template containing three tandemly repeated somatostatin CRE sites inserted upstream of the adenovirus major late promoter. MLP, adenovirus major late promoter construct lacking CRE sequences. Transcription reactions containing affinity-purified αCBP antiserum or control anti-corticotropic-releasing factor binding protein (αCRFBP) antiserum as indicated over corresponding lanes.

CBP interacts with RNA polymerase II holoenzyme. In contrast to results of Kwok et al. [6], TFIIIB did not appear to associate detectably with CBP in HeLa extracts. These results would suggest that TFIIIB may interact with CBP only after being recruited to the promoter.

Our results do not address whether CBP interacts directly with RNA polymerase II. In preliminary GST pull-down assays, purified recombinant CBP polypeptides are unable to associate directly with the large subunit of RNA polymerase II, suggesting that the interaction between CBP and RNA polymerase II may either require post-translational modification (i.e., phosphorylation) or may involve other proteins within the RNA polymerase II holoenzyme complex.

In a previous study, we found that the activity of purified phospho-(Ser-133) CREB in cell-free transcription assays was indistinguishable from that of unphosphorylated CREB [9]. In this report, we found the addition of PKA to HeLa nuclear extracts during the transcription reaction was critical for Ser-133 phosphorylation-dependent activity. These results are consistent with recent findings that other PKA-dependent events in addition to CREB phosphorylation are required for transcriptional induction by cAMP in vivo [8]. In this regard, CBP contains a consensus PKA phosphorylation site at Ser-1772, and it is tempting to speculate that the interaction between CBP and RNA polymerase II may itself be regulated by CBP phosphorylation. In yeast, interaction between an upstream activator (GAL4) and a component of the yeast RNA polymerase II holoenzyme complex (GAL11) is sufficient for transcriptional induction [10]. The importance of CBP in mediating not only cAMP but also mitogen-inducible transcription (2) indicates that CBP may similarly provide contact points for recruitment of mammalian RNA polymerase II holoenzyme by multiple signal-dependent activators.

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