Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors

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We have examined the mechanism by which the cAMP-responsive factor CREB stimulates target gene expression following its phosphorylation at Ser-133. Using an in vitro transcription assay, we found that two signals were required for target gene activation: a phospho(Ser-133)-dependent interaction of CREB with RNA polymerase II via the coactivator CBP and a glutamine-rich domain interaction with TFIID via hTAF1130. The adenovirus El A oncoprotein was found to inhibit phospho(Ser-133) CREB activity by binding to CBP and specifically blocking recruitment of RNA Pol II to the promoter. Our results suggest that the recruitment of CBP–RNA Pol II complexes per se is not sufficient for transcriptional activation and that activator-mediated recruitment of TFIID is additionally required for induction of signal-dependent genes.

[Key Words: cAMP responsive activator; CREB; transcriptional activation; RNA Pol II; signal-dependent factors]

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cAMP stimulates the expression of numerous genes via the PK-A-mediated phosphorylation of cAMP response element binding (CREB) protein at Ser-133 (Gonzalez and Montminy 1989). Although phosphorylation has been shown to regulate a number of nuclear factors by enhancing their nuclear-targeting or DNA-binding activities, CREB belongs to a group of regulators whose transactivation potential is affected specifically (Gonzalez et al. 1991; Hagiwara et al. 1992).

The CREB transactivation domain is bipartite, consisting of constitutive and inducible activators, termed Q2 and the kinase-inducible domain (KID), respectively, which function cooperatively to induce target genes in response to cAMP stimulation (Brindle et al. 1993). The glutamine-rich Q2 domain has been shown to stimulate transcription via its association with a component of the TFIID fraction, the TBP-associated factor dTAF110 (Ferrer et al. 1994). In contrast, the KID region appears to recruit the transcriptional apparatus via its phospho(Ser-133)-dependent interaction with the related coactivators CREB-binding protein (CBP) and P300 (Chrivina et al. 1993; Parker et al. 1996).

Current evidence suggests that in addition to mediating CREB activity, CBP and P300 also function as coactivators for other signal-dependent factors. In this regard, CBP and P300 have been shown to interact with a number of phosphorylation-dependent activators such as 

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(Arias et al. 1994), STAT2 (Bhattacharya et al. 1996), and

elk-1

(Janknecht and Nordheim 1996), as well as certain nuclear receptors, including RAR, RXR, and TR

(Chakravarti et al. 1996; Kamei et al. 1996). In most cases, complex formation with CBP is signal dependent and occurs via a transactivation domain in each nuclear factor. The functional importance of these interactions has been illustrated by immunoneutralization studies in which microinjection of CBP antiserum could block agonist-dependent induction of appropriate reporter genes (Arias et al. 1994) and by transient transfection assays in which overexpression of CBP could potentiate activator-dependent transcription (Kwok et al. 1994). The involvement of CBP and P300 in these signaling events has been further supported by studies in which overexpression of the adenovirus E1A oncoprotein was found to repress transcriptional induction of numerous genes following its association with a carboxy-terminal domain in both coactivators (Eckner et al. 1994; Arany et al. 1995; Lunblad et al. 1995).

In a recent study we observed that activation of the ras pathway blocks phospho(Ser-133) CREB-dependent transcription via the mitogen-dependent recruitment of pp90RSK to CBP (Nakajima et al. 1996). Remarkably,
pp90rsk binds to the same region of CBP and P300 as the adenovirus E1A oncoprotein. And like E1A, binding of pp90rsk to that region is sufficient to inhibit phospho(Ser-133) CREB-dependent transcription. E1A and pp90rsk appear to inhibit transcription of cAMP-responsive genes without interfering with complex formation between phospho(Ser-133) CREB and CBP (Nakajima et al. 1996), suggesting that these repressors may interfere specifically with the ability of CBP to associate with proteins in the transcriptional apparatus.

In a recent series of biochemical experiments, we noted that CBP is a component of RNA polymerase II complexes (RPCs) in vivo (Kee et al. 1996). To evaluate the mechanism by which CBP mediates transcriptional induction of cAMP-responsive genes, we have reconstituted PK-A-dependent transcription via CREB in vitro using a partially purified RNA Pol II holoenzyme fraction that contains CBP and P300. We found that CBP mediated the recruitment of functional RNA Pol II activity to CREB in a phospho(Ser-133)-dependent manner. Remarkably, recruitment of CBP–RPCs did not suffice for transcriptional activation of cAMP-responsive genes: Association of CREB with TFIID via the TATA binding protein-associated factor hTAF5l30 was also required. Our results point to a two-component mechanism for activation of cAMP-responsive genes via CREB.

Results

A CBP–RPC mediates induction of cAMP-responsive genes via phospho(Ser-133) CREB

Previous work demonstrating that CBP associates with RNA Pol II (Kee et al. 1996) prompted us to examine whether partially purified complexes of RNA Pol II that contain CBP could support phospho(Ser-133) CREB-dependent transcription in vitro. Following fractionation of a HeLa whole-cell extract over Bio-Rex 70 resin, we obtained an 0.6 M potassium acetate eluate fraction that was enriched eightfold in CBP-associated RNA Pol II activity relative to total protein, as revealed by in vitro transcription and Western blot assay of CBP immunoprecipitates (Fig. 1A,B). Western blot analysis of CBP immunoprecipitates prepared from this 0.6 M fraction indicated that CBP was also associated with the Pol II holoenzyme component SRB7 (Fig. 1B).

To determine whether the CBP–Pol II complex could support phospho(Ser-133)-dependent CREB activity on a cAMP-responsive template, we performed in vitro transcription assays using purified basal transcription factors (TFIIA, TFIIB, TFIE, TFIIF, TFIH) and a phosphocellulose Hela TFIID fraction, plus the 0.6 M Bio-Rex fraction. Under these conditions, phospho(Ser-133) CREB was found to induce transcription from a 3× CRE–adenovirus major late promoter (MLP) template ~10-fold, whereas unphosphorylated CREB protein had no effect on promoter activity (Fig. 1C, cf. lanes 4 and 6). A mutant CREB polypeptide containing a Ser/Ala-133 substitution at the PK-A phosphoacceptor site was unable to stimulate transcription of the 3× CRE reporter in the presence of PK-A (Fig. 1C, lane 12), revealing the importance of Ser-133 for transcriptional induction in vitro. Mutant CREB polypeptides lacking the glutamine-rich Q2 domain were similarly inactive, indicating that, in addition to Ser-133 phosphorylation, Q2 was also important for CREB activity (Ferreri et al. 1994).

To test whether phospho(Ser-133) CREB stimulates target gene expression via a CBP-dependent mechanism in vitro, we added a dominant-negative CBP polypeptide that contains the CREB-binding domain (KIX) and that has been shown to block induction of cAMP-responsive genes when microinjected into fibroblasts (Parker et al. 1996). In vitro transcription reactions containing KIX peptide were unable to stimulate 3× CRE reporter activity via phospho(Ser-133) CREB, indicating that CBP is indeed required for PK-A-mediated transcription in vitro (Fig. 1C, cf. lanes 6 and 7). In control assays performed on immunoprecipitates of RNA Pol II prepared with anti-CTD antiserum, addition of KIX did not inhibit basal transcription from the MLP template, revealing that the KIX peptide does not block RNA Pol II or basal factor activities (Fig. 1D, right panel).

Adenovirus E1A oncoprotein has been shown to inhibit phospho(Ser-133) CREB-dependent activation by binding to a conserved carboxy-terminal domain in CBP and P300 via residues in conserved region 1 (CR1) and amino terminus of E1A (Eckner et al. 1994). Addition of an E1A polypeptide spanning exon 1 [amino acids 1–139] to in vitro transcription reactions also inhibited phospho(Ser-133) CREB-dependent activation of the 3× CRE template (Fig. 1C, lane 9). Phospho(Ser-133) CREB remained fully active, however, following addition of the mutant dl1101 E1A polypeptide (Egan et al. 1988) containing a deletion in CR1 [amino acids 4–25] that abolishes interaction with CBP or P300 (Fig. 1C, lane 10). But neither wild-type nor mutant E1A polypeptides were found to interfere with RNA Pol II or basal factor activities in control assays performed with an MLP template on RNA Pol II immunoprecipitates (Fig. 1D, left panel).

The CBP–RPC contains holoenzyme-associated proteins

To further characterize the CBP–RPC, we fractionated the 0.6 M Bio-Rex 70 column eluate over a 10%–60% sucrose gradient. In vitro transcription assay of CBP immunoprecipitates from individual gradient fractions revealed that the CBP–RPC was concentrated primarily in fractions 11–17, suggesting that these repressors may interfere specifically with the ability of CBP to associate with proteins in the transcriptional apparatus.
Pol II activity and CDK8 protein [Fig. 2A, bottom panel]. In activation assays with a 3x CRE-MLP template, peak sucrose gradient fractions were also capable of mediating phospho(Ser-133) CREB-dependent activation in conjunction with a highly purified TFIID fraction (εTFIID) (Zhou et al. 1992) (Fig. 2B). Consistent with a coactivator requirement for recruitment of both RNA Pol II and TFIID by phospho(Ser-133) CREB, neither core RNA Pol II [Fig. 2B, cf. lanes 1 and 3] nor TBP were able to substitute for the holo-Pol II and TFIID fractions in transcription assays with the 3x CRE–MLP template (Fig. 2B, cf. lanes 2 and 3).

Figure 1. [See facing page for legend.]
Phospho(Ser-133) CREB recruits RNA Pol II and TFIID activities

To test the prediction that phospho(Ser-133) CREB stimulates target gene expression by recruiting RNA Pol II activity via CBP, we performed affinity selection assays on the 0.6 M Bio-Rex fraction with biotinylated CREB and phospho(Ser-133) CREB proteins. Following incubation with the 0.6 M fraction, CREB protein complexes bound to avidin beads were washed, and binding of RNA Pol II activity to CREB and phospho(Ser-133) CREB was examined by an in vitro transcription assay using an MLP template plus all purified basal transcription factors except RNA Pol II. Phospho(Ser-133) CREB protein (P) but not unphosphorylated CREB [U] was found to recruit RNA Pol II activity from the 0.6 M Bio-Rex column fraction (Fig. 3, lanes 5–8).

When incubated with samples containing a phosphocellulose HeLa TFIID fraction, unphosphorylated CREB and phospho(Ser-133) CREB beads were found to associate comparably with TFIID activity, as revealed by in vitro transcription assays containing purified basal factors minus TATA binding protein (TBP) (Fig. 3, lanes 9–12). No TBP recruitment was noted when affinity selection assays were performed using purified TBP in place of TFIID, however, demonstrating the importance of TBP-associated factors (TAFs) for this interaction (not shown).

Following affinity selection assay with the 0.6 M Bio-Rex fraction supplemented with the Hela phosphocellulose TFIID fraction, phospho(Ser-133) CREB resin was found to recruit both TFIID and Pol II activities in vitro transcription assay [Fig. 3, lane 4].

Phospho(Ser-133) CREB associates with RNA Pol II and TFIID via the KID and Q2 domains, respectively

Previous data showing that KID mediates association of phospho(Ser-133) CREB with CBP (Parker et al. 1996) prompted us to examine whether RNA Pol II activity is recruited to CREB via this domain. In affinity selection assays, glutathione S-transferase (GST)–phospho(Ser-133) KID fusion protein was found to recruit RNA Pol II activity from the 0.6 M Bio-Rex fraction but GST–Q2 did not (Fig. 4A, cf. lanes 2 and 3). In Western blot assays, GST–phospho(Ser-133) KID resin also recruited RNA Pol II large subunit, whereas unphosphorylated GST–KID resin did not (not shown). These results indicate that the KID domain mediates recruitment of RNA Pol II activity to phospho(Ser-133) CREB.

Although the KID region mediates transcriptional induction in response to PK-A, the inability of CREB Q2-deletion mutants to stimulate CRE-dependent transcription (Brindle et al. 1993) suggests the requirement for a second signal in addition to recruitment of CBP. Previous data showing that Q2 specifically interacts with one component of the TFIID fraction [dTAF110] but not others (TBP, dTAF40, dTAF80) (Ferreri et al. 1994) prompted us to examine whether TFIID is functionally recruited to CREB via this coactivator. In affinity selection assays with GST fusion proteins containing either the Q2 or KID domains of CREB, GST–Q2 but not GST–phospho(Ser-133) KID resin was found to associate with TFIID by recruitment/in vitro transcription assay (Fig. 4B, cf. lanes 2–5). These results support the notion that KID and Q2 function as independent activators that synergize in response to CAMP stimulation (Brindle et al. 1995).
Nakajima et al.

**Figure 2.** (A) The CBP-RPC cofractionates with certain general transcription factors and components of the mammalian Pol II holoenzyme complex. Purification of the 0.6 M Bio-Rex 70 column fraction over a 35-ml 10%-60% gradient. One-milliliter sedimentation fractions were collected, and numbers above each lane correspond to odd-numbered fractions. (Top) Western blot assay of sucrose gradient fractions using antiserum against mammalian RNA Pol II holoenzyme component CDK8. (Middle) In vitro transcription assay of CBP immunoprecipitates prepared from odd-numbered sucrose gradient fractions. Immunoprecipitates were washed and then analyzed for RNA Pol II activity on an MLP template in reactions containing all purified general transcription factors (TFIIB, TBP, TFIIE, TFIIF, TFIIH) except RNA Pol II. Recovery control (CON) and MLP transcripts as shown. (Bottom) In vitro transcription assay of TFIH activity in individual sucrose gradient fractions using linearized MLP template. In vitro transcription assays were performed with MLP template plus all purified GTFs minus TFIH. (B) CBP-RNA Pol II and TFIID complexes are both required for phospho(Ser-133) CREB-dependent activation of a cAMP-responsive template. In vitro transcription assay of 3x CRE-MLP template in reactions containing purified basal transcription factors (TFIIA, TFIIB, TFIIE, TFIIF, TFIH) plus either highly purified Hela TFIID (cTFIID, IID) or recombinant TATA-binding protein (TBP). RNA Pol II activity was supplied either as a sucrose gradient purified holoenzyme fraction (HOLO) or core RNA polymerase (CORE). Addition of recombinant phospho(Ser-133) CREB or unphosphorylated CREB, as indicated. (CON) The 550-nucleotide ³²P-labeled RNA used as recovery control.

The Q2 domain recruits TFIID activity via hTAF₁₁₃₀

The recent characterization of cDNA clones for hTAF₁₁₃₀ [Tanese et al. 1996], the human homolog of Drosophila dTAF₁₁₁₀, led us to evaluate whether the Q2 domain recruits TFIID via its association with this protein. In affinity selection assays with ³⁵S-labeled hTAF₁₁₃₀, Q2 but not phospho(Ser-133) KID was found to associate with hTAF₁₁₃₀ (Fig. 5A, cf. lanes 3 and 4). Moreover, both CREB and phospho(Ser-133) CREB associated comparably with hTAF₁₁₃₀, demonstrating the phosphorylation-independent nature of this interaction (Fig. 5A, lanes 5,6). To determine whether hTAF₁₁₃₀ was required for recruitment of TFIID activity via Q2, we added affinity-purified hTAF₁₁₃₀ antigen to pull-down reactions containing GST–Q2 resin plus crude TFIID fraction. Although preimmune antiserum had no effect on the ability of GST–Q2 to recruit TFIID activity by in vitro transcription assay, addition of hTAF₁₁₃₀ antiserum strongly inhibited association of TFIID activity with Q2 (Fig. 5B, cf. lanes 2 and 3). Addition of hTAF₁₁₃₀ antiserum to in vitro transcription reactions also blocked the ability of phospho(Ser-133) CREB to stimulate transcription from a 3x CRE MLP (Fig. 5C, cf. lanes 5 and 6). hTAF₁₁₃₀ antiserum did not inhibit basal transcription of MLP template (MLP). Reactions were supplemented with ³²P-labeled 550-nucleotide RNA as recovery control (CON).
transcription from an MLP template, however, demonstrating that this reagent does not block TFIID activity per se. These results reveal that the association of CREB with TFIID via hTAF130 is required for transcriptional induction by protein kinase A [PK-A].

**E1A blocks phosho(Ser-133) CREB activity by disrupting CBP–RPCs**

To evaluate the mechanism by which E1A blocks activation of cAMP-responsive genes via phosho(Ser-133) CREB, we examined the effects of this oncoprotein on recruitment of RNA Pol II via the KID region. Recruitment/in vitro transcription assays with GST–phospho(Ser-133) KID resin revealed that wild-type but not dl 1101 mutant E1A polypeptide blocked association of phospho-KID with RNA Pol II activity (Fig. 6A, cf. lanes 3 and 4). E1A did not appear to interfere directly with RNA Pol II activity, however, as determined by analysis of immunoprecipitates of RNA Pol II recovered from the 0.6 m Bio-Rex fraction with anti-carboxy-terminal domain [CTD] antiserum (Fig. 1D, left panel).

To distinguish whether E1A disrupts recruitment of RNA Pol II activity to phosho(Ser-133) CREB by interfering with CBP–phospho(Ser133) KID or CBP–RNA Pol II interactions, we performed Western blot assays of GST–phospho(Ser-133) KID resins after pull-down assay.
actions containing all purified basal factors minus RNA Pol II. In vitro transcription assay using MLP template in re-
phospho(Ser-133) KID resin. Recruitment of Pol II was exam-
resins following affinity selection assay with 0.6 M Bio-Rex frac-
wild-type but not mutant ElA appeared to block the re-
pho(Ser-133) KID, neither wild-type nor mutant ElA pro-
with the 0.6 M Bio-Rex fraction (Fig. 6B). Although the
KIX domain could block recruitment of CBP to phos-
pho(Ser-133) KID, neither wild-type nor mutant ElA pro-
was found to interfere with the phosphi(Ser-133) KID–CBP interaction (Fig. 6B). Remarkably, addition of
wild-type but not mutant ElA appeared to block the rec-
rent of RNA Pol II protein to phosphi(Ser-133) KID (Fig. 6B), suggesting that ElA represses phosphi(Ser-133)
CREB activity by promoting dissociation of CBP from RNA Pol II.

To evaluate whether ElA blocks the association of CBP with RPCs via a direct mechanism, we performed
affinity selection assays with a series of GST–CBP poly-
peptides spanning the CBP protein (Fig. 7A). Following
incubation with the 0.6 m Bio-Rex fraction, individual
CBP resins were tested for associated RNA Pol II activity
by in vitro transcription assay [Fig. 7A] and by Western
blot analysis (Fig. 7B). Only CBP polypeptides containing
the ElA-binding domain of CBP (amino acids 1805–1890)
were found to recruit functional RNA Pol II activity,
suggesting that ElA may repress phosphi(Ser-133) CREB
activity by directly blocking the ability of RNA Pol II to
associate with CBP. In this regard, addition of ElA
blocked binding of pol II to GST–CBP resins in vitro [not
shown].

Figure 6. Adenovirus ElA oncprotein blocks recruitment of
RNA Pol II activity to phospho(Ser-133) KID domain of CREB.
(A) In vitro transcription assay of GST–phosphi(Ser-133) KID
resins following affinity selection assay with 0.6 M Bio-Rex frac-
tion. Effect of wild-type ElA (wt ElA), mutant dl 1101 ElA (mt
ElA) polypeptide that is unable to bind to CBP, KIX polypeptide
(which contains the CREB-binding domain of CBP [amino acids
591–670]), or BSA on association of RNA Pol II activity with
phosphi(Ser-133) KID resin. Recruitment of Pol II was exam-
ined by in vitro transcription assay using MLP template in re-
actions containing all purified basal factors minus RNA Pol II.
[αCBP and opol II] RNA Pol II activity contained within immu-
noprecipitates of CBP and RNA Pol II, respectively. Anti-CTD
antiserum was used to immunoprecipitate RNA Pol II activity.
MLP transcript and recovery control (CON) as indicated. (B)
Western blot analysis of CBP [CBP, top] and RNA Pol II [pol II,
bottom] retrieved from GST–phosphi(Ser-133) KID resin fol-
lowing affinity selection assay with 0.6 M Bio-Rex fraction [see
Fig. 1A]. (ON) Twenty percent of total CBP and RNA Pol II
activity in 0.6 M Bio-Rex fraction prior to affinity selection
assay. Addition of BSA, mutant ElA (mut ElA), KIX, or wild-type
ElA (wt ElA) polypeptides to 0.6 M Bio-Rex fraction during af-
finity selection assay as indicated at top. Anti-CTD antiserum
was used to detect RNA Pol II large subunit in Western blot
assays. Solid wedges indicate increasing amounts of protein (2
μg or 20 μg). Relative mass (in kD) indicated at left.

Discussion

Association of ElA with P300 has been shown to block
cellular differentiation and to promote cell cycle entry
[for review, see Bayley and Mymryk 1994]. The ability of
ElA to inhibit transcriptional activation via a variety of
signal-dependent factors [Arany et al. 1995; Lunblad et
al. 1995; Bhattacharya et al. 1996] has supported the no-
tion that ElA may block the ability of P300 and CBP to
interact with the transcriptional apparatus.

In this paper we used a partially purified RNA Pol II
holoenzyme fraction to reconstitute phosphi(Ser-133)
CREB activity in vitro. Purified core Pol II does not ap-
pear to contain CBP by Western blot assay [M. Mont-
miny, unpubl.], and, correspondingly, core Pol II is un-
able to support transcriptional induction by phosphi(Ser-133) CREB (Fig. 1D). Thus, the association we
observed between CBP and Pol II is unlikely to be direct
but, rather, via holoenzyme-specific components.

The holo-Pol II complex was found to associate spe-
cifically with the kinase-inducible domain following PK-
A-mediated phosphorylation of CREB at Ser-133 [Fig. 8].
Recruitment of this holoenzyme was not sufficient for
transcriptional induction of a cAMP-responsive gene,
however. Association of CREB with TFIID via the gluta-
mine-rich constitutive activation domain Q2 was also
required. Thus, two signals were critical for target gene expression in response to cAMP stimulation [Fig. 8]. The importance of both signals for transcriptional activation is further illustrated by the CREB-related CREM-α, CREM-β, and CREM-ε proteins that contain a KID region yet appear to function as transcriptional repressors owing to the absence of a Q2 activation domain (Foulkes and Sassone-Corsi 1992; Brindle et al. 1993).

In addition to CREB, a growing number of signal-dependent factors appear to require CBP and P300 for target gene activation. Our results indicate that, by analogy with phospho(Ser-133) CREB, the recruitment of CBP alone does not suffice for transcriptional activation. We speculate that these signal-dependent factors will contain bipartite activation domains that, in addition to associating with CBP, are also capable of recruiting TFIIID.

In addition to E1A, a number of regulators such as the SV40 large T antigen and the mitogen-activated pp90RSK have been shown to repress cellular gene expression by interacting with the E1A-binding domain of P300 and CBP (Eckner et al. 1996; Nakajima et al. 1996). Our data indicate that these regulators may similarly inhibit transcription of signal-dependent genes by regulating the composition of RPCs.

The same surface of CBP that binds to Pol II also appears to interact with a histone acetylase, referred to as pCAF (Yang et al. 1996). pCAF has been proposed to mediate the transcriptional effects of CBP via a nucleosome displacement model in which signal-dependent factors like CREB may gain access to their target sites on chromatinized templates. pCAF did not appear to fractionate with the CBP–Pol II complex during Bio-Rex 70 column chromatography (T. Nakajima, J. Parvin, and M. Montminy, unpubl.), however, suggesting that the CBP–pCAF
complex is distinct from the CBP–Pol II complex. It will be of interest to determine whether CBP mediates different cellular activities depending on its association with the RNA Pol II holoenzyme or with pCAF.

Materials and methods

Expression and purification of general transcription factors and recombinant polypeptides

TFIIA, TBP, TFIIE, and TFIIH cDNAs were expressed in _Escherichia coli_, and the recombinant proteins were purified as described previously [Parvin et al. 1994]. Recombinant TFIIA-expressing constructs were a generous gift of R. Roeder, and TFIIA was prepared as per the published protocol [Dejong and Roeder 1993; Dejong et al. 1995]. TFIIID was obtained from HeLa whole-cell extracts by collecting an 0.5–1.0 m protein peak from a phosphocellulose column. Highly purified epitope-tagged TFIIID was obtained from a stably transfected line of _Hela_ cells expressing influenza HA epitope-tagged TBP as described previously [Zhou et al. 1992]. TFIIH was prepared from _HeLa_ whole-cell extracts as described previously [Parvin et al. 1994]. Core RNA Pol II was purified from calf thymus as indicated previously (Thompson et al. 1990). In assays for basal transcription, TFIIID was replaced by recombinant yeast TBP subunit. Lack of cross-contamination between individual general transcription factors was verified by in vitro transcription assay with an adenovirus MLP template (not shown).

Experiments with E1A were performed with recombinant polypeptides spanning exon 1 (amino acids 1–139) and containing CR1 of the Ad5 E1A gene, a region that is sufficient for binding to and inhibiting CBP/P300 activity [Egan et al. 1988]. The E1A mutant gene _dl_ 1101 contains a deletion from amino acid 4 to 25 that disrupts binding of E1A to CBP and P300 [Egan et al. 1988]. Wild-type and mutant E1A polypeptides were expressed as GST fusion proteins in _BL21_(DE3)_ E. coli_, purified on glutathione-Sepharose beads, and then cleaved from GST with thrombin. For transcription assays, 2 μg or 20 μg of mutant or wild-type E1A polypeptide was used in each reaction. The CREB-binding domain of CBP (KIX, amino acids 591–670 of CBP) was expressed as a GST fusion protein in pGEX-KR and purified as described previously. KIX polypeptide was cleaved with thrombin and concentrated on a centriicon filter. For transcription assays, 2 μg or 20 μg of KIX polypeptide was added to each reaction.

In vitro basal transcription assays with MLP template

In vitro transcription assays were performed in a reaction volume of 25 μl containing 100 mM potassium acetate, 6 mM magnesium acetate, 20 mM Tris-acetate (pH 7.9), 1 mM EDTA, 20% glycerol, 100 μM each ATP and UTP, 50 μM 3′-O-methyl GTP, 3 μM CTP, 1 mM DTT, 2.5 μg/ml of plasmid DNA template, and 10 μCi of [α-32P]CTP (800 Ci/mmole, DuPont NEN) plus transcription factors. For each reaction, 30 ng TFIIH, 8 ng of γTBP, 4 ng of TFIIIE [3 ng of p56 and 1 ng of p34], 100 ng of TFIIH [50 ng each of RAP 30 and RAP 74], 100 ng of core RNA Pol II, and 0.25 μl of the TFIIH fraction was used. Reaction components were mixed on ice and allowed to incubate for 60 min at 30°C.

For transcription reactions containing immune complexes, avidin, or glutathione beads, pull-down assays were performed overnight at 4°C on a rotator in 200 μl of final volume using 20 μg of 0.6 M Bio-Rex 70 or 1 μl of TFIIID fraction with 2 μg [15 μl of beads] of biotinylated CREB or GST fusion proteins plus binding buffer that contained 100 mM potassium acetate, 10 mM Tris-acetate [pH 7.8], 2 mM magnesium acetate, 0.1% NP-40, 1 mM sodium fluoride, 1 mM DTT, and 10 μM PMSF. Pull-down assays for RNA Pol II were performed with 0.1 μg [1 μl] of carboxy-terminal domain antibody (Promega). Prior to transcription reaction, immune complexes, avidin, and glutathione beads were washed four times with 300–500 μl of binding buffer. Transcription reactions were performed on the moist beads.

Reactions were terminated by adding 200 μl of stop mix containing 0.5% SDS, 7 M urea, 2.5 mM EDTA, 100 mM lithium chloride, 380 mM ammonium acetate, and 10 μg of tRNA. Samples were extracted with phenol–chloroform and precipitated with ethanol. Following resuspension with loading buffer, 32P-labeled transcripts were resolved by electrophoresis over 6% urea–polyacrylamide gels.

Transcriptional activation assays with CRE template

The adenovirus MLP used for activation assays with phospho(Ser-133) CREB and CREB (3x CRE–MLP) contained three CRE sites extending from -56 to -32 of the somatostatin gene inserted upstream of the MLP TATA box. Biotinylated recombinant CREB protein was expressed from a Pin Point Xa bacterial expression vector (Promega) and was phosphorylated with purified PK-A catalytic subunit [gift of Susan Taylor, University of California, San Diego]. Following kinase reaction, phospho(Ser-133) CREB beads were washed with 100 mM KCl, 10 mM Tris [pH 7.8] to remove unincorporated ATP and PK-A. For activation assays with 3x CRE–MLP template [2.5 μg/ml], 60 ng of CREB and phospho(Ser-133) CREB was added to transcription reactions for a final concentration of 60 nM. In activation assays, RNA Pol II activity was supplied by addition of 2 μg [1 μl] of the Bio-Rex 70 0.6 M potassium acetate column fraction containing the CBP–RNA Pol II holoenzyme complex. TFIIID activity was supplied by addition of crude Hela TFIIH fraction [1–2 μl]. Recombinant TFIIA was used only for activation assays [1 μl] and was prepared as described [Dejong and Roeder 1993; Dejong et al. 1995]. TFIIH, TFIIE, TFIIF, and TFIIH proteins were added as described above for basal transcription assays with MLP template. CRE–MLP template was preincubated with CREB, 0.6 M Bio-Rex 70 fraction, and TFIIID complex for 20 min on ice prior to the transcription reaction. For immunoneutralization assay with hTAF130 antiserum, preimmune serum or hTAF130 antibody [0.5 μg] [gift of N. Tanese, NYU Medical Center, New York, NY] was added to crude Hela TFIIH fraction [2 μl] on ice for 30 min prior to addition of CREB and 0.6 M Bio-Rex 70 fraction.

Sucrose gradient analysis

For sucrose gradient purification of the RNA Pol II holoenzyme complex, 6 ml [12 ng] of the 0.6 M Bio-Rex 70 column fraction was loaded onto 28 ml of a 10%–60% sucrose gradient containing 0.2 M potassium acetate, 20 mM Tris-acetate [pH 7.9], 1 mM EDTA, and 0.1% NP-40. Samples were centrifuged in a swinging-bucket SW28 rotor at 25,000 rpm for 16 hr [4°C]. Sedimentation fractions [1 ml] were then collected and were used for Western blot and in vitro transcription assays. For activation assays with 3x CRE–MLP template [Fig. 2B], 0.5 μl of fraction 15, containing 0.4 μg of RNA Pol II holoenzyme purified 70- to 100-fold relative to total protein, was added to each transcription reaction. In these assays, the highly purified epitope-tagged TFIIID was used. In vitro transcription assays of the sucrose gradient fractions for TFIIH activity were performed with linearized MLP template plus 3 μl of each sucrose gradient fraction.
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