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Functional Characterization of Core Promoter Elements: DPE-Specific Transcription Requires the Protein Kinase CK2 and the PC4 Coactivator

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Summary

Downstream core promoter elements are an expanding class of regulatory sequences that add considerable diversity to the promoter architecture of RNA polymerase II-transcribed genes. We set out to determine the factors necessary for *d*ownstream *p*romoter *e*lement (DPE)-dependent transcription and find that, against expectations, TFIID and the GTFs are not sufficient. Instead, the protein kinase CK2 and the coactivator PC4 establish DPE-specific transcription in an in vitro transcription system containing TFIID, Mediator, and the GTFs. Chromatin immunoprecipitation analyses using the DPE-dependent IRF-1 and TAF7 promoters demonstrated that CK2, and PC4 are present on these promoters in vivo. In contrast, neither PC4 nor CK2 were detected on the TAF1-dependent cyclin D promoter, which contains a DCE type of downstream element. Our findings also demonstrate that CK2 activity alters TFIID-dependent recognition of DCE sequences. These data establish that CK2 acts as a switch, converting the transcriptional machinery from functioning on one type of downstream element to another.

Introduction

The general transcriptional machinery has largely been considered necessary and sufficient for the transcription of all promoters by RNA polymerase II. This is especially true in vitro, where some levels of transcription can be established with RNA polymerase II and the general transcription factors (GTFs): TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (for a review, see Orphanides et al. [1996]). However, the biochemical analyses of RNA polymerase II promoters has been rather limited if one considers the diversity of promoters and activators. The predominant promoter used to identify the GTFs has been the adenovirus major late promoter (Ad-MLP) or some variation of it. Most of the conclusions derived, at least in mammalian-derived systems in vitro, are based on the use of this promoter and its derivatives, and this has led to the idea that the GTFs are necessary and sufficient for the function of all core promoter elements.

A corollary to this interpretation is that core promoters lack specificity. This means that although there are various combinations of TATA boxes and Inr elements, their function is recapitulated with the GTFs and TFIID (Smale and Kadonaga, 2003). However, more recently, additional core promoter elements have been described, suggesting that a more considerable variety of core promoter architectures exist. This new and emerging class of core promoter elements is collectively referred to as downstream elements (DEs). Thus far there are three classes of DEs: the downstream promoter element (DPE) (Burke and Kadonaga, 1996; Burke and Kadonaga, 1997), the downstream core element (DCE) (D.-H. Lee et al., submitted; Lewis et al., 2000), and the motif ten element (MTE) (Lim et al., 2004). These elements differ in sequence and architecture, with the DPE functioning in TATA-less promoters, while the DCE functions in TATA-containing promoters (Burke and Kadonaga, 1996; Lewis et al., 2000; Smale and Kadonaga, 2003) (D.-H. Lee et al., submitted). The MTE functions in TATA, Inr, and DPE contexts (Lim et al., 2004). This diversity may reflect a level of specificity not previously seen in the core promoter. For example, core promoters may play a role in dictating the functional interactions with upstream enhancers. Enhancers have been shown to have a specificity for either TATA boxes or DPE-containing promoters in *Drosophila* (Butler and Kadonaga, 2001; Ohtsuki et al., 1998), which might imply that different factors interact on these different promoter architectures to establish either TATA-enhancer or DPE-enhancer interactions.

The GTF TFIID is responsible for the recognition of all known core promoter elements, with the exception of the BRE (Lagrange et al., 1998). TFIID contains the TATA binding protein (TBP) and associated TAFs. TBP itself binds the TATA box, while TAF1 and TAF2 bind to and are necessary for initiator function (Chalkley and Verrijzer, 1999; Kaufmann and Smale, 1994; Kaufmann et al., 1996; Smale and Kadonaga, 2003; Verrijzer et al., 1995; Verrijzer et al., 1994). TAF1 has also been implicated in core promoter specific functions, most notably through an element in the cyclin D1 promoter and the DCE (Hilton and Wang, 2003; D.-H. Lee et al., submitted; Li et al., 2002; O'Brien and Tjian, 2000; Shen and Green, 1997; Wang and Tjian, 1994; Wang et al., 1997). Moreover, analysis of DPE function has revealed that TAFs 6 and 9 make direct contact with the DPE (Burke and Kadonaga, 1997).

However, other studies suggest that Inr function may require additional biochemical activities (Martinez et al., 1998). It has also been suggested that DPE function requires the NC2 repressor in a crude in vitro transcription system (Willy et al., 2000). These data suggest that factors in addition to the GTFs and TFIID are necessary for the function of certain types of core promoter elements; thus, core promoter specific transcription is established by these additional activities.

We suggest that there are also more general reasons

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to hypothesize the existence of RNA polymerase II core promoter specificity. It seems inherently contradictory that in eukaryotic organisms there is such considerable specificity and diversity in activators and other regulatory phenomena, but there is no corresponding specificity at the core promoter. If this "correspondence principle" were correct, how would such specificity manifest itself? One might expect that additional factors are to be found at the core promoter, facilitating communication between activators and unique targets within the core promoter. Clearly, bacterial promoters exploit specificity mechanisms via the family of sigma factors, which bind –35 and –10 regions in bacterial promoters (Gross et al., 1998; Gruber and Gross, 2003). One might expect that eukaryotic promoters incorporate activities and core promoter elements functionally analogous to the bacterial sigma factors. Indeed, it has been suggested that TFIID components are analogous to the bacterial sigma factors (Verrijzer et al., 1995).

The data presented herein offer direct evidence challenging the prevailing view of core promoters and their factor requirements. We find that, despite a functional correlation between TFIID and the DPE (Burke and Kadonaga, 1996; Burke and Kadonaga, 1997), TFIID is insufficient for DPE-specific transcription in vitro. Instead, using a functional transcription assay coupled with conventional biochemistry, we found that the protein kinase CK2, in conjunction with the coactivator PC4, establishes DPE-specific transcription. These data provide evidence of the unique requirements for core promoter-specific transcription and establish a level of factor specificity mediated by downstream elements.

Results

The GTFs and TFIID Are Not Sufficient to Reconstitute DPE-Dependent Transcription

Given the correlation between the physical and functional interactions between TFIID subunits and the DCE (D.-H. Lee et al., submitted; Lewis et al., 2000) and the DPE (Burke and Kadonaga, 1996; Burke and Kadonaga, 1997), we surmised that DPE function would be recapitulated using a purified system composed of GTFs and TFIID, as is the case with the DCE (D.-H. Lee et al., submitted). We constructed a DPE-dependent promoter containing Sp1 sites, the β-globin Inr element, with either wild-type or mutant human IRF-1 DPE promoter sequences (Figure 1A) (Burke and Kadonaga, 1997; Lewis and Orkin, 1995). This synthetic promoter construct clearly shows DPE-dependent transcription using nuclear extracts in vitro (Figure 1B, lanes 1 and 2). However, a highly purified reconstituted transcription system composed of recombinant factors (TFIIA, TFIIB, TFIIE, TFIIF, Sp1, and PC4) together with highly purified TFIID, TFIIH, and RNAPII isolated from HeLa cells did not recapitulate DPE-dependent transcription; both wild-type and mutant DPE templates did not behave as they did in nuclear extracts (Figure 1B, compare lanes 1 and 2 to lanes 3 and 4). This observation suggested to us that the nuclear extract contains activity necessary for DPE-dependent transcription. We then initially partially purified this activity (DSA: *D*PE *s*pecificity *a*ctivity), which when added to our reconstituted transcription system established DPE dependent-specific transcription (Figure 1B, lanes 5 and 6). We also assayed the human IRF-1 promoter (Sims et al., 1993), which contains the same DPE sequence (Burke and Kadonaga, 1997) used in our DPE template. Consistent with our studies, we found that DPE-dependent transcription is established using nuclear extracts and the reconstituted transcription system containing DSA (Figure 1C).

Since a previous study isolated the *Drosophila* NC2 as a factor necessary for DPE-dependent transcription using a crude *Drosophila* nuclear extract-based transcription system (Willy et al., 2000), we analyzed our fractions isolated from HeLa cells, such as TFIID, TFIIH, and RNAPII by Western blot and failed to demonstrate the presence of NC2 subunits Dr1 (Inostroza et al., 1992) and Drap1 (Mermelstein et al., 1996) (Figure 1D). We further attempted to assay for a role of NC2 in DPE-dependent transcription. We used nuclear extracts that had been depleted of NC2 (Castano et al., 2000) (Figure 1E) and analyzed these extracts for DPE-dependent transcription. We observed DPE-dependent transcription in the control nuclear extract (NE) (Figure 1F). As expected, we observed little transcription from either the MLP or DPE templates in the NC2-depleted NE (ΔNC2 NE), due to the concomitant loss of RNAPII, as reported previously. However, we were able to restore transcription to the depleted NE by adding purified RNAPII (Castano et al., 2000) (Figure 1F). This addition restored transcription of both the MLP and DPE templates, and furthermore, there was no transcription from the mutant DPE template. Thus, we found that DPE-dependent transcription requires an additional activity and that NC2 is not participating in DPE-dependent transcription either in our purified reconstituted transcription assays or in assays using nuclear extracts.

Purification of DSA

In order to identify the factor(s) necessary for DPE-specific transcription, we used the DPE-dependent transcription system described in Figure 1A as a biochemical complementation assay. The purification scheme is shown in Figure 2A. We found that the DSA activity bound strongly to phosphocellulose and eluted from the column with a 1 M KCl wash. Further fractionation revealed that the DSA activity did not bind to DEAE-cellulose (Figure 2A and data not shown). We found that DSA bound to heparin-agarose and eluted with a 0.4 M KCl wash (Figure 2A and data not shown). Further fractionation of DSA occurred using a monoS column (Figure 2A). Fractionation of the transcriptionally active monoS fractions over a Superose 6 gel filtration column resulted in the loss of DPE-specific transcription (data not shown), suggesting that DSA activity was split into two or more activites of differing sizes. Since less pure DSA fractions contained Mediator (data not shown), we surmised that Mediator might be one active component of DSA. We assayed for Mediator subunits by Western blot and found they resided in the 2 MDa region of the column (data not shown, but see Figure 2D).

Analysis of the Superose 6 fractions in the presence

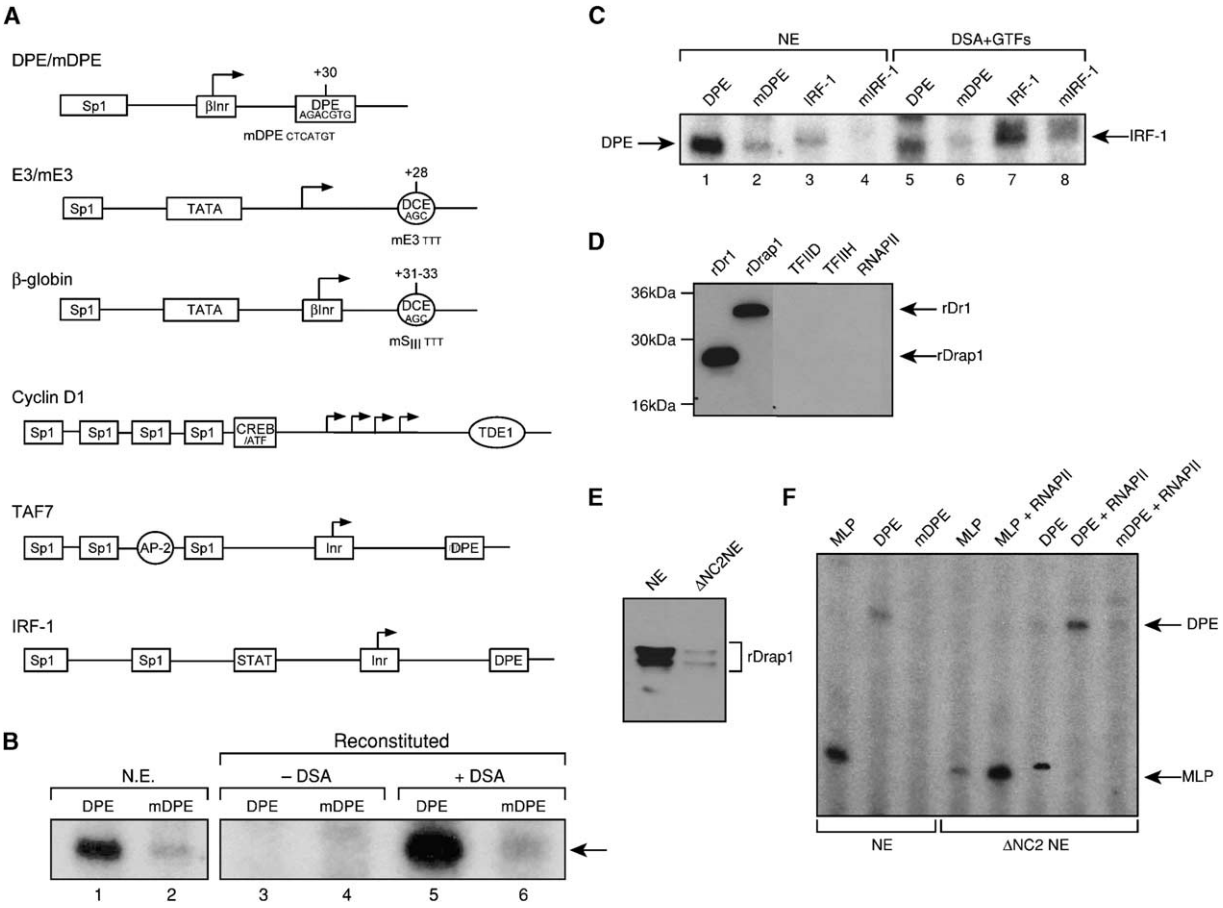

Figure 1. Functional Recapitulation of the DPE Requires Factors in Addition to the GTFs

(A) Schematic illustration of the promoters used in this study including relevant sequences pertaining to the subsequent experiments. The DPE was constructed from pSp1 from Smale and Baltimore (1989) and has been used extensively for Inr-dependent transcriptions. A typical Inr, in this case the β-globin Inr, was inserted downstream of the Sp1 sites (Lewis and Orkin, 1995). From +10 to +40, the human IRF-1 promoter sequence was inserted to obtain the DPE template. The mutant DPE template (mDPE) contains a mutation in the DPE sequence as indicated in the figure and is identical to that used by Burke and Kadonaga (1997). The adenovirus E3 promoter contains adenovirus 5 E3 promoter from –200 to +100. The mutant E3 promoter (mE3) contains a triplet mutation in its DCE subelement (the AGC at +27–29 was mutated to TTT) (D.-H. Lee et al., submitted). The human β-globin promoter template contains sequences from –815 to +100 (Lewis et al., 2000). The β-globin mutant DCE subelement III (mS_{III}) template contains an AGC to TTT mutation (Lewis et al., 2000; D.-H. Lee et al., submitted). The cyclin D1 promoter is from Hilton and Wang (2003) and contains the indicated activator binding sites and start sites and the TAF1-dependent element 1 (TDE1). The DPE-dependent TAF7 promoter was described by Chiang and colleagues (Zhou and Chiang, 2001; Zhou and Chiang, 2002).

(B) An activity derived from the P11 1M fraction is required to establish DPE-specific transcription in vitro. Lanes 1 and 2 show transcription of wild-type (DPE) and mutant DPE (mDPE) promoters in crude HeLa nuclear extracts. Lanes 3 and 4 show a transcription assay using wild-type DPE and mutant DPE templates in a highly purified in vitro transcription assay using GTFs, Sp1, and TFIID. Lanes 5 and 6 utilize the same purified in vitro system supplemented with a partially purified monoS fraction, termed *D*PE-specificity *a*ctivity (DSA).

(C) The IRF-1 DPE sequences establish DPE-specific transcription in vitro using either HeLa nuclear extracts (NE) or the highly purified reconstituted system containing the DSA fraction (DSA+GTFs). The human IRF-1 promoter template contains sequences from –1312 to +50. The mIRF-1 template contains the same DPE mutation as in mDPE (see also Burke and Kadonaga [1997]). The DPE and IRF-1 templates were also assayed in the highly purified in vitro transcription system containing DSA and GTFs.

(D) Affinity-purified TFIID, a purified TFIIH fraction and highly purified RNA polymerase II (RNAPII) were assayed by Western blot for the NC2 subunits Dr1 and Drap1. rDr1 and rDrap1 were added as positive controls. Twenty times the amount of TFIID, TFIIH, and RNAPII that were used in the reconstituted in vitro transcription assays were analyzed here.

(E) Western blot analysis of a nuclear extract and a nuclear extract depleted (ΔNC2) with an NC2/Dr1 antibody as assayed by anti-NC2/Drap1 antibody.

(F) In vitro transcription assays of ΔNC2 NE with adenovirus major late promoter (MLP) and DPE templates. As indicated by Castano et al. (2000), NC2 depletions remove RNAPII, which must be added back to the depleted NE and which was done as indicated (Castano et al., 2000).

of the 2 MDa fractions revealed two peaks of DSA activity: one eluting with an apparent mass of 500 kDa and the other as a 100 kDa species (Figure 2B). Mixing of the 500 kDa or the 100 kDa fraction with Mediator-established DPE-dependent transcription (Figure 2C). Importantly, when the 2 MDa fractions from the gel filtration column were pooled, fractionated over a monoQ column, and analyzed in the presence of the 100

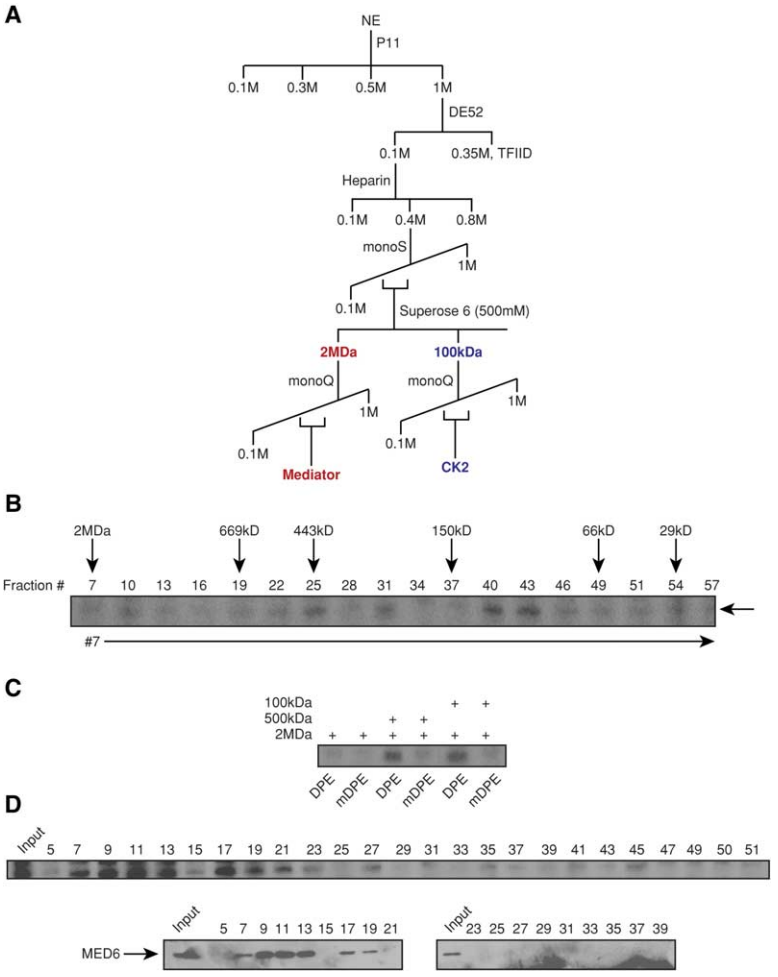

Figure 2. DSA Is Composed of Two Components: A 2 MDa and 100 kDa fraction

(A) Purification scheme of DSA.

(B) Superose 6 gel filtration fractionation of DSA. Shown are in vitro transcriptions in which the large 2 MDa activity has been added to the assay. The Superose 6 fractions are then assayed in that context and a peak activity was found at approximately 500 kDa (fraction 25) and 100 kDa (fractions 40 and 43).

(C) The 500 kDa and 100 kDa peak establishes DPE-specific transcription, along with the 2 MDa activity. Shown are in vitro transcriptions where either the 500 or 100 kDa fraction was assayed in the context of the 2 MDa fraction (fraction 7), and transcription from the wild-type DPE and mutant DPE (mDPE) promoters was compared.

(D) The 2MDa activity cofractionates with Mediator component MED6. The 2 MDa fraction from the Superose 6 column was pooled and fractionated over a monoQ resin. The resulting fractions were assayed in vitro in the context of the 100 kDa fraction and using the DPE template. Shown are the results of the in vitro transcription assay of the column fractions. Below is a Western blot analysis of the monoQ fractions for MED6, a component of the Mediator complex.

kDa fraction, a peak of transcriptional activity coeluted with the Mediator subunit MED6 (Figure 2D). Thus, we fractionated DSA into a 2 MDa Mediator fraction and two other fractions (500 kDa and 100 kDa) that, when independently mixed with the 2 MDa Mediator fraction, reconstituted DPE-specific transcription in vitro.

The 100 kDa Component of DSA Is CK2

Fractionation of the 100 kDa Superose 6 fraction using a monoQ resin yielded an activity peak in fractions 20–24 (Figure 3A). Analysis of the fractions by silver-stained SDS-PAGE indicated that a protein of approximately 40 kDa (indicated by the vertical arrow) correlated with transcriptional activity. The two bands of approximately 20 kDa did not correlate with transcriptional activity and were not examined further. The 40 kDa protein band was excised and analyzed by HPLC-tandem mass spectrometry (LC-MS/MS). Sixteen peptides were identified as the α' subunit of the protein kinase CK2 (Table S1 available in the Supplemental Data available with this article online). We also identified CK2 by mass spectroscopy in the 500 kDa activity (data not shown). To validate the identification of p40 as a subunit of CK2, we asked whether purified recombinant CK2 could replace this activity. Titration of rCK2 into the purified system in the presence of the 2 MDa Mediator fraction stimulated transcription from the DPE promoter; Mediator fraction alone was not functional (Figure 3B).

Since previous studies established a relationship between CK2 and PC4, and PC4 was present in the reconstituted transcription system, we analyzed the contribution of each factor in DPE-dependent transcription. PC4 is phosphorylated by CK2, which inhibits PC4's ability to mediate activator-dependent transcription (Ge and Roeder, 1994; Ge et al., 1994; Kaiser et al., 1995). Surprisingly, the combination of the coactivator PC4 and CK2 was necessary to establish DPE-specific transcription in vitro in a system reconstituted with all GTFs, TFIID, RNAPII, Sp1, and Mediator (Figure 3C). Thus, our results reveal that CK2 and PC4 are necessary and sufficient, in conjunction with Mediator, for DPE-specific transcription in vitro.

CK2 Is Necessary for DPE-Dependent Transcription in Crude Nuclear Extracts

Our results indicated a functional requirement for CK2 in a highly purified in vitro transcription system. We next tested for a CK2 requirement by using a crude nuclear extract assay which, by definition, contains

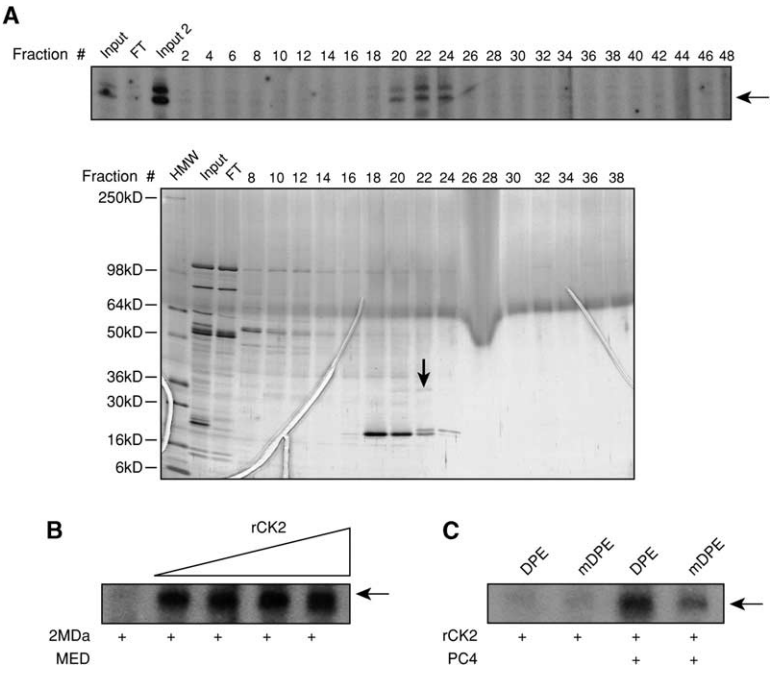

Figure 3. The 100 kDa Component of DSA Is the Protein Kinase CK2

(A) An approximately 40 kDa protein coelutes with the 100 kDa DSA transcription activity. Shown is an in vitro transcription assay (containing the 2 MDa Mediator activity) of the fractionation of the 100 kDa activity with a monoQ anion exchange column. Note the elution of the activity in fractions 20–24. Below is the silver stain PAGE analysis of the monoQ fractions. The arrow points to an approximately 40 kDa band in fractions 20–24 that correlated with the in vitro transcription activity.

(B) Recombinant CK2 (NEB) stimulates transcription from the DPE promoter in the context of the 2 MDa activity. rCK2 was titrated into an in vitro transcription assay using the highly purified system containing rSp1, GTFs, TFIID, and rPC4. The 2 MDa Mediator fraction supplemented each reaction.

(C) Establishment of DPE-specific transcription requires CK2 and PC4. Shown are in vitro transcriptions as in (B), where wild-type and mutant DPE templates were assayed in the context of either CK2 or CK2 and PC4. Mediator, rSp1, GTFs, and TFIID were present in all reactions.

factors necessary for DPE-dependent transcription (see Figure 1). Others have noted that peptides containing the CK2 consensus sequence can inhibit transcription in the CK2-dependent RNA polymerase III transcription system (Johnston et al., 2002). The addition of increasing amounts of a peptide containing the CK2-phosphorylation consensus sequence into HeLa nuclear extracts within a range of concentrations used previously (Johnston et al., 2002) resulted in the decrease of DPE-dependent transcription, whereas the nonspecific FLAG peptide had no effect (Figure 4A).

We next asked if the immunodepletion of CK2 from HeLa nuclear extracts would decrease transcription from the DPE template. Two types of depletion experiments were performed (Figure 4B). In the first experiment, we used antibodies against CK2β to deplete CK2 from nuclear extracts. We then compared transcription from the depleted and untreated extracts. We found that DPE transcription was reduced solely from the wild-type DPE template and not from the mutant template (Figure 4B, lanes 1–4). Importantly, we found that transcription from the Ad-MLP was not affected (Figure 4B, lanes 5 and 6). We then incorporated a second internal control into our experiments by blocking the CK2β antibody (chemically crosslinked to protein A-agarose beads) with either BSA or rCK2β. If the immunodepletion were specific for CK2β, we would predict that the BSA-blocked CK2β antibody would result in decreased transcription from the DPE template while the rCK2β-blocked antibody would not. We found this to be the case (Figure 4B, compare lanes 7 and 8 to lanes 9 and 10; quantitations of lanes 1–6 are indicated in Figure 4C). These results provide strong evidence that in crude nuclear extracts CK2 is indeed required for transcription from a DPE-containing template, but not from the Ad-MLP, a DCE-containing promoter (D.-H. Lee et al., submitted).

We next asked whether the depletion of PC4 from HeLa nuclear extracts would have an effect on DPE transcription. We depleted Hela nuclear extracts by using either protein A-agarose or protein A-agarose conjugated with a PC4 antibody. Equal volumes of the depleted control NE or antibody-depleted NE were analyzed by Western blot (Figure 4D). We were able to effectively remove PC4 from the nuclear extract. We added equal volumes of each extract to in vitro transcription reactions containing either wild-type or mutant DPE templates. As an additional loading control, we assayed for the presence of TBP (Figure 4D). When assaying the PC4-depleted NE, we observed that transcription from the wild-type DPE template was not affected but that transcription from the mDPE template increased. This indicates that the specificity for the DPE was diminished by the loss of PC4.

CK2 Function Is Dependent on Core Promoter Context

We next asked what function CK2 might have on a promoter that did not contain a DPE. The promoters we chose to analyze are the adenovirus E3 and human β-globin promoters, both of which contain a DCE element (D.-H. Lee et al., submitted). These promoters as well as their derivatives containing mutations in the DCE were assayed in the reconstituted transcription system containing TFIID and the GTFs, Sp1, and Mediator either with or without CK2 (Figure 5 and D.-H. Lee et al., submitted). In the absence of CK2, transcription from the E3 promoter (lane 1) was dependent on the presence of a DCE as mutation of this element reduced transcription to less than 40% of the wild-type E3 promoter (Figures 5A and 5B and D.-H. Lee et al., submitted). However, in the presence of CK2, transcription from the mE3 template was now similar to that of the wild-type promoter (Figure 5A, compare lanes 1 and 2

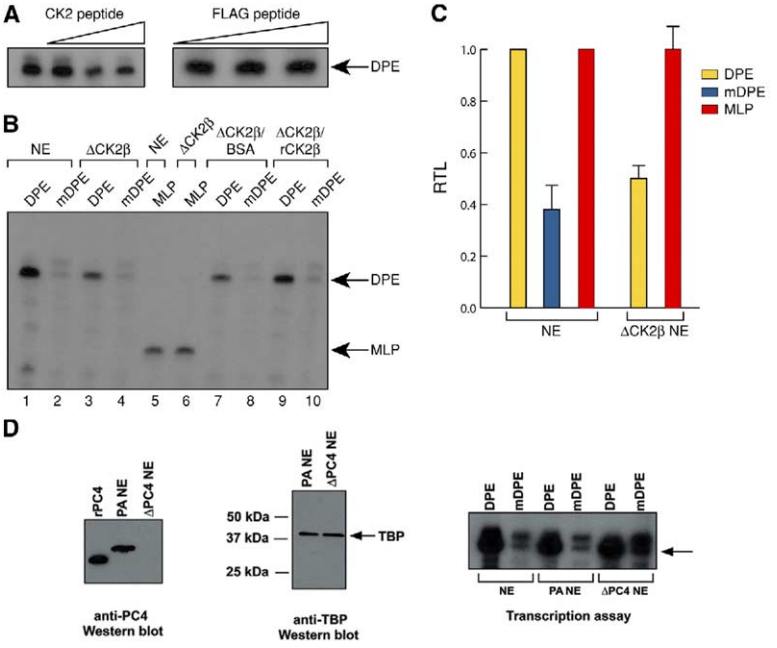

Figure 4. CK2 Is Necessary for DPE-Dependent Transcription in Crude Nuclear Extracts

(A) CK2 or FLAG peptide was titrated into HeLa nuclear extracts and transcription from the DPE-dependent promoter template was assayed by primer extension.

(B) Immunodepletions of the CK2β subunit abrogate DPE-dependent transcription. Wild-type or mutant DPE templates were used as indicated (DPE and mDPE, respectively). HeLa nuclear extracts (NE; lanes 1 and 2) were depleted with an anti-CK2β antibody (ΔCK2β; lanes 3 and 4) and assayed for DPE-dependent transcription. As a control, transcription from the adenovirus MLP was assayed with either the HeLa NE (NE, lane 5) or the CK2β-depleted NE (ΔCK2β, lane 6). Lanes 7 and 8 and lanes 9 and 10 show transcriptions with the indicated DPE templates using HeLa nuclear extracts depleted with CK2β antibody that was blocked with either BSA or rCK2β (ΔCK2β/BSA and ΔCK2/rCK2β, respectively).

(C) Shown are quantitations of the experiment in (B), corresponding to the data in lanes 1–3, 5, and 6 (DPE and mDPE, n = 3; MLP, n = 2). The error bars represent the standard deviations calculated from the relative transcription levels of three experiments. Western blot analysis indicated an approximately 50% reduction in CK2β levels in the depleted NE. Relative transcription levels are indicated (RTL) and are the ratio of the mutant template divided by the wild-type template.

(D) Depletion of PC4 from nuclear extracts reduces DPE-specific transcription. Hela nuclear extracts were depleted with either protein A-agarose alone (PA-NE) or protein A-agarose containing an anti-PC4 antibody (ΔPC4 NE). Equal volumes of the depleted extracts were analyzed by Western blot for the presence of PC4 and TBP. Hela nuclear extracts (NE), protein A-agarose treated nuclear extracts (PA NE), and PC4-depleted nuclear extracts (ΔPC4 NE) were assayed using wild-type DPE and mutant DPE (mDPE) templates. The arrow indicates the correct start site. The higher start site appears in predominantly in the mutant template and is the result of spurious initiation.

to 3 and 4; quantitations are shown in Figure 5B). In no case did the magnitude of transcription from the wild-type E3 promoter change in the presence of CK2. We observed similar effects on the human β-globin promoter. Mutation of the β-globin DCE subelement III (+31/33) abrogated transcription compared to the wild-type promoter in the absence of CK2 (Figure 5, lanes 5 and 6 and D.-H. Lee et al., submitted and Lewis et al., [2000]). In the presence of CK2, however, the effect of these mutations was dramatically reduced (Figure 5, compare lanes 6 and 8). Again, we did not observe any effects on the wild-type templates. These data suggest that CK2 reduces or eliminates the specificity of TFIID for the DCE (D.-H. Lee et al., submitted). This is in contrast to the results seen with the DPE, where CK2 is necessary for establishing DPE-specific transcription.

CK2, PC4, and Mediator Associate on the DPE-Dependent IRF-1 and TAF7 Promoters In Vivo in a Transcription-Dependent Manner

Our analyses established that CK2 and PC4 are necessary to reconstitute DPE-specific transcription. A further test of this hypothesis is to ask whether CK2 and PC4 are associated with DPE-dependent promoters in vivo. We performed chromatin immunoprecipitation (ChIP) experiments on endogenous DPE-dependent promoters by using antibodies specific for different transcription factors. The human IRF-1 promoter is inducible by IFNγ (Sims et al., 1993) and contains a functional DPE (Burke and Kadonaga, 1997). We examined the IRF-1 promoter and coding region in the presence and absence of IFNγ. In agreement with our biochemical reconstitution of DPE-dependent transcription, we found PC4, CK2α and β subunits, Mediator subunits MED10 and MED23/Sur2, as well as components of the transcriptional machinery (TBP, RNA polymerase II, TFIIF, and TFIIH) on the IRF-1 promoter. Importantly, these factors were detected at the promoter only after induction with IFNγ (Figure 6). As expected, only RNAPII was detected in the coding region (Rpb4), consistent with transcription of the IRF-1 promoter upon IFNγ induction (Sims et al., 1993). Under these conditions, we also observed an increase in histone marks that are associated with transcription activity: histone H3 acetylation and H3 lysine 4 tri-methylation within the promoter and coding sequences.

The human TAF7 ($TAF_{II}55$) promoter was shown to contain a functional DPE (Zhou and Chiang, 2001; Zhou and Chiang, 2002). Therefore, we examined this promoter for the presence of CK2 and PC4 using ChIP. Consistent with our in vitro requirements and ChIP analysis of the human IRF1 promoter, we found detectable levels of CK2α and β subunits and PC4 on the TAF7 promoter (Figure 6B). Several positive controls for actively transcribed promoters were included (H3-Ac, H3K4-3Me, and TFIIH).

Several groups have shown that the cyclin D1 promoter is a TAF1-dependent promoter containing a TAF-1-dependent element immediately downstream of the transcriptional start sites (TDE1) (Hilton and Wang, 2003; Suzuki-Yagawa et al., 1997; Wang et al., 1997). We did not find a DPE sequence within this region and thus surmised that the cyclin D1 promoter would serve as a negative control in our experiments. Consistent

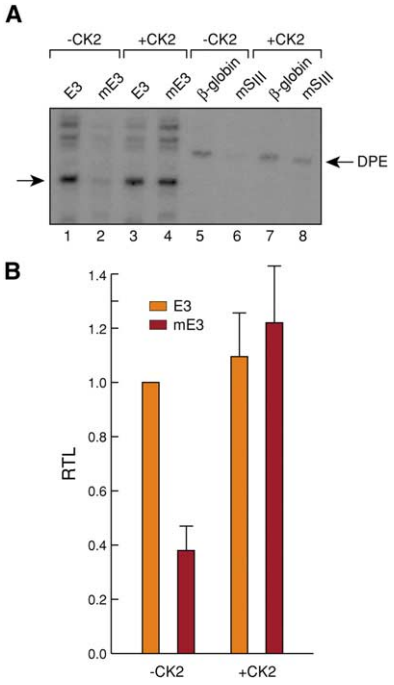

Figure 5. CK2 Activity Abrogates TFIID-Dependent Function of the Inr and DCE Subelements

(A) In vitro transcriptions containing the GTFs, TFIID, Sp1, and Mediator were incubated with the indicated promoter template either in the absence or presence of rCK2 as indicated (–CK2 and +CK2). Shown are primer extension products. E3 is the adenovirus E3 promoter; mE3 is a substitution mutation of the AGC at +27–29 (see Figure 1A and D.-H. Lee et al., submitted). β-globin is the human β-globin promoter (see Figure 1A) (Lewis et al., 2000). mS_{III} is the +31/33 mutation in the β-globin DCE (see Figure 1A, D.-H. Lee et al., submitted, and Lewis et al. [2000]).

(B) The bar graph shows the quantitation of three experiments corresponding to the data in (A), lanes 1–4. The error bars represent the standard deviations calculated from the relative transcription levels of three experiments.

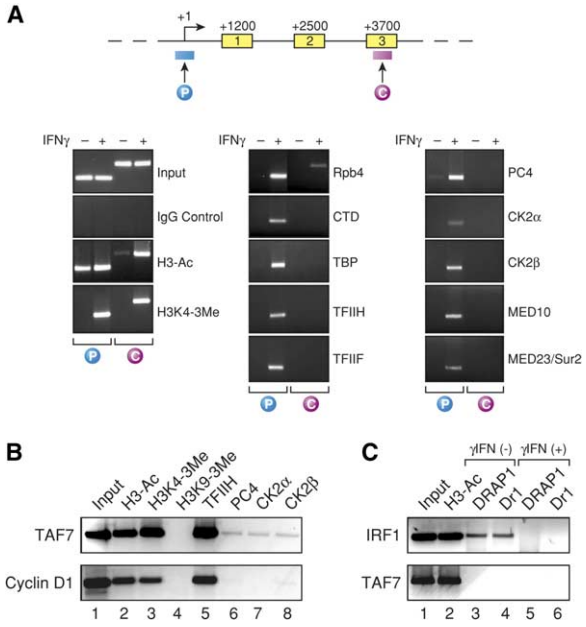

Figure 6. Chromatin Immunoprecipitations Indicate the presence of CK2, PC4, Mediator, and the GTFs on the DPE-Dependent Human IRF-1 and TAF7 Promoters

A schematic of the human IRF-1 promoter is illustrated at the top of the figure (see also Figure 1A). The location of the promoter (blue box and a "P" contained within a circle) and coding region (purple box and a "C" contained within a circle) PCR products are indicated. Shown are the results of ChiP assays of the IRF-1 promoter before (–) and after (+) a 3 hr stimulation of HeLa cells with IFNγ. The antibody (see Experimental Procedures) used in the ChiP is indicated adjacent to the PCR product. Note that the PCR product of the coding region primer set is larger than that of the promoter primer set (for example, see the panel labeled "Input").

(B) Chromatin immunoprecipitations of the human TAF7 and cyclin D1 promoters where performed using HeLa cells. ChiP products were amplified using primers flanking either promoter.

(C) Chromatin immuoprecipitations of the DPE-dependent IRF-1 and TAF7 promoters assaying for the presence of the two subunits of NC2, Dr1, and Drap1. Both promoters were assayed in the absence (lanes 3 and 4) and presence (lanes 5 and 6) of IFNγ as in panel A. All three panels show ethidium bromide-stained agarose gels of the PCR products of the indicated ChiP.

with the absence of a DPE in this promoter, we could not detect CK2 or PC4 by ChIP (Figure 6B). As with the IRF-1 promoter after IFNγ treatment, and the TAF7 promoter, we detected the presence of several protein epitopes known to correlate with actively transcribed genes (TFIIH, H3-Ac and H3K4-3Me; Figure 6B).

We also tested for the presence of NC2 on the two DPE-dependent promoters to address the reported requirement of NC2 for DPE-dependent transcription in vitro (Willy et al., 2000). While we did detect both NC2 subunits (Dr1 and Drap1) on the uninduced IRF-1 promoter, we failed to detect either subunit on the transcriptionally active IRF-1 and TAF7 promoters (Figure 6C). Thus, the presence of NC2 does not correlate with the transcriptional activation of these two DPE-dependent promoters. These results establish that CK2 and PC4 are present at DPE-dependent promoters in vivo.

Discussion

Downstream elements (DEs) are an emerging family of core promoter elements, and thus far, three classes have been described: the DPE, the DCE, and the MTE. Comparison of the DPE and DCE indicates that they each have very unique architectures that imply corresponding differences in the specificity and/or architecture of the transcription initiation complex. The DPE consists of the sequence 5′-A/GGA/TC/TG/A/C-3′ and is located approximately 30 base pairs downstream of the start site of transcription (Smale and Kadonaga, 2003), whereas the downstream core element (DCE) contains a more complex architecture consisting of three subelements located at approximately +10, +20, and +30 of a subset of TATA-containing promoters (Lewis et al., 2000; D.-H. Lee et al., submitted). Both the DPE and DCE elements are bound by specific subunits of TFIID, suggesting that TFIID is necessary for downstream element function. DPE is bound by $dTAF_{II}40$ and $dTAF_{II}60$ (TAFs 9 and 6, respectively) in vitro (Burke and Kadonaga, 1997). Importantly, the DCE is bound by TAF1 (D.-H. Lee et al., submitted). One might have expected that despite the differences in se-

quences, both DEs function in a similar manner by recruiting TFIID and functionally interacting with a specific subset of TAFs. The studies here suggest that the situation is more complex and thus more intriguing. DCE function can be recapitulated in a highly purified transcription system composed of the GTFs including TFIID (D.-H. Lee et al., submitted). In contrast, we found that TFIID and the GTFs are not sufficient to recapitulate DPE function in vitro.

Our biochemical analyses uncovered that the protein kinase CK2 and the coactivator PC4 are necessary to reconstitute DPE-dependent transcription (Figures 1–3). Our biochemical findings are fully supported by additional in vitro studies showing the requirement for CK2 and PC4 in crude nuclear extracts (Figure 4), and our in vivo studies demonstrating the association of CK2 and PC4 with the DPE-dependent IRF-1 and TAF7 promoters (Figure 6). Our experiments also demonstrate a role for CK2 that is promoter-context dependent. Strikingly, the addition of CK2 to an in vitro transcription reaction containing a DCE-dependent promoter had opposite effects to those seen with the DPE (Figure 5). We unexpectedly found that CK2 abrogated the effects of DCE mutations. We have shown elsewhere that TFIID, and specifically TAF1, recognizes DCE sequences (D.-H. Lee et al., submitted). Thus, we hypothesize that TAF1 is phosphorylated by CK2, after which TAF1 loses its ability to bind DCE sequences in the E3 and β-globin promoters. This is discussed below.

Previous studies have suggested that NC2 is necessary for DPE-specific transcription using a *Drosophila* derived system (Willy et al., 2000). Our data do not support this finding, although we cannot rule out the possibility of an indirect effect or that NC2 functions differently in *Drosophila* and humans. Secondly, our highly purified reconstituted transcription system is different than the low-salt nuclear extract utilized to assay for DPE-dependent transcription in the previous studies (Willy et al., 2000). These two disparate systems may therefore reflect differing aspects of DPE-dependent transcription. However, ChIP experiments do not indicate that NC2 is present on two DPE-dependent promoters, IRF-1 and TAF7 (Figure 6). We do find NC2 present on the uninduced IRF-1 promoter, which is entirely consistent with its previously suggested role as a repressor. Immunodepletion of NC2 from HeLa nuclear extracts also does not have any effect on DPE specific transcription (Figure 1).

We also note that we purified Mediator during our establishment of DPE-dependent transcription. Since our transcription reactions contained Sp1, this is not a surprising result, and has been reported by Tjian and colleagues (Ryu and Tjian, 1999; Ryu et al., 1999).

The Coactivator PC4

The second point of this work is that we provide in vivo evidence that PC4 is a bona fide transcription factor. It is required for DPE-dependent transcription and is present on the IRF-1 and TAF7 promoters in chromatin immunoprecipitation experiments in vivo. PC4 has been a somewhat controversial factor. It was initially discovered by virtue of its ability to augment the relative transcriptional stimulation by an activator (Ge and Roeder, 1994; Kretzschmar et al., 1994). Additional data, however, does suggest a true coactivator role wherein activator-dependent transcription was considerably elevated in the presence of PC4 (Ge and Roeder, 1994), and immunodepletion of PC4 was transcriptionally deleterious (Luo et al., 1998). Contrary to reports on the yeast PC4 (Calvo and Manley, 2001), we did not detect PC4 in the coding region of the IRF-1 promoter. This may reflect differences between yeast and human PC4 function or that PC4 function in posttranscriptional events is gene specific.

Protein Kinase CK2

CK2 displays a large number of apparent targets and its pleiotropic effects have made it difficult to assign CK2 a specific biological role. Over 300 CK2 targets have been documented and at least 100 of those have transcription functions (Pinna, 2002). Therefore, the paradox of CK2 is its apparent constitutive activity (Pinna, 2002). However, CK2 does have a significant presence in the nucleus (Filhol et al., 2003; Krek et al., 1992; Penner et al., 1997) and because a large number of its potential substrates are transcription factors, CK2 likely plays an important role in transcriptional regulation. More direct evidence of this is CK2's well-established role in RNA polymerase III transcription (Filhol et al., 2003; Ghavidel et al., 1999; Ghavidel and Schultz, 1997; Ghavidel and Schultz, 2001; Hockman and Schultz, 1996; Hu et al., 2003; Johnston et al., 2002; Penner et al., 1997). Based on our findings here, we suggest that one role of nuclear CK2 is that of a transcriptional regulator of RNAPII genes. Furthermore, CK2's function might not be restricted to IRF-1- and DPE-dependent transcription. Instead, it may be found on a variety of promoters, perhaps playing a more general role in regulating transcription.

The Integration of Downstream Element Function via CK2

Our data, combined with our additional studies of the β-globin DCE (D.-H. Lee et al., submitted), suggest an interesting model which combines our analysis of TFIID and CK2 function on the DPE and DCE classes of downstream elements (Figure 7). Several studies have shown that human and yeast TAF1 is phosphorylated by CK2 (Sawa et al., 2004; Sekiguchi et al., 1991). Buratowski and colleagues have also shown that CK2 copurifies with yeast TFIID (Auty et al., 2004). Sekiguchi et al. (1991) also indicated that TAF1 contains an HMG box, which we suspect is responsible for the TAF1-dependent interactions with the DCE (D.-H. Lee et al., submitted). Notably, our experiments (Figure 5) indicate that CK2 activity disrupts DCE recognition. Finally, several groups have shown that several HMG proteins contain CK2 phosphorylation sites C-terminal to the HMG domain. Phosphorylation of these domains by CK2 alters their conformation and DNA binding activity (Stemmer et al., 2002; Wisniewski et al., 1999). Therefore, we hypothesize that CK2-mediated phosphorylation of TAF1 converts TFIID from a DCE-specific recognition function to a DPE-specific recognition activity. TAF1 phosphorylation results in an overall conformational alteration such that TFIID is capable of DPE recognition

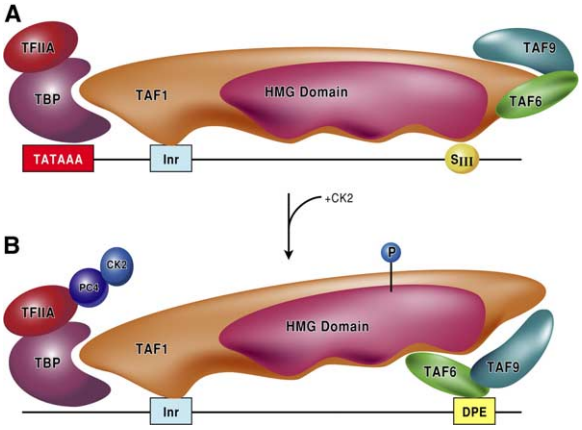

Figure 7. Proposed Hypothetical Model on the Mechanism Establishing DPE-Specific Transcription

(A) TFIID recognizes the DCE class of downstream elements via an interaction with the HMG domain of TAF1 (D.-H. Lee et al., submitted). The three-DCE subelement III is indicated as S_{III}. TAF1 physically interacts with S_{III} in a sequence-specific manner (D.-H. Lee et al., submitted).

(B) CK2 recruitment to the PIC results in the phosphorylation of TAF1, which either alters the DNA binding properties of the TAF1 HMG domain, or it results in a TFIID/TAF1 conformational change, disrupting TFIID interaction with the DCE. TFIID now functionally interacts with DPE sequences via TAF6/TAF9.

via TAF6/9 (Burke and Kadonaga, 1997). Thus, our hypothesis is that CK2 phosphorylation of TAF1 functions as an "on/off" specificity switch, determining whether TFIID has a DCE or DPE function. Although we have not shown that CK2 catalytic activity is necessary, we have preliminary data indicating that TAF1 and PC4 are phosphorylated by CK2 in the context of a PIC (unpublished data). We also suggest that the other TAFs have roles in interacting with additional, unknown core promoter elements and that other TAF posttranslational modifications, such as methylation (Kouskouti et al., 2004) and sumoylation (Boyer-Guittaut et al., 2005), may serve as TFIID-specificity switches as well.

Downstream Element Function

Why do downstream core promoter elements exist? We suspect that the answer lies in considering the promoter in its own natural context, the activators required, and the promoter's regulation in vivo. Secondly, the answer lies in further understanding the role of TAFs in transcriptional regulation. Finally, the additional factor requirements for DPE function (in contrast to DCE function) suggest a level of regulation at the core promoter and that TAF contacts with core promoter elements are not all equivalent. Promoter activation has usually been considered limited to activator binding to an upstream DNA binding site. All activators then target a set of GTFs and coactivators (including Mediator). But the additional core promoter cofactors discovered in this study suggest a level of regulation manifested by the core promoter itself, which has some experimental support (Butler and Kadonaga, 2001; Ohtsuki et al., 1998).

Our results here have implications for the study of promoters in general. We suspect that once other promoters are examined carefully, additional factors and regulators will be found. We suggest too that the complexity of the factor requirements for core promoter function is far greater than has been imagined. Thus, we expect that the continued study of the functional biochemistry of promoter activation will uncover additional novel regulatory factors and mechanisms that will tie into and elaborate on this intricate process.

In conclusion, our studies uncovered a biochemical insufficiency of the GTFs and TFIID in establishing RNAPII-dependent transcription on a core promoter and suggest that core promoter specificity is established by downstream elements. One must now consider that core promoters confer a novel level of transcriptional regulation and that this diversity may play a role in the considerable complexity of promoter regulation. Furthermore, our results, along with the discoveries of the TRFs (for review, see Hochheimer and Tjian [2003]), tissue-specific TAFs (Dikstein et al., 1996; Freiman et al., 2001; Hiller et al., 2001), and activator-specific coactivators such as OCA-B and OCA-S (Luo et al., 1992; Luo and Roeder, 1995; Zheng et al., 2003), underscore the diversity and complexity of regulation at the core promoter itself.

Experimental Procedures

In Vitro Transcriptions

Nuclear extracts were prepared as described (Dignam et al., 1983). Primer extensions and crude nuclear extract in vitro transcriptions are as described (Lewis et al., 2000; Lewis and Orkin, 1995). Reconstituted transcription assays were as described (Akoulitchev et al., 2000; Maldonado et al., 1996a).

DSA Purification

Details of the purification are available in the Supplemental Data.

Identification of CK2 by Mass Spectroscopy

Details of the mass spectroscopy are available in the Supplemental Data.

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed essentially as described (Spencer et al., 2003).

Immunodepletions

Two-hundred and fifty microliters of the Santa Cruz anti-CK2β Mab (SC-12739) or anti-PC4 rabbit serum were conjugated to protein G-agarose beads (Boehringer) as described (Harlow and Lane, 1999). One hundred microliters of the conjugated beads was incubated with 200 μl HeLa nuclear extract for 3 hr at 4°C. This was repeated using a second 100 μl of conjugated beads. In vitro transcriptions were as described above. To block the conjugated beads, 100 μl of anti-CK2β conjugated beads were split into two 50 μl aliquots and incubated with either 2.5 μg of BSA or rCK2β for 2 hr at 4°C. Beads were washed with BC100 and incubated with 50 μL HeLa nuclear extract. Equal amounts of either the parent HeLa extract or its CK2-depleted derivatives were used for in vitro transcriptions as described above.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, a table, and Supplemental References and can be found

with this article online at http://www.molecule.org/cgi/content/full/18/4/471/DC1/.

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