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The coactivator p15 (PC4) initiates transcriptional activation during TFIIA–TFIID–promoter complex formation

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We have analyzed the mechanisms underlying stimulation of transcription by the activator GAL4-AH and the recombinant coactivator p15 (PC4). We show that p15 binds to both double-stranded and single-stranded DNA. Analyses of deletion mutants correlates binding to double-stranded DNA with the ability to mediate activator-dependent transcription. Consistent with this finding, phosphorylation of p15 by casein kinase II inhibits binding to double-stranded DNA and the activity of p15. The functional characterization suggests interactions of p15 with both DNA and components of the TFIID complex. GAL4-AH functions in concert with p15 during formation of TFIIA–TFIID–promoter (DA) complexes, as concluded from order-of-addition experiments. At limiting TFIID concentrations, the number of DA complexes is enhanced. The activator also stimulates transcription moderately after DA complex formation, independently of the concentrations of general transcription factors.

Key words: coactivators/DNA binding proteins/general transcription factors/RNA polymerase II/transcriptional regulation

Introduction

Activation of transcription from class II genes by RNA polymerase II involves several general transcription factors (GTFs) including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and RNA polymerase II (pol II) (reviewed in Conaway and Conaway, 1993; Roy *et al.*, 1994; Zawel and Reinberg, 1994). *In vitro* reconstitution experiments suggested an important role for TFIIA (Lieberman and Berk, 1994; Ozer *et al.*, 1994; Sun *et al.*, 1994; Yokomori *et al.*, 1994) and TBP-associated factors (TAFs) in mediating the response of activators on transcription (Dynlacht *et al.*, 1991; Berkenstam *et al.*, 1992; Zhou *et al.*, 1992; Chiang *et al.*, 1993; Hoey *et al.*, 1993; Chen *et al.*, 1994; Jacq *et al.*, 1994; Thut *et al.*, 1995; Zhou and Berk, 1995). A second and distinct class of coactivators, that do not associate tightly with the TATA-box-binding protein (TBP), contribute significantly to transcriptional activation in reconstituted mammalian transcription systems (Meisterernst *et al.*, 1991; Brou *et al.*, 1993; Merino *et al.*, 1993; Liebermann, 1994). In yeast, several cofactors have been identified (Berger *et al.*, 1992; Swaffield *et al.*, 1995), some of which appear to be associated with RNA pol II (Kim *et al.*, 1994; Koleske and Young, 1994). Several members of the mammalian cofactors have been characterized, among them PC1, PC2, PC3/TopoI/Dr2 and p15 (PC4) (Meisterernst *et al.*, 1991; Kretzschmar *et al.*, 1993; Merino *et al.*, 1993; Ge and Roeder, 1994; Kretzschmar *et al.*, 1994a,b) and some of them have been cloned, among them PC3/TopoI/Dr2 (Kretzschmar *et al.*, 1993; Merino *et al.*, 1993) and p15 (PC4) (Ge and Roeder, 1994; Kretzschmar *et al.*, 1994b; reviewed in Zawel and Reinberg, 1995).

Cloning of the cDNA of human p15 and functional deletion analysis of the coactivator revealed several interesting features. Within its amino-terminal half, p15 contains two short stretches, rich in serine and acidic residues, which were termed SEAC motifs. Similar motifs have also been identified in several viral immediate early regulatory proteins, among them IE62 of varicella zoster virus, IE180 of pseudorabies virus and ICP4 of herpes simplex virus type I. The SEAC motif contributes to cofactor activity and is the target of casein kinase II (CKII) that negatively regulates cofactor activity (Kretzschmar *et al.*, 1994b). The carboxy-terminal portion of p15 harbors a high affinity single-stranded DNA binding domain, whose importance for cofactor activity is unclear. However, this region of p15 alone is not sufficient to mediate the response of activators on transcription (Kretzschmar *et al.*, 1994b).

It has been suggested that the cofactor p15 directly interacts with regulatory proteins such as VP16 (Ge and Roeder, 1994). Further, it has been shown that p15 interacts with TFIIA (Ge and Roeder, 1994), and interaction of p15 with a TBP–TFIIA–promoter complex was abrogated upon phosphorylation of the cofactor with CKII (Kretzschmar *et al.*, 1994b), suggesting that these interactions could be of functional relevance.

Here we investigate mechanisms by which activator and coactivator stimulate transcription, and characterize the DNA-binding properties of p15. We provide evidence that the activity of p15 is correlated to its ability to bind double-stranded DNA. Mechanisms of activator-dependent transcription were analyzed in a reconstituted class II gene transcription system. Consistent with previous observations, showing the interaction of p15 with TBP–TFIIA–promoter complexes, template challenge and order-of-addition experiments suggest that p15 functions initially during TFIIA–TFIID–promoter (DA) complex formation. Both activator and p15 could not function when added to a preformed DA complex. Experiments in which we specifically removed the activator at different steps of preinitiation complex (PIC) formation suggested that the activator, GAL4-AH, in concert with p15, enhanced the number of active DA complexes at limiting TFIID concentrations. In addition, GAL4-AH stimulated transcription

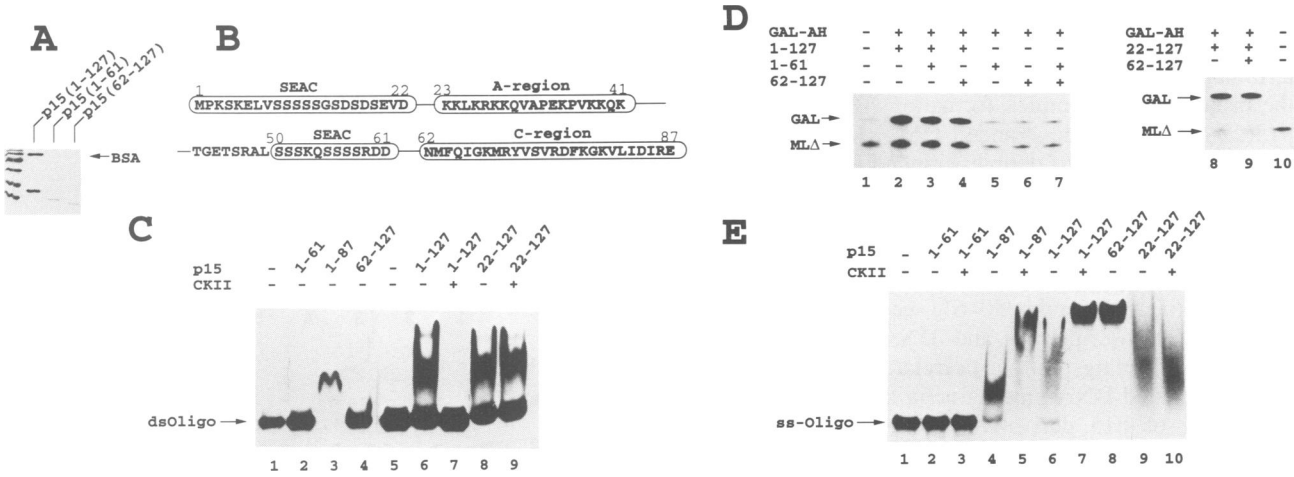

Fig. 1. DNA-binding properties of p15. (**A**) Coomassie-stained SDS–polyacrylamide gel of bacterially expressed and purified p15 derivatives. The preparation of p15(1–127) included BSA (0.4 mg/ml). The left lane contains marker proteins of sizes 97.2, 66.2 (BSA), 45.0, 31.0, 21.5 and 14.4 kDa respectively. (**B**) Amino acid sequence (single letter code) of the amino-terminal 87 amino acids of p15. The major and a minor SEAC motifs, the lysine-rich region (labeled A-region; amino acids 23–41) and the region involved in binding to single-stranded DNA (labeled C-region; amino acids 62–87) are indicated. (**C**) Characterization of DNA-binding properties of p15 derivatives. EMSAs included 200 ng of p15 derivatives, which were treated with CKII and ATP if indicated (CKII), and 25 fmol of double-stranded HIV promoter oligonucleotides. (**D**) Functional analysis of p15(1–61) and p15(62–127). Approximately 100 ng of the indicated deletion mutants were tested in the absence of p15, separately (lanes 5 and 6), in combination (lane 7) and in the presence of active p15 derivatives (50 ng) (lanes 3 and 4). A 24-fold molar excess of p15(62–127) failed to affect activator-dependent transcription in the presence of standard amounts (50 ng) of the active p15 derivative (lanes 7–9). GAL4-AH was included in standard transcription reactions if indicated. (**E**) Phosphorylation of the amino-terminal SEAC motif of p15 by CKII modulates its binding to single-stranded DNA. EMSAs included p15 derivatives (200 ng), which were treated with CKII and ATP if indicated (CKII), and a single-stranded HIV promoter oligonucleotide.

moderately after DA complex formation, independent of concentrations of GTFs.

Results

DNA binding and transcriptional activities of p15

Deletion mutants of p15 were analyzed in DNA binding assays and in a reconstituted class II gene transcription system (Stelzer *et al.*, 1994). All p15 derivatives were expressed in *Escherichia coli* and purified to near homogeneity as exemplified in Figure 1A (see Kretzschmar *et al.*, 1994b for previously employed deletion mutants). Previous deletion analyses revealed that the 40 carboxy-terminal amino acids of p15 (consisting of 127 amino acids in total) could be removed without significant loss of cofactor activity. Deletion of the 21 amino-terminal amino acids, which contain an SEAC motif (Figure 1B), led to reduced but detectable activity (Kretzschmar *et al.*, 1994b), while deletion mutant p15(62–127) was inactive (amino acid composition in parenthesis). Further, it was shown that the inactive mutant p15(62–127) binds stably to single-stranded DNA (Ballard *et al.*, 1988). Binding of active p15 derivatives to either single- or double-stranded DNA was not evident from these studies (Kretzschmar *et al.*, 1994b).

Here we have developed conditions employing high concentrations of p15 derivatives that allowed detection of interaction with both single- and double-stranded DNA in native gels. Under these conditions, all transcriptionally active mutants, p15(1–87), p15(22–127) and p15(1–127), shifted double-stranded oligonucleotides in native gels (Figure 1C, lanes 3, 6 and 8). In contrast, deletion mutants p15(1–61) and p15(62–127) did not bind to double-stranded DNA (Figure 1C, lanes 2 and 4). The latter two mutants also failed to affect both basal and GAL4-AH-dependent transcription (Figure 1D, lane 1 versus lanes 5 and 6), thus, correlating double-stranded DNA binding and functional activities of p15. From both gel shift assays and footprint experiments, we have no evidence for sequence-specific binding of p15 to promoter fragments (data not shown). We conclude that, dependent on amino acids 22–87, p15 binds non-specifically to double-stranded DNA. This portion of p15 contains two positively charged regions (termed A- and C-regions in Figure 1B), one of which comprises an extremely lysine-rich motif (A-region, amino acids 23–41), which could play a role in the recognition of double-stranded DNA.

With the exception of p15(1–61) (Figure 1E, lane 2), all mutants interacted with a single-stranded oligonucleotide (Figure 1E, lanes 4, 6, 8 and 9). Generally, the transcriptionally active p15 derivatives yielded a blurred protein–DNA complex, while p15(62–127) formed a discrete complex with reduced mobility (lane 8). If included together with full-length p15 in transcription reactions, both p15(1–61) and p15(62–127), present in a 4-fold molar excess over full-length p15, could not substantially inhibit GAL4-AH-dependent transcription (Figure 1D, lane 2 versus lanes 3 and 4). Even at a 24-fold molar excess, p15(62–127) did not exert dominant negative effects on either basal or activator-dependent transcription (lanes 8–10). These observations argue against functionally relevant interactions of p15 derivatives with naked single-stranded DNA during stimulation of activator-dependent transcription.

We had previously shown that phosphorylation of p15 by CKII abolished both interaction of p15 with a TBP–TFIIA–promoter complex and cofactor function (Kretzschmar *et al.*, 1994b). Here we have tested the effects of CKII directly on the DNA-binding properties of p15 derivatives. Treatment of p15(1–127) with CKII and ATP

prevented interaction with double-stranded DNA (Figure 1C, lane 6 versus lane 7). In the absence of the amino-terminal SEAC motif (see Figure 1B), CKII did not inhibit the protein–DNA complex formation. Binding of mutant p15(22–127) to single-stranded DNA (Figure 1E, lane 9 versus lane 10) and to double-stranded DNA (Figure 1C, lane 8 versus lane 9) was not inhibited by CKII. These data suggest that the DNA-binding properties of p15 are affected through phosphorylation of the amino-terminal SEAC motif (as opposed to, for example, the second SEAC motif, amino acids 50–61, see Figure 1B), leading to loss of transcriptional and DNA-binding activities. Thus, these findings further correlate the functional and double-stranded DNA-binding activities of p15.

Binding of p15 derivatives to single-stranded DNA was not inhibited by CKII. In contrast, complexes of transcriptionally active mutants that contained the amino-terminal SEAC motif [p15(1–127) and p15(1–87)] displayed reduced mobility (Figure 1E, lane 4 versus lane 5, and lane 6 versus lane 7). This phenomenon could be attributed to an increase in stability of protein–DNA complexes following phosphorylation of p15 by CKII. Non-phosphorylated p15 may dissociate from single-stranded DNA during electrophoresis, resulting in blurred complexes. Interestingly, the protein–DNA complexes that included the phosphorylated p15(1–127) and the transcriptionally inactive mutant p15(62–127) respectively, moved with identical mobility in native gels (lane 7 versus lane 8). One possible explanation for this phenomenon could be a phosphorylation-induced conformational change of the p15 protein (see Figure 5). This may lead to exposure of regions of p15 that are involved in recognition of single-stranded DNA (including, for example, amino acids 62–87, which are contained in all mutants that bind to single-stranded DNA). In any event, and in agreement with the functional characterization of mutant p15(62–127), the conversion of p15 into a molecule that cannot bind to double-stranded DNA, but strongly interacts with single-stranded DNA, coincides with loss of cofactor function (Kretzschmar *et al.*, 1994b).

p15 binds to promoters in the presence of TFIID and TFIIA

Whilst the preceding experiments point to an important role of binding to double-stranded DNA, it might be superficial to conclude that the coactivator functions exclusively through non-specific interactions with DNA. Indeed, previous investigations showed interactions of p15 with both activators (Ge *et al.*, 1994) and TFIID–TFIIA complexes (Ge and Roeder, 1994; Kretzschmar *et al.*, 1994b).

To assess the significance of interactions of the cofactor with both GTFs and DNA, we have subsequently employed template challenge experiments. Circular DNA was added before and after DA complex formation in standard transcription reactions. By increasing the concentration of the internal control template, carrying the adenovirus major late (ML) core promoter (3-fold by addition of 100 ng supercoiled pMLΔ53), activator-dependent transcription was blocked efficiently (Figure 2A, lanes 2 and 3 versus lanes 4 and 5). Inhibition of activator-dependent transcription was not observed if transcription reactions were challenged after preincubation of templates with

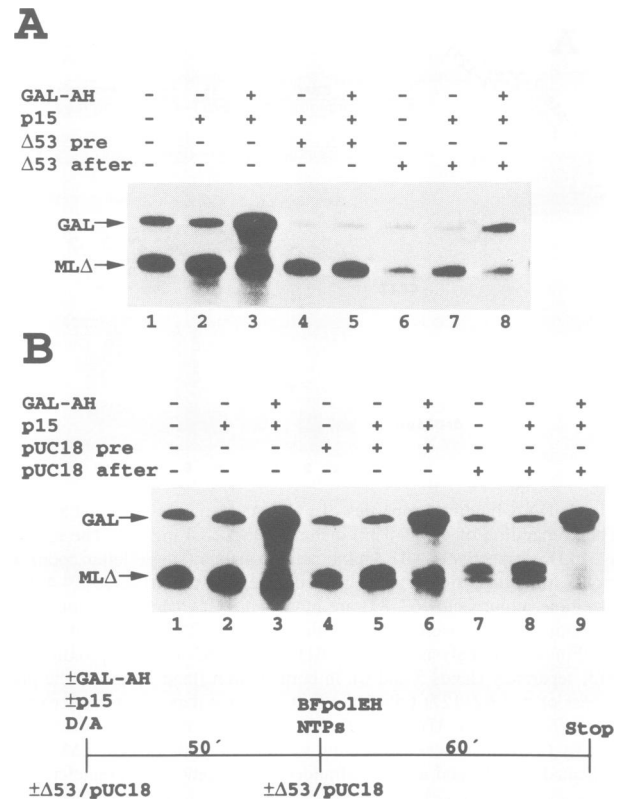

Fig. 2. p15 binds to promoters in the presence of TFIID and TFIIA. (**A**) Core promoters compete for activator-dependent transcription. The transcription protocol for (A) and (B) is schematically shown below (B). ML core promoter template (100 ng) was added during preincubation (if indicated, Δ53 pre), or after preincubation of TFIIA and TFIID (if indicated, Δ53 after) with 50 ng of each template under standard transcription conditions in the presence and absence of p15 and GAL4-AH as indicated. (**B**) Vector pUC18 does not influence activator-dependent transcription. pUC18 DNA (150 ng) were added during (pUC18 pre) or after (pUC18 after) DA complex formation following the scheme shown at the bottom of the figure.

TFIIA and TFIID (lanes 6–8). Although basal transcription was also reduced, an effect which is possibly caused by competition for GTFs (compare lanes 2, 4 and 6), effects on activator-dependent transcription were much more pronounced [compare the ratios of transcripts from activated (top) with basal (bottom) promoters in lanes 3, 5 and 7]. In contrast, 150 ng of the vector pUC18 did not affect activator-dependent transcription, either before or after DA complex formation (Figure 2B). Thus, binding of p15 to the vector is much less efficient than binding to a promoter-containing template. After DA complex formation, the effects of p15 cannot be competed efficiently, indicating that p15 may be bound stably to the DA–GAL4-AH complex. Alternatively, p15 could have acted directly on the formation of DA complexes. Thus, these experiments may indicate simultaneous interactions of p15 with DNA and components of the TFIID complex (these TFIID preparations contain TFIIA; Meisterernst and Roeder, 1991).

p15 initiates activator-dependent transcription during DA complex formation

Further evidence for a functional role of p15 during DA complex formation resulted from order-of-addition

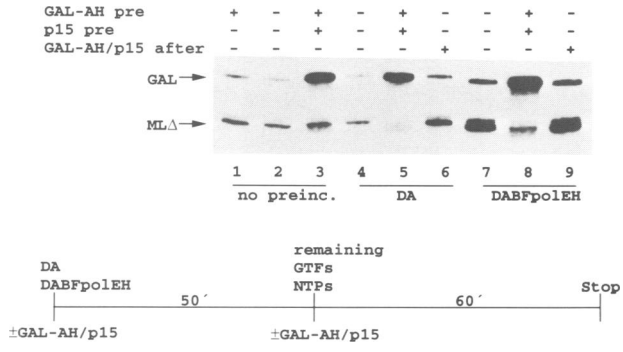

Fig. 3. Activator and p15 function during DA complex formation. The protocol schematically depicted at the bottom of the figure refers to lanes 4–9 in which p15 and activator were added either during preincubation (GAL4-AH pre and p15 pre) or after preincubation (GAL4-AH/p15 after). In lanes 4–6, TFIID (1.2 µl) and additional TFIIA (0.5 µl) were included in the preincubation while, in lanes 7–9, a complete set of GTFs (DABFpolEH) was preincubated with templates under standard conditions. The effects of activators increased as a consequence of the preincubation protocol from 8-fold (lane 1 versus lane 3) to ~12-fold (lane 4 versus lane 5 and lane 7 versus lane 8). Levels of activator-dependent transcription were normalized for the reduction of core promoter activity. Preincubation of templates with a complete set of GTFs generally enhanced basal promoter activity. In lanes 1–3, p15-dependent function of GAL4-AH is demonstrated in standard transcription reactions.

experiments in which we added activator and p15 to preformed intermediate PICs. TFIIA and TFIID (DA), or a complete set of general transcription factors (DABFpolEH), were incubated with templates under transcription conditions. Activator and p15 were either included during the preincubation or added afterwards. As shown in Figure 3, activator and p15 were completely inactive if added after formation of DA complexes (lanes 4–6) while they stimulated transcription efficiently if they were present during the preincubation (lanes 5 and 8). Activator and p15 were consistently inactive if they were added after formation of higher order PICs (DABFpolEH, lane 8). When the activator was included in the preincubation and p15 was added after DA complex formation and vice versa, transcription was not activated (data not shown), indicating that activator and coactivator act in concert during DA complex formation.

GAL4-AH in conjunction with p15 stimulates transcription predominantly during, and moderately after, DA complex formation

Components of the TFIID complex have been identified as direct targets of activators (Sawadogo and Roeder, 1985; Stringer *et al.*, 1990; Hoey *et al.*, 1993; Verrijzer *et al.*, 1994). While the interacting regions correspond in many cases to activation domains, the functional consequences of these interactions are generally unclear. For example, it has not yet been clarified whether interactions of activators with TAFs result either in an increase in the number of TFIID complexes or induce modifications in TFIID. Here, we have attempted to define effects of the activator on the DA complex and subsequent steps of PIC formation. One major obstacle in this analysis resides in the fact that both activator and coactivator are physically present during all steps of PIC formation. To circumvent this problem, we chose to remove the activator during PIC formation by restriction digest. If successful, this procedure should lead to loss of activator function at defined steps of PIC formation. We took advantage of the presence of a *Sph*I restriction site located between the GAL4 recognition sites and the TATA box on the HIV promoter template.

Here we have analyzed the effects of GAL4-AH on the formation of DA and DABFpol complexes respectively (Figure 4A). Templates were digested with *Sph*I for 20 min after preincubation of templates with either TFIIA and TFIID (DA), or with TFIIA, TFIIB, TFIID, TFIIF and RNA pol II (DABFpol). Activator and p15 were included in the preincubations (if indicated, Figure 4A). The remaining general factors, together with nucleotides, were added after the restriction digest. For reasons discussed below, transcription reactions included either limiting (lanes 1–6) or saturating concentrations of TFIID (lanes 7–12).

If templates were digested after DA complex formation, the activator increased transcription 6.5-fold at limiting TFIID concentrations (lanes 1–3), while it did not stimulate transcription at saturating TFIID concentrations (lanes 7–9). GAL4-AH could activate transcription moderately (down from 10- to 3-fold, lanes 4–6 versus lanes 9–12) at saturating concentrations of TFIID, if templates were preincubated with DABFpol. This experiment suggests that GAL4-AH functions in conjunction with p15 predominantly during DA complex formation. It also suggests that the activator moderately stimulates transcription after binding of TFIID to the TATA box. The latter effects were dependent on both the activator and p15 (Figure 4A, lanes 10–12 and data not shown).

Loss of activator-dependent transcription at saturating TFIID concentrations was not caused by the conformational change of the template generated through the restriction digest. Linearization of the vector (pUC19) with *Ssp*I ~600 bp upstream of the GAL4 binding sites did not diminish the induction of transcription by GAL4-AH (Figure 4B, lanes 4–6 versus lanes 10–12). By contrast, linearization of templates with *Sph*I between the TATA box and the GAL4 recognition sites, either prior to the addition of transcription factors (Figure 4B, lanes 1–3), or after formation of DA promoter complexes (lanes 7–9 versus lanes 10–12), led to inactivation of GAL4-AH. The function of restriction enzymes under transcription conditions was also demonstrated by standard agarose gel electrophoresis. The majority of GAL4 reporters were indeed digested by *Sph*I, while the internal control template, which lacks the *Sph*I restriction site, remained in a supercoiled state (Figure 4C). We have considered the possibility that the small number of active promoters (<1%) might not be digested by *Sph*I. However, restriction digest-dependent loss of activator function after DA complex formation at saturating TFIID concentrations provides functional proof for digestion of essentially all active promoters.

In contrast to the effects observed with TFIID, titrations of all other GTFs did not modulate GAL4-AH activity substantially, as exemplified in Figure 4D for TFIIB and TFIIH. This is consistent with the idea that the activator does not simply increase the affinity of isolated GTFs for intermediate PICs. Taken together, our experiments suggest that activator and p15 function predominantly during DA complex formation. The dependency on TFIID

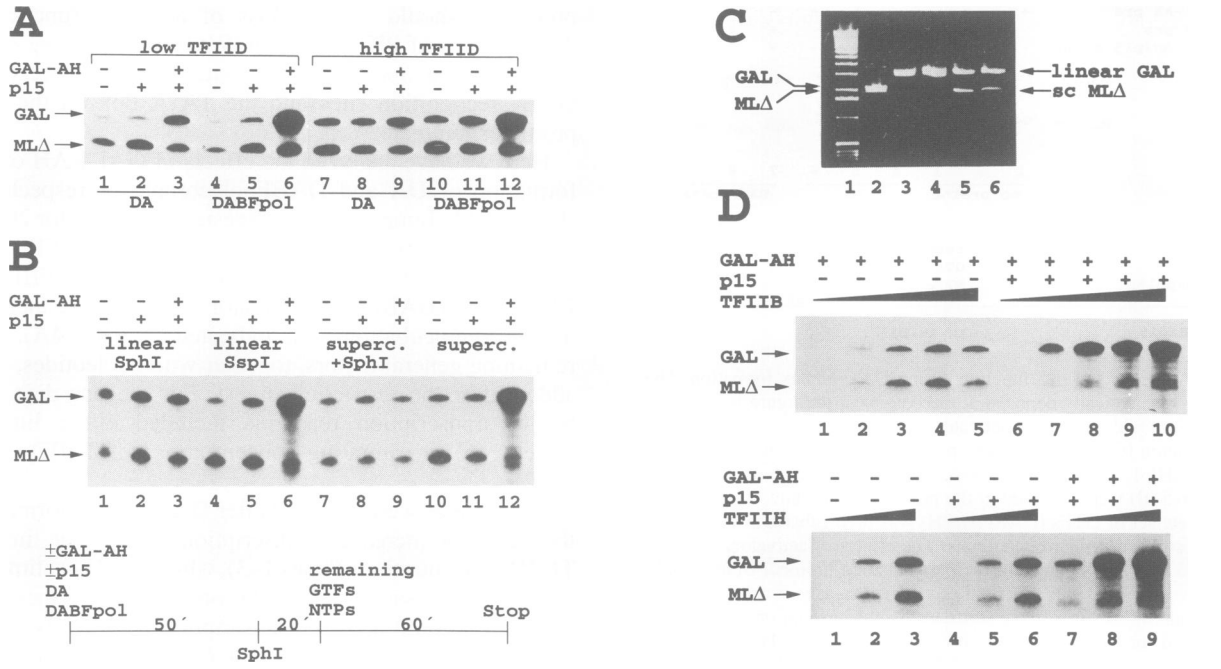

Fig. 4. GAL4-AH functions predominantly during and moderately after DA complex formation. (**A**) DA and DABFpol complexes were preformed employing either limiting (0.8 µl, lanes 1–6) or saturating (3.2 µl, lanes 7–12) concentrations of TFIID and TFIIA (0.5 µl) in the presence of GAL4-AH and p15 (if indicated) in standard transcription reactions. Following a 50 min preincubation period, reactions were digested with *Sph*I for 20 min. Subsequently, transcription was started by addition of the remaining GTFs and NTPs. Reactions were conducted in parallel. However, a shorter exposure (approximately three times shorter) of reactions conducted at saturating TFIID concentrations is shown (lanes 7–12). All reactions contained 80 ng of a single-stranded oligonucleotide, which was added after the 50 min preincubation period, to prevent basal effects of p15 from linearized templates. Similarly to circular double-stranded DNA (Figure 2), the oligonucleotide in some situations caused repression of basal transcription (lane 4 versus lane 5). For calculations of fold stimulation by activators (for numbers see text), levels of transcripts from the ML core promoter were normalized in the absence and presence of GAL4-AH (for example, between lane 2 and lane 3). (**B**) Restriction digest-dependent loss of activator function after DA complex formation. Reactions were performed with *Sph*I prelinearized templates (lanes 1–3), *Ssp*I prelinearized templates (lanes 4–6), supercoiled templates that were digested with *Sph*I after DA complex formation (lanes 7–9) and supercoiled templates (lanes 10–12) respectively. Unique *Ssp*I and *Sph*I restriction sites are located ~600 bp upstream of the GAL4 recognition sites (*Ssp*I in pUC19) and immediately downstream of the GAL4 restriction sites and upstream of the promoter TATA box (*Sph*I), respectively. Reactions were conducted under conditions described in (A) (lanes 7–9), at saturating TFIID concentrations, following the protocol schematically shown below, in the absence and presence of p15 and GAL4-AH as indicated. (**C**) Agarose gel analysis of digested transcription templates. Supercoiled templates (lane 2) were digested with 10 U *Eco*RI, which linearizes both templates, in the absence of all GTFs (lane3), and after DABFpolEH complex formation (lane 4). The restriction enzyme *Sph*I (6 U) linearizes exclusively the GAL reporter pMRG5, as was shown in the absence of GTFs (lane 5), and after DABFpolEH complex formation (lane 6). The reactions were conducted under standard transcription conditions and loaded directly on 1% agarose gels. (**D**) Titration of TFIIB and TFIIH. TFIIB and TFIIH were titrated in standard transcription reactions in the presence and absence of GAL4-AH and/or p15, as indicated on top of the figures.

concentrations provides evidence for the ability of the activator to enhance the number of DA complexes. In addition, independently of the concentration of TFIID and other GTFs, GAL4-AH appears to stimulate transcription moderately after DA complex formation.

Discussion

Here we have characterized a previously identified cofactor of activator-dependent transcription as a DNA-binding protein that initiates stimulation of transcription in concert with activators during DA complex formation. Exceeding previous analyses (Ballard *et al.*, 1988; Kretzschmar *et al.*, 1994b), we have presented functional evidence for interactions of p15 with both DNA and GTFs. In a highly purified reconstituted class II gene transcription system consisting of GTFs and recombinant p15, the activator GAL4-AH stimulates formation of DA complexes at limiting TFIID concentrations. Activation of transcription is not completed with the formation of DA complexes, but seems to include moderate effects of GAL4-AH during subsequent steps of PIC formation.

Relevance of DNA binding for cofactor activity

The p15 protein was shown to interact with both double- and single-stranded DNA. Mutants that bind stably to single-stranded DNA could neither stimulate nor inhibit activator-dependent transcription, arguing against the functional relevance of p15–single-stranded DNA interactions.

At least one other well characterized DNA-binding protein, the adenovirus-encoded DBP, has been shown to stimulate transcription (reviewed in Zijderveld *et al.*, 1994). Like p15, DBP interacts with both single- and double-stranded DNA in an essentially sequence-independent manner. DBP enhances the affinity of several distinct regulatory factors such as USF and NFI for DNA (Stuiver *et al.*, 1990; Zijderveld *et al.*, 1994). While it would be interesting to analyze the effects of p15 on these activators, at the present time we have no evidence for effects of p15 on the affinity of GAL4-AH. At the concentrations used in transcription, both in electrophoretic mobility shift assays (EMSAs) and in footprint experiments, GAL4-AH fully covered the five recognition sites. Protection of GAL4 sites was also seen after preincubation of DNA fragments with TFIIA and TFIID, while activator

and p15 could not function after DA complex formation. Generally, the activator was employed in an ~5-fold molar excess over the number of recognition sites, and higher concentrations of GAL4-AH inhibited transcription (Kretzschmar *et al.*, 1994a).

Three independent observations underline the functional relevance of binding to double-stranded DNA. First, the corresponding regions in p15 (amino acids 22–87) were found to be essential for cofactor activity. Then, modification of p15 by CKII abolished both cofactor activity and binding to double-stranded DNA. Finally, competition with transcription templates in the presence of TFIIA and TFIID eliminated cofactor activity. In fact, the function of all presently characterized cofactors (PC1, PC2, PC3, PC4, PC5 and PC6) was found to be dependent on template concentrations (P.Halle, A.Goppelt and M.M., unpublished observation). On the other hand, circular DNA lacking promoters could not compete as efficiently for both basal and activator-dependent transcription, revealing inefficient binding of p15 to double-stranded DNA. Consistent with this finding, high concentrations of p15 were required to detect binding to double-stranded DNA fragments, and previous analyses demonstrated interactions of p15 with TBP–TFIIA–promoter complexes (Ge and Roeder, 1994; Kretzschmar *et al.*, 1994b). Activation of transcription required an intact TFIID complex that included TAFs. In our present study we used an activation-competent TFIID complex consisting of TBP and TAFs (Dynlacht *et al.*, 1991) and a transcription assay to demonstrate functional interactions between p15 and components of the TFIID complex. Taking into account that core promoters compete efficiently for p15 effects on activator-dependent transcription, functional interactions with activators may require simultaneous contacts of p15 with both GTFs and DNA.

Previously, we could not distinguish effects of CKII on DA–p15 and p15–DNA interactions respectively (Kretzschmar *et al.*, 1994b). Our present data clearly show the direct effects of phosphorylation by CKII on DNA binding properties of p15. As illustrated in Figure 5, our data are consistent with a model implying intramolecular folding of the p15 protein following phosphorylation by CKII. Since binding to single-stranded DNA is not inhibited in this process, it is tempting to speculate that the phosphorylated SEAC motif (amino acids 1–21) interacts with charged regions of p15 contained in the amino-terminal 61 amino acids, for example, the lysine-rich motif (amino acids 23–41; see Figure 1B). This motif, containing nine lysine residues within 19 amino acids, is predicted to fold at least in part into an α-helix, which might expose more than one positive surface. This hypothesis could explain that phosphorylation of p15 by CKII also precludes direct interactions with negatively charged activators (Ge *et al.*, 1994).

Activator and coactivator initiate stimulation of transcription during DA complex formation

Evidence is accumulating that transcriptional activators interact directly with GTFs. Among the potential targets of activators are TBP (Stringer *et al.*, 1990; Horikoshi *et al.*, 1995), the TBP-associated factors (TAFs; Dynlacht *et al.*, 1991; Hoey *et al.*, 1993; Verrijzer *et al.*, 1994; Thut *et al.*, 1995), TFIIB (Lin and Green, 1991; Roberts *et al.*, 1993) and TFIIH (Xiao *et al.*, 1994). Little is known

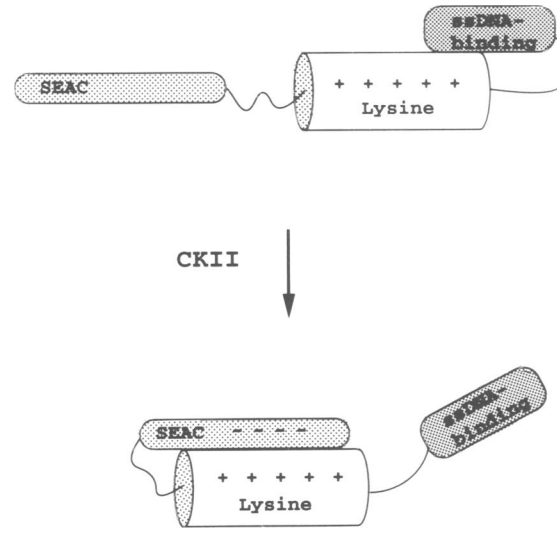

Fig. 5. Model for a phosphorylation-induced conformational change of p15. The model is based on the observation that phosphorylation blocks binding of p15 to double-stranded, but not to single-stranded DNA. Indicated interactions of the lysine-rich region (amino acids 23–41) with the phosphorylated SEAC motif (amino acids 1–21) are purely hypothetical.

about the mechanisms by which these interactions lead to an increase of activity of RNA pol II. One simple explanation would be that activators enhance the affinity of certain GTFs for intermediate preinitiation complexes and/or the promoter. Here we have presented functional evidence for a possible role of GAL4-AH in increasing the number of active DA complexes. In a purified transcription system, the effects of GAL4-AH mediated by p15 were found to be dependent on TFIID concentrations, and removal of the DNA-bound activator after DA complex formation resulted in low levels of activator-dependent transcription. In contrast, the concentration of all other GTFs did not specifically influence activator-dependent transcription. In other investigations (P.Halle, G.Stelzer and M.Meisterernst, in preparation) involving the coactivator PC1 (Meisterernst *et al.*, 1991) we obtained similar results when all GTFs, with the exception of TFIID, were combined and titrated together in activator-dependent transcription.

Choy and Green (1993) proposed a mechanistic model implicating activator-mediated recruitment of GTFs. In their studies several GTFs, but not TFIID, were recruited to the promoter. These studies were performed with nuclear extracts, while we used a reconstituted system which is depleted of other PCs and variables with respect to the TFIID concentration. It is also possible that other PCs, for example PC2, which efficiently activated GAL4-AH-dependent transcription after DA complex formation (K.K., G.S. and M.M., unpublished observation), dominate in nuclear extracts.

To dissect the process of PIC formation, we have introduced a protocol in which the template was digested at defined steps of PIC formation, which leads to spatial delocalization and inactivation of GAL4-AH. This procedure allowed us to distinguish effects of the activator before and after DA complex formation. The effect of the activator was manifested predominantly during DA complex formation. After DA complex formation and/or at

saturating TFIID concentrations, the activator, in conjunction with p15, stimulated transcription only moderately.

At the present time, we do not understand the mechanism by which the activator stimulates transcription after DA complex formation. One possibility is that the activator, independently or together with p15, affects the activity of other GTFs which associate with TFIID. Recent studies by Roberts and Green (1994) have suggested that prototypic acidic activators induce conformational changes in TFIIB which could theoretically account for the effects of the activator observed in our studies after DA complex formation.

However, we also cannot exclude the fact that the activator changes the conformation of intermediate PICs—including the templates—which could increase the activity of the PIC. The cofactor may participate in stimulation of transcription after binding of TFIID, even though it does not function if added after DA complex formation. The latter result could indicate simply that p15 assembles with activator–DA complexes in a way that is precluded after DA complex formation. Based on their DNA binding properties, it is tempting to speculate that p15 and other PCs may indeed remain associated with the PIC, to affect its structure and/or to play a role in other processes during PIC formation such as melting of the promoter, which is a limiting step in the initiation reaction of RNA polymerase II (Wang *et al.*, 1992; Tantin and Carey, 1994; reviewed in Pan and Greenblatt, 1994).

Materials and methods

Expression and purification of transcription factors

Purification of TFIIA, TFIID and TFIIH from HeLa nuclear extracts, of RNA pol II from calf thymus, and expression and purification of recombinant transcription factors RAP30/RAP74, TFIIB, TFIIEα, TFIIEβ, GAL4-AH and p15 derivatives have been described previously (Kretzschmar *et al.*, 1994b and references therein). The expression vector of p15(1–61) was constructed by ligation of PCR fragments into *Nde*I–*Hin*dIII sites of pET11a (Novagen). For purification of p15(1–61), bacterial lysate was adjusted to 0.4 M NaCl by adition of buffer C [20 mM Tris–HCl (pH 7.3 at 25°C), 5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20% (v/v) glycerol, 0.2 mM EDTA], loaded onto P11 and eluted with 0.4 M KCl in buffer C. Protein was precipitated by addition of saturated ammonium sulfate solution to a final concentration of 60%. The precipitate was dissolved in buffer C, dialyzed to 0.1 M NaCl and subsequently loaded onto Mini S (SMART system, Pharmacia). In a linear gradient from 0.1 to 0.5 M NaCl, p15(1–61) eluted between 0.2 and 0.3 M NaCl in a buffer C containing 10% glycerol.

Electrophoretic mobility shift assay

Reactions contained 25 fmol of a single-stranded (coding strand) or double-stranded oligonucleotide comprising HIV-1 core promoter sequences upstream of the initiator region of the adenovirus major late promoter (with the sequence 5′ CCCTCAGATGCTGCATATAAGCAG-CTGCTTTTTGCCCGTCCTCACTCTCTTCCGCATCGC) and 200 ng of p15 derivatives. Reactions were incubated for 30 min at 30°C in a buffer S containing 10 mM Tris–HCl (pH 7.5), 5% (v/v) glycerol, 1 mM DTT, 1 mM EDTA and 0.4 M KCl in a total volume of 20 µl. Binding reactions were subjected to electrophoresis at 120 V on native polyacrylamide gels (10%, 19:1) in TGE buffer [25 mM Tris, 100 mM glycine, 1 mM EDTA (pH 8.3)] for 4 h until the bromophenol blue dye reached the bottom of the gel.

For phosphorylation, prior to addition to oligonucleotides, 200 ng of p15 derivatives were incubated at 30°C for 30 min with 0.06 mU of human recombinant CKII (Boehringer Mannheim) in the presence of 50 µM ATP, 5 mM $MgCl_2$, 25 mM HEPES (pH 8.2) and 1 mg/ml bovine serum albumin (BSA) in a total volume of 10 µl. Then reaction mixtures were diluted to a volume of 20 µl with buffer S.

In vitro transcription assays

Unless indicated otherwise, transcription reactions included supercoiled templates, pMRG5 and pMLΔ53, described previously (Kretzschmar *et al.*, 1994a).

Standard reactions included 50 ng of each transcription template, 10 ng recombinant TFIIB, 0.8 µl TFIID (DE52 fraction, 0.35 mg/ml protein), 10 ng recombinant TFIIEα, 5–10 ng recombinant TFIIEβ,10 ng recombinant RAP30/RAP74, 1.5 µl TFIIH (DE52, 0.5 mg/ml protein), 0.2 µl RNA polymerase II (DE52 fraction, 0.5 mg/ml protein), 40–60 ng p15 derivatives and 30–50 ng GAL4-AH, unless indicated otherwise in the figure legends. TFIID contained substantial amounts of TFIIA, as was reported previously (Meisterernst and Roeder, 1991). In order-of-addition experiments and if indicated in the figure legends, 0.5 µl (Mono Q fraction, 2 mg/ml) of TFIIA were added in addition. Transcription reactions were conducted in a buffer containing 25 mM HEPES (pH 8.2), 10–20% glycerol, 5 mM $MgCl_2$, 60–70 mM KCl, 5 mM DTT, 0.2 mM PMSF, 200 ng/µl BSA, 0.002% NP-40, 100 µM each of UTP and ATP, 5 µM CTP, 20 µM 3′-*O*-methyl GTP, 0.5 µM [α-^{32}P]CTP (3000 Ci/mmol) and 20 U RNase-Block (Stratagene).

GAL4-AH was employed in concentrations that were saturating in transcription and DNA binding. Generally, GAL4-AH was added to buffered transcription templates 1 min prior to the addition of p15 derivatives, immediately followed by pre-mixed GTFs. In order-of-addition experiments (Figure 3), GAL4-AH and p15 were either included in preincubations, or added after formation of intermediate complexes (t = 50 min) as explained in the individual figures. In the combined order-of-addition/restriction digest protocol (Figure 4B), 1.0 µl *Sph*I (40 U/µl, Boehringer Mannheim) was added after a 50 min preincubation period. Mixtures were incubated for 20 min at 28°C under transcription conditions before the addition of nucleotides and the remaining GTFs. Unless indicated otherwise, NTPs were added after preincubation of the remaining components for 50 min. Transcription reactions were carried out for 1 h at 28°C and processed and analyzed as described (Meisterernst and Roeder, 1991).

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