not overlap with each other, interacted with both TFIIH and GAL4-VP16, suggesting that these interactions occur redundantly at multiple sites within the coactivator domain of PC4. The interactions of the different PC4 mutants with GAL4-VP16 were equally strong, whereas those with TFIIH showed variations; for example, PC4(43-127) interacted with TFIIH more strongly than wild-type PC4 did, while PC4(22-127), PC4(1-62), and PC4(1-42) interacted with TFIIH more weakly than wild-type PC4 did (Fig. 4B).

Thus, colocalization of the interaction region to the functionally defined coactivator domain (21, 27) argues that these interactions are functionally relevant for the coactivator activity of PC4. Moreover, the redundancy of these interactions is consistent with the role of PC4 as a coactivator, which is expected to interact with activators and the basal transcriptional machinery at the same time.

Distinct regulation of the interactions of PC4 with GAL4-VP16 and TFIIH. Since the interaction of TFIIH with PC4 mutants appeared to differ slightly from that of GAL4-VP16, we further explored the difference between these two interactions. The interaction between PC4 and GAL4-VP16 was previously shown to be negatively regulated by phosphorylation of the N-terminal region of PC4 (14, 27); therefore, we sought to determine whether the same was true for the interaction between PC4 and TFIIH. To make this determination, we used PC4-GST, in which the C terminus of PC4 is fused to the N terminus of GST, since GST-PC4 could not be phosphorylated efficiently by casein kinase II, presumably because the N-terminal phosphorylation sites of PC4 within GST-PC4 were sterically inaccessible to the casein kinase II (Fig. 5C). PC4-GST, expressed in *E. coli* and retained on glutathione-Sepharose, showed essentially the same binding to TFIIH and GAL4-VP16 as GST-PC4 did (data not shown). As shown in Fig. 5B, PC4-GST could be readily phosphorylated, and the phosphorylation slowed the migration of PC4-GST on the SDS gel, a shift of migration similar to that observed for nonfused PC4 (Fig. 5A), indicating that PC4-GST was phosphorylated in essentially the same manner as PC4 was. Pull-down assays with PC4-GST indicated not only that TFIIH interacted with both phosphorylated and nonphosphorylated PC4, but also that its interaction with PC4 was slightly enhanced by the phosphorylation of PC4 (Fig. 5D). In marked contrast, the interaction between GAL4-VP16 and PC4 was completely abolished upon phosphorylation of PC4, as reported previously (13, 27). Thus, although TFIIH and GAL4-VP16 interact with PC4 through the same coactivator domain, these interactions show markedly distinct regulation through the phosphorylation of PC4.

The number of GAL4 binding sites determines the degree to which each step of the transcriptional process is stimulated upon activation. The multiple interactions of GAL4-VP16 and PC4 with the basal transcription machinery, as demonstrated here and elsewhere, and the observed stimulatory effects before and after initiation suggest that each GAL4-VP16 dimer bound to the five GAL4 sites may have a distinct role in activated transcription. To gain further insight into a potential relationship between each GAL4-VP16 dimer and the effects on distinct steps, as well as the role of PC4 in this process, we determined the degree to which each step of transcription is stimulated in the presence and absence of PC4 when the number of bound GAL4-VP16 dimers was reduced (Fig. 6A). To

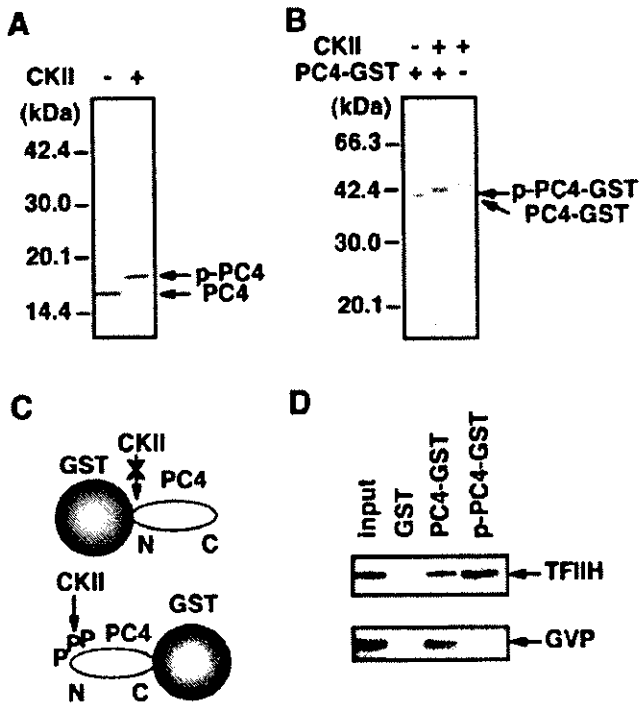


FIG. 5. PC4 interacts with TFIIH in a manner distinct from that of GAL4-VP16. (A) Phosphorylation of PC4 with casein kinase II (CKII). Purified PC4 was phosphorylated by casein kinase II (New England Biolabs), and the shifted mobility of PC4 was observed upon phosphorylation. The positions of phosphorylated PC4 (p-PC4) and nonphosphorylated PC4 (PC4) are shown on the right. (B) Phosphorylation of PC4-GST. The positions of phosphorylated PC4-GST (p-PC4-GST) and nonphosphorylated PC4-GST (PC4-GST) are indicated on the right. (C) As shown schematically, PC4 fused to the N terminus of GST was efficiently phosphorylated by casein kinase II. Casein kinase II phosphorylated PC4 fused to the N terminus, but not to the C terminus, of GST (data not shown), presumably because the phosphorylation sites within the N-terminal region of PC4 were sterically masked by GST. A GST molecule and PC4 are schematically represented, and a phosphate molecule and the amino and carboxyl termini of PC4 are indicated by P, N, and C, respectively. (D) Interaction of phosphorylated PC4 with TFIIH. GST pull-down assays with PC4-GST revealed that TFIIH interacted with both nonphosphorylated and phosphorylated forms of PC4 but that GAL4-VP16 interacted only with the nonphosphorylated form of PC4. Note that approximately twofold more TFIIH bound to p-PC4-GST than to PC4-GST.

this end, we created the templates with one, three, and five GAL4-binding sites (G1, G3, and G5 templates, respectively, binding 2, 6, and 10 GAL4-VP16 dimers) (Fig. 6A) and performed in vitro transcription analyses. As shown in Fig. 6 and quantified in Fig. 7, GAL4-VP16 alone stimulated the level of the 390-nt transcripts from the G1, G3, and G5 templates 2.6-, 2.9-, and 3.5-fold, respectively, showing that increasing the number of bound GAL4-VP16 dimers does not necessarily lead to robust transcriptional activation when PC4 is absent from the reactions. In the presence of PC4, however, stimulation of the 390-nt transcript increased dramatically to 5.3-, 14.3-, and 17.2-fold for the G1, G3, and G5 templates, respectively (Fig. 6B and 7A), revealing that the effect of PC4 becomes more apparent as the number of GAL4-VP16 dimers is increased. Moreover, DNase I footprint analyses showed that

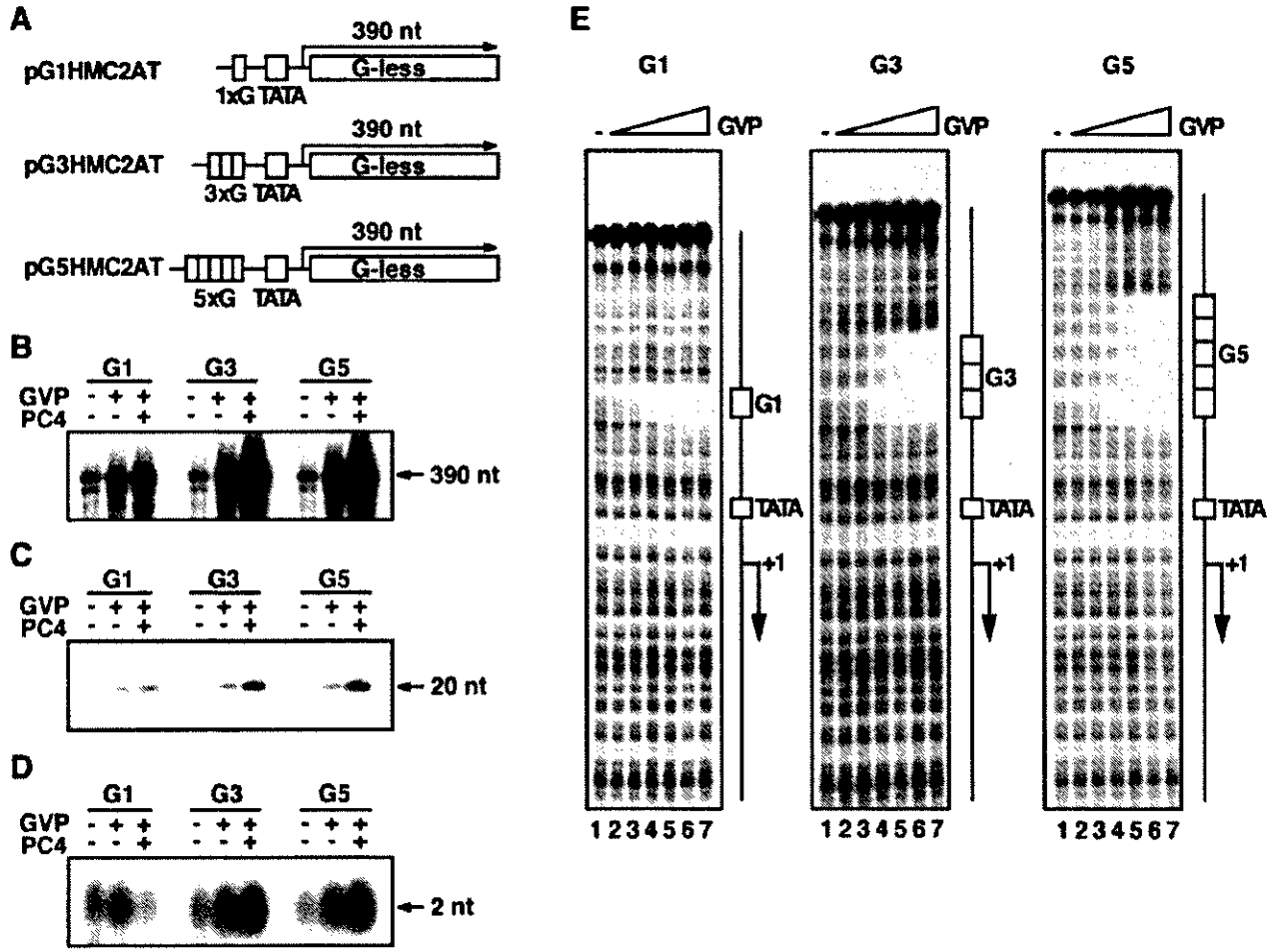


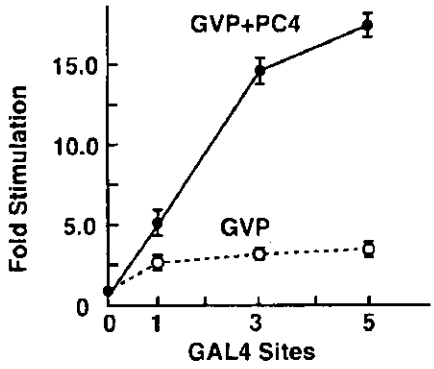
FIG. 6. Effect of the number of GAL4 sites on the degree of stimulation of the 390-, 20-, and 2-nt transcripts. (A) Templates used for in vitro transcription. Three templates, pG1HMC2AT, pG3HMC2AT, and pG5HMC2AT, contained one, three, and five GAL4 binding sites, respectively. For in vitro transcription for 20-nt transcripts, the same set of the templates with a G residue at the +20 position (not shown) was used. The amount of GAL4-VP16 added to the transcription reaction was the same for all reactions (25 ng). (B to D) The effects of one, three, and five GAL4 sites on the stimulation of the 390-nt (B), 20-nt (C), and 2-nt (D) transcripts. G1, G3, and G5 indicate pG1HMC2AT, pG3HMC2AT, and pG5HMC2AT, respectively. (E) Binding of GAL4-VP16 to the G1, G3, and G5 templates. Increasing amounts of GAL4-VP16 were tested for binding to the DNA fragments containing one, three, and five GAL4 sites. The added amounts of GAL4-VP16 were 0 ng (lane 1), 1.6 ng (lane 2), 3.1 ng (lane 3), 6.3 ng (lane 4), 12.5 ng (lane 5), 25 ng (lane 6), and 50 ng (lane 7). The positions of GAL4 binding sites (G1, G3, and G5), the TATA box (TATA), and the initiation site (+1) are indicated on the right.

all of the GAL4 sites on the G1, G3, and G5 templates were occupied almost completely by 25 ng of GAL4-VP16 (Fig. 6E, lane 6), the amount that was used for in vitro transcription reactions. Thus, it is unlikely that transcriptional activation for the G3 and G5 templates derives from the PC4-induced cooperative binding of GAL4-VP16 to its cognate sites. More likely, however, is the possibility that PC4 increases the number, or the effectiveness, of the interactions between GAL4-VP16 and the basal transcription machinery to allow synergistic effects of multiply bound GAL4-VP16 dimers (Fig. 7A).

Next, to determine the relative stimulation of initiation, promoter escape, and elongation in activated transcription from the G1, G3, and G5 templates, we assayed and quantified the amounts of the 2- and 20-nt transcripts from these templates (Fig. 6C and D) and then ascribed the effects of GAL4-VP16 or of GAL4-VP16 and PC4 to three distinct steps (Fig. 7B and C). The analyses of the transcripts from the G1 template in the presence of GAL4-VP16 alone revealed minor stimulation of initiation, with little stimulation of promoter escape and elongation. However, markedly increased levels of stimulation of promoter escape and, to a lesser extent, elongation were observed when PC4 was included in these reactions (Fig. 7C, top panel). Interestingly, no stimulation whatsoever of initiation from the G1 template was observed in the presence of both GAL4-VP16 and PC4 (Fig. 7C, top panel). In contrast, robust activation of transcription from the G3 and G5 templates by GAL4-VP16 and PC4 was attributed largely to the marked stimulation of both initiation and promoter escape (Fig. 7C, middle and bottom panels). Low levels of transcriptional activation for these templates in the presence of GAL4-VP16 alone, however, resulted mainly from the stimulation of initiation.

These data demonstrate the following points. First, GAL4-VP16 alone can effect a low level of stimulation of the initia-

A



B

Transcript		Fold Stimulation	
		+GVP	+GVP, +PC4
G1	2 nt	1.5 ± 0.1	0.9 ± 0.1
	20 nt	2.0 ± 0.3	2.5 ± 0.4
	390 nt	2.6 ± 0.2	5.3 ± 1.8
G3	2 nt	2.1 ± 0.3	2.8 ± 0.2
	20 nt	2.8 ± 0.2	10.2 ± 0.9
	390 nt	2.9 ± 0.1	14.3 ± 1.8
G5	2 nt	2.0 ± 0.4	3.2 ± 0.2
	20 nt	2.5 ± 0.5	10.1 ± 2.4
	390 nt	3.5 ± 0.2	17.2 ± 1.7

C

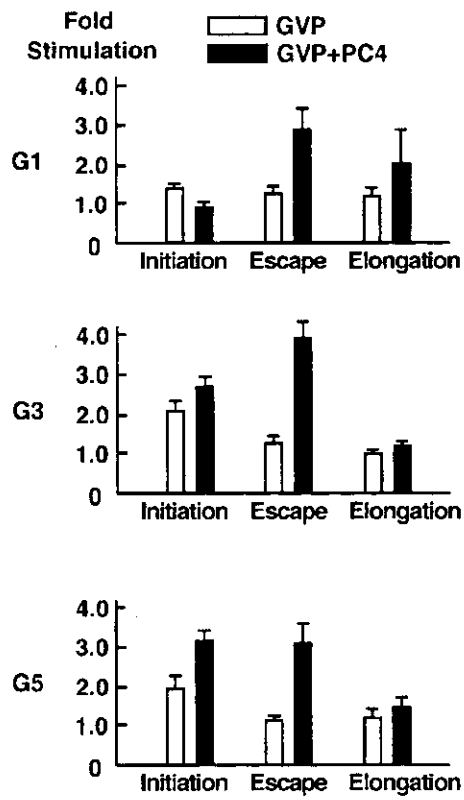


FIG. 7. The stimulation of initiation, promoter escape, and elongation on the templates with different numbers of GAL4-sites. (A) The effect of coactivation by PC4 became more pronounced as the number of GAL4 sites was increased. The levels of the 390-nt transcripts shown in Fig. 6 were quantified by using Fujix BAS 2000, and the values of stimulation (*n*-fold) were calculated. The standard deviations for three independent experiments are indicated. (B) The values of activation (*n*-fold) for the 2-, 20-, and 390-nt transcripts were determined from three independent experiments and are shown as means ± standard deviations. (C) The number of GAL4 sites influences the activation of initiation, promoter escape, and elongation. The values for stimulation (*n*-fold) of initiation, promoter escape, and elongation were calculated as those presented in Fig. 1 were. On the templates with a single GAL4 site, PC4 stimulated promoter escape rather than initiation of GAL4-VP16-dependent transcription, while on the templates with three or five GAL4 sites, PC4 stimulated both initiation and promoter escape to similar extents. There were small but reproducible effects on elongation in all experiments.

tion step but little, if any, promoter escape, regardless of the number of its binding sites. Second, PC4 increases the degrees to which GAL4-VP16 stimulates initiation and promoter escape, having a more pronounced effect on promoter escape than on initiation. Third, promoter escape appears to be preferentially stimulated by GAL4-VP16 in the presence of PC4 when GAL4-VP16 is bound on a single GAL4 site. Together, these observations suggest that each GAL4-VP16 dimer bound on the promoter may stimulate a distinct step of transcription.

DISCUSSION

Although a large body of evidence indicates the functional significance of coactivators in regulating transcription in vitro and in vivo (2, 18, 20, 36, 41), far less is known about the precise mechanism(s) by which these coactivators stimulate transcription in conjunction with activators, especially in the context of naked DNA templates. In the present study, we took advantage of a well-defined reconstituted in vitro transcription system (10, 12) and demonstrated a crucial role for a coactivator, PC4, in stimulating promoter escape in activated transcription, in part through direct interaction with TFIIH.

Figure 8 depicts how PC4 enables GAL4-VP16 to achieve a high level of transcriptional activation. This model postulates at least two targets, termed targets A and B, in the basal transcription machinery, to which signals from activators are transmitted. These signals, in turn, permit target A and target B to regulate the steps leading to initiation (PIC assembly, promoter opening, and initiation) and promoter escape, respectively. Each target postulated in the model is meant to represent multiple factors rather than a single factor, and, conversely, a single factor may constitute a part of more than one target. For instance, since TFIIA and TFIID are important for facilitating both PIC assembly (7, 8, 24, 25) and promoter escape (Fig. 2), each factor must constitute parts of both target A and target B. In addition, PC4 and TFIIH (Fig. 3, 4, and 5), whose ERCC3 helicase activity is also essential for stimulating promoter escape (10), are likely to constitute the target B that regulates promoter escape. This complex network of multiple interactions may induce conformational changes, including

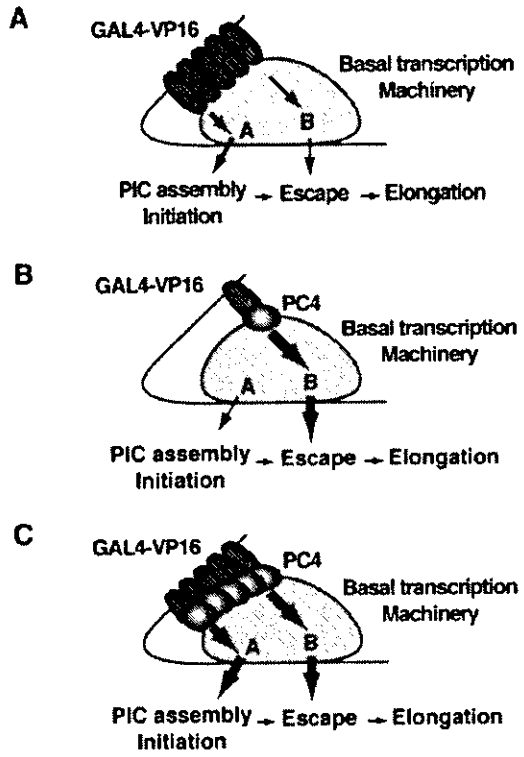


FIG. 8. A model of PC4 coactivator activity. In this model, we postulate that the basal transcription machinery contains at least two targets, termed targets A and B, through which the signals from activators are transmitted either directly or via PC4 to the individual steps of the transcription process. Target A regulates the steps leading to initiation (PIC assembly, promoter opening, and initiation) and is likely to consist of more discrete targets, whereas target B regulates promoter escape. (A) In the absence of PC4, GAL4-VP16 elicits transcriptional activation through the predominant effect on target A, regardless of the number of bound GAL4-VP16 molecules. (B) When PC4 is present, GAL4-VP16 bound at a single GAL4 site provides substantial transcriptional activation through target B. (C) When PC4 is present, multiply bound GAL4-VP16 achieves robust transcription through the synergized effects on both target A and target B, enhanced by PC4.

isomerization of the DA complex (7), that lead to stimulation of individual steps of the transcriptional process.

In the absence of PC4, GAL4-VP16 appears to function mainly through target A, and even increasing the number of GAL4-VP16 dimers bound on the template does not lead to robust transcriptional activation (Fig. 8A). In the presence of PC4, however, GAL4-VP16 can function through target B and also augment the effect through target A. Of the two postulated targets, target B seems to be preferred by the combination of GAL4-VP16 and PC4, since PC4 directs GAL4-VP16 to function predominantly through target B when the amount of GAL4-VP16 is limited, as on the G1 template (Fig. 8B). In contrast, when multiply bound GAL4-VP16 dimers are present, as on the G3 and G5 templates, PC4 permits distinct GAL4-VP16 molecules to function through both target A and target B (Fig. 8C), providing a mechanism for transcriptional synergy (6, 17, 34, 35, 46).

In this model, it is implicitly assumed that GAL4-VP16 and PC4 are capable of multiple interactions with the basal transcription machinery, interactions that obligate GAL4-VP16 and PC4 to adopt different conformations depending upon the target to which they bind. This assumption is supported not only by numerous interaction studies but also by recent structural studies showing that transcriptional activation domains, including that of VP16, are poorly structured in their free form but undergo an induced structural transition when complexed with their targets (5, 9, 30, 45, 51–53, 57, 61). In addition, the VP16 activation domain can adopt different structures whether it is bound to TBP or TFIIB (53). Moreover, this structural flexibility is also displayed by a mediator-like coactivator complex, CSRP (42, 56). Thus, given the lack of a stable three-dimensional structure within its coactivator domain (3), PC4 may form a stable structure only upon binding to activators and the basal transcription machinery. Through these interactions, PC4 could bestow activators with extra surfaces and an added conformational flexibility that permit more functionally effective links between activators and the basal transcription machinery.

Our model of PC4 action appears to contradict the widely accepted notion that PIC assembly is the primary target for activated transcription, as demonstrated by various in vivo and in vitro studies (47). In particular, using a similar in vitro transcription system, Chi et al. (7, 8) demonstrated that PIC assembly, especially DA complex assembly, is necessary and sufficient for activation, an observation supported by others (24, 25, 54, 55). Furthermore, Jacob and Luse (19) failed to detect any stimulatory effect on promoter escape by GAL4-VP16 by using HeLa nuclear extract. We believe, however, that this apparent contradiction can be reconciled for the following reasons. First, the effect on PIC assembly as inferred by the order-of-addition experiments does not necessarily dictate the actual time point at which the assembled PIC acts on steps of transcription. Thus, the effects of the assembled PIC, such as the isomerized DA complex (7), may remain far beyond the time point of their assembly. Second, we also observed the predominant effects on initiation (which may reflect PIC assembly in our assays) to overall stimulation of transcription when the amounts of factors were reduced. We suspect that, under these conditions, the stimulatory effect on promoter escape may be easily overlooked. Third, since PC4 acts as a coactivator only in its nonphosphorylated form (14, 27) and also in a highly concentration-dependent manner (13), PC4 may not have been functional as a coactivator in the transcription systems involving crude fractions (7, 8, 19, 24, 25), in which the majority of PC4 is phosphorylated (14, 27). Given these considerations, our results are not inconsistent with earlier observations that emphasized the predominant role of PIC assembly in transcriptional activation.

The exact mechanism by which PC4 assists the ERCC3 helicase of TFIIH during promoter escape remains an enigma. One attractive possibility is that PC4 stabilizes the ssDNA region exposed during promoter escape through its ssDNA-binding ability (62), thereby indirectly assisting the ERCC3 helicase. It is generally known that ssDNA-binding proteins stimulate the activities of DNA polymerases and helicases (7), and indeed, PC4 facilitates DNA replication mediated by SV40 T antigen (44). However, the possibility of this mechanism seems remote because a PC4 mutant, W89A, which has little ssDNA-binding ability (63), shows essentially the same effect

on promoter escape as wild-type PC4 does (10). Therefore, we favor alternative mechanisms by which PC4 facilitates the recruitment of TFIIH (29) or directly stabilizes the ATP-induced conformational change of TFIIH per se through protein-protein interactions, a mechanism consistent with the fact that TFIIH does not function as a classical helicase (22). Related to this idea, HBx, a coactivator-like transcriptional regulator of the hepatitis B virus (15), stimulates TFIIH helicase activities independently of its ssDNA-binding ability (48).

In conclusion, we have shown that PC4 assists GAL4-VP16 in stimulating the multiple steps of transcription and facilitates synergy by multiply bound GAL4-VP16 dimers. Future studies should address more detailed mechanistic aspects of the coactivator activity of PC4 and identify the precise factors within the basal transcription machinery that are targeted by individual GAL4-VP16 and PC4 molecules bound multiply on a single promoter. These studies may offer a paradigm for further functional analyses of diverse coactivators.

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