**Functional Correlation among Gal11, Transcription Factor (TF) IIE, and TFIIH in *Saccharomyces cerevisiae***

Gal11 AND TFIIE COOPERATIVELY ENHANCE TFIIH-MEDIATED PHOSPHORYLATION OF RNA POLYMERASE II CARBOXYL-TERMINAL DOMAIN SEQUENCES*

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Saccharomyces cerevisiae Gal11, a component of the holoenzyme of RNA polymerase II, interacts through its functional domains A and B with the small (Tfa2) and large (Tfa1) subunits of the general transcription factor (TF) IIE, respectively. We have recently suggested that Gal11 functions through a common pathway with TFIIE in transcriptional regulation (Sakurai, H., and Fukasawa, T. 1997). Here, we report that the activity of the TFIIH-associated kinase, responsible for phosphorylation of the largest subunit of RNA polymerase II at the carboxyl-terminal domain (CTD), is enhanced cooperatively by Gal11 and TFIIE. The enhancement of CTD phosphorylation was observed in the holoenzyme of RNA polymerase II, but not in its core enzyme. The stimulatory effect was completely abolished in the absence of either domain B of Gal11 or the Tfa1 subunit of TFIIE, suggesting that the domain B-Tfa1 interaction is necessary, if not sufficient, for an extensive phosphorylation of the CTD by TFIIH. Stimulation of basal transcription by Gal11 was coupled with enhancement of TFIIH-catalyzed CTD phosphorylation in a cell-free transcription system, suggesting that Gal11 activates transcription by stimulating the CTD phosphorylation in the cell.

In eukaryotes, RNA polymerase II (RNAPII) and a set of general transcription factors (TFs) including TATA-binding protein (TBP), TFIIA, TFIIE, TFIIF, and TFIIH assemble to form the preinitiation complex on the core promoter to initiate transcription from an accurate start site (1–4). The largest subunit (Rpb1) of RNAPII contains a repeated heptapeptide sequence at the carboxyl terminus, which is referred to as the carboxyl-terminal domain (CTD) (for review, see Ref. 5). The CTD is required for transcription of most, if not all, of the genes in vivo (5) as well as for mRNA processing (6). During the transcription cycle, the CTD is phosphorylated by a kinase present within TFIIH (4, 7, 8). Formation of the preinitiation complex requires RNAPII with unphosphorylated CTD, whereas elongation of transcripts is accomplished by RNAPII with phosphorylated CTD (4). These observations suggest that phosphorylation of the CTD by the TFIIH-associated kinase is an obligatory step in the transcription process from initiation to elongation.

In the yeast *Saccharomyces cerevisiae*, partial truncation of the CTD causes defects in expression of various genes (5, 9, 10). Genetic screening for suppressors of truncation mutations of the CTD by Nonet and Young (10) led to identification of a class of genes called *SRB*. Subsequent biochemical analyses suggested that nine Srb proteins (Srb2 and Srb4–11) form a complex, which is tightly associated with RNAPII at the CTD (11–14). The Srb-RNAPII complex also contains the general transcription factors TFIIA, TFIIE, and TFIIH and the global transcription regulators Gal11 and Swi5/Snf. The whole complex has been termed RNAPII holoenzyme since it has been implicated to be a preformed initiation subcomplex (12–17). Another form of RNAPII holoenzyme was isolated by Kornberg and co-workers (18) as a "mediator"-RNAPII complex. The mediator was fractionated from whole cell extracts for the capacity that confers core RNAPII responsiveness to DNA sequence-specific activators in the presence of the general transcription factors *in vitro* (18). The mediator fraction, also associated with RNAPII at the CTD, contains TFIIH and global transcription regulators including Srb2, Srb4, Srb5, Srb6, Gal11, Sin4, Rgr1, Roi3, and Med6 (18–21). Thus, TFIIH, some of the Srb proteins, and Gal11 are common components in both forms of the holoenzyme, whereas the presence of the other components is still controversial. The holoenzyme possesses properties distinct from those of core RNAPII: stimulation of basal as well as activator-induced transcription, interaction with activators, and enhancement of CTD phosphorylation by TFIIH *in vitro* (12, 13, 18, 21).

Loss-of-function mutations of GAL11 result in a wide variety of mutant phenotypes, including inefficient utilization of galactose or nonfermentable carbon sources and temperature-sensitive growth on rich media (22). Purified or recombinant Gal11 stimulates basal transcription not only in cell-free systems consisting of nuclear or whole cell extracts (23), but also in a reconstituted transcription system (22, 24). Gal11 contains two domains (designated A and B) that are essential for Gal11 function in the cell. Domain A, comprising amino acid residues 866–929, is involved in binding to the small subunit of TFIIE, whereas domain B sequences (from 116 to 255) bind to the large subunit of TFIIE (24). Recently, we constructed a mutant form of TFIIE (TFIIEΔΔC) that fails to interact with Gal11 and found that the TFIIEΔΔC mutant shows phenotypes quite similar to those of gal11 null mutations (22). Furthermore, combination of TFIIEΔΔC with a gal11 null mutation did not result in an enhanced effect compared with the respective single mutations. Based on these findings, we have suggested that TFIIE...
and Gal11 function in a common regulatory pathway of transcription (22).

In this work, we addressed the question of how Gal11 and TFIIE regulate transcription in the light of recent findings concerning functional interactions between TFIIE and TFIIH in yeast (25, 26) as well as in mammalian cells (4, 8). Mammalian TFIIE has been shown to regulate the enzymatic activities of TFIIH such as CTD kinase, DNA helicase, and ATPase (27–31). Here, we show that Gal11 stimulates TFIIH-catalyzed phosphorylation of the CTD in the presence of TFIIE only when holo-RNAPII is used as substrate. We further demonstrate that the enhanced phosphorylation of the CTD by Gal11 is associated with stimulation of transcription in a cell-free transcription reaction. In light of these results, the role of Gal11 in the transcription process is discussed.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

Plasmid pSK720 contains polyhistidine-tagged full-length GAL11 in the pQE32 vector (QIAGEN Inc.) (22). Expression constructs of Gal11-ΔA (pSK723) or Gal11-ΔB (pSK724) were created by removal of domain A (amino acids 866–929) or domain B (amino acids 48–326) of Gal11 (24) from pSK720, respectively.

**Protein Purification**

TFIIH purified from yeast (Mono Q fraction) was a gift from Drs. Jesper Svejstrup and Roger Kornberg (32). Holo-RNAPII prepared from a GAL11 wild-type or a gal11 null yeast, core RNAPII, recombinant TBP, and recombinant TFIIA were gifts from Drs. Young-Joon Kim and Roger Kornberg (18). Recombinant proteins (full-length Gal11, Tfs1, and Tfs2) were expressed in *Escherichia coli* and purified as described (22, 33).

Both Gal11-ΔA and Gal11-ΔB were expressed in *E. coli* JM109 cells harboring pSK723 and pSK724, respectively. The respective extract was adsorbed on Ni2+–nitrilotriacetic acid-agarose (QIAGEN Inc.) as described (22), and the slurry was washed with buffer F (0.1 M Heps-KOH, pH 7.6, 10% glycerol, 0.1 M potassium acetate, and 0.1% Nonidet P-40) containing 20 mM imidazole.

**Gal11-ΔA Purification**—After washing the resin with buffer F containing 100 mM imidazole, Gal11-ΔA was eluted with buffer F containing 200 mM imidazole and 0.5 M potassium acetate. The pooled fraction was loaded onto a Sephadex G-25 column (Amersham Pharmacia Biotech) equilibrated with buffer B (containing 0.1 M potassium acetate (buffer B-0.1) and eluted with the same buffer. Proteins were then loaded onto an S-Sepharose column (Amersham Pharmacia Biotech) equilibrated with the same buffer. After washing with buffer B-0.2, the proteins were eluted with buffer B-0.35. The pooled fraction was diluted with buffer B to give a potassium acetate concentration of 0.1 M and fractionated on a Q-Sepharose column (Amersham Pharmacia Biotech). Gal11-ΔA was eluted with buffer B-0.35 after washing with buffer B-0.2.

**Gal11-ΔB Purification**—Gal11-ΔB was eluted from Ni2+–nitrilotriacetic acid-agarose with buffer F containing 100 mM imidazole. The pooled fraction was loaded onto an S-Sepharose column equilibrated with buffer B-0.1. The flow-through fraction was applied to a HiTrap heparin column (Amersham Pharmacia Biotech), and Gal11-ΔB was eluted with buffer B-1.0. The yield of Gal11-ΔA and Gal11-ΔB each was ~0.2 mg/liter of starting culture.

**CTD Kinase Assay**

The reaction mixture (10 μl) contained TFIIH (30 ng) and either holo-RNAPII (100 ng) or its core polymerase (50 ng) in a buffer containing 10 mM Heps-KOH, pH 7.6, 0.1 M potassium acetate, 5 mM MgSO4, 2 mM dithiothreitol, 0.02% Nonidet P-40, 5% glycerol, 50 μg/ml bovine serum albumin, 10 μM ATP, and 1 μCi of [γ-32P]ATP. The reaction was carried out at 24 °C for 40 min and terminated by the addition of SDS-containing loading buffer. After heating at 94 °C for 7 min, the sample was loaded on an SDS-polyacrylamide gel. Labeled proteins were visualized by autoradiography and quantified by a BAS-1000 imaging analyzer (Fuji Film). All experiments were repeated at least three times, and similar results were obtained.

**In Vitro Transcription Assay**

Yeast nuclear extract was prepared from a gal11 null strain (23). A transcription assay (20 μl) was carried out using the GAL7 gene (pSK164, 40 ng) as template as described (23), except that concentrations of nucleoside triphosphates were 0.1 mM each CTP, GTP, and UTP; 20 μM ATP; and 10 μM of γ-32P]ATP. After incubation at 24 °C for 1 h, the mixture was divided into two portions. One was subjected to primer extension to analyze transcripts (22), whereas the other was used for analysis of CTD phosphorylation. The latter sample was incubated at 45 °C for 10 min and then mixed with 200 ng of an anti-CTD antibody (SWG16) and 10 μl of protein A-Sepharose (Amersham Pharmacia Biotech) in 100 μl of a buffer containing 20 mM Tris-Cl, pH 7.6, 150 mM NaCl, and 0.1% Nonidet P-40. After incubation at 4 °C for 3 h on a rotating wheel, the resin was washed three times with the same buffer. Bound proteins were extracted with SDS loading buffer and fractionated on an SDS-polyacrylamide gel, and 32P-labeled proteins were visualized by autoradiography.

**RESULTS**

Enhancement of TFIIH-catalyzed Phosphorylation of the CTD in the Presence of Both Gal11 and TFIIE—First, the effect of TFIIE on the TFIIH-associated CTD kinase activity was determined by using holo-RNAPII purified by the method of Kornberg and co-workers (18) as substrate, which contains TFIIH and transcription regulators including Gal11. A holo-RNAPII preparation from a GAL11 wild-type yeast was incubated with TFIIH in the presence of [γ-32P]ATP, and the phosphorylated proteins were fractionated on an SDS-polyacrylamide gel and visualized by autoradiography. As shown in Fig. 1A, a band with an approximate molecular mass of 205 kDa, corresponding to that of Rpb1 (see Refs. 5 and 7), was phosphorylated by TFIIH (lanes 1 and 2). When TFIIE was added to the reaction mixture, the phosphorylation of Rpb1 was enhanced by a factor of 5.4 ± 0.6 (compare lanes 2 and 3). Further addition of Gal11 did not significantly affect Rpb1 phosphorylation (lanes 4 and 5). By contrast, when holo-RNAPII from a gal11 null yeast was used as substrate (lanes 6–9), TFIIE alone could not stimulate Rpb1 phosphorylation (compare lanes 6 and 8), and a high level of the phosphorylation (11.5 ± 2.1-fold stimulation) was attained only in the presence of both Gal11 and TFIIE (lane 9). The successful stimulation of Rpb1 phosphorylation by TFIIE alone observed in holo-RNAPII from the wild-type yeast (lane 3) was therefore attributed to endogenous Gal11 in the holoenzyme preparation. From these results, we concluded that Gal11 and TFIIE cooperatively enhanced Rpb1 phosphorylation by TFIIH.

Although TFIIH-associated protein kinase is capable of phosphorylating the CTD (7), it has not been determined if the phosphorylation of Rpb1 is restricted within the CTD or not. One might therefore argue that Gal11/TFIIE-enhanced activity could phosphorylate domains other than the CTD. To address this argument, products of the kinase reaction were incubated with a control or an anti-CTD antibody and then treated with phosphatase (Fig. 1B). The samples were electrophoresed on an SDS-polyacrylamide gel, and the labeled proteins were visualized by autoradiography. When the product of the kinase reaction was pretreated with the control antibody, phosphatase treatment resulted in the disappearance of the Rpb1 band on an autoradiogram (compare lanes 1 and 2). By contrast, preincubation with the anti-CTD antibody apparently caused no loss of the Rpb1 band, presumably because the antibody protected phosphorylation groups on the CTD from phosphatase activity (compare lanes 1 and 3). This result indicated that the Gal11/TFIIE-dependent enhancement of Rpb1 phosphorylation occurred at the CTD.

We then studied the effect of Gal11 and TFIIE on TFIIH-catalyzed CTD phosphorylation using core RNAPII as substrate. In accordance with previous reports (18, 21), the efficiency of CTD phosphorylation in core RNAPII was 20–40 times lower than that in holo-RNAPII (data not shown). As shown in Fig. 1A, neither Gal11 nor TFIIE alone affected CTD phosphorylation in core RNAPII (lanes 10–12). The addition of

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both proteins increased the efficacy of phosphorylation by TFIIH, but only by a factor of 1.5 ± 0.2 (lane 13). These results indicated that the cooperative effect of Gal11 and TFIIE was much more pronounced in holo-RNAPII than in core RNAPII.

Tfa1 Subunit of TFIIE Is Required for Enhancement of CTD Phosphorylation—To determine which subunit of yeast TFIIE, Tfa1 or Tfa2 (33), was responsible for the enhancement of CTD phosphorylation by TFIIH, the respective subunit was added to a reaction mixture containing holo-RNAPII prepared from a gal11 null yeast. As shown in Fig. 2A, neither Tfa1 nor Tfa2 affected CTD phosphorylation if Gal11 was not added to the reaction (lanes 3 and 5). The addition of Gal11 enhanced phosphorylation of the CTD when Tfa1 was incorporated into the reaction (12.0 ± 2.5-fold stimulation), whereas no enhancement of phosphorylation was seen when Tfa2 was added (lanes 4 and 6). The presence of both Tfa1 and Tfa2 also caused a significant stimulation of CTD phosphorylation if Gal11 was also added to the reaction (lanes 7 and 8). We therefore concluded that Tfa1 of the TFIIE subunits was responsible for the stimulation of TFIIH-catalyzed CTD phosphorylation in cooperation with Gal11; the conclusion is comparable to the previous finding in mammalian systems that the large subunit of TFIE is sufficient to activate phosphorylation of the CTD by TFIIH (29–31).

Effect of Gal11 Mutants on Enhancement of CTD Phosphorylation—To further assess the cooperative function of Gal11 and TFIIE, Gal11 derivatives with deletions of domain A or B, known to be responsible for the interaction with Tfa2 or Tfa1 of TFIIE, respectively (22, 24), were employed (Fig. 2B). In the presence of TFIIE, a Gal11 derivative lacking domain A (Gal11-ΔA) stimulated CTD phosphorylation, but only 3.0 ± 0.6-fold over the control without Gal11, whereas full-length Gal11 did so more than 10-fold (compare lanes 1–3). Deletion of domain B (Gal11-ΔB) resulted in total loss of the enhancement of CTD phosphorylation (lane 4). These results suggested that the interaction between domain B and Tfa1 was essential in the cooperative function of Gal11 and TFIIE, but might not be sufficient (see “Discussion”).

CTD Phosphorylation in the Preinitiation Complex—To study CTD phosphorylation in holo-RNAPII as incorporated into the preinitiation complex, the kinase reaction mixture contained TBP, TFIIB, TFIIE, TFIIH, holo-RNAPII (which contains TFIIF (18)), and DNA encompassing the promoter region of GAL7 from positions −93 to +43 (34). The latter provided the site of assembly for the preinitiation complex. Aliquots were withdrawn from the reactions at the indicated times for analysis of phosphorylated proteins (Fig. 3A). At all the time points, Rpb1 was two times more efficiently phosphorylated in the mixture in which the preinitiation complex was supposed to be formed than in the mixture in which the complex was not formed. Moreover, phosphorylated Rpb1 migrated slightly
more slowly when the preinitiation complex was formed than when it was not formed, presumably due to an extensive phosphorylation of the CTD at multiple sites in the former (compare odd- and even-numbered lanes). An electrophoretic mobility shift of Rpb1 due to an extensive phosphorylation of the CTD at multiple sites by TFIIH was previously observed using core RNAPII as substrate (7). However, in our assay using holo-RNAPII, a mobility shift of Rpb1 was induced when it was integrated in the preinitiation complex. Phosphorylation of the CTD accompanying the mobility shift of the Rpb1 band was observed even in Gal11-lacking holo-RNAPII. A Gal11 derivative lacking domain B (22, 24) and TFIIH (26), the domain known to encompass the region required for interaction with holo-RNAPII as well (15), it is reasonable to suggest that the phosphorylation of the CTD is necessary for transcription in the nuclear extract. The addition of Gal11 enhanced transcription as well as CTD phosphorylation by factors of 3.6 ± 0.4 and 2.0 ± 0.2 over the background levels, respectively (compare lanes 1 and 3). The stimulatory effect of Gal11 on CTD phosphorylation appeared rather small compared with that on transcription. This may well be due to the presence of nonspecific protein kinases other than TFIIH-associated kinase and free core RNAPII in the nuclear extract, both of which would lower the apparent effect of Gal11 on the CTD phosphorylation. Neither Gal11-ΔA nor Gal11-ΔB exerted appreciable effects on both transcription and CTD phosphorylation (lanes 4 and 5).

**DISCUSSION**

Using yeast RNAPII holoenzyme as substrate, we have demonstrated that CTD phosphorylation catalyzed by TFIIH is significantly enhanced by a cooperative function of Gal11 and TFIIE. The observed stimulatory effect depends on both domain B of Gal11 and the subunit Tfa1 of TFIIE. Since Tfa1 binds both domain B (22, 24) and TFIIH (26), the domain B-Tfα1-TFIIH interaction may be essential for phosphorylation of the CTD in holo-RNAPII. A Gal11 derivative lacking domain A (Gal11-ΔA) stimulated CTD phosphorylation, but less efficiently than wild-type Gal11. Neither Gal11 nor TFIIE led to a significant effect on TFIIH-mediated CTD phosphorylation with core RNAPII as substrate. Since domain A of Gal11 is known to encompass the region required for interaction with holo-RNAPII as well (15), it is reasonable to suggest that the lowered stimulatory ability of Gal11-ΔA is due to its weak...
association with holo-RNAPII and that a tight association of 
Gal11 with holo-RNAPII is a prerequisite for efficient stimula-
tion of CTD phosphorylation by TFIIH. Recent studies by 
Svejstrup et al. (36) have suggested that phosphorylation of the 
CTD causes dissociation of holo-RNAPII into the mediator and 
core RNAPII and that the dissociated core polymerase travels 
along the template to elongate the transcript. Taken all to-
gether, we suggest that the Gal11-TFIIE-TFIIH interaction is 
involved in regulation of the transition of holo-RNAPII to an 
elongation-competent complex in yeast.

The present experiments have demonstrated that neither of 
the mutant Gal11 proteins (Gal11-ΔA and Gal11-ΔB) is capable 
of enhancing transcription in a nuclear extract. These results are 
consistent with those of in vivo analyses showing that 
neither mutant is able to induce the expression of GAL7-lacZ 
(24). Since phosphorylation of the CTD is a key step in the 
transcription reaction (4), we assume that the observed en-
hancement of CTD phosphorylation in cooperation 
with TFIIE, which triggers formation of the elongation 
complex. We previously demonstrated that Gal11 stimulates 
basal transcription in a system reconstituted with core RNAPII 
and the general transcription factors (24). However, we failed 
to show a significant effect of Gal11 on CTD phosphorylation 
in core RNAPII (Fig. 1A). On the other hand, Li and Kornberg 
(35) have shown that CTD phosphorylation is dispensable for 
transcription initiation in the reconstituted system. These 
observations led us to speculate that Gal11 also regulates 
other enzymatic activities such as ATPase and DNA helicase 
of TFIIH besides its CTD kinase activity through TFIIE 
function since mammalian TFIIE has been shown to regulate 
TFIIH ATPase and DNA helicase activities (28–30). Al-
though many more experiments are required to clarify the 
exact role of Gal11 in transcription, this work has clearly 
documented functional interactions among Gal11, TFIIE, 
and TFIIH and consequently further supports a model where 
Gal11 is involved in the transition from initiation to elonga-
tion in the transcription process.

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Functional Correlation among Gal11, Transcription Factor (TF) IIε, and TFIIH in *Saccharomyces cerevisiae*: Gal11 and TFIIε Cooperatively Enhance TFIIH-Mediated Phosphorylation of RNA Polymerase II Carboxyl-Terminal Domain Sequences

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