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Zero-Length Crosslinking Study on Interactions of TBP, GAL4-AH, and TFIIB in the Preinitiation Complex

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A zero-length crosslinking procedure for studying protein-protein interactions in preinitiation complex has been developed. Preinitiation complexes were assembled with immobilized DNA templates coupled to metal beads. Purified complexes were directly crosslinked by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The reaction was stopped by addition of β-mercaptoethanol, and the complexes were isolated from EDC immediately. An application of this method with a preinitiation complex assembled with TBP, TFIIB, and GAL4-AH demonstrated that TBP directly interacts with GAL4-AH and TFIIB in the preinitiation complex. However, crosslinked product between GAL4-AH and TFIIB was not observed. These results indicate that GAL4-AH does not stably interact with TFIIB in the GAL4-AH-TFIIB-TBP-DNA preinitiation complex.

KEY WORDS: TBP, GAL4-AH, TFIIB, Preinitiation Complex, Crosslinking

Transcription initiation by RNA polymerase II is proceed by the assembly of multiprotein-DNA complex with RNA polymerase II (pol II) and general transcription factors (GTFs) TFII-A, -B, -D, -E, -F, -G/J, and -H (reviewed in Zawel and Reinberg, 1993). TATA-box binding protein (TBP) in TFIID recognizes a TATA element, and initiates the assembly of a preinitiation complex (PIC). TFIIA is the second general transcription factor to enter PIC, and then TFIIB follows to form DAB complex. DAB serves as a platform that is recognized by pol II and TFIIF. TFIIE, TFIIH, and then TFIIG/J assemble in an order to complete the formation of PIC.

TFIID consists of a TBP and several TBP-associated factors (TAFs). Although incapable of mediating activator-dependent transcription, TBP alone can initiate the formation of PIC and direct a basal transcription activity (Dynlacht *et al.*, 1991). The association of TBP with the TATA box induces a large DNA bending. This TBP-induced DNA bending allows TFIIB to recognize the preformed TBP-DNA complex through interactions with C-terminal region of TBP and DNA upstream and downstream of the TATA box (Nikolov *et al.*, 1995). TFIIB seems to provide TFIIF binding site to position pol II start sites (Ha *et al.*, 1993). TFIIB also interacts with some transcriptional activator and coactivators (Goodrich and Tjian, 1994).

Transcription activator proteins, that specifically associate with cis-elements located upstream or downstream of TATA-box of the promoter, can dramatically increase the transcription activity of the promoter presumably by increasing preinitiation complex assembly. Because of their high potencies to activate transcription, acidic activators such as GAL4-VP16 or GAL4-AH have been studied to delineate the step(s) accelerated by the activator in preinitiation complex assembly. Many studies indicate that the acidic activator accelerate multiple steps in preinitiation complex assembly. Acidic activators directly interact with TBP (Stringer *et al.*, 1990). Several studies

indicated that activators promote the assembly of a closed complex of TFIID-TFIIA-DNA (Wang *et al.*, 1992; Lieberman and Berk, 1994). The stable entry of TFIIB to preinitiation complex is accelerated by acidic activators in the 'recruitment' assays using immobilized DNA templates (Lin and Green, 1991). Furthermore, acidic activators also increased the stable assembly of TFIIF, TFIIE, and RNA polymerase II only when TAFs are present in preinitiation complex (Choy and Green, 1993). However, the mechanisms by which acidic activators promote the entry of each GTF to preinitiation complex are remained to be elucidated. It is conceivable that some GTFs directly interact with acidic activators and recruited to preinitiation complex and other GTFs more efficiently enter into preinitiation complex in the presence of acidic activators because of the conformational change of preinitiation complex induced by acidic activators.

In order to address this question directly, I have developed a chemical crosslinking assay of preinitiation complex and measured GTFs directly crosslinked to activators in the preinitiation complex. In this study, I specifically asked whether an acidic activator, GAL4-AH, can stably interact with TBP and/or TFIIB in the GAL4-AH-TFIIB-TBP-DNA preinitiation complex. Direct interactions of GAL4-VP16 with TBP or TFIIB were shown by affinity chromatographic assays using GST-GAL4-VP16 (Lin and Green, 1991). However direct interactions of GAL4-VP16 with TBP or TFIIB have not been unequivocally shown to occur in the preinitiation complex. Furthermore GST-GAL4-AH matrix does not bind TFIIB, and the recruitment of TFIIB into preinitiation complex by activators requires the presence of TBP. These results suggest the interaction of acidic activators, specially GAL4-AH, with TFIIB may be kinetic. The results of a chemical crosslinking assay of preinitiation complex in this study indicate that TBP directly interacts with GAL4-AH and TFIIB, but GAL4-AH does not stably interact with TFIIB in the GAL4-AH-TFIIB-TBP-DNA preinitiation complex.

Materials and Methods

Recombinant yeast TBP, GAL4-AH, native TFIIB, and T7-tagged TFIIB were expressed in E. coli and purified as described in Roberts *et al.*, (1993).

A biotinylated T7 primer and a SP6 primer were used for a PCR reaction amplifying a biotinylated 460 bp DNA fragment containing the promoter region of pG5E4T (Lin *et al.*, 1988). The 3.5 pmole of biotinylated DNA fragments were immobilized onto 10 μl of streptavidin-coupled magnetic beads (Dynabead M-280). Preinitiation complexes were formed with 25 pmole of GAL4-AH, 14 pmole of yeast TBP, and 14 pmole of TFIIB at 30°C for 1 hr in 80 μl of a binding buffer (75 mM KCl, 2 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.2 mM EDTA, 2 mM β-mercaptoethanol, 0.003% NP40, 10% glycerol, 20 mM Hepes, pH 8.0). Complexes were magnetically purified, and then resuspended with 76 μl of a crosslinking buffer (75 mM KCl, 2 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.003% NP40, 10% glycerol, 40mM Hepes, pH7.0). Crosslinking reaction was initiated by the addition of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC, final concentration of 2 mM) and allowed to proceed for 12 min at 25°C. The reaction was terminated by the addition of β-mercaptoethanol (final concentration, 20 mM), and the beads were washed once with the binding buffer. The beads were resuspended with SDS sample loading buffer, boiled for 3 min, and then polypeptides were resolved by SDS-PAGE. Enhanced chemiluminescence immunoblotting assay was performed as recommended by the supplier (ECL, Amersham Corp.).

Results

A chemical crosslinking assay of preinitiation complex was developed as Fig. 1. G5E4T DNA template labeled by a biotin at its one end was generated by a PCR reaction employing a biotinylated T7 primer and a SP6 primer, and was immobilized onto streptavidin-coupled magnetic beads. GAL4-AH, TBP, and TFIIB are stably incorporated into immobilized DNA templates,

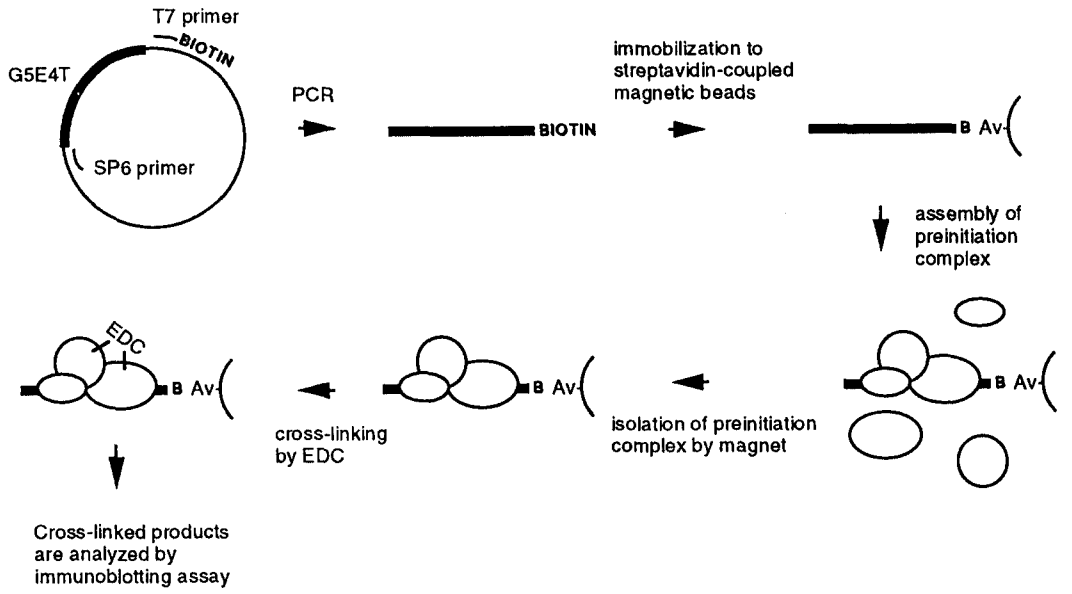

Fig. 1. Experimental scheme for zero-length crosslinking of preinitiation complex.

and the preinitiation complexes were purified from unbound GAL4-AH, TBP, and TFIIB. The purified complexes were directly crosslinked by EDC. After the reaction was stopped by β-mercaptoethanol, the complexes are quickly washed to remove residual EDC completely. Crosslinked products of the complexes were analyzed by immunoblotting assay with anti-GAL4 antibody, anti-TBP antibody, or anti-TFIIB antibody.

EDC was chosen as a crosslinking agent in this study because 1) EDC is water soluble, 2) EDC is a zero-length crosslinker, providing a better chance to link proteins that directly interact each other, and 3) crosslinking step has a specificity for carboxyl group of Glu or Asp, providing preferential crosslinking reactions between activation region of acidic activator and GTFs (Grabarek and Gergely, 1990).

Since the DNA template has five GAL4 binding sites and GAL4-AH is dimer, EDC may introduce multiple crosslinkings between GAL4-AHs. It may result in GAL4-AH polymers and make analysis of crosslinked products difficult. Thus, we first crosslinked GAL4-AHs associated with G5E4T DNA by EDC (Fig. 2A, lane 2). After the crosslinking reaction, only a small amount of crosslinked dimer was observed as heterogeneous in sizes. The formation of GAL4-AH polymers other than dimers was not observed, indicating EDC does not vigorously crosslink GAL4-AHs in dimer and not at all between GAL4-AH dimers. EDC also did not interfere the binding of GAL4-AH to GAL4-binding site because the amount of GAL4-AH associated with DNA was not reduced by the treatment of EDC (Fig. 2A).

Ensuring EDC does not introduce problematic crosslinking between GAL4-AHs, we assembled preinitiation complexes with combinations of GAL4-AH, TBP, and TFIIB. The complexes were isolated from unbound components, and crosslinked by EDC. The presence of each component in crosslinked products was identified by an immunoblotting assay using an antibody recognizing each components specifically. Fig. 2A shows such an assay using an anti-GAL4 antibody. When the complexes were assembled with GAL4-AH and TBP (Fig. 2A, lane 4) or with GAL4-AH, TBP and TFIIB (Fig. 2A, lane 3), a crosslinked product about 55-kDa was observed. When DNA template was omitted from reactions, the formation of the 55-kDa crosslinked product was not observed, indicating the 55-kDa crosslinked product was formed on DNA template (data not shown). When the complexes were assembled with

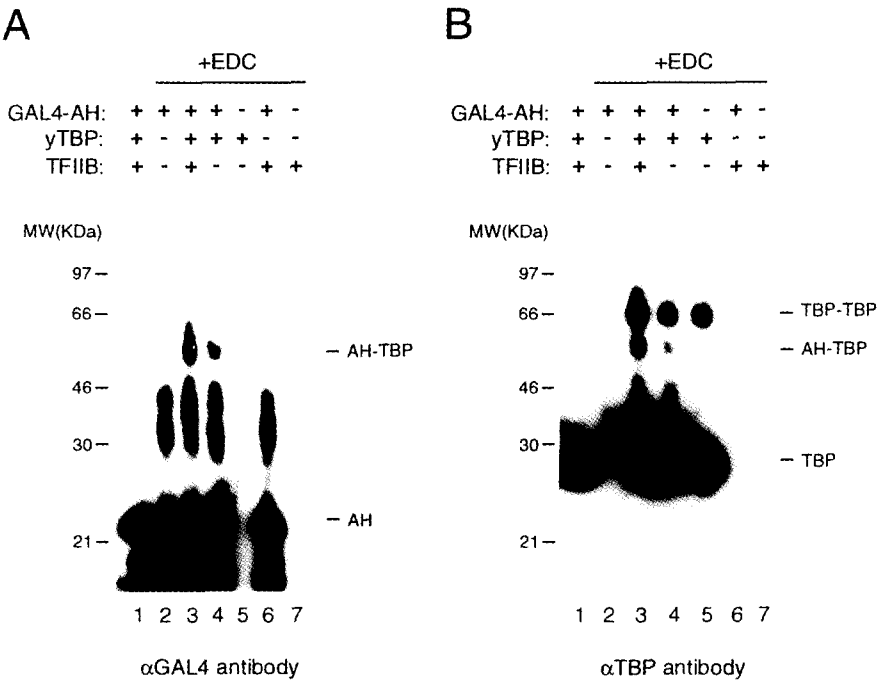

Fig. 2. Crosslinking between TBP and GAL4-AH in preinitiation complex. Purified preinitiation complex was crosslinked with 2 mM EDC for 12 min, and then the reaction was terminated by addition of β-mercaptoethanol. Preinitiation complex was assembled with the indicated factors. (A) Samples were analyzed by immunoblotting assay with an anti-GAL4 antibody. (B) After deprobing, the same membrane was analyzed by immunoblotting assay with an anti-TBP antibody.

GAL4-AH and TFIIB, on the other hand, no crosslinked product was observed (Fig. 2A, lane 6). These results suggest that the 55-kDa crosslinked product is a crosslinked product of GAL4-AH and TBP. In order to confirm this, I deprobed the membrane, and performed an immunoblotting assay using an anti-TBP antibody (Fig. 2B). The 55-kDa crosslinked product was also recognized by the anti-TBP antibody. This result clearly shows that the 55-kDa crosslinked product is a crosslinked product of GAL4-AH and TBP.

A crosslinked product about 70-kDa was also recognized by the anti-TBP antibody (Fig. 2B). However, the 70-kDa crosslinked product was not recognized by either the anti-GAL4 antibody or an anti-T7 antibody which recognizes the T7-tagged TFIIB. This product also formed when only TBP was assembled into DNA templates (Fig. 2B, lane 5). These results indicate that the 70-kDa crosslinked product is a dimer of TBP. The dimer formation of TBP has been previously reported (Taggart & Pugh, 1996). Since the monomeric form of TBP is known to be effective for transcription, it is consistent with the observation that only the monomeric TBP can be crosslinked to GAL4-AH.

When the same membrane was again deprobed, and immuno-blotted with an anti-T7 antibody, no crosslinked product of T7-tagged TFIIB was observed (data not shown). However, TFIIB was found to easily dissociate from the membrane during the deprobing step, and the dissociation of TFIIB during deprobing seems to make difficult the detection of small amounts of crosslinked product. Thus, I performed similar crosslinking reactions, but at this time I divided the SDS-PAGE sample of each reaction into three parts and resolved at the same SDS-PAGE gel. After transferred to PVDF-membrane, the membrane was cut into three

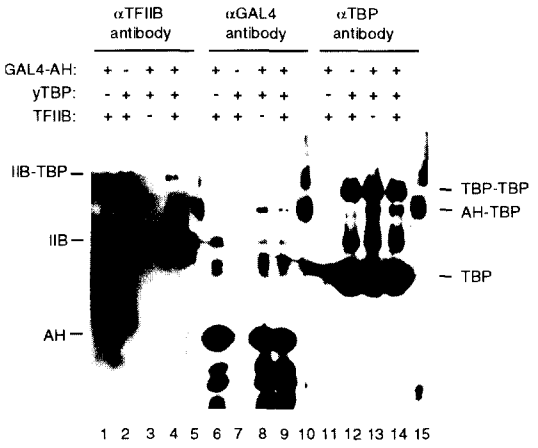

Fig. 3. Crosslinking between TBP and TFIIB in preinitiation complex. As in Fig. 2, except that sample of each reaction was divided into three parts and three parts were separately analyzed by immunoblotting assay with the indicated antibody.

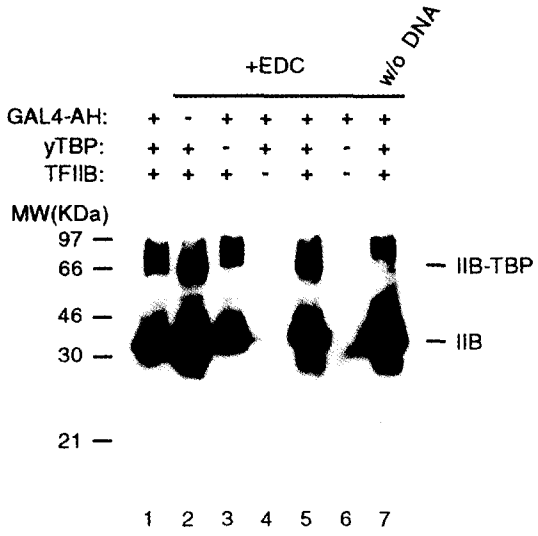

Fig. 4. Crosslinking between TBP and TFIIB in preinitiation complex. As in Fig. 3, except native TFIIB was used instead of T7-tagged TFIIB. DNA template was omitted in the reaction (lane 7).

pieces and analyzed crosslinked products of TBP, GAL4-AH, or TFIIB by separate immnoblotting assays. In this assay, a 75-kDa crosslinked product of TFIIB was observed when the complexes were assembled with TBP and TFIIB or with GAL4-AH, TBP and TFIIB (Fig. 3, lanes 2 and 4). When TFIIB and GAL4-AH was assembled to DNA template, the 75-kDa crosslinked product was not detected. Furthermore, the immunoblotting assay with an anti-GAL4-AH antibody did not show the formation of the 75-kDa crosslinked product (Fig. 3, lanes 6 - 9). Although the immunoblotting assay with the anti-TBP antibody did not resolve the 75-kDa crosslinked product from the crosslinked TBP dimer (Fig. 3, lanes 11 - 14), it is highly likely that the 75-kDa crosslinked product is formed between TBP and TFIIB. With the preparation of TFIIB which was not tagged with T7, the 75-kDa crosslinked product was consistently observed when TFIIB is assembled with DNA only in the presence of TBP (Fig. 4). In these experiments, crosslinked product containing all of GAL4-AH, TBP, and TFIIB was not observed.

Discussion

EDC is a reagent that covalently crosslinks proteins in direct interactions. In this study, preinitiation complexes were assembled to bead-immobilized DNA templates and easily purified away from unbound proteins. Thus, crosslinking reactions could be performed with the purified preinitiation complexes, ensuring only stable interactions in preinitiation complexes result in the formation of crosslinked products. This experimental design is highly likely to exclude possible crosslinking of unbound proteins in transient interactions with proteins of preinitiation complexes. This study was focused to delineate direct interactions between GAL4-AH, TBP and TFIIB in stably formed preinitiation complexes, and demonstrated direct interactions of TBP with TFIIB and GAL4-AH in preinitiation complexes. On the other hand, direct interaction between GAL4-AH and TFIIB was not observed in preinitiation complexes.

Since TFIIB alone dose not bind to DNA and can assemble into the preinitiation complex only after the binding of TBP to TATA box, it is generally accepted that TFIIB makes a direct interaction with TBP. The direct interaction between TFIIB and TBP was demonstrated as the

formation of 75-kDa crosslinked product in this study. TBP also crosslinked with GAL4-AH as the 55-kDa crosslinked product. These results directly demonstrate that GAL4-AH makes an direct interaction with TBP in preinitiation complex.

Interestingly crosslinked product between GAL4-AH and TFIIB was not observed in this study. Several studies suggest that acidic activators make direct interactions with TFIIB and mediate recruitment of TFIIB into preinitiation complex. Immobilized DNA template assays showed the TFIIB binding is a rate limiting step. Protein-protein interaction studies using GAL4-VP16 affinity column have shown GAL4-VP16 can directly interact with TFIIB (Lin and Green, 1991). TFIIB mutants defective in the binding with GAL4-VP16 was unable to support the transcriptional activation by acidic activators without effect on the basal transcriptional activity (Roberts *et al.*, 1993). On the other hand, several evidences support that the interaction between acidic activators and TFIIB is rather transient, if there is. For examples, protein-protein interaction between GAL4-AH and TFIIB is yet to be demonstrated. And the recruitment of TFIIB into preinitiation complex by activators requires the presence of TBP (Lin and Green, 1991). This study employing a direct crosslinking of preinitiation complexes indicates that GAL4-AH is not likely to make direct contact with TFIIB in preinitiation complexes. Since this study is designed to detect only stable interactions between proteins, a transient interaction of GAL4-AH with TFIIB cannot be excluded. The recruitment of TFIIB into preinitiation complex by activators may result from a transient interaction between GAL4-AH and TFIIB that increase effective concentration of TFIIB. However it is still possible that the interaction of GAL4-AH to TBP in preinitiation complex, demonstrated as the formation of 55-kDa crosslinked product, induce a conformational change in TBP increasing binding affinity for TFIIB.

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전사개시전 복합체에서 TBP, GAL4-AH, TFIIB의 상호작용에 대한 Zero-Length Crosslinking 실험

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전사개시전 복합체(preinitiation complex)에서 단백질간의 상호작용을 연구하기 위해 zero-length corsslinking 방법을 이용하였다. DNA template가 결합한 금속 지지체를 이용하여 *in vitro*에서 전사개시전 복합체를 형성시키고, 이렇게 만든 복합체를 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide(EDC)로 crosslinking 시켰다. β-mercaptoethanol를 첨가하여 crosslinking 반응을 멈추게 한 다음, EDC로부터 전사개시전 복합체를 분리하였다. TBP, TFIIB, GAL4-AH 등으로 구성된 전사개시전 복합체에 이러한 방법을 적용함으로써 TBP가 GAL4-AH, TFIIB와 각각 직접적으로 결합하고 있음을 규명하였다. 반면에 GAL4-AH와 TFIIB가 crosslinking된 산물은 확인할 수 없었다. 이러한 결과들은 GAL4-AH, TFIIB, TBP, DNA로 구성된 전사개시전 복합체에서 GAL4-AH는 TFIIB와 안정적인 결합을 하고 있지 않음을 암시한다.