Critical Role of the Second Stirrup Region of the TATA-binding Protein for Transcriptional Activation Both in Yeast and Human*

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We previously identified three TATA-binding protein (TBP) point mutations (L114K, L189K, and K211L) that have severe effects on transcriptional activation by acidic activators, but no effect on basal transcription, in a yeast-derived TBP-dependent in vitro transcription system (Kim, T. K., Hashimoto, S., Kelleher, R. J., III, Flanagan, P. M., Kornberg, R. D., Horikoshi, M., and Roeder, R. G. (1994) Nature 369, 252-255). These activation defects were also demonstrated in vivo in yeast cells (Lee, M., and Struhl, K. (1995) Mol. Cell. Biol. 15, 5461-5469). Here, the transcriptional activities of these and other TBP mutations were examined in human by both in vitro and in vivo assays. Mutations L189K and E188K, which lie in the second stirrup region of TBP, show defective activation by acidic activators both in yeast and human. Somewhat surprisingly, mutations L114K and K211L have almost no demonstrable effect on activation by acidic activators in human, in contrast to their severe effects on defective activator responses in yeast. The implications of these results for TBP structure and function are discussed.

TBP1 has been proposed to be the direct or indirect target of several upstream activators in addition to playing a crucial role in basal transcription (reviewed in Refs. 1 and 2). Amino acid sequence comparisons of TBP species from yeast to human have revealed a highly conserved carboxyl-terminal domain of 180 amino acids and a variable amino-terminal domain (reviewed in Ref. 3). The conserved carboxyl-terminal core is known to be sufficient for binding to the TATA box and for basal transcription in vitro (reviewed in Refs. 1-3). Despite striking structural and functional similarities among eukaryotic TBP, yeast cells are not viable when yeast TBP (yTBP) is replaced with human TBP (hTBP) (4-6). One possibility is that TBP might be involved in species-specific interactions with other transcription factors. However, several other studies showed that yTBP can support both basal and activated transcription by acidic activators in a human in vitro system and that hTBP can mediate responses to acidic activators in yeast cells (7, 8). This interchangeability of yeast and human TBP's argues against species-specific interactions, at least for the response to acidic activators.

Despite this functional interchangeability, hTBP is less active for the response to acidic activators than yTBP in yeast based on levels of reporter gene expression both in vivo and in vitro (7, 8). yTBP and hTBP also differ in another respect. The hTBP is found in a very stable complex (TFIID) containing both TBP and TBP-associated factors (TAFs) with coactivator functions (reviewed in Refs. 1, 2, and 9). In contrast, although yeast contains homologous TAFs that can be isolated in association with yTBP (10, 11), the interactions are apparently much weaker than those reported for the human and Drosophila counterparts. Moreover, although TAFs appear to be obligate coactivators for the in vitro function of activators in human and Drosophila systems (Refs. 12 and 13; reviewed in Refs. 1, 2, and 9), recent studies of yeast TAFs suggest that they are not generally required for the several activators tested (14, 15). In this case activation may be more dependent upon other coactivators, such as components of the holoenzyme mediator complex (reviewed in Ref. 16) or the ADA complex (reviewed in Ref. 17). General coactivators other than TAFs have also been reported in the human system, although they are jointly required with human TAFs for activation (18-20). Given apparent differences in requirements for coactivators (especially TAFs) in yeast and human, one might expect variations in activation mechanisms and in the importance of the well documented interactions of activators with general factors such as TBP (reviewed in Ref. 21) and TFIIB (22).

We previously used a series of point mutants in the conserved carboxyl-terminal core of yeast TBP to define residues important specifically for GAL4-VP16-dependent transcription (23). Mutants L114K, L189K, and K211L were found defective for the response to GAL4-VP16, but nonetheless showed normal basal transcription by RNA polymerase II. A more detailed analysis revealed that activator-induced TFIIB recruitment to (or stabilization within) the preinitiation complex is disrupted by TBP mutations that impair its interaction with VP16 (L114K, TFIIB (L189K), or the TATA element (L114K and K211L). Focusing on these mutations, the objective of the present study was to identify TBP interactions important for transcriptional activation by acidic activators in human as well as yeast, and possible differences in the two systems. In contrast to the behavior of mutants L114K, L189K, and K211L in yeast, where all are severely defective for activator function, two (L114K and K211L) mediated normal activation responses and another (L189K) showed only a modest defect in human systems.

EXPERIMENTAL PROCEDURES

In Vitro Transcription Reactions—Transcription factors TFIIA, TFIIE/F/H, and USA were fractionated from HeLa nuclear extracts by phosphocellulose (P-11) chromatography (24) and further purified as described (19). RNA polymerase II was purified from HeLa nuclear pellet extracts by chromatography through heparin-Sepharose, DEAE-cellulose, and Mono Q columns (25). Flag-tagged TFIID was purified by affinity chromatography (18) and was heat-treated for 5-10 min to inactivate endogenous TBP activity at 47 °C as described (26). Recom-
Results

The previously analyzed TBP mutations, which selectively abolish VP16-activated transcription in yeast (23), occur at residues that are conserved in yTBP and hTBP and located in the conserved carboxyl-terminal domain (Fig. 1A). The corresponding mutant (L114K, L189K, and K211L) and wild-type yTBPs were expressed as hexahistidine fusion proteins in E. coli. The expressed TBPs were purified by nickel affinity chromatography (Fig. 1B) and indicated above each lane of the SDS-polyacrylamide gel.

In Vivo Altered-specificity TBP Assay—HeLa cells were transfected with a c-fos reporter plasmid, TBP and GAL4-VP16 expression plasmids, and an α-globin internal reference plasmid (32). After 48 h, RNase protection analysis was performed to quantitate accurately initiation of transcription. Site-directed mutagenesis was used (33) to introduce the triple amino acid altered-specificity substitutions (8) into TBP, as well as specific substitutions at the Leu-114, Glu-188, Leu-189, or Lys-211 residues (23).

Activation-defective TBP Mutant

The previously analyzed TBP mutants, which have been used to study transcription in yeast (23), occur at residues that are conserved in yTBP and hTBP and located in the conserved carboxyl-terminal domain (Fig. 1A). The corresponding mutant (L114K, L189K, and K211L) and wild-type yTBPs were expressed as hexahistidine fusion proteins in E. coli. The expressed TBPs were purified by nickel affinity chromatography (Fig. 1B) and employed in various in vitro transcription assays. Wild-type or mutant yeast TBPs were purified and indicated above each lane of the SDS-polyacrylamide gel.

In vitro reconstituted transcription was performed and analyzed as described before (19, 23, 30, 31). In vitro reconstituted transcription was performed and analyzed as described before (19, 23, 30, 31).

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The proposed TFIID selectively inactivates TBP, leaving a TAF population that, with ectopic TBP, can mediate activator function (albeit at a level lower than that observed with intact TFIID) (26). The template used for this analysis contains five GAL4 binding sites preceding the HIV TATA element fused to a G-less cassette (Fig. 2A). As shown in Fig. 2B, in the absence of recombinant TBP, no specific transcription could be detected with general factors and heat-treated TFIID either alone (lane 1) or in combination with either the acidic activator GAL4-VP16 or the USA fraction (lanes 2 and 3). A low level of transcription was detected when both GAL4-VP16 and USA were present (lane 4), probably due to a trace amount of TBP contamination in the transcription system (19). In the presence of recombinant wild-type TBP, general factors, and heat-treated TFIID, a low level of basal transcription was observed (lane 5). This basal transcription was unaffected by the sole addition of GAL4-VP16 (lane 6) or USA (lane 7), but both together gave a significant activation (lane 8). This marked activation by GAL4-VP16 was not observed in the absence of a heat-treated (and near homogeneous) TFIID preparation, which presumably supplies essential TAFs that can reassociate with TBP. These results indicate that transcription in this human in vitro reconstituted assay system is dependent upon exogenous TBP.

Using the TBP-dependent human in vitro system described above, we analyzed the response of activation-defective TBP mutants (23) to acidic activators (Fig. 3). Fig. 3A shows an analysis of transcription with different concentrations of TBP in this reconstituted transcription system. Additionally, we analyzed the response of TBP mutants (23) to GAL4-VP16 using non-saturating levels of TBPs (Fig. 3B). While comparable to wild-type TBP with respect to basal activity, the L189K

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mutant was defective for activation (lanes 5 and 6 versus lanes 1 and 2). This is consistent with the effect of the L189K mutation in yeast, although the defect was much less severe in the human system as compared with the yeast system. Somewhat surprisingly, mutants L114K and K211L, which were also severely defective for activated (but not basal) transcription in yeast (23), supported activated transcription by GAL4-VP16 as efficiently as did wild-type TBP. These contrasting activities of TBP mutants in the yeast versus the human system, including the moderately defective activation response with L189K in the human system, were also observed with different acidic activators (e.g. GAL4-AH).

The contrasting activities of TBP mutants in the yeast versus the human system might be due to the different promoters used in the respective assays. The HIV promoter was used in the human system, whereas CYC1 was used in the previous yeast transcription assay (23). To rule out this possibility, we determined the transcription activities of three TBP mutants in the yeast in vitro system with templates containing the HIV human promoter (Fig. 4). In this case, all the mutants (L114K, L189K, and 211L) were shown to be severely defective for GAL4-VP16 activation. These results indicate that the differences in the ability of mutant TBPs to mediate activation in yeast versus human systems are not due to the different promoters used to perform the assays and suggest, along with the data in Fig. 3, that certain interactions might be different for the function of acidic activators in yeast and human. The contrasting responses by TBP mutants appear to be specific for activated transcription, since all of the 34 original mutant TBP tested (see Ref. 23) showed either undetectable activities or basal activities comparable to those displayed by wild-type TBP in both human (Table I) and yeast (23) transcription systems.

To further examine the contrasting responses with TBP, wild-type yTBP and hTBP were compared for their ability to support activated transcription in the TBP-dependent human and yeast in vitro systems. Wild-type yTBP was indistinguishable from wild-type hTBP in support of activation by GAL4-VP16 in the human in vitro system (Fig. 5B). In contrast, wild-type hTBP supported the response to acidic activators much less efficiently than wild-type yTBP in the yeast transcription system (Fig. 5A). This is consistent with the defective response of TBP mutants only in yeast (see Figs. 3 and 4).

Next we assessed the in vivo significance of the in vitro results described above by using an altered-specificity TBP in transcription assays (32). Various point mutations (L114K, L189K, and K211L) were introduced into a TBP derivative that can recognize an altered TATA box (TGTAAA) in a c-fos reporter construct in human (HeLa) cells. Transcription from the c-fos reporter was measured by RNase protection analysis with normalization to transcription from an α-globin internal control plasmid (Fig. 6B).
Expression of each TBP mutant was determined by immuno-
blot analyses from transfected cells, and the amounts of the
TBP expression plasmids used for transfection were adjusted
to give the same level of expression for each.\(^2\) Under these condi-
tions, and consistent with the \textit{in vitro} results, mutants L114K
and K211L supported transcriptional activation by GAL4-
VP16 as well as did wild-type TBP (Fig. 6\(B\)). In contrast the
mutation L189K significantly (over 2-fold) reduced the ability
of TBP to respond to GAL4-VP16 in human cells (lane 2). These
results support the conclusion that TBP shows contrasting
activation responses in yeast and human and that the Leu-189
region plays an important role in transcriptional activation.

In the three-dimensional TBP structure (34, 35), the L189K
mutation maps within the second stirrup region that connects
strands S2\(^'\) and S3\(^'\) (Fig. 8). To further assess the important
role of this region in transcriptional activation in the human
system, we tested a mutation (E188K) in an adjacent residue
(Glu-188) whose side chain points in the same general direction
as the Leu-189 residue. In both the \textit{in vivo} and \textit{in vitro} assay
systems described above, mutant E188K showed a defective
response to GAL4-VP16 that was considerably greater than
that observed for L189K (Fig. 7). In conjunction with the less
dramatic but highly reproducible effect of the L189K mutant,
this compromised activation response by E188K indicates that
the second stirrup region of TBP is critical for transcriptional
activation by acidic activators in the human system.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Comparison of responses to GAL4-VP16 by wild-type
\textit{yTBP} versus \textit{hTBP} in the yeast and the human \textit{in vitro}
transcription systems. Wild-type \textit{yTBP} and \textit{hTBP} were tested for the
ability to support activated transcription by GAL4-VP16 in the TBP-
dependent yeast (\(A\)) and human (\(B\)) transcription systems (3, 7). GAL4-
VP16 was added in increasing amount to transcription reactions. Rel-
ative amounts of GAL4-VP16 assayed are given in multiples of 30 ng.
Radioactivity incorporated into specific transcripts is expressed as rel-
ative transcription and plotted as a function of the GAL4-VP16
concentration.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{Response of TBP mutants to GAL4-VP16 \textit{in vivo} in
human cells. \(A\), the chimeric GAL4-VP16 activator protein contains
the amino-terminal 94 amino acids of GAL4 (with the DNA binding
domain) fused to the carboxyl-terminal 78 amino acids of VP16. The
template DNA for \textit{in vitro} transcription contains an altered TGTAAA
box from the \textit{c-fos} promoter with four GAL4 DNA binding sites. \(B\), RNase
protection analysis was performed with RNA isolated from HeLa
cells transiently transfected with the \textit{c-fos} TGTAAA reporter, GAL4-
VP16 expression construct and expression constructs for wild-type (WT)
or specific TBP mutants (23) as indicated (32). All the TBP's carried the
altered-specificity substitutions as described before (8). Accurately ini-
tiated \textit{c-fos} transcripts, normalized to the \(\alpha\)-globin signal, are shown.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig7.png}
\caption{Defective activation by the E188K mutation, which is
located in the second stirrup region of TBP. \textit{In vivo} (\(A\)) and \textit{in vitro}
(\(B\)) human transcription assays were performed as described in Figs. 6
and 2, respectively. The E188K mutant had 30–50\% the activity of
wild-type TBP for basal transcription \textit{in vitro} (see Footnote 2). Activ-
ated transcription was compared by normalizing the basal level of
transcription. \(B\), numbers below the lanes represent the activation
(\(n\)-fold) in the presence of GAL4-VP16.}
\end{figure}
L189K) were shown to be defective in transcriptional activation by acidic activators as efficiently as wild-type TBP. These contrasting activities of TBP mutants for activator function in the yeast versus human system are also observed with some other TBP mutants (37, 38), including a K138T/Y139A mutant (29) that cannot interact with TFIIA. These mutants can support transcriptional activation in human but are severely defective for activation responses in yeast. Interestingly, all the TBP mutants (23, 37, 38) showing contrasting activation responses have been proposed to affect TBP-TATA element interactions. Since TFIIA can stabilize the interaction of TBP on the TATA element, the K138T/Y139A mutation (29) could also affect the formation of an activation-specific TBP-promoter complex. Thus, one implication from these TBP mutational analyses is that the TBP-TATA element interaction (23, 29, 37, 38) is relatively less important for transcriptional activation in human, compared with the direct interaction of TFIIIB with TBP through the second stirrup region.

Based on the contrasting activities of TBP mutants for activation in yeast versus human systems, there may be activation-specific TBP interactions, which are not conserved from yeast to human or which are not essential in the human systems analyzed (e.g. because of redundant or alternative activation mechanisms). It is known that transcriptional stimulation by activators in yeast and human is dependent upon coactivators distinct from the minimal basal factors (reviewed in Refs. 1–3, 16, 17, and 39). In human, both TBP-associated TAFs (reviewed in Refs. 1 and 9) and USA-derived components (18–20) are obligatory for activation in vitro; these components, as well as basal factors that include TFIIIB (22), have been identified as direct targets for activators, reflecting increased recruitment of TFIIH, TFIIIB, and other general initiation factors. Studies in yeast have identified TBP-interacting TAFs, which can facilitate activation function in vitro (10, 11), as well as a complex of other coactivators (SRBs and other genetically defined coactivators) that form part of a holoenzyme complex with RNA polymerase II (reviewed in Refs. 16, 17, and 39). Importantly, both in vitro (40, 41) and in vivo (14, 15) studies have shown that the yeast TAFs may not be generally required for activation in yeast, in sharp contrast to their critical role in human systems. Possibly related, the TBP-TAF interactions in yeast appear much weaker than those reported for human TFIIID. Mechanistic studies in yeast have shown that activators can interact directly with coactivators to effect holoenzyme recruitment (42; reviewed in Ref. 16) and that TBP recruitment, potentially by direct activator interactions, can also be limiting for activation in yeast (reviewed in Ref. 39). Given that activation through multiply bound activators most likely involves interactions with several of many potential targets in the general transcription machinery (43, 44), the variable effects of TBP mutants L114K and K211L in yeast versus human may simply reflect differential utilization of various activator-coactivator-basal factor interactions and, especially, variations in requirements for the well documented interactions of activators with TBP in yeast and human (reviewed in Ref. 21).

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REFERENCES
1. Burley, S. K., and Roeder, R. G. (1996) Annu. Rev. Biochem. 65, 769–799
2. Roeder, R. G. (1996) Trends Biochem. Sci. 21, 327–335
3. Hernandez, N. (1993) Genes Dev. 7, 1291–1308
4. Cormack, B. P., Strubhan, M., Pontielli, A. S., and Struhl, K. (1991) Cell 65, 341–348
5. Gill, G., and Tjian, R. (1991) Cell 65, 333–340
6. Poon, D., Schroeder, S., Wang, C. K., Yamamoto, T., Horikoshi, M., Roeder, R. G., and Weil, P. A. (1991) Mol. Cell. Biol. 11, 4809–4821
7. S. K. Mahanta and J. Strominger, personal communication.
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7. Kelleher, R. J., III, Planagan, P. M., Chasman, D. I., Ponticelli, A. S., Struhl, K., and Kornberg, R. D. (1992) *Genes Dev.* 6, 296–303
8. Struhl, M., and Struhl, K. (1992) *Cell* 68, 721–730
9. Goodrich, J. A., and Tjian, R. (1994) *Curr. Opin. Cell Biol.* 6, 403–409
10. Poon, D., Bai, Y., Campbell, A. M., Bjorklund, S., Kim, Y.-J., Zhou, S., Kornberg, R. D., and Weil, P. A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 8224–8228
11. Reese, J. C., Apone, L., Walker, S. S., Griffin, L. A., and Green, M. R. (1994) *Curr. Opin. Cell Biol.* 6, 403–409
12. Poon, D., Bai, Y., Campbell, A. M., Bjorklund, S., Kim, Y.-J., Zhou, S., Kornberg, R. D., and Weil, P. A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 8224–8228
13. Reese, J. C., Apone, L., Walker, S. S., Griffin, L. A., and Green, M. R. (1994) *Curr. Opin. Cell Biol.* 6, 403–409
14. Modtaderi, Z., Bai, Y., Poon, D., Weil, P. A., and Struhl, K. (1996) *Nature* 383, 188–191
15. Walker, S. S., Reese, J. C., Apone, L. M., and Green, M. R. (1996) *Nature* 383, 188–191
16. Koleske, A. J., and Young, R. A. (1995) *Trends Biochem. Sci.* 20, 113–116
17. Guarente, L. (1995) *Trends Biochem. Sci.* 20, 517–521
18. Chiang, C.-M., Ge, H., Wang, Z., Hoffmann, A., and Roeder, R. G. (1993) *EMBO J.* 12, 3749–3762
19. Hui, G., and Roeder, R. G. (1994) *Cell* 78, 513–523
20. Kretzschmar, K., Kaiser, K., Lottspeich, F., and Meisterernst, M. (1994) *Cell* 78, 525–534
21. Nikolov, D. B., and Burley, S. K. (1994) *Nat. Struct. Biol.* 1, 621–637
22. Lin, Y.-S., and Green, M. R. (1994) *Cell* 64, 971–981
23. Kim, T. K., Hashimoto, S., Kelleher, R. J., III, Planagan, P. M., Kornberg, R. D., Horikoshi, M., and Roeder, R. G. (1994) *Nature* 369, 252–255
24. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nature* 369, 252–255
25. Meisterernst, M., and Roeder, R. G. (1994) *Cell* 78, 525–534
26. Nakajima, N., Horikoshi, M., and Roeder, R. G. (1988) *Mol. Cell. Biol.* 8, 4025–4040
27. Kim, T. K., and Roeder, R. G. (1994) *J. Biol. Chem.* 269, 4891–4894
28. Kim, T. K., Zhao, Y., Ge, H., Bernstein, R., and Roeder, R. G. (1995) *J. Biol. Chem.* 270, 10976–10981
29. Stargell, L. A., and Struhl, K. (1995) *Science* 269, 75–78
30. Kim, T. K., and Roeder, R. G. (1995) *J. Biol. Chem.* 269, 20866–20869
31. Kim, T. K., and Roeder, R. G. (1994) *Nucleic Acids Res.* 22, 511
32. Tansley, W. P., Ruppert, S., Tjian, R., and Herr, W. (1994) *Genes Dev.* 8, 2754–2769
33. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* 154, 367–382
34. Nikolov, D. B., Hu, S.-H., Lin, J. P., Gasch, A., Hoffmann, A., Horikoshi, M., Chua, N.-H., Roeder, R. G., and Burley, S. K. (1992) *Nature* 360, 40–46
35. Nikolov, D. B., Chen, H., Halay, E. D., Usheva, A. A., Hisatake, K., Lee, D. K., Roeder, R. G., and Burley, S. K. (1995) *Nature* 377, 119–128
36. Kim, T. K., and Roeder, R. G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 4170–4174
37. Arndt, K. M., Ricupero-Hovasse, S., and Winston, F. (1995) *EMBO J.* 14, 1490–1497
38. Lee, M., and Struhl, K. (1995) *Mol. Cell. Biol.* 15, 5461–5469
39. Struhl, K. (1995) *Annu. Rev. Genet.* 29, 651–674
40. Kim, Y.-J., Bjorklund, S., Li, Y., Sayre, M. H., and Kornberg, R. D. (1994) *Cell* 77, 599–608
41. Koleske, A. J., and Young, R. A. (1994) *Nature* 368, 466–469
42. Barberis, A., Pearlberg, J., Simkovitch, N., Farrell, S., Reinagel, P., Bamdad, C., Sigal, G., and Ptashne, M. (1995) *Cell* 81, 359–368
43. Carey, M., Lin, Y.-S., Green, M. R., and Ptashne, M. (1990) *Nature* 345, 361–364
44. Lin, Y.-S., Carey, M., Ptashne, M., and Green, M. R. (1990) *Nature* 345, 359–361