Localization of Subunits of Transcription Factors IIIE and IIIF Immediately Upstream of the Transcriptional Initiation Site of the Adenovirus Major Late Promoter

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The assembly of a preinitiation complex containing RNA polymerase II on promoter DNA is a complex process that involves several general transcription factors. Using 5-N[(-p-azidobenzoyl)-3-aminallyl] photocross-linking, we previously determined the locations of the two large subunits of transcription factor (TF) II A (A35 and A21), TATA box-binding protein (TBP), RNA polymerase II-associated protein (RAP) 30, and TFII B along the Ad2 ML promoter. We have now localized TFII E 34 and RAP 74 just upstream of the transcription start site. The two subunits of TFII F, RAP 74 and RAP 30, cross-linked to nucleotides that probed adjacent spaces on the same face of the DNA helix beginning just downstream of TBP at position −19 and extending to −5. Specific photocross-linking of TFII E 34 required the presence of TFII F. In addition, TFII E and RAP 74 strongly stimulated cross-linking of RAP 30 and the large subunits of RNA polymerase II to position −19. Our topological data support the idea that RAP 74 and TFII E 34 may be involved in melting of the promoter DNA upstream of the initiation site.

Initiation of transcription by RNA pol II is a very complex process, which involves the general transcription factors TFII A, TFII B, TFII D, TFII E, TFII F, and TFII H (reviewed in Refs. 1 and 2). For genes containing a TATA box, promoter recognition is achieved through binding of TBP, the TATA box-binding factor, to the coding strand opposite and the two largest subunits of RNA pol II on the Ad2 ML promoter (14). TFII A cross-links to the coding strand just downstream of TBP at the TATA box and cross-links upstream of TBP at position −40. RAP 30 cross-links strongly and TFII B weakly to the coding strand just downstream of TBP at position −19 (14).

We have now localized two additional polypeptides of the general transcription machinery, RAP 74 and TFII E 34, along the Ad2 ML promoter. Our photocross-linking experiments also provide information on the enzymology of preinitiation complex assembly.

EXPERIMENTAL PROCEDURES

Protein Factors—Recombinant yeast TBP (24), human TFII B (25), human RAP 30 (10, 26), human TFII E 56/34 (27–29), as well as calf thymus RNA pol II (30), were prepared as described previously.

N3R Photocross-linking—The synthesis of N3R-dUMP, the preparation of the probes, and the conditions for binding reactions were as described previously (14). A schematic representation of our various photocrossprobes is shown in Fig. 1. Binding reactions were as we previously described (14). For each probe, the concentration of poly(dG-dC)-(dG-dC) in binding reactions was optimized in order to favor specific binding over nonspecific binding, and was between 0.1 and 1 μg/binding reaction. A typical reaction with all the factors contained 200 ng of each of TFII B, TFII A, RAP 30, TFII E 56, TFII E 34, and purified RNA pol II. Ultraviolet irradiation, nuclelease treatment, and SDS-PAGE analysis of radiolabeled photocross-linking products were as we previously described (14).
Localization of TFII E and TFII F on Promoter DNA

RESULTS AND DISCUSSION

Probes for Photocross-linking—Incorporation of the photoreactive nucleotide N3R-dUMP into DNA probes can be achieved only where T residues are present in the sequence. In this study, we prepared photocross-linking probes that placed the photoreactive nucleotide at positions −19, −9, −8, −5, and −2 on the coding strand using the wild type Ad2 ML promoter (see Fig. 1). In order to probe the space along promoter regions that lacks T residues, we also constructed mutant promoters that contain single T residues at various additional positions, including −14 on the coding strand and −15, −10, and −5 on the non-coding strand. The design of each probe is shown schematically in Fig. 1.

Specific Photocross-linking of TFII E34 in a Region between −14 and −2 in the Presence of TFII E56—In order to map the location of additional GTFs along the Ad2 ML promoter, we first synthesized probe −14/−2, which places 5 photoreactive nucleotides at positions −14, −9, −8, −5, and −2 on the coding strand (see Fig. 1). Using this probe, we observed photo-linking of two large polypeptides of approximately 145–180 kDa and two additional polypeptides of approximately 25–40 kDa in the presence of TBP, TFII B, RAP30/74, RNA pol II, and TFII E56/34 (Fig. 2A, lane 1). To assess whether photocross-linking was promoter-specific, we compared cross-linking reactions performed in the presence or in the absence of TBP. In the conditions that we used (e.g. particular concentrations of salt and nonspecific competitor DNA), we always observed that assembling a reaction in the absence of TBP had the same effect as using a photoprobe with a mutated TATA element (see Ref. 14 and below). As expected for promoter-specific cross-linking events, cross-linking of the various polypeptides to probe −14/−2 was abolished in the absence of TBP (Fig. 2A, compare lanes 1 and 2) or when we used a photoprobe with a mutated TATA element (TAGAGAA instead of TATAAA) (Fig. 2A, compare lanes 1 and 3). The two large cross-linked polypeptides are almost certainly the two largest subunits of RNA pol II because (i) their apparent M, values correspond to those of the two largest subunits of calf thymus RNA pol II (30), (ii) the two largest subunits of our highly purified RNA pol II preparation are the only polypeptides in the range of 145–180 kDa that we used in our photocross-linking experiments, and (iii) photocross-linking of these two large polypeptides was obtained only in the presence of RNA pol II in the reactions (data not shown). The smaller of the two polypeptides in the 25–40-kDa range has not been identified, but may be a subunit of RNA pol II. The larger of the polypeptides in the 25–40-kDa range was immunoprecipitated with a specific antibody directed against TFII E34 (Fig. 2B, lane 5), but not with a control antibody (Fig. 2B, lane 6). Cross-linking of TFII E34 to probe −14/−2 required the presence of TBP in the reactions (Fig. 2B, lane 2). In the absence of TFII E56, we still observed cross-linking of TFII E34 (Fig. 2B, lane 3), but this cross-linking was not specific since it occurred in the absence of TBP (lane 4). Longer exposure of our cross-linking gels also revealed specific cross-linking of RAP74 to the −14/−2 photoprobe (data not shown; see below for a more accurate localization of RAP74). Photocross-linking of RAP74 to probe −14/−2 was also abolished when we used a photoprobe with a mutated TATA element (data not shown), indicating that cross-linking of RAP74 is promoter-specific as well.

Surprisingly, we have not yet been able to cross-link TFII E56 to any of our photoprobes in the presence of TBP, TFII B, RAP30/74, RNA pol II, and TFII E56/34. This was particularly surprising since TFII E1 is a heterotetramer containing 2 molecules of TFII E56 (31). Our photocross-linking data suggest that the large subunit of TFII E may be located quite far away from the promoter DNA or in a region where the chemical arm of N3R-dUMP cannot reach. Alternatively, TFII E56 may act as a molecular chaperone in catalyzing the assembly of the preinitiation complex in the presence of TBP, TFII B, TFII F, RNA pol II, and TFII E34. If this is true, TFII E56 may become stably bound to the preinitiation complex only in the presence of TFII H.

Photocross-linking of RAP74 to Positions −15 and −5, but...
RAP74 strongly stimulates cross-linking of RAP30 and RNA pol II to positions -19 and TATA box (see Ref. 14). These include TBP, TFIIB, RAP30, and the two large subunits of RNA pol II. We reported that TBP cross-links to that position in the context of a TBP:TFIIA-promoter (TATA) complex, while RAP30 is the main cross-linked polypeptide in the context of a TBP:TFIIA:RAP74:RAP30 promoter (TATA) complex (14). In such a complex, we also obtained weak cross-linking of TFIIIB to position -19 (14). This position of TFIIIB agrees with that recently determined by hydroxyl-radical footprinting (32) and x-ray crystallography (33). These observations indicate that position -19 delimits the spatial interface where TBP directs recruitment of RNA pol II to the preinitiation complex through protein-protein interactions with the bridging factors RAP30 and TFIIIB. In addition, we showed that RAP74
The position shown for TFIIB is based on our cross-linking experiments performed in the absence of TFIIE. RAP30 and RAP74 are placed along one face of the DNA between −19 and −5, and TFIIE34 is located between −14 and −2.

Recently, Pan and Greenblatt (23) reported that initiation of transcription on closed, linear templates is limited by melting of the promoter immediately upstream of the transcription start site. Synthesis of a promoter-specific trinucleotide on linear DNA requires TFIIF and is stimulated by TFIIE (23). Artificial melting of the promoter DNA between −9 and −1 on such a template obviates the requirement for TFIIF, suggesting that TFIIF may be involved in melting of the promoter upstream of +1 (23). Photocross-linking of RAP74 and TFIIE34 to nucleotides located between −15 and −2 suggests that these factors may be involved in melting of the DNA helix during transcription initiation.

**Fig. 5. Schematic representation of a TBP-TFIIE-TFIIF-RNA pol II-TFIIE complex.** The structure of the TBP-promoter complex is derived from x-ray crystallographic data on a complex containing the Ad2ML promoter and Arabidopsis thaliana TBP (35, 36). The predicted positions of TFIIE (14), TFIIB (14), RAP74, RAP30, and TFIIE34 are as predicted by our photocross-linking data. Nucleotides −19 (coding strand) and −15 and −5 (non-coding strand) that cross-link to RAP30 and RAP74 are highlighted in red. RNA pol II is not shown because our data on the relative positions of various subunits are still too fragmentary.

Cross-linking of RAP30 and RNA pol II to position −19 (see Ref. 14). Fig. 4A shows that efficient cross-linking of RAP30 and the large subunits of RNA pol II to position −19 requires both TFIIE and RAP74 (compare lanes 1 to lanes 3 and 4). Both subunits of TFIIE were required to promote efficient cross-linking of RAP30 and RNA pol II subunits (data not shown). Photocross-linking of RNA pol II and RAP30 to this position also required the presence of TBP (Fig. 4A, lane 2). Immunoprecipitations using antibodies raised against various subunits of GTFs, including RAP30 (Fig. 4B, lanes 3 and 4), TFIIB (lanes 5 and 6), TBP (lanes 7 and 8), and TFIIE34 (lanes 9 and 10) revealed that RAP30 and RNA pol II are the only proteins that cross-linked specifically to position −19 in the context of a TBP-TFIIB-RAP74/30-RNA pol II-TFIIE36/34 promoter (TATA) complex. Photocross-linking of TFIIE34 to position −19 was also observed, but it was not specific since it occurred in the absence of TBP (compare lane 9 to lane 10). Specific photocross-linking of TFIIE34 was only obtained using probes −14/−2 (see Fig. 2) and −10 (data not shown). Long exposure of the gel shown in Fig. 4B revealed only nonspecific cross-linking of TFIIB (data not shown). These observations suggest that RAP74 and TFIIE help tether RAP30 and RNA pol II to our photoactive nucleotide placed at position −19. There may also be a conformational change in a preinitiation complex that contains RNA pol II that has the effect of making TFIIB inaccessible to the chemical arm of the photoactive nucleotide. Such a conformational change has already been proposed for TFIIE (34).

Fig. 5 shows a schematic representation of our photocross-linking data in the context of a molecular structure for the TBP-promoter complex (35, 36). We propose that position −19 delimits the space where RAP30 and TFIIB stabilize the interaction between TBP and RNA pol II along the promoter DNA.

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**REFERENCES**

1. Zawel, L., and Reinberg, D. (1992) Curr. Opin. Cell Biol. 4, 488–495
2. Conaway, R. C., and Conaway, J. W. (1993) Annu. Rev. Biochem. 62, 161–173
3. Greenblatt, J. (1991) Cell 66, 1067–1070
4. Coulombe, B., Killeen, M., Liljefeld, P., Honda, B., Xiao, H., Ingles, C. J., and Greenblatt, J. (1992) Gene Exp. 2, 99–110
5. Cortes, P., Flores, O., and Reinberg, D. (1992) Mol. Cell. Biol. 12, 413–421
6. Hisatake, K., Roeder, R. G., and Burley, S. K. (1993) Nature 363, 744–747
7. Ha, I., Roberts, S., Maldonado, E., Sun, X., Kim, L.-U., Green, M., and Reinberg, D. (1993) Genes & Dev. 7, 1021–1032
8. Burstowski, S., Hahn, S., Guarente, L., and Sharp, P. A. (1989) Cell 54, 549–561
9. Flores, O., Lu, H., Killeen, M., Greenblatt, J., Burton, Z. F., and Reinberg, D. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 9999–10003
10. Killeen, M., Coulombe, B., and Greenblatt, J. (1992) J. Biol. Chem. 267, 9463–9466
11. McCracken, S. G., and Greenblatt, J. (1991) Science 253, 900–902
12. Usheva, A., Maldonado, E., Goldberg, A., Lu, H., Houbaev, D., Reinberg, D., and Aloni, Y. (1992) Cell 72, 871–881
13. Wang, B. Q., and Burton, Z. F. (1995) J. Biol. Chem. 270, 27035–27044
14. Coulombe, B., Li, J., and Greenblatt, J. (1994) J. Biol. Chem. 269, 19962–19967
15. Flores, O., Maldonado, E., and Reinberg, D. (1989) J. Biol. Chem. 264, 8913–8921
16. Gerard, M., Fischer, L., Moncollin, V., Chipoulet, J. P., Mannon, P., and Egly, J. M. (1991) J. Biol. Chem. 266, 20940–20945
17. Maxon, M. E., Goodrich, J. A., and Tjian, R. (1994) Genes & Dev. 8, 515–524
18. Parvin, J. D., and Sharp, P. (1993) Cell 73, 533–540
19. Tyree, C. M., George, C. P., Lira-Devito, L. M., Wampler, S. L., Dahanus, M. E., Zawel, L., and Kadonaga, J. T. (1993) Genes & Dev. 7, 1254–1265
20. Tomimori, H. T. M. (1994) EMBO J. 13, 396–401
21. Parvin, J. D., Skyhind, B. M., Meyers, R. E., Kim, J., and Sharp, P. A. (1994) J. Biol. Chem. 269, 18414–18421
22. Goodrich, J. A., and Tjian, R. (1994) Cell 77, 145–156
23. Pan, G., and Greenblatt, J. (1994) J. Biol. Chem. 269, 30101–30104
24. Inglis, C. J., Shales, M., Cress, W. D., Trziesnek, S. J., and Greenblatt, J. (1991) Nature 351, 588–590
25. Ha, I., Lane, S., and Reinberg, D. (1991) Nature 352, 689–695
26. Finkelstein, A., Kostrub, C. F., Li, J., Chavez, D. P., Wang, B. Q., Fang, S. M., Greenblatt, J., and Burton, Z. (1992) Nature 355, 464–467
27. Ohkuma, Y., Sumimoto, H., Hoffman, A., Shimasaki, S., Horikoshi, M., and Roeder, R. G. (1991) Nature 354, 398–401
28. Sumimoto, H., Ohkuma, Y., Sinn, E., Kato, H., Shimasaki, S., Horikoshi, M., and Roeder, R. G. (1991) Nature 354, 401–404
29. Peterson, M. G., Inostroza, J., Maxon, M. E., Flores, O., Aloni, A., Reinberg, D., and Tjian, R. (1991) Nature 354, 369–373
30. Hoda, H. G., Illi, and Biatti, S. P. (1977) Biochemistry 16, 1334–1343
31. Ohkuma, Y., Sumimoto, H., Horikoshi, M., and Roeder, R. G. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 9163–9167
32. Lee, S., and Hahn, S. (1995) Nature 376, 609–612
33. Nikolov, D. B., Chen, H., Hua-Hof, D., Usheva, A. A., Hisatake, K., Lee, D. K., Roeder, R. G., and Burley, S. K. (1995) Nature 377, 119–128
34. Robert, S. G. E., and Green, M. R. (1994) Nature 371, 717–720
35. Kim, Y., Geiger, J. H., Hahn, S., and Sigler, P. B. (1993) Nature 365, 512–520
36. Kim, J. L., Nikolov, D. B., and Burley, S. K. (1993) Nature 365, 520–527