Site-Specific Protein-DNA Photocross-Linking of Purified Complexes: Topology of the RNA Polymerase II Transcription Initiation Complex

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The initiation of mRNA synthesis requires the formation of a preinitiation complex containing RNA polymerase II (RNAPII) and the general initiation factors TFIID (or TBP), TFIIB, TFIIE, TFIIIF, and TFIIH on promoter DNA.¹ Because many general initiation factors and RNAPII are multisubunit proteins, the preinitiation complex can comprise up to 50 polypeptides. Some of these are targeted to the promoter through direct interactions with specific sequence elements (such as the interaction of TBP with the TATA box), whereas other factors are recruited mostly through protein–protein interactions with these DNA-bound factors. In the presence of ATP, the preinitiation complex melts promoter DNA in the region of the transcriptional initiation site, thereby making the template strand available for the initiation of RNA synthesis.

Overview of the Procedure

Protein–DNA photocross-linking has proved to be the method of choice to analyze the molecular organization and topology of large nucleoprotein complexes such as those involved in the transcription reaction. In the past, our laboratory used cross-linking probes carrying a photoreactive nucleotide at specific positions along promoter DNA to localize the components of the transcription machinery in the preinitiation complex.^{2–5} A possible limitation of protein–DNA photocross-linking relates to the specificity of preinitiation complex assembly. Because most general initiation factors and RNAPII have an affinity for any DNA (although lower than that for promoter DNA), it is often difficult to set up conditions that will systematically and exclusively allow for the formation of specific complexes on our various photoprobes. In order to circumvent this problem, we developed a method for cross-linking proteins to DNA in purified complexes. The overall procedure is summarized in Fig. 1. Briefly, complexes are first assembled by mixing transcription factors with a photoprobe that juxtaposes one (or a few) photoreactive nucleotide with one (or a few) radiolabeled nucleotide at a specific location in the promoter. The complexes are submitted to an electrophoretic mobility shift assay (EMSA) in a native gel that is then irradiated with ultraviolet (UV) light in order to cross-link the proteins to DNA. The specific complexes are then localized on the gel, purified by cutting out the gel slices, and processed for the identification of cross-linked polypeptides. Because this procedure helps reduce to a minimum the nonspecific cross-linking signals due to aggregation, it has allowed us to define with more precision the topological organization of the RNAPII preinitiation complex.

Preparation of Photoprobes

General Considerations

The nucleotide derivative 5-(*N*-(*p*-azidobenzoyl)-3-aminoallyl)-dUTP (AB-dUTP or N₃R-dUTP) possesses a side chain that places a photoreactive nitrene in the major groove of the DNA helix 10 Å away from the DNA backbone.⁶ For this reason, the cross-linking of a polypeptide to the photoprobe does not require a direct interaction of the polypeptide with the DNA helix.

Chemical Synthesis of Photoreactive Nucleotide AB-dUTP

A volume of 100 µl of a 100 mM 4-azidobenzoic acid *N*-hydroxysuccinimide solution (ABA-NSH) in dimethylformamide (DMF) is added to 1 ml of 2 mM 5-(*N*-(3-aminoallyl))-dUTP (5-aa-dUTP) in 100 mM sodium borate, pH 8.5, and the reaction is incubated at room temperature for 3 h. During this time the reaction mixture frequently forms a precipitate that can be discarded. The reaction mixture is then applied to 0.2 ml DEAE-Sephadex A-25 in a 10-ml disposable column equilibrated in 100 mM TEAB, pH 8.5 (triethylammonium bicarbonate buffer). The column is washed six times with 1 ml of the same buffer and eluted with a step gradient of 0.2 to 1.0 M TEAB, pH 8.5, at intervals of 0.02 M (1 ml per step fraction). Fractions of 1 ml are collected, evaporated to dryness by vacuum centrifugation, and dissolved in 100 µl of deionized water. A volume of 2 µl of each fraction is analyzed by thin layer chromatography on polyethyleneimine (PEI)-cellulose plates (J. T. Baker). The chromatogram is run in 1 M LiCl, and the dried plates are visualized with UV (254 nm). *R_f* values for 5-aa-dUTP and AB-dUTP are 0.54 and 0.098, respectively. Concentrated stocks of AB-dUTP are stable for several years at -70° when protected from ambient light.

Enzymatic Synthesis of Photoprobes

A schematic representation of the procedure for the synthesis of photoprobes is shown in Fig. 2. In this example, one photoreactive nucleotide is placed at position +1 and three radiolabeled guanosines at positions -1, -3, and -4 of the adenovirus major late promoter. Annealing of both specific and upstream primers to single-stranded templates is performed as follows: 500 ng of single-stranded (ss) DNA (approximately 0.5 pmol) is mixed with 40 ng (approximately 5 pmol each) of both specific and upstream primers, 1 µl of 10 × buffer A (300 mM Tris-HCl, pH 8.0, 500 mM KCl, and 70 mM MgCl₂; freshly prepared) is added, and the volume made up to 10 µl with deionized water. The reaction is mixed, incubated for 5 min at 90°, and then for 30 min at room temperature.

Incorporation of the photoreactive AB-dUTP and the radiolabeled nucleotide is achieved via primer extension with T4 DNA polymerase. From this point on, all manipulations must be carried out under reduced light conditions. Bovine serum albumin (BSA) (10 mg/ml, 0.5 µl), AB-dUTP (1 µl; the amount of the nucleotide derivative to be added is determined empirically for each preparation and is generally between 0.5 and 2 µl), 20 µCi of the appropriate [α ³²P]dNTP (3000 Ci/mmol) ([α ³²P]dGTP for the example in Fig. 2), 5–10 U of T4 DNA polymerase, and 10 × buffer A (1 µl) are then added and the volume is made up to 20 µl with deionized water. The reaction is incubated for 30 min at room temperature and is then chased with all four dNTP by adding 5 µl of dNTP mix (10 mM each dATP, dCTP,

dGTP, and dTTP in $1 \times$ buffer A) and incubating for 5 min at room temperature followed by 20 min at 37° . The addition of dNTP in large excess is crucial because it is necessary to limit the incorporation of radiolabeled and photoreactive nucleotides during the extension of the photoprobes. Following probe synthesis, nicks at the 5' end of the primers are repaired by the addition of T4 DNA ligase (5 U) and ATP (1 mM final concentration) and incubating at room temperature for 1 h. The reaction is then heated at 65° for 20 min in order to inactivate the ligase.

The synthesized DNA is digested with restriction endonucleases (10–20 U) for 90 min in order to release the photoprobe (Sma I in the example shown in Fig. 2). The probe is purified on a native gel by adding 5 μ l of a 6X gel loading solution (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in deionized water) to the digest and electrophoresing through a native 8% polyacrylamide gel (40:1 acrylamide:bis) in 1X TBE buffer. The TBE buffer is prepared as a 5X stock by mixing 54 g Tris-base, 27.5 g boric acid, and 20 ml 0.5 M EDTA, pH 8.0, in 1 liter of deionized water. The gel is run at 150 V for about 1 h in 1X TBE buffer.

After the run the gel sandwich is removed from the gel box, and plates are separated such that the gel remains on one of them. The gel/glass plate is then wrapped in plastic and aluminum foil and moved to a dark room (a conventional red light can be used). Kodak X-OMAT AR film is placed on a clean bench, the foil is removed, and the gel is placed on the film with the glass plate facing up (e.g., gel side down) for 2 to 5 min. The gel is removed and rewrapped with the foil and the film is developed (an example of the autoradiogram of a gel used for photoprobe purification is shown in Fig. 3). The band corresponding to the photoprobe can be identified easily because the size of the fragment generated by the restriction enzyme is known. Using a scalpel blade, the film is cut so that the square piece containing the band corresponding to the photoprobe is removed. This operation leaves the film with a window at the position of the photoprobe. The film is superimposed on the gel and an ink marker is used to mark the square corresponding to the photoprobe. The gel slice is then cut out with a clean scalpel blade and chopped into small pieces (six to eight fragments). These are placed in a 1.5-ml microcentrifuge tube and sufficient 10 mM Tris, pH 7.9, added to completely submerge the gel (about 125–200 μ l). The probe is eluted by incubating overnight at room temperature, and the liquid containing the probe is collected and purified on a microspin S-200 HR column (Amersham) in order to remove any salts and other putative contaminants. A 1- μ l aliquot of the photoprobe solution is then counted by liquid scintillation, and the probe is diluted to the appropriate count number (see later) with deionized water. The probe is now ready for use and can be stored in the dark at 4° for 1–2 weeks. Fresh probes (less than a week old) give the best results.

Precautions

- i. AB-NSH and AB-dUTP are manipulated under indirect lighting conditions using a 40-W incandescent lamp. The use of a standard dark room is not necessary. As a rule, we find that conditions providing just enough light to be able to work are acceptable.

- ii. The specific primer must be designed in such a manner that T4 DNA polymerase only adds a few nucleotides. In the example shown in Fig. 2, incorporation during the site-specific labeling is restricted to positions -4 to +1 by omitting dCTP from the reaction. The success of this step can be monitored by analysis of the reaction products on a sequencing gel.

In-Gel Protein–DNA Photocross-Linking

Synthesis of N,N'-Bisacryloylcysamine (Bac)

Bac is a disulfide-containing analog of bis-acrylamide.^{7–9} Its chemical synthesis and use in native gels are essentially as described by Naryshkin *et al.*¹⁰ with minor modifications. Polyacrylamide:bac gels can be dissolved by the use of reducing agents. Acryloyl chloride is highly toxic and all manipulations in this section must be performed in a fume hood. Cystamine dihydrochloride (20 g) is dissolved in 200 ml of 3 M NaOH, whereas acryloyl chloride (21.5 ml) is dissolved in 200 ml chloroform. The resulting solutions are then mixed in a 2-L flask (as acryloyl chloride reacts with water, it is added drop-by-drop). Two phases will form: an upper, aqueous phase and a lower, organic phase. The reaction is stirred magnetically for 3 min at room temperature, followed by 15 min at 50°. Immediately after the latter incubation the reaction mixture is transferred to a 2-L separating funnel and phases are allowed to separate. The lower, organic phase is then transferred to a 1-L beaker. Impure bac is precipitated by chilling on ice for 10 min and is collected by filtration in a Büchner funnel. The crude precipitate is dissolved in 150 ml chloroform in a 1-L beaker and is recrystallized by placing the beaker on a stirrer plate and stirring for 1 min at room temperature, followed by 5 min at 50°, and then placing the beaker on ice for 10 min. The precipitated bac is collected by filtration in a Büchner funnel and transferred to a 250-ml flask. The flask is sealed with Parafilm, piercing the seal several times with a syringe needle, and the flask is placed in a vacuum dessicator and dried under vacuum for 16 h at room temperature. Expect a yield of 7.0–9.0 g.

Preparation of Polyacrylamide:Bac Gels

Polyacrylamide:bac gels for the EMSA are prepared as follows. A 20% acrylamide:bac (19:1) stock solution is prepared by dissolving 19 g of acrylamide and 1 g of bac in 80 ml of water in a 200-ml beaker and stirring for 30 min at 60° (the solubility of bac in water is increased by adding the acrylamide before adding the bac and by performing the addition at 60°). The volume is then adjusted to 100 ml with water and the solution is allowed to cool down to room temperature prior to filtering through a 0.22- μ m filter unit and storing at 4° in the dark (stable for a few weeks). The gel is assembled using one glass plate that has been siliconized by applying 100 μ l of Surfacil siliconizing agent and spreading evenly with a Kimwipe. A 4.5% polyacrylamide:bac gel in 0.5 $\times$ TBE buffer is prepared by mixing 11.25 ml of an acrylamide:bac (19:1) stock solution, 5 ml of 5 $\times$ TBE, 100 μ l MgCl₂, and 33.7 ml water and preheating the slab gel assembly and the gel mixture at 50° in an incubator for 30 min. Polymerization is initiated by adding 250 μ l TEMED and 125 μ l freshly prepared 10% ammonium persulfate, and the gel is poured immediately into the slab gel. Allow 20 min for polymerization at 50° (the TEMED and ammonium persulfate concentrations are critical variables in the preparation of polyacrylamide:bac gels). Finally, the gel and the 0.5 $\times$ TBE

reservoir containing 2 mM MgCl₂ are prechilled by placing them in a 4° cabinet for 3 h (the polyacrylamide:bac is stable for up to 72 h at 4°, but it is better to use it fresh).

Electrophoresis of Protein–DNA Complexes in Native Gels

The reactions are assembled by mixing recombinant TBP (80 ng), recombinant TFIIB (80 ng), RAP30 (300 ng), RAP74 (600 ng), calf thymus RNA polymerase II (300 ng), TFIIE34 (160 ng), and TFIIE56 (380 ng) in a total of 20 μ l of buffer G (12 mM HEPES, pH 7.9, 60 mM KCl, 0.12 mM EDTA, 8 mM MgCl₂, 50 ng/ml BSA, 5 mM 2-mercaptoethanol, and 12% glycerol) and then adding 2.5 mg/ml poly(dIdC.dIdC) (1 μ l) and 6000 cpm of the photoprobe per [α^{32} P]dNTP residue incorporated. All work with the photoprobe should be performed under reduced lighting conditions. The binding reactions (21 μ l) are incubated for 30 min at 30°. The EMSA are performed as described previously.¹¹ The 4.5% acrylamide:bac gels are run in a 4° cabinet for 60 min at 400 V.

UV Irradiation of Gels

UV irradiation of protein–DNA complexes is performed in the gel. One glass plate is removed and the gel is irradiated immediately with a 254-nm UV light for 10 min using a Hoefer UVC 500 ultraviolet cross-linker.

Localization and Isolation of Protein–DNA Complexes

In order to localize and isolate the complexes of interest, one of the glass plates is removed and the gel is covered with Whatman paper. The other glass plate is then removed and the gel is covered with plastic wrap. The gel is exposed to a phosphorimager screen overnight and the image printed. Using a scalpel blade, the print is then cut so that the square piece containing the band corresponding to the complex is removed. This operation leaves the print with a window at the position of the complex of interest. The print is then placed on the gel, and the gel slice corresponding to the square cut is excised with a clean scalpel blade. The excised gel is transferred to a 1.5-ml microcentrifuge tube, taking care to avoid carrying any pieces of Whatman paper and plastic wrap.

Solubilization of Complexes

Gel slices are solubilized by the addition of 10 μ l of 1 M dithiothreitol (DTT) (about 0.4 M final) and heating for 10 min at 37° (2–4 M 2-mercaptoethanol can be substituted for 1 M DTT). Then 40 μ l of ND buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, and 20% glycerol) containing 0.4 M DTT is added and the sample is heated at 37° for 25 min.

Nuclease Digestion and SDS–PAGE Analysis

The gel slice is solubilized by adding 13 μ l of 0.5 U/ μ l DNase in 30 mM CaCl₂ and incubating at 37° for 20 min. A volume of 3.9 μ l 10% SDS is then added and the solution is boiled for 3 min. Finally, 2.5% acetic acid/15 mM ZnCl₂ (5.2 μ l) and 80,000 U/ml S1 nuclease (2.6 μ l) are added and the reaction is incubated at 37° for 20 min. The reaction is stopped by adding 15 μ l 5 $\times$ loading buffer (80 mM Tris, pH 6.8, 12.5% glycerol, 2.5% SDS, 0.9 M 2-mercaptoethanol, and 0.2% bromophenol blue) and the sample is boiled for 5 min.

prior to loading on a standard SDS–PAGE gel along with prestained molecular weight markers. The gel is run at 30 mA while the proteins are in the stacking gel and at 55 mA when they are in the separating gel. Detailed procedures for SDS–PAGE electrophoresis have been described.¹² Finally, the gel is transferred to Whatman paper and dried under vacuum. The dried gel is exposed to Biomax screens and films.

Analysis of RNA Polymerase II Preinitiation Complex

The power of the in-gel photocross-linking procedure to analyze the RNAPII preinitiation complex is illustrated in the example shown in Figs. 4 and 5. Using calf thymus RNAPII and recombinant TBP, TFIIB, RAP74, RAP30, TFIIE56, and TFIIE34 for preinitiation complex assembly on photoprobes –39/–40 (Fig. 4) and +1 (not shown), EMSA analysis revealed the formation of two distinct complexes in the native gels. Assembly of these complexes is promoter specific because a mutation in the TATA box (TATAAA to TAGAGA; not shown) or the omission of TBP in the assembly mixture (Fig. 4, compare + and –) abolishes the formation of the two complexes. The band corresponding to each complex assembled on photoprobes –39/–40 and +1 is excised from the gels and analyzed as described earlier. RPB2, RAP74, TFIIE34, and RAP30 cross-link to positions –39/–40, and RPB1 and RPB2 to position +1 using both complexes A and B. Interestingly, the IIa form of RPB1 with an intact hypophosphorylated CTD (about 220 kDa) is found in the complex of lower mobility (complex A), whereas the IIb form of RPB1 with a proteolyzed CTD (about 180 kDa) is found in the complex of higher mobility (complex B). This result indicates that one major difference between complexes A and B is the form of RNAPII that is associated with the general initiation factors that form the preinitiation complex. In support of this conclusion, the preparation of calf thymus RNAPII used in our experiments contains approximately equal amounts of the IIa and IIb forms of RNAPII.

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Fig. 1.
Overall scheme for in-gel site-specific protein–DNA photocross-linking.

Fig. 2.

Experimental design for the synthesis of photoprobes. In this example, the photoprobe contains one photonucleotide (U) at position +1 and three radiolabeled guanosines (G) at positions -1, -3, and -4 of the adenovirus major late promoter.

Fig. 3.
An autoradiogram of a gel used for the purification of photoprobes. The position of the DNA fragment carrying both photoreactive and radiolabeled nucleotides at a specific location is shown.

Fig. 4.

Electrophoresis of protein –DNA complexes in a native gel. Complexes were assembled with calf thymus RNAPII, TFIIB, TFIIF, and TFIIE in either the presence (+) or the absence (–) of TBP on photoprobe –39/–40. Two complexes, A and B, are resolved using the electrophoretic mobility shift assay (EMSA).

Fig. 5.

SDS-PAGE analysis of the cross-linked polypeptides in complexes A and B. Complexes A and B were assembled on photoprobes -39/-40 and +1. Gels were irradiated with UV light, and gel slices containing each complex were excised and processed as described in the text. Although no difference is observed in polypeptides that cross-link to photoprobe -39/-40, the form of RPB1 that cross-linked to position +1 varies when complex A is compared to complex B. The difference in the molecular weight of RPB1 indicates that the IIa form of RNAPII (e.g., with a hypophosphorylated CTD) is found in complex A, whereas the IIb form (e.g., without the CTD due to its proteolysis during purification) is present in complex B.