Use of Site-Specific Protein–DNA Photocrosslinking of Purified Complexes to Analyze the Topology of the RNA Polymerase II Transcription Initiation Complex

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Summary

A method for the photocrosslinking of proteins to DNA in purified complexes is described. It makes use of the juxtaposition of a limited number of photoreactive nucleotides with a limited number of radiolabeled nucleotides at a specific location in a DNA fragment. Protein–DNA complexes are submitted to an electrophoretic mobility shift assay that is then irradiated with UV light in order to crosslink the proteins to DNA. The specific complexes are localized on the gel, purified, and processed for the identification of the crosslinked polypeptides.

Keywords

Photocrosslinking; Protein–DNA complexes; Photoreactive nucleotides; Multisubunit complexes

1. Introduction

Site-specific protein–DNA photocrosslinking 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 by mammalian RNA polymerase II (RNAPII). A principal advantage of the method is that it yields low-resolution structural information on large multisubunit complexes.

The initiation of mRNA synthesis requires the formation of a preinitiation complex containing 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. Neither X-ray crystallography nor NMR, which can resolve the structure of complexes containing protein bound to small pieces of DNA, could provide any detailed structural information on this complex. This method has also the advantage of presenting sufficient flexibility to allow the rapid analysis of complexes assembled under various conditions. In the past, our laboratory has used crosslinking probes carrying a photoreactive nucleotide at specific positions along promoter DNA to localize the components of the transcription machinery in the preinitiation complex (2–7). Nevertheless, a possible limitation of protein–DNA photocrosslinking 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 crosslinking 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(s) with one (or a few) radiolabeled nucleotide(s) 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 UV light in order to crosslink 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 the crosslinked polypeptides. Because this procedure helps to reduce to a minimum the nonspecific crosslinking signals due to aggregation, it has allowed us to define with more precision the topological organization of the RNAPII preinitiation complex (8).

2. Materials

1. Buffer A (10×): 300 mM Tris-HCl of pH 8.0, 500 mM KCl, and 70 mM MgCl₂, freshly prepared.
2. Bovine serum albumin (BSA) solution: Prepare a 25 mg/mL solution of BSA in deionized distilled water. Store in aliquots at 20°C. Dilute with water to 10 mg/mL prior to use.
3. dNTP mix: 10 mM each dATP, dCTP, dGTP, and dTTP in 1× buffer A, freshly prepared.
4. 6× gel-loading solution: 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in deionized distilled water.
5. 1× TBE buffer: The TBE buffer is prepared as a 5× stock by mixing 54 g Tris-base, 27.5 g boric acid, and 20 mL EDTA 0.5 M (pH 8.0) in 1 L of deionized distilled water.
6. Buffer G: 12 mM Hepes of pH 7.9, 60 mM KCl, 0.12 mM EDTA, 8 mM MgCl₂, 50 ng/mL BSA, 5 mM β-mercaptoethanol, and 12% glycerol.
7. Poly(dIdC.dIdC) stock: Prepare a 25 mg/mL solution of poly(dIdC.dIdC) in deionized distilled water. Store in aliquots at 20°C.
8. Buffer ND: 20 mM Hepes of pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.4 mM DTT, and 20% glycerol.
9. DNase mix: A solution containing 0.5 U/μL DNase I and 30 mM CaCl₂.
10. Acid mix: Mix equal volume of 5% acetic acid and 30 mM ZnCl₂, freshly prepared.
11. 5× Loading buffer: 80 mM Tris-HCL of pH 6.8, 12.5% glycerol, 2.5% SDS, 0.9 M β-mercaptoethanol, 0.2% bromophenol blue.

3. Methods

3.1. Day 1: Preparation of the Photoprobes

The first step of the procedure is the synthesis of the photoprobes. A schematic representation is illustrated 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.

The site-specific incorporation of the photoreactive nucleotide (*see* ^{Note 1}) and the radiolabeled nucleotide is directed through the annealing of a primer, named the specific primer, with a single-stranded DNA template containing the promoter DNA. The promoter is flanked by two restriction sites (in this example *Sma*I). A second primer, named the upstream primer, is annealed a few base pairs upstream of the *Sma*I site. After annealing, the photoreactive and radiolabeled nucleotides are incorporated by primer extension using T₄ DNA polymerase with limiting amounts of dNTPs (*see* ^{Note 2}). After the labeling step, the extension reaction is completed by the addition of an excess of cold dNTPs (*see* ^{Note 3}) and nicks at the 5' end of the primers are repaired by the addition of T₄ DNA ligase. The photoprobe is generated by digestion with the restriction enzyme and gel purified (*see* Fig. 3 for an example of a gel on which the products of a photoprobe synthesis reaction have been separated).

1. Mix 500 ng of single-stranded (ss) DNA (approximately 0.5 pmol) with 40 ng (approximately 5 pmol each) of both the specific and the upstream primers. Add 1 μ L of 10 $\times$ buffer A and complete to 10 μ L with deionized distilled water.
2. Mix well and incubate for 5 min at 90°C.
3. Incubate for 30 min at room temperature.
4. From this point on, all manipulations must be carried out under reduced light conditions (*see* ^{Note 4}). Add 0.5 μ L BSA (10 mg/mL), 1 μ L AB-dUTP (*see* ^{Note 1}), 20 μ Ci of the appropriate (α ³²P)-dNTP (3,000 Ci/mmol) (α ³²P-dGTP for the example in Fig. 2), 5–10 U of T₄ DNA polymerase, and 1 μ L 10 $\times$ buffer A. Complete to a final volume of 20 μ L with deionized distilled water.
5. Incubate for 30 min at room temperature.
6. Add 5 μ L of dNTP mix.

¹The nucleotide derivative we use, namely 5-(N-(p-azidobenzoyl)-3-aminoallyl)-dUTP (AB-dUTP or N₃R-dUTP) (*see* Chap. “Site-Directed DNA Cross-Linking of Large Multi-Subunit Protein–DNA Complexes”), possesses a side chain that places a photoreactive nitrene in the major groove of the DNA helix 10 Å away from the DNA backbone (*see* ref. 10). For this reason the crosslinking of a polypeptide to the photoprobe does not require a direct interaction of the polypeptide with the DNA helix. The amount of AB-dUTP to be added to the reaction is determined empirically for each preparation of the photoreactive nucleotide and is generally between 0.5 and 2 μ L (often 1 μ L).

²The 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, the 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.

³The 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.

⁴The 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.

7. Incubating for 5 min at room temperature.
8. Incubate for 20 min at 37°C.
9. Add 5 U of T₄ DNA ligase and ATP (1 mM final concentration).
10. Incubate for 1 h at room temperature.
11. Incubate at 65°C for 20 min in order to inactivate the ligase.
12. Add 10–20 U of restriction enzyme (*Sma*I in the example shown in Fig. 2).
13. Incubate for 90 min at the temperature recommended by the supplier of restriction enzymes (25°C for *Sma*I).
14. Add 5 µL of a 6× gel-loading solution.
15. Load on a native 8% polyacrylamide gel (40:1 acrylamide:bis) in 1× TBE buffer.
16. Run at 150 V for about 1 h in 1× TBE buffer.
17. Remove the glass plates containing the gel from the gel box.
18. Separate the glass plates and leave the gel on one of them.
19. Wrap the gel/glass plate in plastic wrap.
20. Wrap the entire package in an aluminum foil.
21. Move to a dark room (*see* ^{Note 5}).
22. Place a Kodax X-OMAT AR film on a clean bench.
23. Remove the foil and place the gel on the film with the glass plate facing up (i.e., gel side down).
24. Expose 2–5 min.
25. During the exposition time, mark the film using a sharp tool by tracing the contour of the glass plate (this will be helpful later for the localization of the photoprobe in the gel).
26. Remove the gel and rewrap it with the foil.
27. Develop the film (*see* ^{Note 6}).
28. Using a scalpel, cut the film 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.
29. Superimpose the film on the gel by taking advantage of the marks made in **step 25**, and mark the square corresponding to the photoprobe on the plastic wrap using a pen.

⁵A conventional red light can be used.

⁶An example of the autoradiogram of a gel used for photoprobe purification is shown in Fig. 3. The position of the band corresponding to the photoprobe can be easily identified because the size of the fragment generated by the restriction enzyme is known.

30. Cut out the gel slice containing the photoprobe using a clean scalpel.
31. Cut the gel slice in small pieces (six to eight fragments).
32. Place the gel fragments in a 1.5-mL microcentrifuge tube and add 10 mM Tris–HCL (pH 7.9) in order to completely submerge the gel (about 125–200 μ L).
33. Incubate overnight at room temperature.
34. Collect the liquid containing the probe.
35. Purify the probe on a Micro-spin S-200 HR column (Amersham) to remove any salts and other putative contaminants.
36. Count 1 μ L aliquot of the photoprobe solution by liquid scintillation, and dilute the probe to the appropriate count number with deionized distilled water.
37. The probe is now ready for use and can be stored in the dark at 4°C for 1–2 weeks (*see* ^{Note 7}).

3.2. Day 2: In-Gel Protein–DNA Photo-Crosslinking

The gel-purified photoprobe is used for preinitiation complex assembly with the purified transcription factors (TBP, TFIIA, TFIIB, TFIIE, TFIIIF, and TFIID) and RNAPII. The protein–DNA complexes are submitted to an electrophoresis in a native polyacrylamide:bac gel (*see* ^{Notes 8 and 9}) in order to isolate specific complexes. The gel is immediately irradiated with UV light to crosslink the proteins to DNA. Specific complexes are identified by autoradiography. Examination of the autoradiogram permits identification of the preinitiation complexes that assembled on photoprobes (*see* Fig. 4). For the RNAPII initiation complex, assembly of specific complexes on promoter in the native gels can be assessed by comparing reactions with photoprobes containing a wild-type or a mutated TATA box and/or by comparing reactions performed in either the presence or the absence of TBP (*see* ^{Note 10}). The band corresponding to each complex assembled on photoprobes is excised, solubilized, and treated with DNase I and S1 nuclease. Enzymatic treatments permit to liberate polypeptides that are covalently attached to a short piece of DNA carrying one to four radiolabeled nucleotides. After separation of the photocrosslinked polypeptides by SDS-PAGE, the gel is dried and exposed to X-ray film. Examination of the autoradiogram

⁷Fresh probes (less than a week old) give the best results.

⁸Bac is a disulfide-containing analog of bis-acrylamide (*see* **refs.** 11–13). Its chemical synthesis is essentially as described by Naryshkin et al. (*see* ref. 14). We have used *N,N*-Bis(acryloyl) cystamine from Sigma (product number A4929).

⁹The polyacrylamide:bac gels for the EMSA were 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°C (the solubility of bac in water is increased by adding the acrylamide before adding the bac and by performing the addition at 60°C). The volume is then adjusted to 100 mL with water and the solution allowed to cool down to room temperature prior to filtering through 0.22- μ m filter unit and storing at 4°C 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 Surfactil 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°C 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 poured immediately into the slab gel.

Allow 20 min for polymerization at 50°C (the TEMED and ammonium persulfate concentrations are critical variables in the preparation of polyacrylamide:bac gels). The polyacrylamide:bac is stable for up to 72 h at 4°C, but it is better to use it freshly.
¹⁰Because some of the general transcription factors and RNAPII bind nonspecifically to DNA, it is important to discriminate between specific and nonspecific assembly. Assembly of these complexes is promoter specific because both a mutation in the TATA box (TATAAA to TAGAGA; not shown) and the omission of TBP in the assembly mixture (Fig. 4, compare + and –) abolish the formation of the two complexes.

permits identification of the protein(s) that interact with a particular site. The photocrosslinked polypeptides can be identified according to their molecular weight (*see* Fig. 5).

1. Prechill the 4.5% polyacrylamide:bac gel and the 0.5× TBE reservoir containing 2 mM MgCl₂ by placing them in a 4°C cabinet for 3 h.
2. Mix the proteins (80–600 ng each) and complete the volume to 20 µL of buffer G (*see* ^{Note 11}). Add 1 µL of diluted poly(dIdC.dIdC) (*see* ^{Note 12}) and 6,000 cpm of the photoprobe. The final volume is 21 µL (*see* ^{Note 13}).
3. Mix well and incubate for 30 min at 30°C.
4. Load on a 4.5% acrylamide:bac gel in a 0.5× TBE reservoir containing 2 mM MgCl₂.
5. Run in a 4°C cabinet for 60 min at 400 V.
6. Remove one glass plate and irradiate the protein–DNA complexes in the gel for 10 min using with UV light (*see* ^{Notes 14} and ¹⁵).
7. Cover the gel with Whatman paper.
8. Remove the other glass plate.
9. Cover the gel with a plastic wrap.
10. Expose the gel to a phosphorimager screen overnight and print the image.
11. Using a scalpel, cut the print 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 complex of interest.
12. Superimpose the print on the gel.
13. Cut out the gel slices containing the complex of interest using a clean scalpel.
14. Place the excised gel in a 1.5-mL microcentrifuge tube taking care to avoid carrying any pieces of Whatman paper and plastic wrap.
15. Add 10 µL of 1 M DTT to solubilize gel slices (*see* ^{Note 16}).
16. Incubate 10 min at 37°C.
17. Add 40 µL of ND buffer.
18. Incubate at 37°C for 25 min.

¹¹In the preinitiation complex assembly, we use recombinant human proteins in following quantities: 80 ng of TFIIB, 300 ng of RAP30, 600 ng of RAP74, 160 ng of TFIIE34, 380 ng of TFIIE56, 300 ng of calf thymus RNAPII, and 80 ng of recombinant yeast TBP. The amounts of the different protein factors should be optimized for each different combination of proteins and for each protein preparation.

¹²The poly (dIdC-dIdC) stock should be diluted just prior to use. The exact dilution should be determined experimentally in order to favor specific vs. nonspecific signals without adversely affecting the intensity of the specific signals.

¹³The EMSA were performed as described previously by Wolner & Gralla (*see* ref. 15)

¹⁴Irradiation time with UV light should be optimized by performing a time course with the particular system to be used. We use a Hoefer UVC 500 Ultraviolet Crosslinker with 254-nm bulbs.

¹⁵From this point on, normal light conditions can be used.

¹⁶1 M DTT can be substituted by 2–4 M β-mercaptoethanol.

19. Add 13 μ L of DNase mix.
20. Incubate at 37°C for 20 min.
21. Add 3.9 μ L of 10% SDS.
22. Incubate at 95°C for 3 min.
23. Add 5.2 μ L of acid mix.
24. Add 2.6 μ L of S1 nuclease at 80,000 U/mL.
25. Incubate at 37°C for 20 min.
26. Add 15 μ L of 5 $\times$ loading buffer to stop the reaction.
27. Boil the samples for 5 min.
28. Resolve the photocrosslinked polypeptides by SDS-PAGE gel (run at 30 mA in the stacking gel and at 55 mA in the separating gel) (*see* ^{Note 17}).
29. Transfer the gel to Whatman paper and dry.
30. Expose the dried gel to X-ray film using an intensifying screen (*see* ^{Note 18}).

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¹⁷Detailed procedures for SDS-PAGE electrophoresis have been described (*see* ref. 6).

¹⁸The use of BioMax (Kodak) screens is recommended.

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Fig. 1.
Overall representation of the procedure for in-gel site-specific protein–DNA photocrosslinking.

Fig. 2. Schematic design for the synthesis of the photoprobes. In this example, the photoprobe contains one photonucleotide (U) at position +1, and 3 radiolabeled guanines (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 the photoprobes. The position of the DNA fragment carrying both the photoreactive and radiolabeled nucleotides at a specific location is shown

Fig. 4.

An autoradiogram of protein–DNA complexes electrophoresis in a native gel. The 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 electrophoretic mobility shift assay (EMSA).

Fig. 5.

An autoradiogram of an SDS-PAGE gel showing the crosslinked polypeptides in complexes A and B. Complexes A and B were assembled on photoprobes -39/-40 and +1. No difference is observed in the polypeptides that crosslink to photoprobe -39/-40, the form of RPB1 that crosslinks 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.