Crosslinking transcription factors to their recognition sequences with Pt^{II} complexes

Barbara C.F.Chu and Leslie E.Orgel*

The Salk Institute for Biological Studies, PO Box 85800, San Diego, CA 92186-5800, USA

Received January 31, 1992; Revised and Accepted April 7, 1992

ABSTRACT

We have prepared phosphorothioate-containing cyclic oligodeoxynucleotides that fold into 'dumbbells' containing CRE and TRE sequences, the binding sequences for the CREB and JUN proteins, respectively. Six phosphorothioate residues were introduced into each of the recognition sequences. K₂PtCl₄ crosslinks CRE to CREB and TRE to JUN. The extent of crosslinking is about eight times greater than that observed with standard oligodeoxynucleotides and amounts to 30 – 50% of the efficiency of non-covalent association as estimated by gel-shift assays. Crosslinking is reversed by incubation with NaCN. The crosslinking reaction is specific—a dumbbell oligonucleotide with six phosphorothioate groups introduced into the Sp1 recognition sequence could not be crosslinked efficiently to CREB or JUN proteins with K₂PtCl₄. The binding of TRE to CREB is not strong enough for effective detection by gel-shift assays, but the TRE-CREB complex is crosslinked efficiently by K₂PtCl₄ and can then readily be detected.

INTRODUCTION

The study of the control of transcription is central to much ongoing research on eukaryotic cell biology. It is already clear that it is the rule rather than the exception that gene expression is governed by subtle interactions between a number of protein transcription factors and the short recognition sequences in DNA to which they bind (1–3). The identification and isolation of the proteins that interact with defined short oligodeoxynucleotide sequences in double-helical DNA is, therefore, of considerable importance.

Once the DNA target of a transcription factor is known, multiple 'decoy' copies of its recognition sequence can be transfected into a cell to interfere with gene expression. This approach to the study of the control of transcription has already been adopted successfully in a few cases (4, 5) and is likely to find many more applications in the future. In the long run, when the problem of delivery to cells has been solved, 'decoy' DNA, by controlling the expression of harmful genes, may find uses in medicine.

Techniques for crosslinking proteins to nucleic acids should find many uses in research on transcription factors. Platinum complexes have already been used as crosslinking reagents, but in different contexts (6, 7). Photo-crosslinking of proteins to nucleic acids containing 5-bromouracil has been used extensively, but the efficiency of crosslinking is low (8). Recently, more efficient photo-crosslinking procedures using modified bases have been introduced (9, 10).

In this paper we describe a simple, efficient and specific method for forming covalent crosslinks between short double-stranded DNA sequences and proteins that bind to them. We believe this crosslinking method will find many applications to research, for example in identifying transcription factors that bind specifically, but with such low affinity that they cannot be detected by gel-shift assay. We have chosen platinum complexes as the crosslinking reagents because we know that they function at low concentrations in the nuclear environment.

MATERIALS AND METHODS

Materials

The following were obtained from commercial sources: K₂PtCl₄, Pfaltz and Bauer; trans platinum diammine dichloride (transPt^{II}), cis platinum diammine dichloride (cisPt^{II}) and (poly[dI-dC]·poly[dI-dC]), Sigma; T4 DNA ligase, Gibco-BRL; and TETD/acetonitrile, Applied Biosystems. The purified CREB protein and a CREB containing nuclear extract from PC12 cells were gifts from Dr. Marc Montminy; the purified JUN protein was a gift from Dr. Inder Verma.

Dumbbell oligonucleotides containing the double-stranded CRE, TRE and Sp1 recognition sequences (Figure 1, I, II, III) were obtained by synthesizing linear oligonucleotides (Figure 1, Ia, IIa, IIIa) on an Applied Biosystems model 391 PCR MATE automated DNA synthesizer using phosphoramidite chemistry, and then ligating with T4 DNA ligase. Phosphorothioate linkages were introduced using the sulfurizing reagent TETD/acetonitrile in place of I₂ during the oxidation step in the synthesis cycle. This necessitated synthesizing the oligomer in sequential steps with a break in synthesis when the I₂ reagent was replaced by TETD/acetonitrile and *vice versa*. The synthesizer is reprogrammed before and after each introduction of a

* To whom correspondence should be addressed

phosphorothioate residue, the already synthesized sequence fulfilling the role usually played by the resin-attached initiating monomer. Thus a sequence 5'-N₁N₂N₃N₄(s)N₅N₆N₇-3' would be made by first synthesizing the sequence 5'-N₅N₆N₇-3' in the usual way. The iodine reagent is then replaced by TETD/acetonitrile, and the sequence N₄X is programmed, where X stands for the 5'-N₅N₆N₇-3' sequence that is already attached to the resin and is treated as if it were the resin attached 3'-nucleoside in a standard synthesis. The synthetic program is modified as indicated in the instructions provided with the sulfurizing reagent and N₄ is incorporated into the sequence via a phosphorothioate linkage. The sulfurizing reagent is then replaced by the iodine reagent and the sequence 5'-N₁N₂N₃X-3' is programmed, using the normal synthetic cycle program. The synthesis of 5'-tritylated CRE(s)₆-46mer (Figure 1, Ia), for example, was carried out by synthesizing the following sequences in turn, with the sulfurizing reagent replacing the iodine reagent at the residues indicated by an (s): 1) 5'-ATG-3'; 2) 5'-C(s)X-3'; 3) 5'-GTG-3'; 4) 5'-C(s)X-3'; 5) 5'-GAX-3'; 6) 5'-T(s)X-3'; 7) 5'-AAT TTC TCT CAA ATX-3'; 8) 5'-C(s)X-3'; 9) 5'-GTG-3'; 10) 5'-C(s)X-3'; 11) 5'-GAX-3'; 12) 5'-T(s)X-3'; 13) 5'-GTA ACT CTC TTA CCA X-3'. Here 'X' stands for the resin-attached oligomer.

After deprotection with ammonia, the 5'-tritylated phosphorothioate oligonucleotides were detritylated on an OPC oligonucleotide purification column (Applied Biosystems) and further purified by denaturing gel electrophoresis on 12% acrylamide. (The oligonucleotides were heated at 70°C for 3 minutes prior to loading on the gel.) The phosphorothioate-containing oligonucleotides had longer retention times than the standard oligomers when analyzed by HPLC on an RPC-5 column. They gave multiple peaks on account of the presence of R- and S- isomers at each phosphorothioate group. After oxidation with I₂ (11), they were converted to oligonucleotides containing normal phosphodiester bonds that gave a sharp, single peak on RPC-5. Oligonucleotides were phosphorylated at their 5'-termini using γ -[³²P]-ATP and polynucleotide kinase. The kinased products were purified on a Nensorb DNA purification column (Dupont), but were not separated from the starting oligomer at this stage.

To ligate the nicked dumbbell forms of 5'-[³²P]-oligonucleotides or their phosphorothioate-containing analogues (Figure 1, Ia, IIa, IIIa), ~1–20 pmoles of the linear oligonucleotide was heated at 65°C for 3 minutes in 36 μ l of water. Then 10 μ l of a 5 $\times$ ligase buffer were added so that the final reaction mixture contained 50 mM Tris (pH 7.8), 10 mM MgCl₂, 1 mM ATP, 1 mM DTT and 5% polyethylene glycol. After 10 minutes at room temperature, 4 units (4 μ l) of DNA ligase was added. After overnight incubation at room temperature, the reaction mixture was heated at 75°C for 3 minutes. The ligated product was then separated from non-ligated starting material by denaturing gel electrophoresis on 12% polyacrylamide. The ligated form, which is resistant to the action of alkaline phosphatase, migrates faster than the unligated form. Yields of ligated product ranged from 50–95% for standard oligodeoxynucleotides and from 40–70% for phosphorothioate-containing oligomers.

METHODS

Binding

Binding of the ligated CRE and CRE(s)₆ sequences to the CREB protein was carried out as previously described (12, 13). ~0.015 pmole of the [³²P]-CRE sequences was added to ~200 ng of pure CREB protein in 10 μ l of buffer containing 50 mM KCl, 15 mM Tris, (pH 7.5), 0.1 mM EDTA, 0.5 mM DTT, 180 ng acetylated BSA and 250 ng (poly[dI-dC]·poly[dI-dC]) and then incubated at room temperature for 15–20 minutes.

Binding was detected by gel shift assay on 6% non-denaturing gels using 40 mM Tris-borate at pH 8.2 as the electrophoresis buffer. Binding of the ligated CRE sequences to an aliquot of nuclear extract from PC12 cells (4 μ g total protein) was carried out in the same way. Binding of TRE sequences to the JUN protein was carried out similarly in 10 μ l of buffer containing 50 mM Tris (pH 7.9), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 12.5 mM MgCl₂, 20% glycerol and 250 ng (poly[dI-dC]·poly[dI-dC]). Binding was detected by non-denaturing gel electrophoresis on 6% acrylamide, using 20 mM Tris-borate (pH 8.2) as electrophoresis buffer.

Figure 1. Structures of 46mer phosphorothioate-containing oligodeoxynucleotides that fold into nicked dumbbell DNA containing the double stranded CRE sequence (Ia), TRE sequence (IIa) and Sp1 sequence (IIIa). These are converted to the circular dumbbell forms with DNA ligase to form ligCRE(s)₆ (I), ligTRE(s)₆ (II), and ligSp1(s)₆ (III). The underlined sequences are the core recognition sequences.

Crosslinking

~0.015 pmole of the appropriate [32 P]-labelled dumbbell oligonucleotide was first incubated with 200 ng CREB or JUN protein in 10 μ l of buffer containing 50 mM KCl, 15 mM Tris (pH 7.5), 0.1 mM EDTA, 0.06 mM DTT, 180 ng acetylated BSA and 250 ng (poly[dI-dC]·poly[dI-dC]) as described above. After 15 minutes at room temperature, 1–3 μ l of a freshly prepared solution containing the required amount of K_2PtCl_4 or $transPt^{II}$ in buffer containing 1 mM phosphate (pH 7) and 0.1 mM EDTA was added to the reaction mixture. Incubation was continued at room temperature in the dark for 1 hour. 0.5 μ l of a 5% solution of SDS was then added and the crosslinked product separated from non-crosslinked oligonucleotide on an 8% polyacrylamide gel using buffer containing 90 mM Tris-borate (pH 8.2) and 0.1% SDS (SDS gels disrupt noncovalently associated DNA-protein complexes).

RESULTS

We have previously shown that the double-stranded CRE recognition sequence contained in a ligated dumbbell oligonucleotide binds to the CREB protein just as efficiently as does the normal hybridized double-stranded DNA sequence. Similar results were obtained for the TRE dumbbell sequence and the JUN protein (13). In further studies we found that the introduction of 6 phosphorothioate residues within the octamer recognition sequence of $ligCRE(s)_6$ (Figure 1, I) or $ligTRE(s)_6$ (Figure 1, II) does not diminish their binding efficiency to CREB or JUN, respectively (results not shown). Similar results have been reported for the interaction of phosphorothioate-containing DNA with other proteins (4).

Crosslinking of $ligCRE(s)_6$ (Figure 1, I) to CREB and $ligTRE(s)_6$ (Figure 1, II) to JUN with K_2PtCl_4

Figure 2A shows an autoradiogram of an 8% SDS gel after [32 P] $ligCRE(s)_6$ (I) has been crosslinked to CREB in the presence of 0.3 mM K_2PtCl_4 (lane 1) or 2 mM K_2PtCl_4 (lane 2). No bands are visible when the crosslinking procedure is carried out in the absence of CREB. The 2 bands on the gel correspond to proteins of approximate molecular weights 100,000 and 52,000. We assume that the 100,000 M.W. band corresponds to the dumbbell oligomer (M.W. 16,000) bound to dimeric CREB protein (86,000) (14) and the 52,000 band to the oligomer bound to monomeric CREB protein. The proportion of crosslinked product in the dimeric form increases as the platinum concentration is increased.

Figure 2B compares the crosslinking efficiency of [32 P] $ligCRE(s)_6$ (lane 1) with that of [32 P] $ligCRE$ (lane 2), the same ligated dumbbell sequence, but containing normal phosphodiester linkages and [32 P] $ligSp1(s)_6$ (III) (lane 3), an unrelated dumbbell oligomer containing 6 phosphorothioate residues within the octamer Sp1 recognition sequence. Lane 4 represents a crosslinking mixture that contained [32 P] $ligCRE(s)_6$ and an 80-fold excess of the unligated oligomer without a [32 P]-label; lane 5 represents a crosslinking reaction that contained a hundredfold excess of an unlabelled, unrelated sequence containing 6 phosphorothioate residues.

Comparison of lane 1 with lane 2 (Figure 2B) indicates that the presence of internal phosphorothioate residues within the DNA recognition binding region is responsible for the efficient crosslinking of $ligCRE(s)_6$ to CREB. When the same circular dumbbell CRE sequence contained only normal phosphodiester bonds, the crosslinking efficiency was reduced by 80% (lane 2).

Figure 2. Autoradiograms of 8% SDS gels showing: A. Crosslinking of 200 ng CREB to ~0.015 pmoles of [32 P] $ligCRE(s)_6$ after 1 hour. Lane 1, in the presence of 0.3 mM K_2PtCl_4 ; lane 2, in the presence of 2 mM K_2PtCl_4 ; B. Crosslinking of 200 ng CREB with 2 mM K_2PtCl_4 to: Lane 1, 0.015 pmole [32 P] $ligCRE(s)_6$; lane 2, 0.015 pmole [32 P] $ligCRE$; lane 3, 0.015 pmole [32 P] $ligSp1(s)_6$; lane 4, 0.015 pmole [32 P] $ligCRE(s)_6$ in the presence of an 80fold excess of unlabelled, unligated $CRE(s)_6$; lane 5, 0.015 pmole [32 P] $ligCRE(s)_6$ in the presence of a 100fold excess of a nonspecific DNA sequence containing 6 phosphorothioate residues. Protein size markers are indicated on the left.

Figure 3. A. Autoradiogram of 8% SDS gels showing crosslinking of 200 ng JUN in the presence of 2 mM K_2PtCl_4 after 1 hour to: Lane 1, 0.015 pmole [32 P] $ligTRE(s)_6$; lane 2, 0.015 pmole [32 P] $ligTRE$; lane 3, 0.015 pmole [32 P] $ligSp1(s)_6$; lane 4, 0.015 pmole [32 P] $ligTRE(s)_6$ in the presence of an 80fold excess of unlabelled, unligated $TRE(s)_6$; lane 5, 0.015 pmole [32 P] $ligTRE(s)_6$ in the presence of an 100fold excess of a non-specific unlabelled sequence containing 6 phosphorothioate residues. B. The dissociation of crosslinked products by 0.4 M NaCN. Lane 1, the reaction mixture obtained by crosslinking [32 P] $ligCRE(s)_6$ with CRE (Figure 2B, lane 1, above) after treatment with NaCN; lane 2, the reaction mixture obtained by crosslinking [32 P] $ligTRE(s)_6$ with JUN (Figure 3A, lane 1, above) after treatment with NaCN.

At lower platinum concentrations (0.3 mM), crosslinking was not visible when the non-substituted oligonucleotide was used (results not shown).

Our results also indicate that the crosslinking of the ligCRE(s)₆ to CREB is sequence specific. Addition of an 80-fold excess of the same unlabelled (but unligated) phosphorothioate sequence to the crosslinking reaction decreased the yield of crosslinked product by 85–90% (lane 4), but no decrease in crosslinked product was visible when an unrelated circular sequence containing 6 internal phosphorothioate residues was added to the crosslinking mixture (lane 5). Furthermore, a dumbbell oligomer containing 6 phosphorothioate residues in the Sp1 recognition octamer sequence crosslinked to CREB with less than 10% of the efficiency of the CRE sequence at a high platinum concentration (2 mM) (lane 3). No crosslinking could be detected with an intermediate platinum concentration (0.3 mM) (results not shown).

When the number of pmoles of ligCRE(s)₆ crosslinked to CREB as estimated by SDS gel electrophoresis was compared to the number of pmoles that were bound to CREB as estimated in an independent experiment using non-denaturing gel electrophoresis, the crosslinking efficiency was found to be 30–50% of the binding efficiency when the concentration of K₂PtCl₄ was 2.3 mM. Lowering the K₂PtCl₄ concentration to 0.3 mM resulted in a 15–30% crosslinking efficiency.

The crosslinked products formed by CREB with ligCRE(s)₆ were extracted from SDS gels with a buffer containing 0.05 M Tris, 10⁻³ M EDTA (pH 7.5) and electrophoresed again under the original conditions. About 60% of the radioactivity appeared at the same position as in the original gel, and the remaining counts appeared in the position of the uncomplexed dumbbell DNA. The crosslink, therefore, is not completely stable under the conditions of extraction.

Very similar results are obtained when [³²P]ligTRE(s)₆ (II) is crosslinked to JUN (Figure 3A). [³²P]ligTRE, containing normal phosphodiester bonds (lane 2), crosslinks with approximately 15% of the efficiency of the [³²P]ligTRE(s)₆ (lane 1) when the concentration of K₂PtCl₄ is 2 mM. No crosslinking of [³²P]ligTRE is visible when the concentration of K₂PtCl₄ is 0.3 mM (results not shown). Crosslinking is inhibited by 85% when an 80-fold excess of unlabelled unligated TRE(s)₆ is added to the crosslinking reaction mixture (lane 4), but a 100fold excess of a random oligomer containing the same number of phosphorothioate residues does not inhibit crosslinking (lane 5). A dumbbell [³²P]ligSp1(s)₆ sequence (III) crosslinks to JUN (lane 3) with less than 10% of the efficiency of ligTRE(s)₆. The molecular weight of the crosslinked product indicates that crosslinking occurs between [³²P]ligTRE(s)₆ and monomeric JUN.

TransPt^{II} forms crosslinks between CRE and CREB or TRE and JUN efficiently at concentrations considerably lower than those needed to crosslink with K₂PtCl₄. However, even at relatively low concentrations of transPt^{II} (0.08 mM), aggregates form that stick to the origin of SDS gels. CisPt^{II} was not an effective crosslinking agent.

Dissociation of platinum crosslinked complexes by NaCN

Figure 3B illustrates the result of treating Pt-crosslinked products with 0.4 M NaCN overnight at room temperature. The cyanide ion displaces the platinum complex from the phosphorothioate groups and releases the labelled oligonucleotides ligCREB(s)₆ (lane 1) or ligTRE(s)₆ (lane 2).

Crosslinking of ligTRE(s)₆ to CREB

Weak associations of DNA with protein can be detected more sensitively by crosslinking with platinum than by gel shift binding

Figure 4: A. Autoradiogram of a 6% non-denaturing gel showing binding of 200 ng CREB to: Lane 1, 0.015 pmole [³²P]ligCRE(s)₆; lane 2, 0.015 pmole [³²P]ligTRE(s)₆; lane 3, [³²P]ligSp1(s)₆. B. Autoradiogram of an 8% SDS gel showing crosslinking after 1 hour of 200 ng CREB in the presence of 2 mM K₂PtCl₄ to: Lane 1, 0.015 pmole [³²P]ligCRE(s)₆; lane 2, 0.015 pmole [³²P]ligTRE(s)₆; lane 3, 0.015 pmole [³²P]ligSp1(s)₆.

Figure 5: Autoradiogram of an 8% SDS gel showing crosslinking in the presence of 2 mM K₂PtCl₄ for 1 hour. Lane 1, 0.015 pmole [³²P]ligCRE(s)₆ to 4 μg protein in a PC12 nuclear cell extract; lane 2, 0.015 pmole [³²P]ligCRE(s)₆ to 200 ng CREB; lane 3, 0.015 pmole [³²P]ligTRE(s)₆ to 4 μg protein in a PC12 nuclear cell extract; lane 4, 0.015 pmole [³²P]ligTRE(s)₆ to 200 ng JUN.

assays. Gel electrophoresis indicates that the binding of TRE sequences to CREB is about one tenth as extensive as the binding of the CRE sequence (15). Detection of the association of TRE to CREB is simplified by crosslinking with K_2PtCl_4 . Figure 4A shows an autoradiogram of a gel shift assay on a 6% non-denaturing gel showing the binding of [^{32}P]ligCRE(s)₆ (lane 1), [^{32}P]ligTRE(s)₆ (lane 2), and [^{32}P]ligSp1(s)₆ to CREB. LigCRE(s)₆ is bound ~10 times more efficiently than ligTRE(s)₆. In Figure 5B the same complexes have been crosslinked with K_2PtCl_4 . [^{32}P]ligTRE(s)₆ (lane 2) is crosslinked to CREB with ~40% of the efficiency with which [^{32}P]ligCRE(s)₆ is crosslinked to CREB (lane 1). A survey of the results of several experiments shows that the number of pmoles of ligTRE(s)₆ that crosslinked to CREB was 3–5-fold higher than the number of pmoles that were detected by gel-shift assays. No bands corresponding to crosslinked products could be seen when we attempted to crosslink the [^{32}P]ligSp1(s)₆ (lane 3), a sequence that does not bind CREB (Figure 4A, lane 3). This indicates that the crosslinking of ligTRE(s)₆ to CREB is sequence specific.

Crosslinking of [^{32}P]ligCRE(s)₆ and [^{32}P]ligTRE(s)₆ to proteins in a PC12 nuclear cell extract

When [^{32}P]ligCRE(s)₆ or [^{32}P]ligTRE(s)₆ were added to a nuclear cell extract from PC12 cells and treated with K_2PtCl_4 , they were crosslinked to proteins in the extract (Figure 5). In the case of [^{32}P]ligTRE(s)₆, several bands were present on an SDS gel. The major band (lane 3), as anticipated, had the same mobility as the adduct formed by [^{32}P]ligTRE(s)₆ with pure JUN (lane 4). However, in the case of [^{32}P]ligCRE(s)₆, the major band (lane 1) did not have the same mobility as the [^{32}P]ligCRE(s)₆-CREB adduct (lane 2). Instead it co-electrophoresed with the ligTRE(s)₆-JUN product (lanes 3 and 4). We believe that in a crude nuclear cell extract, ligCRE(s)₆ crosslinks preferentially to the AP-1 binding proteins (FOS, JUN, etc.) to which the CRE sequence is already known to bind (16). Standard gel shift assays using PC12 cell nuclear extracts confirm that the CRE sequence binds more extensively to AP-1 proteins than to the CREB protein.

DISCUSSION

In our studies of the crosslinking of transcription factors to DNA recognition sequences we have used dumbbell DNAs rather than simple double-helical DNAs made up of pairs of complementary strands. We have previously shown that dumbbells bind to transcription factors with undiminished affinity (13). The use of dumbbell DNAs simplifies the crosslinking procedure, and eliminates problems associated with the presence of unhybridized single strands.

Our results show that the transcription factor CREB is crosslinked efficiently by K_2PtCl_4 to double-stranded DNA which contains the octamer recognition sequence CRE, provided the CRE sequence is substituted with appropriate internal phosphorothioate groups. Crosslinking is much less efficient in the absence of phosphorothioate groups. In a similar way the JUN protein is crosslinked efficiently to the TRE recognition sequence if and only if the TRE sequence contains phosphorothioate groups. These differences in crosslinking efficiency reflect the much greater reactivity of the Pt^{II} ion with sulfur-containing ligands than with the nucleoside bases.

The crosslinking reactions are sequence-specific. Crosslinking of CREB to radioactively-labelled ligCRE(s)₆ is inhibited by an 80-fold excess of unlabelled ligCRE(s)₆ but is unaffected by the presence of a 100-fold excess of a randomly-chosen sequence containing six phosphorothioate groups. Similarly, binding of JUN to radioactively-labelled ligTRE(s)₆ is inhibited by an 80-fold excess of unlabelled ligTRE(s)₆ but not by a 100-fold excess of a randomly chosen octamer sequence with equivalent phosphorothioate substitution. Neither CREB nor JUN is crosslinked to a [^{32}P]-labelled dumbbell DNA containing the same number of phosphorothioate residues within the Sp1 octamer recognition sequence.

In this crosslinking procedure, proteins may be modified by Pt^{II} complexes, independently of the nucleic acid, particularly if they contain reduced cysteine residues. The detection of the dimer of CREB crosslinked to ligCRE on SDS gels suggests that CREB may be crosslinked to form a stable dimer by Pt^{II} complexes.

The use of Pt^{II} complexes to crosslink transcription factors to the nucleic acid sequences to which they bind has a number of advantages:-

- (1) The preparation of the modified DNAs containing phosphorothioate groups at chosen positions in the sequence can be carried out routinely with a standard DNA synthesizer using commercially available sulfurizing reagents. This avoids the often time-consuming synthesis of novel protected phosphoramidites.

- (2) The substitution of sulfur for oxygen in the phosphodiester group of DNA does not change significantly the size or stereochemistry of the backbone. Consequently, the affinity of transcription factors for DNA does not decline when phosphorothioate groups are present.

- (3) The crosslinks can be dissociated by treatment of the complex with 0.4 M NaCN. The nucleic acid and protein components are recovered in their native states.

- (4) Crosslinking may provide a more sensitive method of detecting low-affinity protein-DNA association than the gel shift assay which detects non-covalently bonded DNA-protein complexes. The binding of TRE to CREB was only about 10% as extensive as the binding of CRE to CREB when measured by gel-shift assay. However, the crosslinking efficiency of TRE to CREB was about 40% of that of CRE to CREB. Crosslinking may, therefore, be useful in detecting sequence-specific association which is not strong enough to permit detection by electrophoresis.

Since crosslinking can occur between a protein and a DNA sequence even if they do not associate sufficiently strongly to permit quantitation by gel-shift assay, crosslinking analysis is, in some sense, less specific than gel-shift analysis. However, this is generally an advantage rather than a disadvantage, provided crosslinking is not indiscriminate. Crosslinking will be efficient if the transcription factor is associated with its DNA recognition sequence much of the time, and inefficient otherwise. This seems relevant to biological function. The low dissociation rate in the electrophoresis buffer that is required by the gel-shift method is less obviously relevant to biological function.

It must be recognized that cell extracts contain many components that could, in principle, interfere with the crosslinking procedure. Additional studies will be needed to show that the method is generally applicable to cell extracts.

The crosslinking studies reported here were carried out without knowledge of the three-dimensional structures of the protein-DNA complexes. When X-ray structures become available, it should

be possible to choose crosslinking reagents that efficiently bridge phosphorothioate groups in the DNA and reactive cysteine and histidine residues in the protein. This should lead to more efficient crosslinking.

The use of the crosslinking strategies described in this paper to interfere with transcription in cells in tissue culture or in whole animals is our long-term objective. It will be necessary next to study the internalization of these crosslinking reagents into cells, their stability towards intracellular nucleases, and their ability to decoy intracellular transcription factors.

ACKNOWLEDGMENTS

This research was supported by grant No. GM33023-09 from the National Institute of General Medical Sciences. We thank Carol North for technical assistance and Sylvia Bailey for manuscript preparation.

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