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Crosslinking of SRF to the c-*fos* SRE CArG Box Guanines Using Photo-active Thioguanine Oligodeoxynucleotides

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6-Thioguanine (thioG) was chemically incorporated into 25-base oligodeoxynucleotides encoding the c-*fos* serum response element (SRE) at positions corresponding to each guanine of the CArG box, which only slightly impaired DNA binding by the Serum Response Factor (SRF). Upon exposure to long wavelength UV light each thioG-containing SRE could be crosslinked to SRF, with efficiencies ranging from <1 to 25% of the complex depending on the position of thioG in the SRE and on the UV source used. Crosslinking was strongest to the 3′ side of the CArG box, and to the outer rather than the inner CArG box guanines, consistent with hydrogen bonds formed between SRF and the outer guanines in the crystal structure [Pellegrini *et al., Nature* **376,** 490, 1995]. The crosslinked product was found to be chemically unstable. Possible mechanisms of crosslink formation are discussed. © 1996 Academic Press, Inc.

SRF is essential for transcriptional regulation of growth factor-inducible genes, binding homodimerically and with high affinity to SREs such as that of the c-*fos* gene (for review see 1). We chose the well characterised SRF-SRE interaction to investigate the nature of the bond formed upon photochemical crosslinking of protein to oligodeoxynucleotides containing thioG.

Photochemical crosslinking has been widely used to study protein-nucleic acids interactions (for review see 2). Among many photoactive agents, azido derivatives (for review see 3), halobases (4), and thio-bases (mainly 4-thiopyrimidine and 6-thiopurine) (5) are most commonly employed. Thio-base offers unique advantages. Distortion of DNA or RNA structures by thio-bases is similar to that by halobases, but much less than that by azido derivatives. Thio-base absorbs at 330-350 nm, distinct from nucleic acids (260 nm) and proteins (280 nm). Conventionally, DNA and RNA molecules containing thio-base were synthesised by enzymatic incorporation of thio-base triphosphates (2), imposing limitations on specific thio-base incorporation in some sequences. Recent progress in solid phase chemistry has enabled the synthesis of oligodeoxynucleotides containing thio-base uniquely at pre-determined sites. This technology has been applied to study interactions between nucleic acids (5). However less work (6) has examined nucleic acid-protein interactions.

We previously developed an efficient chemical synthesis of oligodeoxynucleotides containing thio-bases (4-thiothymine, 6-thiopurine and 6-thioguanine) at specific positions (7). The precursor thio-base monomers are commercially available (from Glen research), making the technique accessible. Since SRF binds the CArG box consensus binding site $CC(AT)_6GG$ (8), and interacts directly with CArG box guanines (9), we chose thioG to investigate the nature of crosslinking bonds formed upon the photo-crosslinking of SRF and the c-*fos* SRE.

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MATERIALS AND METHODS

Oligodeoxynucleotides containing thioguanine or thiopurine. These were chemically synthesised using the phosphoramidite approach (7b, 7c) and purified by Fast Protein Liquid Chromatography (10). Unmodified oligomers were synthesised using standard phosphoramidite chemistry. The preparation of SRF has been described previously (11).

Preparation of SRE probes containing thioguanine or thiopurine. An oligodeoxynucleotide containing 6-thioguanine, 6-thiopurine, or unmodified guanine was 5′-[^{32}P]-labelled using [γ-^{32}P]-ATP (Amersham) and T4 polynucleotide kinase. The kinase reaction mixture was ethanol precipitated and resuspended in 40 mM Tris, 20 mM $MgCl_2$, 50 mM NaCl, pH 7.5, containing a 5 fold molar excess of unmodified complementary strand as indicated in Figure 1A; however, note that 5′-GATCCCCTGCTGACGCAGATGTCAGCCGATGGACATCCTGTGTAAG-3′ was annealed against mTG2 (Fig. 3A). The annealing reactions were incubated at 37°C for 15 min, ethanol precipitated, and the pellet resuspended in gel loading buffer then purified on a 10% polyacrylamide, 0.5 × TBE gel at 25°C. Double stranded oligodeoxynucleotides were recovered from the gel with equal photoreactivity using standard crush and soak or electroelution protocols at 25°C. Performing the annealing step by heat denaturation and slow cooling sometimes reduced thio-base photoreactivity, however no instability was observed of the modified oligodeoxynucleotides or crosslinked products stored at −20°C for extended periods.

Photo-crosslinking of SRF to SRE probes containing thioguanine or thiopurine. Crosslinking reactions were typically performed in 40-400 μl reaction mixtures, which were either placed in closed 1.5 ml-Eppendorf tubes on ice and irradiated with a UV Stratalinker 2400 (Stratagene) using 365 nm bulbs at a distance of 5 cm, or placed in 1.5 ml Eppendorf tubes shielded by a glass sequencing plate (opaque to light <300 nm and 60 % transparent to light of 330 nm) and irradiated at 25°C at a distance of 0.5-1 cm with the following UV sources: Hanau ''Florotest'' UV lamp (Hanau), ''UV15'' benchtop transilluminator (Desaga), ''UVT-750M'' benchtop transilluminator (IBI) (all set at 366 nm) or Hannovia XBO 200 Watt mercury/xenon lamp of broad emission spectrum. The concentration of SRF dimer was estimated to be 50 nM. Concentrations of SRE probes were between 30-60 nM, but constant within a given experiment. Reaction mixtures were incubated at 25°C for 60-90 min to allow complex formation prior to irradiation. 10 μl aliquots were removed from each of the reaction mixtures after the indicated irradiation times and stored in the dark for analysis by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (12) or by electrophoretic mobility shift assay (EMSA) in a 4% polyacrylamide gel (11). Retarded bound complexes and crosslinked complexes were identified by autoradiography of dried gels and quantified by Cerenkov counting of excised gel slices. Reaction Buffer A comprised of 10 mM Hepes pH 7.8, 25 mM KCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 2 mM spermidine, 2 mg/ml bovine serum albumin (BSA), 10% glycerol and 0.05% Nonidet P-40. Reaction Buffer B was 10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 0.05% skim milk powder and 5% glycerol. Unless stated otherwise reaction mixtures contained 100 μg/ml salmon sperm DNA which was added to SRf in the binding reactions 10 min before the SRE probes.

RESULTS AND DISCUSSION

Crosslinking of SRF to SRE probes containing thioguanine. 25 mer oligodeoxynucleotides were synthesised corresponding to one strand of the c-*fos* SRE, containing thioG as shown in Fig. 1A. Each oligonucleotide posessing a single thioG (designated thioG-SRE) was annealed against its unmodified complementary strand. Their binding and crosslinking to SRF were investigated. All thioG-SREs (TG1, TG2, TG3 and TG4) and the unmodified SRE control (X-SRE) could similarly bind to SRF. The presence of thioG slightly impaired the binding of SRF (Fig. 1B). SDS-PAGE analysis showed that UV irradiation of all thioG-SREs produced a complex of approximately 82 kDa apparent molecular weight, the expected size of one molecule of SRF (67 kDa) crosslinked to the 25 base-paired oligonucleotide duplex (15 kDa). The formation of this crosslink required UV irradiation and the presence of thioG (Fig. 1C).

Effects of the position of thioguanine on crosslinking efficiency. There were minor differences in crosslinking efficiency using different UV sources, and pronounced differences dependent upon the position of thioG in the SRE probes. The outer thioguanines (TG2 and TG3) were more reactive than the inner (TG1 and TG4), and the thioG of TG2 crosslinked better than that of TG3. The strongest crosslinked thioG-SRE in this experiment was TG2 using the Hanau UV source (Fig. 1C), which corresponded to 11% of the amount of TG2 recovered in the band shifted complex. Yields are reproducible for a particular thioG-SRE and UV light treatment, and were similar for several preparations of thioG-SRE.

The crosslinked product contains SRF. The amount of crosslinked complex correlated with the concentration of SRF in the binding reaction (Fig. 2), demonstrating that the crosslinked complex contained SRF and radiolabelled thioG-SRE (TG2).

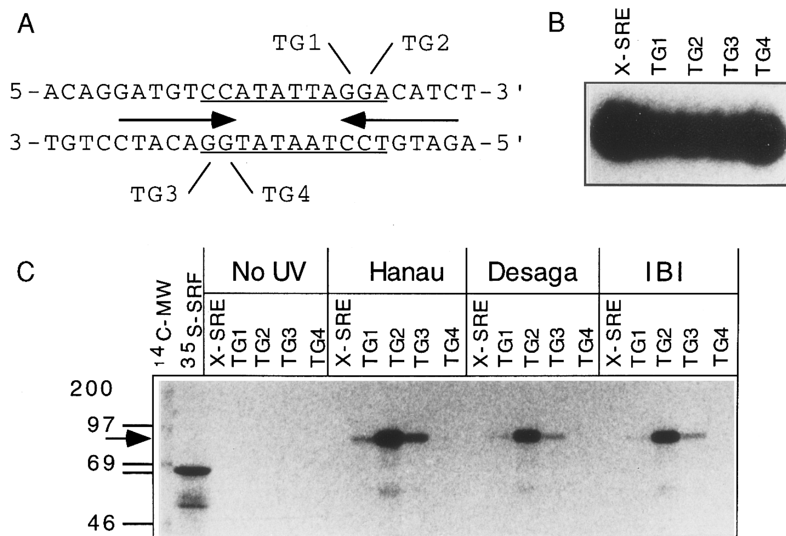

FIG. 1. Photochemical crosslinking of the SRE containing thioG using long wavelength UV light. (A) Nucleotide sequence of the oligodeoxynucleotide duplex in the control SRE probe (X-SRE) and in the thioG-containing probes. The position of thioG in each thioG-SRE (TG1, TG2, TG3 and TG4) is indicated. Arrows show the pseudo diad symmetry of the SRE, with CArG box residues underlined. Each thioG-SRE differs from X-SRE by only one sulphur atom. (B) EMSA analysis of equal aliquots of a binding reaction incubated at 25°C for 90 min. The SRF-SRE complex is arrowed. (C) SDS-PAGE analysis of the above binding reactions exposed at 25°C for 60 min to the indicated UV light source (See Materials and Methods). *In vitro* translated ^{35}S-labelled SRF migrates at 67 kDa. Non-radioactive SRF crosslinked to ^{32}P-labelled SRE is arrowed. Unbound ^{32}P-labelled SRE probes not shown.

Specificity of crosslinking. To further investigate the requirements for crosslinking formation, SRF was bound to equal amounts of either X-SRE, mTG2 [similar to TG2 however containing a mutation which drastically reduces SRF-binding (13)], TG2, or TP2 (similar to TG2 however containing 6-thiopurine instead of thioG, and therefore differing only by a minor groove amino group from TG2) (Fig. 3A). Without non specific competitor DNA, SRF formed a low-specificity complex with mTG2 (Fig. 3B, lane 2), however the crosslinked yield was only 2% of the bound complex, compared with 19% for TG2 in this experiment (using a Stratalinker UV source). The binding affinities of TG2 and TP2 were similar (Fig. 3B lanes 3, 4, 7, and 8), yet the crosslinked yield was markedly lower for TP2 (Fig. 3C) although thiopurine is an effective photo-crosslinking agent (5b). Differences in binding between thioG SREs in Figs. 1 and 3 may reflect conformational changes in the SRE probe structures. This could affect photoreactivity, as could the altered resonance of the purine ring electrons caused by the

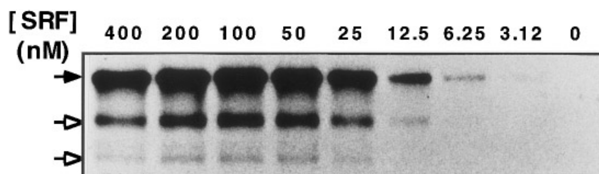

FIG. 2. The appearance of UV-induced crosslinked complex is dependent upon the presence of SRF. A serial dilution of SRF was incubated in Buffer B containing 0.5 μg ssDNA and ^{32}P-labelled TG2, and exposed to UV light for 40 min in a Stratalinker. Samples were analysed by SDS-PAGE. The migration of crosslinked SRF (closed arrow) and degraded crosslinked products (open arrows) is indicated.

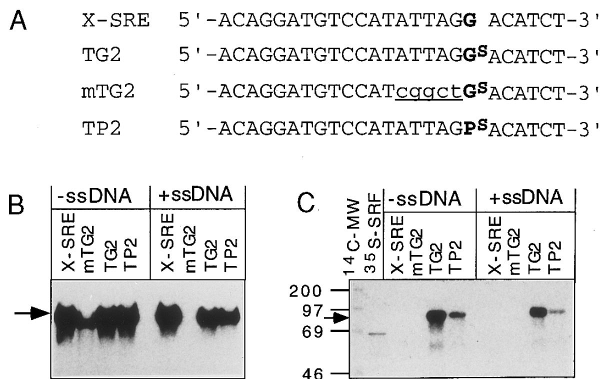

FIG. 3. Crosslinking of SRF to TG2 requires high affinity binding and is influenced by DNA structural modification. (A) Nucleotide sequences of the ^{32}P-labelled upper strands used in the experiment. Note: the central five base-pairs in mTG2 differ from the other duplexes. (B) EMSA analysis of binding reaction in the absence or presence of non specific competitor ssDNA. Bound SRF/SRE complexes are indicated. (C) SDS-PAGE analysis of the above binding reactions after exposure to UV light for 40 min in a Stratalinker.

presence of different purine ring functional groups, or base stacking interactions with surrounding nucleotides. However, taken together the above results suggest that SRF binds the outer guanines more intimately, and the c-*fos* SRE asymmetrically, consistent with findings by x-ray crystallographic studies (9).

Optimising crosslink yield. We then sought to optimise the yield of crosslinking with TG2.

(a) Optimum UV source: A mercury/xenon lamp, filtered through a glass DNA sequencing plate opaque to UV less than 310 nm, provided the highest yield of crosslinked product with yields of approximately 25% of the measured EMSA complex for TG2. This light source emitted a narrow beam of light suitable only for single samples. For multiple samples a Stratalinker gave reproducible yields of 18% to 20% of the bound TG2 complex (data not shown).

(b) Time course: To determine the kinetics of crosslinking formation aliquots were taken from a master mix after various periods of UV irradiation. Typically the yield increased with time to a maximum and decreased upon further irradiation. With a Desaga transilluminator maximum yields were at 60 min (Fig. 4), and 30 min with the Stratalinker (data not shown). The decrease of crosslink at later times indicates complicated kinetics, with lability of the product itself to UV.

Stability of crosslinked products. To further characterise the crosslink and possibly determine

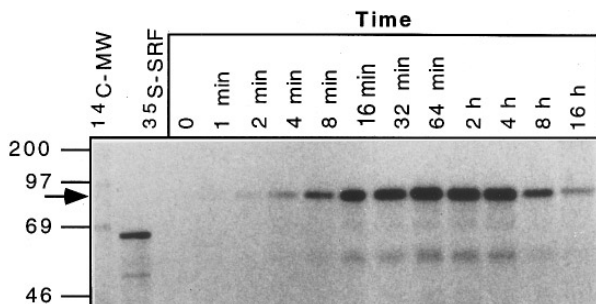

FIG. 4. Time course of crosslinking formation. A typical time course for crosslinking formation with the Desaga transilluminator (see Materials and Methods). Standard binding reactions were exposed for the indicated time to broad wavelength UV light (''366'' nm setting) followed by SDS-PAGE analysis.

TABLE 1
The Crosslinked Product Is Labile to Many Treatments

Treatment	Crosslink stability (estimated)	Treatment	Crosslink stability (estimated)
Acetone precipitation	>80%	0.1% TFA*	50–80%
Ethanol precipitation	>80%	NH_4HCO_3*	50–80%
TCA precipitation	0%	1 M HCl, 30 min*	5%
CNBr treatment	0%	1 M NaOH, 30 min*	20%
Edman degradation§	unstable	(§G. Multhaup and M.A.C. unpublished)	

Note. Crosslink reactions were performed in reaction buffer B, and stability of the crosslinked complex to the indicated treatments was assessed by comparing the band intensity of treated material to that of the nontreated complex after SDS-PAGE and autoradiography. Asterisked treatments resulted in accumulation of radioactivity in the origin or at the interface between collecting and separating gels after SDS-PAGE. These probably represented complex aggregates, in which crosslink stability could not be reliably assessed. Treatments: overnight cyanogen bromide cleavage; 0.1% TFA in acetonitrile, 20 min, 25°C; 50 mM NH_4HCO_3, 37°C, overnight. The HCl and NaOH treatments were for 30 min at 37°C, followed by neutralisation.

the crosslinking sites on SRF at the molecular level, we examined the stability of the crosslinked product of TG2. In general, the crosslinking bond between DNA and protein was unstable to many treatments (Table 1), being labile to mild basic (e.g. NH_4HCO_3) and acidic (e.g. 0.1% trifluoroacetic acid) conditions.

Mechanism of crosslinking formation. Although the mechanism of crosslinking is unknown, the photochemistry of 4-thiopyrimidine and 6-thiopurine within nucleic acids has been documented (5). In a study of photo crosslinking of thymidine-(5′-3′)-6-thiopurine-2′-deoxynucleoside monophosphate, the carbon at the 6-position of 6-thiopurine crosslinked to the carbon at the 6-position of thymine, with the sulphur atom becoming transferred to the 5-position carbon of thymine (5b). Much less is known about the mechanism of thioguanine photo-crosslinked to protein. From the results presented above, the formation of a crosslinking bond was evidenced by the fact that the crosslinked complex could be separated from the bound complex and isolated by SDS-PAGE gel. However, the crosslinking bond was labile (Table 1), and therefore unlikely to be a carbon-carbon bond. TG2 produced the best crosslinking yield. Interestingly, the crystal structure of the SRF-SRE complex (9) shows a hydrogen bond between Lys163 of SRF and the 6-oxygen of base G5 (corresponding to the thioG replacement in TG2), the only hydrogen bond made in the major groove per half-site. Therefore the crosslinking bond may be formed between the amino group of Lys 163 and thio-keto of the thioG in TG2. A resultant N-S crosslinking bond would be chemically labile, consistent with the observed instability of the crosslinked complex. The bond was stable to 2 min boiling in Laemmli buffer, and was therefore probably not a disulfide bond. Site-specific ^{35}S-labelling of thioG in DNA (14) could help elucidate the crosslinking mechanism if the ^{35}S atom in thioG of an SRE probe became attached to crosslinked residue(s) of SRF. The location of ^{35}S may then help determine the interacting residues of SRF. The photo crosslinking approach described here could be of general use in studying nucleic acid-protein interactions.

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