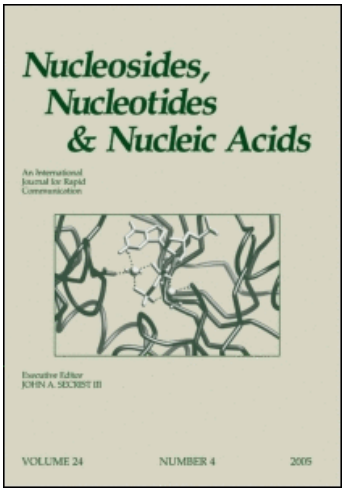

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Photochemical Cross-Linking of λ-Cro Repressor to Operator DNA Containing 4-Thiothymine or 6-Thioguanine

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PHOTOCHEMICAL CROSS-LINKING OF λ-CRO REPRESSOR TO OPERATOR DNA CONTAINING 4-THIOTHYMINE OR 6-THIOGUANINE

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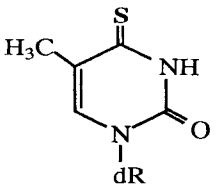
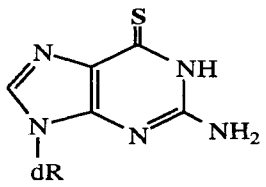
Abstract: Oligonucleotides containing 4-thiothymine or 6-thioguanine cross-link to λ-Cro repressor upon irradiation with long wavelength UV light. The results are consistent with the Cro-DNA interaction model based on its crystal structure.

Photochemical cross-linking of a DNA-protein complex offers a means of probing the interaction interface between the two molecules since irradiation of DNA-protein complex with UV light produces covalent linkages between amino acid residues and nucleic acid bases[1,2]. Because of limitations of the usual procedure of cross-linking protein to normal DNA by short wavelength (254 nm) UV light, notably, damage to both DNA and proteins and low yield of cross-linking, one would prefer to use a DNA specifically containing a photoactive base-analogue absorbing at a longer wavelength to minimize photo-damage to DNA and proteins and to increase cross-linking efficiency.



(A)



(B)

4-Thiothymine (**A**) and 6-thioguanine (**B**) possess several desirable properties for studying DNA-protein interactions[3]. The sulphur atom in the major groove of the DNA is only slightly larger than oxygen, but it otherwise chemically resembles oxygen. Therefore, the introduction of these thiobases into oligonucleotides should not appreciably perturb the binding interaction between the proteins and DNA. Furthermore, they have a λmax at 340

```
          +1 +2 +3 +4 +5 +6 +7 +8 +9 -8' -7' -6' -5' -4' -3' -2' -1'
(T)  T  C  T  A  T  C  A  C  C  G  C  A  A  G  G  G  A  T  A  A  A
(B)  A  G  A  T  A  G  T  G  G  C  G  T  T  C  C  C  T  A  T  T  T
          -1 -2 -3 -4 -5 -6 -7 -8 -9 +8' +7' +6' +5' +4' +3' +2' +1'
```

Fig. 1. Sequence of DNA containing OR3 operator. The two strands have been designated top strand (T) and bottom strand (B) and base pairs in the operator have been numbered within each half-site from 1 to 9 starting at the distal base pair as shown. The sequence of OR3 operator is bold.

Table. Dissociation constants (kd) and cross-linking efficiency of the operator DNAs

[a]DNA	T/B	T_{+1}/B (T^S)	T_{+3}/B (T^S)	T_{+8}/B (G^S)	$T_{-6'}$/B (G^S)	$T_{-4'}$/B (G^S)	$T_{-2'}$/B (T^S)	T/B_{-2} (T^S)
kd (nM)	0.77	0.86	1.31	1.21	1.43	1.58	1.11	0.98
[b]X-link (%)		2.4	2.2	nil	9.0	3.0	nil	nil

[a]DNA	T/B_{-4} (G^S)	T/B_{-5} (T^S)	T/B_{-6} (G^S)	T/B_{-7} (G^S)	T/B_{-9} (G^S)	$T/B_{+8'}$ (T^S)	$T/B_{+3'}$ (T^S)	$T/B_{+1'}$ (T^S)
kd (nM)	1.74	1.05	1.62	1.58	1.31	0.95	1.45	0.91
[b]X-link (%)	3.4	8.6	7.2	7.2	nil	nil	2.6	3.1

[a]T/B refers to the double-stranded nonsubstituted operator DNA. Each substituted operator sequence is referred to by the strand (T and B), the site of the substitution is indicated with subscript numbers shown, and the type of substitution is shown in parentheses.
[b]Fraction of DNA in the cross-linked complex relative to the total DNA (Bound + free).

nm, and are photoactive at 340-350 nm wavelength, which is well away from the usual absorption maxima of protein (280 nm) and DNA (260 nm), thus cross-linking can be carried out at a wavelength which would not appreciably damage proteins and DNA. Upon irradiation with near UV light (340-360 nm) they become chemically reactive through an unknown mechanism, and are capable of covalently linking to a bound protein[3].

We have studied the photochemical cross-linking of Cro protein to its operator DNA (Fig.1) containing 4-thiothymine or 6-thioguanine, prepared with our previously developed methods[4,5]. To ascertain whether the introduction of the modified bases into the DNA affected appreciably the binding affinity between the two molecules we first measured the ability of the substituted operators to bind Cro under equilibrium conditions using the gel retardation method. The results (Table) show that incorporation of the modified bases at a single site within the operator has only minimal affect on the equilibrium binding affinity for Cro.

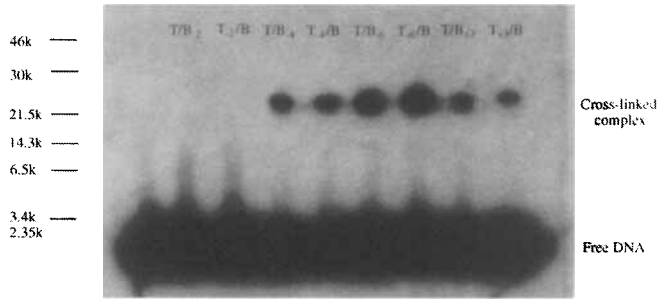


Fig. 2. Analysis by SDS-PAGE of cross-linking between Cro and operator DNAs. Lane 1, DNA (T/B) alone; other lanes, cross-linking between Cro and DNAs (sequences in Table)

The ability of each substituted operator to covalently cross-link with Cro was assessed by irradiation with 360 nm light of a mixture of the protein and the operator DNA at room temperature followed by SDS-PAGE of the irradiated samples. UV irradiation produced an approximate 20 KD ^{32}P-labelled complex (Fig. 2). This is the expected size of one molecule of Cro (Mw 7,350) cross-linked to a 21 bp oligonucleotide (Mw 12,600). There were remarkable differences in the efficiency of crosslinking to the modified bases incorporated at different positions in the operator (Table). Operators substituted by a thiobase at positions +1, +3, -4, -6, (operator T_{+1}/B, T_{+3}/B, T/B$_{-4}$, and T/B$_{-6}$, respectively), and at their symmetry-related positions +1', +3', -4', -6' (operator T/B$_{+1'}$, T/B$_{+3'}$, T$_{-4'}$/B, and T$_{-6'}$/B, respectively) all cross-linked to Cro protein. In addition, cross-link was also detected with operators containing thiobase at positions -5 (T/B$_{-5}$) and -7 (T/B$_{-7}$). In contrast, no cross-link was observed with thiobase substituted operators at position -2 (T/B$_{-2}$) and its symmetry-related position -2' (T$_{-2'}$/B), as well as at positions +8, +8', -9 (T_{+8}/B, B/$_{+8'}$, and T/B$_{-9}$, respectively). These results are consistent with the X-ray crystallographic structure of the Cro-DNA complex[6-8] as thiobases at the positions believed to contact with Cro protein in the crystal were cross-linked, while those where cross-linking were not observed are those postulated not to interact with Cro repressor.

Additional control experiment established (Fig. 3) that the formation of the cross-link was dependent on UV irradiation, and also upon the presence of the thiobase in the Cro operator. The cross-linking required specific Cro-DNA complex formation as cross-linking was not observed when a 22 bp DNA duplex containing thiothymine with sequence of the catabolic repressor protein (CRP) binding site (5' TAATGTGAGTT^{S}AGCTCACTCAT 3') was irradiated in presence of Cro. Furthermore, the cross-linking was severely inhibited by 4-fold excess of operator DNA (T/B) but not by a 4-fold excess of CRP binding site.

An attempt was made to identify the amino acid residues covalently linked to the DNA by separation and proteolysis of the cross-linked complex, separation of the digested DNA-peptides, and determination of the sequence of the peptide covalently linked to the

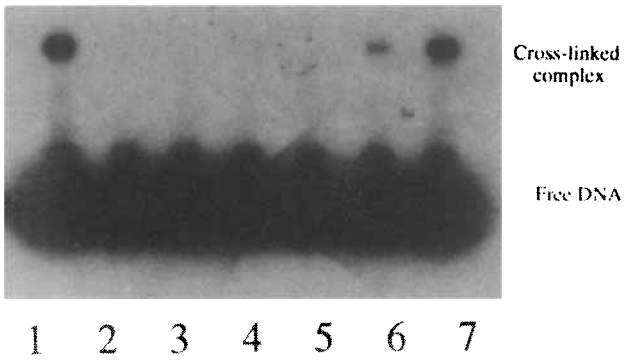


Fig. 3. Specificity of cross-linking between Cro and DNA. Lane 1, cross-linking of Cro with T/B$_{-6}$; lane 2, control reaction omitting UV irradiation; lane 3, control reaction showing no cross-linking to T/B operator; lane 4, control reaction showing no cross-linking to a 22 bp DNA duplex with the sequence of the CRP binding site and containing a thiothymine; lane 5, control reaction omitting Cro; lane 6, control reaction in the presence of a 4-fold excess of T/B operator; lane 7, control reaction in the presence of a 4-fold excess of the 22 bp DNA containing the CRP binding site.

DNA. However this has proved to be more problematic than expected, mainly due to the lability of the cross-linkage between the two molecules. The cross-link was unstable to both acids such as trifluoroacetic acid, and alkaline conditions, for example, the complex was destroyed even in NH_4HCO_3 solution (pH 8.0). The complex was reasonably stable at 20°C, but slow decomposition of the complex was observed at room temperature.

In summary, substitution of 4-thiothymine and 6-thioguanine for thymine and guanine in DNA had minimal effects on the binding affinity for protein, and irradiation of the complex between the protein and DNA containing the thiobases produced significant cross-linking. The irradiated DNA cross-linked to the protein specifically bound to it, and the cross-linking results with Cro were consistent with the Cro-DNA interaction model proposed based on its crystal structure[6-8]. The photochemical cross-linking approach described here could be of general use for investigating DNA-protein interactions.

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