The functional subunit of a dimeric transcription activator protein depends on promoter architecture

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In Class I CAP-dependent promoters, the DNA site for CAP is located upstream of the DNA site for RNA polymerase. In Class II CAP-dependent promoters, the DNA site for CAP overlaps the DNA site for RNA polymerase, replacing the –35 site. We have used an ‘oriented heterodimers’ approach to identify the functional subunit of CAP at two Class I promoters having different distances between the DNA sites for CAP and RNA polymerase [CC(–61.5) and CC(–72.5)] and at one Class II promoter [CC(–41.5)]. Our results indicate that transcription activation at Class I promoters, irrespective of the distance between the DNA sites for CAP and RNA polymerase, requires the activating region of the promoter-proximal subunit of CAP. In striking contrast, our results indicate that transcription activation at Class II promoters requires the activating region of the promoter-distal subunit of CAP.

Key words: activating region/catabolite gene activator protein (CAP)/cyclic AMP receptor protein (CRP)/oriented heterodimers/RNA polymerase

Introduction

Escherichia coli catabolite gene activator protein (CAP; also referred to as the cAMP receptor protein, CRP) is a well characterized transcription activator protein (reviewed by Kolb *et al.*, 1993a). CAP activates transcription at a large set of promoters, the best characterized of which is the *lac* promoter.

CAP functions by binding to a 22 bp two-fold-symmetric DNA site (Berg and von Hippel, 1988; Ebright *et al.*, 1989; Gunasekera *et al.*, 1992). The crystallographic structure of CAP has been determined to 2.5 Å resolution (Weber and Steitz, 1987) and the crystallographic structure of the CAP–DNA complex has been determined to 3.0 Å resolution (Schultz *et al.*, 1991). CAP is a dimer of two identical subunits, each of which is 209 amino acids in length. The CAP–DNA complex is two-fold symmetric: one subunit of CAP interacts with one half of the DNA site; the other subunit of CAP interacts in a two-fold symmetry-related fashion with the other half of the DNA site. The majority of CAP–DNA interactions are mediated

by a helix–turn–helix DNA binding motif present in each subunit of CAP (amino acids 168–188; for reviews of the helix–turn–helix motif, see Brennan, 1991, 1992).

Simple CAP-dependent promoters, those that require only CAP for transcription activation, can be grouped into two classes based on the position of the DNA site for CAP (Ushida and Aiba, 1990; Ebright, 1993).

(i) In Class I CAP-dependent promoters, the DNA site for CAP is located upstream of the DNA site for RNA polymerase. The DNA site for CAP can be located at various distances from the transcription start point, provided that the DNA site for CAP and the DNA site for RNA polymerase are on the same face of the DNA helix. Thus, the DNA site for CAP can be centered at or near positions –61.5, –72.5, –82.5 or –92.5 (Mandecki and Caruthers, 1984; Straney *et al.*, 1989; Gaston *et al.*, 1990; Ushida and Aiba, 1990). The prototype Class I CAP-dependent promoter is the *lac* promoter (DNA site for CAP centered at position –61.5).

(ii) In Class II CAP-dependent promoters, the DNA site for CAP overlaps the DNA site for RNA polymerase, replacing the –35 determinants for binding of RNA polymerase. The prototype Class II CAP-dependent promoter is the *galP1* promoter (DNA site for CAP centered at position –41.5).

Recent work has established that amino acids 156–164 of CAP constitute an activating region essential for transcription activation at Class I and Class II CAP-dependent promoters, but not essential for DNA binding or DNA bending (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991; Williams *et al.*, 1991; West *et al.*, 1993; Zhou *et al.*, 1993a, 1994; Niu *et al.*, 1994; reviewed by Ebright, 1993). It has been proposed that transcription activation at Class I and Class II CAP-dependent promoters requires direct protein–protein contact between the activating region of CAP and RNA polymerase.

Because CAP is a dimer of two identical subunits, CAP has two activating regions, one in each subunit. In previous work, using an approach we have termed ‘oriented heterodimers’, we have shown that transcription activation at the *lac* promoter requires the activating region of only one subunit of CAP: the promoter-proximal subunit (Zhou *et al.*, 1993b). In the work in this report, we have used the oriented heterodimers approach to identify the functional subunit of CAP at two Class I CAP-dependent promoters having different distances between the DNA sites for CAP and RNA polymerase [CC(–61.5) and CC(–72.5)] and at one Class II CAP-dependent promoter [CC(–41.5)] (Figure 1).

Oriented heterodimers: the approach

Our approach had three steps. In step one, we constructed heterodimers consisting of (i) one CAP subunit having a non-functional activating region but having wild-type

Fig. 1. CAP-dependent promoters. CC(-61.5), CC(-72.5) and CC(-41.5) are derivatives of the *E. coli melR* promoter having consensus DNA sites for CAP centered at positions -61.5, -72.5 and -41.5 respectively. CC(-61.5), CC(-72.5) and CC(-41.5) have identical transcription start sites, -10 hexamers and -10/-35 spacers. In previous work CC(-61.5), CC(-72.5) and CC(-41.5) were referred to as CC+20, CC+31 and CC respectively (Bell *et al.*, 1990; Gaston *et al.*, 1990; Williams *et al.*, 1991; Zhang *et al.*, 1992; West *et al.*, 1993; Zhou *et al.*, 1994). In each panel, DNA sites for CAP are boxed; -35 and -10 hexamers are underlined. (A) Class I CAP-dependent promoters CC(-61.5), CC(-72.5) and derivatives. (B) Class II CAP-dependent promoter CC(-41.5) and derivatives.

DNA binding specificity ([Ala158]CAP or [Leu159]CAP; Table I) and (ii) one CAP subunit having a functional activating region but having non-wild-type DNA binding specificity ([Val181]CAP; Table I). In step two, we oriented the resulting heterodimers on promoter DNA by the use of promoter derivatives having DNA sites for CAP consisting of (i) one wild-type DNA half site and (ii) one non-wild-type DNA half site. In step three, we assessed the abilities of the oriented heterodimers to activate transcription.

To orient the [Ala158]CAP/[Val181]CAP and [Leu159]CAP/[Val181]CAP heterodimers on promoter DNA, we used two derivatives of each promoter: CX(*n*) and XC(*n*) [i.e. CX(-61.5) and XC(-61.5), CX(-72.5) and XC(-72.5), and CX(-41.5) and XC(-41.5); see Figures 1 and 2). In each of these derivatives, one of the two DNA half sites of the DNA site for CAP is replaced by a DNA half site having A:T at position 7. In CX(*n*), the promoter-proximal DNA half site is non-wild-type; in XC(*n*), the promoter-distal DNA half site is non-wild-type.

We expect that [Ala158]CAP/[Val181]CAP and [Leu159]CAP/[Val181]CAP heterodimers bind to CX(*n*) in an asymmetric, oriented fashion, since [Ala158]CAP and [Leu159]CAP are able to bind with high affinity to the wild-type DNA half site, but are not able to bind with high affinity to the non-wild-type DNA half site (Figure 2A and C). Each heterodimer has only one functional activating region. At CX(*n*), each heterodimer is oriented such that the functional activating region is in the promoter-proximal subunit. Analogously, we expect that [Ala158]CAP/[Val181]CAP and [Leu159]CAP/[Val181]CAP heterodimers bind to XC(*n*) in an asymmetric, oriented fashion (Figure 2B and D). At XC(*n*), each heterodimer

Table I. CAP subunits used in the analysis

Subunit	Activating region ^a	DNA half site recognized ^{b,c}
CAP	Functional	1 2 3 4 5 6 7 8 9 10 11 A A A T G T <u>G</u> A T C T T T T A C A C T A G A
[Ala158]CAP	Non-functional	A A A T G T <u>G</u> A T C T T T T A C A C T A G A
[Leu159]CAP	Non-functional	A A A T G T <u>G</u> A T C T T T T A C A C T A G A
[Val181]CAP	Functional	A A A T G T <u>N</u> A T C T T T T A C A N T A G A
[Ala158; Val181]CAP	Non-functional	A A A T G T <u>N</u> A T C T T T T A C A N T A G A
[Leu159; Val181]CAP	Non-functional	A A A T G T <u>N</u> A T C T T T T A C A N T A G A

^aBell *et al.* (1990) and Zhou *et al.* (1993a, 1994).

^bEbright *et al.* (1987, 1989) and Gunasekera *et al.* (1992).

^c[Val181]CAP has an amino acid substitution at the amino acid of CAP that makes direct, specificity-determining contact with the base pair at position 7 of the DNA half site (Ebright *et al.*, 1984, 1987). [Val181]CAP binds with equal high affinities to DNA half sites containing G:C, A:T, C:G and T:A at position 7.

is oriented such that the functional activating region is in the promoter-distal subunit.

In control experiments we constructed and analyzed [Val181]CAP/[Val181]CAP homodimers. We expect that [Val181]CAP/[Val181]CAP homodimers bind to CX(*n*) and XC(*n*) in an unoriented fashion, since the [Val181]CAP subunit is able to bind with equal high affinities to the wild-type DNA half site and the non-wild-type DNA half site (Figure 2E and F). The [Val181]CAP/[Val181]CAP

Fig. 2. Oriented heterodimers. The figure illustrates the expected orientations of dimers at the CX(n) and XC(n) promoters. In each panel, the arrow indicates the transcription start point of the promoter. (A, C) The [Ala158]CAP/[Val181]CAP heterodimer and the [Leu159]CAP/[Val181]CAP heterodimer bind to CX(n) in an asymmetric, oriented fashion. There is only one functional activating region, in the promoter-proximal subunit. (B, D) The [Ala158]CAP/[Val181]CAP heterodimer and the [Leu159]CAP/[Val181]CAP heterodimer bind to XC(n) in an asymmetric, oriented fashion. There is only one functional activating region, in the promoter-distal subunit. (E, F) [Val181]CAP/[Val181]CAP homodimers bind to CX(n) and XC(n) in an unoriented fashion. There are two functional activating regions, one in each subunit.

Fig. 3. 'Reverse' oriented heterodimers. (A, C) The [Ala158;Val181]CAP/CAP heterodimer and the [Leu159;Val181]CAP/CAP heterodimer bind to CX(n) in an asymmetric, oriented fashion. There is only one functional activating region, in the promoter-distal subunit. (B, D) The [Ala158;Val181]CAP/CAP heterodimer and the [Leu159;Val181]CAP/CAP heterodimer bind to XC(n) in an asymmetric, oriented fashion. There is only one functional activating region, in the promoter-proximal subunit.

homodimer has two functional activating regions, one in each subunit.

In further control experiments, we performed reciprocal, 'reverse' oriented heterodimer experiments. We constructed, oriented and analyzed heterodimers consisting of (i) one CAP subunit having both a non-functional activating region and non-wild-type DNA binding specificity ([Ala158;Val181]CAP or [Leu159;Val181]CAP; Table I) and (ii) one wild-type CAP subunit. We expect that [Ala158;Val181]CAP/CAP and [Leu159;Val181]CAP/CAP heterodimers bind to CX(n) in an asymmetric, oriented fashion (Figure 3A and C). At CX(n), each heterodimer is oriented such that the single functional activating region is in the promoter-distal subunit. Analogously, we expect that [Ala158;Val181]CAP/CAP and [Leu159;Val181]CAP/CAP heterodimers bind to XC(n) in an asymmetric, oriented fashion (Figure 3B and D). At XC(n), each heterodimer is oriented such that the

single functional activating region is in the promoter-proximal subunit.

Oriented heterodimers: the method

CAP subunits exchange freely on mixing (Brown and Crothers, 1989). To construct [Ala158]CAP/[Val181]CAP or [Leu159]CAP/[Val181]CAP heterodimers, we mixed a defined ratio of [Ala158]CAP or [Leu159]CAP subunits with [Val181]CAP subunits. We used a large molar excess of [Ala158]CAP or [Leu159]CAP subunits over [Val181]CAP subunits (i.e. a 10- to 40-fold molar excess). Under such conditions, only two dimeric species are present in appreciable quantities: (i) [Ala158]CAP/[Ala158]CAP or [Leu159]CAP/[Leu159]CAP homodimers, which bind poorly to CX(n) and XC(n), and which contain no functional activating region, and therefore do not affect the analysis; (ii) [Ala158]CAP/[Val181]CAP or [Leu159]CAP/[Val181]CAP heterodimers, the desired dimeric species.

We performed experiments both *in vivo* and *in vitro*. To perform experiments *in vivo*, we constructed strains that co-express the gene encoding [Ala158]CAP or [Leu159]CAP at a gene dosage of 40 (expression from a high-copy-number plasmid) and the gene encoding [Val181]CAP at a gene dosage of 1 (expression from the chromosome). In such strains, we analyzed transcription activation at CX(n) and XC(n) by measurement of differential rates of β -galactosidase synthesis (see Zhang *et al.*, 1992; Zhou *et al.*, 1993a,b, 1994; Niu *et al.*, 1994).

To perform experiments *in vitro*, we pre-incubated a 10-fold molar excess of [Ala158]CAP with [Val181]CAP under conditions that result in complete subunit exchange (1.0 μ M [Ala158]CAP, 0.1 μ M [Val181]CAP at 37°C for 24 h; see Brown and Crothers, 1989; Zhou *et al.*, 1993b). We then performed abortive initiation *in vitro* transcription experiments (Malan *et al.*, 1984; Zhang *et al.*, 1992; Zhou *et al.*, 1993a,b) using CX(n) and XC(n) as templates.

Results

Transcription activation at Class I promoters, irrespective of distance between the DNA sites for CAP and RNA polymerase, requires the activating region of the promoter-proximal subunit of CAP

The results of oriented heterodimer and 'reverse' oriented heterodimer experiments with the Class I CAP-dependent promoter CC(-61.5) are presented in Tables II–IV. Both *in vivo* and *in vitro*, [Ala158]CAP/[Val181]CAP and [Leu159]CAP/[Val181]CAP heterodimers were functional in transcription activation at CX(-61.5), but were not functional in transcription activation at XC(-61.5). The extent of transcription activation was 20- to 40-fold higher at CX(-61.5) than at XC(-61.5). Reciprocally, [Ala158;Val181]CAP/CAP and [Leu159;Val181]CAP/CAP heterodimers were not functional in transcription activation at CX(-61.5), but were functional in transcription activation at XC(-61.5). In this case, the extent of transcription activation was 20-fold lower at CX(-61.5) than at XC(-61.5). Finally, [Val181]CAP/[Val181]CAP homodimers were functional in transcription activation at both CX(-61.5) and XC(-61.5). In this case, within experimental error, the extent of transcription activation was equal at CX(-61.5) and XC(-61.5). The results indicate

Table II. Transcription activation at CC(−61.5) and CC(−72.5) by oriented heterodimers (*in vivo* data)

Binding-competent dimer	β-Galactosidase synthesis ^a		
	CX(n)	XC(n)	CX(n)/XC(n)
CC(−61.5)			
[Ala158]CAP/[Val181]CAP	330	15	<u>22</u>
[Leu159]CAP/[Val181]CAP	430	12	<u>36</u>
[Val181]CAP/[Val181]CAP	190	130	1.5
CC(−72.5)			
[Ala158]CAP/[Val181]CAP	54	9.9	<u>5.5</u>
[Leu159]CAP/[Val181]CAP	72	9.6	<u>7.5</u>
[Val181]CAP/[Val181]CAP	39	33	1.2

^aSpecific activities (units as in Miller, 1972).**Table III.** Transcription activation at CC(−61.5) and CC(−72.5) by 'reverse' oriented heterodimers (*in vivo* data)

Binding-competent dimer	β-Galactosidase synthesis ^a		
	CX(n)	XC(n)	CX(n)/XC(n)
CC(−61.5)			
[Ala158;Val181]CAP/CAP	20	320	<u>0.063</u>
[Leu159;Val181]CAP/CAP	20	320	<u>0.063</u>
CC(−72.5)			
[Ala158;Val181]CAP/CAP	10	150	<u>0.067</u>
[Leu159;Val181]CAP/CAP	10	150	<u>0.067</u>

^aSpecific activities (units as in Miller, 1972).**Table IV.** Transcription activation at CC(−61.5) by oriented heterodimers (*in vitro* data)

Binding-competent dimer	UMP incorporation (pmol/nmol template/min)		
	CX(−61.5)	XC(−61.5)	CX(−61.5)/XC(−61.5)
[Ala158]CAP/[Val181]CAP	21	<1	<u>>21</u>
[Val181]CAP/[Val181]CAP	47	32	1.5

that transcription activation at the Class I CAP-dependent promoter CC(−61.5), as at the Class I CAP-dependent promoter *lac* (Zhou *et al.*, 1993b), requires the activating region of only one subunit of the CAP dimer: the promoter-proximal subunit.

The results of analogous experiments with the Class I CAP-dependent promoter CC(−72.5) are presented in Tables II and III. The pattern of results was equivalent to the pattern of results with CC(−61.5). Thus, [Ala158]CAP/[Val181]CAP and [Leu159]CAP/[Val181]CAP heterodimers were functional in transcription activation at CX(−72.5), but were not functional in transcription activation at XC(−72.5). [Ala158;Val181]CAP/CAP and [Leu159;Val181]CAP/CAP heterodimers were not functional in transcription activation at CX(−72.5), but were functional in transcription activation at XC(−72.5) and [Val181]CAP/[Val181]CAP homodimers were functional in transcription activation at both CX(−72.5) and XC(−72.5). The results indicate that transcription activation at the Class I CAP-dependent promoter CC(−72.5), as at the Class I CAP-dependent promoters CC(−61.5) and *lac* (Zhou *et al.*, 1993b), requires the activating region

Table V. Transcription activation at CC(−41.5) by oriented heterodimers (*in vivo* data)

Binding-competent dimer	β-Galactosidase synthesis ^a		
	CX(−41.5)	XC(−41.5)	CX(−41.5)/XC(−41.5)
[Ala158]CAP/[Val181]CAP	13	390	<u>0.033</u>
[Leu159]CAP/[Val181]CAP	11	590	<u>0.019</u>
[Val181]CAP/[Val181]CAP	62	57	1.1

^aSpecific activities (units as in Miller, 1972).**Table VI.** Transcription activation at CC(−41.5) by 'reverse' oriented heterodimers (*in vivo* data)

Binding-competent dimer	β-Galactosidase synthesis ^a		
	CX(−41.5)	XC(−41.5)	CX(−41.5)/XC(−41.5)
[Ala158;Val181]CAP/CAP	290	50	<u>5.8</u>
[Leu159;Val181]CAP/CAP	310	20	<u>16</u>

^aSpecific activities (units as in Miller, 1972).**Table VII.** Transcription activation at CC(−41.5) by oriented heterodimers (*in vitro* data)

Binding-competent dimer	UMP incorporation (pmol/nmol template/min)		
	CX(−41.5)	XC(−41.5)	CX(−41.5)/XC(−41.5)
[Ala158]CAP/[Val181]CAP	14	170	<u>0.082</u>
[Val181]CAP/[Val181]CAP	290	210	1.4

of only one subunit of the CAP dimer: the promoter-proximal subunit. We infer that transcription activation at all Class I CAP-dependent promoters, irrespective of differences in promoter sequence and in distance between the DNA sites for CAP and RNA polymerase, requires the activating region of only one subunit of the CAP dimer: the promoter-proximal subunit.

Transcription activation at Class II promoters requires the activating region of the promoter-distal subunit of CAP

The results of oriented heterodimer and 'reverse' oriented heterodimer experiments with the Class II CAP-dependent promoter CC(−41.5) are presented in Tables V–VII. Remarkably, the pattern of results with the Class II promoter CC(−41.5) was precisely the opposite of the pattern of results with the Class I promoters CC(−61.5) and CC(−72.5). Thus, both *in vivo* and *in vitro*, [Ala158]CAP/[Val181]CAP and [Leu159]CAP/[Val181]CAP heterodimers were not functional in transcription activation at CX(−41.5), but were functional in transcription activation at XC(−41.5). The extent of transcription activation was 30- to 50-fold lower at CX(−41.5) than at XC(−41.5). Reciprocally, [Ala158;Val181]CAP/CAP and [Leu159;Val181]CAP/CAP heterodimers were functional in transcription activation at CX(−41.5), but were not functional in transcription activation at XC(−41.5). In this case, the extent of transcription activation was 10-

fold higher at CX(−41.5) than at XC(−41.5). Finally, [Val181]CAP/[Val181]CAP homodimers were functional in transcription activation at both CX(−41.5) and XC(−41.5). In this case, within experimental error, the extent of transcription activation was equal at CX(−41.5) and XC(−41.5). The results indicate that transcription activation at the Class II CAP-dependent promoter CC(−41.5), in striking, unexpected contrast to transcription activation at the Class I CAP-dependent promoters CC(−61.5), CC(−72.5) and *lac* (Zhou *et al.*, 1993b), requires the activating region of only the promoter-distal subunit of the CAP dimer.

Wild-type CAP has a functional activating region and wild-type DNA binding specificity (Table I). We reason that CAP/[Val181]CAP heterodimers bind to CX(*n*) and XC(*n*) in an asymmetric, oriented fashion (as do [Ala158]CAP/[Val181]CAP, [Leu159]CAP/[Val181]CAP, [Ala158; Val181]CAP/CAP and [Leu159; Val181]CAP/CAP heterodimers), but have functional activating regions in both subunits (as do [Val181]CAP/[Val181]CAP homodimers). To confirm the unexpected results with the Class II CAP-dependent promoter CC(−41.5), we performed analogous oriented heterodimer experiments with CAP/[Val181]CAP heterodimers, both *in vivo* and *in vitro*. The experiments with CAP/[Val181]CAP heterodimers yielded results equivalent to those with [Val181]CAP/[Val181]CAP homodimers; thus, CAP/[Val181]CAP heterodimers were functional in transcription activation at both CX(−41.5) and XC(−41.5) (data not shown). The results rule out the possibility that our results with [Ala158]CAP/[Val181]CAP, [Leu159]CAP/[Val181]CAP, [Ala158; Val181]CAP/CAP and [Leu159; Val181]CAP/CAP heterodimers are artefacts of heterodimer formation *per se* or of heterodimer orientation *per se*.

Heterodimers bind in the predicted orientations

The oriented heterodimers approach of the preceding sections assumes that CAP heterodimers bind in a predicted, oriented fashion to DNA sites having one wild-type DNA half site and one non-wild-type DNA half site (Figures 2 and 3). To verify that CAP heterodimers bind in the predicted, oriented fashion, we used phenylazide-mediated protein–DNA photocross-linking (see Pendergrast *et al.*, 1992; Chen *et al.*, 1993).

Fortuitously, [Val181]CAP contains a unique surface cysteine residue at amino acid 10 of the helix–turn–helix DNA binding motif, i.e. Cys178 (see Ebright *et al.*, 1990; Schultz *et al.*, 1991; Zhang *et al.*, 1991; Pendergrast *et al.*, 1992). We used the cysteine-specific chemical modification procedure of Pendergrast *et al.* (1992) to construct a [Val181]CAP derivative having a phenylazide photo-activatable cross-linking agent at amino acid 178, i.e. [(4-azidophenacyl)-Cys178; Val181]CAP ([*Val181]CAP). We then constructed [Ala158]CAP/[*Val181]CAP heterodimers by incubation of a 10-fold molar excess of [Ala158]CAP with [*Val181]CAP under conditions that result in complete subunit exchange, formed protein–DNA complexes, UV irradiated the protein–DNA complexes and identified the nucleotides at which protein–DNA cross-linking occurred. We performed experiments with two synthetic DNA fragments, ICAP-7A-R [analogous to CX(*n*)] and ICAP-7A-L [analogous to XC(*n*)] (sequences in Figure 4A). In each of these synthetic DNA

fragments, one of the two DNA half sites of the consensus DNA site for CAP is replaced by a DNA half site having A:T at position 7. In ICAP-7A-R the right DNA half site is non-wild-type; in ICAP-7A-L the left DNA half site is non-wild-type.

The results are presented in Figure 4B. With DNA fragment ICAP-7A-R, cross-linking occurred exclusively on the top DNA strand (lanes 1 and 3). In contrast, with DNA fragment ICAP-7A-L, cross-linking occurred exclusively on the bottom DNA strand (lanes 2 and 4). With each DNA fragment, the efficiency of cross-linking was 2%, comparable with the efficiency of cross-linking observed by Pendergrast *et al.* (1992). Single nucleotide resolution mapping of cross-linking sites (see Pendergrast *et al.*, 1992; Chen *et al.*, 1993) indicated that with DNA fragment ICAP-7A-R, cross-linking occurred at the adenine residue of T:A at position 9 of the right DNA half site and that with DNA fragment ICAP-7A-L, cross-linking occurred at the adenine residue of T:A at position 9 of the left DNA half site (summarized in Figure 4A). The results show directly that [Ala158]CAP/[*Val181]CAP heterodimers bind in the predicted, oriented fashion, i.e. with the [Ala158]CAP subunit in contact with the wild-type DNA half site and the [*Val181]CAP subunit in contact with the non-wild-type DNA half site.

To further document the DNA binding affinities and specificities of [Ala158]CAP and [Val181]CAP subunits, we performed nitrocellulose filter DNA binding experiments (Gunasekera *et al.*, 1992) with [Ala158]CAP/[Ala158]CAP and [Val181]CAP/[Val181]CAP homodimers and DNA fragments ICAP, ICAP-7A-R and ICAP-7A-L. The results are presented in Table VIII. The results show that, consistent with expectation (Table I), [Ala158]CAP/[Ala158]CAP has a 200-fold higher affinity for DNA fragment ICAP than for DNA fragments ICAP-7A-R and ICAP-7A-L, and [Val181]CAP/[Val181]CAP has equal high affinities for DNA fragments ICAP, ICAP-7A-R and ICAP-7A-L.

Discussion

The results in this report, together with the results of Zhou *et al.* (1993b), establish that transcription activation at all tested Class I CAP-dependent promoters, irrespective of the distance between the DNA sites for CAP and RNA polymerase, requires the activating region of the promoter-proximal subunit of the CAP dimer. We propose that the mechanisms of transcription activation at all Class I CAP-dependent promoters are identical, i.e. that CAP contacts the same target on RNA polymerase (Figure 5A and B) [apparently amino acids 258–265 of the RNA polymerase α subunit (Igarashi and Ishihama, 1991; Zou *et al.*, 1992; Ebright, 1993; Chen *et al.*, 1994; H.Tang, K.Severinov, A.Goldfarb, D.Fenyo, B.Chait, A.Goldfarb and R.Ebright, submitted)].

In striking contrast, the results in this report establish that transcription activation at a Class II CAP-dependent promoter requires the activating region of the promoter-distal subunit of the CAP dimer. We propose that the mechanisms of transcription activation at Class I and Class II CAP-dependent promoters differ. Five additional results are consistent with this proposal.

(i) The profiles of effects of single alanine substitutions

Fig. 4. Protein–DNA photocross-linking with oriented heterodimers. (A) DNA fragments ICAP [analogous to CC(*n*)], ICAP-7A-R [analogous to CX(*n*)] and ICAP-7A-L [analogous to XC(*n*)]. Underlining indicates the DNA site for CAP; rectangles indicate substitutions in ICAP-7A-R and ICAP-7A-L; open circles indicate nucleotides at which protein–DNA photocross-linking occurs with [Ala158]CAP/[*Val181]CAP heterodimers and ICAP-7A-R and ICAP-7A-L. (B) Lane 1, ICAP-7A-R 5′-end-labeled on the top strand; lane 2, ICAP-7A-L 5′-end-labeled on the top strand; lane 3, ICAP-7A-R 5′-end-labeled on the bottom strand; lane 4, ICAP-7A-L 5′-end-labeled on the bottom strand.

Table VIII. DNA binding by CAP, [Ala158]CAP and [Val181]CAP (*in vitro* data)

CAP derivative	K_{obs} (nM ⁻¹)		
	ICAP	ICAP-7A-R	ICAP-7A-L
CAP/CAP	67	0.31	0.37
[Ala158]CAP/[Ala158]CAP	77	0.33	0.48
[Val181]CAP/[Val181]CAP	17	18	27

Fig. 5. Model. The black circle denotes the single essential activating region of CAP. α CTD, α NTD, β , β' and σ denote, respectively, the C-terminal domain of the RNA polymerase α subunit (two per RNA polymerase molecule), the N-terminal domain of the RNA polymerase α subunit (two per RNA polymerase molecule) and the β , β' and σ subunits of RNA polymerase. Recent work regarding the domain organization and structure of the α subunit indicates that (i) the C-terminal domain of the α subunit is capable of independent dimerization and DNA binding and (ii) the C-terminal domain of the α subunit is connected to the remainder of the α subunit, and thus to the remainder of RNA polymerase, through a ≥ 13 amino acid unstructured and/or flexible linker (≥ 44 Å if fully extended; Ross *et al.*, 1993; Blatter *et al.*, 1994). Alternative positioning of the C-terminal domain of the α subunit is proposed to be facilitated by the linker and, possibly, by bending of the intervening DNA. [CAP bends DNA to an angle of $\approx 90^\circ$ (Schultz *et al.*, 1991). RNA polymerase bends DNA to an angle of $\approx 50^\circ$ (Rees *et al.*, 1993; Meyer-Almes *et al.*, 1994).] (A) Class I CAP-dependent promoter having the DNA site for CAP centered at position -61.5 [e.g. CC(-61.5) and *lac*]. (B) Class I CAP-dependent promoter having the DNA site for CAP centered upstream of position -61.5 [e.g. CC(-72.5)]. (C) Class II CAP-dependent promoter [e.g. CC(-41.5)].

within the activating region of CAP differ at Class II and Class I promoters (Zhou *et al.*, 1994).

(ii) CAP derivatives with substitutions at amino acids 52 and 96 exhibit increased transcription activation activity at Class II promoters, but unchanged transcription activation activity at Class I promoters (Bell *et al.*, 1990; Williams *et al.*, 1991; West *et al.*, 1993). [In the three-dimensional structure of the CAP–DNA complex amino acids 52 and 96 are distant from the activating region of CAP (Schultz *et al.*, 1991).]

(iii) Removal of the C-terminal region of the RNA polymerase α subunit has no negative effect on transcription activation at Class II promoters, but strongly decreases transcription activation at Class I promoters (Igarashi *et al.*, 1991; Kolb *et al.*, 1993b).

(iv) Removal of the C-terminal region of the RNA polymerase α subunit suppresses the positive control-defective phenotype of [Ala158]CAP and [Leu159]CAP at Class II promoters, but not at Class I promoters (West *et al.*, 1993).

(v) The kinetic parameters in transcription initiation that are affected by CAP at characterized Class II and Class I promoters differ, being k_f or K_B and k_f at Class II promoters (Gaston *et al.*, 1990; Lavigne *et al.*, 1992; Goodrich and McClure, 1992), but K_B at Class I promoters (Malan *et al.*, 1984; Gaston *et al.*, 1990; see however Straney *et al.*, 1989).

What is the mechanism of transcription activation at a Class II promoter? Recent work suggests that the C-terminal region of the RNA polymerase α subunit contacts DNA sequences immediately upstream of the DNA site for CAP at a Class II promoter (A. Attey, T. Belyaeva, N. Savery, J. Hoggett, A. Ishihama and S. Busby, manuscript in preparation; A. Kolb, personal communication). Therefore, it is likely that the activating region of the promoter-distal subunit of CAP contacts the C-terminal region of the α subunit at a Class II promoter (Figure 5C) [although apparently not the same part of the C-terminal region of the α subunit contacted at Class I promoters (Zhou *et al.*, 1994; H. Tang, T. Heyduk, K. Severinov, A. Goldfarb and R. Ebright, unpublished results)]. One attractive hypothesis is that at Class II promoters the C-terminal region of the α subunit inhibits transcription initiation and the activating region of the promoter-distal subunit of CAP, through protein–protein contact, relieves the inhibition. According to this hypothesis, transcription activation at Class II promoters has two components, (i) ‘anti-inhibition’ by the activating region of the promoter-distal subunit of CAP and

Table IX. Bacterial strains

Strain	Genotype	Source
XE032.1/CX(−61.5)	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−61.5)</i>	This work
XE032.1/CX(−61.5)/158A	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−61.5) pYZ158A</i>	This work
XE032.1/CX(−61.5)/159L	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−61.5) pYZ159L</i>	This work
XE032.1/CX(−61.5)/181V	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−61.5) pPC181V</i>	This work
XE032.1/XC(−61.5)	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−61.5)</i>	This work
XE032.1/XC(−61.5)/158A	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−61.5) pYZ158A</i>	This work
XE032.1/XC(−61.5)/159L	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−61.5) pYZ159L</i>	This work
XE032.1/XC(−61.5)/181V	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−61.5) pPC181V</i>	This work
XE032.1/CX(−72.5)	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−72.5)</i>	This work
XE032.1/CX(−72.5)/158A	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−72.5) pYZ158A</i>	This work
XE032.1/CX(−72.5)/159L	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−72.5) pYZ159L</i>	This work
XE032.1/CX(−72.5)/181V	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−72.5) pPC181V</i>	This work
XE032.1/XC(−72.5)	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−72.5)</i>	This work
XE032.1/XC(−72.5)/158A	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−72.5) pYZ158A</i>	This work
XE032.1/XC(−72.5)/159L	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−72.5) pYZ159L</i>	This work
XE032.1/XC(−72.5)/181V	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−72.5) pPC181V</i>	This work
XE032.1/CX(−41.5)	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−41.5)</i>	This work
XE032.1/CX(−41.5)/158A	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−41.5) pYZ158A</i>	This work
XE032.1/CX(−41.5)/159L	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−41.5) pYZ159L</i>	This work
XE032.1/CX(−41.5)/181V	<i>ΔlacX74 Δgal165 thi crp181V pRW2CX(−41.5) pPC181V</i>	This work
XE032.1/XC(−41.5)	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−41.5)</i>	This work
XE032.1/XC(−41.5)/158A	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−41.5) pYZ158A</i>	This work
XE032.1/XC(−41.5)/159L	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−41.5) pYZ159L</i>	This work
XE032.1/XC(−41.5)/181V	<i>ΔlacX74 Δgal165 thi crp181V pRW2XC(−41.5) pPC181V</i>	This work
X7029/CX(−61.5)	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−61.5)</i>	This work
X7029/CX(−61.5)/158A;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−61.5) pDW158A;181V</i>	This work
X7029/CX(−61.5)/159L;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−61.5) pDW159L;181V</i>	This work
X7029/XC(−61.5)	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−61.5)</i>	This work
X7029/XC(−61.5)/158A;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−61.5) pDW158A;181V</i>	This work
X7029/XC(−61.5)/159L;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−61.5) pDW159L;181V</i>	This work
X7029/CX(−72.5)	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−72.5)</i>	This work
X7029/CX(−72.5)/158A;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−72.5) pDW158A;181V</i>	This work
X7029/CX(−72.5)/159L;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−72.5) pDW159L;181V</i>	This work
X7029/XC(−72.5)	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−72.5)</i>	This work
X7029/XC(−72.5)/158A;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−72.5) pDW158A;181V</i>	This work
X7029/XC(−72.5)/159L;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−72.5) pDW159L;181V</i>	This work
X7029/CX(−41.5)	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−41.5)</i>	This work
X7029/CX(−41.5)/158A;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−41.5) pDW158A;181V</i>	This work
X7029/CX(−41.5)/159L;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2CX(−41.5) pDW159L;181V</i>	This work
X7029/XC(−41.5)	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−41.5)</i>	This work
X7029/XC(−41.5)/158A;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−41.5) pDW158A;181V</i>	This work
X7029/XC(−41.5)/159L;181V	<i>ΔlacX74 Δgal165 thi crp⁺ pRW2XC(−41.5) pDW159L;181V</i>	This work
XE032.1	<i>ΔlacX74 Δgal165 thi crp181V</i>	This work
X7029	<i>ΔlacX74 Δgal165 thi crp⁺</i>	Ebright (1985)

(ii) ‘direct activation’ by a second, as yet uncharacterized, region of CAP.

We conclude that differences in promoter architecture can result in different mechanisms of transcription activation by CAP. We speculate that differences in promoter architecture will also result in different mechanisms of transcription activation by other transcription activator proteins, both prokaryotic and eukaryotic.

Materials and methods

Strains, plasmids, media and microbiological techniques

A list of *E. coli* K-12 strains used in this work is presented in Table IX and a list of plasmids used in this work is presented in Table X. Strain XE032.1 was constructed from strain X7029 by integration and excision of bacteriophage λ Y1079-*crp181V* (the procedure of Ebright *et al.*, 1984). Standard media were prepared and standard genetic manipulations were performed as described by Miller (1972).

CAP derivatives

[Ala158]CAP and [Val181]CAP were purified by cAMP affinity chromatography followed by gel filtration chromatography as described by Zhang *et al.* (1991). [(4-azidophenacyl)-Cys178;Val181]CAP was

prepared by reaction of [Val181]CAP with azidophenacyl bromide (Sigma Chemical Co., St Louis, MO) (the procedure of Pendergrast *et al.*, 1992).

Measurement of transcription activation in vivo

Oriented heterodimer experiments were performed using strains XE032.1/CX(n)/158A, XE032.1/XC(n)/158A, XE032.1/CX(n)/159L, XE032.1/XC(n)/159L, XE032.1/CX(n)/181V and XE032.1/XC(n)/181V (Table IX). ‘Reverse’ oriented heterodimer experiments were performed using strains X7029/CX(n)/158A;181V, X7029/XC(n)/158A;181V, X7029/CX(n)/159L;181V and X7029/XC(n)/159L;181V (Table IX). Differential rates of β -galactosidase synthesis were determined as described by Miller (1972), except that cultures were grown in LB medium containing 20 μ g/ml tetracycline and 100 μ g/ml ampicillin (oriented heterodimer experiments) or LB medium containing 35 μ g/ml tetracycline and 25 μ g/ml kanamycin (‘reverse’ oriented heterodimer experiments). Data reported are means of four independent determinations and have been corrected for CAP-independent β -galactosidase synthesis (0–1 unit).

Measurement of transcription activation in vitro

Subunit exchange reaction mixtures (190 μ l) contained: 1.0 μ M [Ala158]-CAP subunit and 0.1 μ M [Val181]CAP subunit (or 0 μ M [Ala158]CAP subunit and 1.1 μ M [Val181]CAP subunit), 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol and 5% glycerol. Reactions proceeded for 24 h at 37°C.

Table X. Plasmids

Plasmid	Relevant characteristics	Source
pYZCRP	Ap ^R ; ori-pBR322; ori-f1; <i>crp</i> ⁺	Zhou <i>et al.</i> (1993a)
pYZ158A	Ap ^R ; ori-pBR322; ori-f1; <i>crp</i> 158A	Zhou <i>et al.</i> (1993a)
pYZ159L	Ap ^R ; ori-pBR322; ori-f1; <i>crp</i> 159L	Zhou <i>et al.</i> (1993a)
pPC181V	Ap ^R ; ori-pBR322; <i>crp</i> 181V	Ebright <i>et al.</i> (1987)
pDW300	Km ^R ; ori-pSC101; <i>crp</i> ⁺	West <i>et al.</i> (1993)
pDW158A;181V	Km ^R ; ori-pSC101; <i>crp</i> 158A;181V	This work
pDW159L;181V	Km ^R ; ori-pSC101; <i>crp</i> 159L;181V	This work
pDW181V	Km ^R ; ori-pSC101; <i>crp</i> 181V	This work
pRW2CC(-61.5) ^a	Tc ^R ; ori-pRK2; <i>P</i> _{CC(-61.5)} - <i>lacZ</i> YA	Williams <i>et al.</i> (1991)
pRW2CX(-61.5)	Tc ^R ; ori-pRK2; <i>P</i> _{CX(-61.5)} - <i>lacZ</i> YA	This work
pRW2XC(-61.5)	Tc ^R ; ori-pRK2; <i>P</i> _{XC(-61.5)} - <i>lacZ</i> YA	This work
pRW2CC(-72.5) ^a	Tc ^R ; ori-pRK2; <i>P</i> _{CC(-72.5)} - <i>lacZ</i> YA	Zhou <i>et al.</i> (1994)
pRW2CX(-72.5)	Tc ^R ; ori-pRK2; <i>P</i> _{CX(-72.5)} - <i>lacZ</i> YA	This work
pRW2XC(-72.5)	Tc ^R ; ori-pRK2; <i>P</i> _{XC(-72.5)} - <i>lacZ</i> YA	This work
pRW2CC(-41.5) ^a	Tc ^R ; ori-pRK2; <i>P</i> _{CC(-41.5)} - <i>lacZ</i> YA	Bell <i>et al.</i> (1990)
pRW2CX(-41.5)	Tc ^R ; ori-pRK2; <i>P</i> _{CX(-41.5)} - <i>lacZ</i> YA	This work
pRW2XC(-41.5)	Tc ^R ; ori-pRK2; <i>P</i> _{XC(-41.5)} - <i>lacZ</i> YA	This work
pAA121CC(-61.5) ^b	Ap ^R ; ori-pBR322; <i>P</i> _{CC(-61.5)}	Gaston <i>et al.</i> (1990)
pAA121CX(-61.5)	Ap ^R ; ori-pBR322; <i>P</i> _{CX(-61.5)}	This work
pAA121XC(-61.5)	Ap ^R ; ori-pBR322; <i>P</i> _{XC(-61.5)}	This work
pAA121CC(-41.5) ^b	Ap ^R ; ori-pBR322; <i>P</i> _{CC(-41.5)}	Gaston <i>et al.</i> (1990)
pAA121CX(-41.5)	Ap ^R ; ori-pBR322; <i>P</i> _{CX(-41.5)}	This work
pAA121XC(-41.5)	Ap ^R ; ori-pBR322; <i>P</i> _{XC(-41.5)}	This work

^aIn previous work, pRW2CC(-61.5), pRW2CC(-72.5) and pRW2CC(-41.5) were referred to as pRW2CC+20, pRW2CC+31 and pRW2CC, respectively (Bell *et al.*, 1990; Williams *et al.*, 1991; West *et al.*, 1993; Zhou *et al.*, 1994).

^bIn previous work, pAA121CC(-61.5) and pAA121CC(-41.5) were referred to as pAA121CC+20 and pAA121CC, respectively (Gaston *et al.*, 1990).

Abortive initiation *in vitro* transcription experiments (Malan *et al.*, 1984; Zhang *et al.*, 1992; Zhou *et al.*, 1992, 1993a,b) were performed using as templates *Eco*RI–*Hind*III DNA fragments of plasmids pAA121CX(-61.5), pAA121XC(-61.5), pAA121CX(-41.5) and pAA121XC(-41.5). Reaction mixtures (25 µl) contained: 10 nM [Ala158]CAP subunit and 1 nM [Val181]CAP subunit (or 0 nM [Ala158]CAP subunit and 11 nM [Val181]CAP subunit), 0.25 nM DNA fragment, 40 nM *E. coli* RNA polymerase holoenzyme (purified as described by Hager *et al.*, 1990), 0.5 mM ApU (ICN Biomedicals Inc.), 50 nM [α -³²P]UTP (30 Bq/fmol), 40 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol and 0.2 mM cAMP. Reaction components except ApU and [α -³²P]UTP were pre-equilibrated for 10 min at 37°C. Reactions were initiated by addition of ApU and [α -³²P]UTP and were allowed to proceed for 15 min at 37°C. Reactions were terminated by addition of 5 µl 0.5 M EDTA. The reaction product [³²P]ApUpU was resolved by paper chromatography in water: saturated ammonium sulfate:isopropanol (18:80:2 v/v) and was quantified by Cerenkov counting. Data reported are means of four independent determinations and have been corrected for CAP-independent incorporation.

Measurement of DNA binding

Nitrocellulose filter DNA binding experiments were performed as described by Gunasekera *et al.* (1992). Reaction mixtures (500 µl) contained: 2 pM ³²P-labelled DNA fragment (30 Bq/fmol; prepared as described by Ebright *et al.*, 1989), 10–100 000 pM CAP or a CAP derivative, 10 mM MOPS–NaOH (pH 7.3), 200 mM NaCl, 0.1 mM dithiothreitol, 50 µg/ml bovine serum albumin and 0.2 mM cAMP. Reaction mixtures were equilibrated for 60 min at 23°C. Reaction mixtures were then filtered for 10–15 s under suction through 0.22 µm×25 mm nitrocellulose filters (Schleicher & Schuell Inc.) pre-soaked in assay buffer without bovine serum albumin. The filters were dried and radioactivity retained on the filters was determined by Cerenkov counting. All data were corrected for background (i.e. radioactivity retained on the filters in the absence of CAP or CAP derivative).

Values of K_{obs} were extracted by non-linear regression using the program SigmaPlot 4.0 (Jandel Scientific Inc.) and the following equation (Gunasekera *et al.*, 1992):

$$B = \{B_M[\text{CAP}]_F\} / \{(1/K_{obs}) + [\text{CAP}]_F\}$$

where $[\text{CAP}]_F$ denotes the free CAP concentration, B denotes radioactivity retained on the filter and B_M denotes radioactivity retained on

the filter at saturation. $[\text{CAP}]_F$ and B were inputs to the non-linear regression, K_{obs} and B_M were unconstrained outputs.

Protein–DNA photocross-linking

Subunit exchange reaction mixtures (100 µl) contained: 500 nM [Ala158]CAP subunit and 50 nM [(4-azidophenacyl)-Cys178; Val181]CAP subunit, 40 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol and 5% glycerol. Reactions proceeded for 15 h at 37°C.

Protein–DNA photocross-linking was performed essentially as described by Pendergrast *et al.* (1992). Reaction mixtures (100 µl) contained: 500 nM [Ala158]CAP subunit and 50 nM [(4-azidophenacyl)-Cys178; Val181]CAP subunit, 5 nM ³²P-labelled DNA fragment (30 Bq/fmol; prepared as described by Ebright *et al.*, 1989), 0.2 mM cAMP, 10 mM MOPS–NaOH (pH 7.3), 200 mM NaCl and 50 µg/ml bovine serum albumin. Reaction mixtures were incubated 40 min at 22°C in the dark. Reaction mixtures then were UV irradiated for 1 min at 22°C (254 nm, 900 ergs/mm²/s) in polystyrene microcentrifuge tubes in a Rayonet RPR100 photochemical reactor (Southern New England Ultraviolet Inc.). Resulting cross-linked protein–DNA complexes were extracted into 100 µl phenol:chloroform (4:1), washed twice with 100 µl 1 M Tris–HCl (pH 8.0) and 1% SDS and ethanol precipitated.

Nucleotides at which cross-linking occurred were identified by heat/alkali treatment as described by Pendergrast *et al.* (1992). Reaction mixtures (100 µl) contained: ≈5 fmol crosslinked protein–DNA complex, 0.1 N NaOH, 20 mM ammonium acetate, 0.1 mM EDTA and 1% SDS. Reaction mixtures, except NaOH, were pre-incubated for 15 min at 90°C, NaOH was added and reaction mixtures were incubated for 30 min at 90°C. Reactions were quenched by addition of 100 µl 20 mM Tris–HCl (pH 8.0) and 6.5 µl 2 N HCl, and the reaction products were ethanol precipitated and analyzed by 8 M urea–20% polyacrylamide gel electrophoresis.

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