Articles

Trp Repressor Interaction with Bromodeoxyuridine-Substituted Operators Alters UV-Induced Perturbation Pattern in a Sequence-Dependent Manner†

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ABSTRACT: In order to explore DNA sites influenced by the *trp* repressor–operator interaction, bromodeoxyuridine (BrdU) was chemically incorporated into the *TrpEDCBA*, *TrpR*, and *aroH* operators at selected thymidine positions. Different patterns of repressor protection from strand scission in the two halves of complexes with the *TrpEDCBA*, *TrpR*, and *aroH* operators suggest different local environments despite the highly symmetric sequences. Although protection was observed at multiple sites in the operators in the presence of repressor, UV irradiation did not lead to a cross-linked repressor–operator complex. This result indicates the absence of close contacts in the major groove between suitable repressor residues and the 5-methyl of thymidines. Upon *trp* repressor binding and UV irradiation, in addition to protection from strand scission, multiplets were observed at some sites, notably within CTAG sequences in the BrdU-substituted operators. This phenomenon (termed band migration) may result from distortion by the *trp* repressor of the BrdU-substituted operator DNA and consequent exposure of different sites along the backbone to strand scission. Interestingly, UV footprinting of two BrdU-substituted *TrpEDCBA* variant operators showed different patterns when base pair symmetry was matched to each side of the symmetry axis. These observations suggest that alterations in the UV photolysis pattern in response to protein binding result from DNA structural alterations that are sequence dependent.

Specific recognition of DNA sequences by both repressor and activator proteins is required for effective transcriptional regulation in bacteria (Pabo & Sauer, 1984). A number of specific DNA–protein interactions have been characterized in great detail (Steitz, 1990). On the basis of these studies, it has been suggested that both direct and indirect readout mechanisms are important for specific DNA sequence recognition. In direct readout, hydrogen bonds and nonpolar interactions between amino acid residues of proteins and DNA bases in the major groove are critical in the discrimination of DNA sequences. This mechanism has been confirmed by X-ray crystallographic analysis of phage repressors (Jordan & Pabo, 1988; Aggarwal *et al.*, 1988). However, in the indirect readout mechanism, as first proposed for *Escherichia coli trp* repressor (Otwinowski *et al.*, 1988), there are no direct contacts between DNA bases and repressor residues. Both water-mediated contacts between DNA bases and repressor and contacts between the DNA sugar–phosphate backbone and repressor are considered to mediate specific DNA recognition. Questions about the specificity of the crystallized *trp* repressor–operator complex, on which the indirect readout model is based, have been raised (Brennan & Matthews, 1989; Staacke *et al.*, 1990; Carey *et al.*, 1991).

Trp repressor from *E. coli* is a dimeric protein composed of two identical subunits of 107 amino acids (Gunsalus & Yanofsky, 1980; Joachimiak *et al.*, 1983; Arvidson *et al.*, 1986). The function of *trp* repressor is to regulate the expression of several operons, including *TrpR*, *TrpEDCBA*, *aroH* (Yanofsky *et al.*, 1981), *mtr* (Heatwole & Somerville, 1991b), and *aroL* (Heatwole & Somerville, 1992). The regulated genes encode the repressor itself and enzymes responsible for the *de novo* biosynthesis of L-tryptophan and the transport of L-tryptophan into the cell. *Trp* repressor must bind each of these different operators so that expression of each gene is regulated to a different degree (Klig *et al.*, 1988). This regulation may occur in combination with other factors, including binding of tyrosine repressor (Heatwole & Somerville, 1991a,b; 1992) and attenuation (Yanofsky *et al.*, 1981). Structurally, *trp* repressor consists of three functional domains (Zhang *et al.*, 1987; Schevitz *et al.*, 1985). First, the core domain includes the extensively intertwined region that forms the extraordinarily stable dimeric structure (Bae *et al.*, 1988). The L-tryptophan binding pocket of repressor is located at the interface of the two subunits within this core domain (Zhang *et al.*, 1987; Schevitz *et al.*, 1985). The binding of L-tryptophan to the core domain activates the repressor's specific DNA binding activity (Zhang *et al.*, 1987). Second, the NH_2-terminal domain was found to be important for protein–DNA interaction, although direct contacts with operator DNA have not been demonstrated (Carey, 1989). However, the structure for this domain is not well-defined in the crystallographic studies (Otwinowski *et al.*, 1988; Zhang *et al.*, 1987; Schevitz *et al.*, 1985). Third, the functional domain for *trp* repressor's DNA binding activity consists of two helices which form a typical bacterial helix–turn–helix (HTH) DNA binding motif (Otwinowski *et al.*, 1988; Zhang *et al.*, 1987; Schevitz *et al.*, 1985).

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In the analysis of a cocrystal of a *trp* repressor dimer and a symmetric 18 bp operator DNA (Otwinowski *et al.*, 1988), the axis of the recognition helix of *trp* repressor within the DNA binding motif is perpendicular to the *trp* operator DNA helix axis, in an end-on orientation as described by Pabo *et al.* (1990). In contrast, for other bacterial DNA binding proteins, the axis of the recognition helix is parallel to the DNA major groove [e.g., *cro* (Ohlendorf *et al.*, 1982)] or parallel to DNA base pairs [e.g., CAP (Schultz *et al.*, 1991), 434, and λ repressors (Jordan & Pabo, 1988; Aggarwal *et al.*, 1988)]. It appears that *trp* repressor may employ some mechanism for its repressor–operator interaction that differs from other bacterial DNA binding proteins. In contrast to the limited interface shown crystallographically for a 1:1 complex for dimer–operator, both genetic and biochemical studies suggest that there is a very large interface between the repressor and operator DNA (Bass *et al.*, 1987; Kumamoto *et al.*, 1987) and have indicated the importance of the symmetrically disposed CTAG sequences in repressor binding (Bass *et al.*, 1987; Bennett & Yanofsky, 1978). Alternative models for interaction of different numbers of *trp* repressor molecules with different operator sequences are based on this evidence (Kumamoto *et al.*, 1987; Staacke *et al.*, 1990), and recent experiments demonstrate a stoichiometry of two dimers per operator with a number of different sequences (Carey *et al.*, 1991; Haran *et al.*, 1992; Liu & Matthews, 1993).

In the present study, we have examined the interaction of *trp* repressor with chemically synthesized 40 bp *trp* operator DNAs with bromodeoxyuridine (BrdU) substituted for thymidine to increase the sensitivity of the DNA to UV photolysis. This approach has been used successfully in the examination of several DNA binding proteins and not only provides structural information about the interface in the protein–DNA complex, but also has the potential for covalently cross-linking the protein–DNA complex (Lin & Riggs, 1972; Ogata & Gilbert, 1977; Barbier *et al.*, 1984; Wick & Matthews, 1991; Allen *et al.*, 1991; Blatter *et al.*, 1992). UV irradiation of BrdU-containing DNA results in the formation of a radical at the 5-position of BrdU which can subsequently attack susceptible residues (e.g., the sugar–phosphate backbone and amino acid residues) that are in close proximity (Ogata & Gilbert, 1977). By monitoring the perturbation of strand scission upon *trp* repressor binding, information regarding interaction between *trp* repressor and operator DNA can be derived. Our results indicate that BrdU substitution does not alter *trp* repressor–operator interaction in terms of binding affinity or stoichiometry. Although no evidence was found for cross-linking of the repressor–operator complex, UV footprinting patterns of BrdU-substituted *trp* operators reveal different binding environments on either side of the nearly symmetric *trp* operator sequences and demonstrate sequence dependence of the effects of *trp* repressor on reactivity.

MATERIALS AND METHODS

Purification of trp Repressor. Large-scale purification of *trp* repressor was executed as described by Joachimiak *et al.* (1983) and He and Matthews (1990). The bacterial strains, CY15071 containing wild-type repressor (Paluh & Yanofsky, 1986) and superrepressor EK49 (Hurlbert & Yanofsky, 1990), were kindly provided by Dr. Charles Yanofsky, Stanford University, and Dr. B. Hurlbert, University of Arkansas Medical Center. Cells were grown in 14 L of minimal medium in a B. Braun Biostat E fermenter. The expression of *trp* repressor was induced by the addition of IPTG to a final concentration of 1 mM at a cell density of $OD_{550} = 0.2$. After further cultivation at 37 °C overnight, bacterial cells were sedimented by centrifugation at 4000*g* at 4 °C for 30 min and resuspended in a small volume of 0.1 M Tris-HCl, pH 8.0. Bacterial cells were broken by sonication in an ice bath for a total of 6 min (30-s on/off cycle), and cellular debris was removed by centrifugation at 10000*g* at 4 °C for 30 min. The chromosomal DNA was precipitated by the addition of 20% streptomycin to the supernatant to a final concentration of 1%. After incubation at 4 °C for 1 h, the resulting mixture was heated to 62 °C for 5 min, and the denatured proteins and DNA were removed by centrifugation at 10000*g* at 4 °C for 30 min. Repressor protein was precipitated by 45–70% ammonium sulfate. After dialysis against 10 mM potassium phosphate, pH 7.5, 0.1 M KCl, and 0.1 mM EDTA, the protein solution was loaded onto a phosphocellulose column equilibrated with the same buffer. The repressor protein was eluted from the column in 10 mM potassium phosphate, pH 7.5, 0.5 M KCl, and 0.1 mM EDTA. The purity of *trp* repressor was checked by 20% SDS–PAGE and visualized by silver staining; the final purity of *trp* repressor was >95%. The operator binding activity was measured by gel retardation (Carey, 1988). Titration of the operator with repressor under stoichiometric conditions and dual-label experiments as described by Liu (1991) demonstrated that DNA binding activity of the preparations was >90%.

Oligonucleotides. The oligonucleotides were synthesized on a BioSearch 8600 DNA synthesizer. The three 40 bp *E. coli TrpEDCBA, aroH, TrpR* operator DNAs and two *TrpEDCBA* operator variants (*Trp-PR* and *Trp-PL*) used in the present study are shown below with the sites of substitution indicated by "U":

TrpEDCBA	
	TTAAUCAUCGAACUAGUUAACUAGUACGCAAGUTCACGTA^{+11}
	AATTAGUAGCUUGAUCAAUUGAUCAUGCGUUCAAGTGCAT^{+11}
Trp-PR	
	TTAATCATCGUACTAGTTAACTAGTACGCAAGTTCACGTA^{+11}
	AATTAGTAGCATGATCAATTGATCAUGCGTTCAAGTGCAT^{+11}
Trp-PL	
	TTAATCATCGAACTAGTTAACTAGTUCGCAAGTTCACGTA^{+11}
	AATTAGTAGCUTGATCAATTGATCAAGCGTTCAAGTGCAT^{+11}
TrpR	
	GATATGCUAUCGUACUCUUUAGCGAGUACAACCGGGGGAG^{+20}
	CTATACGAUAGCAUGAGAAAUCGCUCAUGUUGGCCCCCTC^{+20}
aroH	
	AGTCGCCGAAUGUACUAGAGAACUAGUGCAUTAGCTTATT^{-18}
	TCAGCGGCTUACAUGAUCUCUUGAUCACGUAATCGAATAA^{-18}
CAP Site	
	CAACGCAAUUAAUGUGAGUUAGCUCACUCAUUAGGCACCC^{-42}
	GTTGCGUUAAUUACACUCAAUCGAGUGAGUAAUCCGUGGG^{-42}

DNAs were synthesized as both BrdU-substituted and nonsubstituted single-stranded 40mer DNAs. For BrdU substitution, 5′-*O*-(4″,4‴-dimethoxytrityl)-2′-deoxy-5-bromouridine 3′-[*O*-(cyanoethyl) *N,N*-diisopropylphosphoramidite] was purchased from ChemGenes Corp., Needham, MA. The same positions were also used for deoxyuridine substitutions for *TrpEDCBA*. These DNAs were purified by 20% gel electrophoresis as described by Wick and Matthews (1991). All BrdU-containing oligonucleotides were protected from exposure to light throughout handling and subsequent experiments. All DNA thus purified was stored in solution form at −70 °C. Concentrations of the purified single-stranded 40mer were determined by absorbance at 260 nm assuming OD_{260} of 1 for 33 μg/mL (Sambrook *et al.*, 1989).

Labeling of DNA. The 5′ end of single-stranded oligonucleotides was labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP as described by Maniatis *et al.* (1982). After phenol and phenol/chloroform extraction, the labeled DNA was separated from the unincorporated [^{32}P]ATP by ethanol precipitation in the presence of 2.5 M ammonium acetate.

Labeled single-stranded DNA was resuspended in 50 μL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.1 M NaCl and an equimolar amount of unlabeled complementary strand. The mixture was heated at 100 °C for 2 min and then slowly cooled to room temperature overnight for the formation of duplex DNA. The 5′-end-labeled BrdU-substituted DNAs were always hybridized with nonsubstituted complementary DNA.

Gel Retardation Experiments. Specific operator binding affinity and nonspecific DNA binding affinity of repressor were measured using a gel retardation assay (also termed mobility shift assay) as originally described by Garner and Revzin (1981) and Fried and Crothers (1981) and modified for *trp* repressor by Carey (1988). The retardation gel consists of 10% acrylamide, 0.27% bis(acrylamide), 10 mM sodium phosphate, pH 6.0, and 0.1 mM L-tryptophan. The running buffer of 10 mM sodium phosphate, pH 6.0, and 0.1 mM L-tryptophan was circulated during electrophoresis to maintain the pH. The repressor protein was diluted in 100 mM NaCl and 20 mM sodium phosphate, pH 6.0, to appropriate concentration on ice. Immediately after each dilution, 2 μL of repressor at each different concentration was mixed with constant operator DNA (<10 pM) under a final binding condition of 20 mM NaCl, 20 mM sodium phosphate, pH 6.0, 5 mM L-tryptophan, 16% glycerol, 0.025% xylene cyanol, and 0.025% bromphenol blue. The repressor–operator binding mixture was loaded onto the well at 300 V. The retardation gel was run at 100 V for 2–3 h at room temperature and dried at 80 °C under vacuum for 1 h on a piece of Whatman filter paper. The dried gel was exposed to Kodak X-Omat AR film at −70 °C with DuPont Lightning Plus intensifying screen. Autoradiograms in the linear range of exposure were further analyzed with a Molecular Dynamics computing densitometer.

UV Footprinting. The UV footprinting experiments were carried out essentially as described by Wick and Matthews (1991). The 5′-end-labeled operator DNAs at 90 nM and 200 nM *trp* repressor in 32 μL were incubated in 20 mM sodium phosphate, pH 6.0, 20 mM NaCl, 0.1 mM DTT, and 0.4 mM L-tryptophan for 30 min at room temperature. The reaction mixtures were transferred individually onto a piece of parafilm and irradiated with UV light (254 nm, 12 800 μW/cm^2) for 90 s in a Rayonet photochemical reactor (Southern New England Ultraviolet Co., Hamden, CT) at room temperature. The irradiated samples were collected and mixed with an equal volume of calf thymus DNA (50 μg/mL) in 5 M ammonium acetate and 2 volumes of 100% ethanol. After incubation at room temperature for 30 min, the DNA was pelleted in a microcentrifuge at 4 °C for 30 min. The DNA pellet was dried in a speed-vacuum device and resuspended in TE. An aliquot of 1 μL from each sample was taken to measure radioactivity in a Beckman liquid scintillation counter (LS1801). Finally, irradiated products were analyzed in a 20% denaturing sequencing gel with the same amount of radioactivity loaded in each well (Wick & Matthews, 1991). The wet gel was exposed to Kodak X-Omat AR film, and autoradiograms were further quantitated with a Molecular Dynamics computing densitometer.

Cross-Linking Experiments. 5′-End-labeled 40 bp operator DNA (94 nM) containing BrdU at specific sites was incubated with *trp* repressor (200 nM) as described above. The binding mixture was UV-irradiated either at 254 nm for 90 s (Wick & Matthews, 1991; Allen *et al.*, 1991) or at 302 nm for 1 h with a Fotodyne illuminator as outlined by Chodosh (1991). After UV irradiation, samples from each reaction mixture were analyzed by Tris–glycine–SDS–20% PAGE, Tris–glycine–20% PAGE (with omission of SDS in both the gel and the running buffer), or 10% PAGE (retardation gel without L-tryptophan) in 10 mM sodium phosphate, pH 6.0. Gels were typically run at 100 V and then subjected to autoradiography. An HPLC system with an anion-exchange column (Allen *et al.*, 1991) was also used in attempts to detect any cross-linked repressor and operator complex.

Table I: Equilibrium Dissociation Constants for 40 bp *trp* Operator DNAs[a]

operator DNA	apparent K_d (M)
TrpEDCBA	$(3.2 \pm 1.1) \times 10^{-10}$
TrpEDCBA/dUper[b]	$(6.0 \pm 0.5) \times 10^{-10}$
TrpEDCBA/BrdUper[c]	$(3.8 \pm 1.7) \times 10^{-10}$
aroH	$(3.4 \pm 1.3) \times 10^{-10}$
aroH/per[c]	$(3.3 \pm 0.9) \times 10^{-10}$
TrpR	$(2.2 \pm 1.6) \times 10^{-9}$
TrpR/per[c]	$(1.9 \pm 0.8) \times 10^{-9}$
Trp-PR[d]	$(3.0 \pm 0.9) \times 10^{-10}$
Trp-PL[d]	$(4.0 \pm 1.2) \times 10^{-10}$

[a] Values were obtained by densitometry of autoradiograms as described in Materials and Methods. The apparent equilibrium dissociation constant was estimated from fitting (Levenberg–Marquardt algorithm) the titration curves of the free DNA bands generated from gel retardation assays according to the following equation $Y = [\text{repressor}]^n/K_d^n + [\text{repressor}]^n$. The values are the average of binding constants from at least three different experiments. [b] Per-substituted dU. See Materials and Methods for sequence and substitutions. [c] Per-substituted BrdU. See Materials and Methods for sequence and substitutions. [d] Both top and bottom strands are singly substituted with BrdU. See Materials and Methods for sequence and substitutions.

RESULTS

Effects of BrdU Substitutions. Using gel retardation, we found that *trp* repressor has binding affinities for both BrdU-substituted and nonsubstituted *trp* 40 bp operators (Table I) similar to those reported for larger operator-containing plasmid DNA fragments (Carey, 1988). For *TrpR* operator sequences, both substituted and unsubstituted, we found that the protein–DNA complex generated was unstable and tended to dissociate during electrophoresis (Liu & Matthews, 1993). Klig *et al.* (1988) observed a higher affinity for a larger DNA fragment containing the *TrpR* operator sequence, suggesting that flanking sequences may contribute to the stability of this complex. The negligible effect of BrdU substitution on *trp* repressor binding affinity suggests that electrostatic interactions do not contribute significantly to the *trp* repressor–operator interaction, since BrdU is presumed to have a larger dipole moment than thymidine (Lin & Riggs, 1972). Similar BrdU-per-substitution in *lac* operator DNA increased *lac* repressor binding affinity up to 5-fold (Wick & Matthews, 1991; Lin & Riggs, 1972). Gel retardation patterns and DNase I footprints (Liu, 1991) for both BrdU-substituted and nonsubstituted operator DNAs are indistinguishable; the DNase I patterns were also similar to those for larger operator-containing plasmid DNAs (Kumamoto *et al.*, 1987). These results indicate that repressor binding to synthetic (both BrdU-substituted and nonsubstituted) 40 bp *TrpEDCBA* and *aroH* DNAs is similar to larger operator-containing plasmid DNA fragments. Furthermore, binding of *trp* repressor to the 40 bp *TrpEDCBA* operator per-substituted with deoxyuridine was only slightly less avid than nonsubstituted 40 bp *TrpEDCBA* DNA (Table I, Figure 1). DNase I footprinting patterns were identical for both dU-substituted and nonsubstituted *TrpEDCBA* 40 bp operator DNAs (Liu, 1991). These results suggest that thymine methyl groups in *trp* operator sequences do not contribute significantly to the binding free energy of the overall holorepressor–operator interaction.

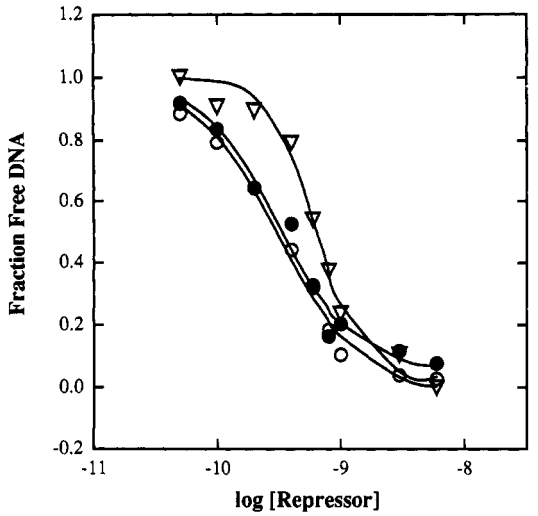

FIGURE 1: Repressor titration of 40 bp nonsubstituted and bromodeoxyuridine- and deoxyuridine-substituted *TrpEDCBA*. The standard retardation gels consist of 10% acrylamide/0.27% bis-(acrylamide) in phosphate buffer at pH 6.0 as described by Carey (1988). The final concentrations in the binding reactions were 5 mM and 5 pM for L-tryptophan and operator DNA, respectively, with varying repressor concentrations from zero to 6 nM. Binding isotherm follows disappearance of free DNA for unsubstituted *TrpEDCBA* (open circle), dU-substituted (triangle), and BrdU-substituted *TrpEDCBA* (solid circle) 40 bp DNA. The values were obtained by densitometric analysis of gel retardation data.

UV Footprinting. The high nonspecific DNA binding activity of *trp* repressor has raised questions regarding the original footprints on *trp* operator (Kumamoto *et al.*, 1987; Fernando & Royer, 1992). For this reason, in our footprinting experiments several controls were employed to avoid potential artifacts that could be ascribed to the high nonspecific DNA binding affinity of *trp* repressor. These included binding reactions with either no repressor or repressor without tryptophan. In addition, 3-indole-4-propionic acid (IPA) at similar concentration was used to replace L-tryptophan in the binding reaction, since the IPA–repressor complex does not bind specifically to operator DNAs (Lawson & Sigler, 1988). Upon UV irradiation of all three controls, a similar pattern of nonuniform sequence-specific DNA cleavages was observed at each of the BrdU incorporation sites (Figures 2, 4, and 5). This nonuniform cleavage is consistent with previous reports for other BrdU-substituted DNAs (Wick & Matthews, 1991; Simpson, 1980). However, in the presence of *both* L-tryptophan and *trp* repressor, suppression of cleavage (protection), abnormal cleavage (including DNA band migration, a term to describe a defined DNA band with different gel mobility in the presence of holorepressor, or double cleavages), and/or enhanced cleavage were observed at different substitution sites in these operator sequences (see Figures 2, 4, and 5). These alterations in DNA strand breakage were dependent on specific repressor binding, while the patterns of cleavage were dependent on operator sequence. Substitution of L-tryptophan with IPA in the binding reaction effectively suppressed any perturbation of the pattern by the protein.

UV footprinting experiments for all DNAs examined were performed under conditions where the protein to DNA ratio ensured the presence of the same electrophoretic species in each case. Furthermore, addition of excess amounts of repressor, up to 100-fold, to the binding reactions did not lead to additional sites of protection, suggesting that the repressor's nonspecific DNA binding activity did not contribute to the observed UV footprinting patterns. In addition, the UV footprinting pattern was independent of the wavelength (254 or 302 nm) employed for the irradiation (data not shown). However, a single base pair change in sequence led to different perturbation patterns under the same conditions (see below). The effects of repressor on the UV footprinting patterns were confirmed by analyses of singly substituted BrdU–*TrpEDCBA* operator DNAs (examples in Figure 3) and selected *TrpR* DNAs. It is noteworthy that alteration in DNA strand scission induced by *trp* holorepressor binding is associated primarily with TpA and ApT dinucleotide steps in all three operator DNA sequences examined.

TrpEDCBA Operator. Protection, band migration, multiplets, and enhanced cleavage were observed upon irradiation of per-substituted *TrpEDCBA* operator only in the presence of both repressor and L-tryptophan (Figure 2). Minor pattern changes sometimes observed with aporepressor alone could be eliminated by the inclusion of IPA in the binding reaction (data not shown). The contact region determined by UV footprinting encompasses about 20 bp and falls within the regions of the operator defined by methylation and DNase I protection reported by Kumamoto *et al.* (1987). The major protection sites observed in the *TrpEDCBA* operator were at B–4 (–4 position in the bottom strand where +1 is the transcription initiation site) and T–5 (–5 position in the top strand) and to a lesser degree at B–10. DNA bands that undergo upward migration with holorepressor binding have been found in both the top and bottom strands of the *TrpEDCBA* operator, specifically at T–8, T–12, T–15, and B–16. Upward migration indicates that a longer DNA fragment was produced upon the holorepressor binding and UV irradiation and suggests that the free radical cleavage is forced 3′ of the original cleavage site. These shifts provide evidence that holorepressor binding may elicit perturbation in DNA structure at these sites. Furthermore, double DNA cleavages (which could also be considered as partial band migration) were found at both B–7 and B–11. Enhanced cleavages were observed at B+2 and T+4. Overall, affected sites occur more frequently on the right-hand half (gene proximal side) of the operator sequence (Figure 2D). To ensure that no abnormal DNA conformation is associated with the BrdU-per-substitution, *TrpEDCBA* 40 bp DNAs that were singly-substituted with BrdU at each site have also been studied in identical UV footprinting experiments. The footprinting data from these singly-substituted *TrpEDCBA* DNAs (examples shown in Figure 3) not only confirmed the protection and DNA band migration pattern obtained with per-substituted DNA, but also clarified some sites where band migration was masked partially in the per-substituted photolysis patterns.

Very similar protection and migration patterns were observed under various binding conditions (e.g., different buffer compositions, varying concentrations of salt or pH of buffers), except for glycerol, whose presence effectively inhibited all DNA cleavage induced by UV irradiation (data not shown; Liu, 1991). A similar protection pattern in the UV footprinting for *TrpEDCBA* per-substituted 40 bp DNA was also observed for different corepressors (such as 5-methyltryptophan, 5-fluorotryptophan, and 6-fluorotryptophan) and for *trp* repressor mutants, such as SC88 (Chou & Matthews, 1989) and superrepressor EK49 (Hurlburt & Yanofsky, 1990) (data not shown; Liu, 1991).

TrpR Operator. Although repressor forms a less stable complex with the *TrpR* 40 bp operator than with the *TrpEDCBA* operator sequence (Table I), *trp* holorepressor still elicited both protection and double cleavage of per-substituted *TrpR* DNA under similar conditions (Figure 4).

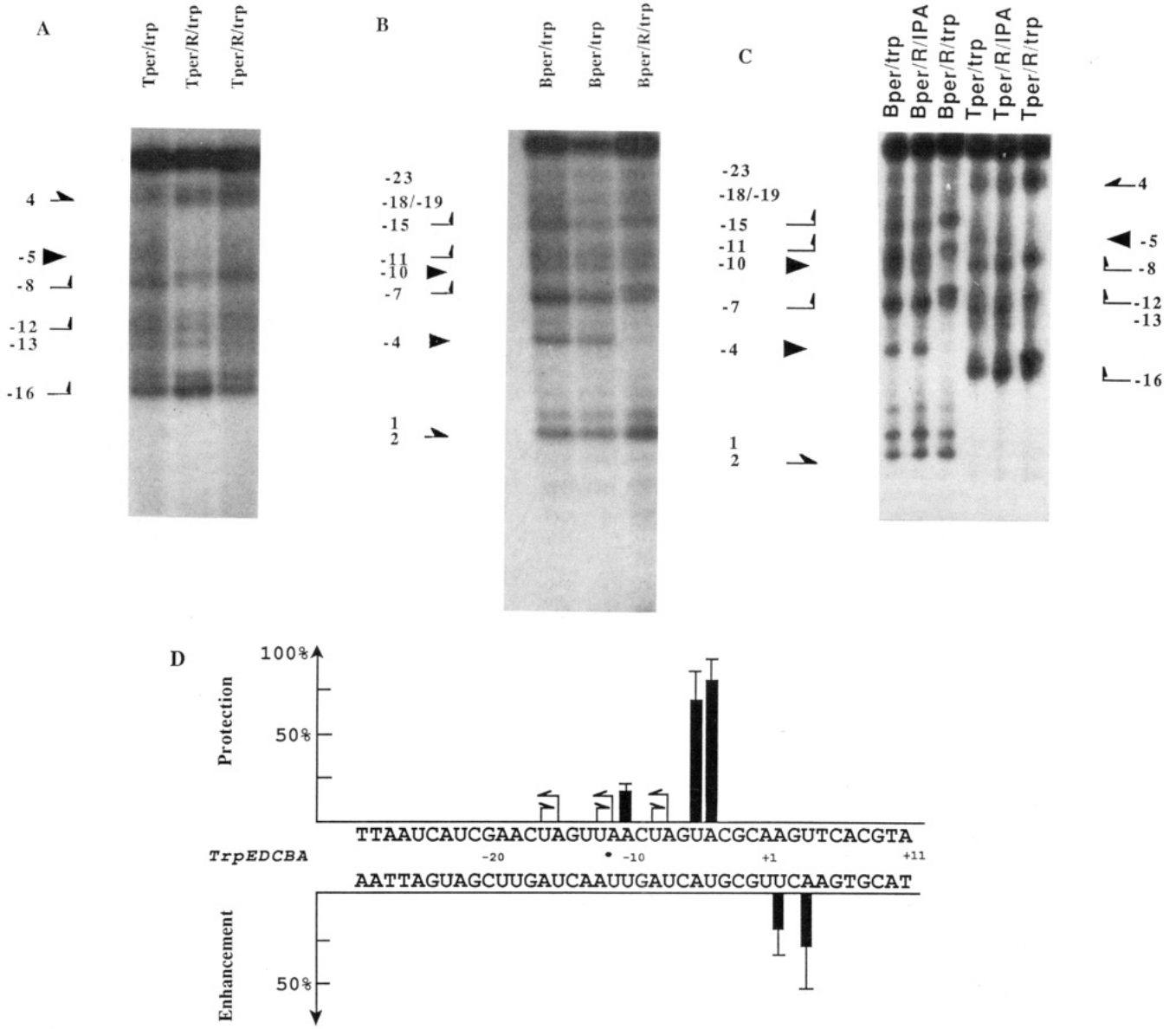

FIGURE 2: UV footprinting profile for *TrpEDCBA* operator DNA containing multiple BrdU substitutions. Autoradiograms for 40 bp $BrdU_{per}$–*TrpEDCBA* operator DNA from different experiments are shown in panels A–C. The final concentrations of repressor and operator DNA were 200 and 94 nM, respectively, in the binding reaction. The UV irradiation was performed and the products analyzed as described under Materials and Methods. The addition of repressor and ligands in binding reactions is indicated at the top of each lane. T_{per} and B_{per} indicate per-substituted top and bottom strands of *TrpEDCBA* DNA, respectively; the addition of *trp* repressor and tryptophan to the binding reactions is indicated as R and trp, respectively. IPA indicates the presence of indolepropionic acid instead of tryptophan as ligand. The arrowheads indicate the protection sites in both top and bottom strands. The straight arrows indicate sites of enhanced cleavage, while bent arrows identify band migration sites, with the arrows pointing in the direction of the migration. Panel D shows a summary of the data derived from densitometric analysis of autoradiograms from three experiments. The BrdU substitution sites are represented by U; the symmetric center is indicated by the solid dot. The protection sites are indicated by bars above the DNA sequence, with the length corresponding to the extent of protection as determined by densitometric analysis. Enhanced cleavage sites are indicated similarly below the DNA sequence. The band migration sites and the orientation of migration are indicated by arrows above the corresponding sites.

The pattern of protection and band migration is simplest for this sequence among the three *trp* operators examined, and the sites of altered photoreactivity are within the binding region determined by methylation and DNase I protection (Kumamoto *et al.*, 1987). The asymmetric protection found for the *TrpEDCBA* operator is also observed for the *TrpR* operator, where the major protection site found at B+8 (+8 position in the *TrpR* bottom strand) is equivalent to the B–4 site in the *TrpEDCBA* operator sequence when their symmetric centers are aligned. In sharp contrast to multiple sites of band migration and double cleavage observed for the *TrpEDCBA* operator sequence, only a limited number of band migration (B+5) and protection sites (B+8, B–7, and B+1) were observed for *TrpR* operator. Results using singly-substituted *TrpR* operators (at B+8 and B+1) confirmed the findings with per-substituted DNA (data not shown). These footprinting data are consistent with the interpretation that *trp* repressor has a simpler interface in its interaction with *TrpR* operator than with *TrpEDCBA* operator, as postulated by Kumamoto *et al.* (1987).

aroH Operator. The UV footprinting pattern observed for *aroH* BrdU-per-substituted 40 bp operator is the most complex of the three operators studied. Protection, double cleavage, DNA band migration (both directions), and enhanced cleavage were observed with this operator (Figure 5). Protection sites (T–27, B–36, and B–44) were observed on both sides of the center of the symmetry. The migrated DNA bands were moved both upward (at B–33, T–34, B–41, B–48, and partially

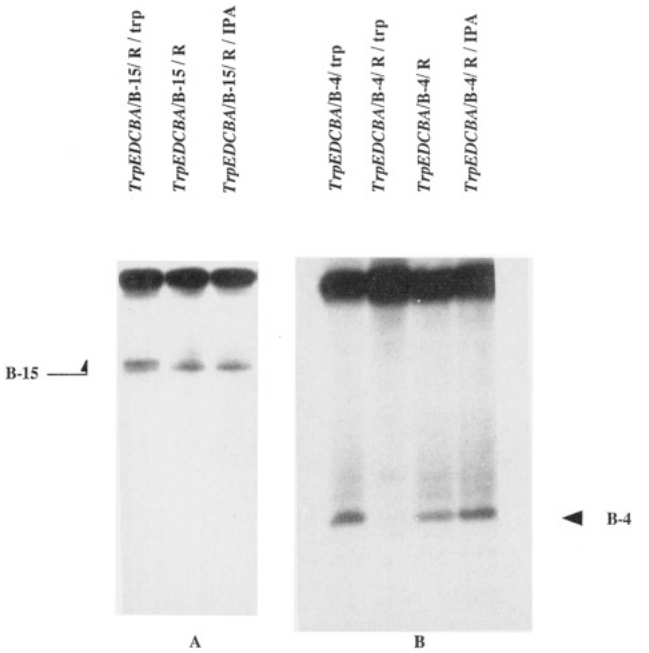

FIGURE 3: UV footprinting of BrdU–*TrpEDCBA*/B-15 (A) and BrdU–*TrpEDCBA*/B-4 (B). DNA sequences singly substituted at –4 or –15 in the bottom strand with bromodeoxyuridine were examined to compare with the UV footprinting pattern observed with the per-substituted DNAs. The final concentrations of repressor and operator DNA were 200 and 94 nM, respectively, in the binding reaction. The addition of repressor and ligands in binding reactions is indicated at the top of each lane. The protection site is indicated by an arrowhead, while the DNA band migration site is indicated by a bent arrow.

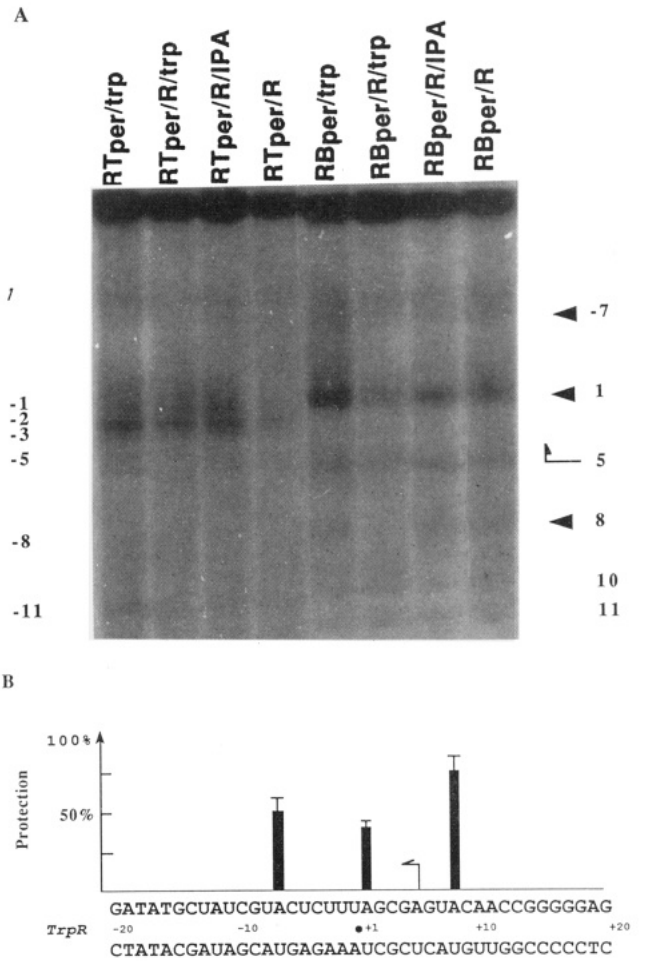

FIGURE 4: UV footprinting of *TrpR* operator. The autoradiogram for UV footprinting of 40 bp BrdUper–*TrpR* operator DNA is shown in panel A. The final concentrations of repressor and operator DNA were 200 and 94 nM, respectively, in the binding reaction. UV footprinting analyses were performed as described in the legend to Figure 2. The addition of repressor and ligands in binding reactions is indicated at the top of each lane. RTper and RBper stand for fully-substituted top strand and fully-substituted bottom strand of *TrpR* operator, respectively; the addition of *trp* repressor and tryptophan to the binding reactions is indicated as R and trp, respectively; IPA indicates indolepropionic acid. The arrowheads indicate the protection sites in the bottom strand, while the bent arrow indicates the DNA band migration site. A schematic summary of the UV footprinting data from three experiments is shown in panel B. The symbols are described in the legend to Figure 2.

at T–42 and B–28) and downward at T–45, instead of only upward as found in the *TrpEDCBA* and *TrpR* operators. Thus, both longer and shorter DNA fragments were produced upon irradiation of *aroH* operator bound to *trp* holorepressor. Furthermore, repressor binding enhanced DNA cleavage at B–39 (Figure 5). Double DNA cleavage was seen at B–28. Compared to the *TrpEDCBA* and *TrpR* operators, the *aroH* operator has a larger region of protection and migration, but this area is still within the segment identified by DNase I footprinting (Kumamoto *et al.*, 1987). An asymmetric photolysis pattern is observed in *aroH* as for the other operators.

Symmetric TrpEDCBA-Based Operators. To further investigate the asymmetry observed in the protection patterns, we changed T to A at B–19 and T to A at B–4 separately in the *TrpEDCBA* operator sequence to make two operators with increased symmetry, *Trp-PR* and *Trp-PL*, respectively (see Materials and Methods for sequences). BrdU was substituted individually at each of the designed symmetric thymidine positions (–4 and –19) in these sequences. These two 40 bp operator variants were found to have similar binding affinity to *trp* holorepressor (Table I) and similar DNase I and methylation protection patterns to the 40 bp *TrpEDCBA* operator (Liu, 1991). However, UV BrdU footprinting demonstrated major differences between the holorepressor–*Trp-PR* and holorepressor–*Trp-PL* operator complexes (Figure 6). Symmetric protection was observed in both *Trp-PR* strands, while symmetric double cleavage was observed in both *Trp-PL* strands. These results suggest a different conformation or protein contact of this AT base pair in the two repressor–operator complexes. These symmetric but distinct patterns are due to specific repressor binding, since the substitution of L-tryptophan with indolepropionic acid in the binding reaction effectively inhibited any protection and band migration (data not shown). These results demonstrate that DNA sequences outside the crucial CTAG motif (Bass *et al.*, 1987; Otwinowski *et al.*, 1988) can influence the *trp* repressor–operator interaction.

Relationship of DNA Structure and Band Migration. A potential source for DNA band migration is a perturbation in the operator DNA structure, such as DNA base pair opening or DNA bending, in response to the *trp* repressor–operator interaction. The *Trp-PR*/*TrpEDCBA* duplex operators provide an opportunity to study the relationship of band migration to base pair opening in the duplex. Specifically, labeled single-stranded *Trp-PR* top strand was hybridized with nonsubstituted *TrpEDCBA* bottom strand to form a duplex operator bearing a 1 base pair mismatch containing BrdU at T–19. This duplex DNA was utilized in UV BrdU footprinting studies. Upon UV irradiation, only a single sharp DNA cleavage was observed at this position in the absence of repressor or in the presence of IPA (Figure 7). Some degree of protection, but no band migration, was observed at T–19 in the *Trp-PR*/*TrpEDCBA* duplex in the presence of holorepressor despite the mismatch at this base pair. Therefore,

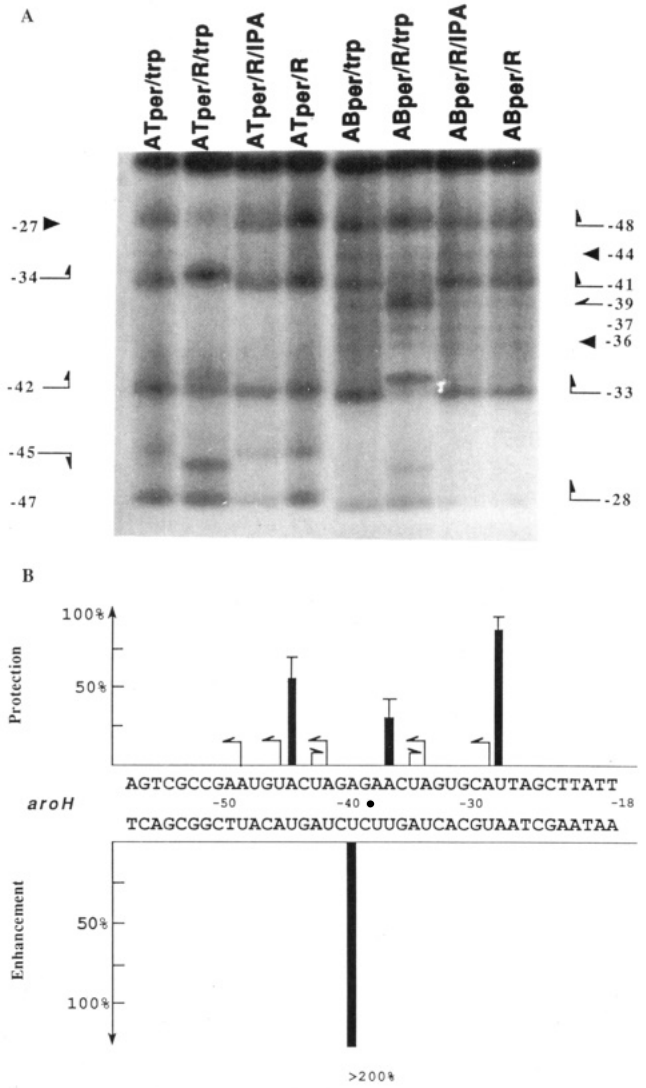

FIGURE 5: UV footprinting profile for *aroH* operator. Autoradiogram for UV footprinting of BrdUper–*aroH* operator 40 bp DNAs is shown in panel A. The final concentrations for repressor and operator DNA were 200 and 94 nM, respectively, in the binding reaction. The addition of repressor and ligands in binding reactions is indicated at the top of each lane. ATper and ABper stand for the fully-substituted top strand and bottom strand of *aroH*, respectively; the addition of *trp* repressor and tryptophan to the binding reactions is indicated as R and trp, respectively. IPA indicates indolepropionic acid. Protection is indicated by arrowheads; DNA band migration is indicated by an upward or downward bent arrow, and enhanced cleavage is indicated by a straight arrow. A schematic summary of UV footprinting data from three experiments is shown in panel B.

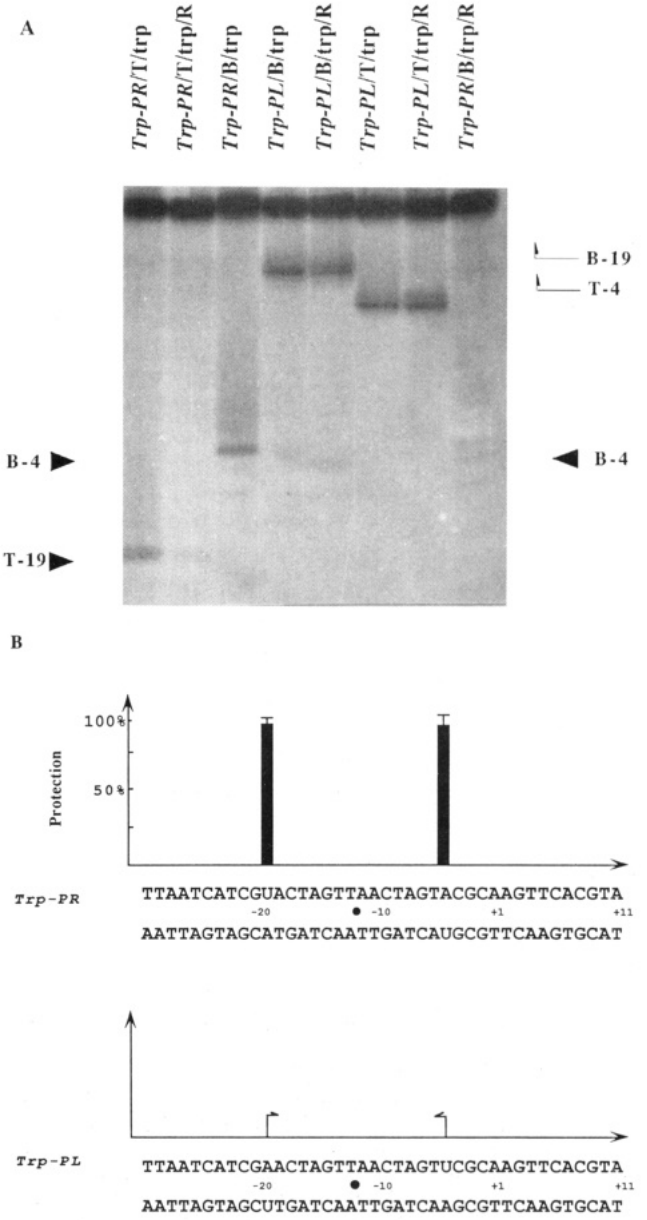

FIGURE 6: UV footprinting profile for two *TrpEDCBA* operator variants. Autoradiogram for UV footprinting of BrdU–40 bp *Trp-PR* and *Trp-PL* operator DNAs is shown in panel A. The final concentrations for repressor and operator DNA were 200 and 94 nM, respectively, in the binding reaction. UV footprinting analyses were performed as described in the legend to Figure 2. The addition of *trp* repressor and tryptophan to the binding reactions is indicated above each lane as R and trp, respectively. The labeled top and bottom strands are indicated by the subdesignation T and B, respectively. The DNA band migration sites are indicated by the bent arrow, while the protection sites are indicated by arrowheads. Symmetric protection is observed for *Trp-PR*, while symmetric band migration is observed for *Trp-PL*. A schematic presentation of the UV footprinting data from three experiments is shown in panel B.

no apparent relationship between base pair opening and DNA band migration or double cleavage was found with specific repressor binding.

CAP Binding Site and DNA Bending. Like *trp* repressor, the catabolite gene activator protein (CAP) can be activated to a species competent for specific DNA binding by a small ligand, cyclic AMP (cAMP) (Simpson, 1980). The binding of the cAMP–CAP complex to its target site causes significant DNA bending (Wu & Crothers, 1984; Schultz *et al.*, 1991). Although previous UV BrdU footprinting studies of CAP and BrdU CAP-binding-site DNA have been presented (Simpson, 1980), the footprinting conditions differed from this study. Therefore, we retested this system to detect any possible DNA band migration; the UV BrdU footprinting experiment was carried out with purified CAP (kindly provided by Dr. M. Fried, University of Texas at San Antonio) and a 40 bp synthetic DNA containing CAP binding site (see Materials and Methods for sequence and BrdU substitution sites). Two major protection sites (T–58 and B–65) reported by Simpson (1980) were also protected under our conditions in a CAP- and cAMP-dependent manner. In addition, some new protection sites were observed (see Figure 8). Although no significant DNA band migration and no doublets were observed in this pattern, the major sites of bending identified in the crystal structure (Schultz *et al.*, 1991) did not undergo significant strand scission even in the absence of protein. Even so, failure to observe band migration in the CAP system indicates that bending likely does not cause this phenomenon in the *trp* system.

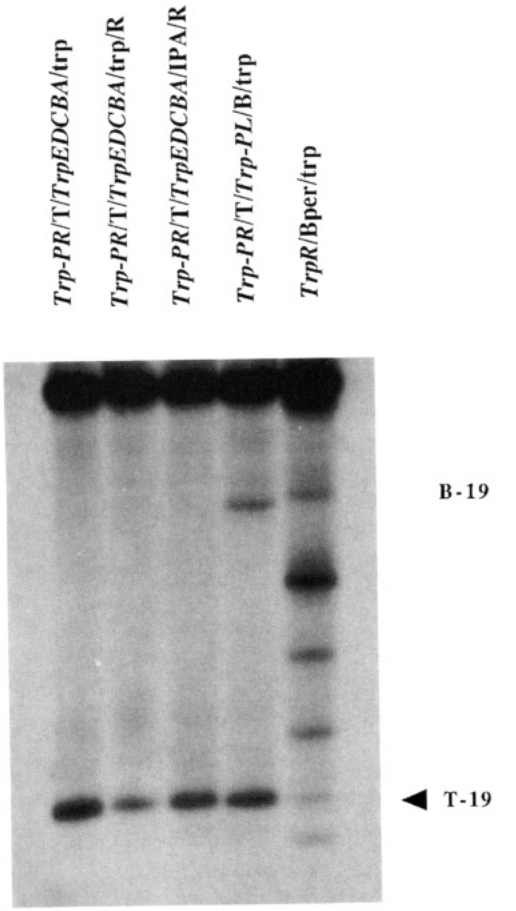

FIGURE 7: Effect of base pair opening on DNA band migration in BrdU UV footprinting. The DNA duplex containing 5′-end-labeled *Trp-PR* top strand and *TrpEDCBA* bottom strand with one mismatch at T–19 was utilized in the same UV footprinting experiments. The final concentrations for repressor and operator DNA were 200 and 94 nM, respectively, in the binding reaction. The addition of repressor and ligands in binding reactions is indicated at the top of each lane. No DNA band migration is observed in this experiment, but protection is noticeable. The DNA duplex containing 5′-end-labeled *Trp-PR* top strand and the 5′-end-labeled *Trp-PL* bottom strand was also used in the same experiment. It has two BrdU substitutions at the mismatched –19 and no substitution at the second mismatched –4 position. Upon UV irradiation, no band migration was observed at position T–19. The *TrpR*/Bper in the last lane produces a band of the same size as the T–19 cleavage product and therefore serves as a marker.

Repressor–Operator Cross-Linking. UV irradiation of BrdU-substituted DNA has been used to reveal close contacts between selected DNA thymidine sites and specific amino acid residues in binding proteins (Barbier *et al.*, 1984; Wick & Matthews, 1991; Allen *et al.*, 1991; Blatter *et al.*, 1992). The success of this method depends on the proximity of appropriate amino acid side chains to the 5-position of thymidine in the protein–DNA complex. Despite use of varied repressor concentrations, alternative binding conditions, and application of multiple detection methods (HPLC, SDS–PAGE, native PAGE, and retardation gel with omission of L-tryptophan), no cross-linked complex has been detected following UV irradiation of *trp* repressor complexed to BrdU-per-substituted *aroH* (Figure 9), *TrpEDCBA*, or *TrpR* operators (not shown). It has been reported that increasing the wavelength of UV irradiation from 254 to 313 nm can increase protein–DNA cross-linking efficiency by several fold (Weintraub, 1973), probably due to different excitation states (Cadet *et al.*, 1986; Saito & Sugiyama, 1990). However, under our conditions increasing the wavelength of UV irradiation from 254 to 302 nm did not produce detectable cross-linked *trp* repressor–operator complex. This result may derive from the nature of the *trp* repressor–operator interaction (e.g., water-mediated interactions) or from proximity of unreactive amino acid residues to the BrdU sites (Wick & Matthews, 1991; Smith, 1969). However, under similar conditions, cross-linked protein and BrdU–DNA complex was observed for *lac* repressor (Wick & Matthews, 1991) and CAP protein (Figure 9).

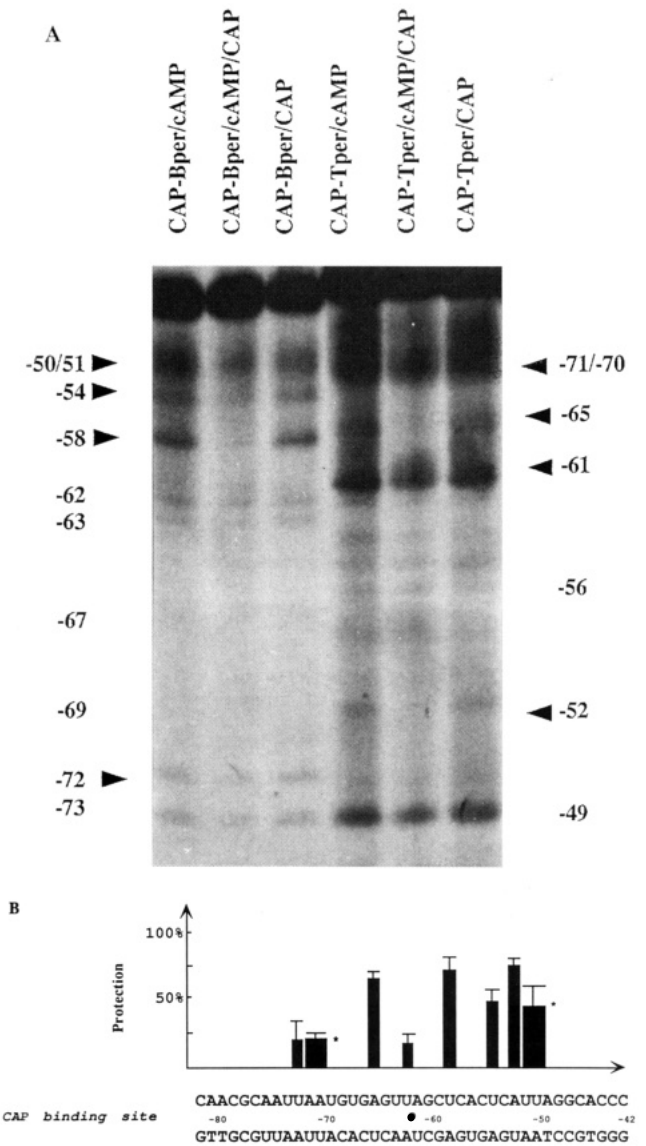

FIGURE 8: UV footprinting profile for CAP binding site DNA. Autoradiogram for UV footprinting of BrdUper–CAP 40 bp binding site is shown in panel A. BrdUper–CAP binding site was synthesized and labeled for UV footprinting as described in Materials and Methods. CAP 40 bp DNA (0.1 μM) was irradiated in the presence or absence of 0.5 μM CAP protein and 20 μM cAMP as described in Materials and Methods. The addition of repressor and ligands in binding reactions is indicated at the top of each lane. Tper and Bper indicate per-substituted top and bottom strands of CAP binding site DNA, respectively. The arrowheads indicate the protection sites in both top and bottom strands. No protection was found when cGMP was used in place of cAMP (data not shown). A schematic presentation of the summary of UV footprinting data from three experiments is shown in panel B. Two protection sites as indicated by an asterisk on the 40 bp CAP binding site DNA are ambiguous in BrdU per-substituted DNA.

DISCUSSION

Using UV footprinting to analyze *trp* repressor effects on operator DNA sequences, several alterations including pro-

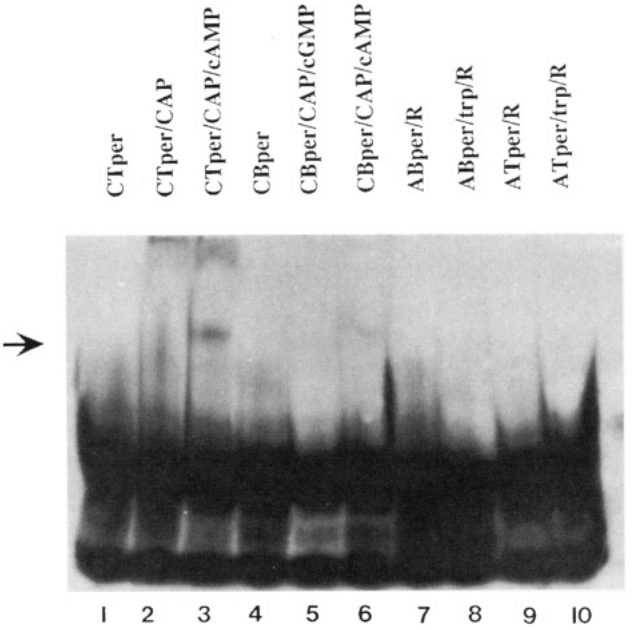

FIGURE 9: Autoradiogram of SDS–PAGE analysis of the cross-linking of *trp* repressor and CAP protein to DNA. The *trp* repressor–BrdU operator and CAP–BrdU CAP binding site 40 bp DNAs were irradiated as described in Materials and Methods. The irradiated products were analyzed by 20% SDS–PAGE. The cross-linked complex (indicated by an arrow) is evident for CAP binding lanes (lanes 3 and 6). However, no cross-linked complex is evident for *trp* repressor binding to BrdUper–*aroH* operator (lanes 8 and 10).

tection, band migration, and double and enhanced cleavages were observed. The *aroH* operator has effects over the longest sequence (~22 bp), while the *TrpEDCBA* operator shows effects over a smaller sequence (~20 bp), and the *TrpR* operator only over 16 bp. All the observed alterations for all three operator DNAs in this study are within the affected regions reported for DNase I and methylation protection experiments (Kumamoto *et al.*, 1987). Our UV footprinting data suggest that the interfaces between the holorepressor–*TrpEDCBA* operator and holorepressor–*aroH* exceed those observed crystallographically (Otwinowski *et al.*, 1988). Under the conditions employed in our studies, *trp* repressor binds to 40 bp *TrpEDCBA* and *aroH* operator DNAs with a stoichiometry of 2 dimers to 1 operator molecule (Liu, 1991; Liu & Matthews, 1983), similar to the results reported recently by Carey *et al.* (1991) and Haran *et al.* (1992). The difference in the size of the interface observed from footprinting (Kumamoto *et al.*, 1987) and crystallographic data (Otwinowski *et al.*, 1988) may, therefore, be due to differences in binding stoichiometry.

Interestingly, the distribution of the protected sites was asymmetric within each of the three operators despite symmetry of sequence. This asymmetric pattern, which is consistent with the lack of symmetry in methylation protection patterns reported by Kumamoto *et al.* (1987), suggests that the protein binding environment is different on the two sides of the nearly symmetrical operator DNA sequence. This result is also consistent with the finding that right-hand half-binding site has a higher binding affinity for *trp* repressor than the other half-binding site (Haran *et al.*, 1992). Typical protection (Ogata & Gilbert, 1977; Wick & Matthews, 1991) of UV-mediated DNA strand scission by binding of *trp* repressor was observed at several positions in all three *trp* operator DNAs. Protection sites were found primarily outside the critical CTAG sequences implicated in operator specificity (Bennett & Yanofsky, 1978; Bass *et al.*, 1987), suggesting that these flanking regions may also participate in the *trp* repressor–operator interaction. *In vivo* studies by Grosfeld *et al.* (1984) showed that a point mutation in the *TrpEDCBA* operator (AT to GC) at –4, a site outside the CTAG motif but protected by holorepressor from strand scission in our studies, resulted in an operator constitutive phenotype. The difference in footprinting observed for *TrpEDCBA*, *TrpR*, and *aroH* operators may further indicate variation in the mode of interaction of repressor with these three operators. The distinct UV footprinting patterns for *Trp-PR* and *Trp-PL*, which differ by only a single base pair from the *TrpEDCBA* operator, indicates that a single base pair change at position –4 influences significantly the repressor–operator interaction. It is interesting that Bass *et al.* (1987) found no effect of mutations either at –4 or at –19 sites in contrast to severe effects that were observed at the critical CTAG sequence. On the other hand, direct comparison of their results to ours is difficult, since Bass *et al.* (1987) started with a symmetrical DNA sequence equivalent to *Trp-PL* and tested only 1 base pair mutation at this site.

An examination of the composite results of BrdU UV footprinting (Figures 2D, 4B, and 5B) indicates that protection and band migration were observed primarily at the dinucleotide TpA and less frequently at ApT. Enhanced cleavage is found only at TpT and TpC sequences in our three operators. Although TpA and ApT dinucleotides are found in both CAP binding site and *lac* operator DNAs, no alteration of UV-induced DNA strand breaks was associated with these dinucleotides in similar experiments (Wick & Matthews, 1991; Simpson, 1980). The only band migration observed at a thymine with no adjacent adenine was at the 5′-CTCG-3′ sequence of the *TrpR* operator, a sequence which occupies a position equivalent to CTAG in the *TrpEDCBA* operator.

From analyses of operator constitutive mutants (Bennett & Yanofsky, 1978) and *in vivo* studies of the DNA specificity determinants of *TrpEDCBA* operator (Bass *et al.*, 1987), the CTAG sequences near the symmetry center are considered to be most important for high-affinity *trp* repressor–operator interaction. In our UV footprinting analyses, these CTAG sequences are consistently associated with DNA band migration, suggesting that the DNA structure in these regions is perturbed in a specific manner by holorepressor binding. The absence of UV protection at the two CTAG motifs in the *TrpEDCBA* operator may suggest the absence of close contact with the thymine methyl group at these positions and is consistent with the recent report by Mazzarelli *et al.* (1992) that the methyl groups of CTAG motifs in the *TrpEDCBA* operator have relatively little effect upon the *trp* repressor binding.

The chemical basis of UV-induced band migration observed with *trp* repressor binding to its operators is unclear at present. One possible explanation for this phenomenon is specific perturbation in the DNA structure elicited by holorepressor binding. It is possible that *trp* holorepressor binding forces the two T·A–A·T base pairs in a TpA step toward each other. Upon UV irradiation, the distorted base pair results in a DNA strand break at the 3′-deoxyribose phosphate linkage, leading to a longer DNA fragment. Otwinowski *et al.* (1988) showed that the 18 bp symmetric *trp* operator DNA bends toward the major groove by 14° at TpA in the CTAG sequence in either half-binding site. The possible relationship of this DNA band migration observed in UV footprinting and the deformation observed in the crystal is unclear at the present, since both *TrpEDCBA* and *aroH* operators have more than two DNA band migration sites in the BrdU UV footprinting and more than one repressor dimer bound to the 40 bp *trp* operator

DNA (Liu & Matthews, 1993). An alternative explanation for specific double cleavages (which could also be considered as partial migration) is that water molecules mediate the interaction between DNA residues and the repressor. Upon UV irradiation, this "fixed" medium molecule in such a special environment could be activated by the free radical, thus generating secondary reaction(s) over a limited distance leading to a mixture of two DNA fragments (double cleavage). Either of these possible mechanisms would result in DNA strand scission without cross-linking repressor to operator. Alternative cleavage by uracilyl-5-yl radical of the duplex DNA phosphate backbone has been reported by Sugiyama *et al.* (1990). Their results also indicate significant effects of DNA sequence and therefore presumably local structure on the products of UV photolysis of BrdU-substituted oligonucleotides.

The UV footprinting patterns and the absence of *trp* repressor cross-linking in this system contrast significantly with other DNA binding proteins (e.g., *lac* repressor and CAP); these distinctions suggest the possibility of differences in the DNA binding mechanism employed by these proteins, which *might* correlate to direct readout *vs* indirect readout mechanisms. The direct hydrogen-bonding and van der Waals interactions between protein residues and DNA bases in the major groove, which are utilized in the specific interactions by both *lac* repressor (de Vlieg *et al.*, 1989) and CAP (Schultz *et al.*, 1991) with their target DNAs, are very important for direct readout. The sequence-dependent ability to adopt a particular, but still ill-defined, structure for specific protein–DNA interaction is very important for the indirect readout mechanism, which has been suggested to be mainly responsible for *trp* repressor–operator interaction by the X-ray crystal structure analysis (Otwinowski *et al.*, 1988). In addition to *trp* repressor, DNA sequence-dependent deformability or bendability has been shown to be important for several other DNA binding proteins (Koudelka *et al.*, 1987, 1988; Gartenberg & Crothers, 1988; Schultz *et al.*, 1991). The DNA band migration observed in our UV footprinting studies provides evidence for the DNA sequence-dependent deformability inherent in this protein–DNA interaction. The possible relationship of the sequence-dependent band migration in UV footprinting reported in this paper and sequence-dependent indirect readout mechanisms warrants further characterization of this phenomenon.

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