DNA Distortion and Base Flipping by the EcoRV DNA Methyltransferase

A STUDY USING INTERFERENCE AT dA AND T BASES AND MODIFIED DEOXYNUCLEOSIDES

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The EcoRV DNA methyltransferase introduces a CH₃ group at the 6-amino position of the first dA in the duplex sequence dGATATC. It has previously been reported that the methylase contacts the four phosphates (pNpNpGpA) at, and preceding, the 5′-end of the recognition sequence as well as the single G in this sequence (Szczelkun, M. D., Jones, H., and Connolly, B. A. (1995) Biochemistry 34, 10734–10743). To study the possible role of the dA and T bases within the ATAT sequence, interference studies have been carried out using diethylpyrocarbonate and osmium tetroxide. The methylase bound very strongly to hemimethylated oligonucleotides modified at the second AT, of the ATAT sequence, bound very strongly to hemimethylated oligonucleotides, that is, the nucleic acid. In contrast, in ternary EcoRV methylase-DNA-sinefungin (an analogue of the natural co-factor, S-adenosyl-l-methionine (AdoMet)) complexes, only small differences in affinity were observed between the normal dA-T base pair and the analogues. These results are almost identical to those seen with DNA dC methylases. In binary EcoRV methylase-DNA complexes, analogues that weakened the base pair caused an increase in affinity between the protein and the nucleic acid. In contrast, in ternary EcoRV methylase-DNA-sinefungin (an analogue of the natural co-factor, S-adenosyl-l-methionine (AdoMet)) complexes, only small differences in affinity were observed between the normal dA-T base pair and the analogues. These results are almost identical to those seen with DNA dC methylases. Dimethylsulfate, which reacts predominantly with the N7 position of dG and has been widely used for footprinting studies is dimethylsulfate, which reacts predominantly with the N7 position of dG and has been widely used for footprinting studies. OsO₄ oxidizes the 5–6 double bond of T resid Manual has not been updated. For further information on footprinting with other reagents, please consult the original literature.

The EcoRV methylase adds CH₃ groups to the first dA in GATATC targets (1) and forms a stable complex with this sequence in the presence of its co-factor, S-adenosyl-l-methionine (AdoMet), or co-factor analogues such as sinefungin (2–5). Tightest binding is observed with hemimethylated GATATC sequences (K_d = 13 nM), and both unmethylated (K_d = 46 nM) and dimethylated (K_d = 143 nM) sequences bind less strongly. Weak binding is observed in the absence of co-factor. DNA footprinting showed that the methylase interacted with four phosphates (NpNpNpGpA), symmetrically disposed on both DNA strands of the duplex, and also with the G in the recognition sequence (3). The protein also caused a 60° bending of DNA in the direction of the major groove (4). These footprinting studies gave no indication of the function that the central ATAT bases played in DNA recognition by the methylase. Experiments using modified bases suggested a critical role for the first AT pair and a much less important function for the second (2, 5). However, it is not known whether these bases contact the protein directly or facilitate the bending of the DNA, particularly at the easily deformed, central, TA step (6–8).

To further define the role of the central ATAT bases, we have used additional interference methods. The best reagent for interference studies is dimethylsulfate, which reacts predominantly with the N7 position of dG and has been widely used (9–11). This reagent also reacts more weakly with the N3 of dA, but in our hands, gave no useful information with the EcoRV methylase (3). Other base-specific chemicals have received much less attention. One potential reagent for dA is diethylpyrocarbonate (DEPC), which causes carboxylation at N7 and N6 of dA and N7 of dG (10, 12–14). However, this reagent shows low reactivity with B-DNA although it is more reactive with the Z-form (15, 16). Binding of small ligands such as echinomycin and quinoxoline antibiotics, which change the conformation of B-DNA, can also cause increased reactivity (17–19). DEPC has also been used as a footprinting agent to study anti-Z-DNA antibodies (20), the tet repressor (21) and HMG box proteins (22). Osmium tetroxide reacts preferentially with T residues although slower reactions with dC and dG have been observed (23, 24). OsO₄ oxidizes the 5–6 double bond of T via the formation of an addition compound and shows a strong preference for single-stranded DNA. Using this reagent, it has been possible to probe DNA structural features such as cruciforms, which show hyper-reactivity (25). In this paper, both DEPC and OsO₄ have been used to study the interaction of EcoRV methylase with the ATAT bases within its recognition sequence.

A key question with EcoRV methylase, and DNA dA methylases generally, is whether they “flip out” the dA base that is the target for methylation. Base flipping was first observed, using x-ray crystallography, with the DNA dC methylase Hhal (26). The target dC is flipped out of the DNA helix by 180° and placed at the catalytic site of the methylase. The structure of a second dC methylase, HaeIII, also indicated base flipping (27). The high sequence homologies seen between dC methylases (28, 29) suggests that base flipping is universal for these enzymes. It has been postulated that base flipping could be a
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common mechanistic feature shared by several enzymes that act on DNA, particularly methylases and DNA repair enzymes (30). However, base flipping can only be unequivocally confirmed by the solution of a structure of an enzyme complexed with double-stranded DNA. Apart from the two dC methylases mentioned above, this has only been done with the repair enzyme endonuclease V (31). Endonuclease V repairs pyrimidine dimers in DNA and flips out one of the dA bases opposite the lesion. Other repair enzymes including uracil-DNA glycosylase (32, 33), the Ado enzyme (34), and Escherichia coli photolyase (35) have had their structures solved, either as apo-enzymes or with short single-stranded oligonucleotides. In these cases, the putative distance between the catalytic apparatus and the inferred position of the target base in double-stranded DNA suggested that base flipping might occur. A similar situation is found with DNA da methylases. Only one structure (Tag I methylase) is available but lacks bound DNA (36). However, the da target (obtained by modelling B-DNA into the structure) is so far from the AdoMet that these authors suggest that base flipping will be necessary for catalysis. Recently, it has been postulated that all da methylases have a common structure, related to dc methylases, and so are likely to share a common mechanism (37).

Although structures of enzyme-DNA complexes provide the ultimate proof for base flipping, other methods have been used. Tighter binding was observed for both HhaI methylase and HpaII methylase using oligonucleotides in which the dG/dC base pair (containing the target dC) was replaced with mismatched bases (38, 39). Furthermore, an inverse correlation was observed between the binding affinity and the strength of interactions between the protein and both the flipped dC and orphan dG. In this publication, the mismatch base approach has been used with the EcoRV methylase to evaluate base flipping with a DNA da methylase.

EXPERIMENTAL PROCEDURES

EcoRV Methyltransferase and Oligonucleotide Preparation—The purification of the EcoRV methyltransferase was as reported (1, 2, 4). Oligonucleotides were prepared using the phosphoramidite method with standard DNA synthesis reagents from Cruachem Ltd. (Glasgow, Scotland). The phosphoramidites of the modified bases were obtained from Cruachem (deoxyinosine), Pharmacia Biotech Inc. (St. Albans, UK) (6-methyloxycyanodinosine), and Cambio Ltd. (Cambridge, UK) (pu- rine-1β-β-2-deoxyriboside, 2-6-diaminopurine-1β-β-2-deoxyriboside, spacer). All oligonucleotides were purified by high pressure liquid chromatography and their concentrations determined by absorbance at 260 nm using the sum of the extinction coefficients of the individual bases (40, 41). Oligonucleotides were 5’-labeled with polynucleotide kinase (Pharmacia) and [γ-32P]ATP (3000 Ci/mmol, Amersham) (42). The non-incorporated radioactivity was removed with two rounds of ethanol precipitation. The pellet was dried in a Savant Speed Vac centrifuge and resuspended in the appropriate volume of 10 mM Tris-HCl, pH 8.5, containing 1 mM EDTA (TE buffer). Duplexes were made by mixing equimolar amounts of the individual strands in TE buffer and heating to 85°C. Annulization was allowed to take place by cooling to room temperature over 6–8 h.

OsO4 Interference—OsO4 (Sigma) was dissolved in distilled water as a 20 mM stock solution (caution OsO4 is very toxic, see Refs. 23 and 24 for safety precautions). Before adding to the DNA, an aliquot was activated by the addition of an equal volume of a 6% (v/v) aqueous solution of pyridine for 5 min. −10 pmol of the duplex oligonucleotide (with the strand under investigation, 5’-labeled with 32P) was incubated in 50 μl of 5 mM Tris-HCl, pH 8.0, and 5 mM of the activated OsO4. After 15 min at 37°C, the reaction was stopped by precipitating the oligonucleotide with ethanol. After two further rounds of ethanol precipitation, the oligonucleotide was suspended in 50 μl of TE buffer. The binding of EcoRV methylase to OsO4-modified DNA was carried out in 20 μl of 50 mM Hepes-KOH, pH 7.5, containing 10 mM EDTA, 100 mM NaCl, 1 μg of acetylated bovine serum albumin, and 1 mM OsO4 (Sigma, Poole, UK). The DNA was titrated with between 50 and 400 μM of the methylase to produce an enzyme concentration that gave approximately 50% bound and 50% free DNA. Samples were incubated at room temperature for 10 min, and the bound and free DNA pools were separated by non-denaturing polyacrylamide gel retardation as described previously (2–4). The two DNA fractions were visualized by autoradiography of the gels, and the appropriate slices were excised. The oligonucleotides were eluted from the gel by shaking overnight in TE buffer at 37°C. After centrifugation, the supernatant was washed three times with n-butanol, and the oligonucleotide was precipitated using ethanol. The pellet was resuspended in 45 μl of water and 5 μl of piperidine and incubated at 90°C for 30 min in order to cleave at the modified positions. The sample was dried, then dissolved in 300 μl of water, and again evaporated to dryness. This step was repeated twice, and the sample was dissolved in 5 μl of loading buffer prior to running on a denaturing DNA sequencing gel (42). Both the free and bound DNA pools, together with a control (consisting of oligonucleotide treated with OsO4 alone) and a Maxam-Gilbert dG chemical sequencing track (43), were run on a 20% denaturing gel and visualized by autoradiography.

Determination of Kd Values for Oligonucleotide Binding to EcoRV Methylase—The protocols used based on band-shift gel retardation analysis have been described in detail (2). Briefly, for the evaluation of Kd values in ternary (enzyme-DNA-sinefungin) complexes, the 5’-la- beled oligonucleotide (0.125 nM) was incubated with EcoRV methylase (0.125 nM) in 20 μl of 50 mM Hepes-KOH, pH 7, 10 mM EDTA, 100 mM NaCl, 20 μg of acetylated bovine serum albumin, and 1.5 mM sinefungin. For binary (enzyme-DNA) complexes, the methylase was pre-incubated with an equimolar amount of GAGCATATGC to remove any traces of tightly bound AdoMet (2). Kd values were then determined as for the ternary complexes with the omission of sinefungin. The methylase concentration in these experiments was varied between 15 and 566 μM, except when the target da was replaced with the spacer; here, 2.1 to 117 μM was used. After incubation at room temperature for 10 min, the free and enzyme-bound DNA fractions were separated by non-denaturing gel electrophoresis (2). The amounts present in each pool were determined using a Fuji BAS-1500 phosphorimager. Kd values were evaluated using “GraFit” (44) to fit the data to binding isotherms. For the evaluation of the Kd values for ternary complexes, only the methylase concentrations above 2.7 μM were used. This ensures that the enzyme concentration is at least 16 times greater than that of the DNA, allowing the approximation [Efree] = [Etotal]. Kd determinations were based on the material present in the free DNA band. This is more accurate than evaluations based on the bound band due to protein-DNA dissociation during electrophoresis (45). Three to five determinations were averaged to give the reported values.

RESULTS

Oligonucleotides Used—The oligonucleotides used in these studies are shown in Fig. 1. All are based on a parent duplex, which contains an EcoRV GATATC site, formed by the hybridization of a 30- and 33-mer (Fig. 1A). All the experiments have been carried out with hemimethylated derivatives of the parent, i.e. containing GATATC on one strand and GMTATC (M = 6-methyldeoxyadenosine) on the other. This is because hemi- methylated oligonucleotides bind most tightly to the methylase (2, 3). For the OsO4 and DEPC interference studies, the two possible hemimethylated oligonucleotides (having M in either the top or bottom strand) have been used (Fig. 1B). To study base flipping, a set of oligonucleotides containing M in the top strand was utilized (Fig. 1C). The target deoxyadenosine base on the bottom strand has been replaced with a variety of
analogues (Fig. 1D), which vary the number of Watson-Crick hydrogen bonds to the partner thymidine. Purine-1-β-D-2'-deoxyribose (P) reduces the number from two to one, whereas 2,6-diaminopurine-1-β-D-2'-deoxyribose (D) increases the value to three. With deoxyinosine (dI) and the spacer (S; a derivative giving an abasic site), no hydrogen bonds can be formed to the thymidine. Thus, these base analogues allow the base pair strength to be correlated with binding affinity, as has been done with HhaI and HpaI (38, 39). In one case, the partner T on the top strand is substituted. For A, B, and C, the dA targets for CH3 group addition (or target dA replacements) are indicated with an asterisk. D, the analogues used.

FIG. 1. The oligonucleotides used in this study. A, parent oligonucleotide that consists of a duplex formed by annealing the top (T) and bottom (B) strands and contains a GATATC (bold) EcoRV site. All oligonucleotides are derived from this parent and have only their EcoRV sites illustrated. B, the hemimethylated oligonucleotides used in interference studies. C, the hemimethylated oligonucleotides used for $K_d$ determinations. In most cases, the target dA on the bottom strand is replaced with a base analogue. In one instance, the partner T on the top strand is substituted. For A, B, and C, the dA targets for CH3 group addition (or target dA replacements) are indicated with an asterisk. D, the analogues used.

Interference Studies—DEPC and OsO$_4$ mainly react with DNA at purines and thymidines, respectively. Nevertheless, their use as DNA footprinting agents has not been extensive, probably due to the low reactivity that B-DNA shows toward the two chemicals. Initially, we attempted protection footprinting by adding the reagent to the pre-formed methylase-DNA complex without success. No reaction was seen between OsO$_4$ and DNA in the buffer used to study the methylase-DNA complex. It is perhaps not surprising that this reagent fails to work under these conditions as the methylase requires double-stranded DNA, which reacts poorly with OsO$_4$ (23, 24). Additionally, OsO$_4$ shows a low reactivity in the presence of NaCl (46), and 100 mM levels of this salt are necessary to keep the methylase active. Addition of DEPC to methylase-DNA complexes caused a rapid, irreversible inactivation of the protein, as assessed by gel shifts (not shown). As DEPC has been used as a protein modification reagent, reacting with lysine and histidine (47), this effect is easily rationalized.

Interference footprinting provided more success. Here, the DNA was modified with the reagent, and the reagent was removed before the addition of the enzyme. This means the reaction conditions can be manipulated to allow, for example, reaction between the DNA and OsO$_4$. The protocols outlined under “Experimental Procedures” gave a reasonable degree of modification of the DNA by both OsO$_4$ and DEPC, as can be seen by examination of the control lanes in Fig. 2. Both reagents also exhibited the expected specificities, i.e. OsO$_4$ reacting most strongly with T and DEPC with dA followed by dG. However, the selectivities are not absolute. In interference footprinting, the reagent used to modify the DNA never comes into contact with the protein. This prevents the irreversible inactivation of the methylase, previously seen using DEPC. The use of DEPC (Fig. 2) produced a clear cut result with the hemimethylated (GATATC/GMTATC) oligonucleotides. Namely the binding of the EcoRV methylase was strongly enhanced by modification to the second dA in the unmethylated strand (GATATC). Better binding occurs for both orientations of the oligonucleotide (i.e. with 6-methyldeoxyadenosine on the top or the bottom strand) and is clearly visible as very pro-
nounced bands at the second dA position for the bound lanes in gels A(I) and B(I) in Fig. 2. No other strong effects involving either reduced or enhanced binding were observed, and in view of the untested nature of DEPC as a footprinting agent, we feel it is unwarranted to draw more conclusions. The results with OsO4 are similar to those observed with DEPC (Fig. 2). The most prominent effect is a very strong enhancement of methylase binding due to modification of the second T in the unmethylated strand (GATATC) of the duplex (Fig. 2, gels A(I) and B(I)). OsO4 is not completely specific and also reacts with dA bases. Reaction of the second dA in the unmethylated strand (GATATC) with this reagent also improves methylase binding, as seen with DEPC. The interference results can be summarized as follows. 1) Modifications to the second AT (GATATC) in the unmethylated strand strongly enhance methylase binding; 2) no strong effects are seen for reaction at the methylated strand (Fig. 2, gels A(II) and B(II)); and 3) in no case does reaction with either OsO4 or DEPC diminish binding.

**Binding of the EcoRV Methylase to Oligonucleotides Having a Modified Target dA Base**—We used the mismatch base approach previously applied to DNA dC methylases (38, 39) with the EcoRV methylase to determine whether or not base flipping takes place. The oligonucleotides shown in Fig. 1, C and D, were used to manipulate the dA/dT base pair by varying the target dA base while keeping the partner T constant. The binding of these oligonucleotides to the methylase, in the absence of sinefungin, i.e., binary complexes, has been investigated by gel retardation analysis as shown in Fig. 3. Quantification of these gel retardation experiments (Fig. 4 and Table I) demonstrated, as expected, rather poor binding of the parent oligonucleotide. The $K_d$ of 181 nM is in good agreement with that of 200 nM found in our earlier study. This is characteristic of the EcoRV methylase, which requires the presence of AdoMet or sinefungin for tight binding to GATATC sequences (2). When the target dA was replaced with 2,6-diaminopurine, the $K_d$ value (177 nM) was unaffected (Fig. 4 and Table I). The presence of the other three analogues (deoxyinosine, purine, and the spacer) increased affinity (Fig. 4 and Table I). With deoxyinosine, the lowering of the $K_d$ was small (about 2.4-fold). However, the oligonucleotides containing purine and the spacer bound particularly tightly with $K_d$ values of 33 and 28 nM, respectively. These values represent about a six-fold improvement in affinity over the wild type.

**When the $K_d$ values for the binding of DNA to the methylase in the presence of sinefungin, i.e., ternary complexes, were evaluated, a different pattern was seen in the gel shifts (not shown). The results are given graphically in Fig. 4 and summarized in Table I. The $K_d$ of 20 nM obtained for the hemimethylated parent is in reasonable agreement with the value of 11 nM reported earlier for the same oligonucleotide (2). Although all the oligonucleotides in which the target dA has been replaced bind to the methylase more tightly than the wild type,
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The results obtained in this paper are consistent with the EcoRV methylase causing distortions to its GATATC cognate sequence. Previously, we showed that the methylase caused a 60° bend toward the major groove on binding to GATATC sequences (4), and this paper extends this observation. The roles of the T and dA bases have been probed by interference studies using OsO4 and DEPC. We found that the reaction of either of the second two AT bases (GATATC) in the non-methylated strand caused a considerable increase in affinity. OsO4 adds across the 5–6 double bond of T (23, 24), and DEPC reacts with the 7-N and 6-NH2 positions of dA (10, 12–14), giving bulky adducts. These modifications would be expected to decrease both base stacking and Watson-Crick hydrogen bonding and so weaken the double helix and facilitate DNA distortion. The increased binding is most likely due to the DNA being able to easily adopt the distorted, bound conformation. The distortion will include DNA bending (4) and base flipping. Earlier studies using OsO4 and DEPC have shown that the presence of base analogues at this position, or an analogue in its position, is indicated by the reaction of purine-1-β-2'-deoxyribose, dI = deoxyinosine, D = 2,6-diaminopurine-1-β-2'-deoxyribose, and S = spacer (see Fig. 1 for the structure of these analogues). The $K_d$ values are the average of three to five determinations and are accurate to at least ±20% (one standard deviation).

**Table I**

| Oligonucleotide                          | $K_d$ (nM) for binary complexes (no sinefungin) | $K_d$ (nM) for ternary complexes (+ sinefungin) |
|-----------------------------------------|------------------------------------------------|-------------------------------------------------|
| --GMTATC--/--CTAT*AG--                 | 181                                            | 20                                              |
| --GMTATC--/--CTAT*1G--                 | 77                                             | 11                                              |
| --GMTATC--/--CTAT*PG--                 | 33                                             | 8                                               |
| --GMTATC--/--CTAT*D6G--                | 177                                            | 12                                              |
| --GMTATC--/--CTAT*SG--                 | 28                                             | 15                                              |
| --GMTATC--/--CTAT*AG--                 | 227                                            | 18                                              |
| --GMTATC--/--CTAT*AG--                 | 153                                            | 14                                              |

where purine replaces dA, binds only 2.5 times more strongly. Given the inherent difficulties in the evaluation of $K_d$ values using gel shift assays (38, 39, 45) and the errors we observe of ±20%, it is unlikely that these small effects are significant.

**DISCUSSION**

The location of the target A base, or an analogue in its position, is indicated by *P = purine-1-β-2'-deoxyribose, dI = deoxyinosine, D = 2,6-diaminopurine-1-β-2'-deoxyribose, S = spacer (see Fig. 1 for the structure of these analogues). The $K_d$ values are the average of three to five determinations and are accurate to at least ±20% (one standard deviation).
The results seen on substituting the target dA and its partner T are quantitatively similar and qualitatively identical to those seen with the DNA dC methylases, *Hha*I and *Hpa*II (38, 39). With the *EcoRV* methylase in enzyme-DNA binary complexes, manipulations of the T/dA base pair that weaken Watson-Crick or stacking interactions result in an increased binding affinity. In the series T/X (replacement of target dA), the *Kd* values followed the order of X = dA ~ D > dI > P > S. The purine and spacer containing oligonucleotides bound about six times more strongly than the control. We did not carry out extensive studies for the X/dA series (replacement of the partner T). However, although the S/dA combination bound more tightly than T/dA, the difference was less pronounced than when S replaced dA. With the dC methylases, a good inverse correlation was observed between the base pair stability and the strength of binding to the enzyme in binary complexes. This trend is also seen with the RV methylase although the correlation is not perfect. Extensive studies on base pair stabilities with modified bases have not been carried out, as has been done with natural base mismatches. Base pair stability depends on several factors including the number of Watson-Crick hydrogen bonds, incorrect alignment of H-bond donor/acceptors, and base stacking. One might expect base pair stability to decline in the following order for the TX series: X = D (three Watson-Crick hydrogen bonds) > dA (two bonds) > P (one bond) > dI (no bonds, misalignment of H-bond donor/acceptors) > S (no bonds, no base stacking). Thus, although the two series representing binding affinities and base pair stabilities do not exactly match, they are a reasonable approximation of each other. It is noteworthy that 2,6-diaminopurine is the only analogue that does not decrease the *Kd* significantly, and this is the only modified base expected to stabilize, rather than weaken, the base pair. All three changes (T/P, T/dI, and T/S) that destabilize the base pair increase binding to the methylase. The change is small for T/dI but a factor of about six for T/P and T/S, similar to the 10-fold increase in binding seen with dC methylases using mismatches (38, 39). With the dC methylases, the results were explained in terms of the base flipping mechanism. Here, as clearly seen by crystallography (26, 27), the target dC is completely extruded from the double helix and placed in the vicinity of the AdoMet co-factor. Base flipping is facilitated by weakening the base pair, and this results in tighter binding. The similarity of the results seen with *EcoRV* methylase, supports the proposition that this dA methylase will also flip its target dA out of the double helix.

Unfortunately, as previously mentioned (38), it is not possible to fully interpret *Kd* changes consequent to base pair alteration as modifications to the target dA and its partner T are likely to have multiple consequences. On the one hand, destabilizing the base pair facilitates base flipping and leads to a lower *Kd*. However, as observed with *Hha*I methylase (26) and *Hae*III methylase (27), both the flipped base and the orphan base, which remains in the helix, make additional stabilizing interactions with the protein. Perturbing these interactions is most likely going to weaken binding and so lead to an increase in *Kd*. The general improvement in binding, seen with *Hha*I
methylase, HpaII methylase, and EcoRV methylase using base mismatches, suggests that the ease of base flipping is the predominant thermodynamic force in binary complexes. However, these complexities are probably responsible for the less than perfect correlation between $K_v$ values and base pair strength, seen with EcoRV methylase, and features such as the T/S base pair binding much more tightly than the S/dA. With EcoRV methylase, the preference for mismatches all but disappears in ternary (enzyme-DNA-sinefungin) complexes, and a similar effect is observed with both HhaI methylase and HpaII methylase (38, 39). In the case of EcoRV methylase, only the T/P base pair binds significantly better than the T/dA, and even here, the effect is not large. The base P may stabilize conformational features other than base flipping, as has previously been seen with the Trp repressor (48) and the EcoRI endonuclease (49).

The increased binding affinity seen with the correct sequence on ternary complex formation occurs because only in the ternary complex are the full complement of energetically favorable interactions to both the flipped and the orphan base made by the protein. This leads to the stabilization of what would normally be a highly unstable and difficult to achieve DNA structure, containing a base fully extruded from the double helix. Further stabilization of mismatches is not possible on ternary complex formation as the bases are either absent (i.e. contain the abasic spacer, S) or of the incorrect structure and so do not interact correctly with the protein.

In conclusion, this paper supports the hypothesis of DNA distortion, including a flipped dA base, on nucleic acid binding to the EcoRV methylase. It has been proposed that methylases may have arisen from base-mismatch binding DNA repair enzymes (30, 38) by the fusion of three protein domains with the ability to recognize a mismatch, recognize a specific sequence, and carry out methylation. In this regard, it is interesting that the RV methylase appears to share some features with the repair enzyme endonuclease V. This enzyme recognizes thymine photodimers and flips out one of the dA bases opposite to the lesion. It also kinks the DNA by 60° (31). Likewise, the EcoRV methylase also flips out a dA base and bends the DNA by 60° (4).

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