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Saturation mutagenesis of the DNA site bound by the small carboxy-terminal domain of γδ resolvase

Vichien Rimphanitchayakit and Nigel D.F.Grindley

Department of Molecular Biophysics and Biochemistry, Yale University, 333 Cedar Street, New Haven, CT 06510, USA

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We have analyzed the sequence requirements for the binding of the carboxy-terminal (DNA binding) domain of γδ resolvase to its recognition site. Using an efficient procedure for saturation mutagenesis we have obtained 31 of the possible 36 base substitutions within the 12 bp minimal binding sequence (using a modified right half of resolvase binding site I as the model sequence). Binding assays *in vitro* with the 43 residue DNA binding domain show that certain substitutions at eight of the 12 positions strongly inhibit complex formation, increasing the dissociation constant by 100-fold or more. The critical positions fall into two groups: the outside 6 bp of the binding sequence (positions *1–6*) and positions *9–10*. These positions correspond to the regions where the DNA binding domain spans the major and minor grooves, respectively, of its binding site. Base substitutions at the intervening positions (*7* and *8*) have more modest (<20-fold) effects on binding while substitutions at the inner two positions (*11* and *12*) are virtually neutral. The hierarchies of base preferences within each critical segment suggest that resolvase makes base-specific contacts in both major and minor grooves.

Key words: γδ resolvase/DNA–protein interaction/site-specific recombination

Introduction

Transposition of γδ, a transposon of the Tn*3* family (class II), occurs by a two step process. First, the transposon-encoded *tnpA* gene product, transposase, catalyzes the joining of two replicons giving rise to a co-integrate with a copy of the γδ transposon at each junction. In the second step, the *tnpR* gene product, resolvase, reduces the co-integrate into two replicons, each with a single copy of γδ. Recombination by resolvase occurs between the two copies of the transposon's *res* site which lies between the divergently transcribed *tnpA* and *tnpR* genes. Resolvase specifically binds to three regions within *res* for its recombination activity. Each binding site consists of a pair of imperfectly conserved 9 bp inverted repeats (consensus 5′-TGTCYR$^{T}_{A}$TA) separated by a variable spacer (for a review, see Hatfull and Grindley, 1988).

The purified 43 amino acid carboxy-terminal domain of resolvase, obtained by mild digestion of intact resolvase with α-chymotrypsin, is able to bind specifically to each half of the resolvase binding sites (Abdel-Meguid *et al.*, 1984). Ethylation interference experiments show that the region defined by the phosphate contacts of the carboxy-terminal domain is slightly smaller than that of intact resolvase and spans an adjacent major and minor groove (Falvey and Grindley, 1987; Rimphanitchayakit *et al.*, 1989). The contact region covers a 12 bp minimal binding segment with the 9 bp inverted repeat (which includes the major groove region) on the outside and an additional 3 bp (and the minor groove region) towards the center of the binding site. This unusually large contact region suggests that there may be specific protein–DNA contacts in the minor groove.

Since the carboxy-terminal DNA binding domain of resolvase possesses the binding specificity of the intact resolvase and binds to half-sites independent of the protein–protein interactions and without the DNA distortions seen with intact resolvase, it is a good subject for the study of the recognition of a binding site by resolvase. In this report, we have used saturation mutagenesis to make base substitutions in the right half of site I and we have tested their effect on the binding of the carboxy-terminal domain.

Results

Mutagenesis of the recognition site of the resolvase carboxy-terminal DNA binding domain

We chose one half of site I for the mutagenesis because site I contains the crossover point and we wanted to retain the option of placing our mutant sites back into site I to test for their effect both on site I binding by intact resolvase and on resolution activity. Site I-R binds the carboxy-terminal domain of resolvase more strongly than I-L; however, the interaction is still a weak one. Since most base substitutions were expected to weaken the interaction, we wanted to start with as strong a binding sequence as possible so as to maximize our ability to distinguish inhibitory effects of different magnitudes. Earlier studies had indicated that substitution of the C·G pair at position 11 of site I with the consensus G·C pair increased binding of the intact resolvase; this can be seen in Figure 4, lanes 1–4 (E.Falvey, V.Rimphanitchayakit and N.Grindley, unpublished data). Since a co-integrate with *res* sites containing this site I substitution is resolved more efficiently than one with wild-type *res* sites (data not shown), and since site I of the Tn*3* *res* site has the consensus G·C pair at this position, it was appropriate for us to use this consensus substitution for our starting material; we call this sequence, site I-R·G*4*C, to designate the change of the fourth position of the binding sequence (reading 5′ → 3′ and from the outside to the inside of a half site) from a G to a C.

Our strategy for mutagenesis and cloning of site I-R·G*4*C was to synthesize chemically a 44 base oligonucleotide containing site I-R·G*4*C (using mutagenic monomer mixtures) and clone it as single-stranded DNA into a duplex vector (M13mp10 replicative form digested with *Sac*I and *Eco*RI) as described by Derbyshire *et al.* (1986). The synthetic oligomer, with ends compatible with the 3′

extension of *Sac*I and the 5′ extension of *Eco*RI, and its relationship to a reconstructed *res* site is shown in Figure 1. The *Eco*RI site between sites I and II (at positions 28−33 of *res*) was derived from the deletion mutant *res*Δ34L (Wells and Grindley, 1984); the sequence changes at this site have no effect on co-integrate resolution (unpublished data). Clones with base substitutions in and around site I-R were identified by directly sequencing single-stranded DNA from random single plaques.

Effect of base substitutions on binding of the resolvase carboxy-terminal domain

All mutants with single base substitutions within or immediately adjacent to the 12 bp minimal binding sequence were tested for their binding affinity for the carboxy-terminal domain (Figure 2 shows representative data). In a few cases, double mutants with one substitution in the region of interest and a second substitution outside this region were included to extend the data base. This was justified because the second mutations occurred in regions which were shown in additional control experiments (see Figure 3) to have no effect on binding.

A 40 bp *Eco*RI−*Sac*I fragment from each mutant was mixed with a 79 bp *Eco*RI−*Hin*dIII fragment containing site I-R·G*4*C as an internal control. Purified resolvase carboxy-terminal domain was added to give a final concentration of ~0.12 μM, at which ~50% of the site I-R·G*4*C internal control fragment was complexed. The protein−DNA complexes were separated from unbound DNA by electrophoresis on 15% non-denaturing polyacrylamide gels and were visualized by autoradiography (Figure 2). A higher concentration of the carboxy-terminal domain (~1.2 μM) and longer exposures of the gel were used for some of the mutants to detect very weak complexes (data not shown). The relative amounts of bound and unbound DNAs were determined by densitometry, and the relative binding affinity of each mutant site (= K_dG*4*C/K_d mutant) was calculated as described in Materials and methods (see Figure 3).

Base substitutions outside the 12 bp minimal binding sequence had essentially no effect on binding of the carboxy-terminal domain. The only significant effects outside this region were seen at the base pair 5′ to the first consensus position (T*1*) and the inhibitory effects here were only ≤2-fold. These results support our previous conclusions regarding the minimal recognition element for the resolvase DNA binding domain (Rimphanitchayakit *et al.*, 1989).

Fig. 1. Top. Sequence of a portion of the γδ *res* site as reconstructed from the cloned oligonucleotide using the *Ssp*I site (within site I) and the *Eco*RI site (between sites I and II) in pVR39. The crossover point is marked ↵ and the 9 bp 'inverted repeats' conserved at all three resolvase binding sites are indicated by horizontal arrows. **Bottom.** Sequence of the synthetic 44 base oligonucleotide with 5′ and 3′ ends complementary, respectively, to *Eco*RI and *Sac*I sticky ends. The diamond indicates the center of the site I sequence. *Italicized* numbers indicate the positions of the bases starting at the outside of the binding sequence. The C at position *4* is the higher affinity G*4*C mutation and replaces the G found at this position in the wild-type site I-R.

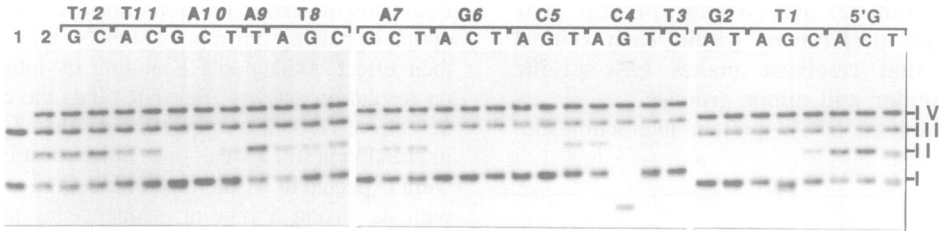

Fig. 2. Assays of complex formation between the resolvase carboxy-terminal domain and mutants of site I-R·G*4*C by polyacrylamide gel electrophoresis. Each non-mutant base and its position is shown above the brackets, the base substitutions are shown below. Bands I and II are free and bound forms of the 40 bp mutant fragments; bands III and IV are free and bound forms of the 79 bp site I-R·G*4*C internal control fragment. **Lanes 1** and **2** are the site I-R·G*4*C DNA fragments with (lane 2) and without (lane 1) resolvase. The free C*4*G 'mutant' fragment migrates faster than the rest because it is derived from wild-type site I-R and is only 31 bp.

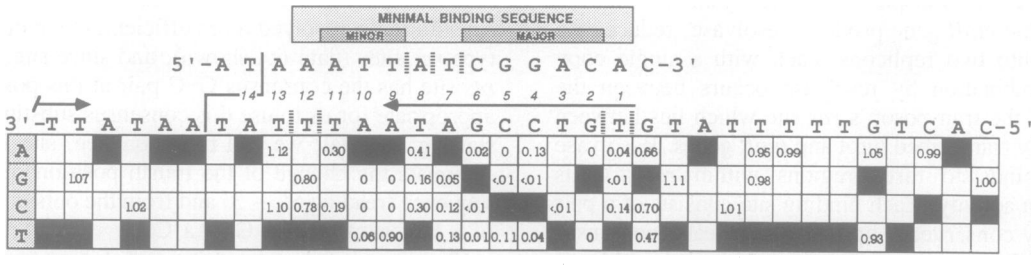

Fig. 3. Binding affinities of site I-R·G*4*C mutants. Relative binding affinities (K_dG*4*C/K_d mutant) were calculated as described in Materials and methods, and are the average of three separate experiments. The '<0.01' indicates that complexes were detected but were present at <1% of the internal control and could not be accurately quantified. Zero indicates that complexes were not detected even upon long exposure of the autoradiograms. The sequence running 3′ → 5′ immediately above the grid is the mutagenized oligonucleotide; substitutions within this sequence are as indicated at the side of the grid. The broken lines between certain bases indicate the positions of phosphate contacts made by the carboxy-terminal DNA binding domain; these are shown on the mutagenized oligonucleotide sequence and its complement (above). Between these phosphates are the major and minor grooves of the DNA helix that lie within the contacted surface of the 12 bp minimal binding sequence (see also Figure 7).

Within the 12 bp minimal recognition sequence, base substitutions that had a strong inhibitory effect (≥100-fold) on binding of the resolvase carboxy-terminal domain (equivalent to a change in the binding free energy of 2.5 kcal/mol or more) fall into two regions. These are from T*1* to C*6* and from A*9* to A*10*, and correspond to the regions where the domain spans the major and minor grooves, respectively, of its DNA binding site. Substitutions at the intervening positions *7* and *8*, the base pairs at which the resolvase domain crosses the sugar–phosphate backbone between the major and minor grooves, had more modest effects (<7-fold at T*8*, 7- to 20-fold at A*7*). At the inner end of the contact region, substitutions at T*11* and T*12* had modest (≤5-fold) or negligible inhibitory effects, respectively. Parenthetically, it can be seen that a G at position C*4* (to give the sequence of the wild-type site I-R) reduced the binding affinity by >100-fold.

Effects of base substitutions on interactions with intact resolvase

Several of the most inhibitory base substitution mutations were used to reconstitute a complete site I within the normal context of the γδ *res* site. In each case the reconstructed site I contains in the right half the high affinity G*4*C substitution (used for the initial mutagenesis) in addition to the substitution under study; the left half of the site is wild-type, as are sites II and III. We refer to these altered sites as site I·G*4*C, *res*·G*4*C and site I (or *res*)·G*4*C·mut (e.g. site I·G*4*C·A*10*C) to indicate the specific mutation. In Figure 4, the binding affinities of intact resolvase for selected site I mutants are compared with its affinity for site I·G*4*C (as an internal control). The results correlate well with the effects on binding of the carboxy-terminal DNA binding domain: the T*1*G mutant shows about the same degree of inhibition relative to site I·G*4*C as the wild-type site I (i.e. with G*4*) (compare lanes 22–24 with lanes 2–4). Greater inhibition is seen with C5A and G2A, while A*10*C shows the strongest inhibition (at least 10^3-fold) and gives a complex with increased electrophoretic mobility. This suggests that site I·G*4*C·A*10*C is less bent than the wild-type (or G*4*C) site I.

We have examined the binding of resolvase to complete *res* sites containing the same four site I mutants (Figure 5). As we have shown previously (Hatfull and Grindley, 1986), at low resolvase concentration an initial complex is formed with wild-type *res* (seen in lane 2 of Figure 5) which contains *res* with a single site occupied by resolvase (about half at site I and half at site II). Increasing amounts of resolvase result in the formation of the fully occupied and condensed complex (the resolvosome) with small amounts of one or two intermediates (with two occupied sites) being detected. A similar pattern is seen with *res*·G*4*C and the four *res*·G*4*C mutants, indicating that in each case an apparently normal

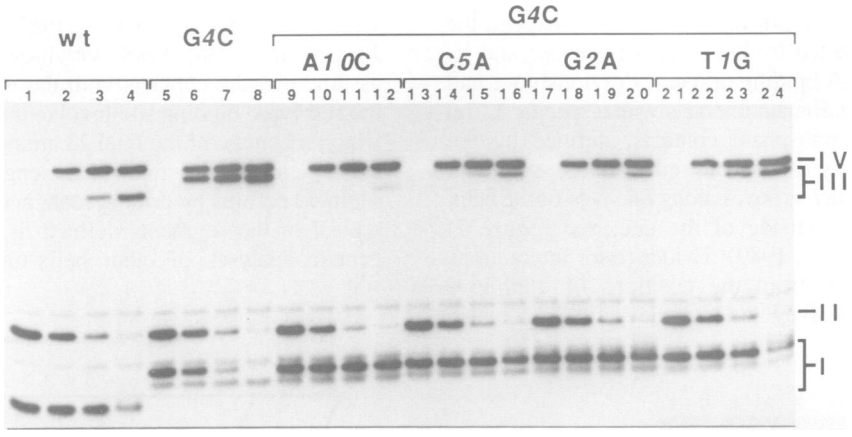

Fig. 4. Binding of intact resolvase to reconstructed site I derivatives assayed by gel electrophoresis. Bands II and IV are free and bound forms of a 80 bp fragment with site I·G*4*C present as an internal control in all reactions. Bands I and III are the free and bound forms of the 50 bp wild-type site I fragment (**lanes 1–4**) or the 63 bp fragments with site I·G*4*C (**lanes 5–8**), and its mutants as indicated (**lanes 9–24**). The concentrations of resolvase used for each set of four lanes were (from left to right) ~0, 3, 30 and 300 nM.

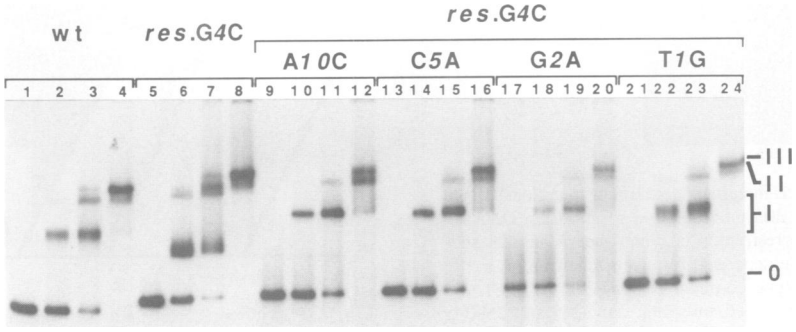

Fig. 5. Gel electrophoresis of complexes between intact resolvase and reconstructed *res* sites. The free DNA is marked O, the first complex formed is marked I and the final complex (the resolvosome) is marked III. For other details see legend to Figure 4.

resolvosome is formed. However, the most severe mutant, A*10*C, results in a reduced yield of the resolvosome and a corresponding increase in accumulation of the intermediate. It is also notable that mobilities of the initial complexes (labeled I in Figure 5) differ—in the case of *res*·G*4*C this complex has a faster mobility, while three of the mutants (A*10*C, C5A and G2A) have a slightly slower mobility than the equivalent complex with wild-type *res*. The faster mobility of the *res*·G*4*C initial complex presumably results from the preference of resolvase for site I·G*4*C over site II while the slower migrating (and sharper) bands seen with A*10*C, C5A and G2A presumably result from the preference for site II. A complex with only one site I occupied migrates faster than the equivalent site II complex because the DNA bend induced by resolvase lies nearer the end of the DNA fragment used in these experiments, and so the fragment is less retarded in the gel.

The A*10*C mutation significantly affects co-integrate resolution. Reactions *in vitro* with co-integrate analogs containing two copies of several mutants of *res*·G*4*C are shown in Figure 6. All gave levels of resolution similar to the *res* and *res*·G*4*C controls with the exception of *res*·G*4*C·A*10*C, which gave a reduced (~5-fold) yield of recombinant products under these standard conditions.

Discussion

The mutational analysis of the resolvase DNA binding site confirms and extends our earlier studies which mapped the region of DNA contacted by both intact resolvase and its carboxy-terminal DNA binding domain. Previously we had shown that the binding domain interacts with a specific 12 bp DNA segment with phosphate contacts, defined by the positions of inhibitory phosphate ethylations, spanning adjacent major and minor grooves along one face of the helix (major groove to the outside of the site; see Figure 7) (Rimphanitchayakit *et al.*, 1989). Evidence for interactions in both grooves came from the positions of inhibitory purine methylations (Falvey and Grindley, 1987) [see also Glasgow *et al.* (1989) for similar studies with the related recombination protein, Hin]. However, because these studies were with intact resolvase, which strongly bends its DNA binding sites (Hatfull *et al.*, 1987; Salvo and Grindley, 1988), it was unclear whether the adenine methylations, in the region spanning the minor groove, inhibited binding either by preventing interactions between resolvase and base pairs, or by inhibiting bending. Here we have probed the base sequence requirements for the resolvase–DNA binding interactions and have bypassed the requirements for major DNA distortions between the contacted regions of each 2-fold symmetric binding site, by studying primarily the carboxy-terminal domain, which interacts with each half-site as a monomer. We have obtained evidence for base-specific interactions in both the major and minor grooves of the contact region.

The most inhibitory base substitutions within the 12 bp binding sequence (those with a loss of 2.5 or more kcal/mol of binding free energy) occur at positions *1–6* and *9–10*. Positions *1–6* correspond to the portion of the binding sequence at which resolvase spans the major groove of the helix (see Figure 7). Resolvase is one of a large number of bacterial DNA binding proteins that contain a conserved helix-turn-helix structural motif (Abdel-Meguid *et al.*, 1984; Pabo and Sauer, 1984; Dodd and Egan, 1987). Studies of a number of protein–DNA co-crystals have shown that the second helix of this motif binds to DNA in the major groove and is responsible for the formation of sequence-specific interactions (Anderson *et al.*, 1987; Aggarwal *et al.*, 1988; Jordan and Pabo, 1988; Otwinowski *et al.*, 1988). We assume that the contacts with the major groove portion of the resolvase binding site involve the same structural motif, which accounts for the final 23 amino acids of the 43 residue carboxy-terminal domain. The length of the major groove segment defined by critical sequence requirements, 6 bp, is typical of the segments defined in similar comprehensive genetic analyses of other helix-turn-helix DNA binding

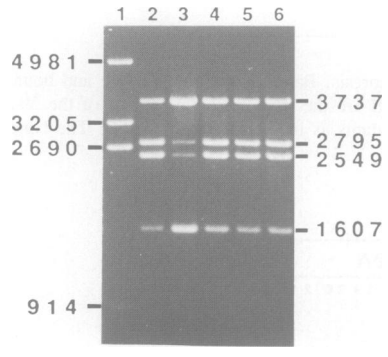

Fig. 6. Resolution *in vitro* of co-integrates containing mutations in site R-I·G*4*C as indicated. After a 30 min resolution reaction the DNAs were digested with appropriate restriction enzymes (see Materials and methods) and the products were separated by electrophoresis on a 1% agarose gel. **Lane 1**: the wild-type co-integrate, pRR51; the 4981 and 914 bp fragments are from unresolved substrate, the 3205 and 2690 bp fragments are from resolved products. **Lanes 2–6**: co-integrates with two copies of *res*·G*4*C (lane 2) and its mutants, A*10*C (lane 3), C5A (lane 4), G2A (lane 5) and T*1*G (lane 6); the 3737 and 1607 bp fragments are from unresolved substrates, the 2795 and 2549 bp fragments are from resolved products.

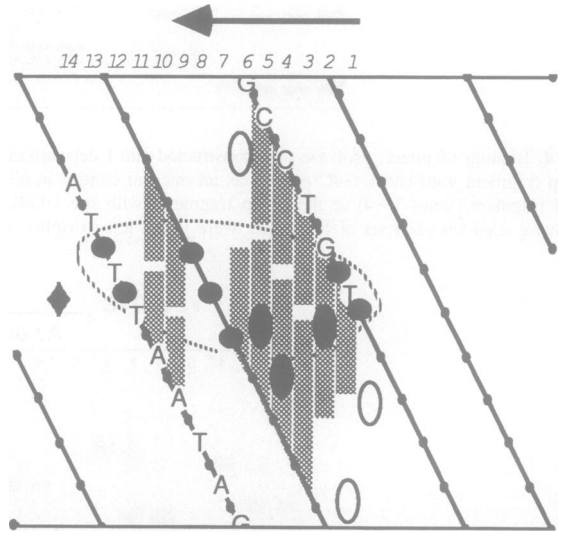

Fig. 7. Planar representation of the binding site for the resolvase carboxy-terminal domain. ●, phosphate contacts (Rimphanitchayakit *et al.*, 1989); ❶, inhibitory guanine methylations; 0, non-inhibitory guanine methylations. Critical base pairs are shown hatched over the region of the groove where resolvase presumably makes its closest approach. The ◆ indicates the position of the dyad axis for site I.

proteins (Bass *et al.*, 1987; Lehming *et al.*, 1987; Benson *et al.*, 1988; Takeda *et al.*, 1989). The complex hierarchies of the base substitutions at positions *1–6* are compatible with major groove interactions and in several cases, following the proposals of Seeman *et al.* (1976), can be used to predict contacts to specific positions of the base pairs, particularly if the inhibitory effects of purine methylations are taken into account (Falvey and Grindley, 1987) (see Figure 7). For example, (i) the hierarchy at position C*4* (C > A > T ⩾ G) and the strong inhibition by methylation of its G complementary base, are compatible with H bonds with the *N*-7, and *O*-6 or *N*-4 positions of the C·G base pair, (ii) the strong preference for a C or T at C*5* accompanied by the inhibitory effect of methylated G is compatible with a contact to the purine *N*-7 position in both C·G and T·A base pairs, (iii) the strong preference for a G at position *6*, together with the failure of *N*-7 methyl G at this position to inhibit binding, suggest contacts to the *O*-6 and/or *N*-4 positions of this G·C pair and rule out an *N*-7 contact. However, the preference for a pyrimidine at position *1* cannot be explained by a hydrogen bond to the purine *N*-7 since methylation of G at the *1′* position of site I-L does not inhibit resolvase binding. Structural studies of the resolvase–DNA complex will be needed to confirm or modify these predictions.

The most unusual feature of the interaction of the resolvase carboxy-terminal domain with DNA is that it extends from the major groove across the sugar phosphate backbone into the adjacent minor groove. This is clear both from our earlier studies of phosphate contacts (Rimphanitchayakit *et al.*, 1989) and from the mutational studies described here. Positions *9–12* of the binding sequence are those at which resolvase spans the minor groove (Figure 7). Base substitutions at *11–12* are essentially neutral, suggesting that there are no specific contacts to these base pairs. Substitutions at *9* and *10*, however, are highly inhibitory to binding. Seeman *et al.* (1976) have proposed that a protein that makes hydrogen bonds to base pairs in the minor groove would be unable to distinguish between an A·T and a T·A base pair since the *N*-3 and *O*-2 hydrogen bond acceptors are symmetrically positioned relative to the helix axis. Substitution with a G·C pair, however, places into the center of the minor groove, a bulky group (the 2-amino group of G), which could interfere with the recognition of an A·T or a T·A pair. This pattern of recognition is exactly that observed at position A*9* where A = T ⩾ G or C. The hierarchy at position A*10* (A > T ⩾ G or C) does not comply with the expectations of Seeman *et al.* (1976), although the A to T transversion is much less inhibitory than either of the other substitutions. A possible explanation for this is that the A*10* to T mutation creates a run of four T residues. Runs of four or more Ts (or As) are implicated in sequence-induced bending of DNA (Koo *et al.*, 1986) and with the progressive narrowing of the minor groove to the 3′ end of the A run (Burkhoff and Tullius, 1987). An introduced bend would alter the relative position of the contacted phosphates 5′ and 3′ to T*12*, while narrowing of the groove would restrict its accessibility to protein.

The effects of base substitutions that we have observed at positions *9* and *10* appear to be significantly different from substitutions at the center of an operator bound by the phage 434 repressor (Koudelka *et al.*, 1987, 1988). These authors found that substitutions of A·T pairs directly across the dyad axis of the operator with G·C or C·G pairs strongly inhibited repressor binding (50-fold for the double substitutions, 7-fold for a single substitution). Biochemical, genetic and structural studies all indicate that these central base pairs are not contacted by the repressor in a sequence-specific manner, but rather that binding of the repressor dimer requires a structural distortion of the DNA (a compression of the minor groove) between the two major groove regions of contact across the operator's dyad axis (Anderson *et al.*, 1987; Koudelka *et al.*, 1987, 1988; Aggarwal *et al.*, 1988). In our case the effects are seen within the binding site of a monomer of the resolvase DNA binding domain, and within the region where the domain clearly spans the minor groove making contacts with phosphates each side of the groove. Although we have not ruled out the possibility that our substitutions at *9* and *10* alter the relative positions of important phosphate contacts across the minor groove or affect bendability (or compressibility) of the minor groove (Satchwell *et al.*, 1986; Koudelka *et al.*, 1987; Gartenberg and Crothers, 1988), we consider that the magnitude of the effects (>100-fold reductions in affinity for a single base substitution) make such an explanation unlikely.

The two sequence blocks with strong base-sequence preferences are separated by base pairs *7* and *8*. The positions of the inhibitory phosphate ethylations in the center of the contact region of the resolvase DNA binding domain clearly show that positions *7* and *8* are those at which resolvase crosses the sugar phosphate backbone between the two DNA grooves (see Figure 7). Base substitutions at these two positions have only a modest effect on binding of the carboxy-terminal domain, suggesting that these bases are not contacted directly by the protein but may affect the geometrical relationship of the two grooves and the phosphate contacts at the borders of the site.

All base substitutions within the 12 bp minimal binding sequence except those at positions *12* and the A to T substitution at position *9* show at least a 2-fold drop in affinity for the resolvase carboxy-terminal domain. Since nearly all possible base substitutions are represented, it appears that we can write the optimal half-site sequence as 5′-TGTCCGAT$^{A}_{T}$ATN. However, we do not know if any elements of the sequence exhibit synergistic effects. Indeed, it seems likely that in a binding sequence as long as 12 bp, several positions will not be directly contacted and that certain combinations of bases at these positions will be inhibitory. In the A·T rich segment *7–12* the bend inducing property of runs of As is also likely to be a factor in producing 'context effects'—three As together occur at three different positions (*7–9*, *9–11* and *11–13*) in the natural sequences of the γδ binding sites, but there are no longer runs overlapping the 12 bp binding sequences.

Comparison of the optimal sequence with the pattern of conservation at the 12 half sites of γδ and Tn*3* (see Grindley *et al.*, 1982) indicates why many substitutions are avoided completely but leaves some puzzles. An A occurs at position *9* at all 12 half sites, yet a T at this position is indistinguishable. Similarly, at position *8*, where none of the substitutions are strongly inhibitory, 10 of 12 sites have T while the other two have A, yet by contrast, at position *6* where all substitutions are highly inhibitory, five of 12 have non-optimal base pairs.

In the context of a reconstructed site I, those mutants tested, with the exception of the A*10*C mutation, generally behave as expected from the binding data with the carboxy-

terminal domain. However, the affinity of intact resolvase for site I·G*4*C·A*10*C is unexpectedly low (~10-fold lower than the G2A and C5A equivalent site I constructs) and the complex exhibits a faster electrophoretic mobility suggesting that it is less bent. A likely explanation for this is that the wild-type A·T pair at position *10* provides a narrow and compressible minor groove which plays an important part in the formation of the resolvase-induced bend at site I; position *10*, at 5 bp from the center of site I is predicted to be on the inside of the site I bend (Hatfull *et al.*, 1987; Salvo and Grindley, 1987). A G·C pair at this position would be expected to widen the minor groove and reduce its compressibility (Satchwell *et al.*, 1986; Gartenberg and Crothers, 1988) causing an additional loss of affinity as well as reduced bending of the complete site. Despite the additional cooperative effects of the accessory sites II and III on the binding of resolvase to *res* (which allow formation of an apparently normal resolvosome), the *res*·A*10*C mutant site is severely inhibited in recombination, possibly as a result of an incorrect structural configuration of site I and the crossover point.

Materials and methods

Materials

Restriction enzymes and DNA ligase were purchased from New England Biolabs. [α-^{32}P]dATP was from Amersham. Deoxynucleoside triphosphates were from P-L Biochemicals. The Klenow fragment of DNA polymerase I was a gift from Cathy Joyce, Yale University. The resolvase carboxy-terminal domain was purified as described previously (Rimphanitchayakit *et al.*, 1989); the concentration in the peak fraction used in our experiment was ~12 μM. Dilutions were made in 20 mM Tris−HCl, pH 7.5, containing 1 mM dithiothreitol, 1 mg/ml bovine serum albumin and 1 M NaCl.

Saturation mutagenesis of site I-R·G4C

The method of Derbyshire *et al.* (1986) was followed. A 44 base oligonucleotide containing site I-R·G*4*C with *Sac*I and *Eco*RI extension at the 3′ and 5′ ends, respectively (see Figure 1) was chemically synthesized using a Biosearch 8600. An equimolar mixture of all four nucleotide monomers was added to each nucleotide reservoir to give ~2.3% of incorrect nucleotides. The mutagenized oligonucleotide mixture was cloned between the *Eco*RI and *Sac*I sites of M13mp10 replicative form (RF) by sequential ligation steps. About 10 pmol of M13mp10 RF was treated with *Eco*RI and alkaline phosphatase. This linear vector was mixed with an ~5000 molar excess of the 44mer in a 10 μl reaction with 1 Weiss unit of ligase at 16°C for 16 h. Ligation was stopped by heating at 75°C for 15 min and the DNA was digested with *Sac*I for 2 h. *Sac*I was heat-killed and the volume of the reaction mixture was adjusted to 0.3 ml with an appropriate amount of ligation buffer and ATP. One Weiss unit of ligase was added and the mixture was incubated at 16°C overnight. The DNA was ethanol precipitated and resuspended in 10 mM Tris−HCl, pH 8, 1 mM EDTA. DNA was transformed into an *Escherichia coli* strain JM101. Random plaques were picked and screened for the presence of base substitutions by standard M13 dideoxy sequencing.

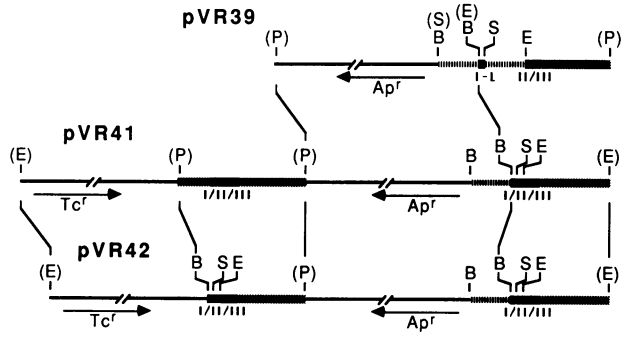

Fig. 8. Linear representation of pVR39, pVR41 and pVR42. Relevant restriction sites are shown for *Eco*RI (E), *Bgl*II (B), *Pvu*II (P) and *Ssp*I (S). Sites in parentheses were modified either by insertion of linkers or by ligation to another blunt ended DNA fragment (Reed, 1981; Wells and Grindley, 1984). ■—*res* segment; ▩—γδ sequences (Reed, 1981); ▥—the *Ssp*I−*Eco*RI fragment of pBR322 (this fragment is duplicated in pVR39 in between site I-L and site II and one copy is replaced by site I-R in pVR40). The rest of each plasmid is essentially pBR322 sequence with modified restriction sites at the ends.

Construction of res and co-integrates containing mutants of site I-R·G4C

A plasmid pRW73, which contains the *res*Δ34L deletion mutant (Wells and Grindley, 1984) and a wild-type *res*, was digested with *Cla*I and the larger fragment religated to give pVR38. pVR39 (see Figure 8) is the plasmid with unique *Ssp*I and *Eco*RI sites used for cloning mutants of site I·G*4*C; it was made from pVR38 by replacing the *Sca*I−*Ssp*I fragment with another that contained the left half of site I and *Bgl*II linkers at the natural *Ssp*I and *Eco*RI sites of pBR322.

The *Ssp*I−*Eco*RI fragment containing site I-R·G*4*C (or its mutants) was prepared as follows. About 0.7 pmol of M13 single-stranded DNA was annealed with excess amounts of the universal sequencing primer. The primer was extended by the action of Klenow in the presence of deoxynucleoside triphosphates and the resulting DNA was digested with *Ssp*I. A 1060 bp *Ssp*I fragment was gel purified and further digested with *Eco*RI. This digest was mixed with *Ssp*I−*Eco*RI-digested pVR39 in a ligation reaction to give a plasmid with a complete reconstituted *res* containing site I·G*4*C (= pVR40).

pVR41, a co-integrate analog with *res*·G*4*C × *res*$^+$ (see Figure 8) was constructed by replacing the *Eco*RI−*Sca*I fragment of pRW73 with that from pVR40. pVR42, a co-integrate analog with two copies of *res*·G*4*C was made by three way ligation of a 710 bp *Bgl*II−*Nde*I fragment from pVR40, a 2940 bp *Nde*I−*Bam*HI fragment from pVR41 and a 1690 bp *Bam*HI−*Bgl*II fragment from pNG105 (Wells and Grindley, 1984). Plasmids similar to pVR41 and pVR42 were constructed with several of the site I-R·G*4*C mutants.

Preparation of labeled DNA fragments

Labeled DNA fragments with site I-R·G*4*C and its mutants were prepared by primer extension from the M13-derived single-stranded templates (~0.1 pmol) in the presence of [α-^{32}P]dATP. For most mutants, the labeled DNA was digested with *Sac*I and *Eco*RI to give a 40 bp fragment. For use as an internal control in the binding reactions, the labeled site I-R·G*4*C DNA was digested with *Eco*RI and *Hin*dIII to give a 79 bp fragment. The wild-type site I-R (with G at *4*) was obtained as a 31 bp *Bst*XI−*Ssp*I fragment from in mGH307, an M13mp11 derivative which contains *res* with a *Bst*XI site between resolvase binding sites I and II. The digested DNAs (~0.002 pmol per reaction) were used directly (without fragment isolation) in the binding assays. Two mutants (A*9*C and A*9*G) had lost their *Eco*RI site (as a result of a second mutation in the first G of the *Eco*RI site) and so we used a 59 bp *Alu*I fragment; these fragments were first purified by polyacrylamide gel electrophoresis.

Labeled DNA fragments containing the reconstructed site I·G*4*C were prepared from pVR42 and its mutant derivatives. The plasmids were digested with *Bgl*II, 3′-end labeled with [α-^{32}P]dATP and finally digested either with *Eco*RI to give a 63 bp fragment with site I, or with *Aha*II to give a 320 bp fragment with *res*. The site I·G*4*C internal control was an 80 bp *Eco*RI−*Sau*96I fragment from pVR42; site I (wild-type) was a 50 bp *Eco*RI−*Bst*XI fragment from pGH309; *res* (wild-type) was a 310 bp *Eco*RI−*Aha*II fragment from pRW20 (a *res*Δ-16L deletion derivative; Wells and Grindley, 1984); all three fragments were 3′-end labeled at their *Eco*RI sites.

Resolvase−DNA binding assays

Appropriate amounts of labeled DNA fragments were mixed with various concentrations of resolvase (see legend to Figure 4) or with the carboxy-terminal domain (~0.12 μM) in 10 μl reactions containing 20 mM Tris−HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg of sonicated calf thymus DNA and 1 μg of bovine serum albumin. Reactions were incubated at 37°C for 10 min (for resolvase) or 25 min (for carboxy-terminal domain), and then equilibrated to room temperature for 5 min. The samples were loaded onto native polyacrylamide gels (5 and 10% for intact resolvase, 15% for the carboxy-terminal domain) in 100 mM Tris−borate, pH 8.3, and 1 mM EDTA (Fried and Crothers, 1981; Hatfull and Grindley, 1986). DNA bands were detected by autoradiography.

Calculation of the relative affinities of the carboxy-terminal domain for site I-R mutants

The autoradiograms were scanned with an LKB Ultroscan XL laser densitometer and the area under each peak was determined. From the appropriate peak areas the ratios of bound to unbound DNA for both the mutant site (B_m/U_m) and the internal control (B_c/U_c) were calculated.

For the reaction:

$$\text{DNA} + \text{Resolvase} \rightleftharpoons \text{DNA}-\text{Resolvase},$$

the dissociation constant is:

$$K_d = \frac{[\text{DNA}][\text{Resolvase}]}{[\text{DNA}-\text{Resolvase}]} = \frac{U[\text{Resolvase}]}{B}$$

Thus,

$$\frac{K_d\,G4C\text{ control}}{K_d\text{ mutant}} = \frac{[\text{Resolvase}]}{[\text{Resolvase}]}\,\frac{U_c/B_c}{U_m/B_m} = \frac{B_m/U_m}{B_c/U_c}$$

(since the resolvase concentration must be identical for a mutant and its *internal* control). This ratio, the relative affinity of each mutant site, is the number given for each mutant in Figure 3. Due to inaccuracies inherent in the densitometric quantitation of autoradiograms there may be systematic errors in the numerical values shown in Figure 3, which increase as the values get smaller. From the data of Swillens *et al.* (1989) and our own data, we estimate that low values in Figure 3 may be underestimated, with a correction faction of between 1.5 (for values ~0.1) and 2–3 (for values ~0.01). Such errors have no effect on the relative order of binding affinities nor do they alter our conclusions.

Resolution reactions

In vitro resolution reactions were performed as described by Reed (1981). After 30 min at 37°C, restriction enzymes were added as required (*Eco*RI for pRR51, the co-integrate with wild-type *res* sites; *Hin*cII for the res·G*4*C·mut co-integrates). Reactions were treated with Proteinase K (20 μg, 5 min) before analysis on 1% agarose gels.

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The present address of V.R. is Department of Biochemistry, Chulalongkorn University, Phythai Road, Bangkok 10500, Thailand.