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Amino acids determining operator binding specificity in the helix-turn-helix motif of Tn*10* Tet repressor

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Each of 22 amino acids in the proposed α-helix-turn-α-helix operator binding motif of the Tn*10* encoded Tet repressor was replaced by alanine and one residue was replaced by valine to determine their role in *tet* operator recognition by a 'loss of contact' analysis with 16 operator variants. One class of amino acids consisting of T27 and R28 in the first α-helix and L41, Y42, W43 and H44 in the recognition α-helix are quantitatively most important for wild-type operator binding. These residues are probably involved in the structural architecture of the motif. A second class of residues is quantitatively less important for binding, but determines specificity by forming base pair specific contacts to three positions in *tet* operator. This property is most clearly demonstrated for Q38 and P39 and to a lesser extent for T40 at the N-terminus of the recognition α-helix. The contacted operator base pairs indicate that the N-terminus of the recognition α-helix is located towards the palindromic center in the repressor-operator complex. Although the orientation of the recognition α-helix in the Tet repressor-*tet* operator complex is inversed as compared with the λ- and 434 repressor-operator complexes, the reduced operator binding of the TA27 mutation in the first α-helix suggests that the hydrogen bonding networks connecting the two α-helices may be similar in these proteins. A novel function is revealed for Q32 in the first α-helix and E37 in the turn preceding the recognition α-helix: repressor mutants with an alanine at either of these positions show a decreased binding of wild-type and increased binding of all mutant operators. Moreover, they are less inducible by tetracycline than wild-type repressor, implying a potential role in the transmission of a conformational change from the inducer binding domain of the protein to the operator binding motif.

Key words: Escherichia coli/mutational analysis/operator-repressor complex/Tet

Introduction

Regulation of gene expression in prokaryotes is often mediated by proteins which bind to specific DNA sequences and thereby either repress or stimulate transcription. It has been shown for a number of proteins that sequence specific protein-DNA recognition is achieved by a secondary structural element termed the 'helix-turn-helix motif' (HTH) (for review and references see Harrison and Aggarwal, 1990; Steitz, 1990). X-ray crystallography of repressor-operator co-crystals including the λcI repressor (Jordan and Pabo, 1988), 434 repressor (Aggarwal *et al.*, 1988), 434 cro (Wolberger *et al.*, 1988) and Trp repressor (Otwinowski *et al.*, 1988) has directly visualized the interaction of this element with the recognized DNA sequence. These studies have established that amino acid side chains protrude from the HTH and interact with the edges of base pairs by hydrogen bonds or van der Waals contacts. The chemical properties of the amino acid side chains therefore play a crucial role in determining specificity for DNA recognition. 'Loss of contact' mutants of some proteins have been used as *in vivo* probes to identify amino acid-base pair contacts (Ebright, 1985, 1986; Hochschild and Ptashne, 1986). This approach relies on the assumption that removal of amino acid side chains participating in sequence specific DNA recognition reduces the ability of the mutant proteins to discriminate between the four possible base pairs at the contacted position in the recognized DNA sequence. The majority of these types of studies has been performed on phage repressor proteins, which are not inducible by effector binding. The only intensively studied inducible system, Lac repressor-*lac* operator recognition, has revealed hints that some features, e.g. the orientation of the recognition α-helix with respect to the center of the operator, are different as compared with the phage repressors (Boelens *et al.*, 1987; Lehming *et al.*, 1990).

In this article we report the analysis of Tet repressor-*tet* operator interactions, another inducible repressor-operator system, by the 'loss of contact' approach. The Tn*10* encoded Tet repressor regulates the tetracycline dependent expression of two divergently oriented genes by binding to two *tet* operators in a central *tet* regulatory region (for review and references see Hillen and Wissmann, 1989). It has been suggested from amino acid homologies and genetic studies with *trans*-dominant mutants that Tet repressor contains a HTH recognizing the *tet* operator (Postle *et al.*, 1984; Isackson and Bertrand, 1985). Additional support has come from the analysis of a mutation in the HTH leading to an altered DNA binding specificity of the repressor (Altschmied *et al.*, 1988).

In the present work we have constructed a set of 23 Tet repressor mutants covering the entire HTH. Twenty-two of these contain a single substitution of a wild-type amino acid by alanine and one a replacement of the wild-type alanine by valine. These mutants were analyzed *in vivo* for binding to a set of 16 *tet* operator variants (Sizemore *et al.*, 1990). Based on this analysis we have defined three classes of Tet repressor mutants. The first class contains repressor mutants with quantitatively severe reductions in operator binding

affinity. A second class defines positions in the HTH involved in base pair specific contacts to the *tet* operator, and a third class identifies residues which enhance discrimination of wild-type *tet* operator from its mutants. Mutations of the latter residues also affect inducibility by tetracycline.

Results

In vivo systems for quantification of Tet repressor–tet operator binding

Twenty-three Tet repressor mutants were tested for their ability to repress the expression of 16 *tetA–lacZ* fusions containing different *tet* operator mutations. The 368 possible combinations yield a wide variety of different binding strengths, which cannot be differentiated in a single *in vivo* system. Therefore, we have used three plasmids which express different amounts of Tet repressor in combination with *tetA–lacZ* fusions on compatible plasmids described earlier (Sizemore *et al.*, 1990) to score *tet* operator binding. pWH520, pWH1411 and pWH510 direct constitutive expression of Tet repressor. A Western blot analysis showed that pWH520 directs at least 5-fold higher level of expression as compared with pWH1411 and at least 20-fold higher level of expression as compared with pWH510. We refer to these expression levels as 'high' (pWH520), 'medium' (pWH1411) and 'low' (pWH510). These properties lead to different efficiencies of repression *in trans* on a *tetA–lacZ* fusion provided on the compatible plasmid pWH1012. With the constitutive β-galactosidase expression set to 100% pWH520 mediates repression to 0.1%, pWH1411 to 0.3% and pWH510 to 48%. The *tetR* mutants TA40 and WA43 gave repressions to 5% and 57% at 'high' and 16% and 81% at 'medium' expression. Thus, the results obtained with these two expression levels are qualitatively similar. The repression exerted by wild-type Tet repressor expressed at high and medium levels for 16 *tet* operator mutants covers the entire activity range and can be used to calibrate any result from one system with the other (see Table I). This was done to produce the data shown in Figure 1. The 'low' expression plasmid served to distinguish the operator affinities of repressor mutants which show near wild-type repression when studied in the high expression system.

Since the *tet* operator sequences overlap with the *tetA* promoter the operator mutations in the pWH1012 derivatives direct the expression of different levels of β-galactosidase in the absence of Tet repressor (Sizemore *et al.*, 1990). To facilitate the comparison of binding efficiencies of Tet

Table I. β-galactosidase expression directed by all possible combinations of 23 Tet repressor variants with 16 *tet* operator variants

TetR variant[a]	*tet* operator variant wt	1G	2A	2C	2T	3C	3G	3T	4A	4C	4G	5C	5G	5T	6A	6C
–b	395[b]	340[b]	652[b]	407[b]	514[b]	324[b]	411[b]	379[b]	585[b]	304[b]	253[b]	379[b]	458[b]	474[b]	194[b]	300[b]
wt	0	19	75	98	22	68	93	73	17	47	86	74	87	9	1	31
wt[c]	0	43	93	109	42	87	100	90	39	72	99	102	97	19	3	60
TA26	0	51	96	104	64	82	102	97	44	77	94	108	103	43	8	64
TA27	45	104	99	95	95	100	100	83	105	106	106	99	94	104	81	104
RA28	99	97	94	99	87	108	102	100	99	100	100	100	101	102	90	104
KA29	0	55	91	101	51	102	99	93	57	86	98	101	99	40	7	77
LA30	1	94	86	102	82	107	102	100	90	96	103	101	101	90	55	89
AV31[c]	8	103	93	103	101	108	105	100	97	100	98	105	102	101	72	78
QA32	0	7	74	92	14	39	81	57	5	23	49	48	83	2	0	12
KA33	0	24	70	95	28	75	91	77	21	43	70	83	92	10	1	30
LA34[c]	1	45	93	108	56	88	99	100	47	74	93	87	96	7	28	67
GA35[c]	1	66	101	100	69	98	102	93	64	87	99	102	96	49	12	75
VA36[c]	8	99	99	111	104	111	102	104	109	104	101	105	100	87	23	100
EA37	0	0	16	92	1	6	45	22	0	1	17	4	42	0	0	5
QA38[c]	21	104	98	105	104	58	15	0	108	103	105	94	107	106	78	105
PA39	0	92	100	104	98	97	105	75	44	1	95	101	98	56	49	98
TA40	5	97	100	102	98	110	97	97	94	102	98	98	95	93	0	72
LA41[c]	94	96	97	103	101	101	93	92	105	87	87	92	93	93	78	101
YA42[c]	105	104	103	105	105	109	102	101	105	97	95	100	98	106	86	96
WA43	57	99	103	98	100	105	96	99	102	95	94	101	100	102	84	94
HA44	91	102	104	110	108	103	101	97	105	95	96	102	104	105	84	97
VA45	0	88	99	96	89	101	98	97	82	99	92	100	99	82	20	85
KA46	0	33	86	97	28	72	92	80	20	47	79	90	90	14	2	47
NA47[c]	0	58	100	99	73	89	98	95	48	65	85	96	93	28	12	47
KA48	4	98	99	100	98	101	98	96	100	97	102	97	95	104	89	99
wt[d]	48	90	91	101	73	108	105	99	89	106	108	104	105	74	58	96
QA32[d]	61	87	nd	nd	82	nd	nd	nd	78	nd	nd	nd	nd	64	62	nd
EA37[d]	66	77	86	99	78	100	100	91	73	82	94	95	95	63	62	89

[a]If not marked otherwise, the results were determined in the 'high' expression system.
[b]The expression levels of operator variants without Tet repressor are given in Units of specific β-galactosidase activities as described by Miller (1972). The expression levels for the TetR variants in each vertical row are given in % of the respective β-galactosidase expression in the absence of repressor.
[c]Determined in the 'medium' expression system.
[d]Determined in the 'low' expression system.
wt: wild-type.
nd: not determined.

repressor to the set of operator mutants the β-galactosidase activity of each pWH1012 derivative determined in the absence of Tet repressor (given in Table I) was set to 100% and β-galactosidase activities obtained in the presence of Tet repressor variants were then related to this value. All results were reproducible with an accuracy of greater than ±5% of the respective activity.

Binding of Tet repressor mutants to wild-type tet operator

The primary structure of the proposed HTH of Tet repressor is depicted in Figure 1. To study the effects of exchanging the wild-type amino acids to alanine on binding to wild-type *tet* operator, 22 single alanine mutants of Tet repressor were constructed. These mutants cover the entire HTH except for position 31 which contains an alanine in the wild-type. This particular residue was mutated to valine. The mutations do not affect protein stability *in vivo* as determined by a Western blot analysis (data not shown). These Tet repressor mutants were assayed *in vivo* for recognition of wild-type *tet* operator. The results are displayed in Figure 1. The mutants TA27 and RA28 (located in the first α-helix), VA36 (located in the turn) and QA38, TA40, LA41, YA42, WA43, HA44 (located in the recognition α-helix) and KA48 (proposed to be located C-terminal of the recognition α-helix) show decreased affinities for *tet* operator. The most pronounced effects are observed for TA27 and RA28 in the first LA41, YA42, WA43 and HA44 in the recognition α-helix. The 22 mutants were also tested for tetracycline induction and all but two were fully inducible. QA32 with 79 ± 0.3% and EA37 with 6 ± 0.1% showed reduced inducibility compared with wild-type Tet repressor with 87 ± 1.0% of the maximal β-Gal expression.

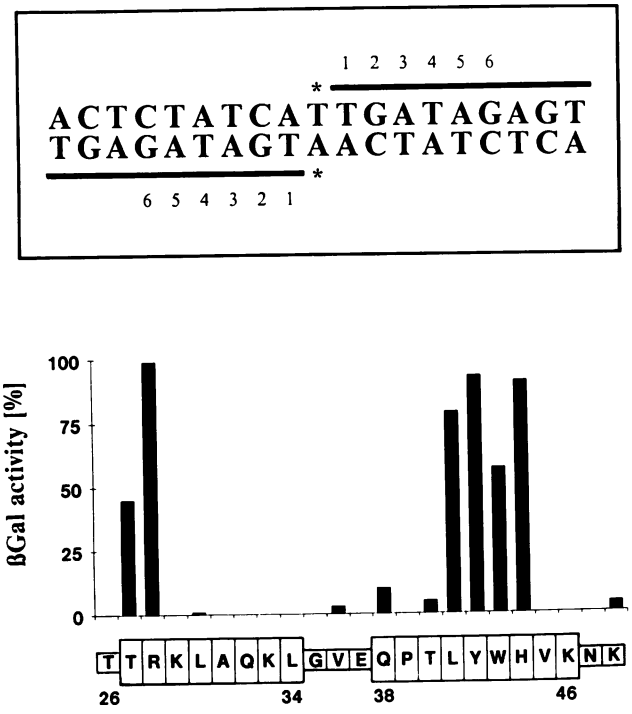

Fig. 1. Binding of mutant Tet repressors to wild-type *tet* operator. At the top of the figure the sequence of *tet* operator O_1 is shown together with the numbering of base pairs. The palindromic half sides are indicated by horizontal bars and the central base pair is marked by asterisks. At the bottom of the figure β-galactosidase activities determined by pWH1012 (*tetA–lacZ* fusion) and plasmids encoding the mutant Tet repressors are given. 100% β-Gal activity corresponds to no detectable repression of *lacZ* expression by Tet repressor, whereas 0% corresponds to maximal repression. The HTH of Tet repressor is shown below the horizontal axis with the proposed α-helical residues depicted by wide and non-α-helical residues depicted by narrower boxes. The wild-type amino acids are indicated using one letter abbreviations. The positions of the residues in the Tet repressor sequence are indicated by numbers at the N- and C-terminal ends of both α-helices. The bars above the wild-type residues show the β-Gal activities obtained with the respective alanine mutant (or in the case of A31, the Val mutant) given as a percentage of the constitutive expression level.

Binding of Tet repressor variants to mutant tet operators

The 23 Tet repressor mutants were tested for their ability to repress expression of *tetA–lacZ* fusions containing *tet* operator mutations at positions 1–6 (see Figure 1). In this study we compare the repression exerted by each Tet repressor mutant with the one exerted by wild-type Tet repressor on *tetA–lacZ* fusions containing the operator variants. The results obtained for all possible combinations are shown in Table I. They reveal that most Tet repressor mutants show similar binding profiles for all operator variants, whereas some mutants show position specific effects.

KA33 and KA46 showed no alteration from wild-type Tet repressor in binding to the set of operator variants. Mutants TA26, KA29, LA30, GA35, VA36, VA45 and NA47 bound to the operator variants with lower affinities as compared with wild-type Tet repressor. Except for VA36 their affinities for wild-type *tet* operator could not be distinguished from that of wild-type Tet repressor in the 'high' expression system. Mutants TA27, WA43 and KA48 showed residual binding to wild-type *tet* operator and no binding to any of the operator variants. Mutants RA28, LA41, YA42 and HA44 bound neither wild-type nor mutant operators.

The most pronounced position specific effect in the binding profile is found for the Tet repressor mutant QA38 as shown in Figure 2. QA38 binds to the wild-type, 3C, 3G, 3T and 6A *tet* operators. Compared with wild-type repressor the binding of 6A is reduced, whereas 3C, 3G and 3T are bound with greater efficiency. The 3T substitution of the wild-type 3A can be considered as a second site revertant, since a decreased affinity of the mutant repressor QA38 for wild-type operator can be compensated by this operator mutation. This proves a direct contact of Q38 at base pair 3 of the *tet* operator.

Another clearly position specific effect is observed in the binding profile of the PA39 mutant (see Figure 2) which binds to the wild-type 3T, 4A, 4C, 5T and 6A operators and has a higher affinity for the 4C operator compared with wild-type repressor, suggesting an interaction with base pair 4 of the operator.

A much smaller position specific effect is found for the TA40 mutant, which recognizes the wild-type, 6A and 6C operators (see Figure 2 and Table I). The 6A operator is slightly better bound than the wild-type operator. In addition, the ratio of 6C operator binding by the TA40 and wild-type repressors is smaller than the ones for 1G, 2T, 4A and 5T operator binding. This indicates a contact of T40 to base pair 6 of *tet* operator, however, the effects are smaller compared with those of the QA38 and PA39 mutants.

Rather marginal but experimentally reproducible position

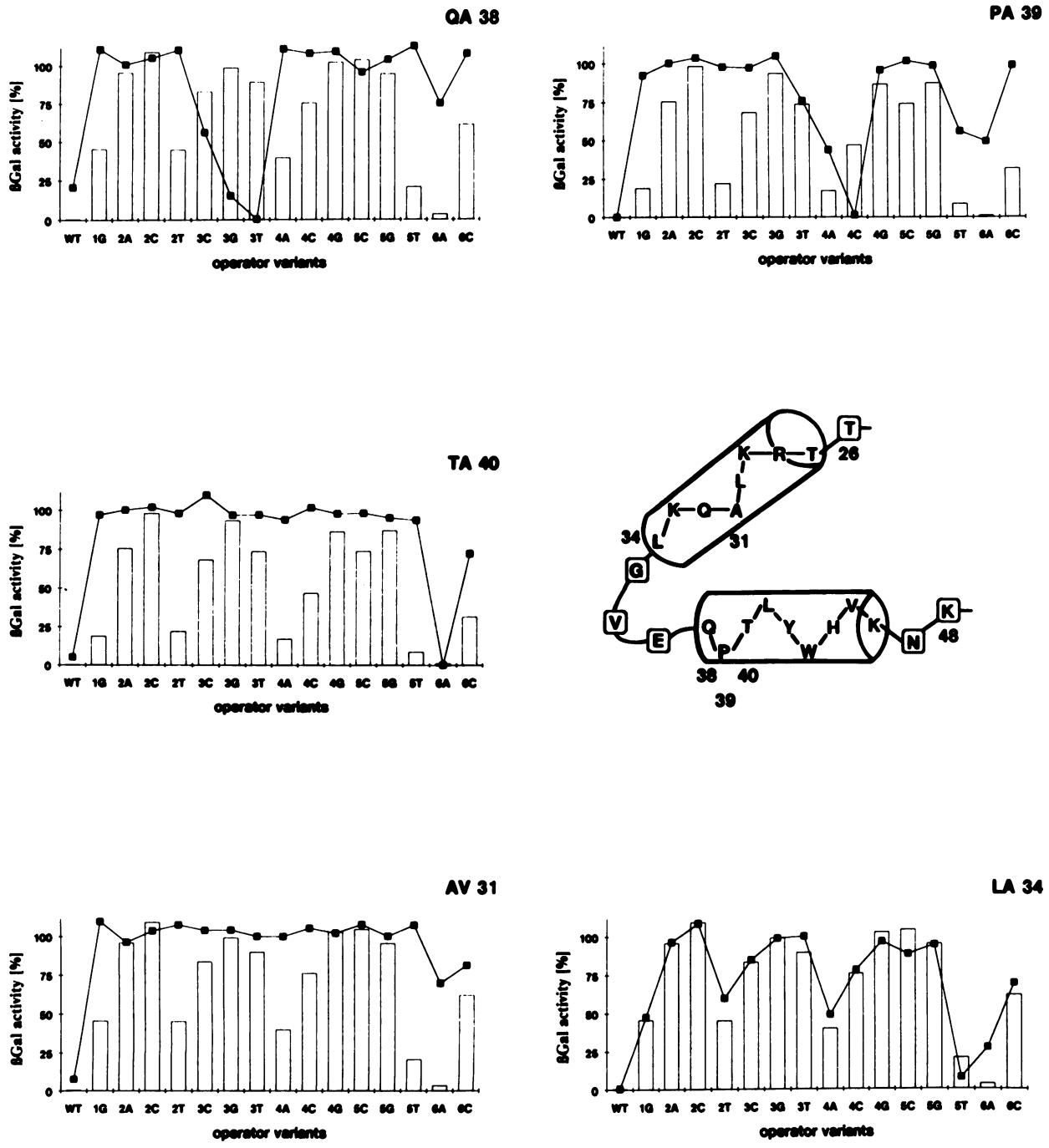

Fig. 2. Binding of mutant Tet repressors of *tet* operator variants revealing base pair specific effects. The results obtained for QA38 (upper left), PA39 (upper right), TA40 (middle left), AV31 (lower left) and LA34 (lower right) (filled squares connected by a line) are compared with those of the wild-type repressor (open columns). The vertical axis defines relative β-Gal activities (see Table I). The wild-type *tet* operator sequence is indicated by wild-type and the operator mutations are denoted by their respective position and nucleotide on the horizontal axis. For example, 2A (i.e. pWH1012-2A, Sizemore *et al.*, 1990) contains palindromic exchanges at position 2 of both operators in the *tet* regulatory region from GC to AT (compare Figure 1). A schematic presentation of the HTH of wild-type Tet repressor is given in the middle row on the right side. Non-helical residues are boxed. Helical residues with numbers are analyzed in this figure.

specific effects are also found for the AV31 and LA34 mutants. AV31 was used since wild-type Tet repressor contains an alanine at position 31. The results shown in Figure 2 indicate that this mutation can still bind *tet* operator, albeit with a reduced affinity. AV31 reproducibly recognizes the 6C operator with a similar efficiency to the 6A operator and with a clearly higher affinity than 1G, 2T, 4A and 5T. Both effects are in contrast to that observed with wild-type Tet repressor. This suggests that A31 may somehow be involved in recognition of the base pair at position 6 of *tet* operator. However, its recognition of base pair 6 shows some similarities to the effect of the QA38 and PA39 mutants at this position (see Table I). Thus, the effect of A31 on base pair 6 may be indirect. LA34 leads to equal or slightly reduced binding of all operator variants, except 5T, compared with wild-type Tet repressor (see Figure 3 and Table I). Binding of the 5T operator is improved compared with wild-type indicating that L34 may influence recognition of the base pair at position 5 of *tet* operator.

Two mutants, QA32 and EA37, led to a general

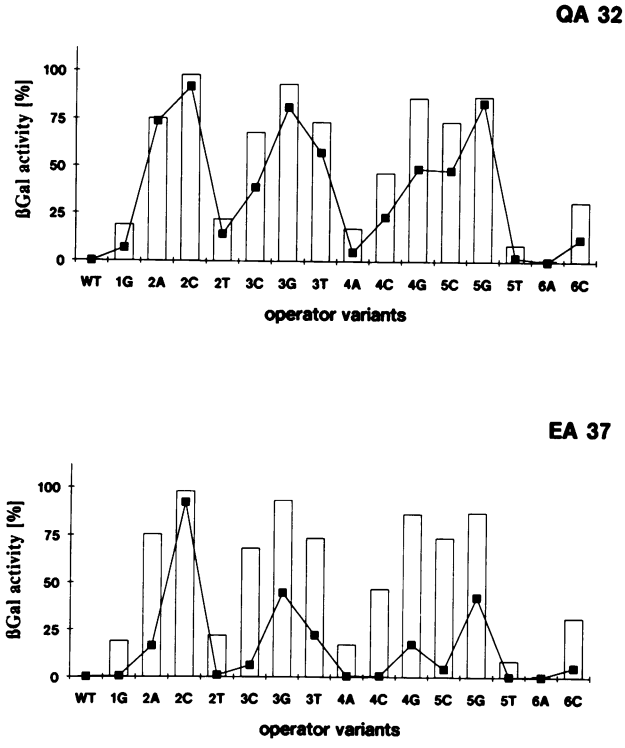

Fig. 3. Repression patterns of mutant Tet repressors showing increased binding to all *tet* operator variants. The results obtained with QA32 and EA37 (filled squares connected by a line) are compared with those of the wild-type repressor (open columns). Graphs are shown as described in the legend to Figure 2.

improvement in binding to all mutant operators. The binding profiles for these mutants are shown in Figure 3. This effect was more pronounced for EA37 than for QA32. Using the high expression system, the binding of wild-type *tet* operator by these repressor mutants was not distinguishable from that obtained with wild-type Tet repressor. With the low expression system it was demonstrated, that wild-type Tet repressor has a higher affinity to wild-type *tet* operator than either of the two mutants (see Table I). This demonstrates that these are not super-repressor mutants. Thus, QA32 and EA37 show decreased recognition of wild-type and increased recognition of mutant operators defining a loss of specificity. Furthermore, they are less inducible than wild-type Tet repressor (see above).

Discussion

The 'loss of contact' approach to study sequence specific recognition of *tet* operator by Tet repressor relies on the fact that in most cases replacement of a wild-type amino acid by alanine leads to (i) a reduction in size and (ii) a change in the chemical nature of the side chain at the mutated position. The even smaller glycine residue was not chosen in order to minimize the probability of secondary structural effects potentially caused by the mutation (Richardson and Richardson, 1988). It is assumed that the alanine substitutions do not interfere with the α-helical structures in the HTH. Therefore, the loss of contact approach allows us to evaluate several aspects of Tn*10* Tet repressor–*tet* operator recognition. First, the analyses of interactions of mutant Tet repressors with wild-type *tet* operator reveal the consequences of exchanging the wild-type side chain for a methyl group for repressor function. Except for GA35 the mutations result in smaller side chains. Therefore, productive interactions of the wild-type side chains at these positions may be eliminated by the mutation. The observed effects clearly identify two residues in the first α-helix (T27, R28) and four residues in the recognition α-helix (L41, Y42, W43, H44) where an exchange to alanine leads to drastic reductions in wild-type *tet* operator binding. The four residues in the recognition α-helix immediately follow three amino acid residues (Q38, P39, T40) for which base pair specific interactions with the operator can be demonstrated (see below). This may indicate that some of these residues are involved in the correct positioning of operator recognizing functions. In particular, L41 is thought to participate in the structural stabilization of the HTH by forming a hydrophobic pocket with L30, A31, L34 and V36 as deduced from a comparison of the Tet repressor sequence with those of other HTH-containing proteins (Efimov, 1984). W43 has been previously shown to be important for recognition of *tet* operator since an exchange to phenylalanine reduces the association constant with *tet* operator by three orders of magnitude (Hansen and Hillen, 1987). The mechanisms by which Y42, W43 and H44 in the recognition α-helix and R28 in the first α-helix contribute to operator recognition are unclear at present. It cannot be distinguished whether they are important for the intact tertiary structure of the HTH or whether they are engaged in contacts to the operator. A possible function of T27 for *tet* operator binding is discussed below.

The analysis of interactions of mutant Tet repressors with *tet* operator variants reveals several positions in the HTH where amino acid side chains participate in base pair specific operator recognition. The QA38 mutant contains an exchange of the first residue in the recognition α-helix and leads to decreased specificity at position 3 in the operator. An AT and a GC base pair are bound about equally well, whereas binding of CG is reduced and that to TA is increased. This clearly proves a contact of residue 38 of Tet repressor to the base pair at position 3 of *tet* operator. Furthermore, this recognition pattern implies a glutamine side chain interaction with the adenine as postulated by Seeman *et al.* (1976). This interpretation is illustrated in Figure 4. Glutamine residues can form two hydrogen bonds with adenine. The alanine replacement puts a methyl group at this position which (i) has a significantly shorter side chain and (ii) has hydrophobic properties. AT and GC base pairs direct similar functions towards the alanine methyl group and are recognized with similar efficiency by QA38. The methyl group of the TA base pair should be directed towards the methyl group of the alanine side chain allowing a hydrophobic interaction. This would explain the reversal of specificity from AT to TA for the QA38 repressor. A similar effect has been described for a QA exchange in bacteriophage 434 repressor which also changed recognition of an AT base pair to TA (Wharton and Ptashne, 1987). Finally, QA38 recognizes the CG base pair at position 3 of *tet* operator with the least efficiency. This may be due to the lack of the methyl group and the structural differences compared with AT and GC.

The PA39 mutant contains an alanine as the second residue in the recognition α-helix and displays small differential binding effects at position 3 and much clearer effects at position 4 of the operator. The high efficiency of binding to the operator variant 4C suggests an interaction with base pair 4 but no hints concerning the mechanisms are available

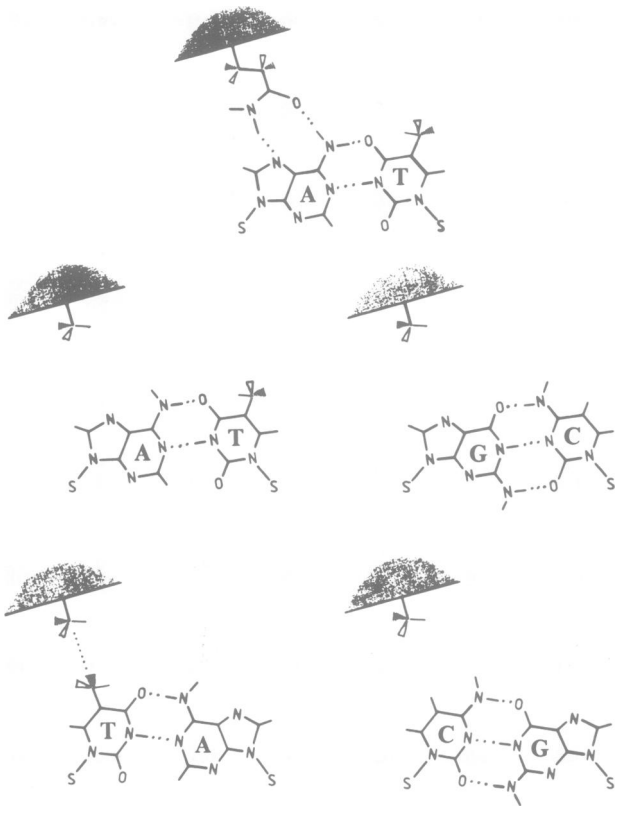

Fig. 4. Potential interaction of wild-type glutamine and mutant alanine residues at position 38 of Tet repressor with base pair 3 of *tet* operator. The proposed interaction of Q38 of Tet repressor with the AT base pair at position 3 of *tet* operator is shown at the top. The protein is represented by grey shading with just the glutamine side chain extruding. The 'S' in the base pairs denotes the sugar residues. Hydrogen bonds and the postulated van der Waals contact are symbolized by dots. In the middle and lower part of the figure our interpretation for the interaction of an alanine residue at this position with the four possible base pairs is shown.

at present. Due to the lack of H-bonding capacity in the proline side chain only hydrophobic contacts should contribute to specificity.

The small effect of the TA40 repressor confirms previous results obtained from the recognition patterns of naturally occurring Tet repressor and *tet* operator variants (Altschmied *et al.*, 1988). In both studies an altered recognition specificity from GC to AT at position 6 of *tet* operator has been observed for the TA40 mutant. However, the low efficiency of TA40 binding to CG base pair suggests that additional functions of the protein could participate in base pair specific recognition at this position.

Q38, P39 and T40 are located at the N-terminus of the recognition α-helix in the HTH (Isackson and Bertrand, 1985; Harrison and Aggarwal, 1990). The results obtained here show that these residues are involved in contacts at positions 3, 4 and 6 of the *tet* operator, respectively. This pattern is summarized in Figure 5 and defines the orientation of the HTH within the major groove of the DNA such that the N-terminus is directed towards the palindromic center of the operator. All other HTH proteins studied in molecular detail so far except for Lac repressor (Boelens *et al.*, 1987) show the opposite orientation of their recognition α-helices with respect to their operators. Both Tet repressor and Lac repressor, are the only thoroughly characterized members of the HTH proteins, in which the operator binding activity

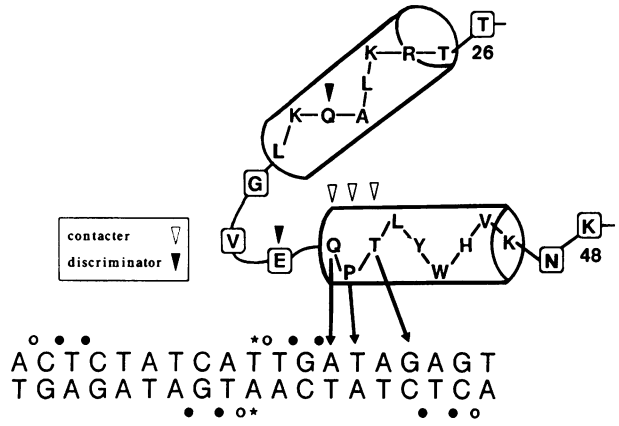

Fig. 5. Interactions of the helix-turn-helix motif of Tn*10* Tet repressor with *tet* operator. The HTH of Tet repressor is schematically shown at the top of the figure. Open and filled triangles in the HTH are defined on the left side. The sequence of *tet* operator O_1 is shown at the bottom of the figure with the central base pair indicated by asterisks. Circles mark phosphates at which ethylation interferes with Tet repressor binding. Stronger and weaker interference are defined by filled and open circles, respectively (Heuer and Hillen, 1988). Arrows between the HTH and *tet* operator indicate contacts between the respective residues.

is abolished upon binding of a ligand (induction). Whether the different orientation of the recognition α-helix in these complexes reflects some aspect of the induction mechanism remains unclear at present.

The effects of the mutations AV31 and LA34 on recognition of mutant operators are rather small and cannot safely be interpreted as contacts to the operator. It may be suspected that A31 and L34 affect Tet repressor binding to *tet* operator either in combination with or via positioning of other residues. However, their altered binding profiles at operator positions 6 and 5 would be in agreement with the hypothesis that the first α-helix folds back upon the recognition α-helix (Harrison and Aggarwal, 1990; Steitz, 1990) of Tet repressor (see Figure 5).

The phosphate contacts of the Tet, λ and 434 repressors with their respective operators have been identified (Heuer and Hillen, 1988; Pabo *et al.*, 1990 and references therein). The phosphate contacts of the *tet* operator–Tet repressor complex are shown in Figure 5. These data are consistent with a pattern expected for a symmetrical Tet repressor dimer binding to one face of the DNA helix in adjacent major grooves (Heuer and Hillen, 1988). Interestingly, the position of these contacts relative to the adenine–glutamine contact is identical in all three complexes. According to these results Tet repressor fits in the recently proposed general operator recognition model of other repressors (Kisters-Woike *et al.*, 1991). Furthermore, we suggest an additional level of similarity for these protein–DNA interactions. In addition to the contact with adenine, glutamine residues at the N-termini of the recognition α-helices of λ and 434 repressor also form hydrogen bonds to glutamine side chains at the N-termini of the previous α-helices in the HTH (Aggarwal *et al.*, 1988; Jordan and Pabo, 1988). These glutamines in the first α-helix in turn form hydrogen bonds to two phosphates of the DNA backbone: one of them is made by the side chain amido group and the other by the peptide amido function. This particular arrangement of a hydrogen bonding network consisting of the two glutamines, two phosphates and the adenine residues would allow a direct correlation of phosphate and base pair contacts and may be

critical for sequence specific DNA recognition (Pabo *et al.*, 1990). At the equivalent position in the first α-helix of the HTH, Tet repressor contains a threonine residue, which upon mutation to alanine in TA27 leads to a large reduction in operator binding (see Figure 1). We propose that this threonine residue also participates in a network correlating phosphate and base specific contacts as outlined above. This could be achieved by hydrogen bonds to two phosphates donated by the hydroxyl side chain group and the peptide amido group of T27 and a hydrophobic interaction of the side chain methyl group of T27 with the hydrophobic part of the Q38 side chain.

We have identified two residues in the HTH of Tet repressor, Q32 and E37, where the mutation to alanine leads to a reduced ability to discriminate between wild-type *tet* operator and the operator variants. These residues contribute to sequence specificity of the wild-type protein by reducing the affinity for mutant operators. Such an effect has not been observed before. We have termed these residues discriminators. One potential mechanism might be that these residues contribute to the structural rigidity of the DNA binding domain which may be optimized for wild-type operator recognition. The mutations might render the HTH more flexible and, therefore allow some adjustment of the interacting elements between Tet repressor and mutant base pairs. The QA32 and EA37 mutants also show reduced inducibility by tetracycline. Since it has been shown that tetracycline binds to a different part of Tet repressor (Hansen *et al.*, 1988; Smith and Bertrand, 1988) it is believed that induction requires an allosteric conformational change in the protein which affects the DNA binding motif. Our results suggest that E37 in particular, and Q32 to a lesser extent, may be involved in transmission of this conformational change in the HTH. A similar interpretation has been described previously for residue R49 (Wissmann *et al.*, 1991) at the C-terminal side of the recognition α-helix. If our hypotheses hold true, hinges for induction would be located on both sides of the recognition α-helix.

Taken together, our analysis reveals three different classes of functional amino acids in the HTH of Tet repressor as defined by the operator recognition effects of the alanine mutations. They are summarized in Figure 5 with respect to their location in the HTH. The first class shows the most severe effects on *tet* operator recognition and binding, but no base pair specific effects were detected. Of course, these residues may well engage in specific DNA contacts in a way which is not sensitive to the loss of contact analysis. However, it is noteworthy that the alanine mutations of all residues showing base pair specific operator recognition effects, which define the second class of functional amino acids, lead to much less severe reductions in wild-type *tet* operator binding. Finally, residues in the third class which we have termed discriminator amino acids, enhance discrimination between wild-type and mutant operators and, furthermore, are relevant for inducibility of Tet repressor. This latter class may be particularly interesting because to our knowledge no linkage of specific operator binding and inducibility has previously been identified.

Materials and methods

Bacterial strains and methods

Escherichia coli WH207, which is Δ*lac* and *recA*, has been described by Wissmann *et al.* (1991). Cultures for β-galactosidase assays were grown in Luria broth supplemented with the appropriate antibiotics to OD_{600} of 0.4 at 37°C. β-galactosidase activities were determined at least twice in independent experiments and done as described by Miller (1972). Inducibility of Tet repressor was tested using 0.2 μg/ml of tetracycline in both the overnight and log cultures.

Plasmids

The *tetA–lacZ* fusion plasmid pWH1012 and its 15 derivatives containing *tet* operator mutations have been described by Sizemore *et al.* (1990). Plasmids pWH1200, pWH1201 and pWH510 has been described by Altschmied *et al.* (1988) and pWH520 by C.Berens, L.Altschmied and W.Hillen (in press). pWH1411 has been described (Wissmann *et al.*, 1991). mWH819 was constructed by inserting an *Eco*RI–*Hin*dIII *tetR* fragment from pWH510 into the respective sites of M13mp19.

Construction of Tet repressor mutants

One set of alanine mutants (TA26, TA27, RA28, KA29, LA30, QA32, KA33, EA37, PA39, TA40, WA43, HA44, VA45, KA46 and KA48) was constructed by mutagenesis as described (Kunkel *et al.*, 1987) using appropriate oligonucleotide primers on mWH819. The resulting in *tetR* mutants were recloned by pWH1201 leading to derivatives of pWH520. The *tetR* mutants QA32 and EA37 were also cloned in pWH1200 yielding derivatives of pWH510. Another set of mutants (LA34, GA35, VA36, QA38, LA41, YA42, NA47 and AV31) of Tet repressor was the result of cassette mutagenesis using plasmid pWH1411 (Wissmann *et al.*, 1991).

Preparation of protein extracts

Total cell protein was prepared from 40 ml LB + Amp (100 mg/ml) + Km (60 mg/l) log cultures at OD_{600} = 0.6. After harvesting cells by centrifugation at 14 000 *g*, the pellets were resuspended in water, boiled for 5 min and 1 vol of sample buffer according to Laemmli (1970) was added. Cell extracts corresponding to 1 OD_{600} of log cultures were heated up to 65°C for 10 min, centrifuged for 10 min at 14 000 *g* and electrophoresed.

Western blotting

The general procedure was performed as described by Towbin *et al.* (1979). Protein extracts were fractionated on a 10% SDS–polyacrylamide gel. Transfer of proteins to Fluorotrans transfer membrane (Pall, England) was done in a Multiphore II electrophoresis apparatus from Pharmacia (Freiburg, FRG) for 150 min at 250 mA. The polyclonal antibody was a gift of Dr C.P.Beck (Freiburg, FRG), a protein A–peroxidase conjugate purchased from Sigma (Munich, FRG) was used as the secondary antibody. The complex was visualized by incubation with 3,3′-diaminobenzidine and hydrogen peroxide obtained from Sigma.

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