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A threonine to alanine exchange at position 40 of Tet repressor alters the recognition of the sixth base pair of *tet* operator from GC to AT

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The *tet* operators of two naturally evolved tetracycline resistance determinants differ by a G·C to A·T transition at the sixth base pair. This mutation prevents heterologous recognition of these *tet* operators by their respective two Tet repressor proteins. The amino acid side chains responsible for this sequence-specific distinction of operators were determined. For this purpose *in vitro* recombinants of the two *tet*R genes were constructed. Restriction sites were introduced by oligonucleotide-directed mutagenesis in both genes followed by the exchange of different coding segments between them. The encoded chimeric Tet repressor proteins were expressed and their operator recognition specificity was scored *in vivo*. Exchanging gradually smaller coding segments led finally to a single amino acid exchange in both genes at position 40 of the primary structures. Each Tet repressor containing Thr at this position recognizes the G·C operator while those with Ala recognize the A·T operator regardless of the rest of the sequences. This result demonstrates clearly that the amino acid 40 of Tet repressor contacts and recognizes base pair 6 of *tet* operator. Sterical interference of the large Thr side chain with the methyl group of A·T and a possible involvement of the hydroxyl in hydrogen bonding to the operator are discussed as the molecular basis of this differentiation between A·T and G·C base pairs.

Key words: tetracycline resistance/transcription control/operator recognition/protein engineering/repressor specificity

Introduction

Tetracycline resistance genes are widespread among enteric bacteria. Based upon differences in their phenotypes and hybridization efficiencies of the resistance genes (*tet*A) they have been divided into four classes named A–D (Mendez *et al.*, 1980). With only one recently described exception (Heuer *et al.*, 1987) the expression of all of them is negatively regulated on the level of transcription by repressor proteins (TetR) and induced by tetracycline. The transcription control sequences as well as the amino acid sequences of the repressor proteins from the four classes share extensive sequence homology (Waters *et al.*, 1983; Postle *et al.*, 1984; Unger *et al.*, 1984a,b; Klock *et al.*, 1985). Despite this observation the class A *tet* operators are only poorly recognized by the class B repressor and vice versa (Klock *et al.*, 1985; Klock and Hillen, 1986).

Each *tet* transcriptional control sequence consists of at least two divergently oriented promoters which are overlapped by two *tet* operators (Klock *et al.*, 1985). Figure 1 displays the four *tet* operator sequences found in the Tn*10*-(class B) and Tn*1721*- (class A) encoded tetracycline-resistance control regions (Bertrand *et al.*, 1983; Waters *et al.*, 1983). These are recognized with high affinity by their respective $TetR^A$ and $TetR^B$ proteins (Klock and Hillen, 1986; Kleinschmidt *et al.*, 1988). The heterologous recognition is in both cases reduced by roughly three orders of magnitude (Klock *et al.*, 1985; Klock and Hillen, 1986). The nucleotide sequence differences between the operators occur at positions 0, 6, 8 and 9 (see Figure 1). A saturation mutagenesis of the Tn*10*-encoded *tet* operator has revealed that the central base pair does not contribute at all and the positions 8 and 9 contribute only weakly to repressor recognition (Wissmann *et al.*, 1988). The A·T to G·C exchange at position 6 is, therefore, the crucial mutation for the reduced heterologous recognition.

In this article we identify the amino acids of the class A and B Tet repressor proteins mediating recognition of the sixth base pair of *tet* operator. Both repressors share ~44% identical amino acids in their primary structures (Unger *et al.*, 1984b). Thus, it is not straightforward to determine the molecular basis for their different operator recognition. In order to localize the important region we used oligonucleotide-directed mutagenesis to introduce restriction sites at equivalent positions in both genes, constructed chimeric *tet*R genes from these mutants, expressed the chimeric proteins and studied their operator recognition specificity *in vivo*.

Results

Construction of $tetR^{B,A}$ genes

To identify the amino acid side chains of the two repressor proteins responsible for the A·T versus G·C distinction at base pair 6 of the *tet* operator we have constructed chimeric genes from both *tet*R sequences. The strategy of the experiment is outlined in Figure 2. The $tetR^A$ and $tetR^B$ nucleotide sequences were searched for positions where restriction sites could be introduced by oligonucleotide-directed mutagenesis at identical positions with respect to the reading frames in both genes. Of all the possibilities, only those which are either silent with respect to the encoded amino acid sequence or introduce changes from the $TetR^A$ to the $TetR^B$ primary structure or vice versa were considered further. The mutagenic oligonucleotides along with the created restriction sites and amino acid exchanges are listed in Table I. Inspection of Figure 2 reveals that these restriction sites subdivide both *tet*R genes in cassettes which can be mutually exchanged leading to the chimeric genes as indicated.

The $tetR^B$ gene was cloned into M13mp9 to yield mWH508 and the gapped duplex approach for mutagenesis was used followed by selection for mutants on the

M13mp9*rev* derivative (Kramer *et al.*, 1984). All five mutagenic oligonucleotides were used in a single experiment and the multimeric mutations shown in Figure 2 were identified by spot hybridization of the single-stranded candidate M13 phage DNAs with the mutagenic oligonucleotides. Using this approach (see Materials and methods for details) 3% of the candidates contained five, 10% four, 7% three, 38% two, 13% a single and 28% no mutation. The yields of mutants for each oligonucleotide are given in Table I. The mutant *tet*R^B genes displayed in Figure 2 were used to construct the chimeric genes. In addition, two *tet*R^B mutants containing amino acid substitutions at positions 22, 23 and 40 respectively were used to determine their operator recognition specificity directly.

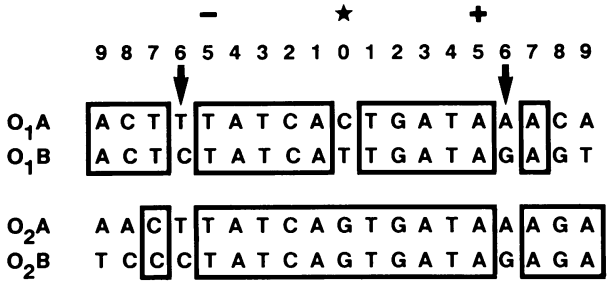

Fig. 1. Comparison of the class A and B *tet* operator sequences. The nucleotide sequences of the *tet* operators O_1 and O_2 from the Tn*1721*-(A) and Tn*10*-(B) encoded tetracycline resistance determinants are shown. Identical nucleotides are boxed. The sequence differences relevant for operator differentiation by Tet repressors are marked with arrows. The numbers and +/− on top of the figure indicate the designation of positions and the star identifies the centre of palindromic symmetry.

The *tet*R^A gene was also cloned in M13mp9 to yield mWH507 and the same mutagenesis protocol was used except that the ssDNA of mWH507 was grown for three passages on *Escherichia coli* RZ1032 to introduce uracil residues which were then selected against by transfection to *E.coli* BMH71-18 *mut*S (Kunkel, 1985). Using this procedure (see Materials and methods for details) no candidates with five mutations were obtained, 13% had four, 8% had three, 9% had two, none had one and 70% had no mutations. The ones displayed in Figure 2 were used to construct chimeric *tet*R^{B,A} genes. Furthermore, a mutant converting the Ala codon at position 40 to a Thr codon was made and tested for operator recognition specificity.

As outlined in Figure 2 the mutant genes were used to exchange different coding segments from *tet*R^B by the respective *tet*R^A sequences. First, codons 8−216 of the *tet*R^A gene were fused to codon 7 of *tet*R^B to yield *tet*R$^{BA8\text{-}216}$. Then the codons 8−92 of *tet*R^B were replaced by the *tet*R^A codons to yield *tet*R$^{BA8\text{-}92}$ followed by exchange of codons 22−54, 22−40 and 40−54 respectively. Finally, the two mutants having Thr40 converted to Ala and Ile22-Glu23 to Val22-Asp23 were also used to study operator recognition. The encoded N-terminal amino acid sequences of the chimeric *tet*R genes are displayed in Figure 3, which indicates the strategy of the experiment. First almost the entire reading frame was exchanged followed by only the N-terminal half of *tet*R^B. The next step involved exchange of the entire proposed α-helix−turn−α-helix operator recognition motif (Isackson and Bertrand, 1985), which was then subdivided in the two α-helices. Finally, a point mutant in the recognition α-helix was studied. Figure 3 also shows the *tet*R$^{BA22\text{-}23}$ mutant containing two amino acid exchanges that has also been checked for operator binding specificity.

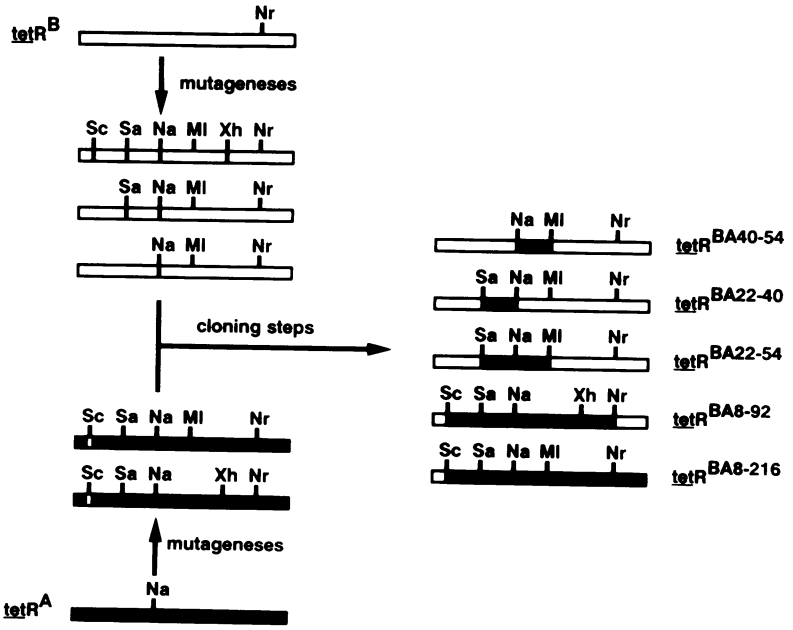

Fig. 2. Mutagenesis and strategy for the construction of mixed *tet*R^{B,A} genes. *tet*R sequences are represented by boxes. White areas correspond to *tet*R^B sequences and black areas to *tet*R^A sequences. The abbreviations are: Na, *Nae*I; Nr, *Nru*I; Ml, *Mlu*I; Sa, *Sal*I; Sc, *Sca*I and Xh, *Xho*I. The mixed *tet*R^{B,A} genes constructed from the mutants are displayed on the right side of the figure along with their designation.

Construction of single copy indicator genes for Tet repressor–tet operator recognition in vivo

The *in vivo* test system for the efficiency of repressor–operator recognition contains *tet–lacZ* fusion genes located on a recombinant λ phage which has been integrated as a single copy into the chromosome of *E.coli* CSH26. The indicator gene for the *tet*^{B} operators contains a *tet*A^B–*lacZ* transcriptional fusion and has been described previously (Wissmann *et al.*, 1986). In order to score recognition of the *tet*^{A} operators a similar system was constructed containing a *tet*R^A–*lacZ* translational fusion. This fusion was generated and characterized using M13mp11. Since problems with cloning the bidirectional *tet* promoter system in M13 derivatives were frequently observed (unpublished observations) we decided to protect the M13 *ori* by a transcriptional terminator from the interfering effects of a *tet* promoter. In addition, the *lac* promoter was deleted to allow only *tet*-specific transcription of the indicator gene. Therefore, the bidirectional *tet* terminator (Schollmeier *et al.*, 1985) was cloned between the *Bam*HI and *Nar*I sites of M13mp11 to yield mWH250 which was then used to construct the *tet*R^A–*lacZ* translational fusion (see Materials and methods for details). Finally, this construction was recombined with λplac5·T743 (Yu and Reznikoff, 1984) to yield λWH100 which was integrated into the chromosome of *E.coli* CSH26 (see Table III). This construction expressed β-galactosidase constitutively in the absence of a *tet*R gene.

Transcription of the *tet–lacZ* fusions was regulated by Tet repressor provided *in trans*. The *tet*R^B gene and the chimeric *tet*R genes containing the *tet*R^B translational initiation sequence were cloned in pACYC177 (see Materials and methods for details) similarly as described for pRT241 and transformed to the indicator strains (see Table III). These constructions contain the *tet*R reading frame in opposite orientation to the *bla* gene and express Tet repressor at a low level (Wray and Reznikoff, 1983; Bertrand *et al.*, 1984; Wissmann *et al.*, 1986; Meier *et al.*, 1988). Efficient expression of *tet*R^A and its mutants is hampered by the fact that the *tet*R^A gene exhibits identical starts for transcription and translation (Klock and Hillen, 1986). Thus, wild-type and mutant *tet*R^A mRNAs do not contain Shine–Dalgarno sequences (Shine and Dalgarno, 1974) leading to a low level of expression (Ptashne *et al.*, 1976). Therefore, these genes were expressed from pACYC177 derivatives containing the *tet*R reading frames in the same orientation as *bla* and probably employ the *bla* promoter for an increased level of transcription as compared to the constructions described above (Wray and Reznikoff, 1983; Bertrand *et al.*, 1984). The technical details are outlined in Materials and methods.

Table I. Properties of mutagenic oligonucleotides and yields of directed mutagenesis

No.	Restriction site created	at codon no.	Mutagenic oligonucleotide	Amino acid mutation	Yield (%)
*tet*R^A					
1	*Sca*I	7	gcagccgaGtacTgtgatc	*tet*R^{AB7}	30
2	*Sal*I	22	ggtcggcgtCgacggtctg	None	26
3	*Mlu*I	54	ctgctcgacgcGTtggccgaagcc	None	17
4	*Xho*I	70	cttcggtgccTCgagccgacgac	None	4
5	*Nru*I	94	gctcgcctaTcgcgatggcgc	None	17
6	*Hin*f	40	ggttcagcagccgAcTctttactggc	*tet*R^{AB40}	33
*tet*R^B					
7	*Sca*I	7	ttagataaaagtaCTgtgattaac	*tet*R^{BA8}	42
8	*Sal*I	22	ggtcggaGtcgaCggtttaac	*tet*R$^{BA22-23}$	19
9	*Nae*I	39	gtagagcagccGGcattgtattgg	*tet*R^{BA40}	26
10	*Mlu*I	54	cttgctcgacgcGttagccattg	None	58
11	*Xho*I	70	cttttgccctCGagaaggggaa	*tet*R^{BA70}	23

Operator recognition properties of TetR^A, TetR^B and mixed TetR^{B,A} proteins measured in vivo

The expression of β-galactosidase from both *tet–lacZ* fusions was measured in the presence of each *tet*R variant as an indicator of repressor–operator recognition. The results are presented in Table II. The TetR^B protein represses the *tet*^{B}–*lacZ* fusion to 4% of its maximal activity, while the expression of the *tet*^{A}–*lacZ* fusion is not affected at all. The TetR^A protein, in contrast, represses the *tet*^{A}–*lacZ* fusion to 10% of the maximal expression and the *tet*^{B}–*lacZ* fusion to ~60% of the maximal level. This result indicates that both repressors are able to distinguish between the class A and B operators in this *in vivo* assay. Distinction between *tet* operators is more efficient for TetR^B compared to TetR^A as indicated by the differentiation factors given in Table II.

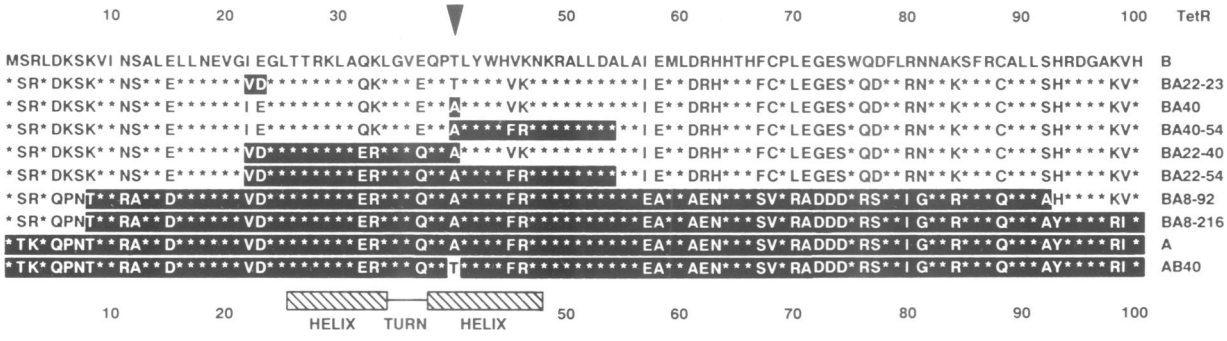

Fig. 3. N-terminal amino acid sequences of mixed TetR proteins. The first 100 amino acids of the class B Tet repressor are shown on top of the figure in one-letter abbreviations. The second line from the bottom shows the sequence of the class A Tet repressor on a black background. Identical amino acids with the class B sequence are indicated by stars. The other lines display the sequences of the mixed proteins along with their designations. The arrow on top of the sequences indicates position 40 relevant for recognition of the sixth base pair of the *tet* operator. The proposed α-helix–turn–α-helix (Isackson and Bertrand, 1985) operator recognition motif is indicated on the bottom.

When the entire C terminus from amino acids 8–216 of $TetR^A$ is fused to the N-terminal seven amino acids of $TetR^B$ the tet^B-lacZ fusion is repressed to 27% while the tet^A-lacZ fusion is repressed to 9%. Thus, the apparent recognition of the tet^A operators by this mutant is 3-fold better than binding to the tet^B operators. The same difference in operator specificity is found when amino acids 8–92, 22–54, 22–40 and 40–54 are from the $TetR^A$ sequence. This result indicates that all of the mixed Tet repressors are able to bind and distinguish the class A and B operators with the $TetR^A$ specificity. The efficiency of β-galactosidase repression increases roughly for both *tet–lacZ* fusions with the length of the $TetR^A$ portion in the mixed proteins (see Table II). When 0.25 μg/ml tetracycline were present in the cultures the β-galactosidase expression was between 80 and 100% for all wild-type and mixed *tet*R genes shown in Table II (data not presented). It is concluded that all TetR derivatives exhibit normal inducer-dependent operator binding.

Table II. Operator recognition specificity of $TetR^A$, $TetR^B$ and mixed $TetR^{B,A}$ proteins *in vivo*

Plasmid	TetR	% β-galactosidase activity[a]		Differentiation factor[b]
		tet^B-lacZ	tet^A-lacZ	
pWH510	B	4 ± 0.6	97 ± 10	<0.05
pWH511	BA22-23	5 ± 1.5	96 ± 5.3	<0.07
pWH512	BA40	92 ± 6.9	57 ± 5.3	>1.4
pWH513	BA40-54	65 ± 3.0	30 ± 4.1	2.2 ± 0.5
pWH514	BA22-40	52 ± 2.8	14 ± 1.9	3.7 ± 0.8
pWH515	BA22-54	45 ± 2.8	11 ± 2.1	4.1 ± 1.3
pWH516	BA8-92	48 ± 1.5	15 ± 1.6	3.2 ± 0.5
pWH517	BA8-216	27 ± 1.9	9 ± 1.2	3.0 ± 0.7
pWH1211	A	58 ± 2.6	10 ± 1.0	5.8 ± 0.9
pWH1212	AB40	1 ± 0.1	55 ± 1.3	0.02 ± 0.002
pWH1200	None	98 ± 7.9	101 ± 4.6	
pWH1201	None	102 ± 6.2	99 ± 4.0	
None	None	105 ± 7.3	104 ± 10	

[a]Determined in units as defined by Miller (1972) and given as a percentage of the non-repressed activity with their standard deviation. The non-repressed expression of the tet^B-lacZ fusion is 3200 ± 200 U and that of the tet^A-lacZ fusion is 100 ± 10 U.
[b]The differentiation factor is defined as the ratio of % β-galactosidase activity from the tet^B-lacZ fusion over the % β-galactosidase activity from the tet^A-lacZ fusion.

Only the $TetR^A$ amino acid at position 40 is common to all of these mixed proteins. Therefore, the $TetR^B$ mutant containing the single Thr40 to Ala exchange was also tested for *tet* operator recognition (see Figure 3). The $TetR^{BA40}$ protein does not repress expression from the tet^B-lacZ fusion while the tet^A-lacZ fusion is repressed to ~60%. This leads to a differentiation factor of >1.4 (see Table II). It is thus very clear that the single amino acid exchange of Thr40 to Ala40 prevents complete recognition of the class B operators with the G·C bp at position 6 under these *in vivo* conditions. The class A operators with the A·T bp at position 6, on the other hand, are recognized after this single amino acid exchange. This result establishes a specific contact of the amino acid 40 with the sixth base pair of *tet* operator. Quantitatively the wild-type $TetR^B$ represses the tet^B-lacZ fusion to 4% while the $TetR^{BA40}$ mutant represses the tet^A-lacZ fusion only to 57%. This indicates that the Thr at position 40 forms stronger interactions with the class B operator than the Ala with the class A operator.

The results obtained with the substitution of amino acids in $TetR^B$ with those from $TetR^A$ suggest that an exchange of Ala40 in $TetR^A$ for Thr should alter the recognition specificity from the tet^A to the tet^B operator. In order to test this assumption the respective mutant $TetR^{AB40}$ protein (see Figure 3) was also analysed in the *in vivo* assays. The results are given in Table II. While the wild-type A repressor shows a differentiation factor of 5.8 in favour of the tet^A operator, the $TetR^{AB40}$ protein recognizes the tet^B operator 50-fold better than the tet^A operator. Thus, the exchange of the amino acid 40 in either TetR protein for the respective other

Table III. Bacterial strains

Strain	Genotype	Reference
BMH71-18	Δ(*lac-pro*AB), *sup*E, *thi*; F′: *lac*I^{q}ZΔM15, *pro*A$^+$B$^+$	Kramer *et al.* (1982)
BMH71-18*mut*S	Δ(*lac-pro*AB), *sup*E, *thi*, *mut*S215::Tn*10*(tet^r); F′: *lac*I^{q}ZΔM15, *pro*A$^+$B$^+$	Kramer *et al.* (1984)
CSH26	Δ(*lac-pro*), *ara*, *thi*	Wissmann *et al.* (1986)
JM101	Δ(*lac-pro*AB), *thi*, *sup*E; F′: *lac*I^{q}ZΔM15, *pro*A$^+$B$^+$, *tra*D36	Yanisch-Perron *et al.* (1985)
JM109	Δ(*lac-pro*AB), *rec*A1, *end*A1, gy*r*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1; F′: *lac*I^{q}ZΔM15, *pro*A$^+$B$^+$, *tra*D36	Yanisch-Perron *et al.* (1985)
K12Δ*H*1Δ*trp*	M72Sm^r, *lac*Zam, Δ(*bio-uvr*B), Δ*trp*EA2; λ:Nam7-Nam53, cI857, ΔH1	Remaut *et al.* (1981)
MK30-3	Δ(*lac-pro*), *rec*A, g*al*E, *str*A; F′: *lac*I^{q}ZΔM15, *pro*A$^+$B$^+$	Kramer *et al.* (1984)
RR1	*hsd*S20(r_B^-,m_B^-), *ara*-14, *pro*A2, *lac*Y1, g*al*K2, *rps*L20, *xyl*-5 *mtl*-1, *sup*E44, *leu*	Bolivar *et al.* (1977)
RZ1032	HfrKL16 Po/45, *thi*, *relA*, *sp*^{o}T, *dut*, *ung*, *sup*E44, Tn*10*(tet^r)	Kunkel (1985), T.Kunkel, personal communication
WH201	Δ(*lac-pro*AB), *thi*, *sup*E; F′: *pro*A^{2}B$^+$, Δ*lac*X74	L.V.Wray, Jr, personal communication
XA103	Δ(*lac-pro*), *nal*A, *met*B, *arg*Eam, *rif*, *sup*F	Yu and Reznikoff (1984)

residue reverts the operator recognition pattern *in vivo* between A·T and G·C. TetR^{A} represses the *tet*^{A}−*lac*Z fusion to 10% and TetR^{AB40} represses the *tet*^{B}−*lac*Z fusion to 1%. This also indicates that Thr may form stronger interactions with the B operator than Ala with the A operator.

Discussion

Tetracycline resistance genes provide a source of naturally evolved Tet repressor−*tet* operator recognition mutants. Particularly striking is the poor heterologous recognition among the classes A and B because only a single relevant position in each *tet* operator half side is different (Wissmann *et al.*, 1988). This sequence difference in the recognized DNA is compensated by the respective repressor proteins. When their amino acid sequences are compared without allowing for gaps in the primary structure, ~44% homology is found (Waters *et al.*, 1983; Postle *et al.*, 1984; Unger *et al.*, 1984b). When one gap in the C terminus is allowed for in this comparison, the homology increases to 50%, and 67% of the amino acids have isofunctional side chains in this case. This result suggests that the two proteins have similar secondary and tertiary structures. On the basis of this assumption it seems quite likely that the recognition of the sixth base pair may be achieved by different side chains of amino acids at a given position in the proteins. Furthermore, it seems feasible to use protein engineering methods to construct chimeric proteins from the two wild-type sequences and study their operator recognition specificities with respect to the sixth base pair.

To construct the chimeric proteins the nucleotide sequences of the two *tet*R genes were searched for positions where restriction sites could be introduced by oligonucleotide-directed mutagenesis. The possible sites were then reduced to those mutations, which are either silent or encode exchanges of the sequence between the two genes with respect to the encoded amino acids. Comparison of these sites on both genes led to the identification of common sites at identical positions with respect to the two reading frames. The next qualifying condition was that the sites must facilitate a cloning strategy to exchange gene segments between them. Finally, they were selected so that they disrupt the two genes logically as indicated in Figure 2. This strategy would identify any important amino acid by distinguishing between the C-terminal and N-terminal half in the first step and then subdividing the relevant portion of the gene into small pieces. If necessary the different amino acids in these small segments could each be checked by making the respective point mutations.

It is essential for this approach to obtain multiple oligonucleotide-directed mutations in the same gene efficiently. The gapped duplex approach followed by the selection for M13mp9 revertants used here (Kramer *et al.*, 1982, 1984) yielded all necessary combinations of five individual mutagenesis events in a single experiment.

The test system for *tet*^{A,B} operator recognition is based upon *tet*−*lac*Z fusion genes which are integrated as single copies on recombinant λ phages in the chromosome of the host cell (Wray and Reznikoff, 1983; Bertrand *et al.*, 1984; Wissmann *et al.*, 1986). The respective fusion of the *tet*A^{B} promoter was already available while a fusion of the *tet*R^{A} gene with *lac*Z had to be constructed. It expressed only low levels of β-galactosidase because the mRNA lacks the ribosome binding site (Klock and Hillen, 1986). This leads to a maximal expression of 100 U β-galactosidase while the most repressed expression is 10 U. Within this window expression differences can be measured with great accuracy as indicated by the standard deviations in Table II.

The recognition and distinction of operators by the wild-type and mutant TetR proteins *in trans* is scored by an *in vivo* test involving expression of the respective genes. Thus, the interpretation of the results in terms of operator binding depends on the assumption that the concentrations of the proteins in the cells do not vary much among the mutants. This is certainly the case when the recognition of the class A and B operators by the same mutant protein is studied. The most reliable results are thus obtained from horizontal comparisons of the data in Table II. Whenever the functions of different mutants are compared, possible differences in expression and stability of the proteins must be considered. Potential problems may increase within the *tet*R^{BA} series with the number of exchanged amino acids. Thus, vertical comparisons of the data in Table II need to be discussed. Comparisons between proteins translated from the *tet*R^{B} initiation codon with the ones starting from the *tet*R^{A} codon are impossible due to the different translation efficiencies discussed above. A quantitative comparison of the binding properties of different mixed Tet repressors would require *in vitro* methods. Nevertheless, the molecular basis for the distinction of the A·T and G·C base pairs at position 6 of *tet* operators by these repressors can be determined unambiguously from the *in vivo* data presented here.

Direct comparison of the effects of *tet*R genes provided *in trans* to the *tet*−*lac*Z fusions leads to the differentiation factors given in Table II. These indicate clearly that the position 40 of the primary structure of TetR is critical for distinction of the A·T and G·C base pairs at position 6 of *tet* operators. Starting either from the TetR^{B} wild-type and replacing the Thr by Ala or from the TetR^{A} wild-type and replacing the Ala by Thr yields the same differentiation between operators compared to the respective wild-types. It is concluded that the distinction of position 6 in *tet* operator is achieved by a single amino acid side chain. For control purposes the double-exchange TetR$^{BA22\text{-}23}$ was also tested and behaves exactly as TetR^{B} wild-type.

It would be interesting to unravel the mechanism of recognition of the sixth base pair. The A·T base pair provides a methyl group in the major groove of the DNA. It is apparent that this base pair is recognized by the amino acid Ala with the smaller side chain compared to Thr. This would indicate that steric hindrance may be important for sequence distinction in this case. However, hydrogen bonding of the OH of Thr may also be important for operator binding. A comparison of the results obtained with the wild-type TetR proteins with the ones from the single amino acid mutants may be suggestive of this hypothesis because large differences in expression and stability of both proteins are very unlikely. This is proven to be true for the comparisons of TetR^{B} with TetR^{BA40} and TetR^{A} with TetR^{AB40}. Recognition of specific operators in both cases is more efficient when Thr is present instead of Ala. Extensive mutagenesis of position 40 in both proteins may contribute to the clarification of the mechanisms of operator binding and differentiation.

Table IV. Plasmids and phages

Plasmid or phage	Marker	Construction or purpose	Reference
M13mp9	*lacZ*$^+$	Cloning vector	Messing and Vieira (1982)
M13mp9rev	*lacZ*$^+$	Used for gdDNA mutagenesis	Kramer *et al.* (1984)
M13mp11	*lacZ*$^+$	Cloning vector	Messing (1983)
pACYC177	amp^r,kan^r	Cloning vector	Chang and Cohen (1978)
pIC-20R	amp^r	Source of polylinker region	Marsh *et al.* (1984)
pWH1200	kan^r	pACYC177 with polylinker in *bla*	This work
pWH1201	kan^r	Polylinker with reversed orientation as in pWH1200	This work
mWH501		*Xba*I–*Hin*dIII (truncated *tet*R^B) from pWH305 in M13mp11	This work
mWH505		*Eco*RI–*Sal*I (*tet*R^A) from pWH321 in M13mp11	This work
mWH507		*Eco*RI–*Hin*dIII (*tet*R^A) from mWH505 in M13mp9	This work
mWH508		*Xma*I–*Hin*dIII (truncated *tet*R^B) from mWH501 in M13mp9	This work
mWH503		mWH508 with introduced *Sal*I site	This work
mWH524		mWH508 with introduced *Sca*I, *Sal*I, *Nae*I, *Mlu*I and *Xho*I sites	This work
mWH531		mWH508 with introduced *Sal*I, *Nae*I and *Mlu*I sites	This work
mWH535		mWH508 with introduced *Nae*I site	This work
mWH536		mWH508 with introduced *Nae*I and *Mlu*I sites	This work
mWH256		mWH507 with introduced *Hin*fI site	This work
mWH556		mWH507 with introduced *Sca*I, *Sal*I, *Mlu*I and *Nru*I sites	This work
mWH557		mWH507 with introduced *Sca*I, *Sal*I, *Xho*I and *Nru*I sites	This work
mWH561–564		Truncated *tet*R$^{BA8\text{-}92,\ BA22\text{-}54,\ BA22\text{-}40,\ BA40\text{-}54}$ in M13mp9rev	This work
pWH305	amp^r	*tet*R^B under control of the λ P_L promoter	Oehmichen *et al.* (1984)
pWH321	amp^r	*tet*R^A under control of the λ P_L promoter	Klock (1985)
pWH504	amp^r	*tet*R$^{BA22\text{-}23}$ under control of the λ P_L promoter	This work
pWH565-570	amp^r	*tet*R$^{BA40,\ BA8\text{-}216,\ BA8\text{-}92,\ BA22\text{-}54,\ BA22\text{-}40,\ BA40\text{-}54}$ under control of the λ P_L promoter	This work
pWH510-517	kan^r	*tet*R$^{B,\ BA22\text{-}23,\ BA40,\ BA40\text{-}54,\ BA22\text{-}40,\ BA22\text{-}54,\ BA8\text{-}92}$ and *tet*R$^{BA8\text{-}216}$ in pWH1200, reverse orientation to *bla*	This work
pWH1211,1212	kan^r	*tet*R$^{A,\ AB40}$ in pWH1201, same orientation as *bla*	This work
mWH250		*lac* promoter/operator region of M13mp11 replaced by a bidirectional transcription terminator	This work
mWH255		*tet*R^A–*lacZ* translational fusion in mWH250	This work
pWH912	ColE1^{imm}	Source of *tet*^{A} regulatory region	K.Tovar *et al.* (in preparation)
pWH951	amp^r	Source of bidirectional transcription terminator	Schollmeier *et al.* (1985)
λplac5·T743	*lacZ*$^-$	*lac* operon with a mutation destroying the *lac* promoter	Yu and Reznikoff (1984)
λRZ5	*lacZ*$^+$	*tet*A^B–*lacZ* transcriptional fusion	Wissmann *et al.* (1986)
λWH100	*lacZ*$^+$	*tet*R^A–*lacZ* translational fusion	This work

Materials and methods

Materials

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, calf intestine phosphatase and *E.coli* DNA polymerase I Klenow fragment were obtained from Boehringer (Mannheim, FRG), [α-^{32}P]ATP and [γ-^{32}P]ATP from Amersham (Braunschweig, FRG) and SeaPlaque LGT Agarose from FMC (Rockland, ME). Reagents for oligonucleotide synthesis were from Pharmacia (Freiburg, FRG), and all other chemicals were of the highest purity available from Merck (Darmstadt, FRG), Serva (Heidelberg, FRG), Roth (Karlsruhe, FRG) or Sigma (St Louis, MO).

Bacterial strains, plasmids and phages

All bacterial strains used were derivatives of *E.coli* and are listed in Table III. RR1 (Bolivar *et al.*, 1977) was generally used for cloning experiments with plasmids. When vectors contained a λP_L promoter, K12Δ*H1*Δ*trp* (Remaut *et al.*, 1981) was used. JM101, JM109 (Yanisch-Perron *et al.*, 1985) and BMH71-18 (Kramer *et al.*, 1982) served as hosts for M13 derivatives. In the course of oligonucleotide-directed mutagenesis BMH71-18 *mut*S, MK30-3 (Kramer *et al.*, 1984) and RZ1032 (Kunkel, 1985; T.Kunkel, personal communication) were used as published. Homologous recombination between M13 derivatives and λ phages was done in WH201 (gift of L.V.Wray, Jr). For propagation of λ phages the strain XA103 (Yu and Reznikoff, 1984) was used and λ lysogens were made in CSH26 (Wissmann *et al.*, 1986). The plasmids and phages with their relevant properties are listed in Table IV.

Oligonucleotide-directed mutagenesis of tetR^B

An *Xba*I–*Hin*dIII fragment from pWH305 containing the truncated *tet*R^B gene (Oehmichen *et al.*, 1984) was inserted in M13mp11 (Messing, 1983) to yield mWH501, recloned as an *Xma*I–*Hin*dIII fragment into M13mp9 (Messing and Vieira, 1982) and the resulting phage was called mWH508. Oligonucleotide-directed mutagenesis was performed using the gapped duplex method as described (Kramer *et al.*, 1982, 1984). The mutants were derived from single-stranded mWH508 hybridized with the *Eco*RI–*Hin*dIII vector fragment of M13mp9*rev* (Kramer *et al.*, 1984). The five mutagenic oligonucleotides, numbers 7–11, shown in Table I were employed in a single experiment carried out otherwise as described (Kramer *et al.*, 1984).

Oligonucleotide-directed mutagenesis of tetR^A

An *Eco*RI–*Sal*I fragment from pWH321 (Klock, 1985) with the *tet*R^A gene was inserted in M13mp11 to yield mWH505 and recloned as an *Eco*RI–*Hin*dIII fragment into M13mp9 to give mWH507. Single-stranded mWH507 grown on *E.coli* RZ1032 was hybridized with the *Eco*RI–*Hin*dIII vector fragment of M13mp9*rev* and the five mutagenic oligonucleotides, numbers 1–5, shown in Table I were added. After fill in and ligation, *E.coli* BMH71-18 *mut*S was transformed with the reaction mixture and the resulting phages were analysed (Kunkel, 1985). The mutant mWH256 was generated from single-stranded mWH507 and the *Eco*RI–*Hin*dIII fragment of M13mp9*rev* using oligonucleotide 6 shown in Table I as described (Kramer *et al.*, 1984). The mutant was identified by sequencing.

Analysis of mutants

Phage particles were grown, precipitated with PEG and suspended in 1 ml of 10 mM Tris−HCl, pH 8.0, 0.1 mM EDTA. DNA from 96 candidates resulting each from 30 μl of the original culture were spotted on 10 cm × 10 cm Biodyne A nylon membranes, and hybridized with 4×10^6 d.p.m. 5′-^{32}P-labelled mutagenic oligonucleotide with sp. act. 9000 Ci/mmol as described (Gatz *et al.*, 1986). Membranes were washed in 6 × SSC for 1 min at T_d −10°C, T_d −2°C and T_d +2°C and autoradiographed after each wash. T_d values were calculated as published (Wallace *et al.*, 1979). Mutants could be unambiguously distinguished from wild-type sequences at either T_d −2°C or T_d +2°C. *tet*R^B mutants with the desired combination of restriction sites (see Figure 2) were completely sequenced (Davies, 1982), while the *tet*R^A mutants (see Figure 2) were sequenced from the *Eco*RI site to codon 95. The designation of the mutant phages are given in Table IV.

Construction of plasmids for constitutive expression of wild-type and mixed tetR genes

Constitutive expression of *tet*R genes was achieved in using plasmids similar to pRT240 and pRT241 (Wray and Reznikoff, 1983; Wissmann *et al.*, 1986). To facilitate the cloning procedures the 65-bp *Hae*III−*Nru*I fragment from the polylinker of pIC-20R (Marsh *et al.*, 1984) was inserted into the *Hin*cII site of pACYC177 (Chang and Cohen, 1978) to yield pWH1200 with the *Nru*I end proximal to the *bla* promoter and pWH1201 with the opposite orientation.

Since the mutant *tet*R^B genes lack the translation start codons they were first assembled to complete genes on pWH305 derivatives (Oehmichen *et al.*, 1984) and then recloned into pWH1200. First truncated mixed genes of *tet*R$^{BA8\text{-}92,\ BA22\text{-}54,\ BA22\text{-}40,\ BA40\text{-}54}$ were constructed by exchanging the appropriate fragments in the respective M13 derivatives to yield mWH561−564 (see Table IV). *Xba*I−*Hin*dIII fragments of these phages were cloned into pWH305 replacing the wild-type *tet*R^B sequence to yield pWH567-570 (see Table IV). Replacement of the *Sal*I fragment spanning the *tet*R$^{BA8\text{-}92}$ from codon 22 into the vector with the *Sal*I fragment from mWH556 yielded the *tet*R$^{BA8\text{-}216}$ gene on pWH566 (see Table IV). The *Xba*I−*Hin*dIII fragments from mWH535 and mWH503 were inserted in pWH305 to yield the *tet*R^{BA40} and *tet*R^{BA22,23} genes on pWH565 and pWH504 respectively (see Table IV).

The *tet*R^B gene and its derivatives containing the *tet*R^B start codon were cloned as *Eco*RI−*Ssp*I (except for *tet*R$^{BA8\text{-}216}$ from pWH566, which was cloned as an *Eco*RI−*Fsp*I) fragments in pWH1200 digested with *Eco*RI−*Hin*cII. The resulting plasmids are named as in Table IV. They express the TetR proteins at low level (Bertrand *et al.*, 1984; Wissmann *et al.*, 1986; Meier *et al.*, 1988).

*tet*R^A from pWH321 and *tet*R^{AB40} from mWH256 were cloned as *Eco*RI−*Sal*I fragments into pWH1201, where they are probably transcribed from the *bla* promoter (Wray and Reznikoff, 1983; Bertrand *et al.*, 1984; Wissmann *et al.*, 1986; Meier *et al.*, 1988). The resulting plasmids pWH1210 and 1211 are given in Table IV.

Construction of the tetR^A−lacZ translational fusion

First the *lac* promoter in M13mp11 was deleted by digestion with *Nar*I and *Sal*I and religation after fill in of the protruding ends to regenerate the *Sal*I site (gift of L.V.Wray, Jr). Then the *Hpa*I−*Sal*I fragment from pWH951 (Schollmeier *et al.*, 1985), containing the bidirectionally active *tet* terminator fused with a *Bam*HI linker on the *Hpa*I side, was inserted between the *Bam*HI and the *Sal*I sites to yield mWH250. The regulatory region from Tn*1721* was isolated from pWH912 (K.Tovar *et al.*, manuscript in preparation) as a 78-bp *Eco*RI−*Tha*I fragment and cloned between the *Eco*RI and *Sma*I sites of mWH250. This generated a *tet*R^A−*lacZ*′ translational fusion with the sequence 5′-ATG ACA AAG TTG CAG AAT TCA-3′, in which the fusion gene is transcribed from the *tet*P$_R$ promoter and regulated by the tandem *tet* operators from Tn*1721* (Klock and Hillen, 1986). The phage was named mWH255.

Escherichia coli WH201 (see Table III) was infected with mWH255 and then superinfected with λplac·T743 (Yu and Reznikoff, 1984) to cross the *tet*R^A−*lacZ*′ fusion to the λ phage by homologous recombination. Resulting λ phages were plated on *E.coli* XA103 to separate them from M13 phages and the plaques screened for β-galactosidase activity. A *lacZ*$^+$ λ phage was isolated, verified by restriction analysis, named λWH100 and used to produce lysogens in *E.coli* CSH26. The β-galactosidase expression of 10 resulting strains was determined and single lysogens were identified.

β-Galactosidase assays

Escherichia coli CSH26/λRZ5 and CSH26/λWH100 were transformed with the repressor expressing plasmids given in Table II. Four to eight independent colonies were selected and the β-galactosidase activity determined as described (Miller, 1972) from cultures grown in LB medium supplemented with the appropriate antibiotics. In the case of *E.coli* CSH26/λWH100 strains, β-galactosidase activities were determined from overnight cultures, whereas *E.coli* CSH26/λRZ5 strains were used as log-phase cultures.

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