Quantitative analysis of Tn*10* Tet repressor binding to a complete set of *tet* operator mutants

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ABSTRACT

A saturating oligonucleotide-directed mutagenesis of both *tet* operators in the *tet* regulatory sequence was performed yielding mutants with four identical base pair exchanges at equivalent positions in the four *tet* operator half sides. The mutants were cloned between bipolar *lacZ* and *galK* indicator genes on a multicopy plasmid allowing the quantitative analysis of their effects *in vivo*. In the absence of Tet repressor the mutations lead to considerably different expression levels of both genes. They are discussed with respect to the promoter consensus sequences. In particular, the −10 region of the *in vivo* active *tet*P_{R2} promoter is unambiguously defined by these results. In the presence of Tet repressor most of the mutants exhibit a lower affinity for that protein as determined quantitatively by their reduced expression levels. In general, *tet* operator recognition is most strongly affected by alterations of base pairs near the center of the palindromic sequence. The most important position is the third base pair, followed by base pairs two, four, five and six, the latter showing similar effects as base pair one. At each position, the four possible base pairs show different affinities for Tet repressor. They are discussed according to their exposure of H-bond donors and -acceptors in the major and minor grooves of the B-DNA. The results are in agreement with major groove contacts at positions two, three and five. At position four a low potential correlation of efficiencies with the H-bonding in the minor groove is found, while mutations at position six seem to influence repressor binding by other mechanisms.

INTRODUCTION

Many procaryotic DNA binding proteins recognize their target sequences using an α-helix-turn-α-helix supersecondary structure (1). Genetic (2,3) and biochemical studies (4,5) suggest that the Tn*10* Tet repressor belongs to this group (6). It is assumed that the recognition α-helix makes sequence specific contacts to *tet* operator in the major groove of the DNA double helix. This model has been supported by modification interference and protection studies of Tet repressor-*tet* operator binding (7,8) which indicated that the two *tet* operator half sides are identically recognized by a Tet repressor dimer (9). Saturation mutagenesis of a *tet* operator half side suggested that the inner base pairs of *tet* operator make the most important contacts to Tet repressor (10).

Recognition of the *tet* operator sequence by Tet repressor is thought to be achieved by interactions between functional groups of amino acid residues with those of the base pairs. Therefore, a systematic sequence variation of the *tet* operator DNA followed by a quantitative analysis of repressor binding should yield information about the nature of these contacts. In a previous study the quantification of Tet repressor binding to the *tet* operator variants was not possible when their affinity was severely reduced (10). In this study we present an *in vivo* system allowing the quantitative differentiation of Tet repressor affinity for these mutants. It is used to determine the effects of symmetrical mutations in both *tet* operators of the wild type *tet* regulatory region (11) on Tet repressor binding. This can be quantified by measuring *tet* promoter P_A driven expression of β-galactosidase and *tet* promoter P_R driven expression of galactokinase (12,13). The results are interpreted with respect to possible contacts of Tet repressor to *tet* operator. Differential effects in repressor binding can be explained by altered exposures of H-bond donors and acceptors on the surface of the DNA double helix (14).

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were obtained from Boehringer (Mannheim, FRG), Sequenase™ (modified T7 polymerase) was purchased from USB (Cleveland, OH). [α-^{32}P]d-ATP was purchased from Amersham (Braunschweig, FRG). 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 (München, FRG).

Bacterial strains, phages and plasmids

All bacterial strains used are derivatives of *Escherichia coli*. JM101 served as a host for M13 derivatives and is F'*tra*D36 *lac*I^q ZΔM15 *pro*AB$^+$/Δ(*lac pro*AB) *sup*E *thi* (15). In the

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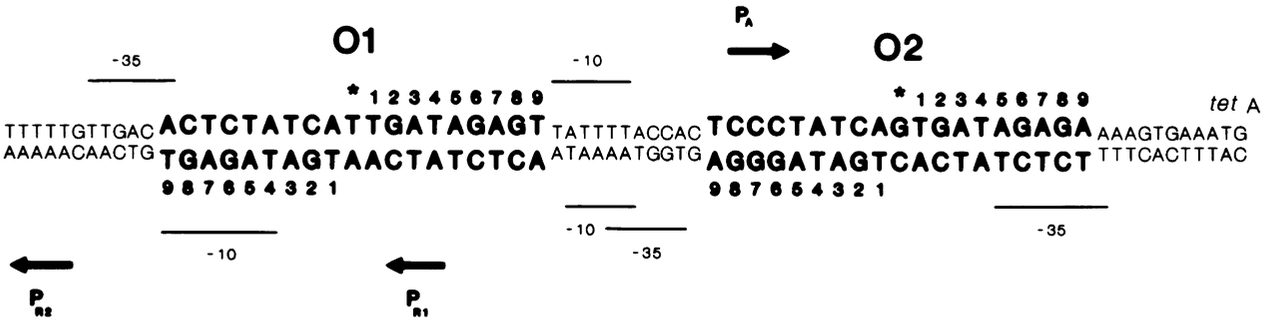

Fig.1 Nucleotide sequence of the Tn*10* encoded wild type *tet* regulatory region. The sequence of the tandem *tet* operators O_1 and O_2 is displayed in bold and enlarged print, the palindromic center is marked with a star. The numbering above and underneath the sequence designates the positions within each operator. The tandem, overlapping promoters P_{R1} and P_{R2}, responsible for transcription of the *tet*R gene are shown in the lower half of the figure, the promoter P_A, responsible for transcription of the *tet*A gene is shown in the upper half of the figure. The bold arrows define the direction, their blunt ends correspond to the respective starting points of transcription.

course of oligonucleotide directed mutagenesis, RZ1032 was used as a host for producing uracil containing DNA. It is HfrKL 16 PO/45 [*lys*A(61−62)], *dut*l, *ung*l, *thi*l, *rel*Al, Zbd-279::Tn*10*, *sup*E44 (16). GM1853 served as a *dam*$^-$ host for determining *lac*Z and *gal*K activities of one operator mutant containing a *dam* methylation site and is *dam*$^-$, *dcm*$^-$, Δ(*lac-pro*), *thi*, *gal*, *ara*, *ton*R, *tsx* (M.G. Marinus, personal communication). WH207 served as the strain, in which β-galactosidase and galactokinase assays were generally performed and is Δ(*lac*X74), *gal*K, *rec*A (details will be published elsewhere). The phage mWH460 is a derivative of M13mp9 in which a 140bp DNA fragment, containing the *tet* regulatory region was cloned (17), and served as the vector for oligonucleotide directed mutagenesis of the tandem *tet* operators. The constructions of the plasmids pRT240 and pWH1200 were described previously (3,18). pRT240 contains the Tn*10* *tet*R repressor gene which is constitutively expressed at a high level. pWH1200 does not encode for Tet repressor and serves as a control plasmid for determining the maximal expression level for all operator mutants.

Oligonucleotide directed mutagenesis of the tandem *tet* operators

The 140bp *tet* fragment in mWH460 contains the tandem *tet* operators and served as the vector for site directed mutagenesis according to the protocol of Kunkel et al. (16). For the mutagenesis, a 28b oligonucleotide for O_1 and a 24b oligonucleotide for O_2 were used simultaneously in the same experiment. The desired mutants with four base alterations were identified by sequencing. The mutant mWH460 derivatives as well as wildtype mWH460 were restricted with *Bam*HI and the resulting 140bp fragments were cloned into the *Bgl*II site of pCB302b (13). The orientations of the 140bp *tet* fragments were determined by restriction analysis and plasmids containing the *tet* regulatory regions as *tet*A-*lac*Z and *tet*R-*gal*K transcriptional fusions were used in the further analysis. The construct containing the wild type *tet* regulatory region was named pWH1012. All *tet* operator mutants are therefore derivatives of pWH1012. In order to determine β-galactosidase and galactokinase activities *in vivo*, the mutant plasmids, as well as pWH1012 and pCB302b, were cotransformed into the respective *E.coli* host strains with either pRT240 or pWH1200.

β-galactosidase and galactokinase assays

Cells were grown at 37°C in LB medium for the β-galactosidase assays or glucose minimal medium for galactokinase assays, supplemented with the appropriate antibiotics. Mid-log phase cultures were assayed for β-galactosidase activity exactly as described by Miller (19) and for galactokinase activity as described by McKenney et al. (20). Three independent cultures were assayed for each strain and measurements were repeated at least twice.

The expression levels are presented as percent of the wildtype in the absence of Tet repressor. The repression efficiency of each mutant in the presence of Tet repressor is given in percent of the non-repressed expression.

Table I. β-galactosidase activities expressed from the *tet*P_A-*lac*Z fusion of the *tet* operator mutants in the presence and absence of Tet repressor

Plasmid[1]	β-galactosidase activity[2] (%)		Repression[3] (%)
	−*tet*R	+*tet*R	
pCB302b	0±0	0±0	–
pWH1012	100±3	0,1±0	100±1
pWH1012-1G	88±2	12±0.9	87±2
pWH1012-2A	164±7	68±2	59±3
pWH1012-2C	87±3	84±4	8±6
pWH1012-2T	108±4	16±0.8	86±2
pWH1012-3C	83±3	49±2	45±5
pWH1012-3G	102±3	89±6	13±6
pWH1012-3T	94±3	61±3	36±6
pWH1012-4A	155±4	29±0.5	82±1
pWH1012-4C	87±1	37±1	58±2
pWH1012-4G	70±2	55±2	22±5
pWH1012-5C	111±4	90±5	20±8
pWH1012-5G	136±4	130±4	5±6
pWH1012-5T	134±3	10±0.3	93±0
pWH1012-6A	41±4	0.3±0.1	99±0
pWH1012-6C	83±2	23±0.6	73±2

[1] in *E.coli* WH207 transformed with pWH1200 (−*tet*R) or pRT240 (+*tet*R), respectively.
[2] determined as described by Miller (19). 100% β-galactosidase activity corresponds to 398 ± 11 units whereas 0% β-galactosidase activity corresponds to values below 0.4 units.
[3] a value of 0% corresponds to no repression, a value of 100% to maximal repression.

RESULTS

Fig.1 shows the nucleotide sequence of the Tn *10* encoded wild type *tet* regulatory region with the overlapping promoters and tandem *tet* operators indicated (7,11). The sequence of the operators is numbered to facilitate the designation of the mutations. Only mutations that showed severe effects on Tet repressor binding which could not be quantified in a previous

Table II. Galactokinase activities expressed from the *tet*P_R-*gal*K fusion of the *tet* operator mutants in the absence and presence of Tet repressor.

Plasmid[1]	galactokinase activity[2] (%)		Repression[3] (%)
	− *tet*R	+ *tet*R	
pCB302b	0 ± 0.03	0 ± 0.01	−
pWH1012	100 ± 4	2 ± 0.4	98 ± 0
pWH1012-1G	56 ± 3	5 ± 0.3	91 ± 2
pWH1012-2A	86 ± 2	25 ± 2	71 ± 3
pWH1012-2C	7 ± 0.5	6 ± 0.4	14 ± 10
pWH1012-2T	20 ± 2	3 ± 0.1	85 ± 2
pWH1012-3C	80 ± 4	39 ± 3	52 ± 7
pWH1012-3G	66 ± 5	49 ± 4	26 ± 10
pWH1012-3T	88 ± 6	52 ± 4	41 ± 9
pWH1012-4A	16 ± 0.9	2 ± 0.3	87 ± 3
pWH1012-4C	7 ± 0.3	2 ± 0.1	72 ± 3
pWH1012-4G	6 ± 0.1	4 ± 0.1	34 ± 3
pWH1012-5C	4 ± 0.1	4 ± 0.08	0 ± 5
pWH1012-5G	5 ± 0.5	4 ± 0.3	19 ± 14
pWH1012-5T	8 ± 0.7	1 ± 0.3	87 ± 5
pWH1012-6A	143 ± 11	3 ± 0.1	98 ± 1
pWH1012-6C	72 ± 0.1	20 ± 1	73 ± 2

[1] in *E.coli* WH207 transformed with pWH1200 (−*tet*R) or pRT240 (+*tet*R), respectively.
[2] determined as described by McKenney (20). 100% galactokinase activity corresponds to 260 ± 10 units whereas 0% galactokinase activity corresponds to values below 0.5 units.
[3] a value of 0% corresponds to no repression, a value of 100% to maximum repression.

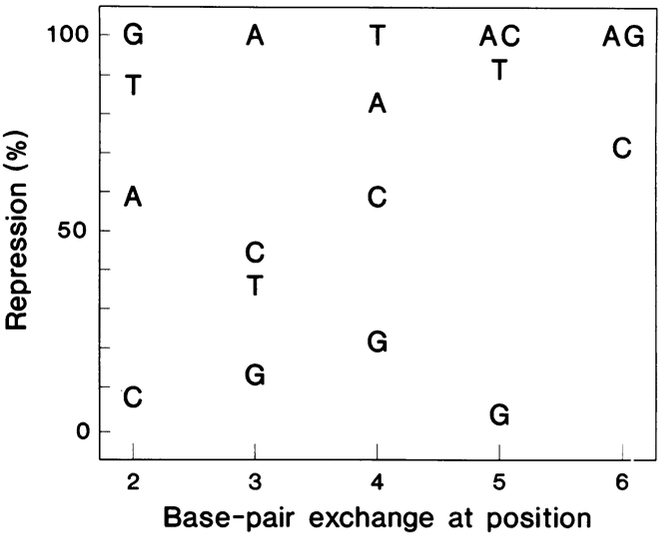

Fig.2 Diagram of the repression results obtained with each operator mutant. The nucleotides studied at each position are shown. All data are taken from Table I, except for mutation 5C, where the data are taken from Table III (see DISCUSSION section) . Each nucleotide beginning from position 2 to position 6 is shown with respect to its position and% repression. The results at position 1 were omitted.

experiment (10) were included in this study. Each mutant contains four nucleotide replacements at equivalent positions in the four operator half sides as indicated by the numbering, e.g. the mutant 1G contains replacement of TA by GC at the four positions termed 1. In addition to this mutant all possible mutants for positions 2, 3, 4, 5 and 6 were constructed except for the replacement of GC by TA at position 6 which could not be obtained in several independent experiments. We therefore assume that this mutation is not viable in the M13 phage used for mutagenesis. After construction and verification, the mutant *tet* regulatory regions were cloned as transcriptional fusions of the P_A promoter to *lac*Z and the P_R promoters to *gal*K. The expression data of the *tet*P_A-*lac*Z fusions with the different *tet* operator mutations are given in Table I. The non-repressed activities vary between 41% and 164% of the β-galactosidase expression directed by the wild type *tet* regulatory region. This indicates that the mutations studied here affect either *tet*P_A promoter activity or the efficiency of translation of the *lac*Z gene, since *tet* operator O_2 is part of the *tet*A-*lac*Z mRNA (21). Combined effects may also be possible. The expression data of galactokinase directed from the *tet*P_R-*gal*K fusion are shown in Table II. The activities vary between 143% and 4% of the wild type. This is most likely due to altered promoter strengths because (i) the *tet*R mRNA is not affected by these mutations (11) and (ii) the −10 region of the most effective P_{R2} promoter accounting for about 95% of the total P_R activity (GUlland et al., in preparation) overlaps entirely with *tet* operator O_1 (7).

For the quantification of Tet repressor binding to the mutant *tet* operators *in vivo*, pRT240 was cotransformed as a source of constitutive expression of Tet repressor (18). Since pWH1012 and its mutants are high copy number plasmids and pRT240 leads to high level expression of Tet repressor, this *in vivo* system is able to distinguish Tet repressor binding to *tet* operator mutants in a different affinity range than a previously used *in vivo* system (10). This fact is clearly demonstrated by the β-galactosidase activities given in Table I. Out of the fifteen *tet* operator mutants examined, only one, namely 6A, shows nearly the same efficiency of repression as the wild type operator. All the other mutants exhibit differential effects on Tet repressor binding. The wild type *tet*P_A-*lac*Z fusion is repressed from 398 ± 11 U in the absence, to 0.35 ± 0.03 U in the presence of Tet repressor. This corresponds to 100% ± 1% repression. The repression efficiencies obtained for the mutant operators are also presented in Table I. Fig.2 shows a diagram of the effects of these mutants on the binding affinities of Tet repressor. The data presented in Table I and Fig.2 indicate that indeed all mutations studied here exhibit different quantitative effects on repressor binding.

The expression data for galactokinase from the *tet*P_R-*gal*K fusions are also used to quantitate the *in vivo* binding of Tet repressor to the *tet* operator mutants (see Table II). They provide an independent control of the β-galactosidase expression results shown above. As anticipated, the relative repression of each *tet* operator mutant is almost the same in both experiments.

The wildtype TA at position 1 is only slightly more effective than a GC base pair. At position 2 a mutant TA base pair is almost recognized as well as the wild type GC. The same is true for the AT to TA exchange at position 5 and, in particular, the GC to AT exchange at position 6. All of these mutants were clearly classified as down mutations in the previously used *in vivo* system (10). Thus, these results demonstrate the different affinity ranges of the two *in vivo* systems.

The CG mutation at position 5 creates a GATC *dam* methylation site in each *tet* operator half side (22). In order to distinguish the *dam* methylation effect on repressor binding in this operator mutant from the effect exerted by the base pair substitution, all mutations at this position were assayed in *E. coli* GM1853, a *dam*$^-$, *lacZ*$^-$, *gal*$^-$ strain. The results for β-galactosidase and galactokinase activities are given in Tables III and IV, respectively. Although high standard deviations were obtained for the β-galactosidase and galactokinase activities in this strain, exchange of the wild type AT by CG or TA showed

Table III. β-galactosidase activities expressed from the *tet*P_A-*lac*Z fusions of the *tet* operator mutants at position 5 in the absence and presence of Tet repressor using a *dam* $^-$ host strain

Plasmid[1]	β-galactosidase activity[2] (%)		Repression[3] (%)
	− *tet*R	+ *tet*R	
pCB302b	0 ± 0	0 ± 0	−
pWH1012	100 ± 15	3 ± 0.3	97 ± 0
pWH1012-5C	274 ± 10	3 ± 0.5	99 ± 0
pWH1012-5G	336 ± 20	200 ± 15	41 ± 9
pWH1012-5T	536 ± 52	199 ± 41	63 ± 12

[1] in *E.coli* GM1853 transformed with pWH1200 (−*tet*R) or pRT240 (+*tet*R), respectively.
[2] determined as described by Miller (19). 100% β-galactosidase expression correspond to 3121 ± 468 units whereas 0% β-galactosidase expression corresponds to values below 0.1 units.
[3] a value of 0% corresponds to no repression, a value of 100% to maximum repression.

Table IV. Galactokinase activities expressed from the *tet*P_R−*gal*K fusions of the *tet* operator mutants at position 5 in the absence and presence of Tet repressor using a *dam* $^-$ host strain

Plasmid[1]	galactokinase activity[2] (%)		Repression[3] (%)
	− *tet*R	+ *tet*R	
pCB302b	0 ± 0	0 ± 0	−
pWH1012	100 ± 8	21 ± 3	79 ± 5
pWH1012-5C	28 ± 1	8 ± 0.6	72 ± 2
pWH1012-5G	32 ± 2	22 ± 0.8	31 ± 7
pWH1012-5T	35 ± 10	9 ± 1	72 ± 12

[1] in *E.coli* GM1853 transformed with pWH1200 (−*tet*R) or pRT240 (+*tet*R), respectively.
[2] determined as described by McKenney (49). 100% galactokinase expression corresponds to 47 ± 4 units whereas 0% galactokinase corresponds to values below 2 units.
[3] a value of 0% corresponds to no repression, a value of 100% to maximum repression.

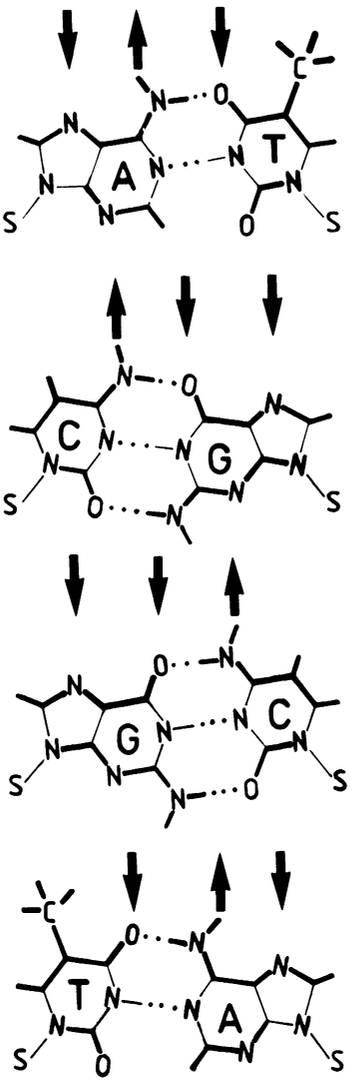

Fig.3 Structures of the four base pairs aligned according to their positioning in the B-form structure of DNA. S denotes the sugar residue. The arrows above each set of structures correspond to the possible H-bond donors (up) and H-bond acceptors (down). The bold lines represent the segments of the nucleotides which are exposed to the minor and major grooves of the DNA double helix.

better repression than exchange by a GC. Furthermore, all three mutations showed a much higher β-galactosidase expression in the absence of Tet repressor in the *dam*$^-$ strain than in the *dam*$^+$ strain.

The galactokinase expression data presented in Table II generally show the same repression efficiencies for most of the mutant operators with only a few exceptions. The AT mutation at position 2 leads to a more efficient repression of the P_R-*gal*K fusion than the P_A-*lac*Z fusion. The mutations at position 4 and 5 exhibit severe promoter down effects of P_R. We, thus, think that the results from the P_A-*lac*Z fusions are more reliable.

DISCUSSION

The analysis of the *tet* operator mutations yields information on two aspects of the *tet* regulatory region. We will first discuss their effects on promoter activity and then the differential affinities of the mutant operators for Tet repressor.

The expression levels of β-galactosidase (Table I) and galactokinase (Table II) in the absence of Tet repressor are clearly affected by most of the mutations. The most obvious explanation, namely alterations in copy number of pWH1012 caused by the mutations, can be clearly ruled out because they would affect both indicator genes to the same extent. This is only observed for the 3C mutant where both expression levels are about 80% of the wild type. Thus, possible copy number effects are not important and the differential expression of both indicator genes must be due to regulatory effects of the mutations. The *tet* regulatory sequence is shown in Fig.1. For the case of β-galactosidase expression it cannot be unambiguously distinguished whether these effects reflect different promoter strengths or whether they are related to changes in the 5′ end of the mRNA, which could result in altered efficiencies of translation (21). However, since these are transcriptional fusions, the major expression differences should result from variation of the promoter strength. The different P_R-*gal*K expressions should clearly reflect promoter mutations because the transcribed mRNA sequence is not affected by the mutations. None of the mutations affect the promoter consensus boxes of P_A or the initiation nucleotide of the mRNA and yet, the expression of β-galactosidase differs between 41% (AT position 6) and 164% (AT position 2). This could be due to translational coupling (23),

competition between the promoters P_A and P_R (24) for RNA polymerase or some as yet unidentified factors. In contrast some of the mutations affect the *tet* P_R consensus sequences which direct galactokinase expression. Inspection of Fig.1 reveals that *tet* operator O_2 overlaps with P_{R1} and *tet* operator O_1 overlaps with P_{R2}. The O_1 positions 4, 5 and 6 have been assigned to the first three nucleotides of the P_{R2} −10 consensus region (11). In agreement with this assumption any mutation of the highly conserved first T of the box is a severe P_R down mutation (27, see Table II). The same is true for all mutations affecting the A at position 5. The residual P_R activity may be ascribed to P_{R1} (Gülland et al., in preparation). At position 6 of *tet* operator O_1 P_{R2} differs from the −10 consensus sequence by containing a G instead of a T. Consequently, the replacement of this G by A is a promoter up mutation (25). A replacement of G by T was not obtained. We suspect that a TA at position 6 would increase P_R activity substantially by creating a much better −10 consensus box for P_{R2} (25). This may be the reason for the apparent lethality of this mutation in the M13 mutagenesis vector, where P_R is directed towards the origin of replication (26). Taken together, the mutations discussed so far confirm the assignment of the −10 consensus box for the P_{R2} promoter.

In addition, mutations outside the −10 region influence P_R activity to different extents. While mutations at position 3 do not affect P_R activity much, the G at position 2 seems to be quite important because replacement by C reduces P_R activity 14-fold and replacement by T and A reduce it 5- and 1.5-fold, respectively. Therefore, this position is clearly important for P_R promoter activity although it is not located in any known consensus region (25).

The *tet* operator O_1 is a perfect palindrome (see Fig.1) with each half side consisting of nine base pairs. With respect to the interaction with Tet repressor positions 2 to 6 were identified as the primarily recognized base pairs in a previous study (10). Here we clearly distinguish further among these positions. At present we cannot relate all of the results from both *in vivo* systems to binding constants which probably do not show a linear relation to the expression of the indicator genes presented here and in ref. 10. However, binding constants for two mutations have been determined previously *in vitro*: the GC to AT transition at position 6 distinguishes the class A from the class B *tet* operators (3). It has been shown that this difference markedly reduces cross-recognition of class A and B *tet* operators by the respective Tet repressors (3,27). The effect of this mutation on binding of Tn*10* Tet repressor has been clearly seen in the previously used repressor titration system (10) while it is recognized at the wild type level in the indicator system used here (see Fig.2 and Tables I and II). This indicates that the *in vivo* system used here distinguishes only between mutants with severely reduced affinity for repressor. The same conclusion is reached by comparing the effects of the GC to AT transition at position 2. It has been concluded from footprinting titration studies that this *tet* operator mutant binds Tet repressor with an association constant of approximately $4\times10^8\ M^{-1}$ (17). This is at least three orders of magnitude less compared to the wild type *tet* operator (28). In the repressor titration system this mutation appears as a non-binder (10) while it shows 59% repression in the system used here (see Fig.2). This gives an indication of the range of affinities distinguished in this study.

The affinity of Tet repressor for the *tet* operator mutants studied here can be derived from either the β-galactosidase or the galactokinase expression data (see Tables I and II). Both determinations yield nearly the same repression efficiencies for most operator mutants and are, therefore, independent repeat experiments.

The relative importance of the base pairs in *tet* operator for protein recognition may be derived from the average of their repression efficiencies for the three mutants at a given position. This procedure identifies position 3 as the most critical one, followed by positions 2, 4, 5, 6 and 1. Thus, the most important contacts clearly occur at base pairs oriented towards the palindromic center of the *tet* operator. Thereby they are somewhat displaced from the center between the contacted phosphates as defined by ethylation interference sites (8).

The quantitative differentiation of Tet repressor affinity for the complete set of operator mutants at a given position allows speculations concerning the functional groups of each base pair involved in interaction with repressor. This interpretation assumes that base specific chemical interactions can either be made in the major or minor groove of DNA (14). A correlation of repressor affinities with the location of the exocyclic functions in the respective base pairs may reveal hints about the contacted sites. The structure of the four base pairs are shown in Fig. 3 and compared with respect to their H-bonding abilities.

At position 2 of *tet* operator the wild type base pair is a GC and the most active mutation a TA (see Tables I and II). Inspection of Fig.3 reveals that both base pairs show a similar location of H-bond acceptors and donors in the major groove. The next best fit is obtained for AT which shows the second best recognition. The worst fit of the CG base pair correlates well with the lowest repression observed for this mutant. Thus, the results presented here are in good agreement with the assumption that the major groove at position 2 is contacted by Tet repressor. In addition, the similarity of GC and TA seem to indicate at least one contact in the center of the major groove at this position. In the minor groove, on the other hand, no correlation between the similarity of functional groups and repression is found.

For the mutant base pairs at position 3, the differences in repressor affinity are smaller, ranging only from 45% for CG and 36% for TA to 13% for GC compared to the wild type AT. This order shows a good correlation of recognition with H-bonding potential in the major groove. It suggests that a potential contact of the N(7) in the wild type AT base pair should be accompanied by other interactions (compare AT and GC). No correlation with H-bonding potential in the minor groove can be established.

The repression of *tet* operator mutants at position 4 varies between 82% (AT) and 22% (GC) of the wild type (TA). For this position the best fit of the major groove shows the lowest and the worst fit the highest repression. This result does not identify the major groove as the site of interaction with the repressor. No correlation of functional groups in the minor groove with repression efficiencies is found either (see Fig.3).

At position 5 the CG mutation creates a *dam* methylation site. Therefore, the relative repression has to be compared in a *dam*$^-$ strain. In this background the CG mutation shows a greater repression than in the *dam*$^+$ strain. This indicates that *dam* methylation at the N(6) of adenines at positions 3 and 4 interferes with Tet repressor binding. The order of repression efficiencies is similar to the order determined for position 3, with CG and TA being most effective. Thus, this indicates contacts in the major groove of the DNA double helix to Tet repressor, although in general, contacts at this position seem to be quantitatively less important than at position 3. The GC mutation, which should

not be affected by *dam* methylation, shows a much better repression in the *dam*$^-$ compared to the *dam*$^+$ strain. The reasons for this observation are not clear.

The contacts at position six are least important as indicated by the high repression found for the two mutations studied. The replacement of wild type GC by AT does not result in any loss of repression while a CG still yields 73% repression. The TA has not been obtained but showed the same effect as the CG substitution in the previous study (10). Taken together, the correlation of repressor binding with functions in the major or minor groove is not perfect. It may be that this base pair influences repressor recognition by other mechanisms or that only part of the functions are contacted.

Finally, at position 1 only one mutant was studied (replacement of TA by GC). It should be noted that the GC base pair at position 1 of *tet* operator O_1 leads to methylation of the C's at position 1 by *dcm* methylase. Nevertheless, the repression is nearly as efficient as in the wild type, showing that the methyl group at N(6) of C does not interfere with repressor binding. However, it has been previously proposed that the N(7) of the adenine residue may be contacted by Tet repressor.

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