Purification of the TET Repressor and TET Operator from the Transposon Tn10 and Characterization of Their Interaction*

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The TET repressor binds ['H]tetracycline with an apparent association constant greater than 10^7 liters•mol^-1. The formation of the TET repressor•tetracycline complex is optimal between pH 7.5 and pH 12 and is reduced below pH 7.5. At pH 5.0, the TET repressor is inactive. The thermal stability extends up to 55 °C and drops sharply to zero between 55 °C and 65 °C. Specific binding of the TET repressor to the TET operator is demonstrated by the protection of a single recognition site for the restriction endonuclease HinfI out of two sites on the respective DNA fragment. This function of the TET repressor is inhibited in the presence of 4 µM tetracycline. The location of the operator is determined within 100 base pairs by the lack of protection of other restriction sites. The interaction of the TET repressor with the TET operator is characterized in vitro using short, homogeneous DNA fragments containing the Tn10-encoded TET gene control sequences, which were cloned under reconstruction of the Eco RI sites into pVH51. These fragments contain the TET gene control elements and different parts of flanking sequences. The 187-base pair (bp) fragment was prepared in milligram amounts from multiple insertion plasmids and radioactively labeled by filling in the protruding Eco RI ends using [γ-32P]ATP. The TET repressor binds the 187-bp fragment with an apparent association constant greater than 10^7 liters•mol^-1. The stoichiometry is 4 TET repressor molecules/187-bp DNA. A 300-fold molar excess of a 180-bp fragment prepared from a HaI II digest of pVH51 does not compete with the 187-bp DNA for TET repressor binding. This result is interpreted as specific interaction of the TET repressor with the TET operator in the 187-bp fragment. This specific interaction is inhibited in the presence of tetracycline. The concentration dependence of the inhibition reveals that the association constant for the TET repressor•tetracycline complex exceeds the one for the TET repressor•TET operator complex. The thermal stability of the TET repressor•TET operator complex extends up to 60 °C and drops sharply between 65 °C and 70 °C. The TET repressor•TET operator complex is stable between pH 6.9 and pH 8.9. These results are discussed with respect to the molecular mechanism and the biological activity of the TET repressor•TET operator interaction.

The tetracycline resistance-encoding transposon Tn10 is 9.300-bp long (1, 2) and carries the genetic information for three tetracycline-inducible proteins (3-5). The 36-kDa protein has been called TET protein (3). It is regulated by a tetracycline-sensitive repressor (3) which also controls its own synthesis (6). Extensive analysis of deletion mutations of Tn10 allowed the assignment of the structural genes coding for the 36-kDa (TET) and the 25-kDa (TET-repressor) proteins (4, 6). Their regulatory regions consist of overlapping promoters and possibly two operators (6). Transcription proceeds in opposite directions from starting points that appear to be very close to each other (6). Fig. 1 presents a schematic drawing of the TET gene control region derived from data available at present (3, 4, 6, and this article). Similar genetic control regions were observed for the transposase gene in Tn3 (7, 8), bacteriophage Λ (9-12), and in plasmids such as pBR322 and pACYC (13).

A considerable amount of recent research is focused on the molecular basis of gene expression (reviewed in Ref. 14). The study of the mechanism of a genetic control region with divergent transcription which is co-regulated by a repressor would be of particular interest. Therefore, we purified the molecular components of the TET gene control system and characterized their interactions. This article describes the purification of the TET repressor protein, characterizes its interaction with tetracycline and demonstrates its specific binding to the TET operator sequence. Furthermore, the construction and analysis of recombinant plasmids that allow large scale purification of TET operator-containing DNA fragments with various amounts of flanking sequence upstream as well as downstream from the TET operator is described. The construction of the plasmids makes use of the reconstruction of Eco RI sites by ligating fragments resulting from digests of Tn10 DNA with HaI II, Alu I, or both into the single Eco RI site of pVH51 (15) after filling in the protruding ends (16-18). Multiple insertion plasmids were constructed for the 187-bp fragment to increase the yield of TET operator preparations (19). Finally, the 187-bp fragment, which contains the TET operator sequence, was prepared in

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The abbreviations used are: bp, base pairs; kDa, dalton 10^3; kbp, base pairs 10^3; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.
The repressor. The result is that only about association constant for the TET repressor. tetracycline com-
binds to the TET repressor regardless of its molar excess binding curve. With this specific activity of the \[^{3}H\]tetracy-
line bound to the nitrocellulose fiter is always less plex. However, it can be concluded that the apparent binding titrated a fmed amount of it with increasing amounts of TET detection and cannot be decreased to determine the precise repressor is presented in this article.

fold excess of unlabeled tetracycline. The half-lifetime at time of TET repressor. \[^{3}H\]tetracycline complexes by moni-
toring the exchange of prebound \[^{3}H\]tetracycline by a
complex to allow removal of free \[^{3}H\]tetracycline from
the fiter by rinsing. Therefore, we determined the half-life-
time of TET repressor-\[^{3}H\]tetracycline complexes by moni-
toring the exchange of prebound \[^{3}H\]tetracycline by a 500-
fold excess of unlabeled tetracycline. The half-lifetime at 20 °C is 65 min, which is long compared to a few seconds needed for the rinsing procedure. Fig. 6 shows the binding of \[^{3}H\] tetracycline to the TET repressor at different concentrations of tetracycline. At low concentrations of tetracycline, complex formation increases linearly with the concentration which is indicative of quantitative binding. In contrast, the amount of tetracycline bound to the nitrocellulose filter is always less than one-half of the input as determined by radioactivity. In order to determine the active fraction of \[^{3}H\]tetracycline, we titrated a fixed amount of it with increasing amounts of TET repressor. The result is that only about 35% of the tetracycline binds to the TET repressor regardless of its molar excess (data not shown). The instability of tetracycline in aqueous solutions has been reported previously (41). Therefore, we conclude that the active fraction of \[^{3}H\]tetracycline is quantitatively bound to the TET repressor at concentrations below 0.6 \muM in the experiment displayed in Fig. 6. At tetracycline concentrations greater than 1.4 \muM, a clear plateau is observed, and between 0.6 and 1.4 \muM, some curvature occurs in the binding curve. With this specific activity of the \[^{3}H\]tetracy-
cline, the concentrations in Fig. 6 are the lower limit of detection and cannot be decreased to determine the precise association constant for the TET repressor-tetracycline complex. However, it can be concluded that the apparent binding

constant of the TET repressor-tetracycline complex is at least 10^7 liters/mol, but probably greater. From the plateau value, the amount of active TET repressor can be determined. Using the apparent molecular weight of 25 kDa, this experiment reveals that 40.5% of the protein is active (Table II).

Fig. 7 displays the temperature dependence of the TET repressor-tetracycline interaction. Around 55 °C, a sharp decrease in activity occurs which probably reflects the thermal denaturation of the protein. At 62 °C, the tetracycline-binding activity is already close to zero. Denaturation of the TET repressor appears to be a highly cooperative process taking place in a temperature range of 10 °C between 55 and 65 °C.

Fig. 8 shows the pH dependence of the TET repressor-tetracycline interaction. The complex is stable at high pH up to a value of 12. Below pH 8.0, the complex stability decreases and the binding of tetracycline is completely inhibited at pH 5.0. The result in Fig. 8 is derived from nitrocellulose-binding experiments. This method cannot distinguish between an influence of pH on the TET repressor-tetracycline complex formation and an influence on the absorption of the complex on the nitrocellulose filters. Therefore, the result displayed in Fig. 8 was confirmed by equilibrium dialysis (not shown).

The binding of tetracycline was found to be independent on the concentrations of MgCl_2 and NaCl (data not shown). In protein mixtures the specificity of tetracycline binding de-

pends on the NaCl concentration. Therefore, it is possible that in nearly homogeneous TET repressor samples, the binding of \[^{3}H\]tetracycline at low concentrations of NaCl is an artifact, although the amount of bound \[^{3}H\]tetracycline remains unchanged.

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Fig. 6. Binding of \[^{3}H\]tetracycline to the TET repressor. 2 \mu g of TET repressor were incubated in 100 \mu l reaction mixtures with the indicated amounts of \[^{3}H\]tetracycline for 15 min at 37 °C. The amount of \[^{3}H\]tetracycline-TET repressor complex was determined by binding of the protein to nitrocellulose filters (see "Materials and Methods"). The binding curve demonstrates quantitative binding at these concentrations and it may be concluded that the association constant is at least 10^7 liters/mol, but most likely greater than that.
Interactions of the TET Repressor with the TET Operator—In order to demonstrate the specific interaction of the TET repressor protein with its operator, we isolated the 1450-bp Eco RI fragment from pRT29. The top panel in Fig. 9, displays the location of the two HincII sites and the Xba I and the Sau IIIa sites which are close to the proposed TET genetic control region (6). It has been reported that the HincII site next to this control region is protected from cleavage when RNA-polymerase is bound to the TET promoter (4) (see Fig. 1). Because the location of the operator should be close to the promoter, we tested whether the TET repressor is also able to prevent cleavage at this HincII site. The second HincII site in the 1450-bp fragment displayed on the left side of the top panel in Fig. 9 serves as an internal control of the specificity of TET repressor binding. In the case of specific binding to the operator, only the HincII site next to the Xba I site should be protected, whereas one would expect equal protection of both HincII sites in the case of nonspecific binding of the TET repressor to the DNA. According to the mechanism presented in Fig. 1, the presence of tetracycline should prevent the binding of TET repressor to the operator and, thus, result in the loss of cleavage protection of the HincII site.

The result of this experiment is displayed in Fig. 9. The top panel shows the location of the relevant restriction sites on the 1450-bp fragment. B shows the position of the operator as determined below. The gel picture gives the results of a HincII restriction digest of the 1450-bp fragment under various conditions. Lane A shows the marker fragments produced by a digestion of pBR322 with Hae III (39). Lane D is the 1450-bp fragment incubated without protein and lane C shows the result of a HincII digest of 0.7 µg 1450-bp fragment. In agreement with the restriction map in the top panel in Fig. 9, the 1450-bp fragment is cleaved twice by HincII resulting in 695-, 620-, and 138-bp fragments. Lane D shows the result of this restriction digest in the presence of 1 µg of TET repressor. It is apparent that the HincII cleavage site resulting in the 695- and 620-bp fragments is protected from cleavage under these conditions, whereas the 138-bp fragment occurs with nearly the same intensity as in lane C. Lane E shows the reaction products when incubation with HincII is done in the presence of 4 µM tetracycline in addition to the components of lane D. The presence of tetracycline results in the loss of cleavage protection by the TET repressor. These data demonstrate clearly that the TET repressor binds specifically to an operator sequence which is located close to this HincII site and that the presence of tetracycline prevents TET repressor binding to the operator.

Similar experiments were performed using the Xba I site 28-bp upstream and the Sau IIIa site 69-bp downstream of the HincII site (6) to monitor the distance covered by the TET repressor when bound on the DNA. Fig. 9 shows the location of these sites on the 1450-bp fragment in A. Neither the Xba I nor the Sau IIIa sites were protected from cleavage in the presence of the TET repressor (data not shown). Therefore, the specific binding site for the TET repressor appears to be within the 97 bp between the Xba I and the Sau IIIa sites on the 1450-bp fragment.

Interaction of the TET Operator with the TET Repressor—Fig. 10 displays the binding of the 187-bp DNA to the TET repressor with increasing amounts of the 187-bp fragment. The DNA is bound quantitatively until the total amount of TET repressor is saturated at these concentrations. It may be taken from Fig. 10 that the TET repressor-TET operator complex is retained to about 85% on the nitrocellulose filters. The concentration of TET repressor in this experiment was 2 × 10⁻⁷ M. Therefore, it may be concluded that the association constant for the 187-bp DNA-TET repressor complex is greater than 10¹⁰. When the total amount of repressor is saturated with DNA, no further increase of binding is observed. The amount of DNA bound to the TET repressor can be used to calculate the stoichiometry of the TET repressor-187-bp DNA complex. When the fraction of active TET repressor is determined by the tetracycline-binding assay (above), the analysis of the stoichiometry reveals, that 4 TET repressor molecules bind to one DNA fragment. This result is very similar to the observations made for the lac repressor-operator complex. It has been speculated that two different
operators exist for the TET gene and the TET repressor gene (6). If we assume that each operator binds a tetramer of the TET repressor, the results in Fig. 10 show that one of the two operators is occupied preferentially. This observation resembles the result of transcription of the genes in minicells, where the TET repressor gene was translated uninduced to a small extent whereas the TET gene was not (6).

In order to determine the specificity of the TET repressor-TET operator interaction, we performed competition experiments using a 180-bp fragment isolated from a Hae III digest of pH51 (19). Up to a 300-fold molar excess of the 180-bp fragment over the 187-bp DNA, we did not observe a reduced binding of the 187-bp DNA. Therefore, we conclude that the binding of the TET repressor to the 187-bp DNA reflects the specific interaction with the TET operator.

The dependence of the TET repressor-TET operator interaction on the concentration of MgCl₂ does not lead to clear results when measured by binding of the complex to nitrocellulose. Below 8 mM MgCl₂ only 30% to 60% of the radioactivity is retained on the filter. Thus, one cannot distinguish between direct Mg²⁺ influence on the TET repressor-TET operator complex or an unspecific effect on the binding of this complex to nitrocellulose. Furthermore, we tested if the binding of the 187-bp DNA to the TET repressor becomes unspecific at low MgCl₂ concentrations. At 2 mM MgCl₂, we did not find any competition of the 180-bp DNA with the 187-bp fragment. Thus, we conclude that the interaction is specific at low concentrations of Mg²⁺; however, for the nitrocellulose filter assay at least 8 mM Mg²⁺ should be present in the reaction mixture.

**Effect of Temperature on the TET Repressor-TET Operator Interaction**—Fig. 11 shows the effect of temperature on the TET repressor-TET operator interaction. A sharp drop in the activity occurs between 85 °C and 70 °C for the TET repressor-TET operator complex. This cooperative transition reflects probably the thermal denaturation of the TET repressor. The 187-bp fragment does not denature near 70 °C under these conditions.³

This result is rather similar to the one obtained above for the stability of the TET repressor-tetracycline complex. In the presence of the operator sequence, the TET repressor remains functional to about 5 °C higher temperatures than the presence of tetracycline. In order to verify this difference in thermal stability, a parallel incubation of the TET repressor-TET operator complex with the TET repressor-tetracycline complex was carried out at 60 °C. After 15-min incubation, the binding of tetracycline to the TET repressor was reduced to 50% as compared to the 37 °C value, whereas the binding of the 187-bp fragment to the TET repressor was not affected. From this result, we conclude that the native structure of the TET repressor is more stabilized against thermal denaturation in the complex with the TET operator than with tetracycline.

**Effect of pH on the TET Repressor-TET Operator Interaction**—Fig. 12 shows the effect of pH on the TET repressor-TET operator interaction. There is a clear optimum between pH 6 and pH 9 for complex formation. At pH 5.0, the stability is decreased to about 50%. At pH 8.9, the loss of activity is not significant, but at pH 10 it is decreased to about 50%. Around pH 11.1, the TET repressor is inactive in binding the TET operator. The influence of higher pH than 11 cannot be measured because the 187-bp DNA denatures, which leads to increased binding of the DNA to nitrocellulose. The pK value of the ε-ammonium group in lysine is 10.5 which may well be altered by a few tenths of a pK unit in a polypeptide. The drop of activity around pH 10 may suggest the involvement of coulomb interactions between lysines and phosphate residues in the TET repressor-TET operator interaction.

Major differences are observed in the pH dependence of the binding of tetracycline and TET operator by the TET repressor. At low pH, the binding activity toward the TET operator extends about 1 pH unit further down then the tetracycline binding. At high pH, the binding of tetracycline is not affected at all, whereas the binding of TET operator drops sharply above pH 9. There appears to be a rather narrow pH range from about 7.5 to 9.0 where both functions are optimal.

**Inhibition of TET Repressor-TET Operator Binding by Tetracycline**—In order to approximate the ratio of the association constants of the TET repressor-TET operator and TET repressor-tetracycline complexes, we measured the dependence of the TET repressor-TET operator complex formation on the concentration of tetracycline. The result of this experiment is displayed in Fig. 13. It may be derived from the data presented in Fig. 13 that, at tetracycline concentrations below 10⁻⁹ M, the effect of tetracycline on the TET operator binding cannot be measured. Around 10⁻⁷ M, about half of the TET operator in this experiment is released, and at concentrations greater than 4·10⁻⁷ M, all TET operators are depleted of the TET repressor. In the experiment shown in Fig. 13, the total concentrations of the components are 2·10⁻⁷ M TET repressor, 5·10⁻⁸ M TET operator, and the total tetracycline concentration is as indicated. Tetracycline removes the TET repressor from the TET operator by forming the TET repressor-tetracycline complex. At a total tetracycline concentration of 10⁻⁷ M, the TET repressor concentration, which is removed...
**DISCUSSION**

The Tn10-encoded tetracycline resistance gene is regulated by a repressor protein which, in addition, blocks its own synthesis (3, 6). As a result, even analytical amounts of the repressor are found only, when expression of the resistance and repressor genes is induced by tetracycline (3, 6). For preparative purposes, it is advantageous to use a recombinant plasmid which contains the repressor gene under the control of an active promoter which lacks the operator function. The construction of pRT211 was reported earlier (6) and Table I shows that the lack of the operator indeed results in an enhanced level of TET repressor in cells harboring the plasmid pRT211.

The second requirement for the preparation of the TET repressor is a fast and sensitive method to assay for the presence of the protein. An in vitro protein-synthesizing system has been used for this purpose previously (3). In order to develop a more rapid procedure, we evaluated conditions that allow us to use the binding of [3H]tetracycline·TET repressor complexes to nitrocellulose filters to measure TET repressor activity. Assaying crude samples of proteins succeeds at an ionic strength of 0.2 M. At low ionic strength, nonspecific binding of tetracycline to proteins is observed. This finding is not surprising because tetracycline contains a number of various functional groups as well as hydrophobic and hydrophilic domains in its molecular structure.

Fig. 2 displays that one contaminating protein is present in the TET repressor preparation. According to the intensity on the gel in Fig. 2, it should not amount to more than 15% of the total protein assuming that both proteins are stained by Coomassie blue to nearly the same extent. We did not detect any DNase activity in the preparation. Therefore, we believe that the functional investigations of this study are not influenced by this contamination. The apparent molecular weight determined from the SDS gel in Fig. 2 agrees well with previous results (3, 5, 6).

The determination of an accurate binding constant from the measurement displayed in Fig. 6 is hampered by the sensitivity of measuring the radioactivity at the given specific activity of the [3H]tetracycline. Because quantitative binding of active tetracycline to the TET repressor is found in Fig. 6, the conclusion is that the equilibrium constant is at least $10^{7}$ liters·mol⁻¹ but is probably greater. This result is supported by the inhibition of operator binding at concentrations of tetracycline in the range of $10^{-7}$ M in the reaction mixture (Fig. 13).

The steep decrease of tetracycline-binding activity around 60 °C is probably the result of denaturation of the protein at this temperature. The cooperative transition occurring in a small temperature range (Fig. 7) supports this conclusion. The decrease in tetracycline binding around pH 6 could suggest the involvement of a histidine from the TET repressor in the binding mechanism. It is also possible that a Schiff's base is involved in the binding of tetracycline which offers a number of carbonyl functions. A Schiff’s base is expected to be stable in an alkaline medium and should become unstable in the same pH range shown in Fig. 8 for the TET repressor.

The exclusive protection of the HincII cleavage site next to the Xba I site in Fig. 9 demonstrates the specificity of the TET repressor·TET operator interaction. The second HincII cleavage site serves as an internal control to support this conclusion. In the presence of tetracycline, no protection from HincII cleavage can be observed. This result proves the inactivation of the operator-binding function of the TET repressor by tetracycline. Thus, the two TET repressor functions drawn in Fig. 1 are demonstrated very clearly by this result. The Xba I and the Sau IIIa site shown in Fig. 9 are close to the HincII site which is protected by TET repressor binding. Cleavage with neither of these restriction endonucleases is affected by TET repressor binding which leaves about 100 base pairs between these sites as the possible location of the operator. The position of the operator should be somewhere in the center of this 100-bp region because the restriction enzymes as well as the TET repressor protect probably a greater part of the DNA than their actual binding sites. It should be noted that this result agrees very well with published data reporting protection of this HincII site by RNA polymerase binding (4).

The association constant for the binding of the TET operator to the TET repressor is greater than $10^{7}$ liters·mol⁻¹. Currently, the specific activity of the 187-bp fragment limits the detection of TET repressor·TET operator complexes to the concentration range in Fig. 10, and the determination of precise equilibrium constants will have to await the development of other methods to measure complex formation or the preparation of DNA fragments with a higher specific activity. The lack of binding competition of the 180-bp fragment from
pVH51 (19) clearly demonstrates that the interaction between the TET repressor and the 187-bp DNA is the specific TET repressor-TET operator complex rather than unspecific binding of the protein to DNA.

The result displayed in Fig. 10 allows the determination of the complex stoichiometry. The result, that 4 repressor molecules bind to the 187-bp fragment, is similar to the observations made for the lac repressor-operator interaction (20). However, it should be kept in mind that the 187-bp fragment may contain two operators (6). Evidence from minicell transcription assays suggests that one out of two operators is occupied preferentially (6). Considering this result, it may be concluded that the stoichiometry for the TET repressor-TET operator interaction is 4 repressor molecules per operator. The answer to this question will be determined, when other methods are available to monitor complex formation.

It was shown before that the specific TET repressor-TET operator complex is not formed in the presence of tetracycline. The concentration dependence of this inhibitory effect of tetracycline reveals that the association constant for the TET repressor-tetracycline complex is greater than the association constant for the TET repressor-TET operator complex. This result differs from the findings for the lac repressor where the repressor-operator interaction is more stable than the repressor-lactose interaction (42). This difference is related to the biological functions of these regulatory systems. Because the lactose genes code for metabolizing enzymes, it is only reasonable that these genes are not derepressed before a sufficient amount of substrate is present. This is achieved by the rather low association constant for the lac repressor-lactose complex.

The TET gene, on the other hand, mediates resistance to a lethal drug. Therefore, it should respond to minimal amounts of the drug, which is achieved by the high association constant of the TET repressor-tetracycline complex. It will be interesting to evaluate the molecular details, which result in such a strong binding.

The TET repressor-TET operator complex remains stable to about 5 °C higher temperatures than the TET repressor-tetracycline complex. Two alternative explanations may be given for this result. First, the TET repressor may consist of two domains with different and independent thermal stabilities. In this case, the thermal denaturation experiment would monitor different parts of the protein. This explanation gains support from results concerning the lac repressor, where the different functions are located on different parts of the protein (43). Secondly, the thermal denaturation experiment may monitor the same part of the TET repressor, which may gain some thermal stability from either the interaction with the DNA or with neighboring TET repressor molecules in the complex consisting of four TET repressors per TET operator. The similar cooperative denaturation of the TET repressor in both cases may lend some support to the latter explanation.

The obvious differences in the pH dependence of the tetracycline and the TET operator binding of the TET repressor leave only a rather narrow pH range for optimal interaction with both molecules. Whereas the steep decrease of TET operator-binding activity between pH 9 and 11 may result from the contribution of coulomb interactions between ε-ammonium groups of lysines with the negative charges of the operator sequence, the decrease in activity around pH 5 may reflect a denaturation of the protein in this pH range. This interpretation is suggested by similar results found for the interaction with the TET operator and with tetracycline. Again, the stability of the TET repressor-TET operator complex extends to a slightly lower pH than the TET repressor-tetracycline complex due to the possibilities discussed above for the thermal stability of these complexes.

The preparation of the TET repressor protein and DNA restriction fragments containing the TET gene regulatory sequences offer the possibility of studying an overlapping bidirectional promoter-operator system in vitro. It may be anticipated that our knowledge of molecular mechanisms of gene regulation will be extended by the results from this new system in the near future.

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REFERENCES

To purification of the TET repressor and TET operator from the transcription TET assay and characterization of their interaction

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Lea A. Vayy, and William E. Renkonen

Materials and Methods

General Methods. Restriction enzymes, 74 DNA lines and 74 DNA polymerase were obtained commercially, and which was prepared by a published procedure (20). Restriction enzyme digests were performed as described previously (18). DNA samples were done as described (17, 19). Screening of transformed cells was done by the silver-staining procedure as the pure form of the DNA sample was done as described (22). SDS-PAGE was performed as described previously (18). The following proteins were used as markers: plasmids, chloramphenicol, and 0.1 M NaCl. 10% SDS-PAGE was performed as described previously (18). The following proteins were used as markers: plasmids, chloramphenicol, and 0.1 M NaCl. 10% SDS-PAGE was performed as described previously (18). The following proteins were used as markers: plasmids, chloramphenicol, and 0.1 M NaCl. 10% SDS-PAGE was performed as described previously (18). 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TABLE I

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<th>Strain/plasmid</th>
<th>MO+</th>
<th>MO/pRT29</th>
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<td>3'-tetracycline bound, pmol/ml of culture</td>
<td>1.1</td>
<td>2.2</td>
<td>11.2</td>
</tr>
</tbody>
</table>

Each of the strains was grown in L-broth until the absorption at 650 nm reached 1.4. Cells were harvested, sonicated, precipitated at 20,000 rpm and to the supernatant ammonium sulfate was added until 35% of saturation. The precipitated proteins were pelleted by centrifugation and resuspended in 5 ml 0.2 M NaCl, 10 mM Tris HCl pH 8.0, 0.1 mM mercaptoethanol, and 0.1 mM EDTA. 10-2 aliquots from these solutions were tested for tetracycline binding as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>total amount</th>
<th>fraction</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>cleared supernatant of sonication</td>
<td>1170</td>
<td>14.3</td>
<td>96.5</td>
</tr>
<tr>
<td>55% ammonium sulfate precipitation</td>
<td>1346</td>
<td>13.8</td>
<td>1.0</td>
</tr>
<tr>
<td>QAE Sephadex A25</td>
<td>61.5</td>
<td>2.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Sephadex G100</td>
<td>17.3</td>
<td>2.2</td>
<td>12.7</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>3.7</td>
<td>1.5</td>
<td>40.5</td>
</tr>
</tbody>
</table>

The total amount of protein was determined by the method of Bradford (14)

(1) The total amount of active repressor was determined from saturation curves of tetracycline binding using the apparent molecular weight of 25 kDa

RESULTS

The results of the restriction analysis with big III digest is also shown in Fig. 5. The purpose of the construction of these plasmids is the preparation of restriction fragments of sufficient yield by constructing plasmids with multiple insertions of the same fragment (17). This was done for the big III fragment and the results are displayed in figures 4 and 5. plasmid pWTH15 and pWTH14 contain five and six copies of the 187-bp fragment, respectively, to observe orientations. The orientations of the insertions within the same plasmids are always parallel because only two small DNA fragments result from the big III restriction analysis in figure 5. This result is in agreement with previous observations (5, 11, 24, 40).

TABLE II

<table>
<thead>
<tr>
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<th>yield</th>
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<td>40.5</td>
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</tbody>
</table>

Fig. 4 Schematic presentation of the recombinant plasmids constructed for the preparation of DNA fragments containing the TET gene regulatory elements. The boxes represent the DNA. Striped boxes denote the inserted sequences. The recognition sites are marked by vertical lines. Only sites important for the analysis of the orientation and the preparation of the inserted sequences are shown. The letter R denotes big III sites, S is for the 187-bp sites, and p is for the 66-bp sites. The designations of the plasmids are given on the right side. The orientation of the insert in the box derived from big III digests of the respective plasmids as shown in Fig. 5.

Fig. 5 Restriction analysis of the plasmids. The restriction analysis for each plasmid is shown for two endonucleases. The plasmids analyzed is given in the top panel over the gel photograph. E denotes a lane with an Eco RI digest and S denotes a lane with an SnaI digest of the respective plasmid. M denotes lanes containing the reaction products of Hpa II digestion with big III. The reaction products of the respective digestes were electrophoresed on a 4% polyacrylamide gel for 4 h at 250 V. The boxes represent the DNA. The letter R denotes big III sites, S is for the 187-bp sites, and p is for the 66-bp sites. The designations of the plasmids are given on the right side. The orientation of the insert in the box derived from big III digests of the respective plasmids as shown in Fig. 5.

Fig. 2 SDS-polyacrylamide gel electrophoresis of the TET repressor. This figure shows two lanes with different amount of proteins loaded on an SDS-polyacrylamide slab gel (21). Lane A shows a load of 12.6 and in lane B an apparent molecular weight of the two proteins was determined as described in Materials and Methods. The identity of 64 kDa is assumed to be identical for the two bands. The letter XH is visible on the original gel. Due to their little intensity we failed to reproduce them in the photograph.

Fig. 3 Location of the DNA fragments cloned between Eco RI sites in pWK1. The three DNA fragments chosen for cloning between Eco RI fragments are shown with respect to their biological functions in the TET gene control region. The dashed lines denote the mRNAs for the two proteins indicated in the figure (4). It makes the SnaI- and the 66-bp site (4). The big III site is protected from cleavage by the binding of TET protein (c). The boxes represent the DNA. The numbers in the boxes refer to the distance in bp from the SnaI site. The DNA fragment from pWK1 with the restriction endonuclease indicated on the right side of the box was used to prepare the respective fragment (14).