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Interdomain Hybrid Tet Proteins Confer Tetracycline Resistance Only When They Are Derived from Closely Related Members of the *tet* Gene Family

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Inner membrane Tet proteins encoded by *tet* genes in gram-negative bacteria mediate resistance to tetracycline (Tc^r) by directing its export. Total sequences for class A, B, and C *tet* genes demonstrate that their products have a common ancestor, with Tet(A) and Tet(C) being more closely related (78% identical) than either is to Tet(B) (45% identical). The N- and C-terminal halves of Tet(B) and Tet(C) appear to comprise separate domains, and *trans*-complementation observed between tetracycline sensitive mutants in either domain of Tet(B) suggests separate but interactive functions for these domains. In this present study, interdomain hybrid genes were constructed to express hybrid *tet* products whose N- and C-terminal halves were derived from different family members [Tet(A/C), Tet(B/C), and Tet(C/B)]. Tet(A/C) specified a level of Tc^r comparable to wild-type Tet(C) and 60% that of Tet(A), indicating that domains from these closely related *tet* products can function in *cis*. Although neither Tet(B/C) nor Tet(C/B) hybrids conferred significant Tc^r, cells producing both of these types of hybrid proteins expressed substantial Tc^r, indicating that productive interactions can occur in *trans* between Tet(B/C) and Tet(C/B). Taken together, these results suggest that highly specific interactions between the N- and C-terminal domains are necessary for Tc^r and do not occur in individual hybrids derived from the more distant relatives, Tet(B) and Tet(C). This requirement for specific interactions suggests that N- and C-terminal domains have coevolved in each member of the Tet family.

High-level resistance to tetracycline (Tc^r) in gram-negative bacteria is mediated by members of a family of related Tc^r determinants, designated as classes A through E (23, 25), each of which specifies an energy-dependent export of tetracycline (23, 24). Genetic and biochemical analyses (1, 3, 5, 14, 15, 17, 19, 21, 35, 39, 40), together with total DNA sequences of classes A to C (14, 29–31, 33, 35, 38), have revealed that each of these determinants contains a repressor gene [*tetR*(A), *tetR*(B), and *tetR*(C)] and a resistance gene [*tet*(A), *tet*(B), and *tet*(C)] and that their divergent expression from overlapping promoter-operator regions is induced by tetracycline. For classes D and E, genetic analyses (23, 36) and partial DNA sequences encompassing repressor genes and central promoter-operator regions (34, 36) are consistent with the same organization. Repressor gene products from all five determinants are 43 to 63% identical (34, 36), establishing that they have a common ancestor. The resistance gene products of *tet*(A) and *tet*(C) [Tet(A) and Tet(C), respectively] are closely related (78% identical [38]), whereas both are more distantly related to Tet(B) (45% identical [29, 38]).

Genetic studies of *tet*(B) have demonstrated the presence of two complementation groups, α and β, within its single reading frame (10–12). These groups correspond approximately to the N- and C-terminal halves, respectively, of the *tet*(B) product; both groups are required for efflux and therefore should have distinct functions. These studies and others have suggested that native Tet(B) exists as a multimer in the cytoplasmic membrane in which α and β domains on different polypetides interact (13). For *tet*(C), although analysis to establish two complementation groups has not been reported, the results of in-phase insertion mutagenesis (2) are consistent with the same two-domain model as proposed for *tet*(B).

Construction of interdomain hybrid *tet* genes specifying hybrid Tet proteins containing intact α and β domains from different family members can provide insights into the function and evolution of these domains. For example, if interdomain hybrid Tet proteins between closer relatives (e.g., A/C, C/A) as well as more distant relatives (e.g., B/C, C/B) confer Tc^r, this would indicate that α and β domains are relatively independent or that their necessary interactions have been preserved despite considerable sequence divergence. Finding active hybrids only between closer relatives (e.g., A/C) would strongly suggest that necessary specific interactions exist, which are possible only with domains from closer relatives, and hence that the α and β domains have coevolved in each member of the Tet family.

We describe the construction of hybrid Tet determinants in which the repressor gene *tetR*, central promoter-operator regions, and N-terminal half (α domain) of the hybrid resistance gene were derived from one *tet* family member, while the *tet* β domain was taken from a different member. Production of active repressor by hybrid determinants would thus control the expression of potentially toxic hybrid *tet* products. The properties of these hybrid *tet* products provide evidence that specific interactions between α and β domains are required for Tc^r.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Escherichia coli* strains and plasmids used in this study are described in Table 1. Cultures were routinely grown in L broth, supplemented when appropriate with ampicillin or chloramphenicol (50 μg/ml each) for plasmid retention. M9 minimal medium (22) plus supplements was used for maxicell analysis (see below).

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant genotype[a]	Source or reference
E. coli		
BC30	*hsdR thi endA* = MM294	B. Bochner and B. Ames
BC32	BC30 Δ(*srlR-recA*)*306*	B. Bochner and B. Ames
Plasmid		
pBR322	Ap^r *tet*(C)$^+$ *ori*pMB9	6
pFB69	pBR322 with *Eco*RI site replaced by *Xho*I-*Bgl*II-*Xho*I linker and with a 1.2-kilobase deletion between *tet*(C)$^+$ and *ori*; Ap^r Tc^r	2
pFBI1	Tc^r derivatives of pFB69 with insertion of dGAATTC after *tet*(C) codon no. 189, 206, or 203, respectively	2
pFBI11		2
pFBI37		2
pSC101	*tetR*(C) *tet*(C)$^+$	9
pLR1068	Cm^r *tetR*(B) *tet*(B)$^+$ *ori*P15A	11
pJOE398	Ap^r *tetR*(A) *tet*(A)$^+$ *ori*pMB9	1
pSP72	Ap^r *ori*pUC(pMB9)	Promega Biotec

[a] Repressor genes *tetR*(A), etc. are wild type in all cases; *tet*( )$^+$ denotes a wild-type resistance gene. Ap^r, Tc^r, and Cm^r indicate resistance to ampicillin, tetracycline, and chloramphenicol, respectively.

Enzymes and reagents. Restriction enzymes and phosphorylated oligonucleotide linkers were obtained from New England BioLabs, Inc. Klenow DNA polymerase I and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. Antibiotics, bovine serum albumin (fraction V), cycloserine, and protease inhibitors (phenylmethylsulfonyl fluoride and *N*-tosyl-L-lysyl-chloromethyl ketone [TLCK]) came from Sigma Chemical Co. [^{35}S]methionine (ca. 10^3 Ci/mmol) was supplied by Du Pont, NEN Research Products.

Plasmid constructions. Protocols used for transformation, DNA isolation, and recombinant DNA manipulation have been described previously (22).

(i) Construction of vectors. Construction of vectors is described in Fig. 1A and B and Table 2. Since the Tn*10* class B Tc^r determinant on pLR1068 (Table 1) occurs conveniently on an *Xho*I-*Bgl*II restriction endonuclease fragment (Fig. 2A), restriction fragments harboring class C determinants to be used for hybrid construction were converted to the same termini as follows. The *Pvu*II restriction site downstream of *tet*(C) in pBR322 was changed to a *Bgl*II site to produce pRAR1009 (Fig. 1A). The *Xho*I-*Bgl*II-*Xho*I linker sites upstream of *tet*(C) in pFB69 (Fig. 2A) were transferred by *Pst*I-*Eag*I fragment replacement into pRAR1009 to product pRAR1010. *Bgl*II digestion and religation of pRAR1010 produced an Ap^r, *tet*-deleted vector (pRAR1011) bearing adjacent, unique *Xho*I and *Bgl*II sites into which *tet* determinants could be introduced.

tetR(C) was introduced into pRAR1010 by replacing its *Xho*I-*Bam*HI segment with an *Xho*I-*Bam*HI fragment from pSC101 (Fig. 2A) to produce the tetracycline-inducible pRAR1012. The *Eco*RI site between *tetR*(C) and *tet*(C) in pRAR1012, which interferes with use of *Eco*RI sites in the pFBI plasmids (see below), was eliminated by *Eco*RI cleavage, fill-in of termini with Klenow polymerase, and religation to produce an *Xmn*I site, generating pRAR1013. Although this alteration increases the distance between the putative ribosome-binding site and start codon for *tetR*(C) from 9 to 13 base pairs, regulation of *tet*(C) was found to be comparable for *E. coli* BC32 harboring pRAR1012 or pRAR1013: addition of 50 μg of tetracycline per ml to mid-log-phase cultures at 37°C halted the growth of both strains (as monitored by the increase in A_{600}) in <1 h, whereas growth of cultures exposed for 1 h to 1 μg of tetracycline per ml was not affected by challenge with 50 μg of drug per ml (data not shown).

Derivatives of pRAR1010 containing *Eco*RI sites inserted into the interdomain region of *tet*(C) were constructed by *Pst*I-*Eag*I fragment replacement from pFBI1, pFBI11, and pFBI37 (Table 1; Fig. 1A and 2A) to yield pRAR1014, pRAR1015, and pRAR1016, respectively. Inducible versions of pRAR1014 through pRAR1016 were made by replacing the *Xho*I-*Bam*HI segment with that from pRAR1013 to yield pRAR1017, pRAR1018, and pRAR1019, respectively. The interdomain location of the single *Eco*RI site in each of these *tet*(C) derivatives allows the expression of substantial Tc^r (Table 3).

Transfer of the Tn*10* Tc^r determinant as an *Xho*I-*Bgl*II fragment from pLR1068 into pRAR1011 (or substitution of this fragment for the corresponding one in pRAR1013 [Fig. 2A]) yielded pRAR1020. Replacement of this determinant in pLR1068 with the *Xho*I-*Bgl*II polylinker from pSP72 (Promega Biotec) produced a pBR322-compatible Cm^r, *tet*-deleted vector (pRAR1021) suitable for cloning determinants as *Xho*I-*Bgl*II fragments.

(ii) Construction of *tet* hybrids. Construction of *tet* hybrids is described in Fig. 1A and C. Suitable restriction endonuclease sites presumed or known to be located between the two domains in *tet*(A) (*Sma*I site), *tet*(B) (*Eco*RI site [12]), and *tet*(C) (*Eco*RI sites of pFBI mutants [2] [see above]) were used. [The overall homology of these *tet* sequences reveals substantial divergence in the central interdomain region of *tet*(B) without apparent change in length (38) (Fig. 3)].

A *tet*(A)α-*tet*(C)β hybrid was made as follows. The *Eco*RI site downstream of *tetR*(A) in pJOE398 (Table 1) was changed to *Xho*I by *Eco*RI cleavage, fill-in of the termini with Klenow DNA polymerase, and addition of an *Xho*I linker (dCCTCGAGG; New England BioLabs), producing pRAR1022. The *Xho*I-*Nru*I segment of pRAR1022 (Table 2) was then inserted in place of the corresponding region in pRAR1013 to produce pRAR1023 (Fig. 2B), which contains *tetR*(A) and the first 296-1/3 codons of *tet*(A) fused perfectly to the C-terminal 99-2/3 codons of *tet*(C). The *Xho*I-*Sma*I [*tetR*(A) plus *tet*(A)α] fragment of pRAR1023 (Fig. 2B) was inserted into pSP72 to produce pRAR1024, removed as an *Xho*I-*Eco*RI fragment (adding 14 base pairs beyond the *Sma*I site), and cloned by fragment replacement into pRAR1018 to produce pRAR1025 (Fig. 2B). This plasmid contains *tetR*(A) and specifies an interdomain hybrid *tet*(A/C) product which is detailed in Results (Fig. 3).

Hybrids between *tet*(B) and *tet*(C) were prepared by exchanging the appropriate *Eco*RI-*Bgl*II fragments from pRAR1020 with either pRAR1018 or pRAR1019 (Table 2; Fig. 1C and 2B). In hybrids pRAR1026 and pRAR1027, derived from pRAR1018 and pRAR1020, both halves of the hybrid reading frame are in the same register. In hybrids pRAR1028 and pRAR1030, derived from pRAR1019 and pRAR1020, frameshifts occurring at the *Eco*RI junctions were adjusted to put both halves of a reading frame into the same register (as outlined in Table 2 and Fig. 1C and 2B and

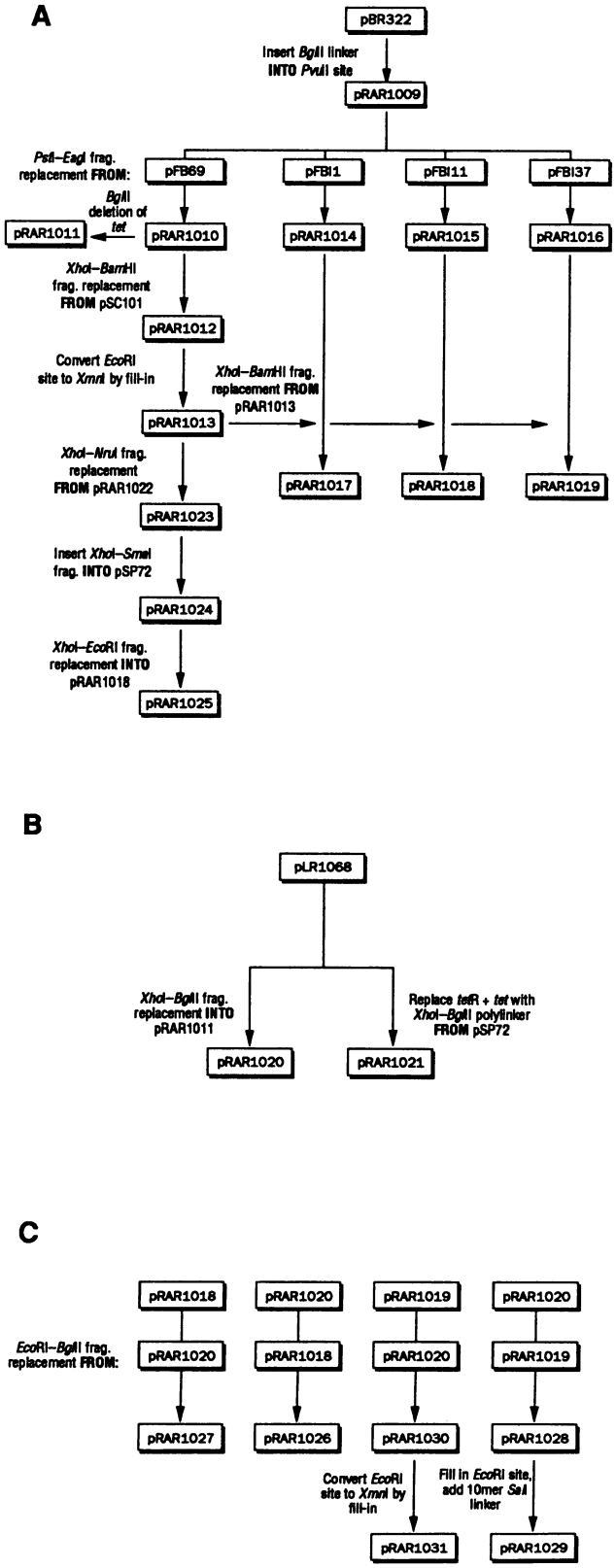

FIG. 1. Construction of plasmids to express hybrid Tet proteins (see Table 2 and Materials and Methods). (A) Construction of interdomain *tet*(C) mutants (pRAR1017, pRAR1018, and pRAR 1019) and a *tet*(A)α/*tet*(C)β hybrid (pRAR1025). (B) Preparation of a pBR322 derivative carrying the class B Tc^r determinant (pRAR1020) and of a Cm^r Δ*tet ori*P15A vector (pRAR1021). (C) Use of interdomain *tet*(C) mutants and pRAR1020 to construct plasmids expressing full-length Tet(B/C) hybrid proteins (pRAR1026 and pRAR1029) or full-length Tet(C/B) hybrids (pRAR1027 and pRAR1031). pRAR1029 and pRAR1031 were produced by adjustment of the hybrid *tet* reading frames of pRAR1028 and pRAR1030, respectively.

detailed in Fig. 3), producing pRAR1029 and pRAR1031, respectively.

For complementation studies, determinants carrying *tet* hybrids of pRAR1026, pRAR1027, pRAR1029, and pRAR 1031 were inserted as *Xho*I-*Bgl*II fragments into the Cm^r vector pRAR1021, generating pRAR1032, pRAR1033, pRAR 1035, and pRAR1037, respectively (Table 2).

Measurement of tetracycline susceptibility. The MIC of tetracycline for *E. coli* strains was determined by a gradient plate method (11). Plasmid-bearing strains were grown in L broth containing the nonbacteriostatic (gratuitous) inducer autoclaved chlortetracycline at 50 μg/ml (prepared fresh weekly and stored at 4°C in the dark), supplemented when appropriate with ampicillin or chloramphenicol at 50 μg/ml for plasmid retention. Induced cultures were centrifuged, suspended in buffered saline (0.067 M KPO_4 [pH 7.2], 0.85% [wt/vol] NaCl), and swabbed across the gradient plates. Linear tetracycline gradients contained ampicillin (25 μg/ml) or chloramphenicol (20 μg/ml) when appropriate. After incubation at 37°C for 24 h, the MIC of tetracycline was estimated from the position in the gradient at which confluent growth ceased.

Detection of plasmid-encoded proteins. To prepare maxicells (32) by using the *recA* host BC32, we grew plasmid-bearing strains at 37°C to an A_{600} of 0.6 in M9 minimal medium plus 0.5% Casamino Acids (Difco Laboratories), 1% glucose, 5 μg of thiamine per ml, and 50 μg of the appropriate antibiotic per ml. After UV irradiation for 1 min, cultures were shaken at 37°C for 2 h in foil-covered tubes. Cycloserine was added to 100 μg/ml, and shaking was continued for 16 h. Cultures were washed twice in M9 minimal medium (containing $MgCl_2$ in place of $MgSO_4$) plus glucose and thiamine. Cells were suspended in the same medium containing (when *tet* induction was desired) 50 μg of autoclaved chlortetracycline per ml (see above) and shaken at 37°C for 2 h. [^{35}S]methionine (5 μCi/ml) was then added, and shaking was continued for 1 h. Cultures were centrifuged at 4°C, washed twice in 50 mM Tris (pH 8)–10 mM trisodium EDTA (pH 8)–1 mM phenylmethylsulfonyl fluoride–1 mM TLCK, and resuspended in the same buffer. After addition of lysozyme (100 μg/ml) and incubation at room temperature for 10 min, maxicells were sonicated at 4°C. Membrane and supernatant fractions were separated by centrifugation at 40,000 × *g* for 1 h at 4°C. Membrane pellets were solubilized in Laemmli (20) sample buffer by incubation at 42°C for 1 h and at 100°C for 30 s. Supernatant proteins were precipitated with 10% trichloroacetic acid at 0°C, washed with acetone at 0°C, and solubilized at 100°C for 4 min in the same volume of Laemmli sample buffer as used for membrane samples. After removal of insoluble material by centrifugation, samples were subjected to electrophoresis (20) in 12.5% polyacrylamide gels. Equivalent amounts of total protein for each membrane fraction were used, based on concentrations estimated by the procedure of Bradford (7) (as modified in Bio-Rad Bulletin no. 1069), with bovine serum albumin as the standard. Gels were fixed with 7% acetic acid–5% methanol, soaked in a fluor (1 M sodium salicylate [pH 6.7], 5% glycerol), dried, and exposed to Kodak X-ray film with a Du Pont intensifying screen.

TABLE 2. Plasmids constructed for this study[a]

Plasmid	Description
pRAR1009	Insertion of *Bgl*II linker (dGGAAGATCTTCC) into *Pvu*II site of pBR322; Ap^r *tet*(C)$^+$
pRAR1010	pRAR1009 with *Pst*I-*Eag*I fragment replacement from pFB69, replacing *Eco*RI site with *Xho*I-*Bgl*II-*Xho*I linker, Ap^r *tet*(C)$^+$
pRAR1011	*Bgl*II deletion of pRAR1010; Ap^r Δ*tet*(C)
pRAR1012	pRAR1010 with *Xho*I-*Bam*HI fragment replacement from pSC101; *tetR*(C) *tet*(C)$^+$
pRAR1013	Fill-in, religation of *Eco*RI site in pRAR1012 to *Xmn*I site; *tetR*(C)$^+$ *tet*(C)$^+$
pRAR1014	Construction as for pRAR1010 but using *Pst*I-*Eag*I fragment from pFBI1; *tet*(C) (pFBI1)
pRAR1015	Same construction, but using fragment from pFBI11
pRAR1016	Same construction, but using fragment from pFBI37
pRAR1017	pRAR1014 with *Xho*I-*Bam*HI [*tetR*(C)] fragment replacement from pRAR1013; *tetR*(C) *tet*(C) (pFBI1)
pRAR1018	Same replacement in pRAR1015; *tetR*(C) *tet*(C) (pFBI11)
pRAR1019	Same replacement in pRAR1016; *tetR*(C) *tet*(C) (pFBI37)
pRAR1020	Insertion of Tn*10* Tc^r determinant as *Xho*I-*Bgl*II fragment from pLR1068 into pRAR1011; *tetR*(B) *tet*(B)$^+$
pRAR1021	Replacement of *Xho*I-*Bgl*II *tetR*(B) *tet*(B)$^+$ segment in pLR1068 with *Xho*I-*Bgl*II polylinker of pSP72; Cm^r Δ*tet* *ori*P15A
pRAR1022	Fill-in of *Eco*RI site of pJOE398 and addition of *Xho*I linker (dCCTCGAGG); *tetR*(A) *tet*(A)$^+$
pRAR1023	pRAR1013 with *Xho*I-*Nru*I fragment replacement from pRAR1022; *tetR*(A) *tet*(A/C) *Nru*I fusion
pRAR1024	*Xho*I-*Sma*I *tetR*(A) *tet*(A)α segment from pRAR1023 cloned into pSP72
pRAR1025	pRAR1018 with *Xho*I-*Eco*RI fragment replacement from pRAR1024; *tetR*(A) *tet*(A/C)
pRAR1026	*Xho*I-*Eco*RI segment from pRAR1020 with *Eco*RI-*Bgl*II from pRAR1018; *tetR*(B) *tet*(B/C)
pRAR1027	*Xho*I-*Eco*RI segment from pRAR1018 with *Eco*RI-*Bgl*II from pRAR1020; *tetR*(C) *tet*(C/B)
pRAR1028	*Xho*I-*Eco*RI segment from pRAR1020 with *Eco*RI-*Bgl*II from pRAR1019; *tetR*(B) *tet*(B·C) (with frameshift at *Eco*RI junction)
pRAR1029	Fill-in of *Eco*RI site in pRAR1028 and addition of *Sal*I linker (dCGGTCGACCG); *tetR*(B) *tet*(B/C)
pRAR1030	*Xho*I-*Eco*RI segment from pRAR1019 with *Eco*RI-*Bgl*II from pRAR1020; *tetR*(C) *tet*(C·B) (with frameshift at *Eco*RI junction)
pRAR1031	Fill-in of *Eco*RI site in pRAR1030, generating *Xmn*I site; *tetR*(C) *tet*(C/B)
pRAR1032	*Xho*I-*Bgl*II fragment carrying hybrid *tet* determination of pRAR1026 cloned into pRAR1021; Cm^r *tetR*(B) *tet*(B/C) *ori*P15A
pRAR1033	As for pRAR1032, using *Xho*I-*Bgl*II from pRAR1027; Cm^r
pRAR1035	As for pRAR1032, using *Xho*I-*Bgl*II from pRAR1029; Cm^r
pRAR1037	As for pRAR1032, using *Xho*I-*Bgl*II from pRAR1031; Cm^r

[a] Coding sequences for all repressor genes are wild type. All plasmids except pRAR1021, pRAR1032, pRAR1033, pRAR1035, and pRAR1037 are Ap^r pBR322 derivatives. A/C, B/C, and C/B indicate in-phase hybrid reading frames. B·C and C·B denote hybrids in which the two halves are not in the same register and cannot express full-length hybrid proteins.

RESULTS

Activity of *tet*(A/C) hybrids. Although *tet*(A) and *tet*(C) specify products which are 78% identical, *tet*(A) confers a substantially higher level of Tc^r in *E. coli* (Table 3). The complete class A Tc^r determinant [*tet*(A) plus *tetR*(A)] was used to first construct a fusion at the *Nru*I site conserved in *tet*(A) and *tet*(C). The hybrid determinant produced (on pRAR1023 [Fig. 2B]) specifies a "3:1" *tet*(A/C) product (exchange of the first 296 codons) which confers roughly 60% as much Tc^r as wild-type *tet*(A) (Table 3). This finding indicates that the remaining portion of Tet(C) (the distal 100 residues) can largely, but not entirely, replace the corresponding part of Tet(A). By using pRAR1023, a "1:1" hybrid *tet*(A/C) (on pRAR1025 [Fig. 1A]) was constructed. It represents exchange of codons 1 to 206 and addition of 7 new codons at the junction (Fig. 2B and 3), followed by the C-terminal 190 codons of *tet*(C). A high level of Tc^r was conferred by pRAR1025, similar to that of *tet*(C) (on pRAR1013 [Table 3]). This level of Tc^r shows that *tet*(A)α and *tet*(C)β sequences used in this construction specify intact *cis*-active domains which are not strongly affected by extraneous amino acid sequences at their junction.

Activity of *tet*(B/C) and *tet*(C/B) hybrids. *tet*(B/C) and *tet*(C/B) hybrids capable of expressing full-length hybrid proteins were constructed by first exchanging *Eco*RI-*Bgl*II fragments between pRAR1020 and either pRAR1018 or pRAR1019 and subsequently manipulating sequences at the *Eco*RI site if necessary to adjust the relative register for the halves of a hybrid reading frame (Fig. 1C and 2B; Table 2). Details of the junction sequences for hybrid genes and products of these plasmids (pRAR1026, pRAR1027, pRAR 1029, and pRAR1031) are shown in Fig. 3. Only pRAR1027 [*tet*(C/B)] was able to confer detectable Tc^r in *E. coli* (Table 3); however, this level of Tc^r was no more than three times the background level of the host strain. To study the properties of this hybrid determinant at a lower gene dosage and in a vector different from the Ap^r pBR322 derivative used for Table 3, we transferred it to the *ori*P15A Cm^r plasmid pRAR1021, thus producing plasmid pRAR1033. Then pRAR1027 and pRAR1033 were tested for Tc^r individually (in the presence of a compatible Δ*tet* vector to ensure constant plasmid replicon content and allow testing on the same drug gradient plate). In each case a low level of Tc^r was expressed, although the level for pRAR1033 was lower than that for pRAR1027 (Table 4, line 8 versus line 14). Finally, pairing pRAR1033 with pRAR1027 gave no significant increase in resistance over pRAR1027 alone (Table 4, line 6 versus line 14). These results confirm that low-level Tc^r is expressed by this *tet*(C/B) hybrid product; the difference in net resistance may reflect the differing copy numbers of the plasmids. [All the pBR322-derived Ap^r plasmids used are *rop* (8) mutants, owing to a *Bgl*II linker inserted into the *Pvu*II site in *rop* or to fusion of foreign DNA at this *Bgl*II site in *tet*(C/B) hybrids. Their copy numbers estimated from yields of plasmid preparations are roughly five times those of the *ori*P15A Cm^r plasmids used, with neither vector showing a detectable increase in number in the presence of the other vector (data not shown)].

The results suggest that the α and β domains in either Tet(B/C) or Tet(C/B) hybrids, if intact, cannot work together effectively. To verify that *tet* sequences of pRAR1026 and pRAR1027 are intact, we used them to reconstitute pRAR1018 and pRAR1020. Both plasmids were digested with *Xho*I and *Eco*RI nucleases, mixed, and ligated. Screening of *E. coli* transformants resistant to 10 μg of tetracycline

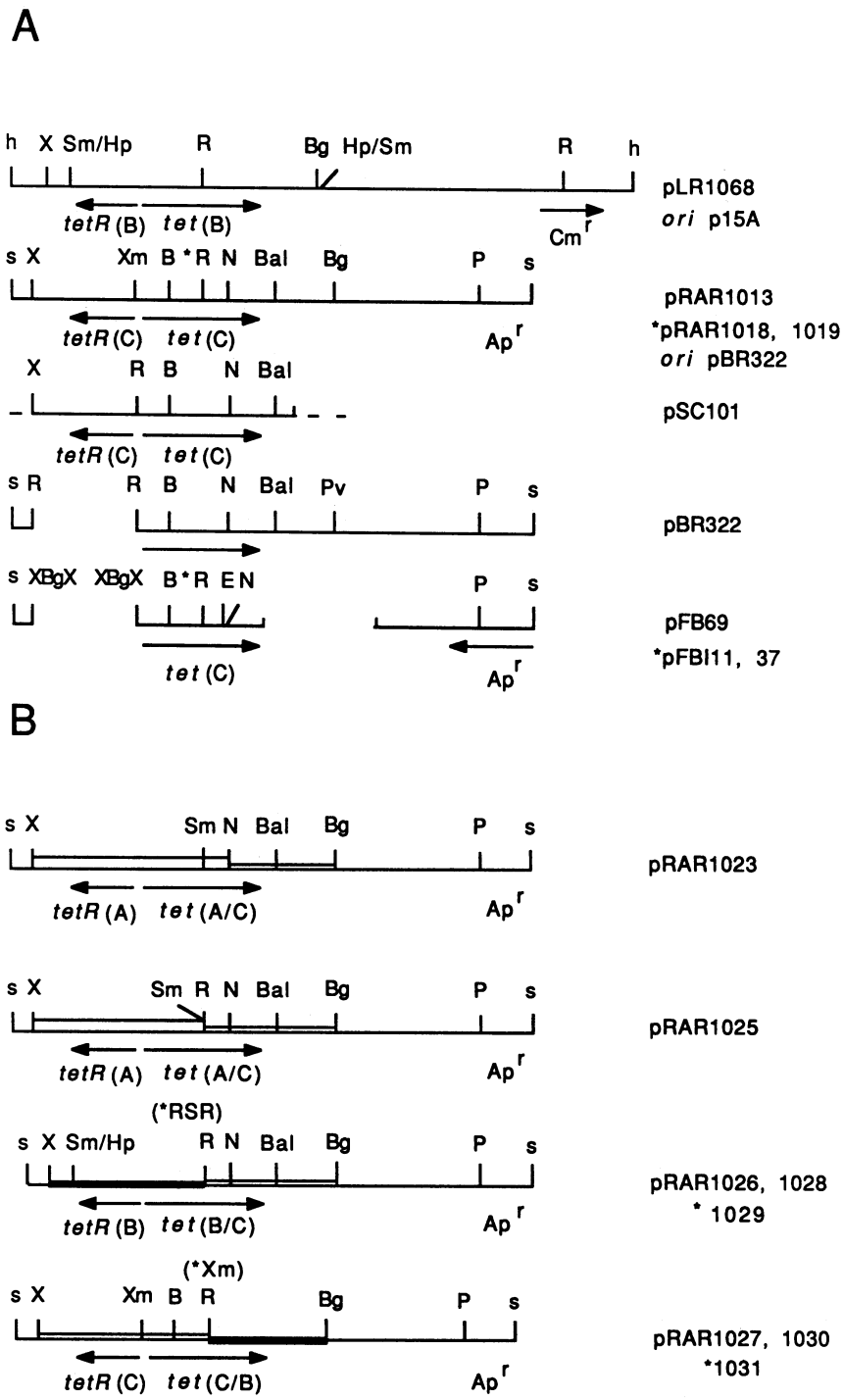

FIG. 2. Restriction enzyme maps of major plasmids used in and constructed for this study. (A) Plasmids harboring wild-type *tet*(B) and *tet*(C) or interdomain *tet*(C) mutants. Locations and orientations of reading frames for *tetR* and *tet* genes, the Cm^r gene of pLR1068, and the Ap^r gene (in all pBR322-derived plasmids but displayed only for pFB69) are shown. Symbols for restriction enzyme sites: X, *Xho*I; Bg, *Bgl*II; B, *Bam*HI; R, *Eco*RI; E, *Eag*I; N, *Nru*I; P, *Pst*I; s, *Ssp*I; Bal, *Bal*I; Pv, *Pvu*II; Xm, *Xmn*I; h, *Hae*II; Sm, *Sma*I; Hp, *Hpa*I. pBR322-derived plasmids are displayed by linearization at the unique *Ssp*I site. The *Eag*I site present in all *tet*(C) genes is indicated only in pFB69. Coincident conceptual gaps have been introduced into maps of pBR322 and pFB69 to emphasize the absence of *tetR*(C). The right-hand gap in pFB69 indicates a 1.2-kilobase-pair deletion spanning the *Bal*I and *Pvu*II sites (2). For pSC101, only the portion identical to pBR322 (sequences from the *Eco*RI site to 195 base pairs downstream of the *Bal*I site [4]) and the *tetR*(C) segment flanked by an *Xho*I site are shown. In pRAR1013, the *Eco*RI site, present in pBR322 and pSC101, has been converted to *Xmn*I. *tet* interdomain *Eco*RI sites marked by an asterisk (*R) occur only in the indicated plasmids. pLR1068 (11) is shown linearized at one of the five *Hae*II sites. Construction of pRAR1013, pRAR1018, and pRAR1019 is outlined in Fig. 1, Table 2, and Materials and Methods. (B) Restriction enzyme maps of hybrid *tet* determinants (see Table 2 and Materials and Methods). Sequences derived from *tet*(A) (▭) *tet*(B) (■) and *tet*(C) (▭) determinants are shown. Symbols and abbreviations are the same as in panel A. (*RSR) indicates that a *Sal*I linker (S) has been added at the junction in pRAR1029, regenerating flanking *Eco*RI sites (see Fig. 3). In pRAR1031, the *Eco*RI junction has been filled in to generate an *Xmn*I site.

TABLE 3. Tetracycline resistance conferred by native, mutant, and hybrid *tet* determinants

Plasmid	Genotype[a]	MIC of tetracycline (μg/ml)[b]
pRAR1011	Δ*tet*	1.2
pRAR1013	*tet*(C)$^+$	90
pRAR1017	*tet*(C) (from pFBI1)	66
pRAR1018	*tet*(C) (from pFBI11)	54
pRAR1019	*tet*(C) (from pFBI37)	36
pRAR1020	*tet*(B)$^+$	150
pRAR1022	*tet*(A)$^+$	182
pRAR1023	*tet*(A/C) "3:1"	114
pRAR1025	*tet*(A/C) "1:1"	99
pRAR1026	*tet*(B/C)	1.5
pRAR1027	*tet*(C/B)	3.1
pRAR1029	*tet*(B/C)	1.1
pRAR1031	*tet*(C/B)	1.1

[a] See Table 2. A wild-type repressor gene (not indicated) is present in each case, derived from the same determinant which provides the resistance gene or the α domain of a hybrid *tet* gene. The vector in all cases is pBR322 derived (Ap^r), and the host is *E. coli* BC32.

[b] The MIC, i.e., the concentration which prevented confluent growth after 24 h of incubation at 37°C, was determined by a gradient technique (see Materials and Methods). Values are averages of two or more determinations. The approximate range of values from the average was as follows: MIC ≤ 5 μg/ml, ± 20%; 5 < MIC < 50 μg/ml, ± 10%; MIC > 50 μg/ml, ± 5%. Ampicillin (25 μg/ml) was present throughout the gradient to ensure plasmid retention.

per ml identified recombinant plasmids with restriction enzyme digestion patterns appropriate for pRAR1018 and pRAR1020, and those plasmids conferred the same levels of Tc^r as the original isolates when tested as described in Table 3 (data not shown). Therefore, the *tet* sequences of pRAR1026 and pRAR1027 are hybrids of the intact sequences of their parents, pRAR1018 and pRAR1020.

Complementation analysis of *tet*(B/C) and *tet*(C/B) hybrids. In an effort to verify directly that all Tet(B/C) and Tet(C/B) hybrid proteins contain intact domains which are potentially functional despite their apparent lack of activity in *cis*, we performed *trans*-complementation analysis as described previously (11). To test two Tet hybrids in the same cell, we transferred the determinants of Ap^r, pBR322-derived pRAR 1026, pRAR1027, pRAR1029, and pRAR1031 to the multicopy, pBR322-compatible, Cm^r Δ*tet* vector pRAR1021, generating pRAR1032, pRAR1033, pRAR1035, and pRAR1037, respectively (Table 2). Various combinations of Ap^r plasmids with Cm^r plasmids in the same *E. coli* strain were then tested for expression of Tc^r. Only pairings capable of expressing in *trans* both domains of Tet(B) and Tet(C) showed substantial Tc^r (Table 4), demonstrating Bα-Bβ and/or Cα-Cβ *trans* complementation. Unexpectedly, despite the intrinsic Tc^r shown by pRAR1027, complementations involving this plasmid resulted in Tc^r levels lower than or equal to those observed for similar complementations involving pRAR1031 [*tet*(C/B)] (Table 4, line 1 versus line 2 and line 9 versus line 10). Furthermore, although pRAR1033 harbors the same hybrid Tet determinant as pRAR1027 and also shows intrinsic Tc^r (see above), it yielded the lowest levels of complementing Tc^r observed.

Expression and stability of hybrid Tet proteins. The preceding observations on complementation and on intrinsic Tc^r (pRAR1027 and pRAR1033) imply that *tet*(B/C) and *tet*(C/B) hybrids express hybrid proteins. Production of plasmid-specified proteins was verified by using the maxicell method (32) (Fig. 4). Observation of all labeled membrane proteins required prior induction by the nonbacteriostatic inducer heat-inactivated chlortetracycline (results not shown), indicating that they are *tet* specific. Calculated sizes for the Tet hybrids and wild-type Tet proteins range from 41.5 to 43.3 kilodaltons (kDa) (pRAR1025, 42,466 Da; pRAR1013, 41,516 Da; pRAR1027, 43,343 Da; pRAR1026, 41,646 Da; pRAR1031, 43,199 Da). However, all of the class A to C Tet proteins have consistently yielded lower apparent molecular masses, of 34 to 36 kDA (1, 14, 33, 40) in Laemmli (20) gels [e.g., Tet(C) expressed from pRAR1013 (34.5 kDa) (Fig. 4)], possibly owing to their hydrophobic nature. Therefore, the induced polypeptides with apparent sizes of 36 kDa [pRAR1025, *tet*(A/C)] and 32 kDa [pRAR1027, *tet*(C/B), and pRAR1026, *tet*(B/C)] (Fig. 4) are consistent with the expected Tet hybrids. Apparent sizes of the hybrid proteins when compared with the wild-type proteins are also consistent with their calculated size differences, except for Tet(C/B); it appears to be smaller than Tet(C), although it is calculated to be larger. Since stability studies do not reveal a larger precursor for Tet(C/B) (see below), the size discrepancy may reflect an increased electrophoretic anomaly. The polypeptides at 26 kDa (Fig. 4, lane 1) and 27 kDa (lanes 2 through 8) are most probably the corresponding *tetR* products, since the sizes are those expected (1, 35) and more than 90% of each protein was found in the soluble fraction (Fig. 5A). Chlortetracycline-inducible labeled material migrating below 14 kDa appeared in both membrane and soluble fractions for all *tet* determinants tested. Its origin is obscure and may represent abortive synthesis, aberrant initiation, or degradation peculiar to the maxicell system.

Pulse-chase experiments examined the stability of wild type and hybrid proteins. Maxicells were labeled for 10 min with [^{35}S]methionine followed by a chase with excess unlabeled methionine. No instability was observed for wild-type Tet(B) expressed by pRAR1020 (not shown). For pRAR1027 [*tet*(C/B)], considerable label persisted in the 32-kDa species during the chase, and neither qualitative change nor a precursor-product relationship was evident. However, the chase led to a severalfold general decline of label to intensities below those observed for steady-state (1-h) labeling (Fig. 4). Although pulse-chase of pRAR1031 was not performed, its steady-state profile (not shown) suggested that the abundance and stability of its *tet*(C/B) product were very similar to those for pRAR1027.

For Tet(B/C) hybrids produced by pRAR1026 (Fig. 4) and pRAR1029 (Fig. 5B), incorporation after 10 min of labeling was substantial and, for pRAR1026, clearly exceeded that for 1-h labeling. Chase conditions caused a severalfold loss of label in the 32-kDa (Fig. 4) or 33-kDa (Fig. 5B) species within 30 min, to a level below that seen for steady-state labeling. In both cases, a possible precursor-product relationship with signals at lower molecular weights was observed. Although the relatively lower abundance and stability observed for both *tet*(B/C) products in maxicells may be related to the lack of Tc^r, the more substantial persistence of the Tet(C/B) hybrids suggest that their effective lack of Tc^r is not due to protein degradation.

To test whether complementation reflected an increase in the stability of the hybrid proteins, we compared membrane-localized labeled polypeptides in maxicells for strains harboring plasmids expressing a Tet(B/C) and Tet(C/B) protein separately or together (Fig. 5A). When both plasmids were present in maxicells, a general increase in the amount of labeled polypeptides in both membrane (Fig. 5A, lane 5) and soluble (note chloramphenicol acetyltransferase in lane 2 versus lane 1) maxicell fractions was observed. No obvious

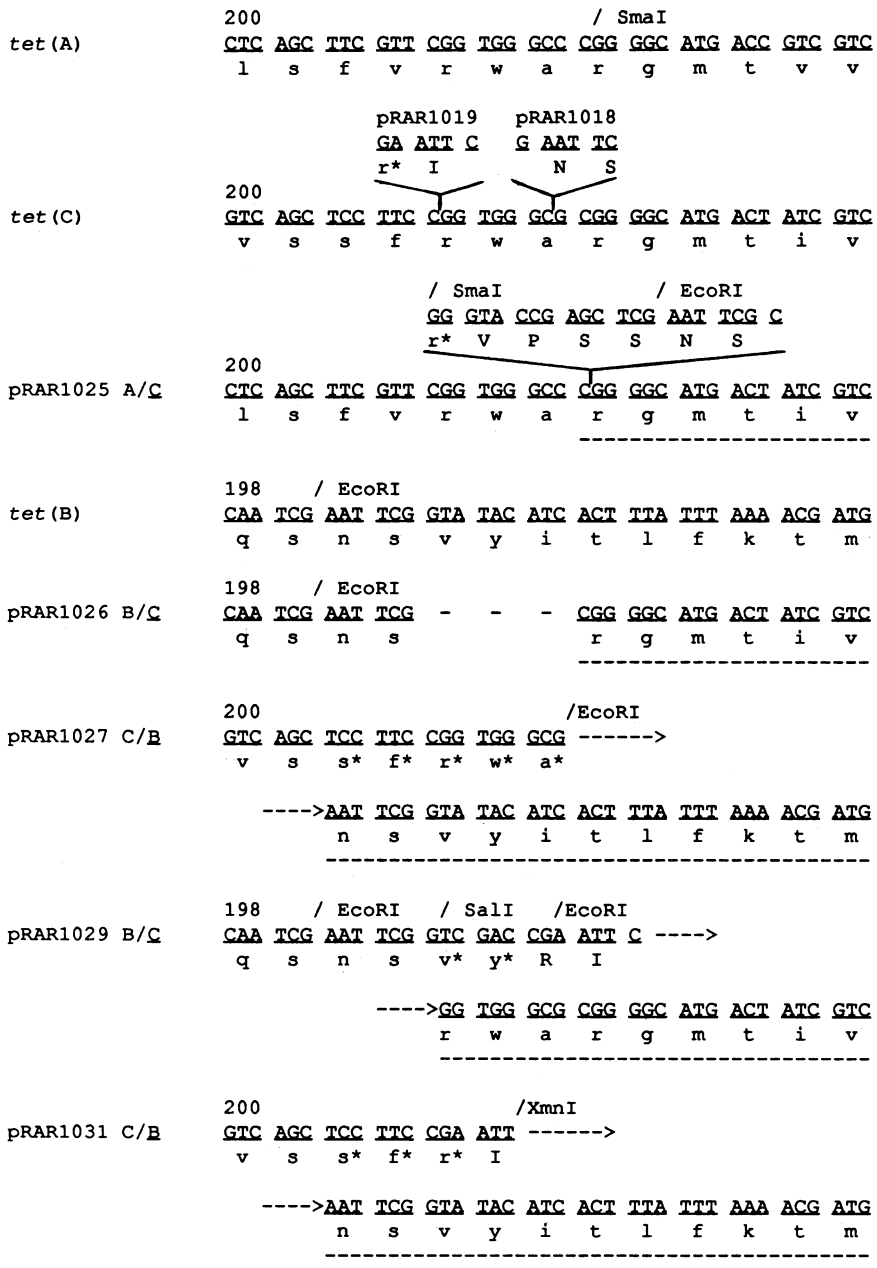

FIG. 3. Sequences of wild-type, mutant, and hybrid *tet* genes in the central interdomain region aligned according to the overall amino acid identities (38). Junctions for hybrid genes are displayed in the same alignments, with arrows indicating a continuous sequence. Published sequences of *tet*(A) (38), *tet*(B) (14, 29), and *tet*(C) (30, 33) are shown. Sequences of the pFBI37 and pFBI11 mutations present in pRAR1019 and pRAR1018, respectively, are from Barany (2). Restriction enzyme sites used in hybrid construction are shown. In pRAR1025, the 10 base pairs between the *SmaI* and *Eco*RI sites are derived from the pSP72 polylinker. Codons numbered as shown are translated by using the single-letter amino acid code. Native amino acids are shown in lowercase letters, and those duplicated in a hybrid are marked with an asterisk. Residues contributed by the distal half of a hybrid are underlined, and those missing from pRAR1026 are indicated by hyphens. New amino acids in hybrids, introduced by DNA manipulations and neither present in nor redundant with either contributor to the hybrid, are shown in capitals. Thus, the pRAR1026 product lacks three amino acids (vyi or, alternatively, rwa); pRAR1027 product is redundant for five residues (sfrwa or, alternatively, nsvyi); and pRAR1029 product is redundant for two residues (vy or rw) and has two completely new residues (RI).

qualitative change occurred in Tet(C/B), but its prominent signal prevented accurate monitoring of Tet(B/C). To eliminate this interference, plasmids expressing a Tet(B/C) protein were paired in the same cell with pRAR1030 (Table 2), which confers no Tc^r but successfully complements, despite its frameshift, which prevents expression of a full-length Tet(C/B) hybrid (data not shown). Again, a nonspecific increase for labeled proteins was observed, with no preferential enhancement of either Tet(B/C) hybrid (pRAR1032 [Fig. 5B]; pRAR1035 [not shown]). In addition, since a similar effect occurred when pRAR1030 was replaced by the noncomplementing Ap^r Δtet vector pRAR1011 (Fig. 5B), this increase in incorporated label was not mediated by *tet* sequences on pRAR1030. (The presence of two compatible

TABLE 4. Complementation analysis of hybrids between *tet*(B) and *tet*(C)

No.	Cm^r plasmid	*tet* genotype[a]	Ap^r plasmid	*tet* genotype[a]	MIC of tetracycline (μg/ml)[b]
1	pRAR1032	*tet*(B/C)	pRAR1027	*tet*(C/B)	19
2	pRAR1032	*tet*(B/C)	pRAR1031	*tet*(C/B)	21
3	pRAR1032	*tet*(B/C)	pRAR1029	*tet*(B/C)	1.3
4	pRAR1033	*tet*(C/B)	pRAR1026	*tet*(B/C)	11
5	pRAR1033	*tet*(C/B)	pRAR1029	*tet*(B/C)	16
6	pRAR1033	*tet*(C/B)	pRAR1027	*tet*(C/B)	2.8
7	pRAR1033	*tet*(C/B)	pRAR1031	*tet*(C/B)	2.2
8	pRAR1033	*tet*(C/B)	pRAR1011	Deleted	2.1
9	pRAR1035	*tet*(B/C)	pRAR1027	*tet*(C/B)	18
10	pRAR1035	*tet*(B/C)	pRAR1031	*tet*(C/B)	19
11	pRAR1037	*tet*(C/B)	pRAR1026	*tet*(B/C)	18
12	pRAR1037	*tet*(C/B)	pRAR1029	*tet*(B/C)	20
13	pRAR1021	Deleted	pRAR1011	Deleted	1.2
14	pRAR1021	Deleted	pRAR1027	*tet*(C/B)	2.7

[a] Described in Table 2. Repressor gene (not indicated) and *tet*α domain are always from the same determinant.

[b] MIC was determined as outlined for Table 3. Values are averages of two or more determinations (see Table 3). Ampicillin (25 μg/ml) and chloramphenicol (20 μg/ml) were present in the medium to ensure retention of both plasmid vectors.

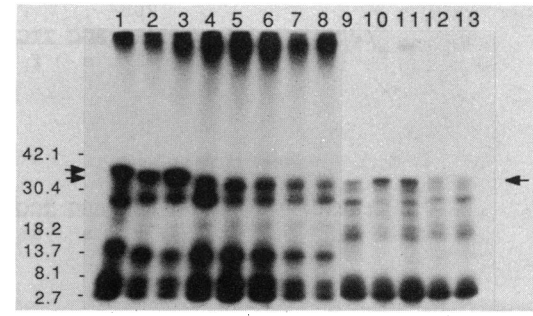

FIG. 4. Expression and stability of Tet mutants and hybrids in maxicells. Autoradiograms are shown for polyacrylamide gels of [^{35}S]methionine-labeled polypeptides present in membrane fractions of plasmid-containing maxicells produced from *E. coli* BC32. *tet* expression was induced by heat-inactivated chlortetracycline prior to labeling. Lanes: 1, pRAR1025 [*tet*(A/C)]; 2, pRAR1018 [*tet*(C) from pFBI11]; 3, pRAR1013 [*tet*(C)$^+$]; 4 through 8, pRAR1027 [*tet*(C/B)]; 9 through 13, pRAR1026 [*tet*(B/C)]. Lanes 5 through 8 and 10 through 13 represent 10-min labeling followed by a chase with 1,000-fold excess of unlabeled L-methionine as follows: lanes 5 and 10, no chase; lanes 6 and 11, 10-min chase; lanes 7 and 12, 30-min chase; lanes 8 and 13, 50-min chase. Lanes 1 through 4 and 9 are the results of 60-min labeling. Equivalent amounts of total membrane protein were loaded in each lane (see Materials and Methods). Positions of molecular mass standards and their sizes in kilodaltons are indicated. Exposure for lanes 1 through 8 was 1 day; exposure for lanes 9 through 13 was 2-1/2 days. Arrows designate location of Tet proteins (32 to 36 kDa).

plasmids increases the number of targets for UV irradiation and the number of surviving plasmids per maxicell. However, the nonspecific increase in labeled proteins noted above appears to exceed that anticipated for increased plasmid survival, suggesting a synergistic process.)

Although it is not known how closely the maxicell analyses reflect the situation in whole cells, the studies do not reveal any enhanced stability of Tet(B/C) hybrids in the presence of a complementing hybrid and suggest that adequate levels of Tet(B/C) for *trans*-complementation exist in its absence. However, these levels of Tet(B/C) do not confer Tc^r. Neither Tet(B/C) hybrids nor Tet(B) protein was evident after gel electrophoretic separation and silver staining of membrane fractions from induced whole cells (data not shown). This finding indicates that relatively low levels of these native and hybrid Tet proteins are produced in vivo.

DISCUSSION

In this study, an α/β interdomain hybrid *tet* gene product [Tet(A)α/Tet(C)β)] derived from two close relatives in the Tet family was shown to confer high-level Tc^r. This finding extends the two-domain model for Tet(B) to other family members and verifies the interdomain nature of the restriction sites in *tet*(A) and *tet*(C) used for hybrid *tet* gene construction. However, fusing the DNA sequence for the intact Tet(C)β domain to *tet*(B)α sequences, shown previously (12) to express an intact Tet(B)α domain, resulted in *tet*(B/C) hybrid genes which do not confer Tc^r. Furthermore, fusing *tet*(B)β sequences known to express an intact domain (12; R. A. Rubin, unpublished results) to intact *tet*(C)α sequences yielded *tet*(C/B) hybrid genes which confer little or no Tc^r. However, when *tet*(B/C) and *tet*(C/B) are present in the same cell, Tc^r is expressed at a significant percentage of wild-type levels. These findings suggest that hybrid *tet* genes whose domains are derived from more distant relatives do not express Tc^r, although each product must contain at least one potentially active domain to permit the observed *trans* complementation. Successful reconstitution of pRAR 1018 [*tet*(C)] and pRAR1020 [*tet*(B)$^+$], from pRAR1026 [*tet*(B/C)] and pRAR1027 [*tet*(C/B)] (see Results), demonstrated that lack of Tc^r for these *tet* hybrids is not due to a mutation in the *tet* sequences.

The failure of *tet*(B/C) and *tet*(C/B) to express Tc^r reflects the apparent inactivity of their respective hybrid gene products. Tet(C/B) hybrid proteins appear both relatively abundant and relatively stable as assessed in maxicells. Increasing their gene copy number in the cell did not increase Tc^r. The very low but detectable level of Tc^r expressed by *tet*(C/B) on pRAR1027 (about twofold above background) also suggests intact domains which are unable to function together efficiently.

Tet(B/C) hybrid proteins, although subject to more significant breakdown in maxicells (Fig. 4 and 5B), appear by several criteria to exist at steady-state levels sufficient to suggest they also lack intrinsic Tc^r. First, labeled polypeptides of appropriate size persist in maxicells after pulse-chase. Second, these hybrids can complement Tet(C/B) hybrids to yield Tc^r in vivo (Table 4). Third, simultaneous expression of a complementing frameshifted *tet*(C · B) hybrid (p6RAR1030 [Table 2]) does not selectively enhance the stability of Tet(B/C) hybrids in maxicells (Fig. 5B). Finally, the simultaneous presence of two different *tet*(B/C) genes in a strain does not result in Tc^r (Table 4, line 3).

Whether the successful *trans* complementations of Tet(B/C) with Tet(C/B) reflect Bα-Bβ interactions, Cα-Cβ interactions, or both processes remains to be determined. The former are to be expected, since *trans* complementation of appropriate *tet*(B) mutants has already been established (10, 11). The existence of two complementation groups for the *tet*(K) gene, from the distinct class K and L family found in gram-positive bacteria, has recently been reported (28). Although genetic analyses for *tet*(C) also favor a two-domain

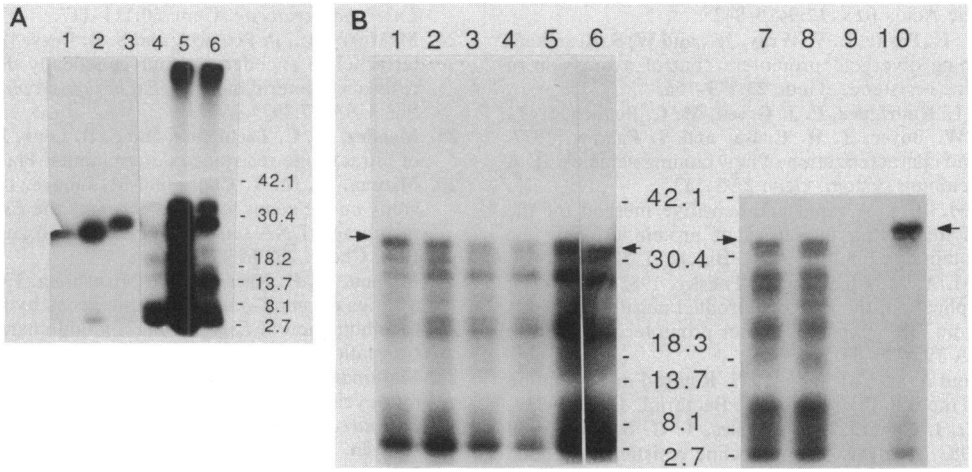

FIG. 5. Effects of pulse-chase or expression of complementing *tet*(C)-*tet*(B) fusions on expression of Tet(B/C) hybrids in maxicells. *tet* expression was induced by heat-inactivated chlortetracycline prior to labeling. The mobilities of molecular mass standards and their sizes in kilodaltons are shown. See the legend to Fig. 4. (A) Labeling for 60 min. Lanes 4 through 6, membrane fractions; lanes 1 through 3, corresponding supernatant fractions diluted 10-fold relative to lanes 4 through 6. Plasmid content: lanes 1 and 4, pRAR1032 [Cm^r *tet*(B/C)]; lanes 2 and 5, pRAR1032 plus pRAR1027 [Ap^r *tet*(C/B)]; lanes 3 and 6, pRAR1027. Both Tet hybrids migrate with an apparent size of 32 kDa. Exposure for lanes 1 through 3 was 12 h; exposure for lanes 4 through 6 was 30 h. (B) Lanes 1 through 4, 10 min of labeling followed by chase (0, 10, 30, and 50 min, respectively) as described in the legend to Fig. 4. Lanes 5 through 10, 60 of min labeling. Plasmid content: lanes 1 through 5, pRAR1029 [Ap^r *tet*(B/C)]; lanes 6 and 9, pRAR1026; lane 7, pRAR1032 [Cm^r *tet*(B/C)] plus pRAR1011 (Ap^r Δ*tet*); lane 8, pRAR1032 plus pRAR1030 [Ap^r *tet*(C·B)]; lane 10, pRAR1020 [*tet*(B)$^+$]. Calculated sizes of *tet* products are 42,592 Da (pRAR1029); 41,646 Da (pRAR1026 and pRAR1032), and 43,273 Da (pRAR1020). Arrows indicate Tet(B/C) hybrids (33 kDa, lanes 1 through 5; 32 kDa, lanes 6 through 8) and Tet(B) (34 kDa). Exposure for lanes 1 through 6 was 4-1/2 days; exposure for lanes 7 through 10 was 16 h.

structure (2) (see above), complementation studies have not been described.

Although Tet(B/C) and Tet(C/B) hybrids confer little or no Tc^r, rare mutational events occurred at frequencies of 10^{-6} to 10^{-9} in plasmids harboring these hybrid *tet* genes to partially restore Tc^r (≥6 μg/ml; Rubin, unpublished). These mutations may represent single amino acid changes in one domain that allow the domains of previously inactive hybrid proteins to interact productively. Most of the Tc^r mutations mapped to date in these plasmids have been localized to the restriction fragment specifying the β domain. For other systems, those who wish to produce hybrids or perform complementations by using apparently related gene sequences may observe inactivity which does not reflect a lack of common ancestry or of conserved function, but rather a failure of constituents to interact productively. As with Tet hybrids, spontaneous active mutants may be selectable, confirming similar functions for the sequences analyzed and identifying important mutations leading to productive interaction.

trans complementation (18, 41) and the properties of hybrids or chimeras (16) continue to be actively investigated for enzymes and regulatory proteins. However, very few such studies for integral membrane proteins have been reported. In the "family" of outer membrane porin proteins OmpC, OmpF, and PhoE, which are approximately 60% identical (26), chimeras (OmpC with OmpF [27], OmpC with PhoE [37]) have recently been constructed. These have allowed preliminary mapping of antibody-binding determinants and regions involved in receptor activity for phages specific for each porin. The constructions with OmpC and PhoE have also permitted preliminary mapping of regions contributing to the contrasting substrate preferences of PhoE (anionic solutes) and OmpC (cationic solutes) (37). To our knowledge, no examination of inner membrane proteins has been performed which is comparable to the *tet* analysis described here. The combination of genetic data on domain structure and the ability to construct hybrids from related but distinct determinants has allowed us to evaluate function (Tc^r) as it is affected by the compatibility of the individual domains. This approach may also prove useful for analysis of the gram-positive Tet family (classes K and L).

Our studies strongly suggest that active interdomain hybrid Tet proteins can be produced only by hybrid *tet* genes derived from more closely related members of the *tet* family, and they do not support any model for Tet function in which the α and β domains are largely autonomous or self-contained modules. Such observations suggest, in turn, that highly specific α-β domain interactions are prerequisites for substantial levels of Tc^r and hence that the α and β domains have coevolved in each member of the family. These findings must also be viewed in the context of recent computer-aided analyses which suggest that α and β domains arose from a common ancestor by gene duplication (31a).

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LITERATURE CITED

1. **Altenbuchner, J., K. Schmid, and R. Schmitt.** 1983. Tn*1721*-encoded tetracycline resistance: mapping of structural and regulatory genes mediating resistance. J. Bacteriol. **153**:116–123.
2. **Barany, F.** 1985. Two-codon insertion mutagenesis of plasmid genes by using single-stranded hexameric oligonucleotides. Proc. Natl. Acad. Sci. USA **82**:4202–4206.
3. **Beck, C. F., R. Mutzel, J. Barbe, and W. Muller.** 1982. A multifunctional gene (*tetR*) controls Tn*10*-encoded tetracycline resistance. J. Bacteriol. **150**:633–642.
4. **Bernardi, A., and F. Bernardi.** 1984. Complete sequence of

pSC101. Nucleic Acids Res. **12**:9415–9425.
5. **Bertrand, K. P., K. Postle, L. V. Wray, Jr., and W. S. Reznikoff.** 1983. Overlapping divergent promoters control expression of Tn*10* tetracycline resistance. Gene **23**:149–156.
6. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multi-purpose cloning system. Gene **2**:95–113.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
8. **Cesareni, G., M. A. Muesing, and B. Polisky.** 1982. Control of ColE1 DNA replication: the *rop* gene product negatively affects transcription from the replication primer promoter. Proc. Natl. Acad. Sci. USA **79**:6313–6317.
9. **Cohen, S. N., and A. C. Y. Chang.** 1977. Revised interpretation of the origin of the pSC101 plasmid. J. Bacteriol. **132**:734–737.
10. **Coleman, D. C., I. Chopra, S. W. Shales, T. G. B. Howe, and T. J. Foster.** 1983. Analysis of tetracycline resistance encoded by transposon Tn*10*: deletion mapping of tetracycline-sensitive point mutations and identification of two structural genes. J. Bacteriol. **153**:921–929.
11. **Curiale, M. S., and S. B. Levy.** 1982. Two complementation groups mediate tetracycline resistance determined by Tn*10*. J. Bacteriol. **151**:209–215.
12. **Curiale, M. S., L. M. McMurry, and S. B. Levy.** 1984. Intracistronic complementation of the membrane protein of Tn*10*. J. Bacteriol. **157**:211–217.
13. **Hickman, R. K., and S. B. Levy.** 1988. Evidence that TET protein functions as a multimer in the inner membrane of *Escherichia coli*. J. Bacteriol. **170**:1715–1720.
14. **Hillen, W., and K. Schollmeier.** 1983. Nucleotide sequence of the Tn*10* encoded tetracycline resistance gene. Nucleic Acids Res. **11**:525–539.
15. **Hillen, W. K., K. Schollmeier, and C. Gatz.** 1984. Control of expression of the Tn*10*-encoded tetracycline resistance operon: interaction of RNA polymerase and TET repressor with the *tet* operon regulatory region. J. Mol. Biol. **172**:185–201.
16. **Houghton, J. E., G. A. O'Donovan, and J. R. Wild.** 1989. Reconstruction of an enzyme by domain substitution effectively switches substrate specificity. Nature (London) **338**:172–174.
17. **Jorgensen, R. A., and W. S. Reznikoff.** 1979. Organization of structural and regulatory genes that mediate tetracycline resistance in transposon Tn*10*. J. Bacteriol. **138**:705–714.
18. **Kelley, R. L., and C. Yanofsky.** 1985. Mutational studies with the *trp* repressor of *Escherichia coli* support the helix-turn-helix model of repressor recognition of operator DNA. Proc. Natl. Acad. Sci. USA **82**:483–487.
19. **Klock, G. B., B. Unger, C. Gatz, W. Hillen, J. Altenbuchner, K. Schmid, and R. Schmitt.** 1985. Heterologous repressor-operator recognition among four classes of tetracycline resistance determinants. J. Bacteriol. **161**:326–332.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Levy, S. B., and L. McMurry.** 1974. Detection of an inducible membrane protein associated with R factor mediated tetracycline resistance. Biochem. Biophys. Res. Commun. **56**:1060–1068.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 250–251. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Marshall, B., S. Morrissey, P. Flynn, and S. B. Levy.** 1986. A new tetracycline resistance determinant, class E, isolated from *Enterobacteriaceae*. Gene **50**:111–117.
24. **McMurry, L., P. Petrucci, and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**:3974–3977.
25. **Mendez, B., C. Tachibana, and S. B. Levy.** 1980. Heterogeneity of tetracycline resistance determinants. Plasmid **3**:99–108.
26. **Mizuno, T., M. Y. Chou, and M. Inouye.** 1983. A comparative study on the genes for three porins of the *Escherichia coli* outer membrane: DNA sequence of the osmoregulated *ompC* gene. J. Biol. Chem. **258**:6932–6940.
27. **Mizuno, T., H. Kasai, and S. Mizushima.** 1987. Construction of a series of *ompC-ompF* chimeric genes by in vivo homologous recombination in *Escherichia coli* and characterization of their translational products. Mol. Gen. Genet. **207**:217–223.
28. **Mojumdar, M., and S. A. Khan.** 1988. Characterization of the tetracycline resistance gene of plasmid pT181 of *Staphylococcus aureus*. J. Bacteriol. **170**:5522–5528.
29. **Nguyen, T. T., K. Postle, and K. P. Bertrand.** 1983. Sequence homology between the tetracycline resistance determinants of Tn*10* and pBR322. Gene **25**:83–92.
30. **Peden, K. W. C.** 1983. Revised sequence of the tetracycline-resistance gene of pBR322. Gene **22**:277–280.
31. **Postle, K., T. T. Nguyen, and K. P. Bertrand.** 1984. Nucleotide sequence of the repressor gene of the Tn*10* tetracycline resistance determinant. Nucleic Acids Res. **12**:4849–4863.
31a. **Rubin, R. A., S. B. Levy, R. L. Heinrikson, and F. J. Kézdy.** 1990. Gene duplication in the evolution of the two complementing domains of Gram-negative bacterial tetracycline efflux proteins. Gene **87**:7–13.
32. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-encoded proteins. J. Bacteriol. **137**: 692–693.
33. **Sutcliffe, J. G.** 1978. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
34. **Tovar, K., A. Ernst, and W. Hillen.** 1988. Identification and nucleotide sequence of the class E *tet* regulatory elements and operator and inducer binding of the encoded purified Tet repressor. Mol. Gen. Genet. **215**:76–80.
35. **Unger, B., J. Becker, and W. Hillen.** 1984. Nucleotide sequence of the gene, protein purification and characterization of the pSC101-encoded tetracycline-resistance-gene-repressor. Gene **31**:103–108.
36. **Unger, B., G. Klock, and W. Hillen.** 1984. Nucleotide sequence of the repressor gene of the RA1 tetracycline resistance determinant: structural and functional comparison with three related Tet repressor genes. Nucleic Acids Res. **12**:7693–7703.
37. **van der Ley, P., P. Burm, M. Agterberg, J. van Meersbergen, and J. Tommassen.** 1987. Analysis of structure-function relationships in *Escherichia coli* K12 outer membrane porins with the aid of *ompC-phoE* and *phoE-ompC* hybrid genes. Mol. Gen. Genet. **209**:585–591.
38. **Waters, S., P. Rogowsky, J. Grinsted, J. Altenbuchner, and R. Schmitt.** 1983. The tetracycline resistance determinants of RP1 and Tn*1721*: Nucleotide sequence analysis. Nucleic Acids Res. **11**:6089–6105.
39. **Wray, L. V., Jr., R. A. Jorgensen, and W. S. Reznikoff.** 1981. Identification of the tetracycline resistance promoter and repressor in transposon Tn*10*. J. Bacteriol. **147**:297–304.
40. **Yang, H.-L., G. Zubay, and S. B. Levy.** 1976. Synthesis of an R plasmid protein associated with tetracycline resistance is negatively regulated. Proc. Natl. Acad. Sci. USA **75**:1509–1512.
41. **Zabin, I., and M. R. Villarejo.** 1975. Protein complementation. Annu. Rev. Biochem. **44**:295–313.