Structural and Functional Analysis of Surface Domains Unique to Bacteriophage T4 Thymidylate Synthase†

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ABSTRACT: The bacteriophage T4 genome encodes most of its own enzymes for dNTP synthesis, which form a complex in infected *Escherichia coli*. The T4 thymidylate synthase (TS) and the T4 deoxycytidylate deaminase (CD) catalyze sequential reactions and are physically linked within this complex [McGaughey, K. M., Wheeler, L. J., Moore, J. T., Maley, G. F., Maley, F., and Mathews, C. K. (1996) *J. Biol. Chem. 271*, 23037−23042]. From the crystal structure of T4TS [Finer-Moore, J. S., Maley, G. F., Maley, F., Montfort, W. R., and Stroud, R. M. (1994) *Biochemistry 33*, 15459−15468], it appears that three regions corresponding to insertions relative to *E. coli* TS lie on one side of the enzyme surface. We have investigated the residual activity of T4TS in response to complete deletion or substitution mutagenesis of these insertions. Two deletions generated in the small domain (residues 70−103) reduced the TS activity to 0.2% and 0.7% of the wild-type activity, with the latter able to complement growth of a thyA$^-$ *E. coli* strain in the absence of thymidine. By insertion of exogenous sequences variable in length and in sequence into these deletion mutants, enzyme activity increased to 44% that of the wild type. Restoration of the TS activity depended mostly on the hydrophobicity of the inserted residues. The sites of insertions also displayed distinct permissiveness for accommodating the exogenous insertions. Deletions and substitutions near the C-terminus resulted in complete inactivation of the T4TS. Proteolysis experiments revealed that the modified surface loops of the small domain were still accessible and flexible for protein−protein interactions. We have used ELISA to detect a physical association between T4TS and T4CD and compared the binding affinity of WT T4TS for two purified insertion mutants of T4CD. The results obtained showed that the native sequences of the small domain inserts are not required for T4TS/T4CD complex formation.

Thymidylate synthase (TS,[1] EC 2.1.1.45) is an enzyme essential for synthesis of the DNA precursor dTMP, by catalyzing the reductive methylation of dUMP. Alignment of over 20 known sequences shows that TS is one of the most highly conserved enzymes (*1*). Three-dimensional structures of TS from various species, including the bacteriophage T4 (*2*), the prokaryotes *Lactobacillus casei* (*3*) and *Escherichia coli* (*4*), and the eukaryotes such as *Leishmania major* (*5*) and humans (*6*), have been determined and indicate that their tertiary structures are also conserved. However, despite the high sequence conservation, inserts specific to certain organisms exist and are localized in surface loops of the TS (Figure 1). These insertions have been considered to not be involved in the core structure or in the catalytic site (*3*). Nevertheless, the presence of such insertions could imply other roles such as mRNA binding (*7*) or interaction with other proteins (*8*).

Bacteriophage T4 thymidylate synthase (T4TS) interacts with a dNTP synthetase complex which contains several phage-coded proteins and at least two enzymes of the bacteriophage's natural host, *E. coli* (*9*). This organization of enzymes permits rapid DNA synthesis and thus maintains very high rates of DNA replication in infected cells (*10*). For example, T4TS and deoxycytidylate deaminase (T4CD) catalyze sequential reactions and have been shown to be functionally associated with one another (*11*). The advantage conferred by such a dNTP synthetase complex could explain why the bacteriophage T4 encodes most of its own enzymes for dNTP synthesis. It remains unclear, however, why the T4 and *E. coli* enzymes have diverged so that only 48.6% of the *E. coli* TS residues are conserved in T4TS (*2*). Three unique natural insertions of 7−12 amino acids are found in the T4TS structure, designated as B, C, and F (Figures 1 and 2). The B and C inserts are located in the small domain of T4TS which spans residues 70−103 and is poorly conserved in all other TS species. The F insert is located near the C-terminus, which undergoes a large conformational change to form part of the folate binding site (*12*). The T4TS X-ray structure (*2*) showed that the F insert is closely packed against a portion of the small domain so as to define a surface of moderate hydrophobic character (Figure 2c) that is

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[1] Abbreviations: TS, thymidylate synthase; T4TS, thymidylate synthase from bacteriophage T4; T4CD, deoxycytidylate deaminase from bacteriophage T4; dTMP, thymidine 5′-monophosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; dNTP, deoxyribonucleoside 5′-triphosphate; IS, insertion site; HIV-1, human immunodeficiency virus type 1.

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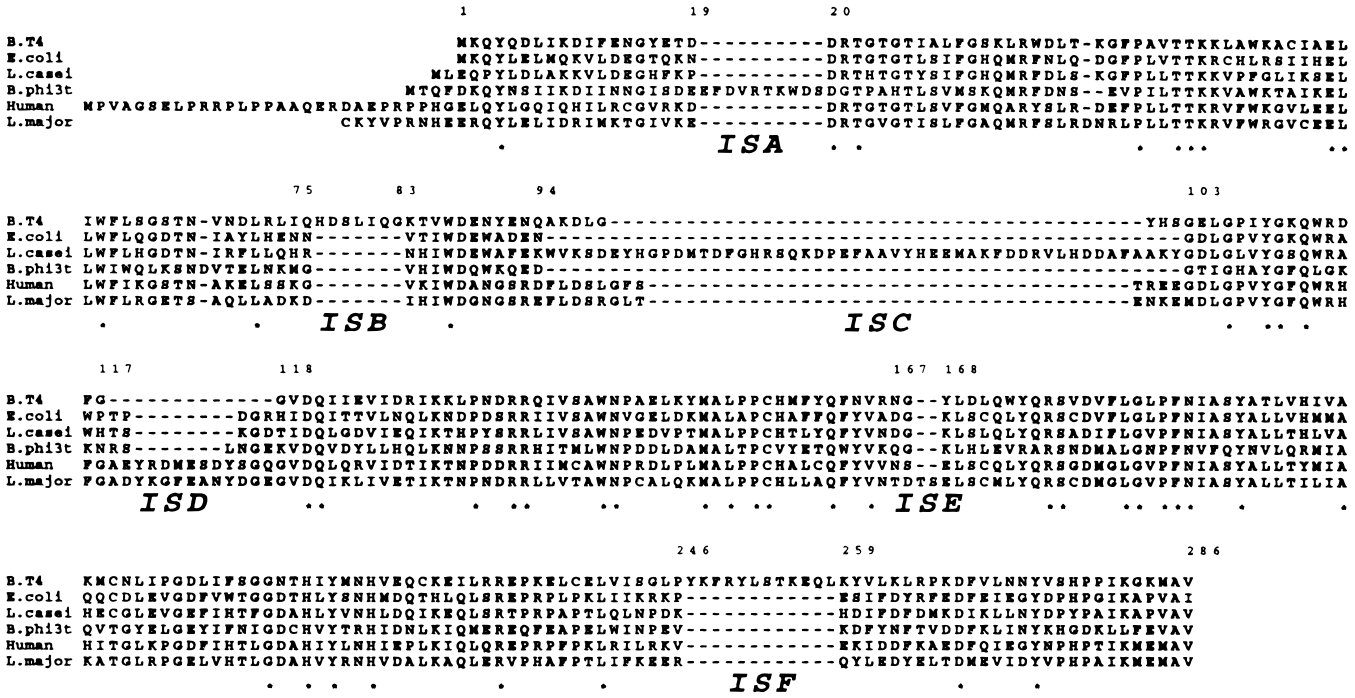

FIGURE 1: Aligned sequences of thymidylate synthases from various sources. Sequences were extracted from the Swissprot Data Bank, and the alignment was performed using the clustal software. Asterisks indicate conserved amino acids. *B.T4*, bacteriophage T4; *E. coli*, *Escherichia coli*; *L. casei*, *Lactobacillus casei*; *B.phi3t*, bacteriophage phi-3T; *L. major*, *Leishmania major*. The six sites (A−F) where insertions have spontaneously occurred in some species were shown to be tolerant for insertion of HIV-1 protease target sequences in *E. coli* TS (*13*). The numbering scheme used is that of the bacteriophage T4, and numbers indicate the amino acid immediately preceding the insertions.

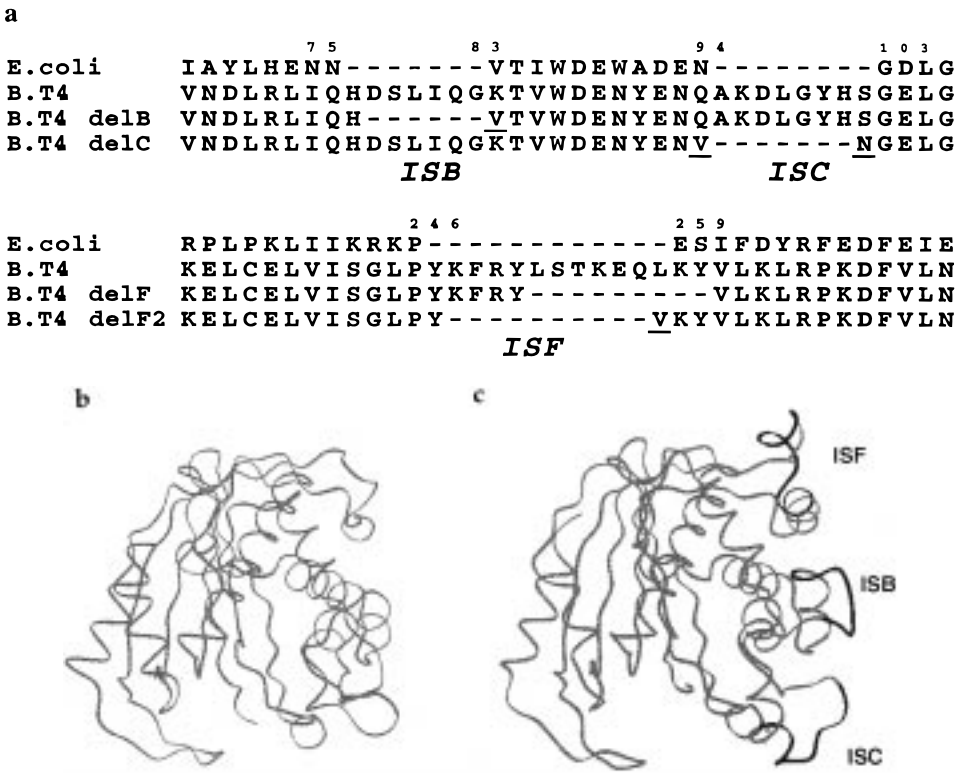

FIGURE 2: (a) Aligned sequences of TS from E. coli, bacteriophage T4, and the deletion mutants. Residues that have been modified for construction of the deletion mutants are underlined. (b) α-Carbon tracing of the *E. coli* TS structure. (c) α-Carbon tracing of the T4TS structure. Locations of the native insertions are indicated in black. The inserts, which are exposed at one side of the T4TS, could provide an interface favorable for protein−protein interactions.

potentially involved in protein−protein interactions within the T4TS complex.

The enzymology of TS has been extensively studied, and mutagenesis of TS has provided evidence for a high degree of tolerance for substitutions of conserved residues (*1*). In a previous study, we have performed extensive insertional mutagenesis of the *E. coli* TS gene and found several sites which tolerated insertion of exogenous sequences (*13*).

Table 1: Amino Acid Sequences of Insertions[a]

Insertion	Amino acid sequence
A8 sense	S F N F ↓ P Q I T
A8 antisense	S D L R E V K A
A10 sense	V S F N F ↓ P Q I T L
A10 antisense	Q S D L R E V K A Y
A20 sense	D R Q G T V S F N F ↓ P Q I T L W Q R P L
A20 antisense	E G S L P Q S D L R E V K A Y S S L S I
A50 sense	Q V W G R D N N S L S E A G A D R Q G T V S F N F ↓ P Q I T L W Q R P L V T I K I G G Q L K E A L L
O10 sense	I R K V L ↓ F L D G I
O10 antisense	D A I Q E Q H F A D

[a] Arrows indicate sites of cleavage in sense peptides that are substrates for HIV-1 protease.

Nevertheless, insertion of such sequences resulted in markedly reduced *E. coli* TS activity. Because of the presence of unique inserts in the T4TS structure relative to *E. coli* TS, we thought that the residues flanking the T4TS insertions might accommodate changes in amino acid sequence more easily than the corresponding residues of the *E. coli* TS. Such plasticity would be exploited for the creation of TS mutants with new properties, improving a system previously described for the selection of protease inhibitors and the study of protease specificity (*13*). We could also investigate the role of the native insertions by replacing them with exogenous sequences to study their involvement in complex stabilization.

The present work describes the deletion of these regions coding for inserts in T4TS relative to *E. coli* TS. Two of the deletions did not completely abolish enzymatic activity, while a similar deletion of a 50 amino acid insert in *L. casei* TS resulted in total inactivation of the enzyme (*14*). Substitution of the native insertions by unrelated sequences led to the partial restoration of TS activity up to 44% of the wild-type activity. In particular, it appeared that one site of insertion could tolerate the introduction of up to 50 amino acids, underscoring the remarkable permissiveness of this region. Moreover, we verified the surface exposure of the modified domains since the HIV-1 protease recognized and cleaved its target sequences substituted for the native insertions. Finally, two mutants were purified to near homogeneity and were used to develop an ELISA to study their binding affinity to T4CD. It appeared that these mutants could form the complex as efficiently as wild-type TS.

MATERIALS AND METHODS

Plasmids. The 1.1 kb T4 *thyA Eco*RI−*Dra*I fragment was subcloned into the phagemid pTZ18R (Pharmacia Biotech Inc.) under control of the *lac* promoter. IPTG induction was not necessary because pTZ is a high copy number plasmid.

pSU*prt*$^+$. The 2865 base pair HIV-1 *pol* gene fragment, corresponding to the protease and reverse transcriptase domains, was subcloned from pBRT1*prt*$^+$ (*15*) into pSU 19 (*16*), using the *Eco*RI and *Sal*I sites.

pSU*prt*$^-$. The 1470 base pair HIV-1 *pol* fragment, corresponding solely to the reverse transcriptase domain, was also subcloned from pBRT3*prt*$^-$ (*15*) into pSU 19 using the *Eco*RI and *Sal*I sites. pBRT1*prt*$^+$ and pBRT3*prt*$^-$ clones were kindly provided by the National Institutes of Health (AIDS Research and Reference Reagent Program).

Construction of Deletion Mutants. Mutagenesis was performed according to the method of Kunkel et al. (*17*). Oligonucleotides were chosen to introduce unique, blunt-end restriction sites to facilitate subsequent insertions in the deleted segment: these were a *Pml*I site at ISB, a *Hpa*I site at ISC, and a *Sna*BI site at ISF and F2 (Figure 2). Annealing and extension reactions were performed on the single-stranded form of the pTZ T4TS phagemid DNA. The resulting plasmids were called pTZ T4TS delB, pTZ T4TS delC, pTZ T4TS delF, and pTZ T4TS delF2, respectively.

Insertion Sequences and Nomenclature of T4 thyA Mutants. Complementary oligonucleotides coding for HIV-1 protease targets were synthesized, hybridized, and cloned in both orientations (designated as sense and antisense) within the deletion mutants. To eliminate the wild-type clones, the ligation mixtures were digested with the corresponding blunt-end enzyme which had no recognition sequence within the desired clone. The ligation mixture was used to transform competent *E. coli* DH5α cells. Mutants were identified by nucleotide sequencing using a commercial kit (Amersham).

The three first letters designate the site of insertion (i.e., ISC means insertion site C), the following character designates the sequence inserted (Table 1), and final number corresponds to the size of insertion (i.e., ISC A10 sense means the 10 amino acid A sequence in sense orientation inserted at site C). The nucleotide sequence corresponding to the 50 amino acids of insert A is CAGGTCTGGGGTAGAGACAACAACTCCCTCTCAGAAGCAGGAGCCGATAGACAAGGAACTGTATCCTTTAACTTCCCTCAGATCACTCTTTGGCAACGACCCCTCGTCACAATAAAGATAGGGGGGCAACTAAAGGAAGCTCTATTAGAT. The nucleotide sequence corresponding to insert O is ATCCGCAAAGTGCTGTTCCTGGATGGCATC.

E. coli Strains, Media, and Phenotypes. Expression of the mutants was performed in β-1308, Δ-*thyA*::Em derivative of the wild-type MG 1655 *E. coli* K12 ($F^- \lambda^-$). The ability of the T4TS mutants to complement growth of the thyA$^-$ strain was determined on Mueller−Hinton (Difco) medium lacking thymidine and supplemented with ampicillin (50 μg/mL). This selection medium was designated as "plus medium" since only thyA$^{+\text{"plus"}}$ strain can grow on it. Cells which present a thymidylate synthase-deficient phenotype were selected on Mueller−Hinton medium supplemented with thymidine (100 μg/mL) and trimethoprim (200 μg/mL). This selection medium was designated as "minus medium" since only thyA$^{-\text{"minus"}}$ strains can grow on it. These two media permit the characterization of mutants which present

a mixed phenotype. Such mutants display a TS activity sufficient to restore growth in the absence of thymidine (on plus medium) but insufficient to inhibit growth in the presence of trimethoprim (on minus medium).

Antibodies and Protein Extracts for Immunoblotting Experiments. Bacteria (1.5 mL, OD = 0.6−1) were centrifuged, resuspended in 50 μL of 15% sucrose, 50 mM Tris-HCl, 5 mM EDTA, and 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pH 7.5, and mixed with 13 μL of 10 mg/mL lysozyme. After 40 min on ice, 150 μL of 0.1% Triton X-100 and 50 mM Tris-HCl, pH 7.5, was added. The suspension was left again on ice for 15 min. After centrifugation at 12000*g* for 30 min, the supernatants were analyzed by SDS−PAGE and western blotting. Antibodies against T4TS were from a polyclonal serum of a rabbit injected with purified TS protein (*18*). Total protein concentration was determined in the extracts by the biuret method (BCA kit, Pierce).

Measurement of Thymidylate Synthase Activity. TS activity was determined spectrophotometrically (*19*). One unit of activity is 1 μmol of dUMP converted to dTMP in 1 min at 30 °C. Pellets corresponding to 1.5 mL cultures of β-1308 bacteria carrying one of the pTZ T4TS mutants (OD = 1) were resuspended in 150 μL of buffer (0.5 mM AEBSF, 4 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.5) and frozen in liquid nitrogen and thawed three times. Cellular debris was removed by centrifugation (30 min at 12000*g*).

The concentration of thymidylate synthase in unpurified supernatants was determined by a quantitative sandwich ELISA, using the same antibodies to capture and to detect T4TS. Microtitration plates were coated with 10 μg/mL antibodies against T4TS in carbonate buffer, pH 9.6, overnight at 4 °C. After blocking wells with 3% BSA in PBS, 50 μL of samples diluted with 1% BSA and 0.05% Tween 20 in PBS (at two dilutions: 1.6 μg/mL and 200 ng/mL of total protein) was added to duplicate wells and incubated for 2 h at room temperature. After three washes with PBS containing 0.05% Tween 20, 50 μL of a 1 μg/mL quantity of the same polyclonal antibodies against T4TS, biotinylated with NHS−LC−biotin (Pierce), was added for 1 h at room temperature. After five washes, 50 μL of peroxidase-labeled extravidin (1:5000 in dilution buffer) was incubated for 1 h at room temperature. Plates were washed six times, and the reaction was visualized with 2,2′-azinobis-(3-ethylbenzothiazolinesulfonic acid) (ABTS, 0.2 mg/mL) and read at 405 nm after 20 min. The use of antibodies to quantitate the TS mutants in crude extracts might be misleading if the mutated domains constitute important epitopes. However, we verified that the polyclonal antibody recognized with similar affinity all our mutants by comparing their reactivity at several dilutions in the ELISA test (data not shown).

Protein Purification. Recombinant mutants of T4TS were purified as described by LaPat-Polasko et al. (*18*), except that the last step, which consisted of an affinity column chromatography, was found unnecessary.

Measurement of Binding Affinity. Purified T4CD was used as the solid phase in an ELISA to measure complex formation with the T4TS. Wells were first coated with excess (0.5 μg) purified T4CD in PBS overnight at 4 °C. After blocking wells with 3% BSA in PBS, various amounts of purified T4TS or crude extracts diluted with 1% BSA and 0.1% Tween 20 in PBS (in 100 μL) were added, incubated for 1.5 h at room temperature, and then washed with PBS containing 0.1% Tween 20. Biotinylated polyclonal antibodies directed against the T4TS and peroxidase-labeled extravidin were used to detect bound T4TS as described previously.

Table 2: Results of Complementation of T4TS Mutants in the thyA$^-$ *E. coli* Strain[a]

	plus medium		minus medium
	24 h	72 h	24 h
T4TS wt	++	++	−
no TS	−	−	++
T4TS delB	−	−	++
T4TS delC	−	(+)	+
T4TS delF	−	−	++
T4TS delF2	−	−	++
T4TS ISB A10 sense	−	(+)	++
T4TS ISB A10 antisense	++	++	−
T4TS ISB O10 sense	−	−	++
T4TS ISB O10 antisense	++	++	−
T4TS ISC A8 sense	++	++	−
T4TS ISC A8 antisense	++	++	−
T4TS ISC A10 sense	++	++	−
T4TS ISC A10 antisense	++	++	−
T4TS ISC A20 sense	++	++	−
T4TS ISC A20 antisense	++	++	−
T4TS ISC A50 sense	−	(+)	+
T4TS ISC O10 sense	(+)	+	−
T4TS ISC O10 antisense	(+)	+	−
T4TS ISF A8 sense	−	−	++
T4TS ISF A8 antisense	−	−	++
T4TS ISF2 A10 sense	−	−	++
T4TS ISF2 O10 sense	−	−	++
T4TS ISF2 O10 antisense	−	−	++

[a] Cells were plated on plus (growth in the absence of thymidine) and minus (growth in the presence of thymidine and trimethoprim) media as well as on nonselective medium and titrated by serial dilution after 24 and 72 h at 37 °C. The number of colonies growing on the different selective media was calculated relative to the number of colonies growing on a nonselective medium: ++, 10−100% (full growth); +, 1−10% (intermediate growth); (+), <1% (weak growth); −, no colony.

RESULTS

Deletions in T4TS. TS deletion mutants lacking inserts at sites B, C, or F were prepared by cassette mutagenesis using oligonucleotides coding for blunt-end restriction sites. We chose restriction sites so as to minimize modification of the residues bordering the inserts (Figure 2a). These mutants were designated as T4TS delB, T4TS delC, T4TS delF, and T4TS delF2 (see Materials and Methods).

T4TS delB corresponded to the deletion of insert B (77−84) and the introduction of a valine residue which is found at the same position in the *E. coli* enzyme (Figure 2a). For construction of T4TS delC, the sequence coding for insert C (94−102) was deleted and a *Hpa*I site coding for valine and asparagine was created. Deletion of the insert near the C-terminus was attempted at two positions. The first construction, T4TS delF, lacked the sequence coding for the 251−261 segment, thereby creating a *Sna*BI site while conserving the naturally occurring flanking residues, tyrosine and valine. The T4TS delF2 mutant corresponded to the deletion of the 247−258 fragment and the creation of a valine residue.

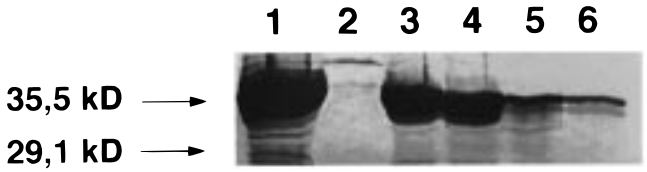

FIGURE 3: In vivo expression of the T4 thymidylate synthase deletion mutants. Analysis by immunoblotting using T4TS-specific antibodies. Lane 1, wild-type T4TS; lane 2, no T4TS (vector control); lane 3, T4TS delB; lane 4, T4TS delC; lane 5, T4TS delF; and lane 6, T4TS delF2.

The phenotypes of these mutants, carried by the high copy number pTZ18R plasmid, were analyzed in the β-1308 *E. coli* strain grown on plus and minus media. Mutants were scored for full growth, intermediate growth, weak growth, and no growth (see legend to Table 2). With the exception of the T4TS delC mutant, the deletion mutants were unable to complement growth of the thyA$^-$ strain in the absence of thymidine. T4TS delC displayed a level of TS activity that allowed the initiation of weak growth after 72 h of incubation on plus medium without thymidine and the inhibition of growth in the presence of trimethoprim on minus medium.

SDS−PAGE and densitometry analysis of cell extracts prepared from *E. coli* containing the wild-type T4 *thyA* gene on the pTZ18R plasmid showed that TS accounted for approximately 13% of soluble cell proteins. We evaluated the level of expression and/or the stability of deletion mutants by immunoblotting experiments. The T4TS delF and delF2 proteins were present in very small amounts whereas bands corresponding to the T4TS delB and delC presented intensities about half that of the wild-type extract (Figure 3, Table 3).

To obtain a more quantitative estimate, TS activity was measured in crude extracts of proteins prepared from bacteria expressing the different TS mutants (Table 3). Enzymatic activities found in the crude extracts were expressed in relation to the amount of the enzyme actually synthesized as estimated by sandwich ELISA. We previously verified that the antigenicity of the mutants was similar to that of the T4TS wild type (see Materials and Methods). The specific activity of the mutants was then expressed as the percentage of the wild-type specific activity.

T4TS delB, which presented a totally defective phenotype, exhibited a measurable activity corresponding to about 0.2% of wild-type activity. T4TS delC displayed an activity attaining 0.7% of that of the wild type. T4TS delF and delF2, like all the mutants constructed in these sites, were undetectable by enzymatic activity assays or by TS-specific ELISA, supporting the results obtained by immunoblotting experiments.

Substitution Mutants. Two double-stranded adapters corresponding to two distinct HIV-1 protease target sequences, called A and O, were separately introduced in both orientations into each of the deletion mutants (Table 1). Sense insertions coded for processing sites present in the HIV-1 Gag−Pol polyproteins whereas antisense sequences corresponded to totally unrelated peptides that served as negative controls for HIV-1 protease cleavage. The permissiveness of the C insertion site was further investigated by introducing 8, 10, 20, and 50 amino acid inserts. Complementation results appeared to be in good correlation with the total enzymatic activity measured in crude extracts (Table 3). The T4TS mutants displayed specific activities ranging from 44.4% to 6.9% of wild-type activity.

Table 3: Activity of T4TS Mutants

T4TS variants	growth phenotype[a]	TS activity[b] (units/mg of extract protein)	TS quantitation[c] (based on ELISA)	specific activity[d] (% wild type)
T4TS wt	++	1.690	1.000	100
T4TS delB	−	0.001	0.305	0.2
T4TS delC	(+)	0.006	0.498	0.7
T4TS delF	−	nd[e]	nd	
T4TS delF2	−	nd	nd	
T4TS ISB A10 sense	(+)	nd	0.008	
T4TS ISB A10 antisense	++	0.276	0.368	44.4
T4TS ISB O10 sense	−	nd	0.035	
T4TS ISB O10 antisense	++	0.251	0.533	27.9
T4TS ISC A8 sense	++	0.100	0.536	11.0
T4TS ISC A8 antisense	++	0.120	0.389	18.3
T4TS ISC A10 sense	++	0.033	0.167	12.0
T4TS ISC A10 antisense	++	0.060	0.214	16.6
T4TS ISC A20 sense	++	0.019	0.171	6.9
T4TS ISC A20 antisense	++	0.057	0.389	8.7
T4TS ISC A50 sense	(+)	nd	0.004	
T4TS ISC O10 sense	+	0.083	0.640	7.7
T4TS ISC O10 antisense	+	0.083	0.600	8.2
T4TS ISF A8 sense	−	nd	nd	
T4TS ISF A8 antisense	−	nd	nd	
T4TS ISF2 A10 sense	−	nd	nd	
T4TS ISF2 O10 sense	−	nd	nd	
T4TS ISF2 O10 antisense	−	nd	nd	

[a] Growth phenotype after 72 h at 37 °C as described in Table 2. [b] Activity of T4TS mutants with respect to total extract protein. A unit is 1 μmol of dTMP formed/min at 30 °C with respect to total extract protein under the conditions described in Materials and Methods. [c] Concentration of thymidylate synthase in the crude extracts as measured by quantitative ELISA. Concentrations were referred to the standard profile obtained by serial dilutions of an extract prepared from *E. coli* containing the wild-type T4 *thyA* gene. The T4TS wild-type concentration was arbitrarily set at 1. [d] Percentage of wild-type specific activity. [e] nd = not detectable.

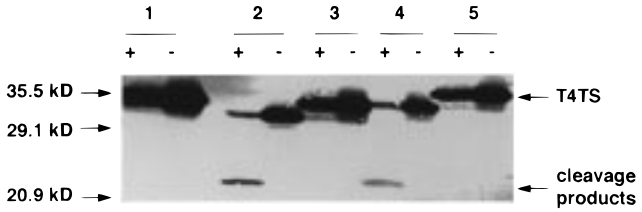

FIGURE 4: In vivo cleavage of insertions within T4TS. Analysis by immunoblotting using T4TS-specific antibodies. β-1308 cells were transformed by two constructs, a pTZ T4TS mutant and a pSU *prt* plasmid expressing either an active (+) or a defective (−) HIV-1 protease protein. Lanes 1, wild-type T4TS; lanes 2, T4TS ISC A10 sense; lanes 3, T4TS ISC A10 antisense; lanes 4, T4TS ISC A20 sense; and lanes 5, T4TS ISC A20 antisense.

ISB substitution mutants displayed a phenotype which seemed dependent on the sequence of the insert. Only antisense insertions were able to restore a wild-type phenotype, as confirmed by the TS activity in crude extracts, which was among the highest observed (Table 3). By inserting A and O antisense sequences, we increased the catalytic activities to 44.4% and 27.9% of the wild-type activity, respectively, compared to 0.2% in the delB mutant. Sense insertions led to enzymes with no activity.

ISC substitution mutants complemented growth in all cases. Nevertheless, we observed a slower growth for the 50 amino acid long insertion mutant, in particular, and for O mutants. Other insertions induced a full restoration of complementation. These results correlated with the activity measured in crude extracts. We observed an inverse correlation between the length of the insertion and enzymatic activity. Mutants with a 20 amino acid insertion exhibited an activity about 2-fold lower than that of the 10 amino acid mutants. The 50 amino acid insertion, equivalent in length to the insert located at the same position in *L. casei*, induced a greater destabilization. Indeed, the ELISA protein level measured in crude extract of the ISC A50 sense mutant was 250 times lower than that of the wild type. As for the ISB A10 sense mutant, we could not measure TS activity in crude extracts, even if some growth could be detected.

Purification of Two Mutants. The mutants T4TS ISB A10 antisense and ISC A10 antisense were purified to near homogeneity (>90% purity). Their respective k_{cat}s of 5.1 and 1.3 s^{-1}, relative to the wild-type T4TS of 10.7 s^{-1}, correlated well with the values found in crude extracts and thus validate the quantitation with the sandwich ELISA (Table 3).

Exposure of the Insertions at the Protein Surface. We verified that the exogenous insertions were correctly exposed at the surface of the protein by testing their susceptibility to cleavage by HIV-1 protease. Protein extracts were prepared from *E. coli* expressing the various T4TS mutants with or without HIV-1 protease and analyzed by immunoblotting with T4TS-specific antibodies. A band of 21 kDa, corresponding to one of the two expected cleavage products, could be seen with ISB mutants (data not shown) and ISC mutants (Figure 4). Cleavage occurred only when TS mutants with exposed sense insertions were recognized as native HIV-1 protease substrates. Antisense insertions were not cleaved by the HIV-1 protease. Susceptibility to the HIV-1 protease also demonstrated the flexibility of the modified loops B and C, which allowed a productive interaction between the target sequence and the binding cleft of the proteolytic enzyme.

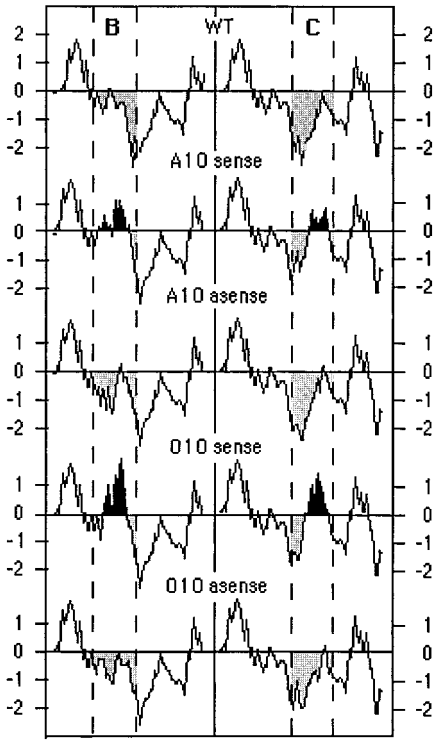

FIGURE 5: Hydrophobic character of the 10 amino acid insertions within the B and C sites. Profiles were calculated by the method of Kyte and Doolittle (*21*). The length for each overlapping segment was set at 11 amino acids. The most hydrophobic region of the graph is at the top. The exogenous 10 amino acid and natural insertions are located between the dashed lines.

Physical Interactions between T4CD and T4TS. Purified T4CD was used as the solid phase in an ELISA to measure complex formation with highly purified preparations of the wild-type T4TS and the ISB and ISC A10 antisense mutants. Biotinylated polyclonal antibodies directed against the T4TS and peroxidase-conjugated secondary antibody were used to detect T4TS bound to immobilized T4CD. The amount of complex formation depended on the concentration of T4TS added, demonstrating that T4TS can bind directly to purified T4CD (Figure 6). It should be noted that nonspecific binding, determined in control assays without immobilized T4CD, was higher for the mutants than for the wild-type TS. Surprisingly, the purified mutants displayed a greater affinity for T4CD than the wild-type TS did. Indeed, half of the maximum binding was achieved at the concentration of 10.3 $\pm$ 2.3, μg/mL for the wild-type TS, 1.7 $\pm$ 0.2 μg/mL for the mutant ISB A10 antisense, and 4 $\pm$ 1.1 μg/mL for the mutant ISC A10 antisense (Figure 6).

DISCUSSION

Three insertions unique to T4TS are exposed at the surface of the T4TS and are thought not to be relevant to fundamental aspects of structure or function (*3*). These inserts are exposed on the same side of the T4TS surface, defining an interface which might allow interactions with other proteins (*2*) (Figure 2c). Indeed, specific interactions between T4TS and several T4 proteins were revealed by means of anti-idiotypic antibodies (*20*) and affinity chromatography using T4TS as the immobilized ligand (*11*). Here we address the function of the unique insertions of T4TS. We investigated the residual activity of T4TS mutants following deletion of the

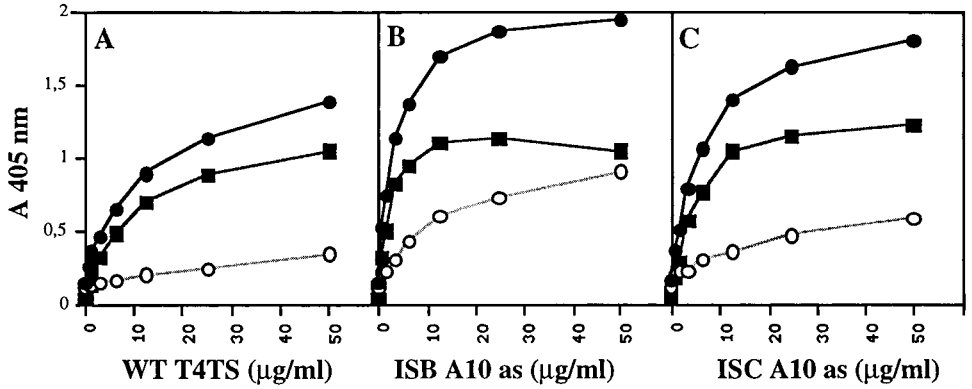

FIGURE 6: Binding of purified wild-type T4TS (A), ISB A10 antisense (B), and ISC A10 antisense (C) to purified T4CD. Purified T4CD immobilized on ELISA plates was incubated with the indicated concentrations of purified T4TS. Bound T4TS was quantited spectrophotometrically (see Materials and Methods). Nonspecific binding was measured in control wells without immobilized T4CD (open circles) and was subtracted from the total binding (closed circles) to obtain specific binding (closed squares). Control assay without T4TS, without primary antibody, or without peroxidase-labeled extravidin gave negligible background. Three independent determinations were performed, with one representative experiment shown.

native inserts and their replacement by exogenous segments, variable in length and in sequence. We then tested these constructs for their ability to bind to T4CD.

First, it appeared that deletion of the inserts profoundly reduced the catalytic activity of the enzyme (delB and delC mutants) or protein stability (delF and delF2 mutants). Deletion of insert B resulted in an almost inactive enzyme, showing that the presence of the native insertion is important for enzymatic activity. However, by inserting unrelated sequences (B A10 antisense and B O10 antisense), we restored a catalytic efficiency of up to 44% and 28%, respectively, of that of the wild-type enzyme (Table 3). Partial restoration of activity did not occur with all insertions. For example, the levels of TS in crude extracts from ISB mutants carrying A10 and O10 sense insertions were very low, suggesting destabilization and rapid degradation. Hydrophobicity of exogenous insertions was calculated in the context of the T4TS structure by the method of Kyte and Doolittle (*21*). The A10 and O10 amino acid sense insertions, which greatly reduced enzyme activity, appeared to be highly hydrophobic whereas the antisense insertions, which retained the TS activity, exhibited a lower hydrophobicity, resembling that of the natural insertion (Figure 5). Exposure of hydrophobic regions at the solvent-accessible domains may have disrupted protein folding and thus substantially decreased the half-life of the sense mutants. It was nevertheless significant that substitutions of two sequences could partially restore TS activity to the mutants, suggesting that the enzyme requires simply a spacer of moderate hydrophobicity in site B to restore activity.

Deletion of the insert located at loop C of *L. casei* TS led to the inactivation of the enzyme, probably due to the loss of interaction with the cofactor 5,10-methylene-5,6,7,8-tetrahydrofolate (*14*). A similar deletion in T4TS also resulted in highly reduced activity, confirming that the insert was required for the integrity of the TS structure and/or for the interaction with the substrate or the cofactor. However, the T4TS delC mutant retained the ability to complement growth of the thyA$^-$ strain in medium lacking thymidine. Since the length of the insert is 5-fold smaller in T4TS than in *L. casei* TS, its deletion has much less of an effect on T4TS activity. In site C, all substitutions complemented thyA$^-$ bacteria, and with the exception of the ISC A50 mutant, they displayed a measurable specific activity ranging from 18.3% to 6.9% of that of the wild type, suggesting that a particular array of surface residues in site C is not essential for activity. The permissiveness of site C was also investigated by inserting 8, 10, 20, and 50 amino acids. It appeared that the longer the insertion the less active the enzyme became. Sense as well as antisense insertions in site C could restore the catalysis of dTMP formation, showing that sites B and C are variably sensitive to changes in their primary structures and, presumably, the hydrophobic profiles of the insertion.

Two mutants lacking insert F located at the C-terminal extremity were constructed in an attempt to obtain a stable deletion mutant. The two deletions resulted in a negligible level of expression as shown by immunoblotting (Figure 3) or by quantitative ELISA (Table 2). This could be due either to inefficient transcription or translation of the mutants or to their rapid degradation. Moreover, exogenous insertions were not accompanied by partial restoration of enzymatic activity. It has been shown that the C-terminal residues of TS are implicated in catalytic activity, cofactor binding, and conformational changes (*12*). Thus, deletion and exogenous insertion near the C-terminus could markedly disrupt the tertiary fold of the protein and abolish enzymatic activity. Nevertheless, we have shown in a previous study on *E. coli* TS that the F site tolerated identical insertions in comparison to those attempted here (*13*). Differences in the conformation during cofactor binding could also explain the higher sensitivity of the T4TS toward C-terminal mutagenesis. Indeed, crystal structures from *E. coli*, *L. casei*, and human TS indicate that their C-termini are relatively unconstrained, whereas the three C-terminal residues of T4TS appear to be already at the bottom of the active site cavity (*2*).

T4TS and T4CD catalyze sequential reactions and appear to be physically associated within the dNTP synthetase complex (*11*). Therefore, we tested the ability of the wild-type T4TS and its mutants to bind to purified T4CD via the ELISA procedure. In this manner, we detected a specific and direct association of the purified wild-type T4TS to purified T4CD. Insertion mutants, ISB and ISC A10 antisense, exhibited a greater affinity than that of the wild type. Interestingly, mutations in T4CD led to a similar result, namely, enhancement of the interaction between T4CD and T4TS. Indeed, mutations in one of the two partners might

result in greater accessibility of the domains involved in the T4TS/T4CD complex.

Mutants created in sites B and C of T4TS exhibited a higher specific activity than that observed for similar mutants engineered in *E. coli* TS (*13*). Accordingly, the selection and the accommodation of native inserts were accompanied by an increased tolerance of the enzyme structure for substitutions at these sites. Our results also strengthen the idea that these native inserts, peculiar to the T4TS and dispensable for structure and enzymatic activity, have been selected to stabilize protein−protein interactions (*2*). The T4TS mutants and the assays described here provided a simple method to define the role of the native insertions by testing their affinity for T4 enzymes linked to dNTP synthesis. By using the ELISA procedure, we have detected a direct association between the T4TS and the T4CD. However, we have shown that the native sequences of the TS loops B and C are not required for the T4TS/T4CD complex formation. Several physical associations with T4TS have been described involving other T4 proteins such as the gp32 (*9*) and the dCMP hydroxymethylase (*20*). Whether the insertions present in the T4TS protein promote the binding to these molecules remains to be elucidated.

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