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# Translational repression by bacteriophage MS2 coat protein does not require cysteine residues

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## ABSTRACT

Previous studies implicated cysteine residues in the translational repressor (i.e. RNA binding) activity of the coat protein of bacteriphage MS2. It has been proposed that a protein sulfhydryl forms a transient covalent bond with an essential pyrimidine in the translational operator by a Michael addition reaction. We have utilized codon-directed mutagenesis methods to determine the importance of each of the two coat protein cysteines for repressor function *in vivo*. The results indicate that cys46 can be replaced by a variety of amino acids without loss of repressor function. Cys101, on the other hand, is more sensitive to substitution. Most position 101 substitutions inactivate the repressor, but one (arginine) results in normal repressor activity. Although the possibility of a transient covalent contact between cys101 and RNA is not categorically ruled out, construction of double mutants demonstrates that cysteines are not absolutely required for translational repression by coat protein.

## INTRODUCTION

The coat protein of the RNA bacteriophage MS2 is a translational repressor and shuts off synthesis of the viral replicase at late times during infection of an *E. coli* host. It does so by binding a small RNA stem-loop structure which contains the ribosome binding site of the replicase cistron (1). The important structural elements of the translational operator have been studied by measuring the affinity of operator sequence variants for the repressor *in vitro*. These studies have revealed that a key feature of the operator structure is a uridine residue in the RNA loop (2). When a purine is substituted at this site, the association constant for repressor is reduced as much as two orders of magnitude from its normal value of about $3\times10^{8}M^{-1}$. On the other hand, when cytidine is substituted, $K_a$ is increased. On the basis of sensitivity of the repressor activity to inactivation by 5-bromouridine, cytosine nucleotides, and certain sulfhydryl reagents, Uhlenbeck and colleagues proposed a transient covalent interaction between the pyrimidine and a protein sulfhydryl group (3). They argued that a reversible Michael adduct forms by addition of the sulfhydryl to C6 of the pyrimidine ring. They suggested that, compared to U, C is more susceptible to this reaction, accounting for the higher $K_a$ with the C variant. Coat protein contains only two cysteines, neither of which, under normal circumstances, is involved in a disulfide bond. Each, therefore, is free potentially to participate in formation of the postulated Michael adduct. A similar interaction has been proposed for some other protein-RNA interactions (4, 5), so this point has significance beyond the narrow question of the mechanisms of RNA binding by coat protein.

I sought to test this model by asking whether the substitution of other amino acid residues for the cysteines normally present at positions 46 and 101 would necessarily result in loss

of translational repression *in vivo*. The construction of plasmids which place the *E. coli lac*Z gene under control of the MS2 translational repressor-operator is being described elsewhere (6). This paper reports the results of mutagenesis of codons 46 and 101 of the coat gene, and the consequences for repressor activity of the resulting amino acid substitutions. The results indicate that a number of different amino acids can be present at position 46, with little or no loss of repression. Although the range of permissible substitutions at position 101 is far more restricted, the presence of cysteine is not absolutely required there either. In fact, certain double mutants, which encode no cysteine residues, are perfectly competent repressors. The implications of these results for the possibility of the involvement of cysteines in Michael adduct formation in the repressor-operator interaction are discussed.

## METHODS

*Recombinant plasmids and site-directed mutagenesis*. The construction and properties of pRZ5 are being described elsewhere (6). The plasmids pCT119, pCT119C46S, and pCT119C101S were constructed by transfer of the coat sequences from pCT1, pCTC46S, and pCTC101S to pUC119 (7) as a Hind III-Kpn I fragment. Plasmid pUC119 contains an origin of replication of the phage M13, which facilitates the production of single-stranded DNA for use as template in mutagenesis reactions, and for dideoxy sequence analysis of mutants. Otherwise, the properties of the pCT119 recombinants are no different from the pCT1 plasmids previously described (6). Site-directed mutagenesis was by the method of Kunkel,et al. (8). Single-stranded templates for mutagenesis were prepared from strain CJ236 ($dut^-$, $ung^-$), containing the appropriate pCT119 derivative, after infection with the helper phage, M13KO7 (7). The mutagenic oligonucleotide was annealed to the template and extended with the large fragment of DNA polymerase I. The DNA was transfected into strain CSH41 containing pRZ5, and plated on LB plates containing ampicillin, chloramphenicol, and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside(X-gal). The next day colonies with the appropriate colony color phenotype were toothpicked into 1ml of LB broth and grown to saturation. Plasmids extracted from each isolate were retransformed into CSH41(pRZ5) to ensure the clonal origin of each isolate. After a second plasmid mini-prep, each plasmid was introduced into a male strain suitable for infection with M13KO7, and isolation of single-stranded DNA for nucleotide sequence determination. The complete sequence of a representative of each mutant coat sequence was determined so as to avoid the possibility of unexpected secondary mutations.

The mutagenic oligonucleotides (produced on an Applied Biosystems model 380A DNA synthesizer) were designed to contain 10 bases of uninterrupted complementarity to the sequences on either side of codons 46 and 101 of the coat gene. The bases opposite the codons to be mutagenized were synthesized from mixtures of phosphoramidites so as to yield the sequence 5'-NNS-3', where N represents any of the four nucleotides and S is G and C. The resulting mixture of 32 codons can specify each of the twenty amino acids and one nonsense codon. This approach has been used previously by others (9). The 23-mer used for mutagenesis of codon 46 was called mut46 and had the sequence 5-GACGAACGCTSNNGGTTACTTTG-3. The other 23-mer, mut101, was 5-CAATAAGCTCSNNGTCGGAATTC-3. These oligonucleotides represent the antisense strand, since the sense strand of the coat sequence was present on the template.

*Analysis of protein products of the various strains*. The presence of coat protein in strains containing wild-type and mutant coat sequences was determined by the western blot method

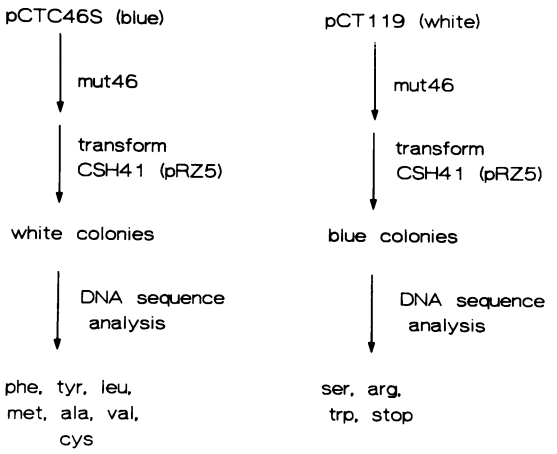


**Figure 1.** An outline of the protocol followed in mutagenesis of codon 46. Mut46 refers to the mutagenic oligonucleotide used to convert codon 46 to any of the possiblities generated by the sequence NNS (see Methods for details). Each of the amino acid replacements isolated by this approach is listed.

(10). Cells containing the mutant coat-producing plasmids were grown in rich medium to $A_{600}=1$, collected by centrifugation and lysed by boiling in SDS gel sample buffer. Equal quantities of the lysates were fractionated by SDS/polyacrylamide gel electrophoresis (11), transferred to nitrocellulose, and probed with anti-MS2 serum and radioiodinated protein A. Coat protein was visualized by autoradiography. The amount of the replicase-β-galactosidase fusion protein was determined by measuring enzymatic activity using o-nitrophenyl-β-D-galactoside (ONPG) as described by Miller (12). For qualitative comparisons of relative repressor efficiencies, colony color on X-gal plates is a sensitive and highly reproducible assay.

## RESULTS

The two-plasmid expression system which places synthesis of a hybrid replicase-β-galactosidase sequence under translational control of the MS2 repressor is described briefly below. The plasmid called pRZ5 contains the hybrid replicase-β-galactosidase gene downstream of the *lac* promoter. Plasmid pRZ5 is derived from pACYC184 and therefore contains a P15A origin of replication and a chloramphenicol resistance determinant. The second plasmid, called pCT119, is derived from pUC119 (7) by insertion of the coat sequence in a manner analogous to the construction of pCT1 (6). Plasmid pCT119 has a ColE1-type origin of replication and an ampicillin resistance determinant. This plasmid also contains an origin of replication from phage M13 and it is a simple matter to prepare single-stranded templates for site-directed mutagenesis and DNA sequence analysis. All of the mutants described below were constructed by mutagenesis of pCT119, pCT119C46S, or pCT119C101S. Since they are derived from different incompatibility groups and contain resistance determinants for different antibiotics, pRZ5 and pCT119 can be maintained stably together in the same host strain. The presence of a wild-type coat sequence in pCT119 leads to repression of translation of the hybrid replicase-β-galactosidase enzyme, and a white colony color phenotype on solid medium containing the chromogenic substrate, X-

**Table I.** The amino acid replacements, their respective codons, and frequencies of occurrence resulting from mutagenesis of codon 46.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| white: | | | | | | | |
| leu | (14) | CUG | (5) | CUC | (7) | UUG | (2) |
| phe | (5) | UUC | (5) | | | | |
| tyr | (5) | UAC | (5) | | | | |
| met | (4) | AUG | (4) | | | | |
| val | (2) | GUC | (2) | GUG | (0) | | |
| ala | (2) | GCC | (1) | GCG | (1) | | |
| cys | (2) | UGC | (2) | | | | |
| blue: | | | | | | | |
| arg | (5) | CGC | (5) | AGG | (0) | CGG | (0) |
| ser | (5) | UCC | (3) | AGC | (2) | UCG | (0) |
| trp | (3) | UGG | (3) | | | | |

gal. The mutant called pCT119C46S is completely defective for repressor function and colonies are blue. A partially defective phenotype is displayed by pCT119C101S, so that colonies on X-gal plates are an intermediate shade of blue. Plasmid pUCter3 is a derivative of pUC19 with synthetic DNA containing translational terminators in all reading frames in the polylinker region. Its relevant property is the absence of the coat sequence. Thus, pUCter3 is used as a control in these experiments.

*Mutations in codon 46.* Previous chemical modification experiments had indicated that reaction of cys46 with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) destroyed the RNA

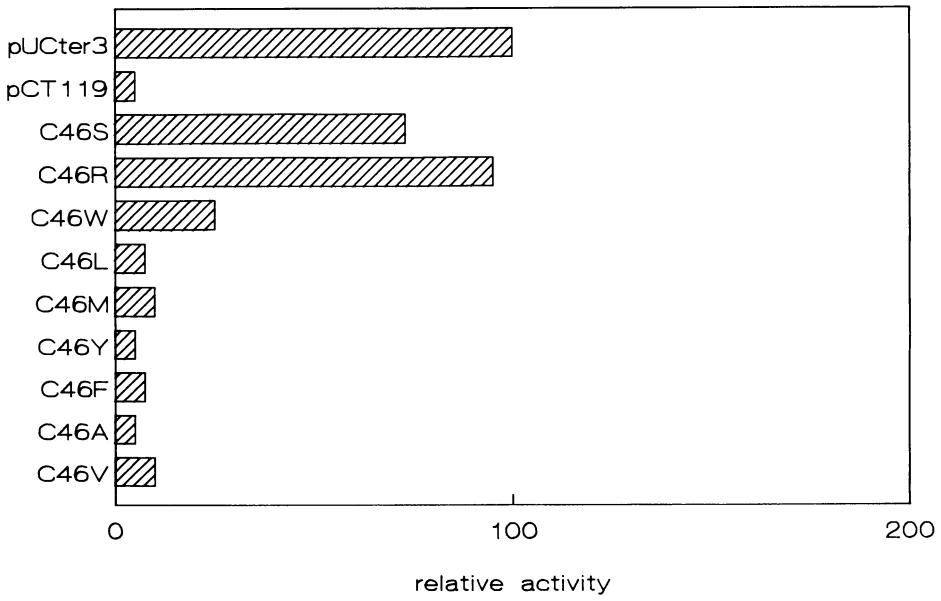


**Figure 2.** Relative β-galactosidase activities produced in strain CSH41(pRZ5) in the presence of each of the coat mutants. High activity represents loss of repressor function. Each value is the average of three separate measurements. The activity produced by CSH41(pUCter3, pRZ5) is arbitrarily defined as 100.

**Table II.** A numerical representation of the data shown graphically in Figures 2, 3, and 5. Also shown is a comparison of colony color on X-gal plates, using an arbitrary scale.

| mutant | β-gal activity | blueness on X-gal | mutant | β-gal activity | blueness on X-gal |
|---|---|---|---|---|---|
| pUCter3 | 100 | +++ | pUCter3 | 100 | +++ |
| w.t. | 5 | +− | w.t. | 5 | +− |
| C46S | 73 | +++ | C101S | 10 | + |
| C46R | 95 | +++ | C101H | 26 | ++ |
| C46W | 25 | ++ | C101L | 18 | ++ |
| C46L | 8 | +− | C101V | 50 | +++ |
| C46M | 10 | +− | C101W | 125 | +++ |
| C46Y | 5 | +− | C101I | 68 | +++ |
| C46F | 8 | − | C101F | 106 | +++ |
| C46A | 5 | +− | C101M | 41 | +++ |
| C46V | 10 | + | C101P | 92 | +++ |
| | | | C101Y | 47 | +++ |
| | | | C101R | 5 | − |
| pUCter3 | 100 | +++ | | | |
| w.t. | 3 | +− | | | |
| C46F-C101R | 6 | − | | | |
| C46L-C101R | 4 | − | | | |

binding activity of the MS2 coat protein (13). Therefore it was of interest to determine how amino acid subtitutions at this position affect repressor activity. We have shown that replacement of cys46 with serine results in loss of repression *in vivo* (6). However, this mutant, called C46S, is also defective for protein folding and/or stability as determined by its inability to form capsids. This compromised the assignment of cys46 as an essential contact residue. Therefore, we set out to isolate a variety of mutations in codon 46.

The mutagenesis was performed by the method of Kunkel, et al. (8), utilizing a mutagenic oligonucleotide (called mut46) whose sequence is shown in Methods. Mut46 is a degenerate mixture of olignucleotides capable, in principle, of converting codon 46 to any of 32 different triplets, representing codons for each of the 20 amino acids and one nonsense codon. As shown in Figure 1, the mutagenesis was performed with two different templates. First I mutagenized pCT119C46S, transformed *E. coli* strain CSH41(pRZ5), and plated on LB plates containing ampicillin, chloramphenicol, and X-gal. Cells containing pCT119C46S are blue. White colonies represented revertants that had regained the ability to repress synthesis of the replicase-β-galactosidase fusion protein. Nucleotide sequence analysis of 34 revertants revealed that at least six different amino acid substitutions at position 46 result in a functional repressor. Most mutations were isolated more that once. Table I lists the codons produced, the frequency of their occurrence, and the corresponding amino acid substitutions. The repressor activity of the mutants was estimated by measurement of β-galactosidase acitivity produced by each mutant strain (12). Figure 2 graphically illustrates these results, which are also summarized numerically in Table II. Also shown is a rough measure of relative colony color on solid medium containing X-gal. The plasmid pUCter3 does not contain the coat sequence and, thus, does not repress at all. It is included here as a control. Most of the mutants are able to repress about as well as wild-type. One of the mutants, C46F, even represses better than wild-type, based on colony color. Thus, phenylalanine, tyrosine, leucine, methionine, valine and alanine are acceptable replacements for cysteine at position 46.

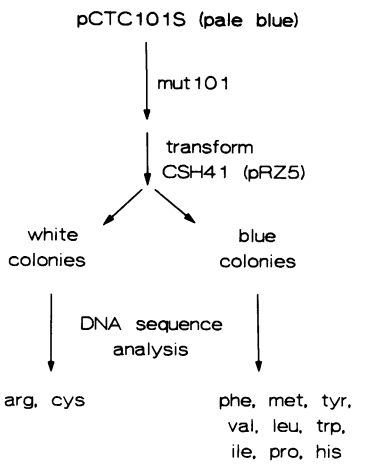


**Figure 3.** The scheme for directed mutagenesis of codon 101. The mutagenic oligonucleotide, mut101, was designed by the same principles as mut46 (see Methods).

It should be noted that comparisons of relative repressor efficiencies in strains that produce low levels of β-galactosidase are more appropriately determined by colony color on X-gal plates than by solution assay. The occasional segregation of the two plasmids results in an artifactual derepression of enzyme synthesis in some cells. This introduces a degree of variability that is especially significant when comparing low level activities. For this reason, the exact level of repressibility varies from experiment to experiment when determined by this means. Blueness on X-gal plates, however, is a sensitive and highly reproducible, if only semi-quantitative, indicator of relative repressor strengths. Even colonies with the wild-type coat sequence turn pale blue with prolonged incubation or at high cell densities, making it possible to distinguish mutants (like C46F) that have increased repressor activity compared to wild-type.

In order to isolate a battery of replacements at position 46 that result in loss of repression, we subjected the wild-type coat sequence in pCT119 to mutagenesis with mut46. This time we screened for blue colonies. Thirty six were picked for nucleotide sequence analysis. Of these, 22 had lost repressor function as a result of large deletions and insertions. It is unclear why such a high fraction of the repressor defects resulted from these rearrangements. One other isolate was defective because it had aquired a nonsense triplet at codon 46. Of the remaining 13 mutants, all contained substitutions at position 46. Five were arginine, five were serine, and three were tryptophan. The levels of β-galactosidase activity produced by each mutant are shown in Figure 2 and in Table II.

*Mutations in codon 101.* A slightly different strategy was utilized in the mutational analysis of postion 101 (see Figure 3). A mutagenic oligonucleotide (mut101, see Methods) similar in design to mut46 was used to mutagenize pCT119C101S. This mutant replaces cys101 with serine and is partially defective for repressor function. It produces pale blue colonies on X-gal plates (6). Analysis of the capsid forming ability of this mutant indicated a potential defect in protein folding or stability. Thus, as with C46S, it was unclear whether the defect was due to loss of a contact with RNA, or to a more generalized perturbation of coat protein structure. However, I took advantage of the intermediate blueness of C101S colonies.

**Table III.** Replacments of amino acids and codons at position 101 and their respective frequencies.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| white: | | | | | | | |
| cys | (9) | UGC | (9) | | | | |
| arg | (12) | AGG | (7) | CGC | (5) | CGG | (0) |
| blue: | | | | | | | |
| phe | (6) | UUC | (6) | | | | |
| met | (2) | AUG | (2) | | | | |
| leu | (1) | CUC | (1) | CUG | (0) | UUG | (0) |
| val | (2) | GUG | (2) | GUC | (0) | | |
| tyr | (5) | UAC | (5) | | | | |
| ser | (1) | AGC | (1) | UCC | (0) | UCG | (0) |
| trp | (3) | UGG | (3) | | | | |
| ile | (1) | AUC | (1) | | | | |
| pro | (1) | CCC | (1) | | | | |
| his | (1) | CAC | (1) | | | | |

By picking white colonies, mutants which restored repressor function were selected. By picking bluer colonies, repressor mutants that were more defective than C101S were isolated. DNA from twenty-two white and thirty-six blue colonies was subjected to nucleotide sequence analysis. Again, a significant fraction (13 of 36) of the repressor defectives were the result of rearrangements. Otherwise, the results are different from those reported above for the position 46 substitutions. Position 101 proves to be more sensitive to substitution. The repressor defective phenotype resulted from the substitution of a variety of different amino acids at postion 101. In addition to serine, nine different substitutions result in loss or reduction of repressor acitivity. They are histidine, leucine, phenylalanine,

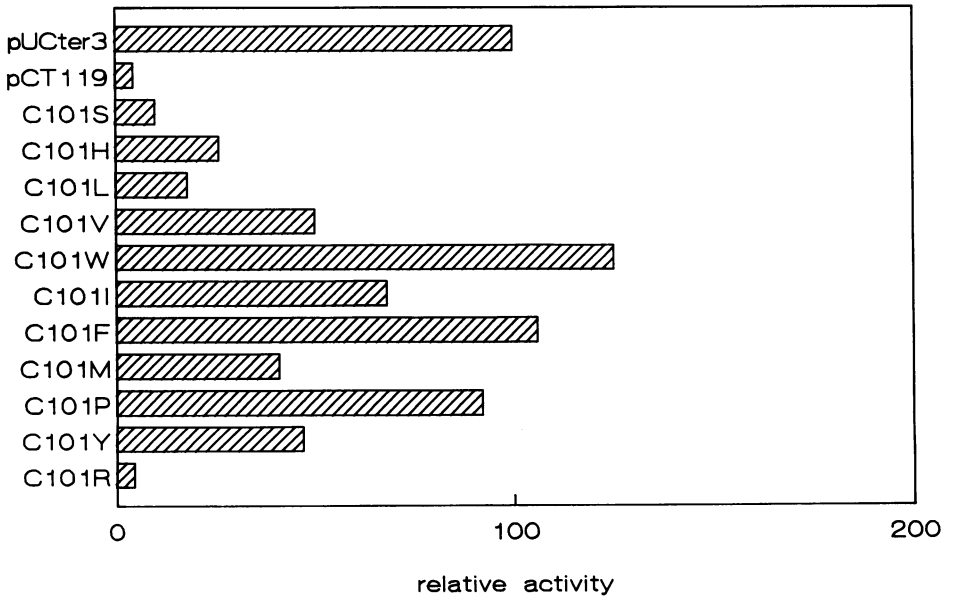


**Figure 4.** β-galactosidase activities of CSH41(pRZ5) strains containing the various codon 101 mutations.

methionine, valine, tyrosine, tryptophan, isoleucine, and proline. On the other hand, of the twenty-two isolates that had reverted to the white colony phenotype, ten had done so by reaquisition of a cysteine residue. The remaining twelve had all aquired an arginine at position 101. By comparison of colony color, the C101R variant is a better repressor than wild-type. This striking frequency of occurrence of arginine is apparently not the result of any serious bias in the mutagenic oligonucleotide, since five of the mutants used CGC, and seven used AGG to encode this amino acid. The codon and amino acid replacements are shown in Table III. A summary of the β-galactosidase assays is shown in Table II and Figure 4.

*Analysis of a double mutant that contains no cysteines.* The combined results of mutational analyses of codons 46 and 101 suggested that in an appropriate double mutant, cysteines would prove to be completely dispensible for repressor function. An *Eco*RI site between the two codons made possible the straight-forward construction of a coat sequence with phenylalanine or leucine at position 46, and arginine at position 101. The resulting double mutants, called C46F-C101R and C46L-C101R, were subjected to nucleotide sequence analysis to verify their structures, and then tested for their ability to repress replicase-β-galactosidase synthesis. CSH41(pRZ5) was transformed with pCT119C46F-C101R and pCT119C46L-C101R. In both cases this resulted in white colonies on X-gal plates, and low levels of β-galactosidase activity as determined by solution assay (see Figure 5). Thus, coat molecules which contain no cysteine residues can function as translational repressors.

*Western blot analysis of the mutant coat proteins.* Sometimes an amino acid substitution results in reduced steady state levels of the altered protein. This seems often to result from a destabilizing effect of the substitution since, proteins that denature readily tend to be degraded rapidly. This was the case in a study of lambda cro protein, for example (14). To determine the relative steady state levels of coat protein produced by the various mutants, cells containing the appropriate plasmids were grown to $A_{600}$ of about one. Cells were collected by centrifugation, resuspended, and then lysed by boiling in SDS-gel sample buffer. Proteins were fractionated by electrophoresis in the presence of SDS (11) and transferred to nitrocellulose (10). Coat protein was visualized using anti-MS2 serum and radioiodinated protein A. The results (Figure 6) show that in most cases, coat protein is produced at levels similar to wild-type. C46S, C46R, and C101H are the exceptions. Moreover, each of these presents a repressor defective phenotype (see Figures 2 and 3). It is not known how much of the defect is due to destabilization and/or underproduction of the repressor.

Note that C46W, C46Y, and C46V produce an additional species whose mobility is substantially less than normal coat protein. This extra band consistently appeared in several repetitions of the experiment shown in Figure 6. Its identity is unknown, but it is possible that these substitutions partially stabilize an intersubunit contact against denaturation by SDS. The extra band may correspond to a dimer, or other oligomeric species.

## DISCUSSION

This paper reports the effects on translational repression of substitution of the cysteine residues at positions 46 and 101 of the MS2 coat protein. In all, nine substitutions at position 46 and eleven at position 101 have been tested. All nineteen possible substitutions at each position have not yet been isolated. There are several potential explanations for this. Since it cannot be guaranteed that each nucleotide in the synthetic mixture was incorporated with equal efficiency, we cannot rule out the possibility that the mutagenic oligonucleotides

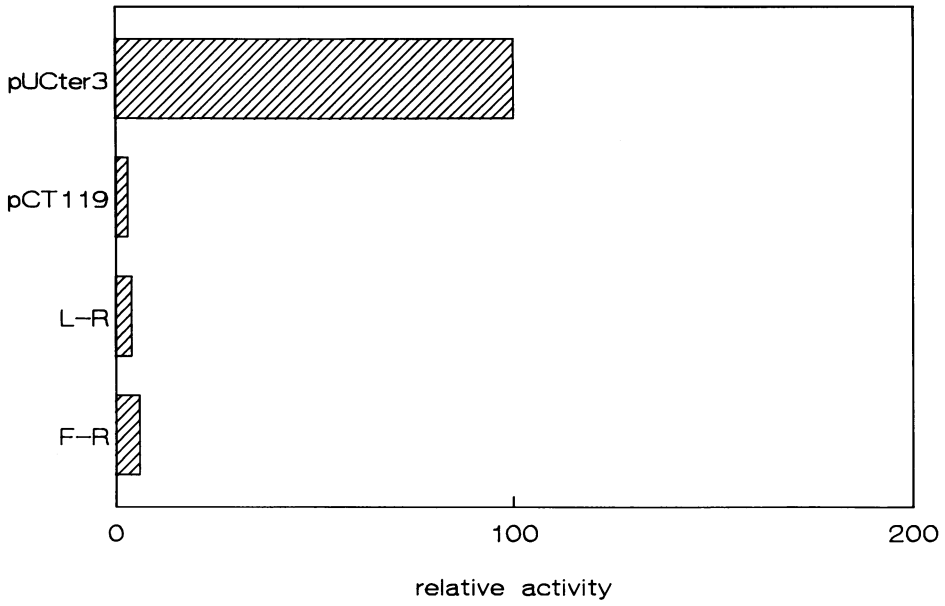


**Figure 5.** β-galactosidase activities produced by CSH41(pRZ5) strains containing coat sequences with mutations in both codons 46 and 101. L-R refers to C46L-C101R, and F-R to C46F-C101R.

are biased toward the introduction of a subset of all the codon possibilities. It also seems likely that the screening procedures may have caused us to overlook certain mutations. Two screens were used for isolation of codon 46 mutations. In the first, only white colonies were picked. In the second, attention was directed to the bluest ones. Thus, some colonies of intermediate color may have been ignored. Such considerations may have similarly biased the screening for position 101 substitutions, since colonies whose phenotype was similar to C101S would have been ignored.

Although the present work should not be regarded as an exhaustive mutational analysis of codons 46 and 101, the results do permit some conclusions. First, it is clear that a variety of substitutions at position 46 are consistent with normal, or near normal, repressor function. The substitutions that were isolated are all relatively hydrophobic in character. Many of these mutants utilize an amino acid whose side chain bears little similarity to the cysteine residue that normally resides there. For example, phe46 functions perfectly well, even though its side chain is considerably bulkier and more hydrophobic than cysteine. Meanwhile, the apparently conservative substitution of serine at this position results in the loss of repressor activity. The presence of arginine or tryptophan at position 46 also results in the repressor defective phenotype. C46S is apparently a folding or stability defect (6). By analogy to the results of Pakula, et al. (14), the western blot data (Figure 6) suggest that C46R may be defective for a similar reason. In any event, it seems unlikely that residue 46 is the site of an important protein-RNA contact, since a variety of amino acids are capable of substituting for cysteine.

In view of the large number of permissible substitutions at postion 46, the previous report that chemical modification of cys46 inactivates the repressor (13) may seem to present

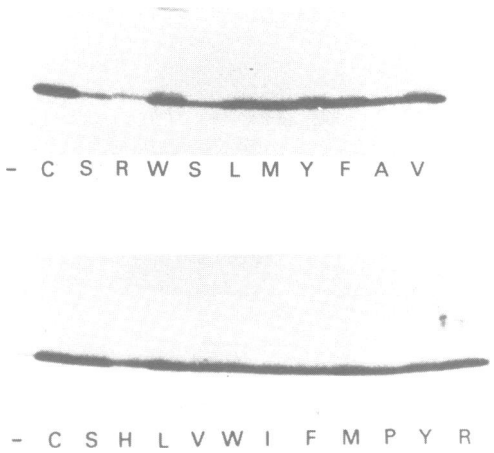


**Figure 6.** Western blot of the coat proteins produced by each of the mutants described in this study. The top panel shows the coat proteins with substitutions at position 46, and the bottom shows those substituted at position 101. Each is labeled using the single-letter amino acid code to specify the substitution. The lanes labeled with a minus sign contain lysates from cells that do not produce coat protein. Note the presence of two C46S mutants. The one at left is the original C46S mutant described previously and contains UCU at codon 46. The second C46S mutant was isolated as shown in Figure 1 and has AGC at codon 46.

a paradox. One must bear in mind, however, that even highly selective chemical modifications can cause long range structural perturbations or steric effects that result in inactivation of a protein. A pertinent example of such a phenomenon was recently described by Profy and Schimmel (15). Reaction of glycine-tRNA-synthetase with N-ethyl-maleimide (NEM) had led to the belief that a subunit of the enzyme contained a single essential cysteine residue. Site-directed mutagenesis experiments demonstrated that cys395 provided the reactive thiol. However, the replacement of cys395 with alanine resulted in an enzyme with essentially wild-type behavior. Thus, it was shown that although cys395 itself was not essential for catalytic activity, the chemical modification of this site was not tolerated. An analogous occurrence may explain the loss of activity of coat protein when cys46 is modified. It is important to recognize this limitation of chemical modification and mutagenesis studies. The affected amino acid need not be an active site residue for its modification or substitution to result in inactivation. Therefore, it is risky to base conclusions on the behavior of only one mutant or chemical derivative.

Mutational analysis of position 101 indicates that it is sensitive to substitution, consistent with the possiblity (among others) that it represents a site of contact with RNA. At least ten different amino acid substitutions result in lost function, but only arginine has so far been found to functionally replace cysteine at this site. Significantly, C101R is a better repressor than wild-type. Moreover, C101R partially suppresses an operator constitutive mutation that substitutes adenine for the uridine residue normally present at $-5$ (unpublished results). On the other hand it has no effect on operator mutants altered at position $-4$. It is possible that substitution of cys46 with arginine results in replacement of a protein-RNA contact with a new interaction. The arginine side chain affords plenty of opportunity for H-bonds or charge interactions with RNA. Whether this is really the case is the subject of future experimentation.

Among the means by which amino acid substitution could influence repressor function are the following: 1. Alterations of a specific protein-RNA contact can directly affect binding behavior. Specific contacts can be eliminated or new ones created. 2. Some mutations may result in an altered ability of the protein to fold into the proper 3-dimensional conformation. 3. The thermodynamic stability of a protein can be affected by mutation. Mutations that increase and decrease thermal stability have been described (for example see ref. 16). 4. Some amino acid substitutions alter the metabolic stability (i.e. degradation rate) of a protein and, thus, alter its steady state level within the cell. Metabolic instability is sometimes a secondary consequence of thermodynamic instability. 5. Since the active repressor is probably a dimer (17), mutations that affect the equilibrium constant for dimerization will have an effect on apparent $K_a$ for RNA.

We seem to have eliminated cys46 as a likely site of protein-RNA interaction. Cys101, however, remains a candidate for a contact site. Does coat protein form a Michael adduct between cys101 and U −5 of the translational operator? Although it has been shown that cysteines are not necessary for *in vivo* repressor activity, it cannot be confidently asserted that such a contact does not occur in the wild-type complex. As suggested above, arg101 may establish new contacts with RNA, compensating for the loss of cysteine at this site. It must also be recognized that, in principle, amino acid side chains other than cysteine could potentially form a Michael adduct, particularly when placed in an appropriate chemical environment. This work only indicates that the cysteines are non-essential. Future work will attempt to resolve these issues.

## ACKNOWLEDGEMENTS

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