functional heterogeneity in protein sources within the gland after a cholecystokinin- or methacholine-stimulated switch from a basal pool to a prestored pool of enzyme; their interpretation of the data, in terms of the equilibrium hypothesis, was that newly synthesized and old enzyme were mixed in the cytosol. In contrast, our data directly confirm that enzymes are secreted in groups, in a linked fashion from prepackaged organelles, exactly as expected of exocytosis. Instead of a unique secretory pathway as postulated in the original exocytosis model, our observations of nonparallel secretion under exocytotic conditions suggest the existence of multiple inter- or intracellular exocytotic pathways.

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8. This was the dose that was originally found to cause enhanced secretion of chymotrypsinogen in the pancreas in vitro (*12*). For a review of the chemistry and purification of chymodenin, see J. W. Adelson *et al.*, in *Gastrointestinal Hormones*, G. B. Jerzy-Glass, Ed. (Raven, New York, 1980), pp. 387–396.
9. The correlation coefficient for the output of each pair of enzymes in vitro was tested for an increase in the chymodenin treatment values over the control value by Fisher's conversion of the r values to a normal z distribution by the formula $z(r) = 1/2\ln[(1 + r)/(1 - r)]$ followed by a t-test of the differences of the z values [G. W. Snedecor and W. G. Cochran, *Statistical Methods* (Iowa State Univ. Press, Ames, 1980)]. The results were significant for chymotrypsinogen-amylase, chymotrypsinogen-lipase, amylase-lipase, amylase–total protein, and lipase–total protein. To further ensure that the high correlation coefficients obtained in vitro after chymodenin treatment were not an artifact, the data were challenged by testing the possibility that the variance of the numerical ratios of the enzyme outputs would decrease, as would be expected. The results were: chymotrypsinogen-lipase, $F = 0.002$; chymotrypsinogen–total protein, $F = 0.02$; amylase-lipase, $F = 0.0001$; amylase–total protein, $F = 0.002$; and lipase–total protein, $F = 0.02$. For chymotrypsinogen-amylase, F was 0.09. The chymodenin-treated population was not different from the control population before treatment: the mean pretreatment correlation for the chymodenin-treated population was $r = 0.73$ and for the control treated population was $r = 0.70$. When the data were divided into two groups, one above and one below the mean output of each enzyme, correlation analysis revealed that the coefficients were significant and comparable in magnitude to those of the entire population, indicating the lack of an anomalous effect of output range on the correlations obtained.
10. A statistical comparison of the mean correlation coefficients for enzyme output in the chymodenin-treated group with the mean correlation coefficients in control group would not be valid because the coefficients are not independent observations; thus the standard error cannot be derived. The increased correlations in the chymodenin-treated group were observed in six of six cases, were of a large magnitude, and were consistent with chymodenin causing the release of enzymes in more tightly linked groups than in the controls.
11. The enzyme output data were not distributed in isolated data clusters on an animal-by-animal basis; the data among individual animals overlapped with each other to a large extent. Further, Adelson and Rothman (*12*) earlier showed that the inter-animal and interperiod variances in enzyme output did not differ significantly. The differences of proportionality in situ were not significantly dependent on an initial pretreatment difference between the populations treated with cholecystokinin only and cholecystokinin plus chymodenin; when the two populations were tested separately for changes in the regression before and after chymodenin treatment, the changes in slope for the chymodenin-treated population were similar to those for time-paired controls.
12. One of us (J.W.A.) and Rothman reported earlier that chymodenin selectively stimulated pancreatic secretion of chymotrypsinogen; this was based on evidence that chymotrypsinogen secretion increased two- to threefold on stimulation by chymodenin whereas lipase secretion remained unchanged in experiments with the rabbit pancreas in vitro and in situ [J. W. Adelson and S. S. Rothman, *Science* **183**, 1087 (1974); *Am. J. Physiol.* **229**, 160 (1975)]. Although it appeared that chymotrypsinogen was secreted independently of other enzymes because its output increased compared with the output of lipase, which did not change, we believe that the original interpretation of the data was incorrect because this conclusion was arrived at without consideration of the link between the secretion of chymotrypsinogen and the secretion of lipase ($r = 0.90$) in both the earlier study and the present ones. Thus the two enzymes were secreted together in a fixed ratio of chymotrypsinogen to lipase, which was greater than that in the basal state.
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15 September 1984; accepted 22 November 1985

Immunogenicity of Synthetic Peptides from Circumsporozoite Protein of *Plasmodium falciparum*

Abstract. *In a study of recombinant proteins that might be useful in developing a vaccine against malaria, synthetic peptides from the circumsporozoite (CS) protein of* Plasmodium falciparum *were found to be immunogenic for mice and rabbits. Antibody to peptides from the repeating region of the CS protein recognized native CS protein and blocked sporozoite invasion of human hepatoma cells in vitro. Antibodies to peptides from regions I and II had no biologic activity, although antibody to region I recognized processed CS protein by Western blot analysis. These data support the feasibility of developing a vaccine against the sporozoite stage of the malaria parasite by using synthetic peptides of the repeating region of the CS protein conjugated to a carrier protein.*

When injected into humans and other animals, irradiated sporozoites of the malaria parasite, *Plasmodium*, provide protection against further challenge with viable sporozoites (*1–3*). This protection is mediated, at least in part, by antibodies to the circumsporozoite (CS) protein present on the sporozoite's surface (*4*). Recently, the CS genes encoding the CS proteins of *Plasmodium knowlesi* and *P. falciparum* were cloned (*5*) and sequenced (*6*). The CS gene for *P. falciparum* encodes for 41 tetrapeptide repeating units flanked by two regions showing homology between *P. falciparum* and *P. knowlesi*. It was suggested by Dame *et al.* (*6*) that these two small conserved sequences might have an important biological function and, along with the repeat region, be useful targets for vaccine development.

We synthesized peptides from the repeating and conserved regions of the CS protein of *P. falciparum* (Table 1) (*7*). Repeating sequences and peptides from region I were conjugated to bovine serum albumin (BSA) with the use of succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), or to thyroglobulin with the use of *m*-maleimi-

dobenzoyl *N*-hydroxysuccinimide ester (MBS). Peptides from region II were conjugated to keyhole limpet hemocyanin (KLH) with the use of MBS, and to BSA with SMCC. The degree of conjugation, quantitated by comparing the amino acid composition of the carrier protein before and after reaction with the peptide, revealed 15 to 25 peptides per molecule of BSA and 25 to 35 peptides per 100 kilodaltons of thyroglobulin. Antisera to peptides from regions I, II, and repeating region conjugated to carriers were raised in mice and rabbits and tested as described (*8*). Each of the peptides produced detectable antibody responses in mice, although the titers against region I and the repeat peptides were higher than against region II (Fig. 1). In contrast, high titers against region II were elicited in rabbits. All repeat peptides produced virtually identical antibody responses to those shown for the 16-residue repeat peptide in Fig. 1.

When these antisera were tested by indirect immunofluorescent assay (IFA) against *P. falciparum* sporozoites, antibodies to region I and the repeat peptides were reactive (Table 2). Mouse and rabbit antisera to region II did not react. We examined the reactivity of the peptide antisera to other malaria sporozoites by IFA. The absence of cross-reactivity of antisera to the repeating peptides of *P. knowlesi* and *P. cynomolgi* was expected, because sequences in the repeat domain are different in each species (*5, 6, 9*). Cross-reactivity with *P. berghei* was consistent with the previous finding that some *P. falciparum* monoclonal antibodies (Mab's) react with *P. berghei* sporozoites (*10*). Since the sequence of the *P. berghei* CS protein is unknown, this may reflect either sequence or conformational homology between the two species. The IFA data for region I showed cross-reactivity with *P. knowlesi* and *P. berghei* and no reactivity with *P. vivax, P. cynomolgi*, and *P. gallinaceum*.

Although it was proposed (*6*) that the conserved sequences in regions I and II might have a vital biological function, the absence of IFA reactivity with antibodies to region I of *P. cynomolgi* and *P. vivax* indicates that this region may not be conserved in all primate malaria species. Furthermore, the fact that antibodies to region I have no blocking effects on sporozoite invasion of liver cells suggests that this region is not a receptor for liver invasion.

The issues with respect to region II are somewhat different. Oligonucleotide probes prepared from region II of *P. falciparum* hybridize with *P. vivax* and *P. knowlesi* (*11*), indicating that this region has been conserved. Antibody to region II peptide does not react with intact CS protein from *P. falciparum* by IFA or Western blot analysis, nor does it cross-react with other malaria species (Table 2). The lack of reactivity was somewhat unexpected since short flexible peptides frequently induce antibodies that react with more ordered native molecules (*12*). Moreover, polyclonal antisera to the synthetic peptides should recognize the entire range of permissible peptide conformations. High titers of rabbit antibody to region II were no more reactive in these assays than low titers of mouse antibody. The present results indicate that the conserved region II may be part of a highly ordered region in the intact protein. Thus, limitations in the range of conformation in this region of the CS protein may prevent binding by antibodies to region II peptides (*13*).

We previously showed that Mab's to the repeat regions of the CS protein of *P. falciparum* detect three proteins (60, 53, and 51 kD) from extracts of *P. falciparum* sporozoites by Western blot analysis (*6, 10*). Antisera to the repeating peptides detected the same three proteins (Fig. 2). Antisera to region I detected the 53- and 51-kD doublet but not the 60-kD peptide. The higher molecular weight protein (60 kD) is presumed to be a precursor protein (*14*) since internal precursors of higher molecular weight have been identified for the CS proteins of *P. berghei* and *P. knowlesi* (*15*). The recognition of processed but not precursor protein by antisera to region I suggests that processing affects conformation or exposure of region I, that is, the 15 amino acids just proximal to the repeat region. No reactivity with antisera to region II could be detected.

Serum samples from humans and other animals immune to sporozoite challenge produce a circumsporozoite precipitin (CSP) reaction and inhibit sporo-

Table 1. Sequences of synthetic peptides from *P. falciparum* CS protein. Details of the synthesis are described (*7*). Synthetic peptides included 8-, 10-, 12-, 14-, and 16-residues of the repeat region as well as the conserved regions I and II as described by Dame *et al.* (*6*). Regions I and II were conjugated to carrier proteins at the carboxyl terminus; all peptides from the repeat regions were conjugated through an amino terminal cysteine.

Residues	Sequence
8	NPNANPNAC-
10	NANPNANPNAC-
12	NPNANPNANPNAC-
14	NANPNANPNANPNAC-
16	NPNANPNANPNANPNAC-
Region I	-CKHKKLKQPGDG
Region II	-TEWSPCSVTCGNGIQ

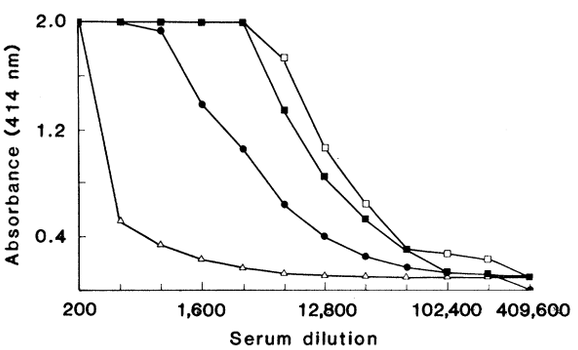

Fig. 1. Immunogenicity of synthetic *P. falciparum* circumsporozoite peptides (*8*). Antiserum to a 16-residue repeat peptide (●); antiserum to a region I peptide (■); mouse antiserum to region II peptide (△); rabbit antiserum to region II peptide (□).

Table 2. Indirect fluorescent antibody (IFA) reactivity of mouse antisera to synthetic peptides containing 8, 10, 12, 14, or 16 residues or to synthetic peptides from regions I and II. The antisera were assayed for cross-reactivity with human, primate, rodent, and avian malaria sporozoites by IFA as described (*23*). Fluorescence was graded from 0 to 4+, with 0 equal to no fluorescence and 4+ equal to fluorescence seen uniformly over the sporozoite. Sera from nonimmunized mice and mice immunized with CFA only were used as negative controls.

Plasmodium	Peptide antisera						
	8	10	12	14	16	Region I	Region II
falciparum	4+	3+	4+	4+	4+	1 to 3+	0
vivax	0	0	0	0	0	0	0
knowlesi	0	0	0	0	0	2+	0
cynomolgi	0	0	0	0	0	0	0
berghei	1+	2+	2+	2+	4+	1+	0
gallinaceum	0	0	0	0	0	0	0

*High titers of rabbit antisera to region II had no activity by IFA.

zoite invasion of a human hepatoma cell line in vitro. Both the precipitin and inhibition assay are thought to be predictive of protection in vivo (*14, 16, 17*). As shown in Table 3, the antisera to the repeating peptides produce strong CSP reactions and completely inhibit sporozoite invasion. Antisera to regions I and II had no activity.

We initiated these studies to determine whether the repeat sequences or one or both of the conserved sequences of the CS protein of *P. falciparum* would be the most appropriate vaccine targets. At issue, as with any synthetic peptide vaccine, is whether such antibodies react with the native protein and, more important, whether these antibodies mediate the desired biological effects. These studies clearly show that antisera to the repeat region and region I, but not to region II, recognize native CS protein. It has been shown that Mab's to the repeat region of *P. falciparum* sporozoites block invasion of hepatocytes in vitro and partially neutralize sporozoites that are injected with the Mab's into chimpanzees (*14*). These Mab's were prepared from spleen cells of mice immunized with irradiated sporozoites. Thus the antigen was presented as a whole protein within the sporozoite and in the sporozoite membrane. In the present study we have shown that peptides of the repeat region linked to a carrier will induce antibodies with similar biologic properties to antibodies induced by immunization with the whole organism. That is, these antibodies to synthetic peptides of the repeat region produce strong CSP reactions and block sporozoite invasion of hepatoma cells.

Gysin *et al.* (*18*) recently demonstrated that, when conjugated with bovine immunoglobulin G (IgG), peptides from the repeat region of the CS protein of the simian parasite *P. knowlesi* neutralized sporozoite infectivity. Our results support the concept that the development of a vaccine to the sporozoite stage of the malaria parasite based on the use of synthetic peptides coupled to a carrier protein is feasible. Although thyroglobulin was used as a carrier protein in these studies, carrier proteins commonly used in humans, such as tetanus toxoid or diphtheria toxoid, could readily be substituted and would probably lead to similar results. The use of a carrier-peptide vaccine is not without potential problems, however, including limitations in the amount of peptide that can be coupled to a carrier, sensitization of the host leading to rapid clearance of antigen, and the phenomenon of epitope-specific suppression (*19, 20*). In epitope-specific regulation, priming with a carrier followed by subsequent immunization with a new epitope (not present in the priming) coupled to the carrier leads to suppression of the antibody response to the new epitope (*19*). Subsequent immunization with a different carrier but the same epitope still results in suppression of the response to the epitope. Epitope-specific suppression has been observed with peptides from streptococcal M protein and diphtheria peptides conjugated to tetanus toxoid (*20, 21*). Thus, peptide-toxoid vaccines may not be immunogenic in individuals previously immunized with toxoid. Even if the peptide is linked to a carrier not commonly used in humans (for example, KLH), the potential requirement for boosting at frequent intervals (if immunity to sporozoites is short lived) may present similar problems.

Table 3. Circumsporozoite precipitin (CSP) reactivity and percentage inhibition of sporozoite invasion of HepG2-A 16 hepatoma cells by mouse antisera to repeat, region I, and region II peptides. The CSP reactions were performed as described (*22*). Twenty-five random sporozoites were examined for each serum sample and the number of CSP positive organisms is indicated. The degree of CSP reactivity is shown in parentheses (0, no CSP reactivity detectable; 2+, a granular precipitate on the surface of the sporozoites; 4+, a long threadlike filament at one end of the sporozoites). Inhibition of sporozoite invasion was performed as described (*17, 22*). Inhibition was the percentage reduction of sporozoite invasion by antisera to the synthetic peptides compared to normal mouse serum controls when CS reactive Mab 2Fl.1 (*6, 10*) gave 100 percent inhibition of sporozoite invasion at a dilution of 1:20. NT, not tested.

Immunogen	CSP	Inhibition (%)
8 residues	23/25 (4+)	100
12 residues	13/25 (4+)	NT
16 residues	21/25 (4+)	100
Region I	0/25 (0)	0
Region II	0/25 (0)	0

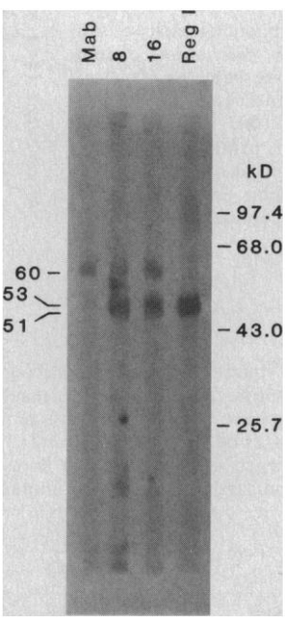

Fig. 2. Western blot analysis of peptide antisera reacted with intact *P. falciparum* CS protein (*24*). A pool of Mab's (2E6.4, 2F1.1, 4D9.1, 4D11.6, and 5G5.3) known to react with *P. falciparum* sporozoites and intact CS protein served as a control (*6, 10*).

Our data showed that the repeat region of the CS protein induced antibodies with biologic activity correlated with protection, and that the other regions we tested did not. As an alternative to the synthetic peptide approach, we elected to produce a recombinant *P. falciparum* CS protein repeat derivative in *Escherichia coli*. This recombinant protein is expressed at high levels, readily purified, highly immunogenic, and a candidate for vaccine trials in man (*22*). Ultimately, whether a vaccine based on synthetic peptides or on recombinant proteins will be protective must await the results of clinical trials in humans.

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8. Six- to eight-week-old C57BL/6 mice (five per group) were injected subcutaneously and intraperitoneally with 0.5-ml doses containing 50 μg of the peptide-carrier conjugate emulsified (1:1) in complete Freund's adjuvant (CFA). Each group received booster injections 4 weeks later with antigen in incomplete Freund's adjuvant. Seven days later, blood was withdrawn, and the blood samples were pooled and clotted at 4°C overnight. The sera were stored at −80°C until tested. Rabbits were immunized subcutaneously and intramuscularly with 1.0-ml doses containing 2.5 mg of the region II-KLH conjugate emulsified 1:1 in CFA. Animals received booster injections of the homologous antigen in CFA 8 weeks later. For the final booster injection, unconjugated region II [2.5 mg in 0.5 ml of phosphate-buffered saline (PBS)] was incubated with 5 μl of 25 percent glutaraldehyde for 1 hour at room temperature. Rabbits received booster injections of this cross-linked region II in incomplete Freund's adjuvant at week 11. Blood was withdrawn 1 week later, and sera were stored at −80°C. Antibody to the peptides was detected by ELISA (enzyme-linked immunosorbent assay) (*6*, *22*). The screening antigen was homologous peptide conjugated to BSA. Rabbit antisera were also tested by ELISA as described (*6*, *22*) except that goat antibody to rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) was substituted for the goat antiserum to mouse IgG.
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23. For IFA, salivary gland sporozoites were suspended in Medium 199 containing 2.5 percent BSA at a concentration of 2×10^4 to 5×10^4 per milliliter. Portions (10 μl) were spread onto each well of a multiwell printed IFA slide, air-dried at room temperature, and stored at −80°C. Portions (10 μl) of sera diluted 1:100 in PBS were spread on each spot of a thawed IFA slide and incubated for 20 minutes at room temperature. Sera were then aspirated and the slides washed with two drops of PBS. Portions (20 μl) of goat antiserum to mouse IgG conjugated to fluorescein isothiocyanate (Kirkegaard and Perry, Gaithersburg, Md.) diluted 1:40 in blocking buffer (1.0 percent BSA, 0.5 percent casein, 0.005 percent thimerosal, and 0.0005 percent phenol red in PBS) plus 0.4 percent ethidium bromide was then added to each spot. After a second 20-minute incubation the spots were washed with two drops of PBS, mounted in glycerol, and examined under ultraviolet light at 500× magnification for fluorescence.
24. Circumsporozoite protein from *P. falciparum* sporozoites isolated from the salivary glands of *Anopheles freeborni* mosquitoes was extracted as described (*6*). Protein from 10^5 sporozoites in sodium dodecyl sulfate (SDS) sample buffer containing 2 percent mercaptoethanol was separated by SDS–polyacrylamide gel electrophoresis according to the method of Laemmli [*Nature (London)* **227**, 680 (1970)] with an 8 to 12 percent gradient gel. Western blot analysis was performed according to a modification of the method of H. Towbin *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)]. The air-dried filter was cut into 4-mm strips and reacted with antisera to repeat peptides (8 and 16 mer), regions I, II, or a pool of five monoclonal antibodies (2E6.4, 2F1.1, 4D9.1, 4D11.6, and 5G5.3) diluted 1:1000 with PBS containing 0.05 percent Tween-20 (PBS-Tw 20). Filter-bound mouse antibody was incubated with ^{125}I-labeled sheep antiserum prepared against whole mouse antibody (2×10^5 cpm/ml in PBS-Tw 20). Rabbit antibody to region II was incubated with ^{125}I-labeled staphylococcal protein A (diluted 3×10^5 cpm/ml in PBS-Tw 20). Antibody was detected by autoradiography with a 48-hour exposure.

25. We thank F. H. Top, Jr., C. L. Diggs, and W. H. Bancroft for support and encouragement. We thank M. Watson for preparation of the manuscript. M.R.H. was supported by AID contract DPE-0453-C-3051-00. J.H.T. and R.L.B. were supported in part by the Naval Medical Research and Development Command, Research Task No. 3M463750D808AD061.

8 February 1985; accepted 21 March 1985

A Catalytic RNA and Its Gene from *Salmonella typhimurium*

Abstract. *The gene for the RNA subunit (M1 RNA) of ribonuclease P from* Salmonella typhimurium *directs the synthesis of an RNA that can cleave transfer RNA precursor molecules. The mature M1 RNA coded for by* Salmonella typhimurium *is 375 nucleotides long and has six nucleotide changes in comparison to M1 RNA from* Escherichia coli. *The regions for promotion and termination of transcription are closely conserved, but adjacent regions of nucleotide sequences show considerable drift.*

RNA molecules that catalyze the cleavage or formation (or both) of covalent bonds have been identified in extracts of *Escherichia coli* (*1*, *2*), *Bacillus subtilis* (*1*), and *Tetrahymena thermophila* (*3*). Ribonuclease P, an enzyme essential for the processing of the 5′ termini of transfer RNA (tRNA) molecules (*4*), is composed of two subunits, an RNA and a protein; the RNA subunit (M1 RNA) is responsible for catalysis. The gene coding for M1 RNA in *E. coli* has been characterized (*5*). We isolated and characterized the corresponding gene and its transcript from *Salmonella typhimurium* to determine (i) whether the catalytic capabilities of the RNA subunit of ribonuclease P are conserved and reflected in extensive primary sequence homology or common higher order structure of the RNA and (ii) whether the regulatory regions adjacent to the gene in *E. coli* are utilized in a closely related organism.

The gene for M1 RNA was isolated by probing a phage library (*6*) containing a digest of the *S. typhimurium* LT2 genome with isotopically labeled M1 RNA from *E. coli* (*5*). We had previously determined that a single DNA fragment in an Eco RI digest of genomic *S. typhimurium* DNA hybridized with the probe even under high stringency conditions. This fragment, about 8.5 kilobases (kb) in length, was isolated from the phage library and cloned into the Eco RI site of pBR329 by standard techniques to make the plasmid pSa17.

We first determined whether pSa17 directed the synthesis of a gene transcript in *E. coli* similar in size to that of M1 RNA from *E. coli*. Cells harboring plasmids pSa17, pRR1 (carrying the gene for M1 RNA from *E. coli*), and pBR329 (carrying a wheat-storage protein gene 12-15) were treated with ^{32}P-labeled phosphate. The RNA was extracted with phenol and analyzed by polyacrylamide gel electrophoresis and autoradiography (Fig. 1A). A transcript with about the same mobility as M1 RNA from *E. coli* (lanes 1 and 2) was seen for pSa17. No

Table 1. Comparison of some single-copy oligonucleotides in fingerprints of M1 RNA from *Salmonella typhimurium* and *Escherichia coli*. The oligonucleotide designations refer to the fingerprints shown in Fig. 1, C (*E. coli*, EC) and D (*S. typhimurium*, ST). Oligonucleotide EC 8 is grouped for comparative purposes with ST P and ST 8 because they are at the same location in their respective M1 RNA sequences. Similarly, EC 35 is grouped with ST 35X. EC 26 is grouped with ST Q because they are similar in composition and chromatographic mobility. Composition of the *E. coli* nucleotides was determined previously (*5*). Composition of the *S. typhimurium* oligonucleotides was determined as described in the text and was checked against the DNA sequence. U, uracil; other abbreviations for bases are as given in the text.

Oligonucleotide	Composition
EC 8	$UUUCACCU_{OH}$
ST P	$UUUCACUU_{OH}$
ST 8	$UUUCACU_{OH}$
EC 35	UCCUCUUCG
ST 35X	UCCUUUCG
EC 26	AACCCG + CAACAG
ST Q	CCCACG