Complementary oligodeoxynucleotide mediated inhibition of tobacco mosaic virus RNA translation *in vitro*

Carol Crum¹, Jerry D.Johnson², Arlen Nelson² and Don Roth^{1,2} *

¹Department of Plant, Soil and Insect Sciences and ²Department of Molecular Biology, University of Wyoming, Laramie, WY 82071, USA

Received September 8, 1987; Revised and Accepted December 23, 1987

ABSTRACT

Two different "antisense" oligodeoxynucleotides and their RNA analogues, each complementary to non-overlapping sequences of 51 bases near the 5' end of TMV RNA, inhibit *in vitro* translation of the genomic RNA in a rabbit reticulocyte lysate. Inhibition is dependent upon complementarity, concentration, and hybridization of the oligomers with TMV RNA. Inhibition is observed at molar ratios of TMV RNA to antisense oligomers as low as 1:1.5. A plateau of inhibition at which 10-25% of the control signal remains is achieved by molar ratios of TMV RNA:antisense DNA or RNA $\geq 1:15$. The extent of inhibition is not increased by the simultaneous presence of both complementary fragments. Oligodeoxynucleotides and their RNA analogues identical to the same regions of TMV RNA have no direct effect on translation, however, they can block inhibition by the antisense fragments. Translation of BMV RNA is not affected by any of the oligodeoxynucleotides. Polyacrylamide gel electrophoresis shows translation of TMV p126 is selectively inhibited. We conclude that the observed inhibition of translation is due to direct interference with ribosome function.

INTRODUCTION

Tobacco mosaic virus (TMV) is a single stranded, plus sense RNA virus 6395 nucleotides in length which has been completely sequenced (1). The RNA has the canonical 5' m7GpppG cap followed by a 69 nucleotide untranslated leader region preceeding the first cistron. TMV RNA is efficiently translated in many *in vitro* systems, however, in eukaryotic systems expression is limited to the 5'-proximal cistron. This region codes for two proteins with molecular weights of 125,941 (p126) and 183,253 (p183). The latter protein is a read-through product containing the entire 3417 nucleotide coding region of the p126 gene plus an additional 1497 nucleotides downstream from the p126 termination codon. At least two other proteins are formed from sub-genomic RNAs *in vivo* (2-4), however, they

are not translated from full length genomic RNA in *in vitro* systems derived from rabbit reticulocytes or wheat germ lysates (2,5).

We have shown that a TMV induced protein of 120,000 Mr from infected tobacco tissue homogenates has nucleotide binding characteristics expected for a replicase (6). Several lines of evidence have suggested that this TMV induced protein is p126 (7). Further, substantial indirect data have implicated p126 in virus replication (7-10). However, conclusive determination of the p126 functional role has been complicated by the inability to purify template dependent TMV specific replicase activity and to identify the associated proteins.

Recent studies using both prokaryotic and eukaryotic models have shown that RNA or DNA antisense sequences, capable of duplex formation with target mRNAs, can be used to inhibit gene expression *in vitro* and *in vivo* (11). Clearly, these results have important implications in the development of plant viral control strategies, but they also offer a mechanism to study viral gene function and regulation. Coleman et al. (12) demonstrated that translation of the *E. coli* bacteriophage SP replicase gene could be effectively regulated by antisense RNA directed against the upstream leader sequences or the 5' initiation sequences of the target gene. Similarly, Rous sarcoma virus gene expression was inhibited by a complementary oligodeoxynucleotide *in vitro* (13) and *in vivo* (14,15). Recently, antisense oligodeoxynucleotides were used to block *in vitro* translation of two Sendai virus mRNAs (16) and encephalomyocarditis virus genomic RNA (17). Several cDNAs complementary to various segments of the TMV p126 coding region have been tested for the ability to inhibit translation in wheat germ and rabbit reticulocyte lysates (18). Variable levels of inhibition were observed both with different fragments and between the two cell-free extracts. Evidence was presented suggesting that endogenous RNase H activity in wheat germ lysates may be responsible for these differences (18). The ability of antisense RNA to regulate gene expression in plants has been suggested by a study where antisense genes cotransfected with a chloramphenicol acetyl transferase construction were capable of inhibiting expression of the reporter gene in tobacco protoplasts (19). One possible way to investigate the role of p126 in TMV development is to attempt to specifically regulate its synthesis and subsequently assay for functional deficiencies. In this study, we have investigated

the effect on translation of duplex formation arising from hybridization of TMV RNA with two oligonucleotides, one complementary to the TMV leader sequence and the other to the 5'-proximal segment of the p126 coding region. We also compare the effects of both the ribo- and deoxyribonucleotide forms of the sequences.

MATERIALS AND METHODS

Viral RNA isolation

TMV RNA was purified from virions of strain U₁ by bringing viral suspensions to 1% SDS-2mM EGTA, extracting twice with equal volumes of H₂O saturated, distilled phenol, extracting once with a 50:50 mixture of phenol-chloroform, once with ether, and then precipitating with two volumes of 95% ethanol. BMV RNA was purchased from Promega Biotech.

Oligodeoxynucleotide preparation

Based upon prior studies which suggested that complementary or antisense oligonucleotides directed at the 5' end of the gene or the 5' leader sequence upstream from the target gene initiation codon are most inhibitory to translation (18-22), we synthesized 51 base oligodeoxynucleotides both complementary and identical to these regions of the p126 gene. In addition, GATC tails were added to the 5' end of each fragment to facilitate cloning. Oligodeoxynucleotides were synthesized on an Applied Biosystems 380A DNA Synthesizer using β -cyanoethyl phosphoramidite precursors. An initial synthesis using O-methyl phosphoramidites yielded 55mers which were unsuitable for cloning, probably due to methylation of bases during deprotection (23). Oligodeoxynucleotides were deblocked and released from the support by treatment with concentrated NH₄OH for 8-12 hr at 55°, desalted by Sephadex G50 chromatography, lyophilized, dissolved in 5mM Tris-Cl (pH 7.5)-0.5mM EDTA, and examined for purity by electrophoresis on 20% polyacrylamide gels (24). Labeling of oligomers with ³²P was done using γ -³²P-ATP (ICN) and polynucleotide kinase (US Biochemicals) as described (25).

Cloning and transcription of oligodeoxynucleotides

Complementary oligodeoxynucleotides were annealed in 25mM Tris-Cl (pH 7.6), 10mM MgCl₂, 10mM DTT by heating in a boiling water bath for 2 min then slowly cooling over 1.5 hrs to 25°. The annealed fragments were ligated into the Bam HI site of a Bluescribe plasmid (Stratagene Cloning Systems) and cloned in *E. coli* JM101. Plasmid DNAs, purified by CsCl density-gradient

centrifugation, were linearized by digestion with Hind III or Eco RI prior to transcription by T7 and T3 RNA polymerases respectively. Following restriction endonuclease digestion, template DNAs were incubated with proteinase K for 30 min at 37°, extracted with chloroform:phenol(1:1) and ethanol precipitated. Transcription reactions consisted of 40 ng/μl DNA template, 400μM each ATP, UTP, GTP and CTP, 30mM DTT, 1U/μl RNase-Block (Stratagene Cloning Systems) and 0.4 U/μl T3 or T7 RNA polymerase in 40mM Tris-Cl(pH 8), 8mM MgCl₂, 2mM spermidine and 50mM NaCl. Following incubation at 37° for 30 min, reaction mixtures were diluted 10-fold with 40mM Tris-Cl(pH 7.5), 6mM MgCl₂, 10mM NaCl and 1U RNase-free DNase/μg DNA template was added. Incubation was continued for 15 min at 37° followed by chloroform:phenol(1:1) extraction and ethanol precipitation of transcripts. Transcript orientation was determined by probing dot blots of the RNAs with ³²P-labeled oligodeoxynucleotides.

In vitro translation

TMV or BMV RNA at 50 and 80 μg/ml respectively and oligonucleotides at molar ratios indicated in the figures were annealed in 10mM Tris-Cl(pH 7.5)-100mM NaCl (final volume of 10 μl) at 45° for 40 minutes. Translations were done immediately following hybridization in micrococcal-nuclease treated, rabbit reticulocyte lysate (ProMega Biotech) at 30° for 60 min. Standard reactions contained 10 μl of lysate, the 10 μl hybridization mix, and 25 μCi of ³⁵S-methionine (1100 Ci/mMole) in a total volume of 25 μl. Incorporation of ³⁵S-methionine into polypeptides was determined by trichloroacetic acid precipitation of proteins and liquid scintillation counting in 5 ml of Biofluor (New England Nuclear). Alternatively, aliquots of the reaction mixtures were electrophoresed on 10% polyacrylamide gels containing 0.6% SDS (26) and bands visualized by autoradiography. The TMV encoded protein, p126, was excised from the gels and quantified by liquid scintillation counting.

RESULTS

Construction of oligodeoxynucleotides and hybridization to TMV RNA

The sequences of the oligodeoxynucleotides and designations used in these experiments are presented in Figure 1. The L (leader) sequences begin 7 bases from the m7GpppG cap and end at base 58. The 5' sequences begin at base 64, 6 bases upstream

A

ORIENTATION	SEQUENCE	NAME
leader-sense	TACAACAATTACCAACAACAACAAA CAACAAACAACATTACAATTACTATT	L - 1
leader-antisense	ATGTTGTTAATGGTTGTTGTTGTTT GTTGTTTGTGTAATGTTAATGATAA	L - 2
5'-sense	ATTACAATGGCATACACACAGACAG CTACCACATCAGCTTGCTGGACACT	5' - 1
5'-antisense	TAATGTTACCGTATGTGTCTGTGCG ATGGTGTAGTCGAAACGACCTGTGA	5' - 2

B

Figure 1. Sequence and orientation of TMV RNA specific oligodeoxynucleotides. Panel A gives the orientation, sequence and designation of the oligodeoxynucleotides used to study antisense inhibition of TMV RNA translation. Panel B is a schematic representation of TMV genomic organization showing the hybridization sites of the complementary oligonucleotides. The identical RNA sequences are represented by the same designations given the DNA fragments.

from the p126 initiation codon, and end at base 115. These regions of TMV RNA are known to be ribosome binding sites (28). Each fragment also has a GATC tail at the 5' end to facilitate cloning.

Figure 1B indicates the expected annealing location of the L-2 and 5'-2, antisense, oligonucleotides with TMV RNA. The hybridization conditions were chosen to achieve >90% annealing of the TMV RNA to the oligomers based on estimates from data of Britten et al. (27). The extent of hybridization was determined by annealing ^{32}P -labeled oligodeoxynucleotides and TMV RNA as described then separating duplexes from single-stranded DNA by Sephadex G-100 chromatography. The amount of labeled DNA present in the RNA peak was determined by liquid scintillation counting. At equimolar DNA:RNA ratios, 90% of the TMV RNA was annealed to the antisense fragments.

Inhibition of TMV RNA translation by duplex formation

Duplex formation between TMV RNA and either L-2 or 5'-2,

Figure 2. Effect of sense and antisense oligonucleotides on *in vitro* translation of TMV RNA. TMV RNA (50 $\mu\text{g}/\text{ml}$) was hybridized with individual oligomers at molar ratios indicated. The entire 10 μl hybridization mixture was added to 10 μl of a micrococcal-nuclease treated, rabbit reticulocyte lysate along with 25 μCi of ^{35}S -methionine (1100 Ci/mMole) and incubated at 30° for 60 min. Aliquots of the reaction mixtures (2 μl) were added to 1 ml of 1M NaOH-1.5% H_2O_2 and incubated for 10 min at 37°. This mixture was then diluted with 4 ml of ice cold 25% trichloroacetic acid plus 2% casamino acids and, after 30 min, passed through GF/C filters. Filters were washed with 20 ml of cold 10% trichloroacetic acid then 1 ml of acetone, air dried, and counted in 5 ml of Biofluor. A) synthetic DNA sequences as depicted in Fig. 1; B) RNA analogues from cloned copies of the DNA sequences

Table I: Translation of plant viral RNA in the presence of oligodeoxynucleotides.

Treatment ^a	% Translation ^b
1. TMV RNA, no hybridization	114
2. TMV RNA, hybridization	-100-
3. BMV RNA, no hybridization	106
4. BMV RNA, hybridization	-100-
5. BMV RNA + 5'-1 (1:300 molar ratio)	96
6. BMV RNA + 5'-2 (1:300 molar ratio)	112
7. BMV RNA + L-1 (1:300 molar ratio)	106
8. BMV RNA + L-2 (1:300 molar ratio)	98
9. TMV RNA + (5'-1 + 5'-2) (1:15 molar ratio)	107
10. TMV RNA + (L-1 + L-2) (1:15 molar ratio)	94
11. TMV RNA + 5'-2, no annealing (1:30 molar ratio)	61
12. TMV RNA + L-2, no annealing (1:30 molar ratio)	56

^a Hybridization treatments, except where indicated, were done prior to *in vitro* translations and consisted of incubation of RNA with or without oligodeoxynucleotides in 100mM NaCl-10mM Tris·Cl(pH 7.5) at 45° for 40 min. *In vitro* translations were done in nuclease-treated, rabbit reticulocyte lysate at 30° for 60 min.

^b The amount of translation occurring in each reaction mixture was determined by measuring trichloroacetic acid precipitable radioactive products as described in Figure 2. The amount of ³⁵S incorporated in each experiment is compared to that of controls subjected to hybridization in the absence of oligomers, lines 1 and 3 for TMV and BMV respectively. Incorporation of ³⁵S-methionine was typically 4-5 x 10⁶ CPM for the TMV control and 5-6 x 10⁶ CPM for the BMV directed reaction.

antisense fragments, inhibits *in vitro* translation of TMV in a concentration dependent fashion (Fig. 2). Both complementary oligodeoxynucleotides inhibit translation 30% to 35% when present in 1.5 fold molar excess (Fig. 2A). Increasing the molar ratio of oligodeoxynucleotides to 1:15 reduced translation to 30-40% of control levels. An additional 20 fold increase had only a slight effect on ³⁵S incorporation. The L-2 and 5'-2 fragments were equally effective in inhibiting translation. Oligodeoxynucleotides with sequences identical to the same regions of TMV RNA, sense fragments, had no significant effect on translation when added to the annealing reactions in place of the antisense oligomers. Further, an equimolar mixture of L-2

and 5'-2 did not inhibit translation to any greater extent than either of the two components separately (Fig. 2A).

A comparable experiment was done using RNA analogues of the synthetic oligodeoxynucleotides (Fig. 2B). Transcripts of the sense and antisense sequences were prepared *in vitro* using T3 and T7 RNA polymerases as described in Materials and Methods. The effects of the RNA fragments on TMV translation are very similar to those seen with the DNA. Only the antisense sequences were inhibitory. The principal difference between results with antisense DNA and RNA being a somewhat greater extent of inhibition with the RNA (Fig. 2A and B).

Results from a number of variables which were tested are presented in Table I. Pretreatment of the TMV or BMV RNA in the hybridization buffer in the absence of oligodeoxynucleotides does not alter their activity in the translation assay (lines 1-4). Neither the sense or antisense DNA fragments affect translation of BMV RNA (lines 5-8). Although the sense oligomers had no effect on TMV translation by themselves, prehybridization of complementary pairs of DNA fragments, L-1:L-2 or 5'-1:5'-2, prior to addition of the TMV RNA can block inhibition by the antisense sequences (lines 9-10). Finally, when mixtures of the complementary oligonucleotides and TMV RNA were put directly in the translation mix without prior annealing, inhibition was noted but not at levels observed with prior annealing treatments (lines 11-12).

Inhibition of p126 synthesis was examined by separating the ³⁵S-labeled polypeptides in various reaction mixtures by electrophoresis on 10% polyacrylamide gels containing 0.6% SDS. Autoradiograms of the dried gels show that TMV RNA directs the synthesis of 126 and 183 KDa peptides as expected (Fig. 3). The L-2 and 5'-2 fragments reduce the amount of radioactivity in these bands. The autoradiogram shown in Figure 3 was chosen to demonstrate inhibition of p126 synthesis. Longer exposures of the same gel show identical effects on p183. The extent of inhibition of p126 by the presence of the antisense oligomers was determined by excising the appropriate gel band and measuring ³⁵S-methionine incorporation by liquid scintillation counting (Fig. 3).

DISCUSSION

The capacity of polynucleotides complementary to mRNAs to modulate viral gene expression was first indicated by the studies of Coleman et al. (12), Zamecnik and Stephenson (14),

Figure 3. Autoradiogram of SDS-polyacrylamide gel analysis of *in vitro* translation reaction mixtures. TMV RNA was incubated at 45° for 40 min in the presence or absence of oligodeoxynucleotides at 15 fold molar excess then used to direct *in vitro* translation reactions. Products were analyzed by electrophoresis on 10% polyacrylamide gels containing 0.6% SDS and bands visualized by autoradiography. The extent of translation of the p126 cistron was determined by excising the band and measuring ³⁵S methionine incorporation by liquid scintillation counting. Lanes A-D were hybridized with the oligomer indicated in the figure. Lane E is the product of TMV RNA only. Background radioactivity from minus RNA controls was 1.8% of lane E.

and Izant and Weintraub (20). More recently, antisense RNA mediated inhibition of gene expression in plant cells has been demonstrated by Ecker and Davis (19). As we are interested in the ability of antisense constructions to inhibit specific viral genes during plant infections, these results encouraged us to explore the principle of antisense inhibition with TMV RNA translation.

To investigate the potential usefulness of this approach, we have synthesized four oligodeoxynucleotides based on sequences present near the 5'-terminus of TMV genomic RNA. Prior studies suggest that these regions are attachment sites for ribosomes and thus appear to represent appropriate sites for antisense oligomer mediated inhibition of translation (28). Previous work in other systems have demonstrated that this region of mRNA is a good target for inhibiting translation with complementary polynucleotides (12,15-17,29-32). Others have also shown that antisense fragments as short a 14-23 bases can inhibit translation as effectively as constructions which cover the full

gene (29). Sense and antisense sequences 51 bases long were produced which correspond to the 5'-untranslated leader and the 5'-end of the p126-p183 cistron including the initiation codon. Each fragment also has a 5'-terminal GATC to facilitate cloning. We have used these DNA fragments and their RNA analogues with a TMV directed, *in vitro* translation system from rabbit reticulocytes to determine whether the presence of double-stranded segments in these regions of the genomic RNA inhibit protein synthesis. The reticulocyte system was chosen because of its apparent lack of RNase H activity (18). This nuclear enzyme, which is present in wheat germ lysates (18), can block translation by cleaving RNA in DNA:RNA hybrids thus enhancing apparent hybrid arrest of translation. This activity is probably not relevant for effective *in vivo* inhibition of translation as the antisense material is usually RNA and the complexes which are important are cytoplasmic.

TMV encodes at least four proteins *in vivo*, however, only two major proteins, p126 and p183, are produced in eukaryotic, *in vitro* translation systems (2,4,5). The p183 results from read through of the normal p126 termination codon. If the oligonucleotides inhibit translation at their common initiation site, a simultaneous decrease in production of both polypeptides is expected. Two sequences complementary to the 5'-proximal region hybridize to TMV RNA under the conditions used and both are potent and specific inhibitors of the *in vitro* expression of the p126-p183 cistron (Fig. 2 and 3).

Previous studies of TMV translation in the presence of cDNA show that a 240 base fragment which covers both the 5' leader segment as well as the 5'-proximal segment of the coding region was the most effective fragment tested with respect to inhibition of translation (18). Our results show that a much shorter antisense fragment, directed only against untranslated leader sequence, is as effective an inhibitor as one which covers the initiator AUG. Several synthetic DNA fragments 30-40 bases in length, directed towards various regions of the untranslated leader segment of encephalomyocarditis virus RNA, effectively inhibited *in vitro* translation (17). Our results support the conclusion that antisense inhibitors do not have to cover the initiator AUG to be effective.

The DNA and RNA antisense sequences are approximately equally effective indicating that the loss of translation is not due to RNase H activity in the reticulocyte extract. We are therefore encouraged to believe that inhibition is due to a

direct affect on the translation process and may be effective *in vivo*. Hybridization of the oligomers with the RNA is essential for efficient inhibition (Table I). However, the quantitative relationship between the extent of duplex formation and inhibition of translation is apparently not simple. Our results indicate that equimolar amounts of antisense DNA result in 90% of the TMV RNA being annealed to an oligomer under our hybridization conditions. Yet, when a hybridization reaction mixture with a 1.5 fold molar excess of DNA is used to direct *in vitro* translation, the extent of inhibition observed is only 30-40%. This indicates that some displacement of duplex from the mRNA may be possible. This observation may also account for the inability of 30 fold molar excesses of complementary RNA or DNA to completely block translation (Fig. 2). The inhibition observed when oligodeoxynucleotides were added directly to the *in vitro* system concomitant with TMV RNA was likely due to hybridization occurring during the 60 min translation reaction.

Oligofibo- or oligodeoxyribo- nucleotides with sequences identical to the same regions of the TMV have no effect on the incorporation of ^{35}S -methionine indicating that neither the hybridization reaction *per se* nor noncomplementary sequences inhibit translation (Fig. 2 and Table I). Others have observed a non-specific inhibition of translation by concentrations of single-stranded DNA higher than those used in these experiments (18). Our results demonstrate that the presence of single-stranded oligomers, either DNA or RNA, at moderate levels does not give rise to measurable inhibition of translation. The sequence requirements for inhibition were further examined by substituting BMV for TMV RNA. The BMV RNA has no sequence homology with any of the four oligodeoxynucleotides and, as expected, no inhibition of translation was seen with any of these fragments (Table I).

The ability of the antisense fragments to block translation could be eliminated by titrating the L-2 or 5'-2 fragments with the corresponding sense strand prior to addition of TMV RNA (Table I). This further indicates that the inhibition is dependent on the ability of the antisense fragments to form duplex structures with the RNA.

The extent of inhibition was not materially affected by using mixtures of the two antisense fragments (Fig. 1). The possibility that the simultaneous presence of two antisense constructions might give rise to a synergistic or additive inhibition was attractive as a potential means of improving *in*

vivo effectiveness. From the results seen in these studies, it would appear that this is not a useful strategy, however, additional variables such as the stability of antisense RNA (33) may lead to different conclusions from *in vivo* studies. Wilson et al. (34) have suggested that TMV uncoating *in vivo* is linked to translation initiation at or near the p126 initiation region. This raises the interesting possibility that antisense constructions directed against this region may have additional effects on viral infection and gene expression. We therefore wish to defer conclusions regarding the interactive effects of the two different target sequences pending completion of *in vivo* studies.

ACKNOWLEDGEMENTS

The work was supported by NSF grants RII-8610680 and DMB-8611424. We also wish to thank our colleagues Gregory Raymond, Joe DeParasis and Jay Hiddinga for their assistance.

* To whom correspondence should be addressed

REFERENCES

1. Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J. and Karn, J. (1983) Proc. Natl. Acad. Sci. USA. 79, 5818-5822.
2. Guilley, H., Jonard, G, Kukla, B. and Richards, K. E. (1979) Nucleic Acids Res. 6, 1287-1308.
3. Beachy, R. M., Zaitlin, M., Bruening, G., and Israel, H.W. (1976) Virology 73, 498-507.
4. Goelet, P. and Karn, J. (1983) J. Mol. Biol. 154, 541-550.
5. Pelham, H. R. B., and Jackson R. J. (1976) Eur. J. Biochem. 67, 247-256.
6. Evans, R. K., Haley, B. E. and Roth, D. A. (1985) J. Biol. Chem. 260, 7800-7804.
7. Scalla, R., Romaine, P., Asselin, A., Rigand, J. and Zaitlin, M. (1978) Virology 91, 183-193.
8. Ahlquist, P., Strauss, E. G., Rice, C. M., Strauss, J. H., Haseloff, J., and Zimmern, D. (1985) J. Virology 53, 536-542.
9. Paterson, R. and Knight, C. A. (1975) Virology 64, 10-2210.
10. Siegel, A. Hari, V. and Kolacz, A. (1978) Virology 85, 494-498.
11. Green, P. J., Pines, O., and Inouye, M. (1986) Ann. Rev. Biochem. 55, 569-597.

12. Coleman, J., Hirashima, A., Inokuchi, Y., Green, P. J., and Inouye, M. (1985) *Nature* 315, 601-603.
13. Stephenson, M. L. and Zamecnik, P. C. (1978) *Proc. Natl. Acad. Sci. USA* 75, 280-284.
14. Zamecnik, P. C. and Stephenson, M. L. (1978) *Proc. Natl. Acad. Sci. USA* 75, 280-284.
15. Chang, L. J. and Stoltzfus, C. M. (1987) *J. Virology* 61, 921-924.
16. Gupta, K. C. (1978) *J. Biol. Chem.* 262, 7492-7496.
17. Shih, D., Park, I-W., Evans, C.L., Jaynes, J.M., and Palmenberg, A.C. (1987) *J. Virol.* 61, 2033-2037
18. Minshull, J. and Hunt, T. (1986) *Nucl. Acids Res.* 14, 6433-6451.
19. Ecker, J. R. and Davis, R. W. (1986) *Proc. Natl. Acad. Sci. USA* 83, 5372-5376.
20. Izant, J. G. and Weintraub, H. (1984) *Cell* 36, 1007-1015.
21. Kawasaki, E. S. (1985) *Nucleic Acids Res.* 13, 4991-5004.
22. Pestka, S., Daugherty, B. L. V., Hotta, K., and Pestka R. K. (1984) *Proc. Natl. Acad. Sci. USA* 81, 7525-7528.
23. Urdea, M.S., Ku, L., Horn, T., Gee, Y.G., and Warner, B.D. (1985) *Nucl. Acids Res. Symp.* Series 16, 257-260.
24. Maxam, A., and Gilbert, W. (1980) *Meth. Enzymol.* 65, 499-560.
25. McGraw, R. A. (1984) *Anal. Biochem.* 143, 298-303.
26. Owens, J. R. and Haley, B. E. (1976) *J. Supramol. Struct.* 5, 91-102.
27. Britten, R. J., Graham, D. E., and Neufeld, B. R. (1974) *Meth. Enzymol.* 29, 363-418.
28. Filipowicz, W. and Haenni, A. L. (1979) *Proc. Natl. Acad. Sci. USA* 76, 3111-3115.
29. Melton, D. A. (1985) *Proc. Natl. Acad. Sci. USA* 82, 144-148.
30. Lawson, T. G., Ray, B.K., Dodds, J. T., Grifo, J. A., Abramson, R. D. Merrick, W. C., Betsch, D. F., Weith, H. L., and Thach, R. E. (1986) *J. Biol. Chem.* 261, 13979-13989.
31. Blake, K. R., Murakami, A., and Miller, P. S. (1985) *Biochem.* 24, 6132-6138.
32. Liebhaber, S. A., Cash, F. E. and Shakin, S. H. (1984) *J. Biol. Chem.* 259, 15597-15602.
33. Loesch-Fries, L. S., Halk, E., Merlo, D., Jarvis, N., Nelson, S., Krahn, K., and Burhop, L. (1987) in *Molecular Strategies for Crop Protection*, A. R. Liss, Inc., pp 221-234.
34. Wilson, T. M. A. (1984) *Virology* 137, 255-265.