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Complementary oligonucleotide sequence inhibits both Vmw65 gene expression and replication of herpes simplex virus

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Summary

The virion tegument protein, Vmw65, of herpes simplex virus is a transacting molecule which induces immediate early gene transcription. We show that an oligodeoxyribonucleotide which is complementary to the translation initiation region of Vmw65 mRNA inhibited the expression of Vmw65 biological activity in a Vmw65-expressing cell line and reduced the yield of HSV-1 in tissue culture. The levels of oligomer required to effect viral replication resulted in no observable cellular toxicity.

Herpes simplex virus; Oligonucleotide inhibition

Introduction

The regulation of genetic expression from RNA by complementary nucleic acid sequences has been described in both procaryotic and eucaryotic cells (Mizuno et al., 1983). This natural phenomenon has been mimicked successfully to inhibit the translation of selected mRNAs in both in vitro translation systems (Stephenson and Zamecnik, 1978) and tissue culture (Izant and Weintraub, 1984). The level of inhibition achieved by these methods depends upon the chemical structure of the complementary sequence (Maher and Dolnick, 1988), the site of complementarity (Melton, 1985) and the system in which the inhibition is observed (Blake et al., 1985). We have used complementary oligodeoxyribonucleotide sequences (oligos)

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to inhibit the expression of an important transcriptional activating factor of herpes simplex virus (HSV), the virion tegument protein known as Vmw65.

In the present study we assessed the ability of two related oligos to inhibit expression of the HSV-1 Vmw65 gene product in MTX5 cells. Previously, we demonstrated that these cells express Vmw65 mRNA and specifically activate transient gene expression from plasmid constructs containing HSV immediate early promoters (Kmetz et al., 1988). Oligos which are complementary to putative translation initiation regions of the Vmw65 mRNA (Pellett et al., 1985; Dalrymple et al., 1985) were synthesized and introduced to cultures of MTX5 cells. One of these oligos (Oligo 293) specifically inhibited the transient expression of β-galactosidase (β-gal) from a plasmid-borne *lacZ* gene, of which expression is controlled by the HSV-1 ICP4 promoter. The molecular events underlying the inhibitory activity of Oligo 293 were determined using an in vitro transcript of RNA (pEM-2 RNA) which contains both the targeted RNA sequence and a majority of the Vmw65 protein coding sequence. Preincubation of Oligo 293 with selected RNA targets inhibited the in vitro translation of only the pEM-2 RNA. When Oligo 293 and pEM-2 RNA were incubated together under conditions which were similar to those used for in vitro translation, the oligo was protected from digestion in a subsequent S1 nuclease assay. When considered together, these results suggested that the oligo and target RNA combine in a sequence specific manner which could be used to block viral replication of HSV. In fact, subsequent exposure of replicating cultures of HSV to Oligo 293 did result in reduced yields of infectious virus.

Materials and Methods

Cells and virus

L TK$^-$ cells, originally from the American Type Culture Collection, were maintained in Eagles Minimal Essential Medium (EMEM) supplemented with glutamine, penicillin, streptomycin and 10% fetal calf serum. MTX5 cells (Kmetz et al., 1988) were maintained in supplemented EMEM containing 1 × HAT (Wigler et al., 1979). VERO cells were maintained in Medium 199 containing glutamine, penicillin, streptomycin, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8.0), and 5% fetal calf serum. HSV-1 (strain KOS) stocks were grown in VERO cells and harvested by standard procedures.

Plasmid constructs

Restriction endonucleases and DNA ligase were purchased from New England Biolabs, Inc. Restriction endonuclease digestion and DNA fragment ligation were performed by established procedures (Maniatis et al., 1982) using buffers recommended by the manufacturer.

The HSV-1 *Sal* F′ fragment (0.681–0.688 map units [m.u.]), which contains the amino terminal 83% of the Vmw65 translational reading frame (Pellett et al., 1985),

was generated from plasmid *Hin*d Bgl DG (0.647–0.698 m.u., Hall et al., 1982) by digestion with *Sal*I restriction endonuclease. The 1.2 kb *Sal* F′ fragment was separated from other DNA fragments by electrophoresis through 1% Low Melting Point agarose, extracted according to prescribed methods (Maniatis et al., 1982) and cloned into the *Sal*I site of the transcription vector pGEM-2 (Promega Biotech) to produce the recombinant plasmid pEM-2. The orientation of the *Sal* F′ fragment within pEM-2 was determined by a double digestion with restriction enzymes *Bam*HI and *Kpn*I, which recognizes a unique site within the inserted fragment.

Plasmid *Hin*d Bgl DG was obtained from E. Wagner, University of California, Irvine, California. Plasmid pON 105, which contains the HSV-1 ICP4 promoter located immediately 5′ of the *E. coli lac Z* gene, was constructed by E. Mocarski, Stanford University, Stanford, California.

Oligodeoxyribonucleotide preparation

Using an Applied Biosystems Synthesizer Model 280A, we synthesized two oligodeoxyribonucleotide sequences, 5′-GTCCGCGTCCATGTCGGC (Oligo 293) and 5′-CAAGAGGTCCATTGGGTG (Oligo 294). Both of these sequences are complementary to regions of the HSV-1 mRNA sequence which encode putative translation initiation codons for the Vmw65 protein. Following synthesis, the oligos were treated with thiophenol to remove methoxy groups on the phosphates, then cleaved from the silica support with ammonium hydroxide. Deprotection was completed by heating the oligos in 14.5 M ammonium hydroxide at 55°C for 8–16 h. The solution was dried in a Speed Vac Concentrator (Savant), redissolved in water and extracted with *n*-butanol to remove benzamides formed during protection. The crude oligo preparation was dried, resuspended in water, and electrophoresed on a denaturing polyacrylamide gel according to established procedures (Maxam and Gilbert, 1980). The oligo bands were visualized by UV shadowing using a fluorescent TLC plate, then cut from the gel and eluted by overnight incubation at 37°C in 0.5 M ammonium acetate. Salts were removed from the oligo preparation by filtration through a C-18 Sep-pak (Waters). The concentration of oligomer was determined as A_{260} units in the solution ($1A_{260}$ unit = 40 μg oligomer). An oligonucleotide length of 18 residues was chosen to achieve a nucleotide sequence which would be theoretically unique from any cellular DNA sequence. For any nucleotide sequence which is n residues in length, the probability of occurrence in a given genome is once in every 4^n nucleotides. A value of 4^{18} is clearly larger than any estimates of the nucleotide content of mammalian genomes.

In vitro transcription

Transcription reagents were obtained from Promega Biotech and protocols were performed as recommended by the manufacturer. To produce pEM-2 RNA encoding the truncated Vmw65 reading frame, plasmid pEM-2 was linearized by digestion with *Bam*HI and used as template for in vitro transcription with T7 RNA

polymerase. In vitro transcripts were purified by digestion of the template DNA with RQ1 DNase (20 min, 37°C), two extractions with phenol:chloroform:isoamyl alcohol (25:24:1), extraction with chloroform:isoamyl alcohol (24:1), precipitation in 0.3 M sodium acetate and 70% ethanol, and resuspension in diethyl pyrocarbonate (DEPC)-treated water.

In vitro translation/hybrid arrest

In vitro translation reagents were purchased from Promega Biotech and used according to the manufacturer's specifications. Translation reactions contained 1 μg of an appropriate RNA sample and 38 μl of translation cocktail in a total volume of 50 μl. (One milliliter of translation cocktail contains: 790 μl of rabbit reticulocyte lysate, 26 μl of a methionine-free amino acid mixture, 39 μl of RNasin, 130 μl of [^{35}S]methionine [50 μCi, >400 Ci/mmol, Amersham] and 15 μl of DEPC-treated water.) The translation mixture was incubated for 2 h at 30°C, then digested for 20 min at 37°C with RNase A at a final concentration of 370 μg/ml. For hybrid arrest experiments, 1 μg of RNA was combined with an appropriate amount of oligo in DEPC-treated water to yield a total volume of 12 μl. This mixture was incubated at room temperature for 90 min before addition to 38 μl of translation cocktail.

Analysis of in vitro translation products

To determine the total [^{35}S]methionine incorporation, the equivalent of 2 μl of translation mixture was added to 1 ml of a 1 N NaOH/1.5% hydrogen peroxide solution and incubated at 37°C for 10 min. Following the addition of 4 ml of cold 25% TCA/2% casamino acids, samples were incubated on ice for 30 min, then filtered through Millipore AP filters. Filters were washed under vacuum with ethanol and acetone, air dried, and added to 4 ml of Aquasol (Dupont). TCA-precipitable counts were quantified in a Packard model 2000CA Liquid Scintillation Analyzer.

For size analysis of the in vitro translation products, the equivalent of 2 μl of translation mixture was diluted into an equal volume of 2 × Laemmli Loading Buffer (1 × = 88 mM Tris-HCl, pH 6.8; 2% sodium dodecyl sulphate [SDS]; 5% β-mercaptoethanol; 10% glycerol, and 0.001% bromphenol blue), heated in a boiling water bath for 5 min, and resolved by electrophoresis in either a 10% polyacrylamide-SDS (Laemmli) gel or a prepoured 10–20% polyacrylamide-SDS gel (Integrated Separation Systems). The resultant gels were dried under vacuum and autoradiography was performed using Kodak Xomat RP or Bluescribe film.

S1 nuclease analysis of RNA-oligodeoxyribonucleotide hybrid formation

Oligo used for S1 nuclease analysis was end labelled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England Biolabs) according to published protocol (Maxam and Gilbert, 1980). Hybridization solutions (12 μl) containing 1.2 μg of pEM-2 RNA and 60 ng of ^{32}P end-labeled oligo [Oligo 293 (4.3×10^7 cpm/μg) or

Oligo 294 (3.6×10^7 cpm/μg)] in water. Parallel samples which each contained 60 ng of ^{32}P end-labeled oligo in 12 μl of water were used to determine the sensitivity of unhybridized oligo preparations to S1 nuclease digestion. Before digestion with S1 nuclease, all nucleic acid solutions were incubated at room temperature for 90 min. After incubation 200 μl of S1 digestion buffer (30 mM NaOAc, pH 4.6; 250 mM NaCl; 1 mM $ZnSO_4$; 5 μg/ml denatured calf thymus DNA and appropriate amounts of S1 nuclease) was added to each tube and the tubes were incubated for 1 h at 37°C. All reactions were terminated by the addition of 50 μl of S1 stop buffer (0.3 M NaOAc, pH 4.6; 10 mM EDTA; and 0.2 μg/ml yeast RNA). The solutions were extracted twice with an equal volume of water saturated phenol and twice with an equal volume of chloroform:isoamyl alcohol (24:1). Finally, two volumes of ethanol (100%) were added and the mixtures were evaporated to dryness under vacuum. Dried pellets were counted (Cerenkov) and 20 μl of gel loading buffer [90% formamide, 1 × TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3)] were added to each tube. Samples were heated for 5 min at 70°C and placed on ice. Approximately 15000 cpm were added per lane on a 15% polyacrylamide (acrylamide:bis acrylamide ratio = 29:1), 8 M urea gel. The samples were resolved by electrophoresis at 200 volts for 3 h and visualized by autoradiography using Kodak XAR film after an exposure time of 19 h at −70°C.

Transient expression of β-galactosidase in transfected cells

MTX5 cells were seeded into 96 well microtiter plates and allowed to grow to 80% confluency (approximately 8×10^4 cells/well). One half microgram of plasmid pON 105 DNA was introduced into the cells of each well by the DEAE dextran precipitation technique (Graham and Van der Eb, 1973). After 4 to 6 h, the cells were rinsed with Hank's balanced salt solution (BSS) and overlaid with supplemented EMEM containing 1 × HAT (Wigler et al., 1978). Forty-eight hours after transfection the cells were assayed for β-gal expression. The intracellular level of β-gal activity in each well was determined from cell lysates of the monolayer cultures. Aliquots were assayed by incubation in the presence of the β-gal substrate, 4-methylumbelliferyl-β-D-galactoside (MUG, 125 μg/ml, Sigma), for 2 h at 37°C. The generation of fluorescent product was quantified on a Microfluor microfluorimeter (Dynatech) after addition of 0.1 M glycine, pH 10.3 (Spaete and Mocarski, 1985).

The effect of oligo on the expression of Vmw65 activity was examined by growing MTX5 cells in the presence of either Oligo 293 or Oligo 294 (at a concentration of 25 μg/ml of medium) for two passages prior to the transfection of pON 105 plasmid DNA. When appropriate, oligo solutions were added to the medium of cell cultures at six hours after plasmid transfection. Control cell cultures were preincubated in the absence of oligo. β-Gal activity was assayed as above.

Oligodeoxyribonucleotide inhibition of HSV replication

L TK$^-$ cells were seeded at 3.0×10^6 cells per 25 cm^2 T flask. Cells were overlaid with 5 ml of medium and incubated at 36.5°C for 18–24 h. When appropriate, Oligo 293 or Oligo 294 was present in the culture medium. Following this incubation, cells were rinsed with Hank's balanced salt solution (HBSS) and infected with HSV-1 (0.5 PFU/cell) suspended in 1 ml of serum-free EMEM. Virus and cells were incubated at 36.5°C for 1 h with occasional rocking. Following adsorption, 4 ml of medium (EMEM + 2% fetal calf serum) containing an appropriate concentration of oligo were added to each flask and the cells were incubated for 48 h at 37°C.

Cells were harvested in medium and centrifuged at $300 \times g$ and 4°C for 10 min. Supernatants were collected and centrifuged at $14\,500 \times g$ and 4°C for 30 min. Cell pellets from the two centrifugations were combined and resuspended in 1.5 ml of 15% fetal calf serum, 10% glycerol in EMEM. The suspension was frozen and thawed four times, then drawn through a 20-gauge needle four times and stored at −80°C. Virus titer was determined by plaque assay on VERO cells. Dilutions of each virus preparation were adsorbed as above, after which the virus inoculum

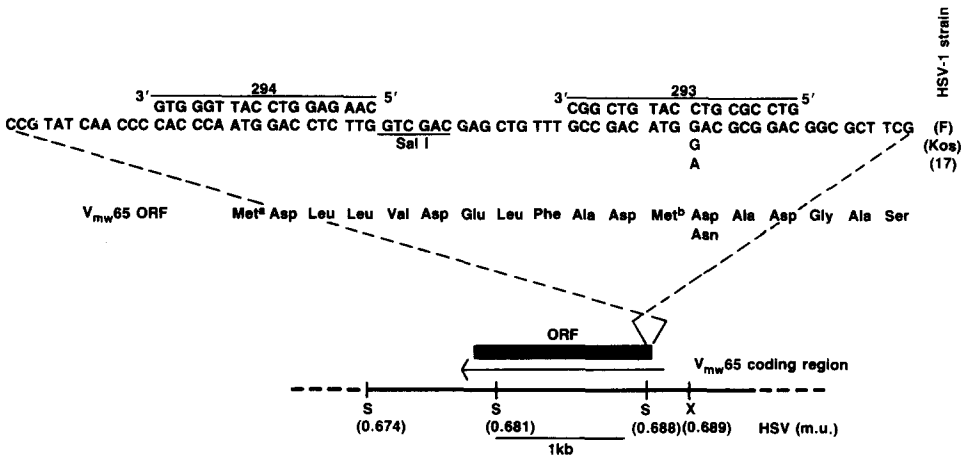

Fig. 1. Schematic of the Vmw65 gene region. A selected restriction map of the HSV-1 region encoding the Vmw65 gene is illustrated at the bottom of the figure. X, *Xho*I restriction site; S, *Sal*I restriction site. The location of the Vmw65 mRNA coding region and the direction of transcription on the prototypical arrangement of the HSV-1 genome are depicted by an arrow. The open reading frame (ORF) of the Vmw65 protein is represented as a black rectangle and the DNA sequence spanning the putative translation initiation region of the gene is given. For reference, the *Sal*I site at 0.688 m.u. is shown under the nucleotide sequence. Differences in the DNA sequence of this region are shown for three strains of HSV-1. The DNA sequence of the F strain is from Pellett et al. (1985), and the sequence from strain 17 is from Dalrymple et al. (1985). The KOS strain sequence was determined in our laboratory (unpublished data). [a]The translation initiation codon proposed by Pellett et al. (1985). [b]The translation initiation codon proposed by Dalrymple et al. (1985). The predicted amino acid sequences of the N-terminal region of the Vmw65 proteins are shown. The nucleotide sequences of the complementary oligos (293 and 294) are shown above their respective regions of complementarity.

was removed by aspiration and cells were overlaid with 3 ml of EMEM containing 1% fetal calf serum and 0.75% methylcellulose. Cells were incubated at 36.5°C for 72 h before plaques were counted.

Results

Inhibition of Vmw65 transactivation function by Oligo 293

The nucleotide sequence of the translation initiation region of the Vmw65 gene is highly conserved among different strains of HSV-1 (Fig. 1). We synthesized two oligodeoxyribonucleotides (Oligo 293 and Oligo 294) which are complementary to target sites within this region of the Vmw65 mRNA. The ability of these complementary oligos to inhibit transcriptional activation by the Vmw65 polypeptide was examined in MTX5 cells. Transient expression assays conducted in these cells indicated that activation of gene expression occurs only with plasmid constructs which contain HSV immediate early promoters (Kmetz et al., 1988). Using β-gal levels to quantitate transcriptional activity, we found that preincubation of MTX5 cells

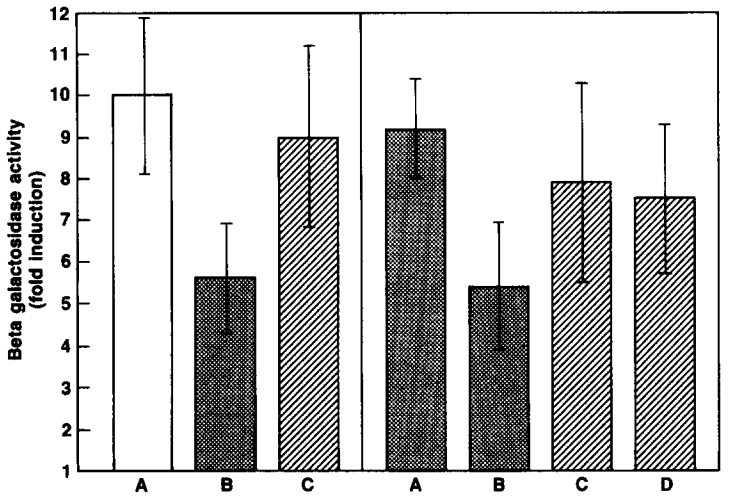

Fig. 2. Inhibition of Vmw65 function by complementary oligos. The transient activation of the ICP4 promoter was assayed in MTX5 cells as described in Materials and Methods. This activity is expressed quantitatively as fold induction in the levels of β-gal activity. The value given represents the mean of at least three experimental values. Error bars represent the standard deviation of the mean value. For the experiments represented in the left panel, (column B and C) cells were pretreated with oligo as described in Materials and Methods. Column A represents values in untreated cells; column B represents values after Oligo 293 treatment; column C represents values after Oligo 294 treatment. The data of the right panel illustrate the levels of β-gal induction in cells which were treated with Oligo 293 (A and B) or Oligo 294 (C and D) after transfection with the expression plasmid pON 105. When appropriate, cells were pretreated with either Oligo 293 (B) or Oligo 294 (D). All β-gal values are relative to the background values expressed in untransfected MTX5 cells.

with Oligo 293 significantly decreased the level of ICP4 promoter expression in these cells (Fig. 2, left panel). Addition of Oligo 293 to the culture medium after the time of transfection did not affect the expression of ICP4 promoter activity in either control or pretreated cells (Fig. 2, compare values in lanes A and B, right panel with values in lanes A and B, left panel). Thus, Oligo 293 specifically inhibited Vmw65 protein expression without significantly affecting cellular protein synthesis.

In contrast to the results observed with Oligo 293, preincubation of MTX5 cells with Oligo 294 only minimally inhibited ICP4 promoter expression in our transient assays (Fig. 2, left panel). When Oligo 294 was added to either pretreated or untreated cells after the time of transfection with indicator plasmid, levels of β-gal dropped significantly (Fig. 2, right panel). The inhibitory effect of Oligo 294 under these conditions was shown to be a result of Oligo 294 toxicity to MTX5 cells. The toxic effect of this oligo was demonstrated by [^{3}H]leucine assays, which revealed that exposure of MTX5 cells to Oligo 294 reduced the level of cellular translation by 15–20% (data not shown).

The toxic effects observed with Oligo 294 in the reported experiments were not observed in a number of subsequent experiments. We judged the purity of the original oligonucleotide preparations by end-labelling the sample with ^{32}P-ATP and electrophoresis of an aliquot in a polyacrylamide-urea gel. Because the oligomer preparations were judged to contain no appreciable amount of failure sequences (<1%) from oligonucleotide synthesis, we believe that the toxicity reported in these experiments must be a result of organic contaminants which were carried through the oligomer sample preparation.

Inhibition of HSV replication by Oligo 293

We examined the relative abilities of the complementary oligos to inhibit the replication of HSV in L TK$^-$ cells. The yields of infectious HSV-1 were reduced

TABLE 1

Effect of complementary oligonucleotide on yield of infectious HSV

Experiment	Treatment	Virus yield (PFU/ml)	Reduction in titer (%)
1	− Oligo	5.5×10^7	
	+ Oligo 293	2.0×10^7	64
2	− Oligo	3.9×10^7	
	+ Oligo 293	2.8×10^7	28
3	− Oligo	5.7×10^7	
	+ Oligo 293	3.4×10^7	40
4	− Oligo	3.1×10^7	
	+ Oligo 294	3.3×10^7	0
5	− Oligo	3.1×10^7	
	+ Oligo 294	3.0×10^7	3

Oligonucleotide preparations were added to the medium at a final concentration of 25 μg/ml. All virus infections were performed at an MOI = 0.5 PFU/ml. Virus titrations were performed in duplicate. Data points represent the average of at least two replicate cultures.

TABLE 2

Cell association of radiolabeled oligo by L TK$^-$ cells

Incubation time (h)	Cell associated radioactivity (% of total)	
	Oligo 293	Oligo 294
1	0.004	0.007
3	0.012	0.018
5	0.037	0.033
7	0.090	0.045
24	0.27	0.34

These values are the mean of at least two independent determinations. The method of determining cell association of oligo was described elsewhere (Ceruzzi and Draper, 1989).

by the treatment of infected cells with Oligo 293, but not by treatment with Oligo 294 (Table 1). An analysis of oligo uptake by L TK$^-$ cells revealed that similar amounts of Oligo 293 and Oligo 294 became cell-associated during incubation (Table 2). Thus, it appears that the inability of Oligo 294 to inhibit HSV replication was not attributable to a diminished amount of this oligo in infected cells.

In a dose response study of viral inhibition by Oligo 293 we observed an antiviral effect even at an oligo concentration of 0.25 μg/ml of medium (Table 3). At concentrations above 1.25 μg/ml, the level of viral inhibition remained fairly constant. This result suggested that there are limits to the amount of oligo which will be internalized from the medium.

Inhibition of translation of pEM-2 RNA by Oligo 293

We subcloned a portion of the Vmw65 gene into the pGEM-2 expression vector as described in Materials and Methods. The recombinant clone pEM-2, was used for the in vitro synthesis of an RNA whose sequence encodes the initiation codon and amino terminal 83% of the Vmw65 protein. The in vitro translation of pEM-2 RNA in a cell-free rabbit reticulocyte system produced a polypeptide with an M_r of approximately 48000. The observed size of the translation product was slightly greater than the size which was predicted from the nucleotide sequence of the gene (45000, Pellett et al., 1985). The difference between the observed and predicted

TABLE 3

Inhibition of HSV replication by various concentrations of Oligo 293

Concentration of Oligo 293 (μg/ml)	Virus yield (PFU/ml)	Inhibition (%)
0.00	5.5×10^7	–
0.25	3.5×10^7	36
1.25	1.8×10^7	67
6.25	2.1×10^7	62
25.0	2.0×10^7	64

All virus infections were performed at a MOI = 0.5 PFU/cell. Data points represents the average viral titers from two replicate cultures.

TABLE 4

Inhibition of in vitro translation by Oligo 293

Input RNA	Molar ratio (Oligo 293/RNA)	Experiments run (*N*)	Mean inhibition[a] (%)
pEM-2 RNA	400	4	21 ± 18
pEM-2 RNA	800	2	50 ± 2.8
pEM-2 RNA	1600	9	73 ± 7.1
BMV mRNA	1600	4	0 ± 0
BMV mRNA	3200	2	5.1 ± 4.9
Rabbit globin mRNA	1600	1	0 ± 0

[a]Inhibition values are based on TCA-precipitable cpm from cell-free translations. Control values (not shown) were obtained for each experiment by the translation of parallel samples of unhybridized pEM-2, globin or BMV RNA preparations.

M_r may be attributable to the high proline content of the polypeptide (18%) in the N-terminal 100 amino acid residues.

Preincubation of pEM-2 RNA with Oligo 293 reduced the yield of Vmw65 poly-

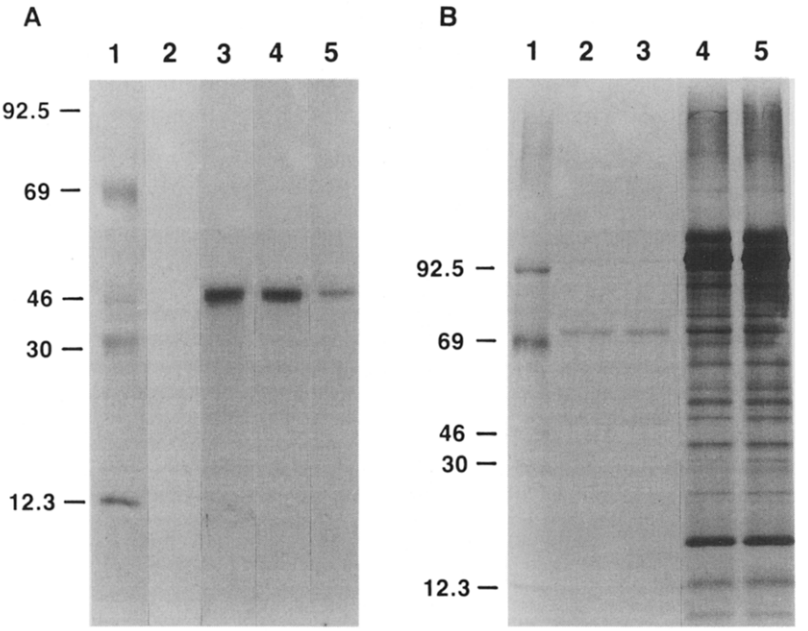

Fig. 3. SDS-PAGE of in vitro translation products. Aliquots of the products synthesized by the in vitro translation of various RNA molecules were subjected to SDS polyacrylamide gel electrophoresis as described in Materials and Methods. The migration of ^{14}C-labeled proteins of known size (Amersham Corp.) are shown in lane 1 of each panel. The molecular weights of the marker proteins are shown to the left of the protein bands. The following RNA species were used for translation. Panel A: lane 2, endogenous RNA; lanes 3–5, pEM-2 RNA. Panel B: lanes 2 and 3, rabbit globin mRNA; lanes 4 and 5, BMV RNA. Where appropriate, RNA was incubated with Oligo 293 prior to the in vitro translation. The ratios of oligo to RNA were as follows: panel A: lane 4, 800:1; lane 5, 1600:1; panel B: lanes 3 and 5, 1600:1.

peptide produced by in vitro translation. The level of translational inhibition increased as the concentration of Oligo 293 increased (Table 4, Fig. 3A). Analysis of the in vitro translation products by SDS-PAGE revealed that the inhibition of Oligo 293 did not result in the synthesis of truncated polypeptides. Further, preincubation with Oligo 293 did not reduce the level of in vitro protein synthesis from either brome mosaic virus (BMV) or rabbit globin mRNAs (Table 4, Fig. 3B). These results suggest that the mechanism of translation inhibition by Oligo 293 involved an interaction of oligo with pEM-2 RNA but not the in vitro ribosomal assembly.

Protection of Oligo 293 from nuclease digestion by pEM-2 RNA

We determined the specific interaction of Oligo 293 with the target pEM-2 RNA by co-incubating samples of the two molecules and subjecting the mixture to digestion with S1 nuclease. The titration of S1 nuclease in these reactions showed that optimum digestion of unhybridized Oligo 293 occurred at a nuclease concentration of 6.5 units/ml (Fig. 4A). Incubation with pEM-2 RNA ([oligo]/[pEM-

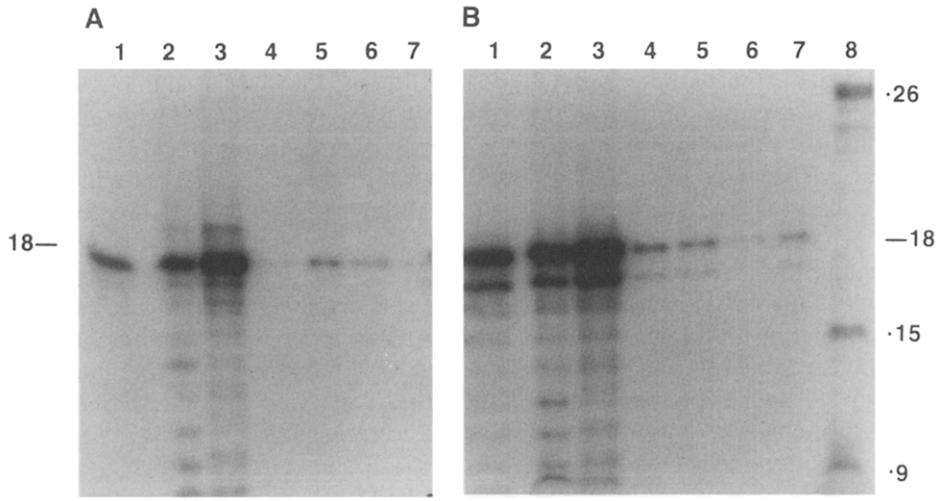

Fig. 4. S1 nuclease digestion of oligo. Samples of ^{32}P-labeled oligo were digested with S1 nuclease as described in Materials and Methods and aliquots of the digestion products (1×10^4 cpm for Oligo 293 and 1.5×10^4 cpm for Oligo 294) were electrophoresed in 8 M urea 15% polyacrylamide gels. Size markers were generated by digesting pBR322 DNA with the restriction enzyme *Msp*I and end labeling of the fragments according to established procedures (Maniatis et al., 1982). The results of nuclease digestion of Oligo 293 (panel A) and Oligo 294 (panel B) are shown. When appropriate, (lanes 3, 5 and 7), oligo preparations were incubated with pEM-2 RNA prior to nuclease digestion. The concentrations of S1 nuclease used for the digestions were 0.65 units/ml (lanes 2 and 3), 6.5 units/ml (lanes 4 and 5), and 65 units/ml (lanes 6 and 7). The migration of undigested oligo is shown in Lane 1 of each panel. Lane 8 of panel B shows the relative migration of the *Msp*I-digested pBR322 size markers. The sizes of the marker fragments are listed to the right of panel B. The size of undigested oligo (18) is shown for each panel.

2]=1.1/1) protected radiolabeled Oligo 293 from digestion with S1 nuclease (Figure 4A). By contrast, unhybridized Oligo 294 was quite resistant to digestion by S1 nuclease, and incubation with pEM-2 RNA did not increase the resistance of Oligo 294 nuclease digestion (Fig. 4B). The specific hybridization of pEM-2 RNA to Oligo 293 was expected, because the nucleotide sequence of the RNA is complementary to Oligo 293 but not to Oligo 294 (Fig. 1).

Discussion

In the present study we demonstrate that complementary oligodeoxyribonucleotides can specifically inhibit HSV gene expression in tissue culture. Our target gene product was the virion tegument protein (Vmw65) of HSV-1, which services a transactivating factor for immediate early gene transcription (Campbell et al., 1984). We used this transactivating property of the Vmw65 protein to quantitate the inhibitory effect of complementary oligos in a Vmw65-expressing cell line. In these cells, the transient expression of β-gal from plasmid constructs is dependent upon the transcriptional activation of the HSV-1 ICP4 promoter by Vmw65. Two oligos which are complementary to the predicted translation initiation regions of the Vmw65 mRNA (Pellett et al., 1985; Dalrymple et al., 1985) were assayed for their ability to inhibit β-gal expression in this assay. One of these oligos (Oligo 293) inhibited β-gal activity, the other (Oligo 294) did not. Lacking an amino acid analysis of the N-terminal portion of the Vmw65 protein, these data provide good evidence that the translation initiation codon proposed by Pellett et al. (1985) is correct for viral mRNA (see Fig. 1). The identification of the functional translation initiation codon for the Vmw65 mRNA will aid in designing antiviral molecules which inhibit gene expression at this locus.

Although the level of Vmw65 activity in MTX5 cells was reduced after treatment with Oligo 293, complete inhibition of this activity was not seen with concentrations up to 25 μg of oligo/ml of medium. Two independent factors may limit the inhibitory activity of oligos in these assays. One factor is the intracellular ratio of Oligo 293 to Vmw65 mRNA and the other is the molecular stability of the Vmw65 protein. The importance of the oligo to target RNA ratio has been demonstrated both in cells (Bevilacqua et al., 1988) and in cell free extracts (Blake et al., 1985). The determination of an effective oligo to RNA ratio must consider the size, nucleotide composition and molecular stability of both interacting molecules. We determined the percentage of Oligo 293 which is stably associated with L TK^- cells after a 24 h incubation (at an extracellular oligo concentration of 25 μg/ml, Table 2). When this value was compared to our estimates of Vmw65 mRNA abundancy during HSV-1 infection of L TK^- cells (data not shown), we calculated the oligo to RNA ratio to be approximately 880:1. We have observed that the extracellular oligo concentration directly affects intracellular oligo concentrations (Ceruzzi and Draper, 1989). Because we observed a reduction in virus yield at extracellular concentrations which were much lower than the 25 μg/ml concentration used in our calculations, (1.25 μg/ml, Table 2), we believe that an intracellular oligo

to RNA ratio of less than 880:1 may still be inhibitory to the translation of Vmw65 mRNA during viral infection. Of course, these estimates of intracellular levels change throughout infection and the inhibitory effect of Oligo 293 can be overcome by either the degradation of intracellular oligo or by the synthesis of greater amounts of Vmw65 mRNA.

The inhibition of Vmw65 expression in MTX5 cells was detected only when the cells were preincubated with Oligo 293 for 4 days. We believe that the necessity for this long exposure to the inhibitory molecules reflects a slow rate of decay for the Vmw65 protein which is constitutively expressed in these cells. A stable Vmw65 polypeptide may be important for the successful induction of immediate early gene transcription during HSV infection. Alternatively, the Vmw65 protein may serve a bifunctional role in the replication of this virus. The abundance of the Vmw65 polypeptide in the tegument of the virus (390–580 copies/virion; Heine et al., 1974) identifies this protein as a putative structural component of the virion. If the Vmw65 protein plays a structural role in the mature virus, then the stability of this protein is important to the integrity of the virion.

We have observed that an oligo molecule which is complementary to the translation initiation region of the HSV-1 Vmw65 gene can inhibit the replication of HSV-1 in tissue culture (Tables 1 and 3). This oligo (Oligo 293) exhibited better inhibition of viral replication than previously described molecules which are complementary to the splice junction of the ICP22 and ICP47 mRNAs of HSV-1 [50% inhibition of virus growth at an Oligo 293 concentration of 150 nM (Table 2) compared to 50% inhibition at 25 μM concentration of the splice junction oligo (Smith et al., 1986)]. In our studies, inhibitory concentrations of Oligo 293 were not toxic to the host cells. Thus, we have shown the feasibility of defining a molecular target and synthesizing complementary oligonucleotides which will specifically inhibit viral gene expression without exerting deleterious effects upon the host cell.

We have extended these studies to determine the effects of Oligo 293 and related oligos on the replication of other strains of HSV. Our preliminary results indicate that strain-specific patterns of inhibition exist. An analysis of these patterns will be helpful in determining the essentiality of Vmw65 gene expression for viral replication. By examining the contribution of the Vmw65 protein to virion structure and elucidating ways by which Vmw65 gene structure regulates expression of this important gene product, we hope to define additional regions within the Vmw65 gene which would be good targets for binding of inhibitory molecules.

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