Inhibition of Mouse Hepatitis Virus Multiplication by an Oligonucleotide Complementary to the Leader RNA

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ABSTRACT. An oligonucleotide complementary to a leader RNA of positive-stranded mouse hepatitis virus (MHV) was tested for the effect on the viral replication in mouse DBT cells. A 14-mer antisense oligonucleotide contained a sequence complementary to the conserved pentanucleotide sequence, UCUAA, of the leader RNA. A treatment of MHV-infected cells with the antisense oligonucleotide at concentrations from 5 to 25 µM had an inhibitory effect on the viral multiplication and reduced the synthesis of viral specific mRNA and proteins. No inhibitory effect was observed when the cells were treated with sense oligonucleotide and oligonucleotide which contained unrelated sequences at concentrations from 1 to 10 µM. These results showed that antisense oligonucleotide against the leader RNA reduced the multiplication of positive-stranded RNA virus, MHV.

Key words: antisense oligonucleotide, leader RNA, MHV, viral multiplication.


Mouse hepatitis virus (MHV) is a member of Coronaviridae, which causes a variety of diseases including hepatitis and encephalomyelitis in laboratory mice [9, 34]. MHV is an enveloped virus containing a helical nucleocapsid structure composed of a single-stranded, positive-polarity RNA of approximately 30 kilobase (kb) in length [23]. During infection, virion RNA is initially transcribed into a full-length negative-stranded RNA [5, 13]. In turn, the negative-stranded RNA is transcribed into a genomic RNA and six species of subgenomic mRNAs [15]. The mRNAs form a 3'-coterminal nested-set extending for different lengths in 5' direction [16]. The 5'-ends of each mRNA and the genomic mRNA contains an identical leader sequence of approximately 70 nucleotides, which are encoded only at the 5'-end of genomic RNA [14, 30]. The free leader RNA species is synthesized initially, dissociates from the negative-stranded template, and rebinds to the full-length negative-stranded RNA at the initiation sites of the six subgenomic mRNAs. The leader RNA thus takes part in a leader-primed transcription [4]. Recently it is suggested that subgenomic negative-stranded RNA is also synthesized as a template for the transcription of mRNAs [26]. In MHV-infected cells, three major viral proteins are detected. The glycoproteins, M and S, of ca. 23,000 and 90,000 to 180,000 Da, are translated from mRNA 6 and 3, respectively [33]. A nucleocapsid (N) protein of ca. 60,000 Da is the most abundant and translated from mRNA 7 [31]. It has been shown that a specific interaction occurs between the N protein and the sequence in the leader RNA [3, 32].

Although the molecular mechanisms underlying the phenomenon are still unclear, antisense oligonucleotides and their analogues have been used as tools for inhibiting viral replication and for regulating specific gene expression [1, 2, 6, 8, 11, 18, 25]. In infected cells with MHV, both positive- and negative-stranded virus-specific RNAs are synthesized. Thus, to investigate the effect of antisense oligonucleotide on the gene expression of MHV will provide the insight on the molecular mechanisms underlying the inhibition of gene expression and viral multiplication with antisense oligonucleotides in the cells. However, there have not been reported the effects of antisense oligonucleotides on the gene expression and multiplication of positive-stranded RNA virus in infected cells. In this report, we investigated the effect of antisense oligonucleotide against the leader sequences on MHV multiplication. We selected the leader sequence including the sequence, UCUAA, as a target region of antisense oligonucleotide. The sequence, UCUAA, is conserved at initiation sites for each of the six subgenomic mRNAs and is thought to be associated with the interaction be-

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between the leader RNA and negative-stranded RNA [27].

MATERIALS AND METHODS

Cell, virus and cultivation: The JHM strain of MHV [20] was used in this study. DBT cells [10] were cultivated in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS) at 37°C in a humidified atmosphere containing 5% CO₂.

Oligonucleotides: The oligonucleotides were synthesized using phosphoramidates method on Beckman system Plus-I DNA Synthesizer and purified by HPLC. AL-oligo (5'GATTTAGATTAGATT3') contained a sequence complementary to the consensus sequence, UCUAA, of MHV leader RNA. SL-oligo (5'AATCTAATCTAAAC3') was complementary to AL-oligo. An oligonucleotide with repeated sequence, 5'GATAGATAGATAA3', designated as (GATA)₄, was also synthesized as a control oligonucleotide unrelated with the leader RNA.

Incorporation of oligonucleotides into cells: An oligonucleotide was dephosphorylated by bacterial alkaline phosphatase (Takara Shuzo Co.) and then radiolabeled with [γ-³²P]ATP (4500 Ci/mmol; ICN Radiochemical, U.S.A.) using T4 polynucleotide kinase (Takara Shuzo Co.) [21]. After DBT cells were incubated with radiolabeled oligonucleotide at concentrations from 1 to 25 μM for 1 hr at 37°C, incorporation of oligonucleotides into cells was determined according to the method of Miller et al. [22]. After the addition of 10 ml of ACS-II (Amersham Co.) to an aliquot of cell lysate, the radioactivity was counted with Aloka LSC-750 liquid scintillation counter. After the oligonucleotide was washed out from the medium, the cells were incubated for 0, 3 and 5 hr and the radioactive compounds in the cell lysate was analyzed by 4% agarose gel electrophoresis (Nusieve GTG agarose; FMC Bioproducts). The gel was dried and subjected to autoradiography.

Inhibition of virus multiplication by oligonucleotides: DBT cells were infected with MHV at a multiplicity of infection (m.o.i.) of 0.1 in the absence or presence of oligonucleotide at concentrations from 1 to 25 μM for 1 hr at 37°C under the condition of CS-free MEM. After the incubation, oligonucleotide and virus were removed, and then cells were washed twice with CS-free MEM. After the addition of MEM with CS, plaque assays were performed to titrate infectious progeny at each time post infection (p.i.) according to the method of Hirano et al. [10]. The infectivity was expressed in plaque forming units (PFU). Percentage of inhibition =

\[
\frac{\text{PFU obtained from the cells treated with oligonucleotide}}{\text{PFU obtained from control cells untreated with oligonucleotide}} \times 100(\%)
\]

Northern blot hybridization: Cellular RNA was prepared from DBT cells infected with MHV at m.o.i. of 1 in the absence or presence of oligonucleotide at 10 μM at 4.5 h.p.i. according to the method of Silver et al. [28]. The RNA samples were electrophoresed in 1% agarose gels containing formaldehyde, and blotted onto nitrocellulose membranes [21]. A cDNA of MHV mRNA 7 which was kindly provided by Dr. Siddell [29] was ³²P-labeled by nick-translation as a probe [24]. Prehybridization was carried out for 2 hr at 45°C in 25 mM KPO₄ (pH 7.4), 5×SSC (1×SSC is 0.15 M NaCl, 0.015M sodium citrate), 5×Denhardt's solution [7] (1×.Denhardt's solution is 0.05% bovine serum albumin, 0.05% Ficoll and 0.05% polyvinylpyrrolidone), 0.5% SDS, 100 μg/ml of yeast tRNA and 50% formamide. Hybridization was carried out at 45°C for 15 hr in fresh hybridization solution containing ³²P-labeled cDNA of MHV-mRNA 7. Autoradiography using Fuji RX X-ray film (Fuji Photo Film Co.) was carried out with an intensifying screen at ~70°C. The X-ray film was scanned with densitometer (Type G3300, Hoeffer Scientific Inst.).

Labeling of proteins with [³⁵S]methionine: DBT cells were infected with MHV (0.1 m.o.i.) in the absence or presence of oligonucleotides at 5 μM for 1 hr at 37°C. After the removal of MHV and oligonucleotide from the medium, the cells were incubated for 5.5 hr at 37°C. After the replacement of the medium with methionine-free MEM medium and the incubation for 30 min at 37°C, [³⁵S]methionine (50 μCi/ml; ICN Radiochemical, U.S.A.) was added to the medium and the cells were incubated for 3 hr at 37°C. The cells were washed twice with phosphate-buffered saline (PBS) and resuspended with 0.5% SDS in PBS. The cell lysate was vortexed and centrifuged at 10,000 rpm
for 10 min. The sample buffer (1% SDS, 1% 2-mercaptoethanol, 20% glycerol, 10 mM Tris-HCl, pH 6.8) was added to the supernatant. After boiling the sample solution for 2 min, the samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed by the method of Laemmli [12]. The gel was run at 4 mA for 22 hr and exposed to Fuji RX X-ray film at −70°C. The X-ray film was scanned with densitometer (Type G3300, Hoeffer Scientific Inst.).

RESULTS

Inhibition of the multiplication of MHV by AL-oligo: A genomic organization of MHV was shown in Fig. 1. To investigate the effects of the treatment of cells with AL-oligo on the viral multiplication, DBT cells (5×10⁵) were incubated with MHV-JHM (0.1 m.o.i.) in the presence of oligonucleotides at concentrations from 1 to 25 μM for 1 hr at 37°C. A typical result of the plaque assay was shown in Table 1. The yield of infectious virion particles from the cells treated with AL-oligo at concentrations from 5 to 25 μM for 1 hr reduced from 6 to 15 h.p.i. (Fig. 2a), compared with the yield from control cells untreated with oligonucleotides. The inhibitory effect by AL-oligo at 5 μM on the viral multiplication gradually reduced with incubation time. In the case of the treatment with AL-oligo at concentrations of 10 and 25 μM, the inhibitory effect was sustained over 12 h.p.i. (Fig. 2a). No inhibitory effect on the viral multiplication was observed at 1 μM. When DBT cells were infected with MHV-JHM at 1 m.o.i., the similar inhibitory effect by AL-oligo at a concentration of 10 μM on the viral multiplication was observed (data not shown). In the case of the treatment of cells with SL-oligo, no inhibitory effect was observed at concentrations from 1 to 10 μM (Fig. 2b). On the contrary, a significant inhibition (20% inhibition) of viral multiplication was observed at high concentration (25 μM). No inhibitory effect on the viral multiplication was observed in the cells treated with (GATA)₄ at concentrations from 1 to 10 μM.

Incorporation of oligonucleotide: Since the presence of oligonucleotide in the virus suspension may interfere the exact evaluation of the plaque assay, we examined the effects of AL-oligo on the viral multiplication after the cells were treated with oligonucleotides for 1 hr and the oligonucleotides were removed from the medium. When the DBT cells were incubated for 1 hr at 37°C in CS-free medium, approximately 1.7% of the oligonucleotides was incorporated into the cells (2–50×10⁶ molecules/cell, Fig. 3a). During this incubation time, no change in the size of oligonucleotides was observed in the medium (data not shown). When the radioactive compounds in the cell lysates were analyzed by 4% GTG-agarose gel electrophoresis (Fig. 3b), slow migrating band was observed at 3 and

![Fig. 1. Genomic organization of MHV and the sequences of oligonucleotides. The relationship between the 3'-coterminal nested-set of mRNAs and the viral genome is shown, together with the coding regions for the structure proteins, nucleocapsid (N), membrane (M) and surface (S), specified by mRNA 7, 6 and 3, respectively. The leader RNA contains conserved sequence, UCUAA.](image)

Table 1. Inhibitory effects of AL-oligo on MHV multiplication

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Concentration (μM)</th>
<th>h.p.i.</th>
<th>PFU/plateᵃ)</th>
<th>PFU/ml</th>
<th>Percentage of inhibition(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>6</td>
<td>156±11</td>
<td>3.12×10⁷</td>
<td>-</td>
</tr>
<tr>
<td>AL-oligo</td>
<td>5</td>
<td>6</td>
<td>62±16</td>
<td>1.24×10⁷</td>
<td>60.3</td>
</tr>
<tr>
<td>SL-oligo</td>
<td>5</td>
<td>6</td>
<td>153±30</td>
<td>3.06×10⁷</td>
<td>2.0</td>
</tr>
<tr>
<td>(GATA)₄</td>
<td>5</td>
<td>6</td>
<td>167±32</td>
<td>3.34×10⁷</td>
<td>- 7.0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>12</td>
<td>254±15</td>
<td>5.07×10⁶</td>
<td>-</td>
</tr>
<tr>
<td>AL-oligo</td>
<td>5</td>
<td>12</td>
<td>196±21</td>
<td>3.92×10⁶</td>
<td>22.7</td>
</tr>
<tr>
<td>SL-oligo</td>
<td>5</td>
<td>12</td>
<td>245±16</td>
<td>4.89×10⁶</td>
<td>3.6</td>
</tr>
<tr>
<td>(GATA)₄</td>
<td>5</td>
<td>12</td>
<td>264±22</td>
<td>5.28×10⁶</td>
<td>- 0.4</td>
</tr>
</tbody>
</table>

ᵃ) Each value represents an average of three plates of one experiment.
Fig. 2. The effect of the treatment of DBT cells with oligonucleotides on the viral multiplication. (a) DBT cells were infected with MHV-JHM (0.1 m.o.i.) in the presence of AL-oligo at concentrations of 1 (○—○), 5 (●—●), 10 (△—△) and 25 (▲—▲) μM for 1 hr at 37°C. (b) DBT cells were infected with MHV-JHM (0.1 m.o.i.) in the presence of AL-oligo (○), SL-oligo (△, ▲) and (GATA)_2 (□, ■). Plaque assays were performed to titrate infectious progeny at 6 h.p.i. (○, △, □) and 12 h.p.i. (●, ▲, ■). Each point represents an average of four separate experiments.

5 h.p.i. The intensity of the slow migrating band from the infected cells at 5 h.p.i. was weaker than that from the mock-infected cells at 5 h.p.i.

Effect of AL-oligo on the synthesis of viral specific mRNA and proteins: To investigate the effect of AL-oligo on the synthesis of viral specific mRNA, cellular RNA was prepared from infected cells with MHV (1 m.o.i.) in the presence or absence of oligonucleotides at a concentration of 10 μM at 4.5

Fig. 3. Incorporation of oligonucleotides into the cells. (a) After incubation of the cells with radiolabeled AL-oligonucleotide at 5 μM, the radioactivity in the cell lysate was counted. Each point was an average of four separate experiments; bars indicate standard deviation. (b) After incubation of mock-infected (lanes 1 to 3) and MHV-infected (0.1 m.o.i.) (lanes 4 to 6) cells for 0 (lanes 1 and 4), 3 (lanes 2 and 5) and 5 (lanes 3 and 6) hr with radiolabeled AL-oligonucleotide at 5 μM, the radioactive compounds in the cell lysates were analyzed by 4% agarose gel electrophoresis.
h.p.i. and analyzed by Northern blot hybridization using cDNA of mRNA 7 as a probe (Fig. 4). Since genomic RNA and six subgenomic mRNAs contain the sequence complementary to the mRNA 7 (Fig. 1), all mRNA species hybridize with cDNA of mRNA 7. The synthesis of all viral mRNAs in the cells treated with AL-oligo was reduced, compared to that in control cells untreated with oligonucleotides (lanes 2 and 3). On the contrary, no inhibitory effect on the synthesis of viral mRNA was shown in the cells treated with SL-oligo and (GATA)$_4$.

N protein is most abundant MHV-specific protein in the infected cells [31] and only N protein was apparent under the conditions used in this study. The band of N protein was confirmed by using monoclonal antibody against N protein (data not shown). The synthesis of viral N protein also reduced in the cells treated with AL-oligo during 6 to 9 h.p.i., compared to that in untreated control cells (Fig. 5, lanes 2 and 3). On the contrary, the treatment of cells with SL-oligo or (GATA)$_4$ did not inhibit the synthesis of N protein significantly (lane 4 and 5, respectively).
DISCUSSION

It has been reported that antisense oligonucleotides and their analogues against viral genes inhibited the expression of the genes and viral multiplication. The percentage of inhibition of viral multiplication by the treatment with antisense oligonucleotides was in the range from about 25 to 99% [1, 2, 6, 8, 11, 18, 25]. The degree of the reduction of viral multiplication by the treatment of cells with antisense oligonucleotides seems to be dependent on the type of virus, sequences of oligonucleotides as a target, the modification of oligonucleotide and the experimental conditions. However, the molecular mechanisms underlying the phenomenon are still unclear. Since positive-stranded RNA virus synthesizes negative-stranded RNA in its replication cycle, the investigation of the effect of antisense oligonucleotide on gene expression of positive-stranded RNA virus may provide the insight on the molecular mechanisms underlying the inhibition of gene expression and viral multiplication. Therefore, we examined the effect of the treatment of cells with oligonucleotide complementary to the leader RNA on the multiplication of positive-stranded RNA virus, MHV.

The cells were treated with oligonucleotide at 37°C for 1 hr during MHV adsorption and the oligonucleotide was removed from the medium. During the incubation for 1 hr, an approximately 1.7% of oligonucleotide was incorporated into the cells and radioactivity in the cells increased with incubation time (Fig. 3a). This result was in good agreement with the reports by Loke et al. [19] and Kawamura et al. [11]. The yield of infectious virion particles from the cells treated with AL-oligo from 5 to 25 μM was reduced from 25 to 36% level at 6 h.p.i., compared with the yield from control cells untreated with oligonucleotides. When DBT cells were infected with other MHV strain (A59) in the presence of AL-oligo at 10 μM, the inhibitory effect on the viral multiplication was also observed (data not shown). The pentanucleotide sequence, UCUAA, in the leader RNA is conserved among different MHV strains [27]. Therefore, the leader sequence including the sequence, UCUAA, may be adequate targets for the study of inhibition of MHV multiplication by AL-oligo. The treatment of cells with SL-oligo and (GATA), at concentrations from 1 to 10 μM showed little inhibitory effect on the viral multiplication. When the cells were treated with SL-oligo at 25 μM, the significant inhibition (20% inhibition) of the viral multiplication was observed. These results indicated that the inhibitory effect by AL-oligo on the viral multiplication was specific for the antisense sequence of the leader RNA of MHV at 5 and 10 μM. However, the somewhat of nonspecific inhibitory effects by oligonucleotides on the viral multiplication might occur at 25 μM. Alternatively, the interaction between negative-stranded RNA and sense oligonucleotide might interfere the viral transcription. It has been reported that the treatment of cells with mismatched oligonucleotides at high concentration showed nonspecific inhibitory effects on the human immunodeficiency virus type 1 [2] and vesicular stomatitis virus (VSV) [6], and that oligonucleotides against negative strand of VSV showed the inhibitory effect on the viral multiplication [6]. Therefore, the oligonucleotide complementary to the negative-stranded RNA may not inhibit the transcription of mRNA from the negative-stranded RNA by RNA-dependent RNA polymerase at low concentration. On the contrary, the interaction between SL-oligo and the negative-stranded RNA may interfere the transcription of mRNA from MHV negative strand at high concentration of SL-oligo.

The synthesis of virus-specific mRNA and N protein was also reduced in the cells treated with AL-oligo at 5 and 10 μM, compared with that in the control cells untreated with oligonucleotides. Densitometric analysis of autoradiographs indicated that MHV-specific mRNA and N protein synthesized in the MHV-infected cells treated with AL-oligo were reduced to approximately 20% level, compared with those in the control cells untreated with oligonucleotides. Thus, the level of reduction of the synthesis of MHV-specific mRNA and N protein was similar to that of the yield of virus particles. Since the yield of virus particles was low till 5 h.p.i., we could not determine the effects by AL-oligo on the viral multiplication exactly till 5 h.p.i. by plaque assay. However, it was shown that the synthesis of viral mRNA in the cells treated with AL-oligo was reduced at 4.5 h.p.i., compared to that in control cells (Fig. 4). The amounts of incorporated oligonucleotides into the cells were dependent on the concentration of oligonucleotide in the medium and the degree of reduction in the yield of viral particles in the cells treated with AL-oligo at 6 h.p.i. seemed to be dependent on the concentration of AL-oligo (Fig. 2 and 3). The treatment of MHV-infected cells
with AL-oligo at 10 μM at 3 h.p.i. for 1 h showed little inhibitory effect on the viral multiplication (data not shown). The presence of AL-oligo at the initial stage of the viral infection may be important for obtaining the inhibitory effect.

Since no change in the size of oligonucleotide was observed (data not shown), the oligonucleotide remained intact in the medium during the incubation for 1 h. After incubation for 3 and 5 h, slow migrating band appeared in the cell lysates. This result suggested that most of oligonucleotides might be associated with cellular component. This result was in agreement with the recent report [11, 15]. Since the intensity of the band from infected cells at 5 h.p.i. was weaker than that from mock-infected cells at 5 h.p.i., AL-oligo might react with the leader RNA of MHV and be degraded in infected cells.

In this study, it was shown that antisense oligonucleotide against the leader RNA reduced the viral multiplication and the synthesis of virus-specific mRNAs and N protein. The treatment with SL-oligo showed no inhibitory effects on the viral multiplication and the synthesis of virus-specific mRNAs and N protein at low concentration. On the contrary, somewhat of inhibitory effect was observed by sense oligonucleotide at high concentration. Further investigation concerning the interaction between oligonucleotide and positive- and negative-stranded RNA of MHV should be required for the elucidation of the molecular mechanisms underlying the inhibition of viral multiplication by antisense oligonucleotide and such a study is now in progress.

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