Inhibition of Simian virus 40 DNA replication in CV-1 cells by an oligodeoxynucleotide covalently linked to an intercalating agent

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ABSTRACT

An octathymidylate covalently linked via its 3'-end to an acridine derivative inhibited the cytopathic effect of Simian Virus SV40 on CV-1 cells in culture. Control experiments revealed that this effect was virus-specific and did not arise as a result of oligonucleotide degradation by nucleases. A photoactive probe was covalently attached to the 5'-end of the oligonucleotide-acridine conjugate. Upon UV-irradiation, photo-crosslinking was shown to occur at the A.T-rich region within the viral origin of replication. A local triple helix can form at moderate salt concentrations with two octathymidylate-acridine conjugates bound to the octaadenylate sequence. Alternatively the octathymidylate-acridine conjugate can bind to the major groove of duplex DNA forming a local triple helix. Different mechanisms are discussed to explain the inhibition of viral DNA replication.

INTRODUCTION

The regulation of gene expression usually involves proteins that bind strongly and selectively to nucleic acid sequences either at the level of DNA where they control transcription, or at the level of messenger RNAs where they control translation (for a review see reference 1). Additional steps, such as pre-mRNA splicing or mRNA export from the nucleus, are also controlled by proteins. It has recently been discovered that RNAs could play a regulatory role by hybridizing to mRNA, thereby preventing protein synthesis (for a review see reference 2). This finding has been the basis for the development of a new strategy to artificially control gene expression (3 — 7). Oligodeoxynucleotides can be added to culture media and do not require either transfection or microinjection procedures. It has been reported that oligodeoxynucleotides can inhibit the development of Rous Sarcoma virus (RSV) (3) or of the human immunodeficiency virus (HIV) (8). However the penetration of oligonucleotides across cell membranes is not very efficient. In addition, oligodeoxynucleotides are very sensitive to nucleases. Several attempts have been made to overcome these two difficulties. Oligomethylphosphonates have been synthesized and shown to be moderately active at inhibiting mRNA translation (9). They are resistant to nucleases and seem to penetrate better into cells in culture, where they may inhibit mRNA splicing (10). Oligonucleotides synthesized with the a-anomers of nucleotide units instead of the natural b-anomers are also resistant to nucleases (11,12). However they are poor inhibitors of mRNA translation (13,14). Oligomethylphosphonates and oligo-[a]-deoxynucleotides do not form RNase H-sensitive complexes when hybridized to mRNAs. In contrast oligophosphorothioates form RNase H-sensitive complexes with mRNAs and are very efficient inhibitors of mRNA translation (14,15).

We have previously described a family of molecules in which oligodeoxynucleotides are covalently linked to intercalating agents (16 — 19). The intercalating agent provides an additional binding energy without perturbing the specificity of recognition of the complementary sequence. The resulting stabilization allows strong complexes to be formed with short oligonucleotides. In addition cellular uptake is facilitated for two reasons: i) shorter oligonucleotides have less negative charges and ii) the intercalating agent drags the oligonucleotide inside cells (19). The intercalating agent also prevents degradation of the oligonucleotide by exonucleases (20). We previously showed that oligodeoxynucleotide-acridine conjugates could prevent RNA translation in both in vitro systems (21) and microinjected Xenopus oocytes (22). An oligodeoxynucleotide-intercalator conjugate targeted to the 3'-end of influenza virus (-) RNAs prevented the viral transcriptase complex from synthesizing (+) RNAs and consequently inhibited the cytopathic effect of the virus (23). Trypanosomes in culture were killed by oligonucleotide-
intercalator conjugates targeted to the common sequence that is present at the 5'-end of all mRNAs in these parasites (24). Protein synthesis was completely blocked in the presence of such conjugates. Biological effects associated with oligodeoxynucleotides targeted to RNAs are correlated with the ability of endogenous RNase H to cleave the RNA in RNA-oligodeoxynucleotide hybrids (22,25). Therefore it was of interest to determine whether an oligonucleotide-intercalator conjugate could elicit biological effects when targeted to a DNA sequence, a situation in which RNase H could not be involved. Here we show that an octathymidylate covalently linked to an acridine derivative blocks the cytopathic effect of Simian Virus 40 (SV40) in monkey CV-1 cells in culture. This effect arises as a result of an inhibition of viral DNA replication. Using a photocrosslinking reaction we show that this oligothymidy late-acridine conjugate binds to the octadeoxyadenylate sequence within the A.T-rich region of the viral origin of replication.

MATERIALS AND METHODS

Inhibition of the cytopathic effect of SV40 in CV1 cell cultures

CV1 cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 2% fetal calf serum (FCS), penicillin and streptomycin sulfate. For anti-SV40 studies, 3x10^5 cells were seeded in 3.5 cm diameter Falcon Petri dishes and incubated for 24 hours at 37°C in a 5% CO₂ atmosphere. The medium was then discarded and 0.2 ml of SV40 inoculum were added corresponding to a multiplicity of infection (moi) of about 0.1 pfu/cell. After 90 minutes at 37°C, the viral inoculum was discarded and 2 ml of medium containing the compounds undergoing evaluation were added. Mock-infected cultures were used to test the cytototoxicity of the compounds. Cultures were examined under the microscope at day 6, at which time the cytopathic effect (CPE) was virtually complete (90–100% cellular destruction in infected controls). Inhibition of the CPE was quantified by staining the residual cell monolayer with crystal violet and further extraction of the dye with monomethylic ether (26). Absorbance at 600 nm was measured to determine the extent of cell destruction.

Inhibition of SV40 DNA synthesis

CV1 monkey cells were maintained in Dulbecco-modified Eagle medium (DMEM) containing 5% foetal calf serum and glutamin. SV40 virus stocks were obtained by infecting CV1 cells at a m.o.i. of 0.01 pfu/cell. The cells were scraped off the dish when the cytopathic effect was about 50%. After freezing and thawing, the virus suspension was clarified by centrifugation (1 h, 15,000 g, 4°C) and aliquots were titred by plaque formation on CV1 monkey cells. This effect arises as a result of an inhibition of viral DNA replication. Using a photocrosslinking reaction we show that this oligothymidy late-acridine conjugate binds to the octadeoxyadenylate sequence within the A.T-rich region of the viral origin of replication.
The concentration giving 50% cell killing was about 5 μM. No effect on the cytopathic effect of the virus was observed at sub-cytotoxic concentrations. The acridine-substituted octathyminidate did not exhibit any cytotoxic effect on uninfected CV1 cells in culture up to 60 μM nor did it inhibit cell division. The same results were obtained on MRC5 and primary monkey kidney cells. These observations seemed to eliminate the possibility that CPE inhibition was due to release of the acridine derivative.

Two additional experiments were carried out to demonstrate that the effect of (Tp)m₆Acr was not due to a release of acridine which could have resulted from oligonucleotide hydrolysis by nucleases. First, a tetramathyminidate covalently linked to acridine did not exhibit any effect on the cytopathic effect of the virus even though it contained both acridine and thymines as in (Tp)m₆Acr. Second, a heptanucleotide covalently linked to acridine, d(AAAGGAG)m₆Acr, which had no target in SV40 DNA did not inhibit virus development (data not shown). These experiments demonstrated that the effect of (Tp)m₆Acr was not due to the release of acridine from the oligonucleotide.

As control experiments for the specificity of the effect, the oligonucleotide (Tp)m₆Acr was tested in three other systems: MDCK cells infected by influenza virus A/PR8 and Vero cells infected by HSV-1 herpes virus or rhinovirus 1 B. No inhibition of the cytopathic effect of these viruses was detected at concentrations where SV40 was nearly 100% inhibited.

Inhibition of viral DNA synthesis

As the complementary sequence of (Tp)m₆Acr is part of the origin of replication of SV40 DNA (figure 1), this oligonucleotide might interfere with DNA replication. To test this hypothesis, the viral DNA was selectively extracted as described in (35) at different times after infection of CV1 cells. After agarose gel electrophoresis and transfer to nitrocellulose filters, the viral DNA was revealed by hybridization with 3²P-labelled SV40 DNA.

Table 1: Effect of oligonucleotides, oligonucleotide-acridine conjugates and acridine derivative alone on the cytopathic effect (CPE) of SV40 in CV1 cell cultures (see Materials and Methods for CPE measurements).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>Inhibition of CPE (average ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Tp)m₆Acr(a)</td>
<td>30</td>
<td>95 ± 4 (1)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>67 ± 32 (1)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>(Tp)m₆OE(b)</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>(Tp)m₆Acr(a)</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>(Tp)m₆OE(b)</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Ac₆OH(c)</td>
<td>10</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Results obtained on: (1) 8 cultures (4 exp) (2) 4 cultures (2exp) ; (3) 12 cultures (6 exp) (4) 8 cultures (4exp) , (5) 4 cultures (2exp)

(a) Oligothyminidylates covalently linked to 2-methoxy, 6-chloro, 9-amino-acridine (Acr) via a hexamethylene (m₆) or pentamethylene (m₅) linker between the 9-amino group of acridine and the 3'-phosphate group of the oligonucleotide.

(b) Oligothymidylates whose 3'-phosphate group was substituted by an ethyl group

(c) Ac₆OH is 2-methoxy, 6-chloro, 9-(ω-hydroxypropylamino)acridine.
Inhibition of Simian Virus 40 DNA replication in CV1 cells

Figure 2. Effect of (Tp)m8Acr on SV40 DNA synthesis in CV1 cells. Viral DNA was extracted from control infected CV1 cells (lanes 1-3) or infected cells treated with different concentrations of the oligonucleotide (3, 10 and 30 µM in culture medium; lanes 4-6, 7-9 and 10-12, respectively). Samples were harvested at different times after infection: 1 h (lanes 1, 4, 7 and 10), 23 h (lanes 2, 5, 8 and 11) and 38 h (lanes 3, 6, 9 and 12). After electrophoresis and membrane transfer, the filters were hybridized with [32P] labelled SV40 DNA as described under Materials and Methods. The lower band corresponds to supercoiled SV40, the upper band to relaxed DNA.

Two bands could be detected on the gels corresponding to supercoiled and relaxed DNA (figure 2). As shown on figure 2, addition of (Tp)m8Acr to CV1 cells infected by SV40 at 1 pfu/cell strongly reduced the synthesis of viral DNA. The effect was concentration-dependent with more than 90% reduction at an oligonucleotide concentration of 30 µM as determined from densitometric analysis of hybridized gels analogous to that of figure 2 but with a shorter exposure time.

The acridine derivative bearing a pentamethylene linker was also tested for its ability to affect viral DNA synthesis. At sub-cytotoxic concentrations (1-5 µM) no effect could be detected on SV40 DNA replication. The shorter oligonucleotide (Tp)m4Acr had no effect on DNA synthesis nor did (Tp)m8OEt where the 3'-phosphate group was substituted by an ethyl group to mimic part of the linker but without acridine attached to it. Also an oligonucleotide with an unrelated sequence covalently attached to acridine (d(TCGTCG)m8Acr) did not inhibit SV40 DNA synthesis. Figure 2 presents the results obtained when the oligonucleotide (Tp)m8Acr was added to CV1 cells before infection. Similar results were obtained when infection preceded addition of the oligonucleotide-acridine conjugate. These conditions are similar to those under which inhibition of the cytopathic effect (CPE) of the virus was observed (see above) except that CPE inhibition was measured at a lower m.o.i. than that used in the experiments where DNA synthesis was analyzed. Only slightly higher oligonucleotide concentrations were required to observe the inhibition of DNA synthesis (figure 2) as compared to inhibition of CPE (table 1).

Hybrid formation between (Tp)m8Acr and SV40 DNA

In order to test the ability of (Tp)m8Acr to hybridize to SV40 DNA, a 346 bp-fragment containing the potential target sequence (A8) was prepared by Hind III digestion of a plasmid (pSVECAT) containing a SV40 DNA fragment. To analyze the formation of hybrids, a photoactive probe (p-azidophenacyl) was used to prepare (Tp)m8Acr. This compound will be abbreviated as Acr via a covalently attached to the 5'-end of (Tp)m8Acr (see Materials and Methods). Upon excitation with light of wavelengths longer than 300 nm the azido derivative is converted to highly reactive species. Covalent bonds are formed with nucleic acid bases, leading to a photocrosslinking reaction (36-38). Upon alkaline treatment, these photoinduced crosslinks are converted to strand breaks which can be revealed by polyacrylamide gel electrophoresis followed by autoradiography using 32P end-labelled DNA fragments as described previously (37,38).

The SV40 DNA fragment was 3'-labelled using deoxyxuridyl terminal transferase and α-32P-dideoxy ATP. The A8 sequence starts 248 nucleotides from the 3'-end of one strand. When the 346 bp-fragment was heat-denatured before addition of the oligonucleotide at 1 µM concentration, the strand containing the A8 sequence was cleaved at the expected position after UV-irradiation and alkaline treatment (figure 3). The length of the cleaved fragment (3'-end labelled) was 247 ± 3 bases as determined by comparison with size markers. These experiments clearly demonstrated that the single-stranded fragment could hybridize to the oligonucleotide at a concentration of 1 µM in a pH 7 buffer containing 10 mM Na phosphate and 0.25 M NaCl. When the salt or the oligonucleotide concentrations were increased, a second band appeared on the gels. The same result was obtained at 1 µM concentration of the oligonucleotide when 1 mM spermine was added to the buffer containing 0.1 M NaCl. The distance between the two bands was estimated from a plot of the logarithm of the distance migrated by a set of markers versus the length of these markers. The two bands had estimated lengths of 247 ± 3 and 255 ± 3 nucleotides, respectively. This result indicated that two oligonucleotides could bind to the single-stranded A8 sequence forming a triple helix as previously shown when a 27-mer single-stranded fragment containing an A8...
Acr = $\text{N}_3\text{O} - \text{C-CH}_2\text{S-P-O}-(5')$

$\text{N}_3\Phi = \text{N}_3\text{O} - \text{C-CH}_2\text{S-P-O}-(3')$

Figure 4. Sequence of the SV40 single-stranded DNA fragment with two derivatized oligothymidylylates bound to the A$_8$ target sequence. In the presence of 0.1 M NaCl only oligonucleotide 1 is bound (double helix with antiparallel strands). At higher NaCl concentrations (≥ 0.25 M) or in the presence of 1 M NaCl and 1 mM spermine the second oligonucleotide 2 binds in parallel orientation with respect to the target (see figure 3 and reference 38).

The sequence was used as a target for N$_3\Phi$(sp)(T)p$_n$Acr (38). The location of the cleavage sites at low and high salt concentrations showed that the first oligothymidylate was bound in an antiparallel orientation with respect to the A$_8$ sequence as expected for a Watson-Crick double helix while the second oligothymidylate (in the triple helix) was bound parallel to the A$_8$ sequence (figure 4). Triple helix formation involves hydrogen bonding of thymine to an A.T base pair within the major groove as shown on figure 6.

These results suggested that (T)p$_n$Acr could also bind to a double helix containing eight adenines on one strand and eight thymines on the other strand. We previously reported that photocrosslinking and cleavage could be observed on both strands of a 27-mer duplex when irradiated in the presence of N$_3\Phi$(sp)(T)p$_n$Acr (38). The location of the cleavage sites indicated that the oligothymidy late was bound parallel to the A$_8$-containing strand in agreement with local triple helix formation involving binding of the oligothymidy late to the major groove of duplex DNA. Part of the interaction of N$_3\Phi$(sp)(T)p$_n$Acr with duplex DNA could involve the azidophenacyl group. We tried to use the acridine group of (T)p$_n$Acr as a photosensitizer. Unfortunately neither photocrosslinking nor cleavage were observed due to the low photochemical reactivity of 2-methoxy, 6-chloro, 9-aminoacridine. Another acridine derivative (2,6-diamoacridine or proflavine, abbreviated Pf) was covalently attached to a 3'-thiophosphate group of the octamethylylate. Proflavine has been shown previously to induce photocrosslinking of (T)p$_n$Pf when this oligonucleotide was hybridized to the 27-mer single-stranded DNA fragment containing the A$_8$ sequence (33). A 27 bp duplex DNA fragment was used as a substrate to determine whether (T)p$_n$Pf could bind to the double helix thereby forming a local triple helix. Irradiation of (T)p$_n$Pf in the presence of the 27-mer duplex induced photocrosslinking on both strands of the double helix as revealed in two separate experiments where each of the two strands was 5'-end labelled (figure 5). The crosslinking sites were revealed by electrophoresis of the irradiated fragments on denaturing polyacrylamide gels after treatment with 1 M piperidine at 90°C for 15 minutes. The results are summarized in figure 5 where the arrows represent the sites of cleavage, with efficiencies proportional to the arrow lengths, after correction for background cleavage at guanines due to piperidine treatment (compare lanes 1 and 2 in figure 5). It can be seen that cleavage (and therefore crosslinking) occurred on the two strands of the double helix at positions indicating that (T)p$_n$Pf was bound parallel to the A$_8$-containing strand as expected for triple helix formation, with the oligonucleotide binding to the major groove. It should be noted that these reactions were observed either at high salt concentration (1 M NaCl) or at lower salt concentration (0.1 M NaCl) in the presence of 1 mM spermine which was previously shown to enhance triple helix formation (39). The location of the cleavage sites indicated that (T)p$_n$Pf did not bind to an open double helix forming a D-loop. If that were the case (T)p$_n$Pf should have been bound in an antiparallel orientation with respect to the A$_8$-containing strand instead of the parallel orientation observed in the present experiments.
**DISCUSSION**

The results reported in this study demonstrated that an octathymidylate covalently linked to an intercalating agent, \((\text{Tp})_8\text{m}_6\text{Acr}\), could block the cytopathic effect of SV40 in CV1 cells in culture. This inhibition was specific for the SV40 virus. It was not observed with another DNA-containing virus (HSV-1) nor with two RNA-containing viruses (influenza virus and rhinovirus IB). An octathymidylate lacking the acridine substituent had no effect on virus development. Previous work from our laboratory has shown that covalent attachment of the acridine derivative to an oligonucleotide results in a strong stabilization of the complex formed with a complementary sequence, especially for short oligonucleotides (16–18). This stabilization is due to the additional binding energy provided by intercalation of the acridine ring in the mini-double helix formed by the oligonucleotide with its complementary sequence. In addition attachment of the acridine at the 3'-end of the oligonucleotide protects it against 3'-exonuclease digestion and favors uptake by living cells (19,20,24).

A shorter oligothymidylate covalently linked to the acridine derivative, \((\text{Tp})_4\text{m}_5\text{Acr}\) did not inhibit the cytopathic effect of the virus. This observation demonstrated that the effect obtained with \((\text{Tp})_8\text{m}_6\text{Acr}\) was not due to intermediate degradation products of the oligonucleotide, nor was it due to the acridine derivative itself which could have been released upon complete digestion. The acridine derivative was strongly cytotoxic at 5 \(\mu\)M whereas concentration up to 60 \(\mu\)M of the various acridine-substituted oligonucleotides were not cytotoxic.

The octathymidylate sequence can bind to the poly(A) tails of mRNAs. Experiments carried out in microinjected Xenopus oocytes showed that at concentrations of \((\text{Tp})_8\text{m}_6\text{Acr}\) up to 10 \(\mu\)M inside the oocyte, protein synthesis was not shut off (C. Cazenave, unpublished results). The penetration of \((\text{Tp})_8\text{m}_6\text{Acr}\) in CV1 cells in culture could be followed by fluorescence microscopy. The green fluorescence of the acridine derivative was seen to accumulate inside vesicles in the cytoplasmic compartment. This localization was identical to that seen with the free acridine derivative, which might indicate that it is the acridine which drags the oligonucleotide inside cells. However the intracellular concentration of the oligonucleotide-acridine conjugate remained low as compared to acridine itself. Therefore it is very unlikely that the concentration of \((\text{Tp})_8\text{m}_6\text{Acr}\) inside CV1 cells reached a concentration which could block protein synthesis in an unspecific way. As a matter of fact, this oligothymidylate-acridine conjugate was not cytotoxic to CV1 cells up to a concentration of 60 \(\mu\)M in the culture medium as would have been expected if all protein synthesis was shut off.

The octathymidylate-acridine conjugate efficiently blocked SV40 DNA replication in CV1 cells. The inhibition could be due to several phenomena. SV40 DNA replication is dependent upon the synthesis of T-antigen. Immunofluorescence studies revealed that T-antigen synthesis was not markedly affected in CV1 cells infected in the presence of \((\text{Tp})_8\text{m}_6\text{Acr}\) (data not shown). This seems to rule out an indirect effect of this oligonucleotide on DNA replication mediated by an inhibition of T-antigen synthesis. However we cannot exclude the possibility either that a slight reduction in T-antigen synthesis could result in a drastic effect on viral DNA replication or that the oligonucleotide-acridine conjugate binds to T-antigen thereby inhibiting its stimulatory effect on DNA replication (40). An interaction of \((\text{Tp})_8\text{m}_6\text{Acr}\) with DNA polymerase(s) leading to a general inhibition of DNA replication is also possible.
synthesis seems unlikely, as no effect was seen on CV1 or HL60 cell division at the concentrations where the cytopathic effect of SV40 was blocked. The choice of (Tp)_mACr as a possible inhibitor of SV40 development rested upon the possibility that this oligonucleotide could interfere with SV40 DNA replication because of its complementarity to the octaadenylate sequence which is found within the SV40 DNA origin of replication. This \((A\cdot T)_b\) sequence has been previously shown to play a crucial role in the replication of both polyoma (41) and SV40 DNAs (34,42,43). It also constitutes a nuclease S1-hypersensitive site in supercoiled molecules (44). During the replication process, the two strands are separated in order for DNA polymerase to copy each strand of the double helix. Using 346 bp and 27 bp DNA fragments containing the \((A\cdot T)_b\) sequence, we showed that i) \((T)p_mACr\) binds to the \(A_8\) sequence when the two strands of the 346 bp fragment are separated, ii) two molecules of \((T)p_mACr\) can bind to the single-stranded \(A_8\) sequence and form a local triple helix and iii) \((T)p_mACr\) can bind to the major groove of DNA at the \(A_8\cdot T_8\) sequence. It is difficult to know what the local environment and concentration of \((T)p_mACr\) are in CV1 cells, at the stage where this oligonucleotide may interact with the infecting SV40 DNA. However, it is certainly present in large excess with respect to the viral DNA. Therefore formation of a triple helix with the \(A_8\) sequence is likely when the two strands of the origin of replication are separated. Such an interaction could result in the observed inhibition of DNA replication. Recent experiments have shown that duplex SV40 DNA is unwound from the origin of replication by T antigen binding (45). It is also possible that formation of a double helix stabilized by the intercalating agent is sufficient to inhibit DNA replication. The additional binding energy provided by the intercalating agent leads to a strong increase in the residence time of the oligonucleotide on its target sequence (16–18). This is true also when the oligonucleotide binds to the major groove of duplex DNA thereby forming a local triple helix (46). These three possibilities are summarized on figures 6. Double and triple helix formation could be stabilized by formation of a ternary complex involving one of the components of the replication machinery, viral DNA and the oligonucleotide-intercalator conjugate. A study of SV40 DNA replication in an in vitro system (34,47) should help to determine what are the prerequisites for an efficient inhibition of DNA replication by oligodeoxynucleotides covalently linked to intercalating agents.

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