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Inhibition of HSV-1 Proliferation by Decoy Phosphodiester Oligonucleotides Containing ICP4 Recognition Sequences

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Transcriptional control in eukaryotes results from the interplay between DNA sequences in promoters, enhancers, or silencers and transcription factors. Selective control of gene expression can thus be achieved by inhibiting specific transcription factor/DNA interactions. Transcriptional activity of DNA binding transcription factors can be inhibited by competition with double-stranded oligonucleotides (decoys) that contain their specific recognition sequences. The immediate early protein ICP4 of herpes simplex virus type 1 (HSV-1) is a sequence-specific DNA binding protein that is essential for viral replication. We synthesized double-stranded hairpin phosphodiester oligonucleotides carrying ICP4 sites and demonstrated their ability to specifically titrate ICP4. Upon addition to Vero cells, ICP4 hairpin decoys significantly reduced HSV-1 titers (IC_{50} = 0.3 μM), whereas a control hairpin oligonucleotide had no activity. Antiviral activity of ICP4 hairpin decoys was correlated to their relative binding affinities. These results show that phosphodiester oligonucleotides can compete for binding of specific transcription factors within cells, thus providing a potential therapeutic tool to control disease-causing genes.

Transcription control Gene expression Transcription factors Sense approach
Oligonucleotide decoy DNA binding competition Herpes simplex virus ICP4

THE ability of synthetic oligonucleotides to hybridize specifically with complementary sequences of RNA or DNA (antisense or antigene strategies) provides a useful tool by which gene expression can be exogenously manipulated at the transcriptional or translational levels (Hélène and Toulmé, 1990). Succesful antisense applications have been reported for a wide variety of viral, cellular, and animal models (Stein and Cheng, 1993; Hélène and Saison-Behmoaras, 1994).

The decoy or "sense" approach represents an alternative use of synthetic oligonucleotides as transcriptional modulators (Blumenfeld and Vasseur, 1994). In this strategy, oligonucleotides carrying the recognition sites for specific DNA or RNA binding transcription factors are used as competitive inhibitors to titrate the targeted proteins and, thus, to decrease or activate the transcription of genes controlled by those factors.

Although transcriptional inhibition by oligonucleotide decoys has been frequently verified in vitro, the efficiency of this competition approach in cells has not been much explored, and no viral models have been studied. The few examples include the block of normal cell cycle functions by microinjection of CRE or AP-1 decoys in 3T3 or human Hs68 fibroblasts, respectively (Berkowitz et al., 1989; Riabowol et al., 1992), as well as the inhibition of the transcriptional activity of NFκB or Oct-1 by the corresponding phosphorothioate (PS) decoys, when added at micromolar concentrations to a transiently transfected B-cell line (Bielinska et al., 1990). More recently, PS decoys were used at 20 μM concentration to study the role

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of NFκB or PU.1 in the expression of cell adhesion molecules in HL-60 and HUVEC cells (Eck et al., 1993) or human bone marrow $CD34^+$ cells (Voso et al., 1994), respectively.

We are interested in investigating the potential use of oligonucleotide decoys as drugs for controlling the expression of cellular or viral genes. To ensure that oligonucleotides will be efficient inhibitors of transcription factors, the oligonucleotide-protein interaction must have high affinity and specificity, and oligonucleotides must be stable in the biological environment. Although chemical modifications such as phosphorothioates may overcome the degradation problems, they are often counteracted by the loss of critical properties of decoys, such as specificity (Chu and Orgel, 1992) or affinity (Eck et al., 1993). On the other hand, phosphodiester (PO) oligonucleotides are natural candidates for biological applications both in terms of affinity and specificity properties, but their in vivo use may be severely hampered by their sensitivity to nuclease degradation. However, decoy stability and efficiency can be further increased by using structural instead of chemical modifications. For example, we have previously shown that covalently closed dumbbell oligonucleotides completely and specifically inhibited transcription in an ex vivo transient expression system at nanomolar concentrations (Clusel et al., 1993). On the contrary, double-stranded PO oligonucleotides, while being very effective in inhibiting transcription in an in vitro assay, were inactive when used under the same conditions in the ex vivo model (Clusel et al., 1993). Because both types of oligonucleotides were introduced into cells by transfection, their different inhibitory activities seem not to be related to a differential uptake, but to a higher in vivo stability of the dumbbell oligonucleotides. These results strongly suggested that PO oligonucleotides may still be considered as valuable therapeutic tools, and prompted us to explore the use of PO decoys to inhibit viral proliferation in an acute infection model.

The herpes simplex virus type 1 (HSV-1) immediate early protein ICP4 (the product of IE3 gene; also called Vmw175 or IE175) is the major transcriptional regulator of the virus. ICP4 is a sequence-specific DNA binding protein (Faber and Wilcox, 1986; Everett et al., 1991; DiDonato et al., 1991) required to activate transcription of early (E) and late (L) genes and to repress immediate early (IE) gene expression (Preston, 1979; Dixon and Schaffer, 1980; DeLuca and Schaffer, 1985).

Because ICP4 inactivation results in failure to transcribe E or L genes, and thus inhibits HSV-1 replication, targeting ICP4 by a decoy approach provides a sensitive test for studying the ability of oligonucleotide decoys to titrate a specific transcription factor in vivo.

We present results concerning the inhibition of HSV-1 proliferation by hairpin PO oligonucleotides carrying the binding sites for the viral transcription factor ICP4.

MATERIALS AND METHODS

Cells and Virus

Vero cells (green monkey kidney cells) were grown in Earle's MEM supplemented with 5% (v/v) fetal calf serum (FCS) (both from GIBCO BRL). Stocks of HSV-1 strain F (kindly provided by Professor J. M. Huraux, Paris) were prepared from low-multiplicity passages in Vero cells. Viral titrations were performed by virus plaque assay on Vero cell monolayers (see below).

Preparation of Nuclear Extracts From Vero Cells

Vero cells (2×10^7 cells) were either mock infected or infected with HSV-1 at a multiplicity of 10 pfu/cell. After 1 h of adsorption at 37°C, the cells were washed twice with phosphate-buffered saline (PBS), and medium containing 2% FCS was then added. Infections were allowed to proceed for 5 or 16 h at 37°C, as indicated, before cells were harvested for extract preparation. Nuclear extracts from uninfected or infected cells were prepared essentially as described in Cereghini et al. (1987), except that all solutions contained 5 μg/ml aprotinin and leupeptin. Protein concentration was determined by the BCA protein assay (Pierce).

Oligodeoxynucleotides

All syntheses were performed by Genset (Paris, France), using an automated DNA synthesizer (Applied Biosystems 394/8). Full-length oligonucleotides were purified by reverse-phase HPLC, ethanol precipitated three times, and resuspended in sterile water to a 100-fold concentrated stock solution.

Sequences of the oligodeoxynucleotides used in this study are depicted in Table 1. Double-stranded dsIE3 contains the cap site sequence (−8/+16) from HSV-1 IE3 gene that codes for ICP4 (Faber and Wilcox, 1988). This sequence encompasses an ICP4 binding site that has been shown to mediate the autoregulation of IE3 transcription by ICP4 itself (Roberts et al., 1988).

TABLE 1
STRUCTURE AND SEQUENCE OF THE OLIGONUCLEOTIDES USED IN THIS STUDY

DOUBLE STRANDED OLIGONUCLEOTIDES		HAIRPIN OLIGONUCLEOTIDES	
dsIE3	5' CCCCGATCGTCCACACGGAGCGCG 3' GGGGCTAGCAGGTGTGCCTCGCGC	5'CCCCGATCGTCCACACGGAGCGCG T T T 3'GGGGCTAGCAGGTGTGCCTCGCGC T T	hpIE3
dsIEX	5' CTGCC ATCGTCTCTCCGGAGAGCG 3' GACGGTAGCAGAGAGGCCTCTCGC	5' CTGCC ATCGTCTCTCCGGAGAGCG T T T 3' GACGGTAGCAGAGAGGCCTCTCGC T T	hpIEX
dsControl	5' CGTaCTgTTGGTAAAATGGAAGAC 3' GCAtGAcAACCATTTT ACCTT CTG	5' CGTaCTgTTGGTAAAATGGAAGAC T T T 3' GCAtGAcAACCATTTT ACCTT CTG T T	hpControl

The ICP4 consensus sequence (DiDonato et al., 1991) is underlined in each oligonucleotide. dsIE3 contains the ICP4 binding site present at the cap site of the IE3 gene (−8/+16; Faber and Wilcox, 1988). dsIEX contains the ICP4 binding site of the IEX promoter (DiDonato et al., 1991; Bohenzky et al., 1993). dsControl corresponds to a mutant site as defined by DiDonato et al. (mutated positions are indicated by lower case letters), which is no longer recognized by ICP4 (see Figs. 1 and 2). hpIEX, hpIE3, and hpControl are hairpin-structured oligonucleotides, in which strands of dsIEX, dsIE3, and dsControl, respectively, are connected by a nucleotidic loop.

dsIEX contains an ICP4 binding site described by DiDonato et al. (1991) that is located 740 bp upstream from the HSV-1 IE1 gene. This site was recently shown to be part of a functional promoter of the IE/E class (Bohenzky et al., 1993). Control oligonucleotide, dsControl, contains an ICP4 mutant binding site, changed at four positions with respect to the ICP4 consensus sequence defined by DiDonato et al. (1991). hpIE3, hpIEX, and hpControl are the hairpin oligonucleotides corresponding to dsIE3, dsIEX, and dsControl, respectively (Table 1).

Band Shift Assays

5′ End-labeled oligonucleotides were obtained by phosphorylation with [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase, following standard procedures. Binding reactions were performed in 20 μl of reaction mix containing: 10 mM HEPES, pH 7.9, 10% (v/v) glycerol, 100 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.25 mM PMSF, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 1 ng (25,000 cpm) of ^{32}P end-labeled probe, 1 μg sonicated salmon sperm DNA, 1 μg poly(dI-dC)·poly(dI-dC), and 2–10 μg of nuclear proteins. Where indicated, competitor oligonucleotides were added at the same time as the radioactive probe. Reaction mixtures were incubated 10 min on ice, and then loaded on a 6% polyacrylamide nondenaturing gel containing 0.25 × TBE. Samples were run at 12 V/cm until a suitable separation was achieved. The gel was fixed, dried, and exposed to X-ray film.

Oligonucleotide Degradation in Serum

Single-stranded IEX (ss; corresponding to the upper strand of dsIEX in Table 1) and hairpin IEX (hpIEX, Table 1) were 5′ end-labeled as described above. Labeled dsIEX (Table 1) with the same specific activity of ssIEX and hpIEX was prepared by annealing radioactive ssIEX with an excess of unlabeled ds oligonucleotide.

Radioactive oligonucleotides (4 μg, 200,000 cpm) were incubated for 24 h at 37°C in 40 μl of MEM medium containing 10% fetal calf serum. At different times aliquots were removed, mixed with an equal volume of formamide loading buffer, and analyzed by electrophoresis on a 20% denaturing polyacrylamide gel. After migration, gels were exposed to X-ray film, and the bands corresponding to full-length single-stranded, double-stranded, or hairpin oligonucleotides were located, excised from the gel, and quantified by Cerenkov counting. Values were normalized by the total radioactive counting of each sample, measured before loading the gel.

Oligonucleotide Treatment of Cells

Oligonucleotides were diluted to the appropriate final concentration by adding Opti-MEM (GIBCO-BRL) containing no added FCS. Where indicated, lipofectin (GIBCO-BRL) was added to the oligonucleotide solution to a final concentration of 10 μg/ml. The solution was gently mixed and the lipofectin–oligonucleotide complex formation was allowed to proceed for 20 min at room temperature.

Vero cells were plated in 24-well tissue culture plates (1×10^5 cells/well) and incubated 24 h at 37°C as described above. Unattached cells were removed by rinsing with PBS, and the monolayer was exposed to free oligonucleotides or oligonucleotide–lipofectin complexes for 4 h under standard culture conditions. The cells were then rinsed and fed with fresh Opti-MEM for an additional incubation at 37°C during 1 h before infection. Infection was carried out with HSV-1 strain F at 3 pfu/cell in Opti-MEM. Cells were incubated 1 h at 37°C to allow virus adsorption. Excess virus was then removed and cells were given fresh Opti-MEM containing 2% FCS and the appropriate concentration of oligonucleotides. After 24 h incubation, cell lysates were prepared by three freeze–thawing cycles, and the amout of virus present in each lysate was measured by virus plaque assay on Vero cell monolayers.

Virus Plaque Assay

HSV-1 titration on Vero cell monolayers was performed in duplicate wells, as described by Poddevin et al. (1994). The number of plaques in each well was counted and decoy antiviral activity was evaluated as follows: % inhibition = [1 − (plaques in treated cells/plaques in control cells)] × 100. Control wells were treated in the absence of oligonucleotides.

RESULTS

ICP4 Binding Activities in Vero Cells

ICP4 is a transcriptional regulatory protein essential for productive HSV-1 infection that is involved in both the activation of early and late viral genes, as well as in the repression of its own expression and probably in that of other viral immediate-early genes (Preston, 1979; Dixon and Schaffer, 1980; Watson and Clements, 1980; DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a,b). ICP4 is a sequence-specific DNA binding protein that associates with a number of different sites, some of which include the consensus core ATCGTC (Faber and Wilcox, 1988; DiDonato et al., 1991; Everett et al., 1991).

Most of the studies characterizing the sequence requirements for ICP4 binding have been performed with long oligonucleotides or promoter fragments (>45 bp) and, in addition, using partially purified ICP4 (Faber and Wilcox, 1988; DiDonato et al., 1991; Everett et al., 1991). In the case of the ex vivo use of decoy competitors, oligonucleotide length is a major concern, short decoys being preferred in terms of target specificity, cellular uptake, and ease of synthesis. On the other hand, protein–protein interactions between ICP4 and cell or infected cell factors seem to modulate the differential recognition of ICP4 targets in HSV-1 promoters (Gelman and Silverstein, 1987; Papavassiliou et al., 1991). Thus, the definition of the decoy sequences to be used in the ex vivo experiments must be based on in vitro studies performed with infected cell nuclear extracts and not with purified proteins. Therefore, we were first interested in checking that the 24-bp decoys that we have chosen to target ICP4 (dsIE3 and dsIEX, Table 1) were specifically bound in HSV-1-infected Vero cell extracts.

ICP4 binding properties were studied by mobility shift assays, using dsIE3, a probe derived from the well-characterized ICP4 cap site region (Faber and Wilcox, 1988), and nuclear extracts from Vero cells prepared 5 or 16 h after infection with HSV-1. As shown in Fig. 1, incubation of labeled dsIE3 with infected nuclear extracts led to the formation of one HSV-1-specific complex, absent in either mock-infected cells (compare lanes 4–7 to lanes 2–3) or when a control ds oligonucleotide was used (lanes 9–14). Similar amounts of ICP4-specific complex were formed in extracts corresponding to 5 h (Vi.5) or 16 h (Vi.16) postinfection (Fig. 1, compare lanes 4 and 5 to lanes 6 and 7). These results are consistent with previous reported data concerning the time course of ICP4 expression after HSV-1 infection (Honess and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985).

Two faster migrating bands were additionally detected. Both of them were nonspecific cellular (nsVm) or viral (nsVi) binding activities, because they were also formed with a control oligonucleotide (dsControl, Table 1; Fig. 1, compare lanes 2–7 to lanes 9–14).

A similar band shift profile was observed when infected Vero cell extracts were incubated with another ICP4 probe, dsIEX (Fig. 2, lanes 2, 3, 10). This probe was derived from an ICP4 binding site characterized by DiDonato et al. (1991), which

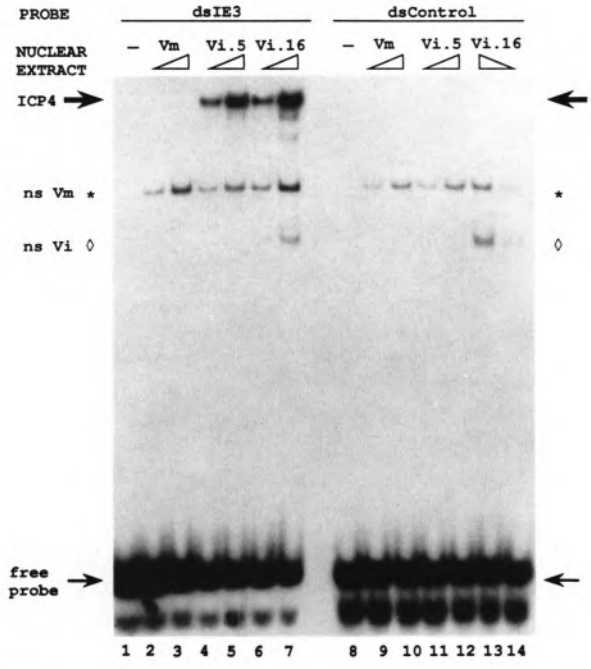

FIG. 1. Short ICP4 binding sites are specifically recognized in Vero cells infected with HSV1. Autoradiograph of a band shift assay using the 24-bp dsIE3 (lanes 1–7) or dsControl (lanes 8–14) probes and the following amounts of nuclear proteins: Vero mock-infected extract (Vm), 3 μg (lanes 2, 9) or 6 μg (lanes 3, 10); Vero infected cell extract prepared 5 h after infection (Vi.5), 2 μg (lanes 4, 11) or 4 μg (lanes 5, 12); Vero infected cell extract prepared at 16 h after infection (Vi.16), 2 μg (lanes 6, 13) or 4 μg (lanes 7, 14). Lane 1 contained no added protein. Oligonucleotide sequences are described in Table 1. Band shift assays were carried out as described in Materials and Methods. The ICP4-specific band is marked with an arrow; nsVm and nsVi indicate cellular or viral nonspecific binding activities, respectively.

was shown to present a higher binding affinity for partially purified ICP4 than the IE3 cap site. We further investigated the relative binding affinities of ICP4 towards the 24-bp dsIE3 and dsIEX sites by performing binding competitions with the corresponding unlabeled oligonucleotides.

As shown in Fig. 2A, binding of ICP4 to radioactive dsIEx was substantially reduced by a 10-fold excess of unlabeled dsIEX (lane 4), whereas it was almost unchanged upon addition of the same excess of unlabeled dsIE3 (lane 11). This difference was even more clear when a 100-fold excess of competitor was added to the ICP4 binding reaction: whhereas cold dsIEX almost completely competed the ICP4-bound probe (lane 5), a significant amount of the ICP4 complex was still present when the same molar excess of dsIE3 competitor was added (lane 12). The fact that comparable amounts of ICP4-specific complex were obtained for 100-fold dsIEX and 1000-fold dsIE3 excess (Fig. 2A, lower panel, compare lane 5 to lane 13), indicated that the affinity of ICP4 for the IEX site is about 10-fold higher than that for the IE3 binding sequence. These results correlate well to the data reported by DiDonato et al. (1991), who found a four- to sixfold difference in affinity.

The specificity of the ICP4 binding activity was further confirmed by the fact that addition of a 1000-fold molar excess of the unlabeled control oligonucleotide did not compete for the ICP4-bound dsIEX probe (Fig. 2B, compare lanes 3–5 to lane 2). On the contrary, as expected from the observed binding to radioactive dsControl (Fig. 1, lanes 9–14), the nonspecific Vero complex nsVm readily dissapeared upon addition of unlabeled dsControl (Fig. 2B, compare lanes 4 and 5 to lane 2).

Specific Binding of ICP4 on Hairpin Oligonucleotides

We have previously shown that oligonucleotide stability seems to play an essential role in the ex vivo efficiency of decoy molecules, and that one way of getting that stabilization in the case of phosphodiester oligonucleotides is by using covalently closed, dumbbell-structured molecules (Clusel et al., 1993). Hairpin oligonucleotides represent an interesting alternative for studying phosphodiester decoy activities in cellular assays for several reasons. First, the presence of the loop in the oligonucleotide prevents strand separation during the incubation in the culture medium, as might happen with double-stranded oligonucleotides. Second, under culture conditions, the half-life of hairpin-derived oligonucleotides with protein binding activity (intact hairpins plus double-stranded molecules originated by endonucleolytic cleavage in the hairpin loops) is higher than that of double-stranded oligonucleotides (Fig. 3). And finally, large quantities of hairpin PO oligonucleotides can be easily synthesized and purified.

We were then interested in studying the binding properties of the hairpin-structured molecules derived from dsIEX and dsIE3 oligonucleotides (hpIEX, hpIE3, Table 1). To do so, we compared the competition properties of ds and hp oligonucleotides on ICP4 binding. As shown in Fig. 2A, the competition profiles of dsIEX and hpIEX, as well as those of dsIE3 and hpIE3, were very similar (compare lanes 4–6 to lanes 7–9, and lanes 11–13 to lanes 14–16, respectively). Only a very slight difference between dsIEX and hpIEX was observed at a 100-fold excess addition (Fig. 2A, lower panel, compare lane 5 to lane 8). Finally, the gel shift patterns obtained with radioactive hpIEX, hpIE3, or hpControl did not differ from

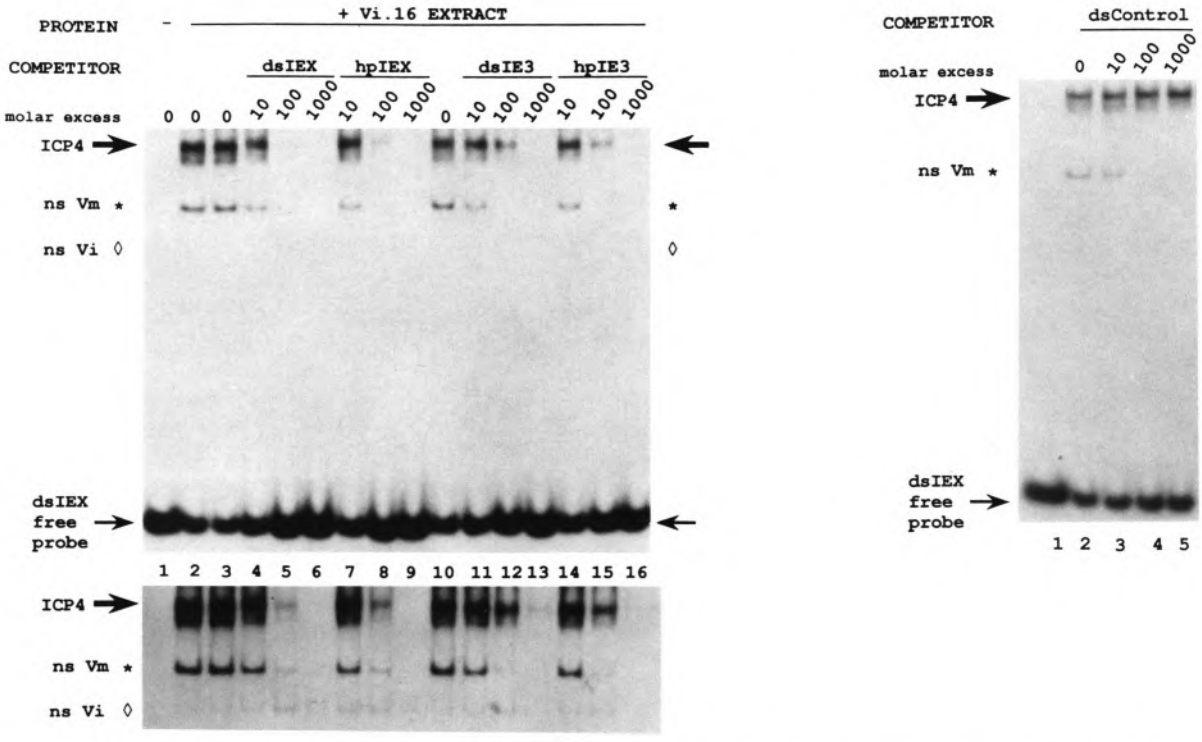

FIG. 2. Hairpin oligonucleotides display similar binding affinities for ICP4 as their double-stranded counterparts. (A) Competition assay with dsIEX, dsIE3, hpIEX, and hpIE3. Binding reactions were carried out as described in Materials and Methods, using labeled dsIEX and 10 μg of Vi.16 nuclear proteins, in the absence (lanes 2, 3, and 10) or the presence of the indicated amounts of unlabeled dsIEX (lanes 4–6), hpIEX (lanes 7–9), dsIE3 (lanes 11–13), or hpIE3 (lanes 14–16). The ICP4-specific band is marked with an arrow; nsVm and nsVi indicate cellular or viral nonspecific binding activities, respectively. The lower panel shows a longer exposure of the same autoradiograph. (B) Competition experiment with dsControl. Binding reactions were performed as in (A), in the absence (lane 2) or in the presence of the indicated excess of unlabeled dsControl (lanes 3–5). Symbols are the same as those used in (A).

those observed with the corresponding ds oligonucleotides (data not shown).

These data indicated that the presence of a loop in the oligonucleotide molecule does not interfere with the proper recognition of binding sites by ICP4. On the other hand, the loops in the hairpin structures seem not to bind any additional protein in Vero or infected Vero cell extracts. These results prompted us to test the ex vivo effects of hpIEX and hpIE3 on HSV-1 proliferation upon acute infection of Vero cells.

Inhibition of HSV-1 Replication by Hairpin Decoys

It was previously shown that cationic lipids such as DOTMA (Felgner et al., 1987) greatly enhance the cellular uptake and activity of antisense oligonucleotides in cell culture (Chiang et al., 1991; Bennett et al., 1992; Capaccioli et al., 1993). Therefore, we set up conditions to deliver the ICP4 decoy oligonucleotides in Vero cells using the commercial cationic lipid lipofectin as a carrier. Experimental conditions were established so that cellular and viral toxicity due to lipofectin were lower than 10% with respect to the untreated controls.

Figure 4 represents the results obtained when Vero cells were exposed to hpIEX, hpIE3, and hpControl at concentrations varying from 0.01 to 10 μM in the presence of 10 μg/ml lipofectin, during 4 h before infection as described in Materials and Methods. Incubation of Vero cells with either hpIEX/lipofectin or hpIE3/lipofectin complex produced a dose-dependent decrease in HSV-1 titer, reaching a maximum at 1 μM concentration in both cases. HSV-1 inhibition by ICP4 decoys was sequence specific, because under the same conditions the control oligonucleotide hpControl was completely inactive (Fig. 4). In addition, hpIEX was more active than hpIE3 at all concentrations tested, probably reflecting the higher binding affinity of the IEX decoy for ICP4 (Fig. 2A).

Concerning the extent of hpIEX competition, 40% inhibition was obtained at 0.1 μM and the

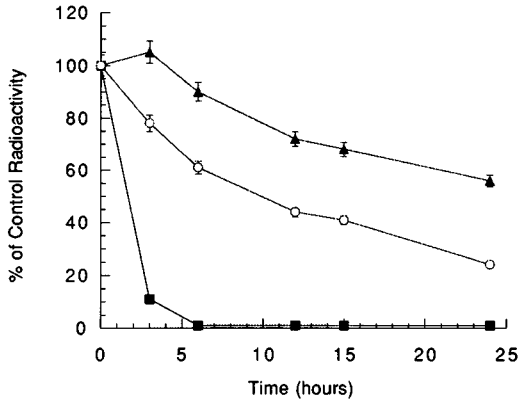

FIG. 3. Hairpin oligonucleotides are more stable than double-stranded decoys. Incubation of ^{32}P-end-labeled single stranded (ssIEX, crosses), double-stranded (dsIEX, circles) or hairpin (hpIEX, triangles) oligonucleotides for the indicated times at 37°C in MEM containing 10% FCS was performed as described in Materials and Methods. The data were analyzed by taking as 100% the full-length band of a nonincubated sample. For ssIEX and dsIEX, the values plotted represent the remaining full-length oligonucleotides. In the case of hpIEX, each point corresponds to the sum of the remaining hairpin oligonucleotide plus the 24-bp double stranded structure generated by endonucleolytic cleavage in the hairpin loops. This sum represents the actual available structures that retain the ability of binding to the target protein at the indicated incubation time.

calculated IC_{50} for hpIEX was 0.3 μM (Fig. 4). Maximum inhibition at 1 μM hpIEX was about 60%. In the case of hpIE3, viral proliferation was reduced by 40% at 1 μM (Fig. 4). Increasing decoy concentration to 10 μM did not increase HSV-1 inhibition; in fact, the concentration-dependence curves were biphasic, a lower titer reduction being systematically observed at higher concentrations of both hpIEX and hpIE3 oligonucleotides (Fig. 4). Similar results were previously reported by Chiang et al. (1991) for ICAM-1 antisense inhibition in HUVEC cells. Because cationic lipids interact with cellular membranes through electrostatic interactions, the biphasic profile probably may be due to the saturation of the cationic lipids at high oligonucleotide concentration.

The fact that hpIEX specifically inhibited HSV-1 replication with an IC_{50} of 0.3 μM strongly suggests that inhibition of viral replication in Vero cells by titration of ICP4 with hairpin decoys might be a very efficient process, provided proper intracellular delivery of the oligonucleotide decoy is obtained.

DISCUSSION

Much work on oligonucleotide-based approaches has been devoted to producing nuclease-resistant oligonucleotide analogues, because degradation of unmodified PO oligodeoxynucleotides in cells or culture media might hamper their use in ex vivo or in vivo experiments (Stein and Cheng, 1993; Milligan et al., 1993). One advantage of the decoy approach, compared to antisense or antigene strategies, is that double-stranded PO oligonucleotides are considerably more stable than single-stranded molecules (Fig. 3; Chu and Orgel, 1992). However, double-stranded PO oligonucleotides are probably not stable enough to exert a specific inhibition in an ex vivo system (Clusel et al., 1993). On the other hand, stabilization of the double-stranded molecules, by connecting both ends with nucleotidic loops (dumbbell structures), allows detecting the specific inhibition of the expression of a transfected promoter at nanomolar decoy concentrations (Clusel et al., 1993).

We have demonstrated that biological activity of decoy oligonucleotides also can be detected with hairpin-structured PO oligonucleotides. We have first shown that two different ICP4 binding sequences, dsIE3 and dsIEX, present in two genes regulated by ICP4 (Roberts et al., 1988; Bohenzky et al., 1993), were specifically recognized by an HSV-1 cell-infected protein with a 5-10-fold difference in relative affinities (Fig. 2A). Moreover, the hairpin-structured PO oligonucleotides, hpIEX and hpIE3, displayed the same relative binding affinities as the corresponding double-

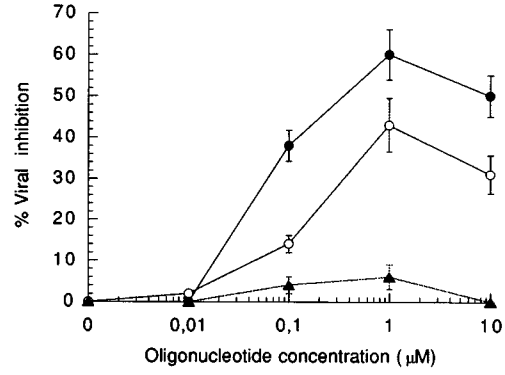

FIG. 4. Hairpin decoys can inhibit HSV1 proliferation in Vero cells. Vero cells were treated in duplicate wells with the indicated concentrations of hairpin oligonucleotides in the presence of 10 μg/ml lipofectin during 4 h before infection, and then in the absence of lipofectin during the postinfection period, and then in the absence of lipofectin during the postinfection period, as described in Materials and Methods 100% activity (0% inhibition) refers to the titer of HSV-1 obtained in the presence of 10 μg/ml lipofectin alone. Under the experimental conditions, this control value was at least 90% of the viral titer obtained in the absence of lipofectin. Data shown represent the mean of three independent experiments. hpIEX (●), hpIE3 (○), hpControl (▲).

stranded molecules (Fig. 2A). Similar results were obtained with both hairpin and dumbbell oligonucleotides containing the binding sites for proteins, such as HNF-1, NF-Y, Sp1, NFκB, or HPV E2 (Clusel et al., 1993; C. Clusel, M. Blumenfeld, E. Ugarte, A. Israel, and F. Thierry, unpublished results). These data indicate that the presence of interstrand connecting loops to stabilize the decoy molecule does not modify the protein/oligonucleotide interactions. This is not the case when double-stranded oligonucleotides are stabilized by replacement of the PO linkages by chemical analogues. For example, PS double-stranded NFκB oligonucleotides have lower binding affinities than the corresponding PO molecules (Eck et al., 1993). In addition, sequence-independent effects of PS oligonucleotides have been frequently reported (Stein and Cheng, 1993; Yaswen et al., 1993), one of such nonspecific effects being the rapid induction of Sp1 transcription factor (Perez et al., 1994). The additional advantage of duplex stabilization by structural and not by chemical modification is that strand separation of PO hairpins or dumbbells is prevented by the presence of the intramolecular loops, thus reducing the possibility of nonspecific, single-stranded effects.

Concerning the biological activities of the ICP4-binding decoys, we showed that delivery of submicromolar concentrations of hpIEX using the cationic lipid carrier lipofectin specifically reduced HSV-1 titer by more than 50% (Fig. 4). Most interestingly, antiviral activity of hpIE3 and hpIEX correlated with their ICP4 binding affinities as determined by gel shift competition experiments (Fig. 2A). However, incubation of Vero cells with oligonucleotides in the absence of lipofectin produced no significant specific inhibition of HSV-1 (data not shown).

The expression of the ICP4 gene is an early event in HSV-1 life cycle: synthesis of immediate early polypeptides reaches peak rates at 2–4 h postinfection, and early genes controlled by ICP4 are already detected at 4 h postinfection (Honess and Roizman, 1974). Thus, to compete with ICP4 in an efficient way, decoy oligonucleotides must be present in the relevant intracellular compartment (the nucleus) early enough after infection to titrate ICP4 before it activates gene transcription. It is therefore obvious that cellular uptake and intracellular compartmentalization of decoy oligonucleotides are as critical as decoy stability in determining the efficacy of the sense approach. It is thus possible that oligonucleotide uptake and compartmentalization in Vero cells constitute the limiting step for the efficient inhibition of ICP4 activity. The fact that association of oligonucleotides to lipofectin allowed detecting decoy inhibition seems to support this hypothesis. Nevertheless, oligonucleotide stability seems to be partly involved in hairpin biological activity, because dumbbell decoys, which are considerably more stable than hairpins to both endo- and exonucleolytic activities, are more active inhibitors of HSV-1 proliferation (C. Clusel, S. Meguenni, and M. Blumenfeld, manuscript in preparation).

Concerning ICP4 activity, the correlation between ICP4 DNA binding and transcriptional regulation has recently been questioned, some data supporting a transactivation function mediated by protein–protein interactions and not by direct DNA binding to promoter sites (Shepard and DeLuca, 1991). If this is case, the decoy oligonucleotides can still be very efficient, acting not as competitive inhibitors but as noncompetitive ones (e.g., inducing a conformational change that interferes with the protein–protein interaction).

Finally, the use of stable PO decoy oligonucleotides, active at low submicromolar concentrations, together with the development of appropriate delivery carriers devoid of toxicity should provide a powerful tool for specifically controlling gene expression both for research or therapeutic purposes.

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