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Biological Activity of Hexitol Nucleic Acids Targeted at *Ha-ras* and Intracellular Adhesion Molecule-1 mRNA

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ABSTRACT. Hexitol nucleic acid (HNA) is a new steric blocking oligonucleotide, hybridizing sequence selectively with RNA. The biological activity of HNA was evaluated in an *in vitro* translation arrest system targeting *Ha-ras* mRNA and in a cellular system targeting intracellular adhesion molecule-1 (ICAM-1) expression. HNA very efficiently and selectively inhibited *Ha-ras* mRNA translation (IC_{50} of 50 nM) when targeted at the translation initiation region. When targeting at the 12th codon region, a gap-mer approach was needed to inhibit mRNA translation. Similarly, HNA inhibited ICAM-1 expression in keratinocytes when targeting at codon sequences. In this test system, HNA is less active but more selective than phosphorothioates, but needs lipofection to become active in keratinocytes. This new steric blocker may be an efficient antisense agent providing that enough material can be brought into cells. BIOCHEM PHARMACOL **59**;6:655–663, 2000.

KEY WORDS. hexitol nucleic acids; antisense; *Ha-ras*; ICAM-1; phosphorothioates; translation arrest; lipofection

ASO¶ are presumed to act by down-regulating gene expression by selective hybridization with mRNA sequences within cells. The exact mechanism of action of ASO, however, is dependent on the synthetic modification and the target site, and remains a field of active research. Generally, two mechanisms are proposed to explain the biological activity of ASO, i.e. degradation of the targeted mRNA by RNase H or steric blocking of the translation machinery. In the latter case, the protein level is reduced but not the mRNA level. When targeting against coding regions, an RNase H-dependent mechanism is preferred, because the ASO–mRNA complex is generally not strong enough to avoid dissociation by the translation machinery. However, this is not an absolute rule, as peptide nucleic acids (PNA), targeted to codon 12, were recently demonstrated to inhibit translation of *Ha-ras* mRNA [1]. As the 5′-untranslated regions of mRNA are involved in several metabolic processes of mRNA, this region is preferred as a target site for both RNase H-dependent and-independent mechanisms. Recently, it has been demonstrated that a 2′-*O*-(2-methoxy)ethyl ASO, targeting the 5′ cap region of the human ICAM-1, inhibits protein expression with an IC_{50} of 2 nM [2]. This oligonucleotide interferes with the formation of the 80S translation initiation complex and functions by steric blocking the target.

HNA [3–5] are oligonucleotide analogues composed of a phosphorylated anhydrohexitol backbone and the four natural nucleobases (Scheme 1). Together with phosphoramidate oligonucleotides [6], 2′-*O*-(2-methoxy)ethyl oligonucleotides [7] and 2′,3′-bridged bicyclic oligonucleotides [8], they belong to a selected group of carbohydrate-modified oligonucleotides that bind tightly in a sequence-selective manner with RNA. None of them, however, activates RNase H. Due to these properties, these oligomers might be suited to function as steric blockers of mRNA. Their biological activity might be explained by hindrance of RNA processing, hindrance of nucleoplasmic transport, or hindrance of translation.

We studied the effects of HNA in both the ICAM-1 and *Ha-ras* models. The compounds were first evaluated in a cell-free translation system (*Ha-ras* model in a rabbit

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¶ *Abbreviations:* ASO, antisense oligonucleotides; HNA, hexitol nucleic acids; ICAM-1, intracellular adhesion molecule-1; IFN-γ, interferon-γ; HLA-DR, human leukocyte antigen class II molecule; RT, room temperature; SFM, serum-free medium; and TNF-α, tumor necrosis factor-α.

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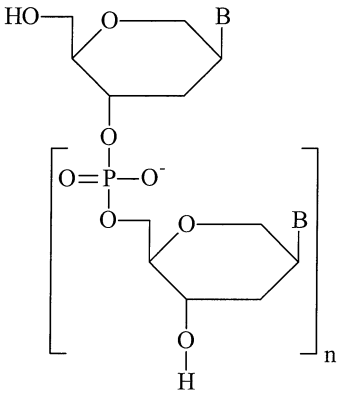

SCHEME 1. Structure of hexitol nucleic acids (HNA).

reticulocyte lysate) in order to determine if they are able to block mRNA translation. In a second step, a cellular system was used to control ICAM-1 expression. Sequences that are targeted either at coding regions or at untranslated regions were studied in both models. For the ICAM-1 model a comparison was made with phosphorothioate oligonucleotides. We and others have reported that selective inhibition of the activated (codon 12 G→T transition) *Ha-ras* mRNA expression can be achieved with phosphodiester or modified oligonucleotides, which induce RNase H cleavage of the target RNA [9–14]. This RNase H mechanism seems to be important for the antisense effect of oligonucleotides directed against codon 12. Phosphorothioate oligonucleotides have also been successfully targeted to the AUG initiation of translation region [15, 12]. These oligomers are believed to act through a translation arrest mechanism. In the present study, anhydrohexitol-modified oligonucleotides bearing a 3′-propanediol modification were targeted both to the region of *Ha-ras* mRNA, including the G→U point mutation at the 12th codon, and to the AUG initiation of translation region.

It has been shown that hybrids formed between HNA and target mRNA are poorly cleaved by RNase H [5]. We report here that although anhydrohexitol-modified oligonucleotides formed very stable hybrids with the target sequence, only the 16-mer targeted to start codon region prevented translation of mutated *Ha-ras* mRNA in rabbit reticulocyte extract. ICAM-1 has been used as an example to investigate how antisense molecules can influence the expression of adhesion molecules [16–19]. Such oligonucleotides are currently being evaluated in the treatment of several important clinical inflammatory diseases such as Crohn's disease, rheumatoid arthritis, psoriasis, acute renal transplant rejection, and ulcerative colitis [17]. Most studies describing the effect of ASO on ICAM-1 expression have been carried out using phosphorothioates, as the thiophosphate moieties increase the nuclease resistance of the ASO. An initial study, carried out by Bennett *et al.* [20], describes a phosphorothioate 20-mer sequence (ISIS 2302) which selectively inhibits ICAM-1 expression. The mechanism of action of ISIS 2302 is mediated by RNase H degradation of the target mRNA. A second phosphorothioate 20-mer (ISIS 3067), endowed with selective biological activity, acts by an RNase H-independent mode of action. Because of their different modes of action, the ISIS 2302 and ISIS 3067 sequences were selected for our comparative studies.

MATERIALS AND METHODS

Reagents

HNA were synthesized, using the phosphoramidite approach, de-protected, and purified as described previously [4, 5]. Two HNA sequences were synthesized targeting the AUG start codon region of *Ha-ras* mRNA (Table I): an 11-mer 4′-CCTCGCTACTG-6′ and a 16-mer 4′-GGACTCCTCGCTACTG-6′. The 12th codon region was targeted with a 12-mer HNA sequence 4′-CGCG-GCAGCCAC-6′ and a 17-mer RNA sequence 4′-CCCGCGGCAGCCACACC-6′. In addition, an HNA–DNA hybrid was synthesized (Table I): 4′-CGCdGdGdC-dAdGdCCAC-6′. A phosphodiester HNA oligomer (GS1190) directed against the 3′-untranslated region of ICAM-1 mRNA was synthesized (Table I). The sequence of GS1190 is identical to that of ISIS 2302 (4′-GC-CCAAGCTGGCATCCGTCA-6′). The second HNA oligomer evaluated (GS1185) was directed against the 5′-untranslated region of ICAM-1 mRNA and is sequence-identical with ISIS 3067 (4′-TCTGAGTAGCAGAG-GAGCTC-6′). In order to evaluate cellular uptake of the HNA, a 6′-fluorescein-labeled HNA 11-mer (GS1187) was also synthesized (4′-fluorescein TAGCAGAGGAG-6′), representing a section (38–48) of GS1185 and targeting the 5′ cap region.

TABLE I. Oligonucleotides targeted at *Ha-ras* and ICAM-1

Sequence (6′-4′)	Target	Hexitol number	PT number
GTCATCGCTCC	AUG start codon	GS1191	—
GTCATCGCTCCTCAGG	AUG start codon	GS1192	—
CACCGACGGCGC	12th codon region	GS1239	—
CCACACCGACGGCGCCC	12th codon region	GS1193	—
CACdCdGdAdCdGdGCGC	12th codon region	GS1196	—
GCCCAAGCTGGCATCCGTCA	3′-UTL (2114-2133)	GS1190	ISIS2302
TCTGAGTAGCAGAGGAGCTC	5′ Cap (32-51)	GS1185	ISIS3067
FITC-TAGCAGAGGAG-propanediol	5′ Cap (38-48)	GS1187	—

In Vitro *Transcription and Translation Using Ha-ras mRNA as Target*

Capped SP6 transcripts were synthesized using an *Eco*RI linearized plasmid which contains the entire coding region of normal and activated *Ha-ras* as described [9]. The transcript was translated in rabbit reticulocyte lysate purchased from Promega. Lysate (17 μL) was added to the reaction mixture (25 μL) supplemented with amino acid mix (1 μL, without methionine), ^{35}S methionine (1 μL, 15 μCi/μL), oligonucleotide, and transcript. The reaction mixture was incubated for 30 min at 37° and 8 μL of the mixture was analyzed on 14% (w/v) Tris–glycine, SDS–PAGE, NOVEX™ pre-cast gel (Promega).

Cells and Cell Culture Conditions

Human foreskin from patients undergoing circumcision was transported in PBS with calcium, magnesium (GIBCO, Life Technologies), and 10 μg/mL gentamycin. The skin was washed three times with this medium, cut into 0.5-cm^2 squares, and incubated upside down overnight at 4° in 10 mL Dispase II (Boehringer) solution at 16 U/mL. Tissues were placed in 15 mL trypsin–EDTA and the epidermal compartment carefully lifted with a forceps. By gently scraping the dermis and epidermis, the keratinocytes were released from the tissue into the trypsin solution. After removal of dermal parts, the remaining cell suspension and stratum corneum was incubated for 20 min at 37°, followed by filtration over a Cell Strainer™ (Falcon). The filter was washed once with complete keratinocyte–SFM™ medium (GIBCO), and 10% fetal calf serum (Sebak) was added to inactivate trypsin. Cells were centrifuged (10 min at 200 *g*), counted, and plated in keratinocyte–SFM™ medium at a cell density of 3×10^6/75 cm^2. Cultures at 80% confluence were subcultured once and frozen at second passage using standard procedures. For *in situ* ELISA, third passage normal human keratinocytes (NHK) were seeded in flat-bottomed 96-well plates (Nunc, Life Technologies) at a density of 2750 cells (NHK4#4) per well and allowed to grow subconfluent. For fluorescent studies, keratinocytes were seeded in 12-well plates (Falcon, Becton Dickinson) at 15,000/mL and allowed to grow subconfluent (75%).

Lipofection, Oligonucleotide, and Cytokine Treatments

An assay for ICAM-1 ablation in keratinocytes was developed that enabled a sufficiently long pretreatment with oligo in the presence of lipofect(am)in, a recovery period in the presence of oligo but in the absence of lipofect(am)in, and finally cytokine treatment and incubation, both in the presence of oligo. A greater reduction in ICAM-1 protein levels was obtained with lipofectin compared to lipofectamin, with the optimum observed at 5–10 μg/mL lipofectin. It was noted that the use of lipofectin negatively affected the viability of keratinocytes in a mitochondrial activity assay [21], but the described treatment provided the only frame within which a significant activity of hexitol oligonucleotides was observed. Lipofectin–oligonucleotide mixtures were allowed to pre-complex for 20 min at ambient temperature prior to application to subconfluent keratinocyte cultures for 5 hr (lipofectin: 10 μg/mL final). Cells were washed with SFM with oligonucleotides and allowed to recuperate overnight prior to cytokine stimulus (TNF-α = 7.5 ng/mL or IFN-γ = 10–30 U/mL) and further incubated in the presence of oligonucleotide for 48 hr.

Mitochondrial Assays for Cell Viability

Effects of treatment on cell viability were assessed with an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [21]. The MTT assay was performed as described in Vandermeeren *et al.* [22]. Briefly, normal human keratinocytes received the same treatments as described for the cell-ELISA experiments. After the incubation time, 25 μL/well of an MTT solution (5 mg/mL MTT [Sigma], in Ca^{2+}/Mg^{2+}-free PBS) was added to the cultures and incubated for 3 hr at 37°. The medium was aspirated and the plates frozen-thawed twice (−20°). By adding 200 μL of ethanol/acetone (60/40, v/v, −20°) and incubating for 30 min at 4° on the shaker, the monolayers were fixed and the formazan crystals dissolved. Optical densities were measured at 540–650 nm on a Thermomax plate reader (Molecular Devices).

Cell-ELISAs for ICAM-1 or HLA-DR

The cell-ELISA for ICAM-1 expression is a modification of the method of Winiski and Foster [23]. Following incubation, the cells were fixed with 2% paraformaldehyde in PBS for 15 min at RT, and after subsequent washing with five changes of PBS (Life Technologies), the fixed monolayers were incubated with 125 μL/well of a 2% normal goat serum solution in PBS/1% BSA for 1 hr at RT to block non-specific binding sites. The blocking solution was replaced overnight at 4° with 100 μL/well of mouse anti-human ICAM-1 monoclonal antibody R6.5 (Bender) at 50 ng/mL final concentration or with mouse anti-human HLA-DR monoclonal antibody (clone L243, Becton/Dickinson) at 1/100 final dilution in PBS/BSA, and an immunoglobulin G1 non-relevant monoclonal antibody was applied as a negative control. After the plates were washed 5 times with PBS, they were incubated for 1 hr at RT with 100 μL/well of a sheep anti-mouse horseradish peroxidase (Life Sciences) antibody solution in PBS/BSA (final dilution 1/1000). The culture plates were washed with PBS and 100 μL/well of an *O*-phenylenediamine substrate solution (Sigma) was added. The reaction was allowed to proceed for a maximum of 15 min in a shaker plate at RT, after which the color development was stopped with 2 N H_2SO_4, 100 μL/well. The plates were then read on a Thermomax (Molecular Dynamics) microtiter plate reader at 490 nm.

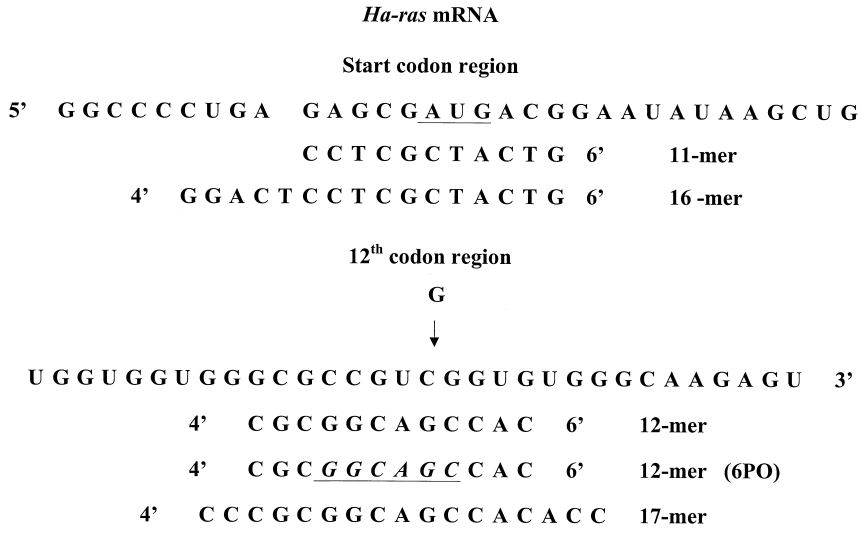

FIG. 1. The sequences of *Ha-ras* mRNA and oligonucleotides targeted to the start codon region (AUG) and around the mutation (G→U) are shown. All the oligonucleotides contain the anhydrohexitol modification. The phosphodiester window in the dodecamer targeted to the 12th codon is underlined.

RESULTS

In Vitro *Arrest of Translation by Anhydrohexitol-Modified Oligonucleotides*

Two series of HNA oligonucleotides were synthesized (Fig. 1). The 11-mer 4′-CCTCGCTACTG-6′ and the 16-mer 4′-GGACTCCTCGCTACTG-6′ are both targeted at the start codon region of *Ha-ras* mRNA. The second series comprises a 12-mer 4′-CGCGGCAGCCAC-6′ and a 17-mer 4′-CCCGCGGCAGCCACACC-6′ fully modified HNA sequence targeted at the 12th codon region. As HNA is not able to induce RNase H, a gap-oligomer was also synthesized. This oligomer contains three hexitol nucleotides at both flanks and a window of six phosphodiester nucleotides: 4′-CGCdGdGdCdAdGdCCAC-6′. We used a rabbit reticulocyte lysate (RRL) for cell-free translation experiments to evaluate the antisense activity of modified oligonucleotides. Inhibition of translation by ASO in RRL commonly results from mRNA cleavage by RNase H. Figure 2 shows that a dose-dependent inhibition of translation was obtained with anhydrohexitol-modified hexadecamer targeted to the translation initiation region. The undecamer targeted to the same region did not inhibit mRNA translation, probably because hybrid stability was not sufficient to compete with 80S translation initiation complex formation. Figure 3 shows that the hexadecamer was very efficient at inhibiting *Ha-ras* mRNA translation. An IC_{50} of 50 nM was determined after quantitation of p21 levels. RNase H activity was not involved in the translation inhibition caused by the modified hexadecamer. This inhibition was specific since the translation of luciferase mRNA, which does not contain the target sequence, was not affected (Fig. 2). In order to further demonstrate the selective antisense effect of the 16-mer directed at the AUG start codon, the inverted HNA sequence as well as a scrambled HNA sequence were evaluated. Neither sequence had any effect on *Ha-ras* mRNA translation (Fig. 4). When targeted to the coding region, modified oligonucleotides should hybridize with their mRNA target stably enough to avoid dissociation by the translation machinery. The anhydrohexitol-modified dodecamer and heptadecamer oligonucleotides targeted at the 12th codon region of *Ha-ras* mRNA were not able to arrest elongation by the ribosomes (Fig. 2). However, when a window of six phosphodiester linkages was introduced into the modified dodecamer, an inhibition of translation was obtained in the presence of RNase H (Fig. 2). Figure 3B shows that micromolar concentrations were needed to inhibit mRNA translation.

Cytokine-Induced ICAM-1 Expression in Human Keratinocyte Cultures

Testing of ASO in normal human keratinocytes was shown to be a relatively simple *in vitro* model. Nestle *et al.* [18] demonstrated that phosphorothioate ASO directed to ICAM-1 were readily taken up by keratinocytes and displayed ICAM-1-ablating activity, even in the absence of lipofectin. It was shown that uptake in the absence of lipofectin was specific for keratinocyte cultures versus fibroblasts and endotheliocytes. In keratinocytes, IFN-γ-induced ICAM-1 expression could be specifically blocked by 50% or 30%, depending on the presence of lipofectin, whereas no effect of IFN-γ on induction of HLA-DR and TNF-α receptor expression was observed. This keratinocyte–ICAM-1 model was used to investigate the activity of HNA.

Pilot experiments confirmed that the phosphorothioate oligos ISIS 2302 and ISIS 3067 effectively ablated ICAM-1 expression in keratinocytes in a dose-dependent way with IC_{50}s of 0.1 mM, a 3-fold higher value than reported by Nestle *et al.* [18]. In contrast, the matched hexitol oligos GS1190 and GS1185 at 1 mM concentrations were not

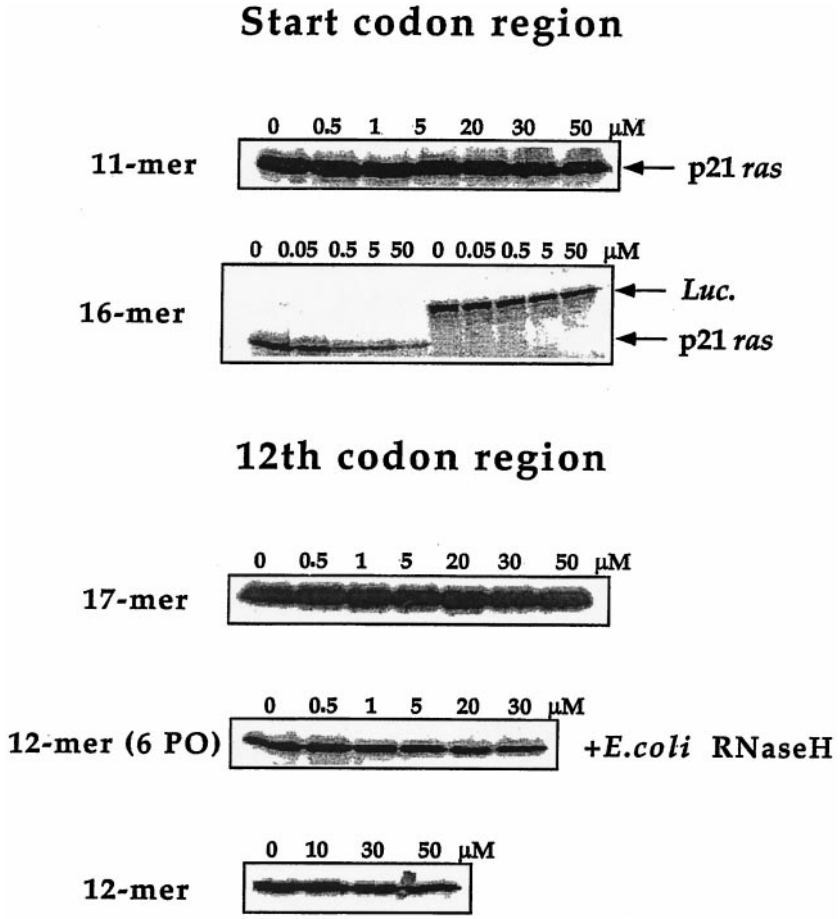

FIG. 2. Effect of oligonucleotides on translation of mutated *Ha-ras* mRNA (G→U mutation at the 12th codon) in rabbit reticulocyte. *In vitro* transcribed *Ha-ras* mRNA was translated for 30 min at 37° in the absence of oligonucleotide (0) or at the concentration indicated above the lanes. Oligonucleotide identity is indicated on the left of the figure (see Fig. 1 for sequences) and the position of the *ras* and luciferase (Luc.) proteins are shown by arrows. One unit of *Escherichia coli* RNase H was added to the translation medium when the dodecamer with a window of six natural phosphodiesters (12-mer (6 PO)) was used (+ *E. coli* RNase H).

capable of ablating ICAM-1 in the absence of lipofectin, suggesting a less favorable cell-directed kinetics than phosphorothioate oligos. This observation for the hexitol variants was confirmed using the fluorescein isothiocyanate-labeled hexitol 11-mer GS 1187, which did not accumulate in keratinocytes over times up to 24 hr in the absence of lipofectin. Given the absence of hexitol uptake under standard conditions, a lipofection protocol was optimized for keratinocytes. In the presence of 10 or 20 μg/mL lipofectin, a fraction of keratinocytes displayed cytoplasmic and nuclear staining. Fluorescence in the nucleoli was more intensive than over the chromatin, while cytoplasmic fluorescence was weaker than the nuclear and most prominent in the vacuome. Keratinocytes pretreated with lipofectin followed by cytokine stimulation displayed a significant ICAM-1 signal on both ELISA and Northern blots, suggesting that despite the moderate inhibitory effect on metabolic activity (in a mitochondrial assay), the cells were capable of responding physiologically to cytokine, with ICAM-1 expression over time periods as long as 2 days. In the presence of lipofectin, both the hexitol and phosphorothioate antisense oligos ablated ICAM-1 expression in *in situ* ELISA (Fig. 5a). The inhibitory effect was 10-fold lower following IFN-γ stimulation than after TNF-α, which correlates with the much higher levels of ICAM-1 mRNA following IFN-γ induction versus TNF-α. The ASO ISIS 3067, GS1185, and ISIS 2302 displayed similar dose titration curves, with IC_{50}s below 500 nM and over 80% inhibition of ICAM-1 protein levels. In contrast, the hexitol oligo GS1190 (to the 3′-UTL region) was found to be less active as indicated by the plateauing curve at 40% inhibition, suggesting that—in contrast to the matched phosphorothioate oligonucleotides—binding to the coding region is required for proper ablation of ICAM-1 expression by hexitol oligonucleotides. Following IFN-γ induction and in agreement with the higher mRNA levels, all oligonucleotides were at least 10-fold less potent, but the 2 phosphorothioate oligos performed better (IC_{50} = 5 μM) than the matched hexitols (Fig. 5a; IC_{50} > 5 μM). To further assess the specificity of the matched antisense anti-ICAM-1 oligonucleotides, we compared their effects on two different proteins, ICAM-1 and HLA-DR, which are inducible in keratinocytes by IFN-γ (*in situ* ELISA, Fig. 5b). In this comparative experiment, the two phosphorothioate oligo-

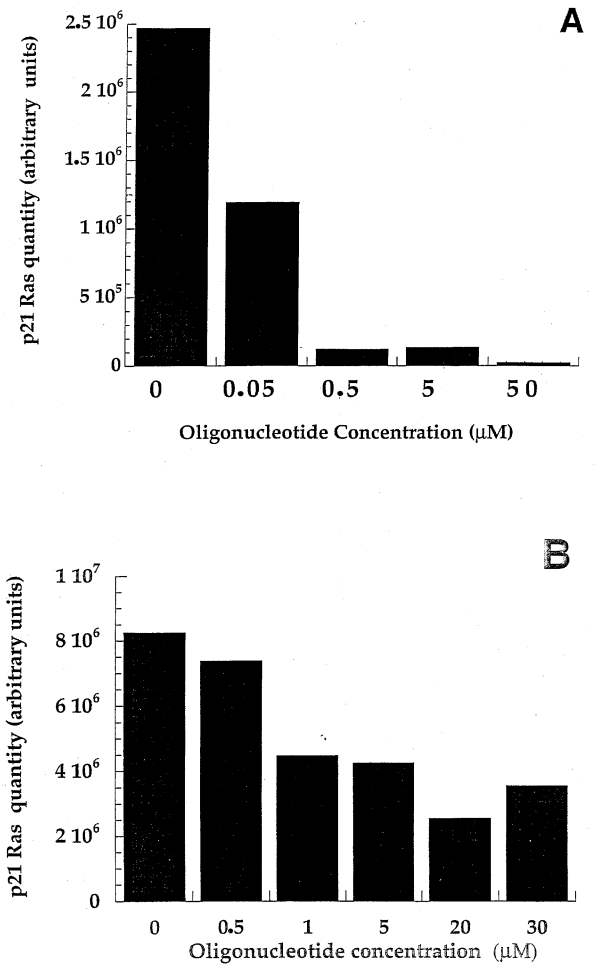

FIG. 3. SDS–PAGE gels were fixed, dried, and exposed to a phosphorimager screen. Protein (p21) was quantitated using a Molecular Dynamics Phosphorimager. The antisense used is the 16-mer targeted to the start codon region (A) and the dodecamer containing six phosphodiester linkages targeting the 12th codon region (B).

nucleotides inhibited HLA-DR expression slightly better than ICAM-1, whereas the hexitol oligonucleotides displayed a clear effect on ICAM-1 expression and little (GS1185) (GS1190) effect on HLA-DR expression.

DISCUSSION

HNA may be considered as a new type of modified oligonucleotides with the potential of functioning as ASO. The backbone structure of HNA is built up from phosphorylated 1,5-anhydro-2,3-dideoxy-D-arabino-hexitol units with the base moiety attached to the 2-position. These oligomers adopt a pre-organized helical structure which fits the A-form of dsRNA [24]. HNA forms very stable duplexes with RNA following the Watson–Crick rules. If these molecules are able to hybridize with RNA within cells, they may be used as steric blocking agents and, hence, cause translation arrest. Indeed, it was demonstrated previously that HNA–RNA duplexes are poor substrates for RNase H [5] and that sequence-selective biological effects of HNA are most probably due to a simple antagonistic action (steric blockage). Many different types of human tumors, including sarcomas, neuroblastomas, leukemias, and lymphomas, contain active oncogenes of the *ras* gene family. Some cancers show a very high frequency of *ras* oncogene expression [9]. A universal feature of *ras*, activation in these tumors is a point mutation leading to a single amino acid substitution either at codon 12, 13, or 61.

In a first *in vitro* evaluation procedure, we tested two HNA sequences targeted against the 12th codon region of *Ha-ras* mRNA in a rabbit reticulocyte lysate for cell-free translation experiments. This *in vitro* model was used to investigate whether translation arrest is attainable using HNA, before proceeding with experiments in a cellular system. The HNA sequences mainly differ in length, i.e. a dodecamer and a heptadecamer. Neither compound was able, however, to arrest elongation by ribosomes, and no biological effect was observed. One reason for this finding might be that ASO targeted to the coding sequence rely on the induction of RNase H for their biological activity. Indeed, Monia *et al.* [13] have shown that uniformly 2′-O-alkyl-modified 17-mer phosphorothioate oligonucleotides targeted to the codon 12 region were ineffective in inhibiting *Ha-ras* gene expression. To restore this activity, a 2′-deoxy residue stretch had to be introduced into the oligonucleotide. Oligonucleotides with different backbone and sugar modifications bearing a phosphorothioate window have been successfully targeted to the *Ha-ras* codon 12 region [14]. A third oligonucleotide was thus synthesized,

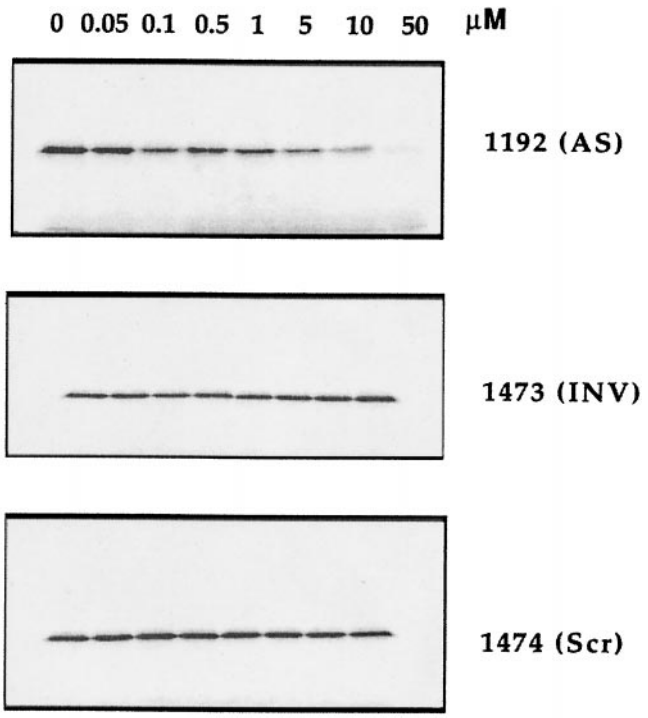

FIG. 4. Effect of the inverted HNA sequence and a scrambled HNA sequence (6′-CGTGATCGCCATGTCC-4′) on translation of mutated *Ha-ras* mRNA (in comparison to the active 16-mer).

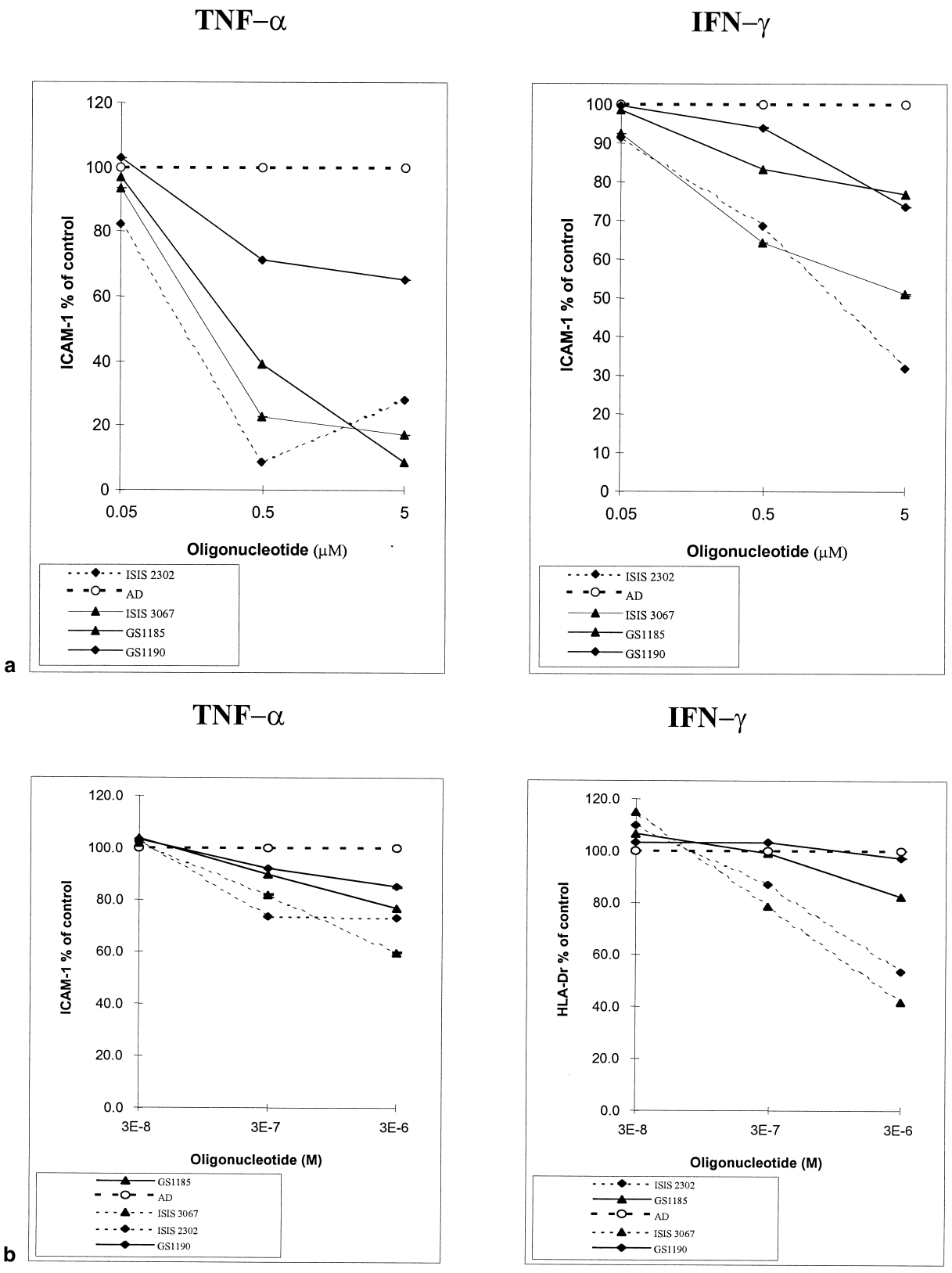

FIG. 5. (a) Ablating effects of matched phosphorothioate and hexitol anti-ICAM-1 ASO in cytokine-induced ICAM-1 expression in human keratinocytes in comparison with the addition of distilled water (AD) as the control. (TNF-α = 7.5 ng/mL and IFN-γ = 10 U/mL). (b) Specificity of matched phosphorothioate and hexitol anti-ICAM-1 ASO in 30 U/mL IFN-γ-induced ICAM-1 and HLA-DR expression in human keratinocytes.

an oligomer with flanking HNA sequences and a window of six deoxynucleotides. In contrast to the above oligomers, this gap-mer was able to inhibit translation in the presence of RNase H with an IC_{50} of 5 μM. Targeting the translation initiation region is a better approach to evaluate the biological potential of steric blockers. Therefore, two HNA sequences (an 11-mer and a 16-mer) directed at the AUG start codon region were synthesized. Upon evaluation in the rabbit reticulocyte lysate, the undecamer did not show any inhibition of mRNA translation. However, the hexadecamer showed a dose-dependent inhibition of translation, with an IC_{50} of 50 nM. It is clear that the HNA hexadecamer is a very potent translation inhibitor. Moreover, its antisense effect is specific, as no translation inhibition of luciferase mRNA was observed and neither an inverted nor a scrambled HNA sequence showed any activity.

These initial results prompted us to carry out a more elaborate study in cellular systems as well as a comparison with phosphorothioates using the ICAM-1 model. Adhesion of leukocytes to endothelial cells is mediated by the expression of ICAM-1. This adhesion is an essential step in the immune reaction against injury or infection. In resting endothelium, the expression of ICAM-1 is low. In response to inflammatory mediators such as IFN-γ and TNF-α, ICAM-1 expression on endothelial cells is increased. Two sets of oligonucleotides were evaluated in the ICAM-1 expression system in keratinocytes. The phosphorothioate 20-mer ISIS 2302 and the HNA equivalent GS1190 are directed against a specific sequence in the 3′-untranslated region of ICAM-1 mRNA and are thought to inhibit mRNA expression mainly by an RNase H-mediated mechanism. ISIS 2302 inhibits TNF-α-induced ICAM-1 expression in human umbilical vein endothelial cells (HUVEC) at 25 nM (IC_{50}) as well as reducing the ICAM-1 mRNA level. The phosphorothioate 20-mer ISIS 3067 and HNA GS1185 have an identical sequence and are targeted at the 5′-untranslated region of ICAM-1 mRNA, starting at the transcription initiation site. ISIS 3067 shows an IC_{50} of 30 nM in the TNF-α-induced ICAM-1 expression system in HUVEC [20]. However, the oligonucleotide did not reduce the ICAM-1 mRNA level, although it did reduce protein synthesis. It was suggested, therefore, that its mode of action might be largely RNase H-independent.

While in our hands both phosphorothioate oligomers were able to ablate ICAM-1 expression in keratinocytes at 0.1 mM, the HNA oligomers were not effective at 1 mM concentrations, due to the low cellular uptake of HNA. The HNA oligomers clearly need lipofection to become active in keratinocytes, which is in contrast to the matched phosphorothioate oligonucleotides. After prolonged lipofection, HNA has both a cytosolic and a nuclear localization. In the presence of lipofectin, GS1185 is more active than GS1190. The difference in biological activity between GS1190 and ISIS 2302 is much more pronounced than between GS1185 and ISIS 3067, which may be explained by the inability of HNA to induce RNase H-mediated cleavage of the target mRNA. The potent activity of GS1185, which is in the same range as that described for ISIS 1185 and ISIS 2302, suggests that targeting HNA oligonucleotides to coding sequences may be a good strategy to inhibit ICAM-1 expression in keratinocytes. At much higher levels of ICAM-1 mRNA, the difference in activity between phosphorothioate oligonucleotides and HNA becomes more striking, in favor of the former oligomers. In contrast, when comparing the effects of the oligomers on two different proteins (ICAM-1 and HLA-DR), the effect of HNA seems to be somewhat more selective than that of phosphorothioate oligonucleotides. Although they share similar non-specific effects, the aspecific effect on HLA-DR in the setting used was somewhat lower for HNA than for the matched phosphorothioates. It is known that phosphorothioate oligonucleotides, besides their sequence-specific antisense effects, demonstrate sequence-specific non-antisense effects and non-sequence-specific effects. The relative importance of these effects on the biological activity of phosphorothioate oligonucleotides may differ considerably from one test system to the other and is largely unknown. The difference in the biological effects of ISIS 3067 and GS1185 may be explained by a) differences in cellular uptake in favor of phosphorothioate oligos, b) a somewhat higher non-specific component in the mode of action of phosphorothioate oligonucleotides, and c) the fact that optimization of the target sequence was carried out with phosphorothioates and not with HNA. The more pronounced difference in activity of ISIS 2302 and GS1190 may be explained by recruitment and activation of RNase H when ISIS 2302 is used, which is not the case when the HNA sequence GS1190 is employed.

In conclusion, HNA may be an effective steric blocking agent *in vivo*, provided that enough material can be brought into the cells.

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