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Inhibition of Dengue Virus by Novel, Modified Antisense Oligonucleotides

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Five different target regions along the length of the dengue virus type 2 genome were compared for inhibition of the virus following intracellular injection of the cognate antisense oligonucleotides and their analogs. Unmodified phosphodiester oligonucleotides as well as the corresponding phosphorothioate oligonucleotides were ineffective in bringing about a significant inhibition of the virus. Novel modified phosphorothioate oligonucleotides in which the C-5 atoms of uridines and cytidines were replaced by propynyl groups caused a significant inhibition of the virus. Antisense oligonucleotide directed against the target region near the translation initiation site of dengue virus RNA was the most effective, followed by antisense oligonucleotide directed against a target in the 3′ untranslated region of the virus RNA. It is suggested that the inhibitory effect of these novel modified oligonucleotides is due to their increased affinity for the target sequences and that they probably function via an RNase H cleavage of the oligonucleotide:RNA heteroduplex.

Dengue virus, a member of the flavivirus family, causes one of the major infectious diseases in the tropical regions of the world, accounting for nearly one hundred million dengue cases of illness each year (5). There are at least four distinct serotypes of dengue virus. Primary dengue virus infection often results in a painful, debilitating, but nonfatal dengue fever. However, the more severe and sometimes fatal forms dengue hemorrhagic fever and dengue shock syndrome have been frequently seen in regions where more than one serotype of dengue virus is circulating. Dengue hemorrhagic fever and dengue shock syndrome are believed to be the result of an antibody-mediated immune enhancement of the viral infection (5). In the presence of subneutralizing amounts of antibody, the virus-antibody complex is believed to enter the target cells (monocytes and macrophages) more efficiently through the Fc receptors on the cell surface. Despite more than three decades of effort, a safe and effective vaccine against dengue virus is not available. Indeed, the phenomenon of antibody-mediated enhancement of infection could actually make a vaccine undesirable unless complete protection against all four serotypes is guaranteed.

The development of therapies based on gene targeting has received widespread attention in recent years. Short oligonucleotides have been used to modulate gene expression both at the transcriptional level by triple helix formation at promoter regions (4, 18) and at posttranscriptional levels either by binding to splice sites and abrogating maturation of mRNA (24) or by binding to ribosome binding sites and inhibiting translation (22, 23). Because phosphodiester (PO) oligonucleotides are readily degraded by nucleases, oligonucleotide analogs containing modified residues have been employed. Phosphorothioate (PS) oligonucleotides, in which an oxygen atom in the PO linkage is replaced by a sulfur atom, and methyl phosphonates, in which a methyl group replaces the oxygen atom, are resistant to nucleases and have been successfully used to inhibit translation from a variety of cellular (7, 22, 23, 26) and viral (8, 16, 28, 31) mRNAs in vitro. The feasibility of developing antisense oligonucleotide analogs into successful therapeutic agents depends on (i) the ability of the oligonucleotide to permeate the cell or development of a delivery system which facilitates transport across the hydrophobic cell membrane barrier, (ii) the intracellular stability of the oligonucleotide, and (iii) the affinity of the oligonucleotide for its intended target sequence. Although PS oligonucleotides are resistant to cellular nucleases and have significantly longer half-lives than unmodified PO oligonucleotides (3), thioation leads to a decreased affinity of the oligonucleotide for its target sequence (10, 30). Greater efficacy might be achieved by further modifying the PS oligonucleotides in order to increase affinity for the target site. C-5 propyne substitution of uridines and cytidines in a PS oligonucleotide was recently shown to overcome the effects of thioation on target affinity (29). The PS oligonucleotide containing modified cytidines and uridines not only had the lost target affinity due to thioation restored but also showed significantly higher affinity than the unmodified PO oligonucleotide (29). Here we demonstrate significant inhibition of dengue virus type 2 in cells microinjected with antisense PS oligonucleotides containing C-5 propyne-substituted uridines and cytidines.

MATERIALS AND METHODS

Virus and antibody. Dengue virus type 2 (strain New Guinea C) was obtained as a suckling mouse brain suspension from the American Type Culture Collection, Rockville, Md., and grown to high titers in C6/36 (*Aedes albopictus*) cells cultured in minimal essential medium containing 10% fetal bovine serum. Virus titers were determined by plaque assay on LLCMK/2 cells (American Type Culture Collection). Purified monoclonal antibody 4G2 specific for dengue virus envelope protein (9) was a gift from James Burans, Naval Medical Research Institute, Bethesda, Md.

Oligonucleotides. PO and PS oligodeoxynucleotides were purchased from Synthecell Corp., Columbia, Md. Propyne-substituted PS (PrPS) oligonucleotides were synthesized at Gilead Sciences Inc. according to the method of Froehler et al. (1). Five antisense oligonucleotides and the corresponding scrambled control oligonucleotides were used (Fig. 1).

Microinjection. LLCMK/2 cells were grown on Cellocator (Eppendorf) coverslips with RPMI 1640 containing 5% fetal bovine serum. Oligonucleotides dissolved in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.1) containing 90 mM KCl were microinjected into the cytoplasm,

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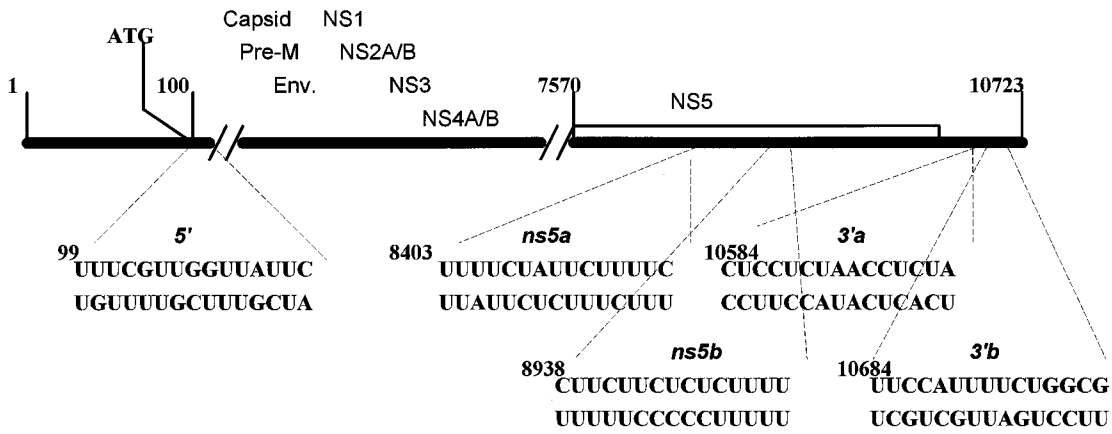

FIG. 1. Oligonucleotides and target regions. The thick line represents the dengue virus type 2 genome. The translation initiation codon (ATG) and the coding regions for various structural (Capsid, Pre-M, and Env.) and nonstructural (NS1 to NS5) proteins are shown at the top. The top and the bottom sequences in each pair of sequences shown represent those of the antisense and the control oligonucleotides, respectively.

using a Leitz micromanipulator under a phase-contrast microscope and an Eppendorf microinjector. Approximately 4×10^{-14} liter (about 1/10 cell volume) of a 10 or 1 μM solution was microinjected into each cell, resulting in final intracellular concentrations of 1 and 0.1 μM, respectively.

Infection and immunofluorescence. Following microinjection, cells were allowed a recovery period of 4 h. Cells were then washed and incubated for 1 h at 37°C with dengue virus type 2 at a multiplicity of infection of 50. Twenty-four hours postinfection, the cells were fixed at −20°C with a 1:1 mixture of acetone and methanol and were processed for indirect immunofluorescence with 4G2 antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Organon-Teknika). In some control experiments, c-Myc was stained by using anti-Myc rabbit antibody (Accurate Chemicals, Westbury, N.Y.) and Texas red-conjugated donkey anti-rabbit immunoglobulins (Jackson Immunoresearch Labs, West Grove, Pa.). The relative fluorescence was quantified with a Nikon Microphot microscope and Metamorph image analysis software (Universal Imaging Corp., West Chester, Pa.) and was expressed as gray value per cell.

In situ hybridization. Twenty-four hours postinfection, microinjected LLCMK/2 cells were processed for in situ hybridization by an adaptation of the method of McCabe et al. (14). Diethyl pyrocarbonate-treated water and reagents were used throughout. Briefly, cells were fixed in 4% paraformaldehyde at room temperature for 10 min; washed in phosphate-buffered saline in 70% ethanol for 2 min and in 2× SSC (1× SSC is 15 mM sodium citrate [pH 7.1] plus 150 mM NaCl) two times for 5 min each; and transferred to 0.1 M triethanolamine, pH 8.0. Acetic anhydride (final concentration, 0.25%) was added dropwise, with vigorous shaking of the slides during the addition. After 10 min, slides were washed sequentially with Tris-glycine (0.1 M, pH 7.0), 2× SSC, 70% ethanol, 95% ethanol, and 100% ethanol and then were air dried. Prehybridization was performed for 2 to 3 h at 42°C with 20 μl of prehybridization solution (Tris, 50 mM [pH 7.2]; EDTA, 1 mM; NaCl, 300 mM; Ficoll, 0.02%; polyvinylpyrrolidone, 0.02%; acetylated bovine serum albumin, 0.5 mg/ml; dithiothreitol, 10 mM; tRNA, 0.5 mg/ml; dextran sulfate, 10%) per slide. Hybridization was accomplished by aspirating the prehybridization solution and incubating the slides for 12 to 16 h at 42°C with the same solution containing 4×10^7 to 8×10^7 cpm of a ^{32}P-labeled riboprobe (complementary to dengue virus type 2 sequence from residues 8762 to 10269) per ml. The probe was generated by SP6 transcription of an *Eco*RI-cut pGEM11Z-f (Promega) clone containing the dengue virus type 2 NS-5 gene. Following hybridization, the slides were washed or treated as follows: buffer A (2× SSC, 1 mM EDTA, 5 mM dithiothreitol, 0.1% Triton X-100), three times for 30 min each; buffer A without Triton X-100, 5 min; 40 μg of RNase A per ml in 10 mM Tris (pH 7.5)–5 mM EDTA–0.3 mM NaCl, 15 min; buffer A, 5 min; buffer A at 42°C, three times for 10 min each; 2× SSC at 42°C, 10 min; and 0.3 M ammonium acetate–ethanol at 1:1, 3:7, 1:9, and 0:10 for 1 min each. The slides were then allowed to air dry. Emulsion autoradiography was done as described by McCabe et al. (14), and the slides were examined microscopically.

Statistical analysis. Experimental results for the negative control (uninfected cells), the positive control (infected cells previously injected with buffer), infected cells previously injected with control oligonucleotide, and infected cells previously injected with antisense oligonucleotide were contrasted. The Kruskal-Wallis nonparametric method (11) was used to contrast the four outcomes simultaneously. When a statistically significant difference ($P \leq 0.05$) arose, each of the six possible pairs was compared by a rank sum test using a sequential Bonferroni approach (15, 21) and a more exact critical error calculation [$1 - (1 - P)^k$] in place of P/k, where P denotes the P value and k denotes the number of comparisons remaining. Paired-comparison methods imply much smaller P values per comparison in order that the P values of several tests will accumulate to 0.05. Thus, a P value of 0.02 on a test of a single pair, for example, may not be significant.

RESULTS

Propyne substitution increases the efficacy of antisense oligonucleotides. Two antisense oligonucleotides (5′-AS [nucleotides 99 to 113] and 3′b-AS [nucleotides 10684 to 10698]) were synthesized as unmodified POs, PSs, and PrPSs containing 5-propyne-C and 5-propyne-U. Three other PrPS antisense oligonucleotides (NS5a-AS [nucleotides 8403 to 8417], NS-5b-AS [nucleotides 8938 to 8952], and 3′a-AS [nucleotides 10584 to 10598]) were also synthesized and tested. The sequences of these oligonucleotides and the regions of the dengue virus genome targeted by them are shown in Fig. 1. Control oligonucleotides had the identical base composition but in random order. No sequence complementarity was found between the control oligonucleotides and either strand of dengue virus RNA or other flavivirus RNAs. For each oligonucleotide, approximately 200 to 300 cells were microinjected to obtain an estimated intracellular concentration of 1 or 0.1 μM. The cells were later infected and analyzed for the synthesis of dengue virus envelope protein by indirect immunofluorescence assay and quantitative image analysis. All experiments were repeated at least three times, and the data shown are from a representative set of experiments. Figure 2 depicts graphically four experiments comparing the abilities of the PO, PS, and PrPS preparations of the 5′ oligonucleotide and the 3′b oligonucleotide to inhibit viral replication. When the PO preparations were used at an intracellular concentration of 1 μM (Fig. 2A), there was no significant difference ($P = 0.8216$) between the groups of cells injected with antisense oligonucleotides (5′-AS and 3′b-AS), their corresponding scrambled control oligonucleotides (5′-CO and 3′b-CO), and the cells injected only with the buffer.

Figure 2B compares viral replication between groups of cells microinjected with the PS preparations at 1 μM. There was no significant change in viral replication between cells microinjected with either antisense oligonucleotide (5′-AS and 3′b-AS) and buffer-injected cells ($0.0201 \leq P \leq 0.0389$) or the corresponding scrambled control oligonucleotides ($0.2623 \leq P \leq 1.0$).

Figures 2C and D show similar comparisons of groups of cells microinjected with the PrPS preparations at 1 and 0.1 μM concentrations, respectively. There was significantly less viral replication in cells injected with either antisense oligonucleotide at 1 μM (5′-AS and 3′b-AS) than in the buffer-injected control ($0.0062 \leq P \leq 0.009$), and there was significantly less viral replication in cells injected with either antisense oligonu-

cleotide than in cells injected with their corresponding control (5′-CO and 3′b-CO) oligonucleotides ($0.009 \leq P \leq 0.0106$). Further, at 0.1 μM, although both 5′ and 3′b antisense oligonucleotides showed significantly less viral replication than cells injected with buffer ($P = 0.0062$), only the cells injected with 5′-AS and not those injected with 3′b-AS showed significantly less viral replication than cells injected with the corresponding control oligonucleotides ($P = 0.009$ and 0.0283, respectively) (Fig. 2D). Representative fields of view for cells injected with buffer, 5′ control, and 3′b control as well as antisense oligonucleotide at 1 μM are shown in Fig. 3A. The specificity of inhibition was also tested by measuring the effect of 5′ and 3′b antisense oligonucleotides on the expression of cellular c-Myc protein (Fig. 3B). Since c-Myc has a very short half-life of only about 30 min (19), measurements made 24 h after microinjection of antisense oligonucleotides reflect the effect of the oligonucleotide on the new synthesis of the Myc protein. There was no significant difference in the expression of c-Myc protein in cells injected with these oligonucleotides (data not shown).

Alternative antisense PrPSs have no effect on viral replication. Three other PrPS antisense oligonucleotides targeted to different regions of the dengue virus RNA and their corresponding control oligonucleotides were tested. NS5a-AS and NS5b-AS were directed against a segment of the dengue virus genome which encodes the nonstructural protein 5, and 3′a-AS was directed against a segment of the genome in the 3′ untranslated region (Fig. 1). Figure 4A compares the viral replication of groups of cells injected with a 1 μM concentration of NS5a-AS, NS5b-AS, their corresponding controls (NS5a-CO and NS5b-CO), or buffer alone. There was no significant decrease in viral replication in cells injected with either of the antisense oligonucleotides compared with cells injected with either buffer ($0.0547 \leq P \leq 0.8782$) or the corresponding control oligonucleotides ($0.0285 \leq P \leq 0.3613$). Similarly, there was no significant difference in viral replication in cells injected with 3′a-AS oligonucleotide compared with those injected with buffer ($P = 0.0176$) or with the corresponding control oligonucleotide, 3′a-CO ($P = 0.2002$) (Fig. 4B).

Antisense microinjected cells contain decreased amounts of viral RNA. Since 5′ and 3′b antisense oligonucleotides showed significant inhibition of virus-specific protein synthesis, we looked at the levels of viral RNA in cells microinjected with either of the antisense oligonucleotides and their corresponding control oligonucleotides. Virus-specific RNA was detected by in situ hybridization to a ^{32}P-labeled riboprobe. It can be seen from Fig. 5 that cells microinjected with 5′ or 3′b PrPS

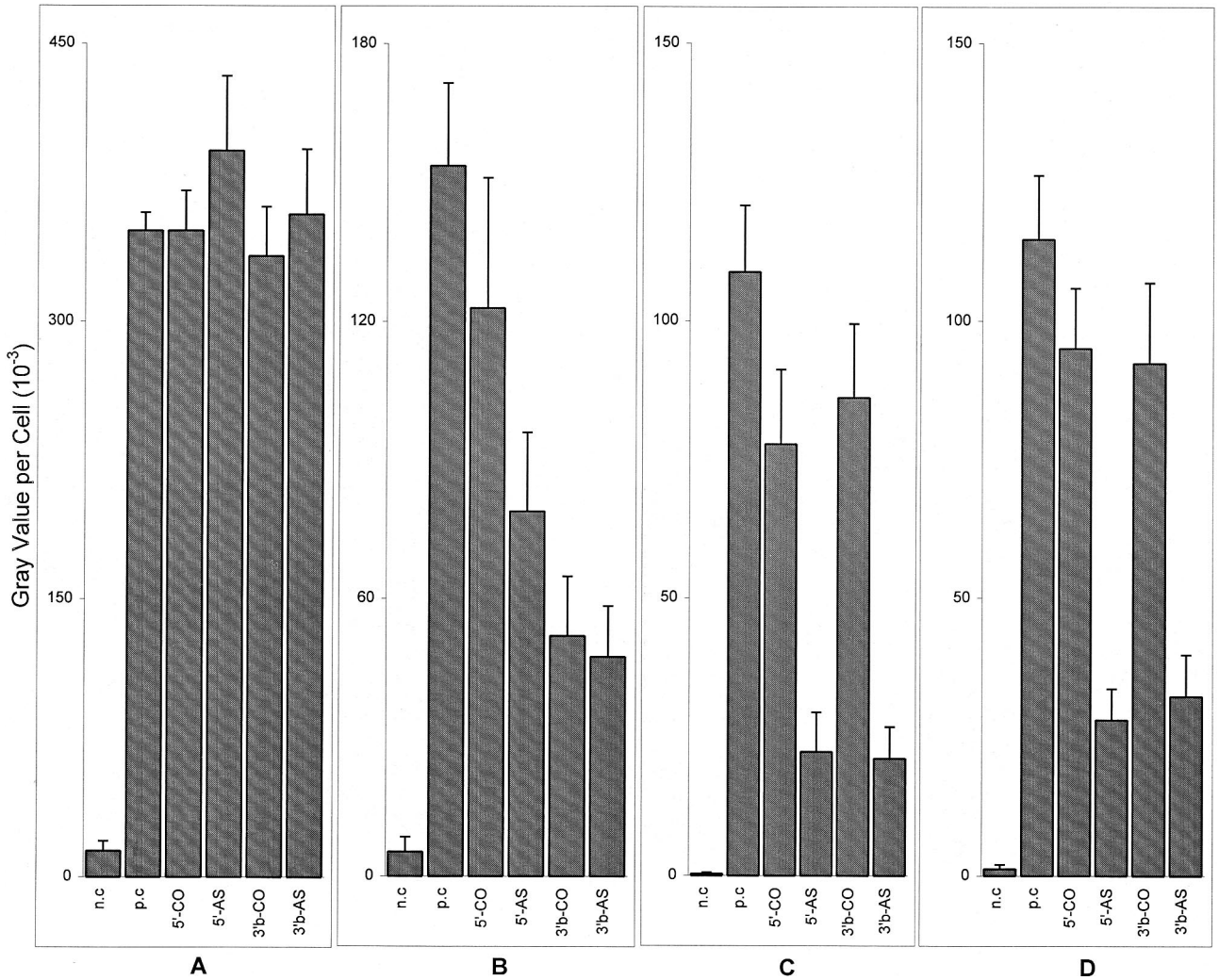

FIG. 2. Comparison of PrPS 5′ and 3′b antisense oligonucleotides (C [1 μM] and D [100 nM]) with their unmodified PS (B) (1 μM) and PO (A) (1 μM) antisense oligonucleotides. The bars on top of the columns indicate the standard errors. n.c, negative control (noninjected, noninfected cells); p.c, positive control (buffer-injected, infected cells); -CO, control oligonucleotide; -AS, antisense oligonucleotide.

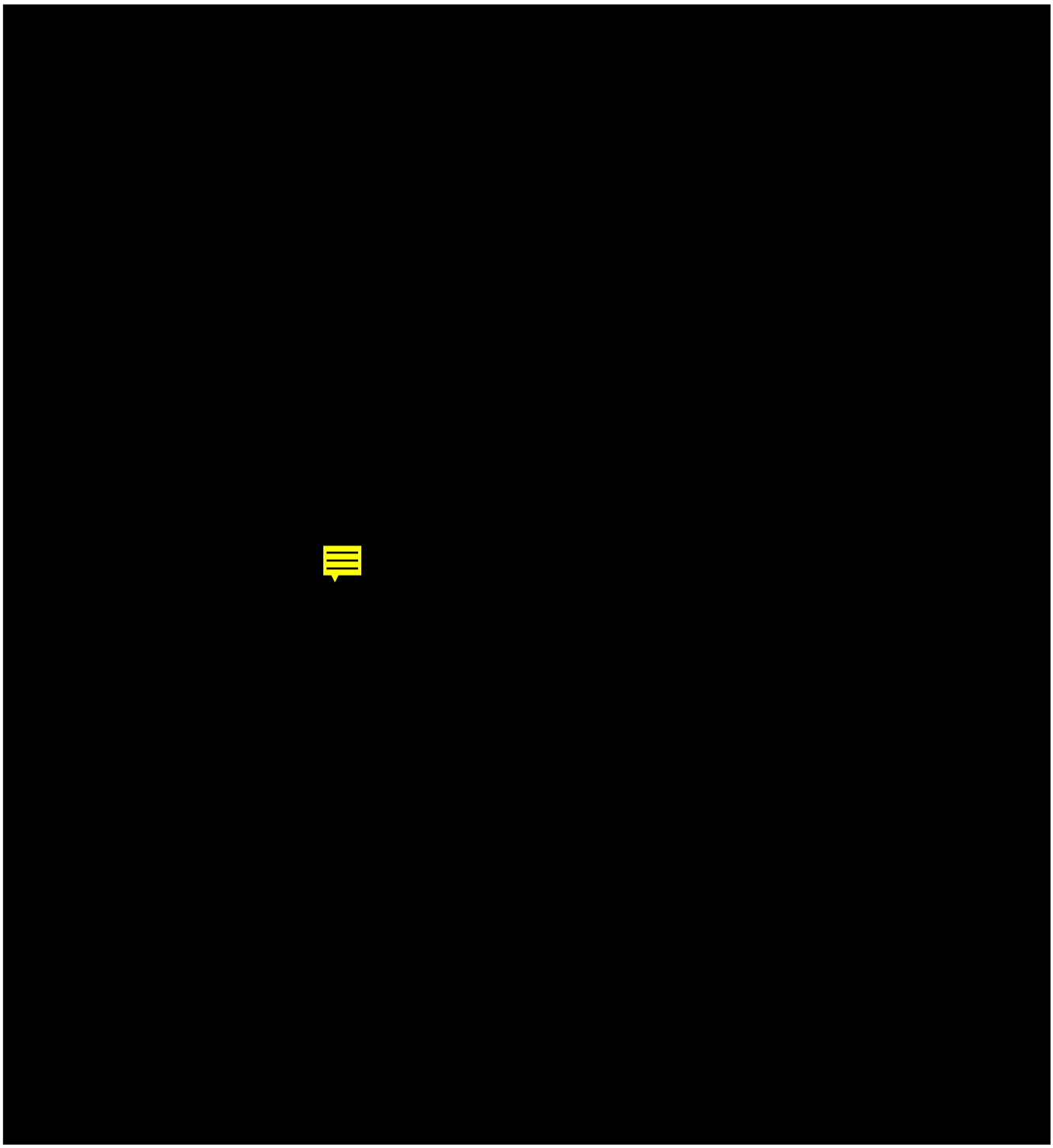

FIG. 3. (A) Inhibition of dengue virus by 5′ and 3′b PrPS antisense oligonucleotides. Buffer-injected and infected LLCMK/2 cells (a) or infected LLCMK/2 cells that had been previously injected with 5′ control (b), 5′ antisense (e), 3′b control (c), or 3′ antisense (f) oligonucleotide were fluorescently labeled with dengue virus-specific antibody as described in Materials and Methods. Panel d represents control uninfected cells. (B) Effect of 5′ and 3′b PrPS antisense oligonucleotides on c-Myc protein synthesis. LLCMK/2 cells were fluorescently labeled with anti-Myc antibody following microinjection of buffer (a), 5′ control (b) or antisense (e) oligonucleotide, or 3′b control (c) or antisense (f) oligonucleotide. Panel d is a negative control in which the cells were treated with nonimmune rabbit serum in place of anti-Myc antibody.

antisense oligonucleotide contain greatly reduced levels of viral RNA compared with cells injected with buffer alone or the corresponding control oligonucleotides, strengthening the evidence obtained by immunofluorescence analyses. Degradation of oligonucleotide:RNA heteroduplexes has been suggested as one of the mechanisms by which antisense oligonucleotides exert their effect (3). Our data suggest that the 5′ and 3′b antisense oligonucleotides may follow a similar pathway.

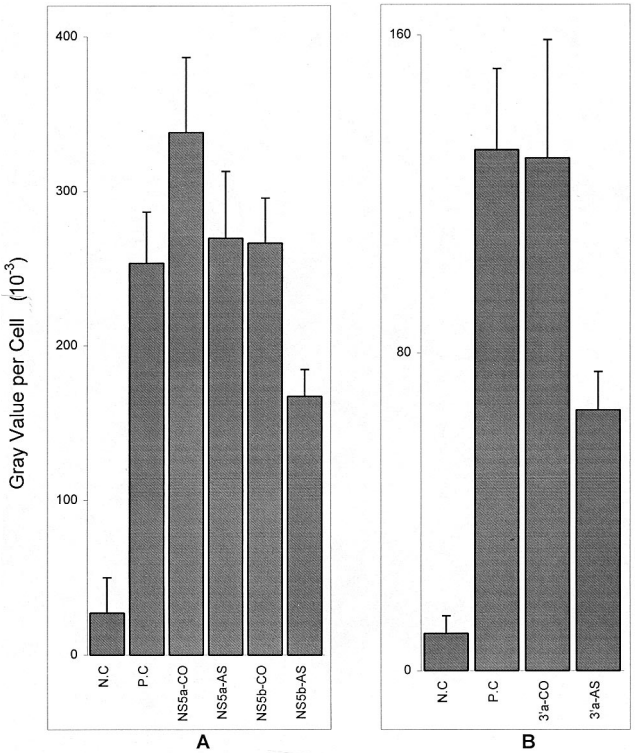

FIG. 4. Effect of PrPS antisense oligonucleotides (1 μM) targeted to regions on the dengue virus genome encoding nonstructural protein NS5 (A) and a region in the 3′ untranslated sequence (B). N.C, P.C, -CO, and -AS are as described in the legend to Fig. 2. The sequences NS5a, NS5b, and 3′a are described in Fig. 1.

DISCUSSION

Dengue virus type 2 contains a positive-strand RNA genome of 10,723 bases (9). The genome contains a single open reading frame starting at nucleotide 97 and ending at nucleotide 10269. This long open reading frame is translated into a large polyprotein from which structural and nonstructural proteins are derived by specific proteolytic cleavage (6). The viral envelope is the major virion protein, and its synthesis can be used as a measure of any inhibition of viral RNA replication or translation. In determining the regions for selection as targets for antisense oligonucleotides, we considered (i) the predicted local secondary structure for dengue virus RNA as analyzed by the Fold program of the Genetics Computer Group software and (ii) the pyrimidine content of the sequence. Pyrimidine-rich sequences were selected so that a large proportion of the residues could be propyne substituted. We chose five different target regions: the 5′ antisense oligonucleotide spanned the translation initiation region, including the last residue of the initiating methionine codon; the NS5a and NS5b antisense oligonucleotides targeted two different regions of the NS-5 gene, which presumably codes for the RNA-dependent RNA polymerase; and the 3′a and 3′b antisense oligonucleotides targeted sequences in the 3′ untranslated region. The 3′a sequence not only is shared by all four serotypes of dengue virus (with the exception of a single-base mismatch in the case of dengue virus type 3) but is also found in other flaviviruses, such as yellow fever virus (20) and Japanese encephalitis virus (27). The 3′b sequence is conserved in dengue virus types 1, 2, and 3 (2, 9, 17). One or both of these two sequences could be important for dengue virus RNA replication.

Liposome entrapment of oligonucleotides has been widely used as a means of successfully delivering the antisense agents into the cell. Following transfection by *N*-(1-(2,3-diolyloxy) propyl)-*N*,*N*,*N*-triethylammonium chloride (DOTMA), there

FIG. 5. In situ hybridization analysis of dengue virus RNA in cells microinjected with 5′ and 3′b PrPS antisense oligonucleotides. LLCMK/2 cells buffer injected and infected (a) or infected LLCMK/2 cells that had been previously injected with 5′ control (b), 5′ antisense (e), 3′b control (c), or 3′ antisense (f) oligonucleotide were analyzed by in situ hybridization with a ^{32}P-labeled dengue virus-specific riboprobe. Panel d is a negative control in which uninfected LLCMK/2 cells were identically treated.

was an enhancement in the uptake of oligonucleotides, and the oligonucleotides rapidly accumulated in the nuclei (29). However, we observed no inhibitory effect on dengue virus following lipofectin (Gibco-BRL) transfection of LLCMK/2 cells (data not shown). Transfections using a fluorescein-labeled oligonucleotide showed that the oligonucleotide rapidly accumulated in the nucleus (data not shown). Since dengue virus replicates exclusively in the cytoplasm, we hypothesize that the failure to obtain inhibition may be because of an inability to achieve a critical cytoplasmic concentration. Higher concentrations of oligonucleotide are required to achieve inhibition of viral genes than are required for cellular genes (13). Oligonucleotides delivered by direct microinjection also accumulate rapidly in the nucleus (12). However, what remains in the cytoplasm is probably readily accessible compared with liposome-entrapped oligonucleotides.

The data presented here show that although both the 5′-AS and 3′b-AS oligonucleotides at an intracellular concentration of 1 μM resulted in significantly less viral replication than the controls, at the lower 0.1 μM concentration the 5′-AS oligonucleotide appeared to be more effective. Although internal RNA sequences and splice sites have been successfully used as targets for antisense inhibition (24), antisense oligonucleotides are generally most effective when directed against translation initiation sites (25). The 3′a oligonucleotide, whose target is only 100 bases upstream of the 3′b target, shows limited efficacy. These differences are probably a reflection of the complex secondary structures presented by the large (>10-kb) dengue virus RNA. Further, in addition to exhibiting inhibition of gene function at submicromolar concentrations, the C-5 propyne-substituted oligonucleotides exert considerably less non-sequence-specific inhibition.

The mechanism of inhibition of gene function by antisense oligonucleotides may be twofold: they can bind the RNA and cause steric inhibition of translation (and/or replication in the case of an RNA virus) or they can lead to RNA degradation by presenting the oligonucleotide:RNA heteroduplex as a substrate for RNase H-like activity. Heteroduplexes containing certain backbone-modified oligonucleotides (such as 2′-alkyl residues) are not cleaved by RNase H (3). However, heteroduplexes formed by PS oligonucleotides are known to be good substrates for RNase H, and RNase H degradation of RNA is believed to be major factor contributing to the efficacy of an antisense oligonucleotide. It appears that the propyne-substituted antisense oligonucleotides follow a similar pathway. These novel modified oligonucleotide analogs may be generally more effective as antisense agents against other viruses.

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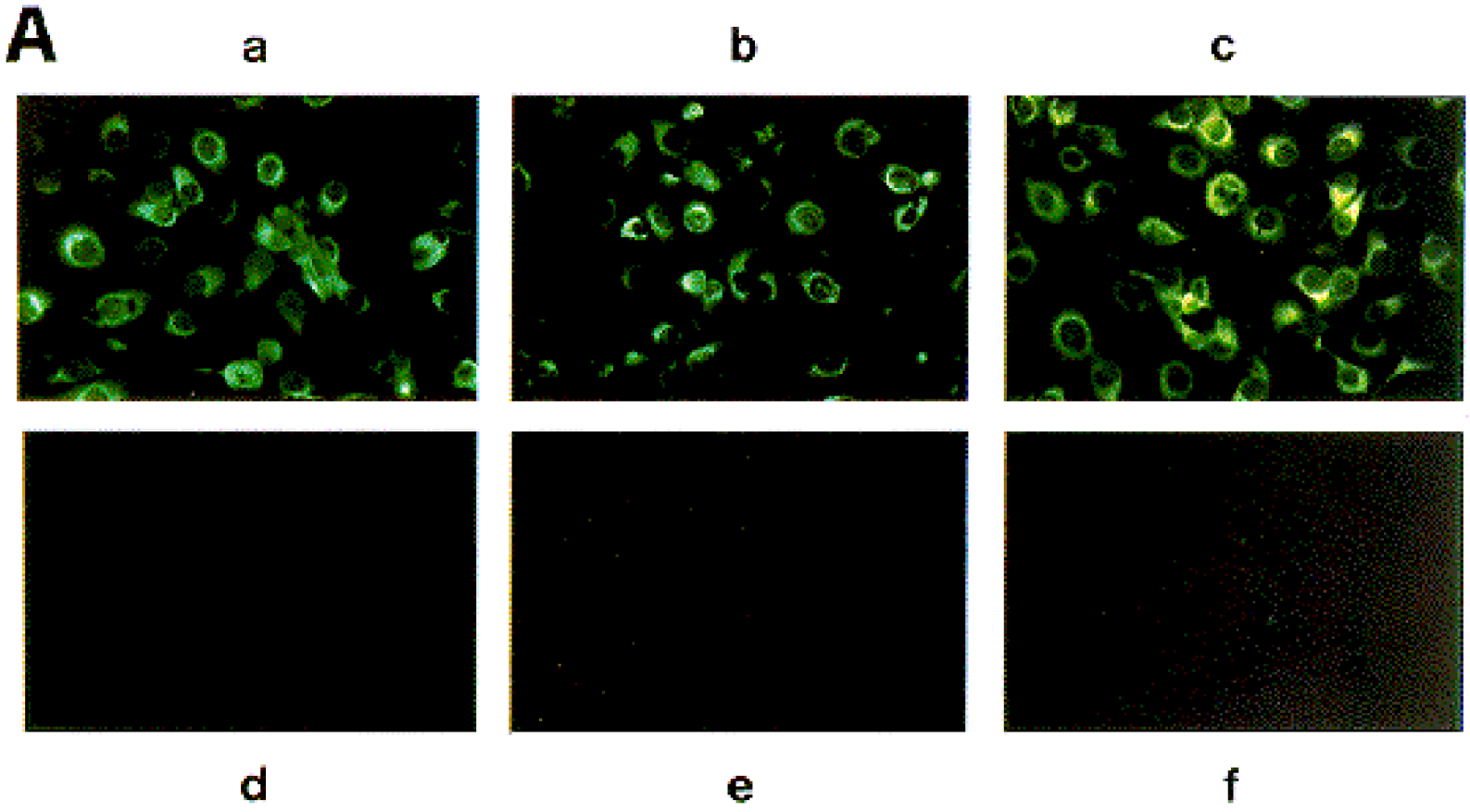
REFERENCES

1. **Froehler, B. C., S. Wadwani, T. J. Terhorst, and S. R. Gerrard.** 1992. Oligodeoxynucleotides containing C-5 propyne analogs of 2′-deoxyuridine and 2′-deoxycytidine. Tetrahedron Lett. **33:**5307–5310.
2. **Fu, J., B.-H. Tan, E.-H. Yap, Y.-C. Chan, and Y.-H. Tan.** 1992. Dengue virus type 1 complete genome. Virology **188:**953–958.
3. **Ghosh, M. K., and J. S. Cohen.** 1992. Oligodeoxynucleotides as antisense inhibitors of gene expression. Prog. Nucleic Acids Res. Mol. Biol. **42:**79–126.
4. **Grigoriev, M., D. Praseuth, P. Robin, A. Hemar, T. Saison-Behmoaras, A. Dautry-Varsat, N. T. Thuong, C. Helene, and A. Harel-Bellan.** 1992. A triple helix forming oligonucleotide-intercalater conjugate acts as a transcriptional repressor via inhibition of NF-κB binding to interleukin-2 receptor α-regulatory sequence. J. Biol. Chem. **267:**3389–3395.
5. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239:**476–481.
6. **Henchal, E. A., and R. Putnak.** 1990. The dengue viruses. Clin. Microbiol. Rev. **3:**376–396.
7. **Higgins, K. A., J. R. Perez, T. A. Coleman, K. Dorshkind, W. McComas, U. M. Sarmiento, C. A. Rosen, and R. Narayanan.** 1993. Antisense inhibition of the p65 subunit of NF-κB blocks tumorigenicity and causes tumor regression. Proc. Natl. Acad. Sci. USA **90:**9901–9905.
8. **Hoke, G. D., K. Draper, S. M. Freier, C. Gonzalez, V. B. Driver, M. C. Zounes, and D. J. Ecker.** 1991. Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection. Nucleic Acids Res. **20:**5743–5748.
9. **Irie, K., P. M. Mohan, Y. Sasaguri, R. Putnak, and R. Padmanabhan.** 1989. Sequence analysis of cloned dengue virus type 2 genome (New Guinea-C strain). Gene **75:**197–211.
10. **Kibler-Herzog, L., G. Zon, B. Uznanski, G. Whittier, and W. D. Wilson.** 1991. Duplex stabilities of phosphorothioate, methylphosphonate, and RNA analogs of two DNA 14-mers. Nucleic Acids Res. **19:**2979–2986.
11. **Kruskal, W. H., and W. A. Wallis.** 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. **47:**583–621.
12. **Leonetti, J. P., N. Mechti, G. Degols, C. Gagnor, and B. Lebleu.** 1991. Intracellular distribution of microinjected antisense oligonucleotides. Proc. Natl. Acad. Sci. USA **88:**2702–2706.
13. **Malcolm, A. D. B., J. Coulson, N. Blake, and L. C. Archard.** 1992. Applications of antisense oligonucleotides in virology. Biochem. Soc. Trans. **20:**762–764.
14. **McCabe, J. T., K. Almasan, E. Lehmann, J. Hanze, R. E. Lang, D. W. Pfaff, and D. Ganten.** 1988. In situ hybridization demonstrates vasopressin gene transcription in hypothalamic neurons of crossbred hypertensive × diabetes insipidus rats. Neuroscience **27:**159–167.
15. **Miller, R. G., Jr.** 1981. Simultaneous statistical inference, 2nd ed. Springer-Verlag, New York.
16. **Offensperger, W.-B., S. Offensperger, E. Walter, K. Teubner, G. Igloi, H. E. Blum, and W. Gerok.** 1993. In vivo inhibition of duck hepatitis B virus replication and gene expression by phosphorothioate modified antisense oligonucleotides. EMBO J. **12:**1257–1262.
17. **Osatomi, K., and H. Sumioshi.** 1990. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology **176:**643–647.
18. **Postel, E. H., S. J. Flint, D. J. Kessler, and M. E. Hogan.** 1991. Evidence that a triplex forming oligodeoxyribonucleotide binds to the c-myc promoter in HeLa cells, thereby reducing c-myc mRNA levels. Proc. Natl. Acad. Sci. USA **88:**8227–8231.
19. **Ramsay, G., G. I. Evan, and J. M. Bishop.** 1984. The protein encoded by the human proto-oncogene c-myc. Proc. Natl. Acad. Sci. USA **81:**7742–7746.
20. **Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss.** 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science **229:**726–733.
21. **Rice, W. R.** 1989. Analyzing tables of statistical tests. Evolution **43:**223–225.
22. **Shi, Y., J. M. Glynn, L. J. Guilbert, T. G. Cotter, R. P. Bissonnette, and D. R. Green.** 1992. Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. Science **257:**212–214.
23. **Simons, M., E. R. Edelman, J.-L. DeKeyser, R. Langer, and R. D. Rosenberg.** 1992. Antisense c-myb oligonucleotides inhibit arterial smooth muscle cell accumulation in vivo. Nature (London) **359:**67–70.
24. **Smith, C. C., L. Aurelian, M. P. Reddy, P. S. Miller, and P. O. P. Ts'o.** 1986. Antiviral effect of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5. Proc. Natl. Acad. Sci. USA **83:**2787–2791.
25. **Stein, A., and J. S. Cohen.** 1988. Oligodeoxynucleotides as inhibitors of gene expression. Cancer Res. **48:**2649.
26. **Stein, C. A., and Y.-C. Cheng.** 1993. Antisense oligonucleotides as therapeutic agents: is the bullet really magical? Science **261:**1004–1012.
27. **Sumioshi, H., C. Mori, T. Fuke, K. Morita, S. Kuhara, J. Kondou, Y. Kikuchi, H. Nagamatu, and A. Igarashi.** 1987. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. Virology **161:**497–510.
28. **Vickers, T., B. F. Baker, P. D. Cook, M. Zounes, R. W. Buckheit, Jr., J. Germany, and D. J. Ecker.** 1991. Inhibition of HIV-LTR gene expression by oligonucleotides targeted to the TAR element. Nucleic Acids Res. **19:**3359–3368.
29. **Wagner, R. W., M. D. Matteucci, J. G. Lewis, A. J. Gutierrez, C. Moulds, and B. C. Froehler.** 1993. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. Science **260:**1510–1513.
30. **Young, S., and R. W. Wagner.** 1991. Hybridization and dissociation rates of phosphodiester or modified oligodeoxynucleotides with RNA at near-physiological conditions. Nucleic Acids Res. **19:**2463–2470.
31. **Zon, G.** 1990. Innovations in the use of antisense oligonucleotides. Ann. N. Y. Acad. Sci. **616:**161–172.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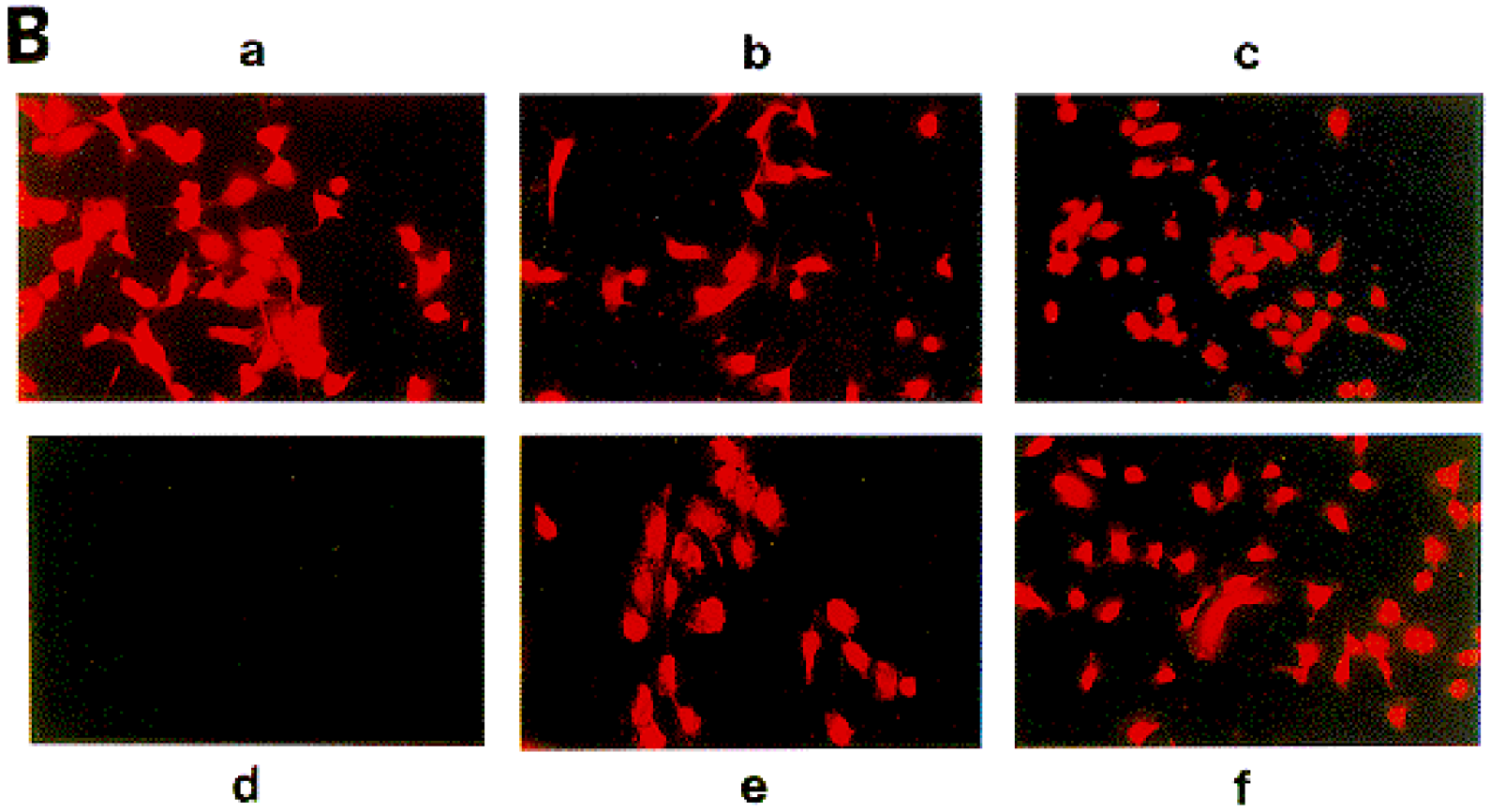
B
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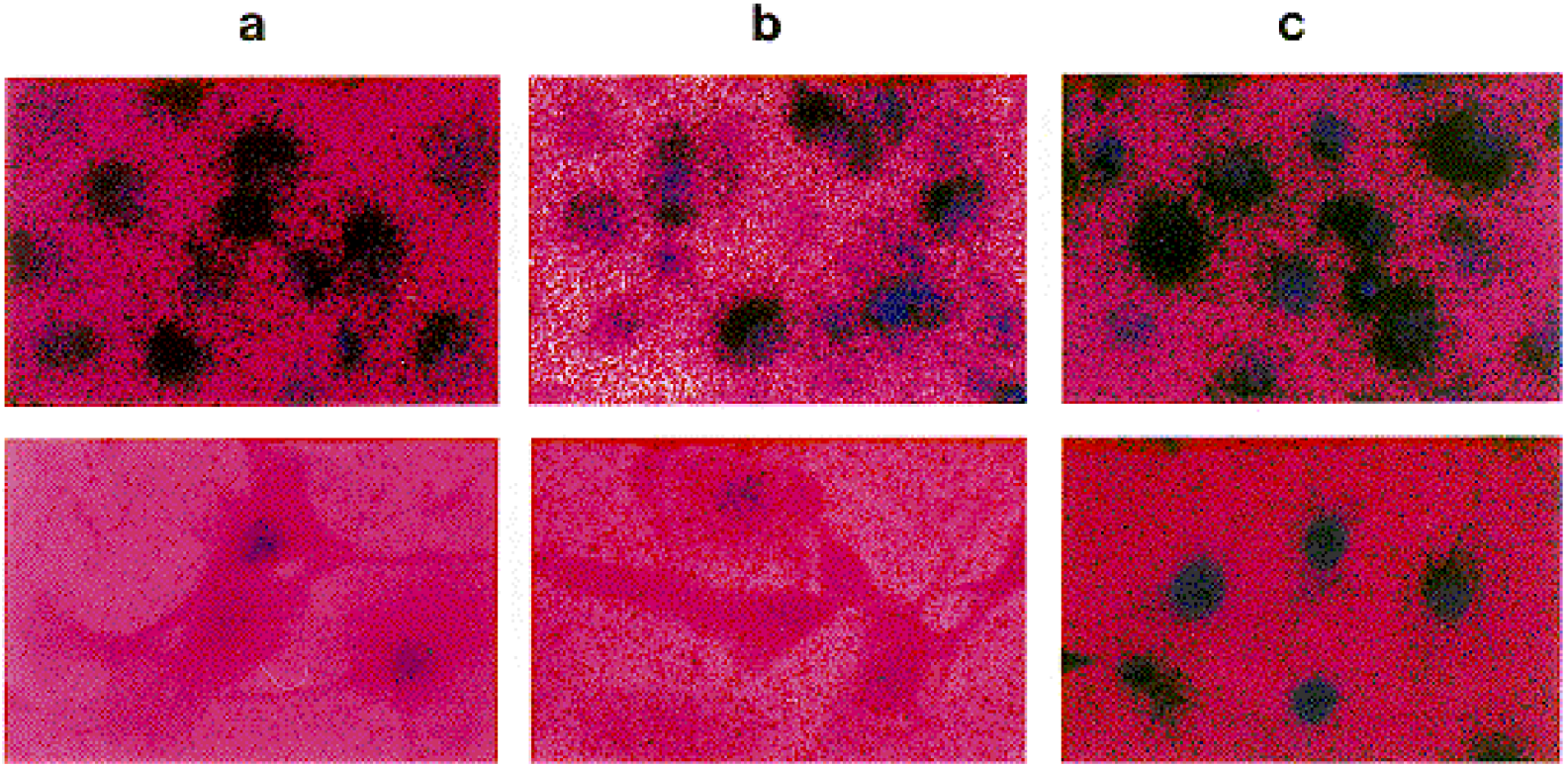
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