BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 305–309 (1996)
ARTICLE NO. 1797

Relative Levels of Inhibition of p24 Gene Expression by Different 20-mer Antisense Oligonucleotide Sequences Targeting Nucleotides +1129 to +1268 of the HIV-1 *gag* Genome: An Analysis of Mechanism

Michael I. Anazodo,*,† Elena Duta,*,† Albert D. Friesen,† and Jim A Wright*,[1]

**Manitoba Institute of Cell Biology and Department of Biochemistry and Molecular Biology, University of Manitoba, 100 Olivia Street, Winnipeg, Manitoba R3E 0V9, Canada; and †Genesys Pharma Inc., 3-117 Victor Lewis Drive, Winnipeg, Manitoba, Canada*

Received October 30, 1996

GPI2A is a 20-mer antisense oligonucleotide sequence that is complementary to a region of the HIV-1 *gag* gene. An analysis of viral core antigen p24 protein synthesis inhibition was performed with cells expressing HIV-1 proteins, following treatment with GPI2A or eight other unique antisense constructs designed to bind to regions of the *gag* gene, at positions that 5′ or 3′ flank the GPI2A target site. GPI2A was found to be the most effective construct, indicating that the GPI2A target region is a particularly sensitive site for antisense activity. An analysis of energy-related parameters important in complementary duplex formation was performed for each antisense construct. Also, the potential of each antisense sequence to exhibit self-complementarity or to self-dimerize was assessed. The results from these analyses provided an explanation for the high specificity and the superior inhibitory characteristics of GPIA when compared to the eight other antisense oligonucleotides. GPI2A exhibited the second most favorable energy-related characteristics for hybridization reactions, and most importantly, unlike the other eight antisense sequences, it did not show the potential to self-complement or to dimerize. The results of this study and a previous investigation of sequence specificity requirements for GPI2A inhibition of HIV-1 gene expression provide strong evidence for an antisense mode of action for this oligonucleotide construct, a useful tool for analysis of viral gene expression and perhaps a potential therapeutic agent.

Antisense oligonucleotides have been very useful tools for studying cellular and viral gene expression, and they have shown promise as potential therapeutic reagents for the treatment of a variety of diseases, including cancer and several viral infections (1-3). One such interesting antisense molecule designed to target the *gag* region of the human immunodeficiency virus (HIV-1) genome has been described recently (4). This antisense sequence, called GPI2A, inhibited viral mRNA and protein synthesis in a sequence specific and dose-dependent manner in cultured monkey kidney cells transfected with an expression plasmid containing the HIV-1 *gag* and *pol* genes (4). The antiviral activity of this compound was also demonstrated with acute and chronically infected human lymphoid cells. Recently, there has been much discussion about the specificity of antisense compounds, since some antisense sequences previously considered to be specific for their target sites, were later found to be less specific than originally proposed (5,6). The specificity and effectiveness of an antisense sequence not only depends upon its ability to recognize the intended target, but also upon its ability to form stable duplexes with the complementary template, and its ability to remain in a non self-complementary conformation. Previous work has demonstrated the sequence specificity of GPI2A (4). The present study was undertaken to examine the hybridization and conformational characteristics of the 20-mer GPI2A oligonucleotide and its ability to inhibit gene expression, relative to

[1] To whom correspondence should be addressed. Fax: (204) 787-2190.

0006-291X/96 $18.00

eight other unique antisense sequences that are complementary to regions flanking the GPI2A target site.

MATERIALS AND METHODS

Cells and culture conditions. A Cos-like monkey kidney cell line, CMT3 (7), was transfected with the expression plasmids pCMV *gag pol*-rre-r and pCMV rev to produce the B4.14 cell line, as described previously (4,8). The pCMV rev plasmid was included to obtain efficient expression of the *gag* gene. The cells were cultured in Iscove's modified Dulbecco's medium in the presence of 10% fetal calf serum as described (4,8).

Antisense oligonucleotides and their synthesis. Oligonucleotides in the antisense orientation were synthesized on an Applied Biosystems 392 automated DNA synthesizer by standard phosphoramidite chemistry, and purified by high-performance liquid chromatography (4,8,9). GPI2A is a 20-mer antisense sequence thioated at seven base positions that targets the *gag* region at nucleotides +1189 to +1208 (4), using the nomenclature recommended by Ratner *et al* (10). Eight other antisense constructs similarly thioated at seven base positions were synthesized to be complementary to regions of the *gag* sequence as follows: +1129 to +1148, +1149 to +1168, +1169 to +1188, +1179 to +1198, +1199 to +1218, +1209 to +1228, +1229 to +1248 and +1249 to +1268. The sequence of GPI2A has been described (4), and the other antisense constructs are shown in Fig. 2.

Antisense treatment. B4.14 cells were exposed to antisense constructs as previously reported (4,8). In brief, 70% confluent cultures on plastic surfaces were pretreated with 6.2 μg/ml antisense oligonucleotides in the presence of 10 μg/ml lipofectin reagent (GIBCO) for 24 hours. The medium was then replaced with fresh medium containing 7% heat-inactivated (55°C for 30 min) bovine serum and 6.2 μg/ml antisense, and the cells were incubated for an extra 24 hours at 37°C as described (4,8).

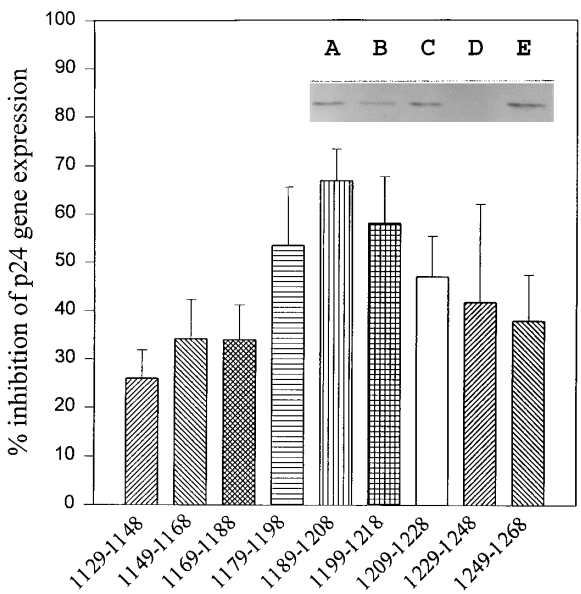

FIG. 1. Densitometry results of Western blot analysis of HIV-1 p24 expression in B4.14 cells (n=4). The axis shows the nucleotide sequence locations of the *gag* genome that are complementary to the various antisense oligonucleotides. Statistical analysis (Students t-test) showed that the results obtained with GPI2A were significantly different (significance set at $p \leq 0.05$) from the results obtained with antisense constructs targeting viral sequences +1129 to +1148, +1149 to +1168, +1169 to +1188, +1229 to +1248 and +1249 to +1268. Significant differences between GPI2A and the other three antisense constructs were not found, although a trend to significance was observed with the construct targeting the sequence +1209 to +1228 ($p=0.1$). The inset shows an autoradiograph of a Western blot analysis from cells treated with antisense oligonucleotides targeting sequences +1129 to +1148 (A), sequences +1189 to + 1208 (GPI2A) (B), and sequences +1249 to 1268 (C). Lanes D and E show data from control experiments, with (D) showing a Western blot result obtained with CMT3 cell extract, which does not contain the viral p24 protein (7), and (E) showing a Western blot result with B4.14 cell extract following treatment with the inverse sequence of GPI2A which is not complementary to any of the sequences in the *gag* genome.

TABLE 1
Characteristics of Nine 20-mer Antisense Oligodeoxyribonucleotide Sequences That Target the HIV-1 *gag* Genome

Target sequence	Tm[a]	dG[b] (Kcal/mol)	dH[c] (Kcal/mol)
1129 to 1148	40.65	−34.2	−147.0
1149 to 1168	35.25	−30.9	−147.0
1169 to 1188	45.05	−34.2	−142.2
1179 to 1198	43.55	−33.2	−137.5
1189 to 1208	47.45	−36.1	−149.8
1199 to 1218	40.35	−33.0	−147.5
1209 to 1228	34.95	−30.0	−138.2
1229 to 1248	35.05	−30.6	−144.1
1249 to 1268	57.75	−41.1	−157.3

[a] Melting temperature of the oligonucleotide duplex formed.
[b] Free energy values for oligonucleotide-complement dimer formation.
[c] Enthalpy values for oligonucleotide-complement dimer formation.

Computer analysis. A computer program (OLIGO, Primer Analysis Software, Version 3.4) was used to calculate for each antisense sequence the melting temperature, free energy (dG), and enthalpy (dH) relevant to oligonucleotide-duplex formation, and to determine potential dimer formation and self-complementary properties (11,12).

Western blot analysis. The potential inhibitory activity of each antisense sequence was assessed by determining the effects on expression of the HIV-1 viral core p24 antigen as described previously (4,8), using mouse monoclonal antibody specific for viral p24 protein (Ab anti-HIV-1 p24, NEN Virus Research).

RESULTS AND DISCUSSION

The monkey kidney cell line, B4.14, efficiently expresses the *gag pol* region of the HIV-1 virus. In keeping with previous observations (4), Fig. 1 shows that treatment of B4.14 cells with GPI2A, an antisense construct that is complementary to nucleotide sequences +1189 to 1208 of the *gag* genome significantly inhibited viral p24 protein synthesis. Eight other antisense sequences that are complementary to regions of the HIV-1 genome that 5′ or 3′ flank the GPI2A target were also capable of inhibiting viral protein synthesis, providing a battery of potentially useful new inhibitory reagents. Interestingly, GPI2A appeared to be the most effective inhibitor, suggesting that the nucleotide region between +1189 and +1208 is a particularly sensitive region for antisense activity and holds promise for future drug design. Three other antisense sequences directly flanking, and in two cases partially overlaping, the GIP2A target site (complementary to viral nucleotide sequences +1179 to +1198, +1199 to +1218 and +1209 to +1228) were also very good inhibitors. Although GPI2A appeared to be a superior inhibitory agent when compared to these latter three antisense constructs (Fig.1), a statistically significant difference was not observed. However, statistically significant differences ($p \leq 0.05$) were found when the results obtained with GPI2A treatment were compared to those obtained with each of the other four antisense constructs.

An analysis of energy related parameters involved in duplex formation for each of the antisense constructs is shown in Table 1. This analysis indicates that the interactions of these oligonucleotide sequences with their complementary templates exhibit at least the minimal Tm, dG and dH values suitable for nucleic acid hybridization reactions, as generally reflected in their abilities to inhibit virus gene expression shown in Fig. 1. Based upon this analysis alone, the most favorable interaction should occur with the antisense construct complementary to nucleotides +1249 to +1268, and the least favorable should be the antisense sequence targeting nucleotides +1209 to +1228. GPI2A has very good properties, although it exhibits

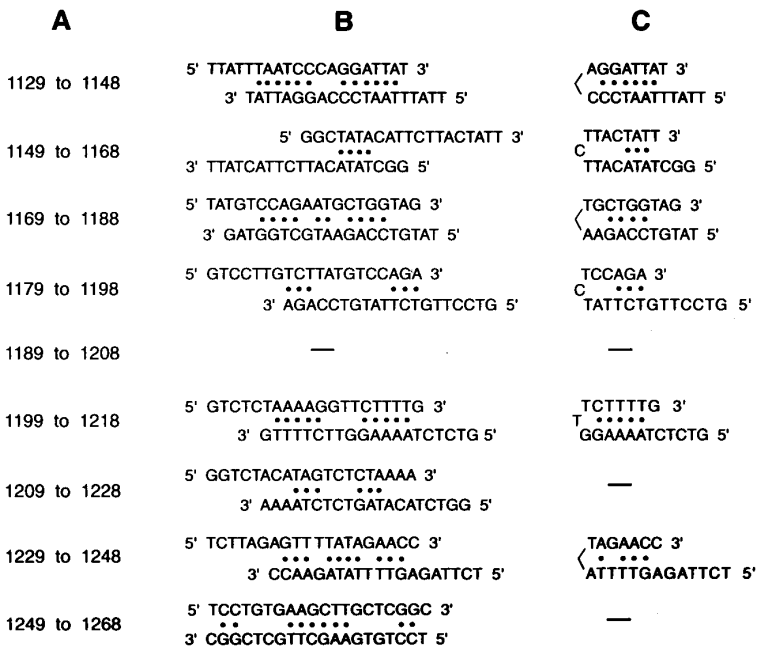

FIG. 2. Antisense conformational analysis. (A) Shows the nucleotide sequence locations in the *gag* genome that are complementary to the antisense oligonucleotides used in the p24 inhibition studies presented in Fig. 1. (B) Shows potential dimer formation of the various antisense oligonucleotides. (C) Shows potential self-complementary interactions of the various antisense oligonucleotides. (−) Antisense oligonucleotides targeting sequences +1189 to +1208 (GPI2A) did not show a potential for dimerization or for self-complementarity, whereas sequences +1209 to +1228 and +1249 to +1268 showed potential for dimerization but not for self-complementarity.

the second most favorable characteristics in this type of analysis. Since the antisense oligonucleotides targeting +1249 to +1268 and +1209 to +1228 were intermediate as inhibitors of gene expression in comparison to the other antisense sequences, and GPI2A appeared to be the best inhibitor (Fig. 1), it is clear that other properties of these antisense constructs are also very important in determining their relative abilities to modulate gene expression.

Fig. 2 shows the potential of each antisense sequence to exhibit self-complementarity, and to form dimers with itself. It is important to note that all antisense sequences except GPI2A have a strong potential to either fold/self-complement, or to form dimers, that would obviously interfere with hybridization reactions at the intended viral target site. This includes the antisense construct designed to bind the nucleotide sequence between +1249 and +1268, which possessed the highest energy-related binding characteristics (Table 1), but has a strong potential for self-hybridization (Fig. 2).

The above analysis provides an explanation for the high specificity and the superior inhibitory properties of GPI2A. The sequence exhibits relatively high energy-related binding potential (Table 1), which is coupled with excellent conformational characteristics (Fig. 2). This analysis, along with previous observations of GPI2A sequence specificity (3,4), provide good evidence for an antisense mode of action for the GPI2A oligonucleotide construct, a potential therapeutic agent for the treatment of HIV-1 infections, and a useful tool for analyzing viral gene expression. Three other antisense oligonucleotides that directly flank or partially overlap the GPI2A target site were also good inhibitors of viral gene expression. Their inhibitory effects were greater than anticipated when considering their conformational properties, which indicated a

strong potential to self-complement and/or self-dimerize . Therefore, it is likely that at least part of the inhibitory characteristics of these latter three constructs are due to non-antisense modes of action as previously described for the activity of antisense sequences targeting a variety of different genes (13). These observations are also consistent with the concept that only a few oligonucleotide regions of a gene are in a proper context suitable for antisense design (14,15).

ACKNOWLEDGMENTS

This study was supported by operating grants awarded to J.A.W. from the Manitoba Health Research Council, the NCI (Canada) and NSERC. J.A.W. is a Terry Fox Senior Scientist of the NCI (Canada).

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