

Transcription Factor Decoy Molecules Based on a Peptide Nucleic Acid (PNA)-DNA Chimera Mimicking Sp1 Binding Sites*

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Peptide nucleic acids (PNAs) are DNA-mimicking molecules in which the sugar-phosphate backbone is replaced by a pseudopeptide backbone composed of *N*-(2-aminoethyl)glycine units. We determined whether double-stranded molecules based on PNAs and PNA-DNA-PNA (PDP) chimeras could be capable of stable interactions with nuclear proteins belonging to the Sp1 transcription factor family and, therefore, could act as decoy reagents able to inhibit molecular interactions between Sp1 and DNA. Since the structure of PNA/PNA hybrids is very different from that of the DNA/DNA double helix, they could theoretically alter the molecular structure of the double-stranded PNA-DNA-PNA chimeras, perturbing interactions with specific transcription factors. We found that PNA-based hybrids do not inhibit Sp1/DNA interactions. In contrast, hybrid molecules based on PNA-DNA-PNA chimeras are very effective decoy molecules, encouraging further experiments focused on the possible use of these molecules for the development of potential agents for a decoy approach in gene therapy. In this respect, the finding that PDP-based decoy molecules are more resistant than DNA/DNA hybrids to enzymatic degradation appears to be of great interest. Furthermore, their resistance can even be improved after complexation with cationic liposomes to which PDP/PDP chimeras are able to bind by virtue of their internal DNA structure.

In vitro transfection of cis elements that decoy against nuclear factors leads to alteration of gene expression and was recently proposed in molecular medicine as a novel tool for the possible therapy of several disorders (1–12). One of the most effective decoy approaches so far described involves nuclear proteins belonging to the NF- κ B¹ superfamily (7–9, 13–20).

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¹ The abbreviations used are: NF- κ B, nuclear factor κ B; Sp1, promoter-specific transcription factor Sp1; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; ODN, oligodeoxyribonucleotide; PNA, peptide nucleic acid; PDP, PNA-DNA-PNA chimera; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; ara-C, cytosine arabinoside; HPLC, high pressure liquid chromatography; Lys₄-Chol, tetralysine-cholesterol; Lys₄-Palm, tetralysine-palmitate; lipo-Lys₄-Chol, liposomal formulation of

Decoy molecules against NF- κ B inhibit the expression of NF- κ B regulated genes (*MHC* complex genes, IL2 receptor α , Igk, IL6, δ opioid receptor, preprogalanin, adhesion molecule-1) (20). More recently, dumbbell DNA decoy elements against NF- κ B were demonstrated to be active in inhibiting *ex vivo* transcription driven by the long terminal repeat (LTR) of human immunodeficiency type-1 virus (HIV-1) (19). In addition to proteins belonging to the NF- κ B superfamily, decoy molecules for other target transcription factors, such as HNF-1, RFX1, nuclear factor YB, E2F, cAMP-response element, and Sp1, were found to be effective (1–6, 10–12, 21).

As to the molecular targets for the decoy approach, proteins belonging to the Sp1 family are of great interest since these transcription factors are involved in the regulation of the expression of several genes relevant to human pathologies, including those encoding vascular endothelial growth factor, plasminogen-activator inhibitor type-1 (PAI-1), COL1A2, urokinase-type plasminogen activator (uPA), and uPA receptor (3, 21–25). Sp1 binding sites are also present in the HIV-1 LTR (26). Thus, the development of experimental approaches based on Sp1 decoys to modulate the transcription of Sp1-dependent genes appears to be of great interest (3).

Recently, Ishibashi *et al.* (3) demonstrated that transfection of oligodeoxynucleotides (ODNs) carrying the consensus sequence for Sp1 binding (Sp1 decoy ODNs) was able to inhibit the tumor necrosis factor- α -mediated expression of both vascular endothelial growth factor and transforming growth factor β 1. These results are appealing since it is well known that the expression of these genes is an important aspect in growth and metastasis of solid tumors. In addition, it was found that the *in vitro* invasiveness, synthesis of mRNA for uPA, and cell proliferation were also inhibited by the transfection of Sp1 decoy ODNs, suggesting that Sp1 decoy strategy could be effective for regulating tumor growth by reducing in cancer cell (*a*) angiogenic growth factor expression, (*b*) proliferation, and (*c*) invasiveness. In another report, Verrecchia *et al.* (21) found that decoy Sp1-binding ODNs inhibited *COL1A2* promoter activity both in cultured fibroblasts and *in vivo*, in the skin of transgenic mice, which have integrated a mouse *COL1A2* promoter/luciferase reporter gene construct, indicating that targeting Sp1 efficiently blocks extracellular matrix gene expression, and suggest that such an approach may represent an interesting therapeutic alternative toward the treatment of fibrotic disorders.

The activity of decoy molecules carrying Sp1 binding sites was also studied by Motojima *et al.* (22) and by Hata *et al.* (25). Motojima *et al.* (22) demonstrated that Sp1 decoys are able to

Lys₄-Chol; lipo-Lys₄-Palm, liposomal formulation of Lys₄-Palm; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; glob, globin.

inhibit angiotensin II-induced up-regulation of *PAI-1* gene expression in mesangial cells (22). Hata *et al.* (25) showed that Sp1 decoys reduced expression of kinase domain receptor, a high affinity, endothelial cell-specific, autophosphorylating tyrosine kinase receptor for vascular endothelial growth factor. This transcriptionally regulated receptor is a critical mediator of endothelial cell growth and vascular development (25).

In a recent study, we have investigated the possible use of peptide nucleic acids (PNAs) (27–30) as alternative reagents in experiments aimed at the control of gene expression involving the decoy approach (20, 31). In PNAs, the pseudopeptide backbone is composed of *N*-(2-aminoethyl)glycine units (27). PNAs hybridize with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson-Crick double helices (28, 29), are resistant to both nucleases and proteases (30, 32), and were found to be excellent candidates for antisense and antigene therapies (33–35). We demonstrated that NF- κ B p52 is able to bind to both DNA/DNA and DNA/PNA hybrids mimicking the NF- κ B target sites present in the HIV-1 LTR. On the contrary, low binding of NF- κ B p52 to PNA/PNA hybrids was found (20). We have also reported a conformational study to explain these binding data using a molecular dynamics approach. These data have underlined that the loss of charged phosphate groups and the different shape of the helix in PNA/DNA and PNA/PNA hybrids drastically reduce binding efficiency to NF- κ B transcription factor (31).

More recently, PNA-DNA chimeras have been described as reagents of great interest in gene therapy. PNA-DNA chimeras are PNA-DNA (30, 36–39) covalently bonded hybrids and were designed on the one hand to improve the poor cellular uptake and solubility of PNAs and on the other hand to exhibit biological properties typical of DNA, such as the ability to stimulate RNaseH activity and to act as substrate for cellular enzymes (for instance DNA polymerases). Finally, PNA-DNA chimeras in which the PNA part is attached to 3'- or 5'-ends of the DNA are expected to be particularly stable against degradation by 3'- and/or 5'-exonucleases, which represent the major type of oligonucleotide degrading enzymes found in serum (30).

In the present report, we determined whether PNA/PNA and PNA/DNA hybrid could act as decoy molecules for transcription factor Sp1. Furthermore, we investigated binding to Sp1 transcription factor(s) and biological activity of PNA-DNA-PNA chimeras mimicking Sp1 binding sites.

MATERIALS AND METHODS

Synthetic Oligonucleotides and Peptide Nucleic Acids—The synthetic oligonucleotides used in this study were purchased from Amersham Biosciences. HPLC-purified PNAs were purchased from ISOGEN Biosciences (Maarssen, the Netherlands).

PNA-DNA-PNA Oligomer Assembly—Chimera synthesis proceeded by sequential elongation of the PNA fragment, to which DNA first and then PNA were attached. Aminomethyl-polystyrene-NH₂ resin (loading 37 μ mol/g), resin functionalized with an hexamethylene bisacetamide linker bound through an ester bond to a Gly, was used. The DNA part of the chimera was prepared on an Amersham Biosciences Gene Assembler. Chain elongation was performed with 15 eq of methyl DNA phosphoramidites using 5-(*o*-nitrophenyl)tetrazole (8 eq) as the activator. Standard DNA capping, washing, oxidation, and detritylation cycles were used. Coupling yields were gauged spectrophotometrically (254 nm) by the absorption of the released trityl cation after each deprotection step. In the last DNA elongation step, cyanoethyl 5-amino-5-deoxythymidine phosphoramidite was used (38, 39). The PNA part of the chimera was prepared on a full automated PerSeptive Biosystems Expedite 8900 Nucleic Acid synthesizer (PerSeptive Biosystems, Foster City, CA) using standard (designed for 2- μ mol scale) PNA coupling cycles and solutions. Fmoc (Bz, benzyl)/(iBu, isobutyl)-protected PNA was used. To improve the coupling efficiency of the first PNA moiety, a double coupling cycle was employed (40). Upon completion of the last elongation cycle, the terminal Fmoc group was cleaved by piperidine treatment, and the primary amine was acetylated. The methyl groups

were removed from the phosphate functions by treatment of the resin with 0.25 ml of thiophenol in 0.5 ml of tetrahydrofuran and 0.5 ml of triethylamine for 45 min. The resin was washed consecutively with tetrahydrofuran, methanol, acetonitrile, and water (5 \times 1 ml for each solvent). The oligomers were cleaved from the support with concomitant deprotection of the remaining protective groups by treatment with 0.1 M sodium hydroxide in water/dioxane (1/1, v/v, 1.5 ml) at 55 $^{\circ}$ C for 16 h. The reaction mixtures were neutralized by the addition of acetic acid, concentrated, and redissolved in 0.15 M ammonium bicarbonate. Desalting was performed using a Sephadex G-25 (superfine, DNA grade) gel filtration column with 0.15 M ammonium bicarbonate buffers. Samples were filtered and then purified by reverse-phase-HPLC on a Li-Crosphere 100 RP-18 endcapped column (4 \times 250 mm) on a Jasco HPLC system. Gradient elution was performed at 40 $^{\circ}$ C, building up gradient starting with buffer A (50 mM triethylammonium acetate in water) and applying buffer B (50 mM triethylammonium acetate in acetonitrile/water 1/1 v/v) with a flow rate of 1 ml/min. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analyses were performed on all chimeras as follows: (a) 5842.6 (M+H)⁺ and (b) 5602.0 (M+H)⁺; (a) cag-³²P-TGA GGC GTG GCCA -ggg-Gly and (b) ccc-³²P-TGG CCACGC CTCA -ctg-Gly.

Electrophoretic Mobility Shift Assay—The electrophoretic mobility shift assay (41, 42) was performed using the double-stranded synthetic oligonucleotides mimicking the Sp1 (nucleotide sequences are presented in Table I and in Fig. 1). The DNA stretches of the target molecules were 5'-end-labeled using [γ -³²P]ATP and T4 polynucleotide kinase (MBI Fermentas, Milano, Italy) in the case of DNA/DNA, DNA/PNA, and DNA/PDP hybrids. ³²P-labeled PDP/PDP molecules were obtained by nick translation, using low concentrations (0.1–0.01 units/reaction) of DNase I and [α -³²P]dCTP. Binding reactions were set up as described elsewhere (43) in a total volume of 25 μ l of binding buffer plus 5% glycerol, 1 mM dithiothreitol, and 0.25 ng of ³²P-labeled oligonucleotides. 12 μ g of crude nuclear extracts isolated from human cell lines were used, and the binding reaction was carried out in the presence of 1 μ g of the nonspecific competitor poly(dIdC)-poly(dIdC) (43). After 20 min of binding at room temperature, the samples were electrophoresed at constant voltage (200 V) under low ionic strength conditions (0.25 \times TBE buffer = 22 mM Tris borate, 0.4 mM EDTA) on 6% polyacrylamide gels. Gels were dried and subjected to standard autoradiographic procedures (43). In competition experiments, the competitor molecules carrying HIV-1 Sp1 binding sites (DNA/DNA, PNA/PNA, DNA/PNA, PDP/PDP, and DNA/PDP) were preincubated for 20 min with nuclear extracts, before the addition of labeled target DNA. Nuclear extracts were prepared according to Dignam *et al.* (42). The nucleotide sequences of competitor double-stranded target DNAs used as controls were 5'-TAA TAT GTA AAA ACA TT-3' (sense strand, NF-IL2A), 5'-CAC TTG ATA ACA GAA AGT GAT AAC TCT-3' (sense strand, GATA-1), and 5'-CAT GTT ATG CAT ATT CCT GTA AGT G-3' (sense strand, STAT-1).

Cell Lines and Culture Conditions—Human erythroleukemia K562(S) cells (44) were cultured in a humidified atmosphere at 5% CO₂ in RPMI 1640 (Flow Laboratories) supplemented with 10% fetal bovine serum (CELBIO), 50 units/ml penicillin, and 50 μ g/ml streptomycin (45, 46). Cell growth was studied by determining the cell number/ml after different days of *in vitro* cell culture (46). Stock solutions of ara-C (100 μ M) were stored at -20 $^{\circ}$ C in the dark and diluted immediately before use. Treatment with the indicated concentrations of DNA- and PNA-based molecules was carried out by adding the appropriate concentrations of the compounds at the beginning of the experiment (cells were usually seeded at 30,000 cells/ml). The medium was not changed during the induction period. K562 cells containing heme or hemoglobin were detected by specific reaction with a benzidine/hydrogen peroxide solution as reported elsewhere (45, 46). The final concentration of benzidine was 0.2% in 5 M glacial acetic acid, 10% H₂O₂ (45, 46).

Stability of Decoy Molecules—The stability of decoy molecules was evaluated after incubation of DNA and PNA-DNA-PNA-based decoys with 3' \rightarrow 5'-exonuclease III, 5' \rightarrow 3'- λ -exonuclease, and DNase I. ExoIII, λ -exonuclease, and DNase I were purchased from MBI Fermentas (ExoIII and λ -exonucleases) and Promega Corp., Madison, WI (DNase I). In addition, serum (fetal calf serum, Eurobio, 30 g/liter of protein concentration) was also employed. After incubation with increasing amounts of the enzymes (for 10 min in the case of ExoIII, for 30 min in the case of λ -exonuclease and DNase I), the decoy molecules were layered on the top of a 2% agarose gel and detected by ethidium bromide staining. Disappearance of the decoy molecule was considered as an evidence of degradation by the employed enzymes. Results were presented as percentage of recovery with respect to control untreated reaction mixtures.

TABLE I
Synthetic oligonucleotides, PNA, and PNA-DNA chimeras

PNA stretches are underlined.

	Gene/genome	Entry	Sequence	Length
Oligonucleotides	HIV-1	HIV-Sp1	5'-TGAGGCGTGGCCT-3'	14-mer
		HIV-Sp1c	5'-AGGCCACGCCTCA-3'	14-mer
		HIV-Sp1-L	5'-CAGTGAGGCGTGGCCAGGG-3'	19-mer
		HIV-Sp1-Lc	5'-CCCTGGCCACGCCTCACTG-3'	19-mer
	uPA receptor	uPAR-Sp1	5'-CCAGCCGGCCGCGCCCGGGAAGGGA-3'	26-mer
		uPAR-Sp1c	5'-TCCCTTCCCGGGGCGCGCCGCGCTGG-3'	26-mer
	γ -globin	γ -glob-Sp1	5'-CTAAACTCCACCCATGGGTT-3'	20-mer
		γ -glob-Sp1c	5'-AACCCATGGGTGGAGTTAG-3'	20-mer
	ϵ -globin	ϵ -glob-Sp1	5'-GGACCTGACTCCACCCTGAGG-3'	22-mer
ϵ -glob-Sp1c		5'-CCTCAGGGGTGGAGTCAGGTCC-3'	22-mer	
PNA	HIV-1	HIV-PNA-Sp1	NH ₂ -TGA GG CGT GGC CT-Ac	14-mer
		HIV-PNA-Sp1c	NH ₂ -AGG CCA CGC CTC A-Ac	14-mer
PNA-DNA chimeras	HIV-1	HIV-PDP-Sp1	NH ₂ -CAGTGAGGCGTGGCCAGGG-gly	19-mer
		HIV-PDP-Sp1c	NH ₂ -CCCTGGCCACGCCTCACTG-gly	19-mer

Liposome Preparation—Egg phosphatidyl choline was purchased from Lipid Products (Surrey, England). Tetralysine cationic lipids, tetralysine-cholesterol (Lys₄-Chol), and tetralysine-palmitate (Lys₄-Palm) were a generous gift of Prof. M. Marastoni (Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy). Positively charged liposomes were produced by a protocol based on reverse phase evaporation followed by extrusion of the liposome suspension through polycarbonate filters with homogeneous pore size. Liposomes were subjected to one extrusion cycle through two stacked 400-nm pore size filters followed by three extrusion cycles through two stacked 200-nm pore size membranes in order to obtain unilamellar liposomes with a homogeneous size distribution. Different cationic detergents were alternatively used for the production of the liposomes, namely Lys₄-Chol and Lys₄-Palm (47). The resulting liposomal formulations were named as follows: lipo-Lys₄-Chol and lipo-Lys₄-Palm. The morphological and dimensional analysis of the produced liposomes was performed by freeze-fracture electron microscopy technique and photo correlation spectroscopy (Zetasizer, Malvern, UK). The freeze-fracture electron micrographs (47, 48) confirmed that the extruded liposomal suspension was mainly constituted by unilamellar vesicles. Photon correlation spectroscopy studies demonstrated that the extruded vesicles present a narrow size distribution with an average diameter reflecting the pore size of the employed membrane.

For analysis of protective effects, ³²P-labeled DNA/DNA and DNA/PNA hybrids were incubated with increasing amounts (2–25 μ g/reaction) of lipo-Lys₄-Chol and lipo-Lys₄-Palm for 30 min at room temperature. A further overnight incubation period was then performed in the absence or in the presence of a 25- μ l reaction of serum, and ³²P-labeled material was phenol-extracted, ethanol-precipitated, and analyzed by polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Design of Synthetic Oligonucleotides, PNAs and PNA-DNA Chimeras—The design of the Sp1 oligonucleotides, PNA, and PNA-DNA-PNA chimeras was conducted, taking into account possible solubility and self-annealing problems related to the chemical properties of the molecules. Indeed, it has been reported that PNAs could exhibit low solubility at high G+C/A+T ratios (29, 30). In addition, oligonucleotides mimicking Sp1 binding sites but exhibiting intramolecular self-complementary stretches should not be considered in order to avoid self-hybridization of the molecules to be used for production of a double-stranded decoy. For these reasons, we decided to synthesize oligonucleotides, PNAs, and PNA-DNA-PNA chimeras mimicking a genomic region present within the HIV-1 LTR and containing Sp1 binding sites exhibiting a G+C/A+T ratio lower than Sp1 binding sites present in the promoter sequences of most eukaryotic genes (Table I and data not shown).

The nucleotide sequences of the employed HIV-1 Sp1 oligonucleotides are shown in Table I, together with the other Sp1 oligonucleotides, PNAs, and PNA-DNA chimeras employed in this study. We synthesized two oligonucleotides, HIV-Sp1 (14-mer) and HIV-Sp1-L (19-mer), whose sequences are identical to HIV-Sp1(PNA) and HIV-Sp1(PDP), respectively. The PNA-

DNA-PNA chimera carries a DNA sequence, identical to HIV-Sp1 DNA and HIV-Sp1(PNA), flanked by two PNA stretches at both ends. Since we wanted to define the decoy activity of these molecules in the double-stranded configuration, the complementary oligonucleotides, PNAs, and PNA-DNA chimeras were also synthesized. Two classes of oligonucleotides were also produced: (a) three DNA molecules carrying the Sp1 binding sites present in the promoters of the human urokinase-type plasminogen activator receptor (uPAR-Sp1), ϵ -globin (ϵ -glob-Sp1), and γ -globin (γ -glob-Sp1) genes and (b) additional oligonucleotides carrying unrelated binding sites for NF-IL2A, STAT-1, and GATA-1 transcription factor proteins. The sequences of these oligonucleotides are shown in Table I or stated under "Materials and Methods."

Fig. 1 shows all the decoy molecules analyzed in our study. The DNA/DNA, PNA/PNA, and PDP/PDP hybrids were produced after annealing of the complementary DNA, PNA, and PNA-DNA-PNA molecules. DNA/PNA and PNA/DNA hybrids were produced after annealing of HIV-Sp1 14-mer DNA and HIV-Sp1(PNA). DNA/PDP and PDP/DNA hybrids were produced after annealing of the HIV-Sp1-L 19-mer DNA and HIV-Sp1(PDP).

The Double-stranded PNA/DNA, DNA/PNA, and PNA/PNA Hybrids Carrying Sp1 Binding Sites Are Unable to Inhibit the Interactions between Sp1 Nuclear Factors and Target DNA/DNA Molecules—When 12 μ g of crude nuclear extracts from human leukemic K562 cells were incubated for 20 min in the presence of the cold double-stranded 14- and 19-bp Sp1 DNA/DNA hybrids, a concentration-dependent inhibition of interactions between ³²P-labeled Sp1 14-mer and nuclear factors was observed (Fig. 2A). As expected, the longer double-stranded HIV-Sp1 oligonucleotide displayed higher decoy efficiency with respect to the 14-mer oligonucleotide. 50% inhibition of Sp1-DNA interactions was obtained with 25 ng of the 14-mer oligonucleotide and 6 ng of the 19-mer oligonucleotide (Fig. 2A, lower part of the panel). The experiments shown in Fig. 2B, left side of the panel, and Fig. 2C, lower side of the panel, clearly indicate that DNA/PNA, PNA/DNA, or PNA/PNA HIV-Sp1 molecules are unable, even when added at 100 ng/reaction, to inhibit the interactions between ³²P-labeled Sp1 14-mer and nuclear factors.

The Double-stranded DNA/PDP, PDP/DNA, and PDP/PDP Molecules Carrying PNA-DNA-PNA Chimeras Mimicking Sp1 Binding Sites Efficiently Inhibit the Interactions between Sp1 Nuclear Factors and Target DNA/DNA Molecules—The effects of putative decoy molecules based on PNA-DNA chimeras were at first determined on the molecular interactions between ³²P-labeled Sp1 14-mer and nuclear factors (Fig. 2, B and C); subsequently, their effects were also assayed using the ³²P-

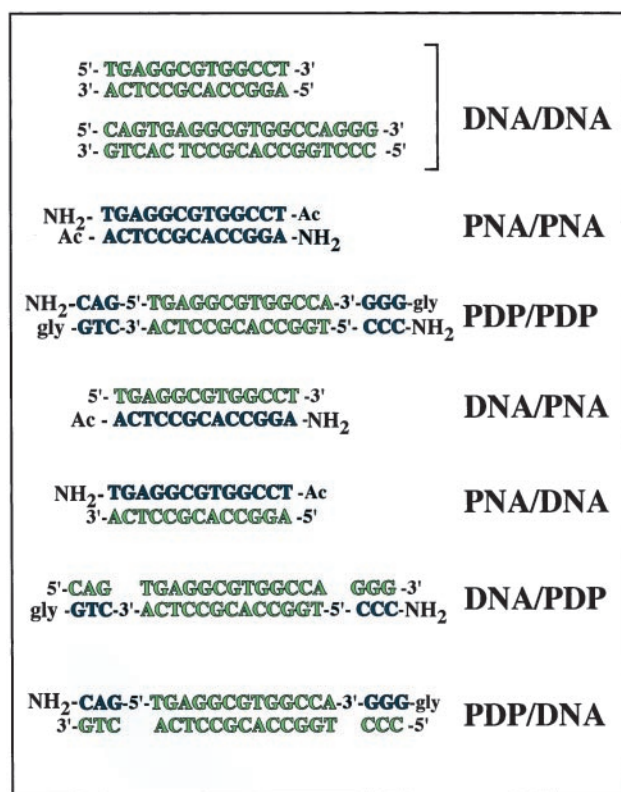


FIG. 1. Structure and sequences of the decoy molecules employed. DNA stretches are in green, PNA stretches are in dark blue.

labeled Sp1 19-mer (Fig. 3). The results obtained clearly indicate that DNA/PDP and PDP/DNA hybrids are efficient decoys (in particular, see Fig. 2C). PDP/PDP molecules are also able to suppress the interactions between ³²P-labeled Sp1 14-mer and nuclear factors, but when added to the binding reaction at a concentration of 25 ng/reaction. These effects were reproducibly obtained also using the ³²P-labeled Sp1 19-mer. In this case, the binding of nuclear factors to the target DNA originates three retarded bands (Fig. 3, arrows). We first performed control experiments demonstrating the decoy effects of the double-stranded HIV-Sp1 19-mer DNA/DNA (Fig. 3A); then, we demonstrated that NF-IL2A and GATA-1 DNA/DNA hybrids had no inhibitory effects on the binding of nuclear factors to the ³²P-labeled Sp1 19-mer (Fig. 3B). Fig. 3C demonstrates that DNA/PDP, PDP/DNA, and PDP/PDP hybrids are able to efficiently inhibit the interactions between the ³²P-labeled Sp1 19-mer and nuclear factors. Fig. 3C (right side of the panel) shows that DNA/PNA, PNA/DNA, and PNA/PNA hybrids carrying Sp1 binding sites do not exhibit any decoy activity even when added at 200 ng/reaction. Finally, HIV-Sp1 PDP/DNA and PDP/PDP hybrids exhibit low ability to inhibit the generation of the fast mobility band. This was reproducibly obtained in independent experiments and tentatively explained by a different affinity of PNA-DNA-based decoy molecules for different proteins (or protein complexes) belonging to the Sp1 family.

The Double-stranded DNA/PDP, PDP/DNA, and PDP/PDP Molecules Carrying PNA-DNA-PNA Chimeras Mimicking HIV-1 Sp1 Binding Sites Do Not Inhibit the Interactions between STAT-1, NF-IL2A, and GATA-1 Transcription Factors to the Relative Target DNA-DNA Sequences—The results reported in Fig. 4, which were performed using ³²P-end-labeled STAT-1, NF-IL2A, and GATA-1 DNA-DNA target molecules and nuclear factors isolated from the K562 cell line, firmly establish that the effects of PNA-DNA chimera-based decoy

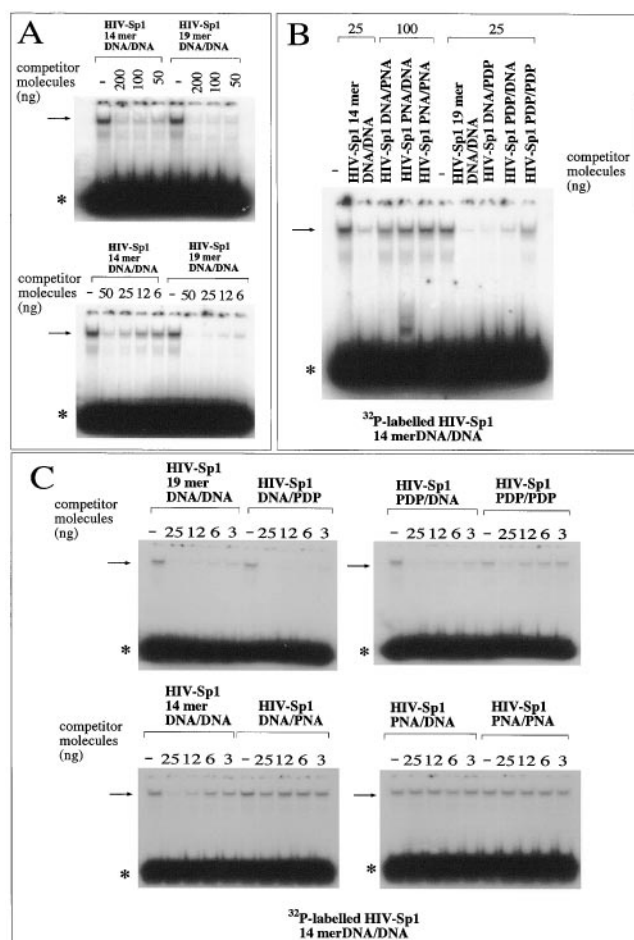


FIG. 2. Effects of DNA-, PNA-, and PDP-based hybrids on molecular interactions between nuclear proteins and ³²P-labeled HIV-1 Sp1 14-mer DNA/DNA target molecules. 12 μ g of K562 nuclear extracts were incubated for 20 min in binding buffer in the absence (–) or in the presence of the indicated amounts of decoy molecules. After this incubation period, a further 20-min incubation step was performed in the presence of ³²P-labeled HIV-1 Sp1 14-mer DNA/DNA target molecules. Protein/DNA complexes were separated by polyacrylamide gel electrophoresis, and autoradiography was performed. Arrows indicate protein/DNA complexes. * indicates the free ³²P-labeled Sp1 14 mer.

molecules are sequence-specific. In fact, although STAT-1, NF-IL2A, and GATA-1 cold oligomers suppress the binding of nuclear factors to the relative ³²P-end-labeled DNA/DNA target molecules, no inhibitory activity was determined by addition of double-stranded PDP/DNA, DNA/PDP, and PDP/PDP chimera mimicking the HIV-1 Sp1 binding sites.

Binding of Nuclear Factors to ³²P-labeled DNA/PDP, PDP/DNA, and PDP/PDP Chimeras—To directly confirm that PNA-DNA chimera-based molecules are recognized by Sp1 factors, we carried out another set of experiments. DNA strands were labeled with [γ -³²P]ATP before annealing to the PNA-DNA-PNA chimera or PNA-relative counterparts (Fig. 5). This allows the generation of ³²P-labeled Sp1 DNA/PNA, PNA/DNA, DNA/PDP, and PDP/DNA molecules. In addition, PDP/PDP molecules were labeled by nick translation reaction, using low amounts of DNase I, as described under “Materials and Methods” and reported elsewhere (56). When these PNA and PDP-based ³²P-labeled hybrids were added to 12 μ g of nuclear extracts from K562 cells, only DNA/PDP and PDP/DNA molecules generated a clearly detectable retarded band (Fig. 5A). The retarded band generated by PDP/PDP hybrids is barely detectable (Fig. 5A, right panel), as expected in consid-

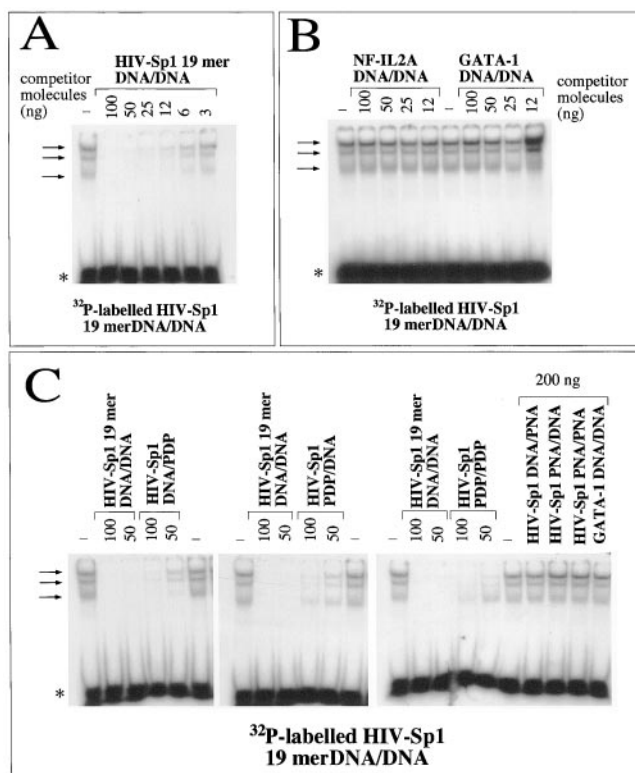


FIG. 3. Effects of DNA-, PNA-, and PDP-based hybrids on molecular interactions between nuclear proteins and ^{32}P -labeled HIV-1 Sp1 19-mer DNA/DNA target molecules. 12 μg of K562 nuclear extracts were incubated for 20 min in binding buffer in the absence (–) or in the presence of the indicated amounts of decoy molecules. After this incubation period, a further 20-min incubation step was performed in the presence of ^{32}P -labeled HIV-1 Sp1 19-mer DNA/DNA target molecules. Protein/DNA complexes were separated by polyacrylamide gel electrophoresis, and autoradiography was performed. Arrows indicate protein/DNA complexes. * indicates the free ^{32}P -labeled Sp1 mer. Control DNA/DNA competitors carrying binding sites for GATA-1 and NF-IL2A were used, as indicated.

eration of the low specific activity of these ^{32}P -labeled molecules (56). On the contrary, Sp1 DNA/PNA and PNA/DNA hybrids were unable to generate retarded bands, as shown in Fig. 5B. In this experimental approach, Sp1 PNA/PNA hybrids were not considered due to the difficulty of obtaining radioactive molecules. The data shown in Fig. 5 confirm on the one hand that Sp1 PNA/DNA hybrids are not efficiently recognized by Sp1 nuclear factors, whereas PDP-based hybrids are recognized by these nuclear proteins. Despite the slightly different retarded patterns obtained, the data shown in Fig. 5A support the hypothesis that the decoy activity of Sp1 PDP-based hybrids (Figs. 2 and 3) is due to the ability of these molecules to interact with transcription factors belonging to the Sp1 family.

The Double-stranded DNA/PDP, PDP/DNA, and PDP/PDP Molecules Carrying PNA-DNA-PNA Chimeras Mimicking the HIV-1 Sp1 Binding Sites Inhibit the Interactions between Nuclear Factors and Sp1 Binding Sites Present within the Promoter of Genes Coding ϵ -Globin, γ -Globin, and uPAR—The experiment reported in Fig. 6 (A–C) was performed using ^{32}P -end-labeled ϵ -globin Sp1 DNA-DNA target molecules and nuclear factors isolated from K562 cells. The results obtained firmly establish that DNA/PDP, PDP/DNA, and PDP/PDP HIV-Sp1 molecules are able to inhibit the binding of nuclear factors to ^{32}P -end-labeled ϵ -globin Sp1 DNA-DNA target molecules when added at high concentrations (50 ng/reaction; Fig. 6C, left side of the panel).

The same extent of inhibition was reached using 6 and 12 ng of ϵ -globin Sp1 DNA/DNA and HIV-Sp1 DNA/DNA hybrids,

respectively (Fig. 6, A and B). Inhibitory effects of DNA/PDP, PDP/DNA, and PDP/PDP HIV-Sp1 molecules were also found on the binding of nuclear factors to ^{32}P -end-labeled γ -globin (Fig. 6D) and uPAR (Fig. 6E) Sp1 DNA-DNA target molecules. These data demonstrate that the HIV-Sp1 decoy is able to inhibit the binding of Sp1 factors to other promoter elements carrying Sp1-like binding sites.

Ex Vivo Effects of PDP-based Decoy Hybrids: Inhibition of ara-C-induced Erythroid Differentiation of K562 Cells—The finding that DNA/PDP, PDP/DNA, and PDP/PDP HIV-Sp1 molecules are able to inhibit the binding of nuclear factors to ^{32}P -end-labeled ϵ -globin and γ -globin Sp1 DNA-DNA target molecules prompted us to determine their effects on the human leukemic K562 cells. This cell line does not produce large amounts of hemoglobin (Fig. 7A) but, after treatment for 5–7 days with a variety of chemical inducers, among which were 5-azacytidine (45), mithramycin (43), tallimustine (44), and cytosine arabinoside (ara-C) (44), they undergo erythroid differentiation, becoming positive to the benzidine stain (benzidine-positive cells as shown in Fig. 7B). Erythroid differentiation of K562 cells is associated with accumulation of Hb Portland ($\zeta_2\gamma_2$) and Hb Gower 1 ($\zeta_2\epsilon_2$) (43). Therefore, activation of both ϵ -globin and γ -globin genes is operated in erythroid-induced K562 cells, as also determined and elsewhere verified by Northern blotting and quantitative reverse transcription-PCR analysis (44). Interestingly, if ara-C-treated K562 are cultured in the presence of HIV-Sp1 DNA/DNA molecules, erythroid differentiation is inhibited (Fig. 7C). In contrast, unrelated double-stranded oligonucleotides are unable to exert this inhibitory effect (Fig. 7C). The finding that Sp1 double-stranded oligonucleotides are able to inhibit erythroid differentiation of K562 cells is in agreement with a number of reports pointing out that Sp1 transcription factor could be involved in transcriptional regulation of the expression of erythroid-specific genes, including the human globin genes (49–54).

Therefore, we determined the activity of HIV-Sp1 DNA/PDP, PDP/DNA, and PDP/PDP molecules on ara-C-treated K562 cells and compared this activity with that of HIV-Sp1 DNA/DNA molecules. Cells were treated for 6 days in the presence or absence of the indicated concentration of Sp1 decoy molecules, and the proportion of benzidine-positive cells was determined. The results obtained are shown in Fig. 7D and demonstrate that all PDP-based Sp1 decoys are able to reduce the ara-C-induced increase of benzidine-positive cells. Among PDP-based decoy molecules, the most active ones on the inhibition of erythroid differentiation were found to be DNA/PDP and PDP/DNA. On the contrary, only slight inhibition was found using PDP/PDP decoy molecules. Control experiments demonstrated that unrelated DNA/DNA molecules were almost ineffective in inhibiting ara-C-mediated increase of the proportion of benzidine-positive cells (data not shown and Fig. 7D). These experiments suggest that PNA-DNA chimera-based decoy molecules for Sp1 nuclear factors are active on *ex vivo* experimental cell systems, their efficiency being very similar to that of Sp1 DNA/DNA decoy molecules, at least in the case of DNA/PDP and PDP/DNA hybrids.

Stability of the Decoy Molecules Based on PNA-DNA-PNA Chimeras—The results of the experiments shown in Figs. 2, 3, and 7 suggest that double-stranded biomolecules based on PNA-DNA chimeras might be considered efficient decoy molecules. To propose these biomolecules for gene therapy, however, two points should be, in our opinion, addressed: stability of the decoy molecules in serum and possible delivery with commonly used vectors, such as liposomes.

To determine the stability of the decoy molecules based on

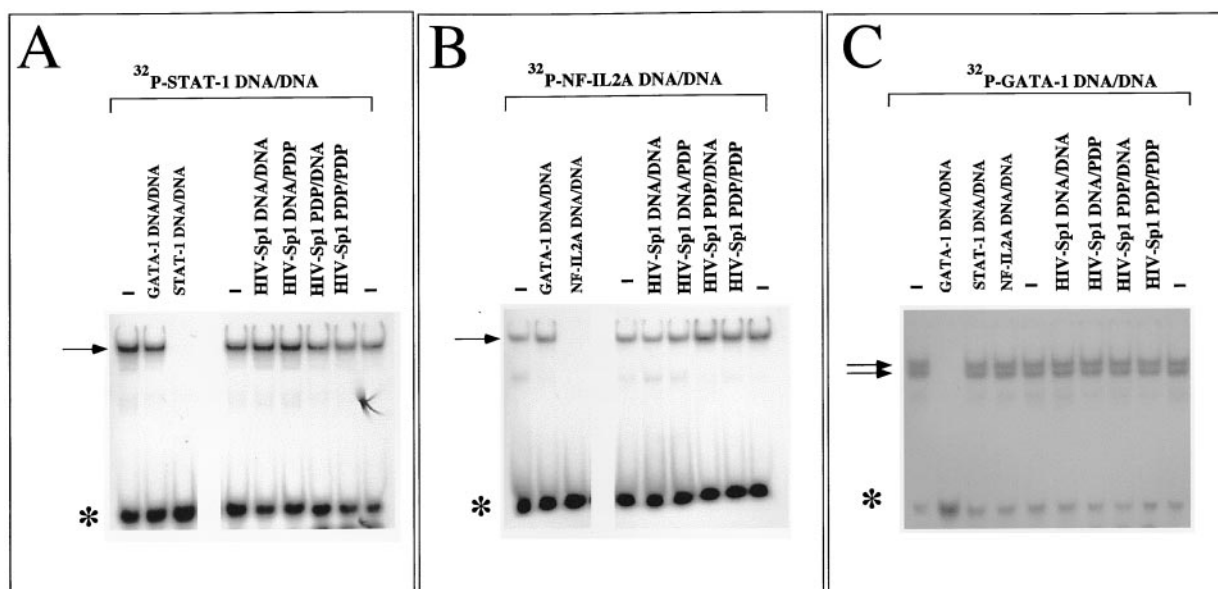


FIG. 4. Effects of DNA/DNA, DNA/PDP, PDP/DNA, and PDP/PDP hybrids carrying Sp1 binding sites on the interaction between crude nuclear extracts from human leukemic K562 cells and ^{32}P -labeled STAT-1 (A), NF-IL2A (B), and GATA-1 (C) DNA-DNA target molecules. 12 μg of nuclear factors were incubated for 20 min in binding buffer in the absence (–) or in the presence of 100 ng of DNA/DNA, DNA/PDP, PDP/DNA, and PDP/PDP molecules. After this incubation period, a further 20-min incubation step was performed in the presence of ^{32}P -labeled DNA/DNA target molecules. Protein/DNA complexes were separated by polyacrylamide gel electrophoresis, and autoradiography was performed. Arrows indicate protein/DNA complexes. * indicates the free ^{32}P -labeled NF-IL2A, GATA-1 and STAT-1 mers. Control DNA/DNA competitors carrying binding sites for STAT-1, NF-IL2A, and GATA-1 were used (100 ng) to verify the specificity of protein/DNA interactions observed.

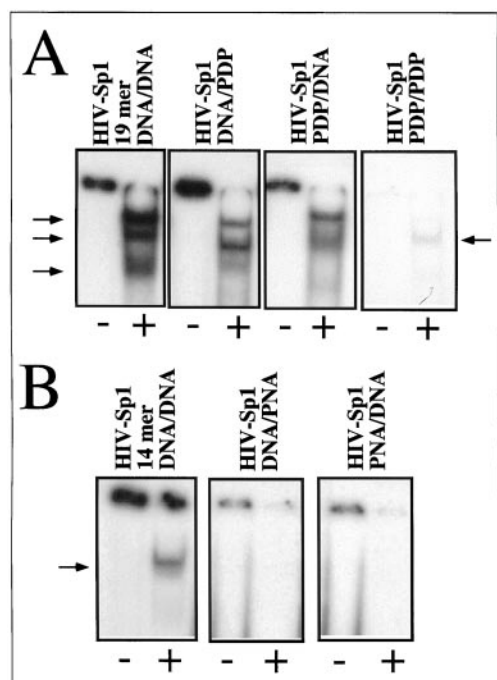


FIG. 5. Direct binding of nuclear factors to PDP- and PNA-based hybrids. ^{32}P -labeled Sp1 DNA/DNA (A and B), DNA/PDP (A), PDP/PDP (A), DNA/PNA (B), and PNA/DNA (B) hybrids were incubated for 20 min in binding buffer in the absence (–) or in the presence (+) of 12 μg of nuclear extracts from K562 cells. Protein/DNA complexes were separated by polyacrylamide gel electrophoresis, and autoradiography was performed. Arrows indicate complexes between proteins and target molecules.

PNA-DNA-PNA chimeras mimicking the Sp1 binding sites, unlabelled PDP/PDP molecules or ^{32}P -end-labeled DNA/PDP and PDP/DNA HIV-Sp1 hybrid molecules were incubated (a) with increasing amounts of 3' \rightarrow 5'-exonuclease (Fig. 8, A and B) and (b) with serum (Fig. 8C).

After incubations, the decoy molecules were isolated, layered on the top of a polyacrylamide (the ^{32}P -labeled decoy molecules) or an agarose gel (the unlabelled decoy molecules), and electrophoresed, and autoradiography was performed in the case of DNA/PDP or DNA/DNA molecules (Fig. 8A). In the case of PDP/PDP hybrids (Fig. 8B) or when serum was employed (Fig. 8C), gels were stained with ethidium bromide. This was necessary due to the 5'-phosphatase activity present in serum, leading to a rapid removal of 5'- ^{32}P (data not shown). Disappearance of the ^{32}P -end-labeled or ethidium bromide-stained bands indicates that degradation of the decoy molecules has occurred. The observed stabilities of PDP/PDP hybrids or DNA/PDP molecules were compared with those of DNA/DNA decoy molecules.

Fig. 8 clearly demonstrates that Sp1 PDP/PDP decoy molecules are resistant to ExoIII 3' \rightarrow 5'-exonuclease (Fig. 8B), unlike the corresponding DNA/DNA hybrid. PDP/PDP chimeras are also resistant to serum (Fig. 8C, lower part of the panel). When experiments were conducted using DNA/PDP hybrids, the results obtained demonstrated that DNA/PDP hybrids exhibit a high level of resistance to both 3' \rightarrow 5'-exonuclease (Fig. 8A) and serum (Fig. 8C, middle part of the panel) as compared with HIV-Sp1 DNA/DNA decoy oligonucleotides. These results were consistently reproduced in three independent experiments; in addition, results similar to those obtained with DNA/PDP molecules were also obtained using PDP/DNA decoy hybrids (data not shown). Furthermore, DNA/PDP and PDP/DNA molecules were consistently found more resistant than DNA/DNA hybrids to 5' \rightarrow 3'-exonuclease and DNase I and when exposed to cellular extracts.² Taken together, these data demonstrate that decoy molecules based on PNA-DNA-PNA chimeras exhibit higher levels of resistance to nucleases with respect to decoy molecules based on DNA/DNA hybrids.

Complexation of PDP-based Decoy Molecules to Cationic Liposomes—Different cationic detergents were used, namely

² M. Borgatti, I. Lampronti, A. Romanelli, C. Pedone, M. Saviano, N. Bianchi, C. Mischiati, and R. Gambari, manuscript in preparation.

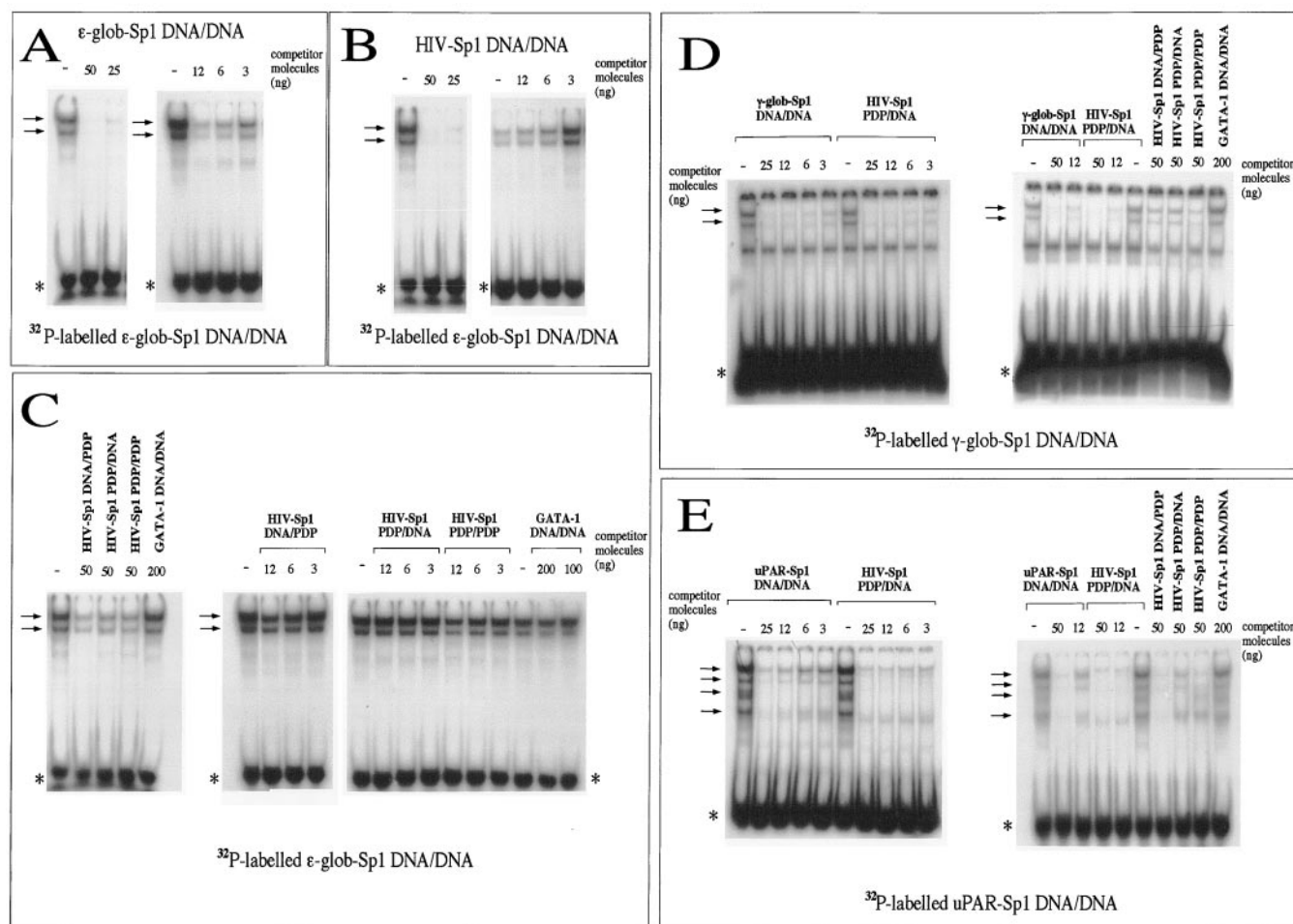


FIG. 6. Effects of DNA-, PNA-, and PDP-based hybrids on molecular interactions between nuclear proteins and ^{32}P -labeled Sp1 binding sites present in the ϵ -globin (A-C), γ -globin (D), and uPAR (E) promoters. 12 μg of K562 nuclear extracts were incubated for 20 min in binding buffer in the absence (-) or in the presence of the indicated amounts of decoy molecules. After this incubation period, a further 20-min incubation step was performed in the presence of ^{32}P -labeled ϵ -glob-Sp1, γ -glob-Sp1, and uPAR target molecules. Protein/DNA complexes were separated by polyacrylamide gel electrophoresis, and autoradiography was performed. Arrows indicate protein/DNA complexes. * indicates the free ^{32}P -labeled Sp1 mer. Control DNA/DNA competitor carrying binding sites for GATA-1 was used, as indicated.

Lys₄-Chol and Lys₄-Palm, for the production of the liposomal formulation (47, 48). The resulting liposomal formulations were named as follows: lipo-Lys₄-Chol and lipo-Lys₄-Palm. The complexation of PNA-DNA chimeras to liposomes was performed just before the analysis, simply mixing the nucleic acid to "preformed" cationic vesicles, resulting in a quantitative association yield.

Fig. 9 (A and B) demonstrates that Lys₄-Chol and Lys₄-Palm are able to complex to ^{32}P -end-labeled HIV-Sp1 DNA/PDP and PDP/DNA hybrids. This is demonstrated by the formation of complexes (identified as bound material) unable to migrate into the gels when high concentrations of liposomes were used (1–2 μg /reaction). It should be noted that complexation of Sp1 DNA/PDP and PDP/DNA to both Lys₄-Palm and Lys₄-Chol is similar to complexation of Sp1 DNA/DNA hybrids. Both Lys₄-Chol and Lys₄-Palm are also able to complex with PDP/PDP HIV-Sp1 (Fig. 9C). In this case, due to the difficulty in obtaining ^{32}P -labeled PDP/PDP chimeras (56), unlabelled PDP/PDP molecules were used, and the gels were stained with ethidium bromide. The data presented in Fig. 9 conclusively demonstrate that Sp1 decoy molecules based on PNA-DNA chimeras can be complexed to cationic liposomes.

These results are relevant for the delivery of PDP-based decoys, as well as for a possible increase of their resistance to nucleases. This could be particularly important for PDP/DNA and DNA/PDP molecules. To determine whether liposome com-

plexation leads to an increase of resistance of PDP/DNA molecules to degradation in serum, the experiment shown in Fig. 9D was performed. Free or liposome-complexed ^{32}P -end-labeled DNA/PDP HIV-Sp1 hybrid molecules were incubated in the presence of serum concentrations causing complete degradation of the PNA/DNA molecules (as shown in Fig. 8C, lane d). The results obtained clearly suggest that complexation of Sp1 DNA/PDP molecules to both Lys₄-Chol and Lys₄-Palm liposomes leads to a protective effect against enzymatic degradation.

DISCUSSION

The transcription factors belonging to the Sp1 superfamily are of great importance for the control of expression of a variety of genes. Members of this family of regulatory proteins bind with varying affinities to sequences designated as "Sp1 sites" (for instance GC and CACCC boxes), making up a transcriptional network playing an important role in the fine-tuning of gene expression. Sp1-dependent transcription can be growth-regulated, and the activity, expression, and/or post-translational modification of multiple family members is altered with cell growth. Furthermore, Sp1 factors are involved in many growth-related signal transduction pathways, apoptosis, and angiogenesis and, therefore, in several aspects of tumorigenesis (51).

With respect to gene therapy, the decoy approach against Sp1 transcription factors has been proposed as a useful tool to

FIG. 7. Effects of DNA-, PNA-, and PDP-based hybrids on ara-C-mediated induction of erythroid differentiation of K562 cells. A and B, benzidine staining of uninduced (A) and ara-C-induced K562 (B) cells. C, increase in the proportion of benzidine-positive cells cultured with 1 μ M ara-C in the absence (closed symbols) or in the presence (open symbols) of 5 μ g/ml HIV-1 Sp1 DNA/DNA molecules. D, effects of DNA/DNA, DNA/PDP, PDP/DNA, and PDP/PDP molecules on increase of benzidine-positive cells after 6 days of cell culture in the presence of 5 μ g/ml decoy molecules. (—), ara-C-treated K562 cells.

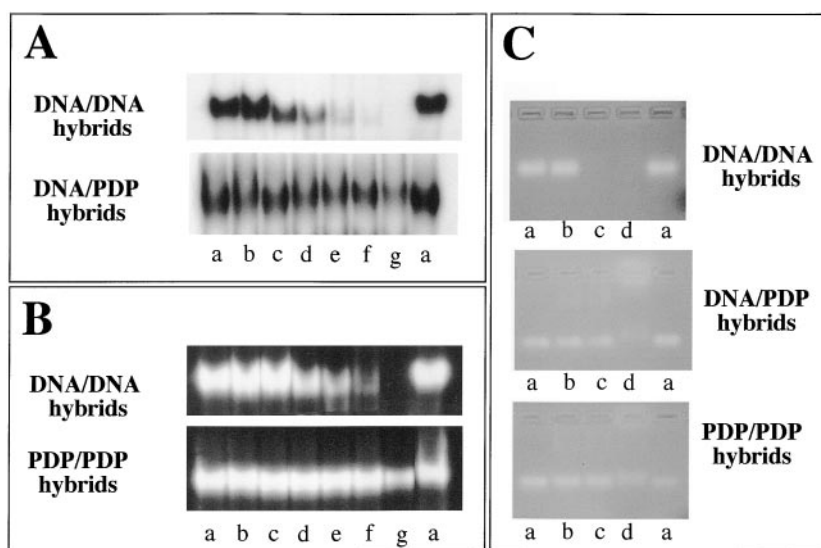
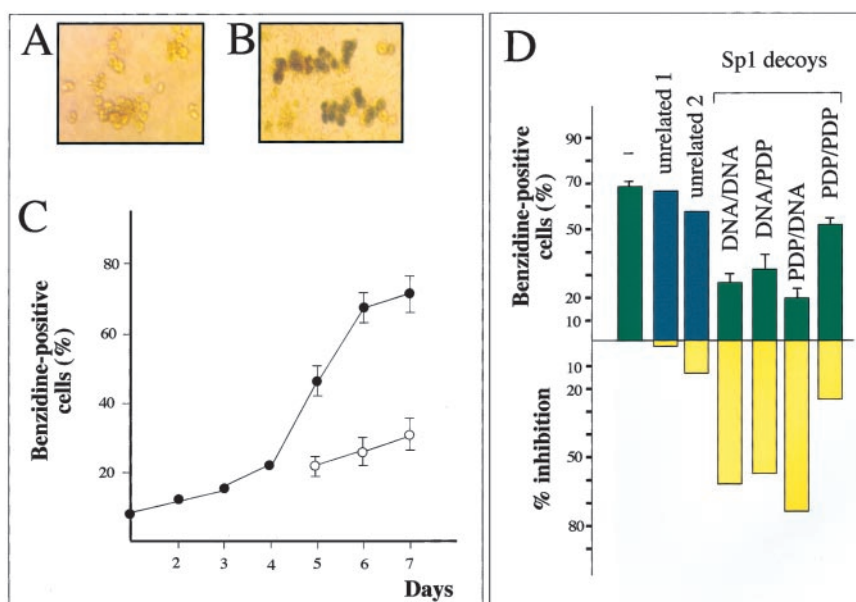


FIG. 8. Stability of decoy molecules. A and B, experiments showing the effects of Exo III on DNA/DNA (A and B), DNA/PDP (A), and PDP/PDP (B) decoy molecules. 250 ng of HIV-1 Sp1 PDP/PDP, DNA/PDP, and DNA/DNA decoys were incubated for 10 min in the absence (a) or in the presence of 0.001 (b), 0.01 (c), 0.1 (d), 1 (e), 10 (f), and 100 (g) units of Exo III in a 20- μ l reaction mixture. After incubation, the decoy molecules were layered on the top of a 20% polyacrylamide gel and detected by autoradiography (A) or on 2% agarose gels and detected by ethidium bromide staining (B). C, differential effects of serum (a = no serum; b = 3 μ l/reaction; c = 12.5 μ l/reaction) on DNA (upper part of the panel) and PNA-DNA-PNA (middle and lower parts of the panel)-based decoys. Incubations were conducted at 37 $^{\circ}$ C for 3 h in a 50- μ l reaction mixture. After incubation, the decoy molecules were layered on the top of a 2% agarose gel and detected by ethidium bromide staining. DNA/DNA and DNA/PDP hybrids were 32 P-labeled in panel A. In panels B and C, unlabelled decoy molecules were used. Disappearance of the decoy molecules was considered as an evidence of degradation by the employed enzymes.

alter Sp1-dependent gene expression (3, 21, 25). This was achieved by using synthetic ODNs carrying Sp1-specific cis elements as decoy molecules.

In fact, Ishibashi *et al.* (3) demonstrated that Sp1 decoy ODNs are able to inhibit the tumor necrosis factor- α -mediated expression of vascular endothelial growth factor, transforming growth factor β 1, and tissue factor by cancer cells. In another report, Verrecchia *et al.* (21) found that decoy Sp1-binding oligonucleotides inhibited *COL1A2* promoter activity both in cultured fibroblasts and *in vivo*. Motojima *et al.* (22) demonstrated that Sp1 decoys are able to inhibit angiotensin II-induced expression of *PAI-1* gene in mesangial cells. Finally, Hata *et al.* (25) showed that treatment with Sp1 decoys reduced expression of kinase domain receptor, a tyrosine kinase receptor for vascular endothelial growth factor. This transcription-

ally regulated receptor is a critical mediator of endothelial cell growth and vascular development. Unfortunately, synthetic ODNs are not stable and, therefore, should be extensively modified to be used *in vivo* or *ex vivo* (1–7).

In a recent study, we proposed PNAs as alternative reagents in experiments aimed at the control of gene expression involving the decoy approach (20). In PNAs, the pseudopeptide backbone is composed of *N*-(2-aminoethyl)glycine units (27–30). PNAs hybridize with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson-Crick double helices (27), and are resistant to both nucleases and proteases (32). We demonstrated that NF- κ B p52 is able to bind to both NF- κ B DNA/DNA and DNA/PNA hybrid, mimicking the NF- κ B target sites present in the HIV-1 LTR. However, the binding of the NF- κ B DNA-PNA to NF- κ B transcription factors

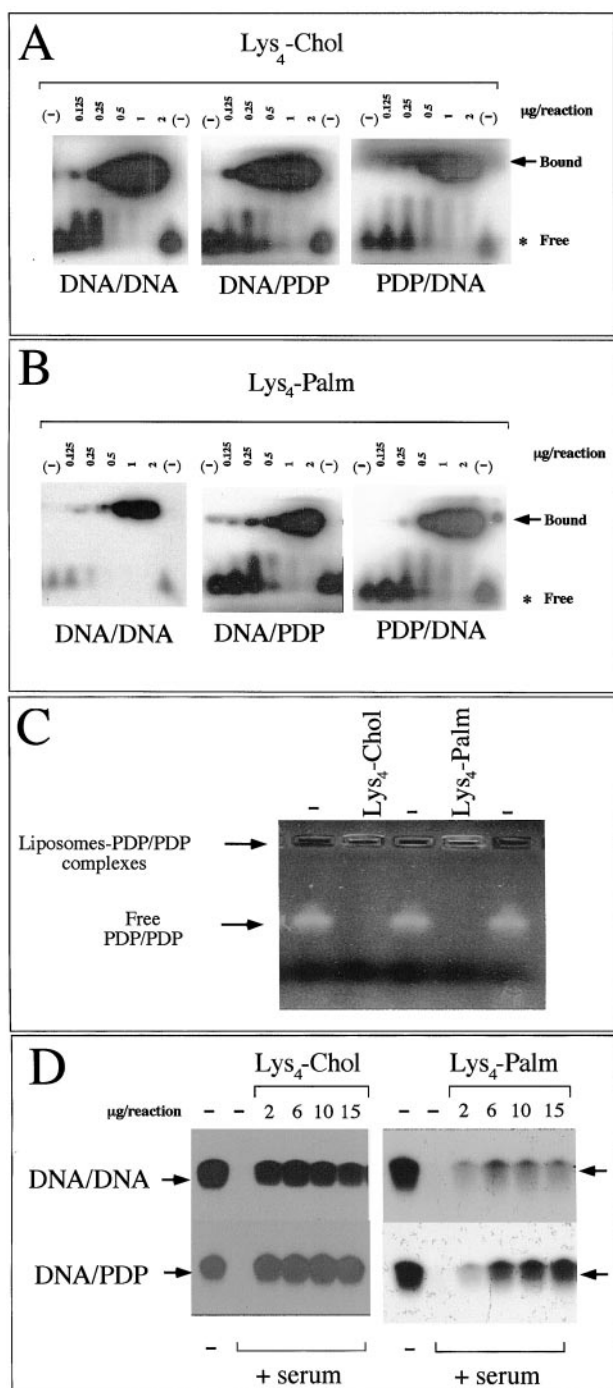


FIG. 9. Comparison of complexation efficiencies of Sp1 DNA/DNA, DNA/PDP and PDP/DNA hybrids to the different liposomal formulations and protective effects. As shown in A–C, the indicated amounts of liposomes were incubated in the presence of radiolabeled hybrid molecules, and formed complexes were electrophoresed through an agarose gel and exposed to autoradiographic procedure. Complexation of Sp1 DNA/DNA, DNA/PDP, and PDP/DNA hybrids with liposomes were performed using the multivalent cationic lipid Lys₄-Chol (lipo-Lys₄-Chol) (A) or Lys₄-Palm (lipo-Lys₄-Palm) (B). C, complexation of Sp1 PDP/PDP molecules to lipo-Lys₄-Chol (Lys₄-Chol) and lipo-Lys₄-Palm (Lys₄-Palm). In this case, detection was performed on unlabelled PDP/PDP hybrids by ethidium bromide staining of molecules after agarose gel electrophoresis. D, protective effects of lipo-Lys₄-Chol and lipo-Lys₄-Palm. ³²P-labeled DNA/DNA and DNA/PDP hybrids were incubated for 30 min with or without the indicated amounts of liposomes (final reaction volume = 50 μl). A further overnight incubation period (final reaction volume = 75 μl) was then performed in the absence or in the presence of serum (25 $\mu\text{l}/\text{reaction}$), as indicated. ³²P-labeled material was phenol-extracted, ethanol-precipitated, and analyzed by polyacrylamide gel electrophoresis and autoradiography.

was found to exhibit low stability and, therefore, this reagent is expected to be unsuitable for a decoy approach (20).

More recently, we determined whether PNA-DNA chimeras mimicking NF- κ B binding sites are capable of stable interactions with proteins belonging to the NF- κ B family (55). DNA-PNA chimeras were originally proposed to improve the poor cellular uptake and solubility of PNAs (30). More recently, they were found to exhibit biological properties typical of DNA, such as the ability to stimulate RNaseH activation and to act as substrate for cellular enzymes (for instance, DNA polymerases) (30). Molecular modeling was employed for the design of PNA-DNA chimeras; prediction of molecular interactions between chimeras and NF- κ B nuclear proteins were investigated by molecular dynamics simulations, and interactions between PNA-DNA chimeras and NF- κ B proteins were studied by gel shifts. We found significant differences between the structure of duplex NF- κ B PNA-DNA chimera and duplex NF- κ B DNA-DNA (31). However, it was found that these differences do not prevent the duplex PNA-DNA chimera from binding to NF- κ B transcription factors, being able to suppress the molecular interactions between HIV-1 LTR and p50, p52, and nuclear factors from B-lymphoid cells. Therefore, these results demonstrate that the designed NF- κ B DNA-PNA chimeras could be used for a decoy approach in gene therapy.

In the present report, our main focus was to determine whether decoy molecules based on PNA and PNA-DNA chimeras are capable of stable interactions with Sp1 and, therefore, could act as decoy reagents able to inhibit molecular interactions between Sp1 and DNA. The results obtained firmly indicate that Sp1-molecules based on PNA-DNA-PNA chimeras are powerful decoy reagents (Figs. 2, 3, and 6). This effect is specific since these decoys do not affect binding of NF-IL2A, GATA-1, and STAT-1 to their binding elements.

A second conclusion to be drawn from the results of this report is that PDP/DNA and DNA/PDP Sp1 decoy molecules exhibit the ability to inhibit ara-C-induced erythroid differentiation of human leukemia K562 cells to an extent similar to that of Sp1 DNA/DNA decoys. This finding supports the hypothesis of a biological activity of decoy molecules based on PNA-DNA chimeras.

Our results are expected to have practical implications. The finding that DNA-PNA chimeras stably interact with Sp1 transcription factors encourages further experiments focused on the possible use of these molecules for the development of potential agents for a decoy approach in gene therapy. In this respect, the finding that PDP-based decoy molecules are more resistant than DNA/DNA hybrids to enzymatic degradation (Fig. 8 and data not shown) appears to be of great interest. Furthermore, their resistance can even be improved, and their delivery could be facilitated after complexation with cationic liposomes or microspheres (56) to which PDP/PDP chimeras are able to bind by virtue of their internal DNA structure.

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