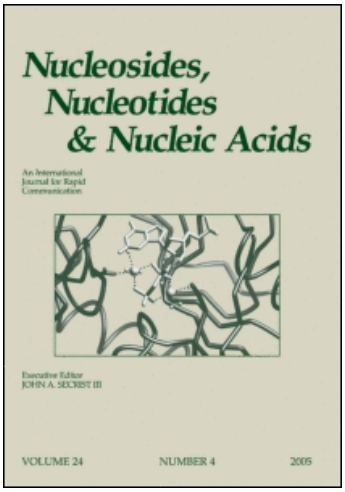

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ANTISENSE PHOSPHOROTHIOATE OLIGONUCLEOTIDES TARGETED TO THE HUMAN CHEMOKINE RECEPTOR CXCR4

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ABSTRACT: The CXC chemokine receptor CXCR4 is used as a major co-receptor for fusion and entry by syncytia-inducing T-tropic (X4) isolates of HIV-1. In the present study, we report the effects of an antisense oligodeoxyribonucleotide on the inhibition of CXCR4 gene expression in X4 HIV-1 infected HeLa-CD4 cells, to find more efficacious therapeutic possibilities for Human Immunodeficiency Virus type 1 (HIV-1) infection. Antisense phosphorothioate oligodeoxyribonucleotides (anti-S-ODNs) corresponding to the sequence of bases 69 to 88 of the human CXCR4 mRNA gene were synthesized. When the naked anti-S-ODN was incubated with HeLa-CD4 cells, the surface levels of this chemokine receptor were reduced up to 50%, indicating sequence-specific inhibition. We also examined the concomitant use of a basic peptide transfection reagent, nucleosomal histone proteins (RNP), for delivery of anti-S-ODNs. The anti-S-ODN encapsulated with RNP had higher inhibitory effects on p24 products than the naked anti-S-ODN.

INTRODUCTION

HIV-1 enters cells by binding to the cell surface CD4 and coreceptor molecules.[1-4] Although the list of possible coreceptors is continuously expanding, the major coreceptors are CCR5 and CXCR4, which facilitate the cell entry of the macrophage-tropic (R5)[1-3,5,6] and X4 strains of HIV-1, respectively. RANTES, macrophage-inflammatory protein

#This article is dedicated to the memory of Professor Alexander Krayevsky.

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(MIP)-1α, and MIP-1ß are ligands for CCR5 and block R5 HIV-1 infection[7], whereas stromal cell-derived factor-1 (SDF-1) is a ligand for CXCR4 and blocks X4 HIV-1 infection.[8,9] The chemokines, chemokine modifications, peptides, and nonpeptide receptor antagonists or agonists have become important agents for the treatment of HIV-1 infection.[7,10,11] Recently, two groups have reported that the horseshoe crab blood cell-derived peptide, T22[12], and the nonpeptide, TAK-779[13], act as CXCR4 and CCR5 antagonists that can inhibit infection by X4 and R5 HIV-1. Based on these findings, we postulate that down regulation of CXCR4 expression may be an effective and harmless way to inhibit HIV-1 infection. The use of antisense ODNs has been regarded as a potential therapeutic approach with reference to the gene levels.[14-16] Their sequences are complementary to the respective target mRNAs, whereby they inhibit the translation of the specific mRNA. One of the most interesting applications of antisense ODNs is anti-HIV-1 gene therapy, which is based on the use of antisense ODNs targeted to viral transcripts.[17-19]

In this study, we present a detailed analysis of the inhibition of functional expression of the HIV-1 coreceptor, CXCR4, by anti-S-ODNs complementary to the CXCR4 mRNA. We also describe the potential of a basic peptide transfection reagent, nucleosomal histone proteins.[20,21] Using fluorescent isothiocyanate (FITC)-labeled-anti-S-ODNs, we assessed the cellular up-take and intercellular distribution of the anti-S-ODNs.

RESULTS AND DISCUSSION

Inhibition of HIV-1 replication by naked anti-S-ODN in HeLa-CD4 cells.

The HeLa-CD4 cells were used as the target cells, since they were infected with the X4 HIV-1 *NL432*. We selected an antisense sequence, including the initiation codon. Since it has been suggested as a target site in several other mRNAs, many investigators have chosen to target the translation initiation site.

We examined the inhibitory effects of the functional expression of the CXCR4 receptor in HeLa-CD4 cells by the naked anti-S-ODN containing the AUG initiation codon at the center of the oligodeoxyribonucleotide. The anti-S-ODN was directly added to the cultured cells, as indicated in the Experimental, and then the cells were incubated at 37°C. The anti-S-ODN had an inhibitory effect on the p24 antigen, and caused 35% inhibition at the high concentration of 10 μM (Figure 1). Furthermore, to clarify the sequence-specificity, we tested S-ODNs containing sen-S-ODN and scr-S-ODN sequences with the same base composition as that of the anti-S-ODN target. The control sequences, the sen- and scr-S-ODNs, had no inhibitory effects (Figure 1). These results suggest that the anti-

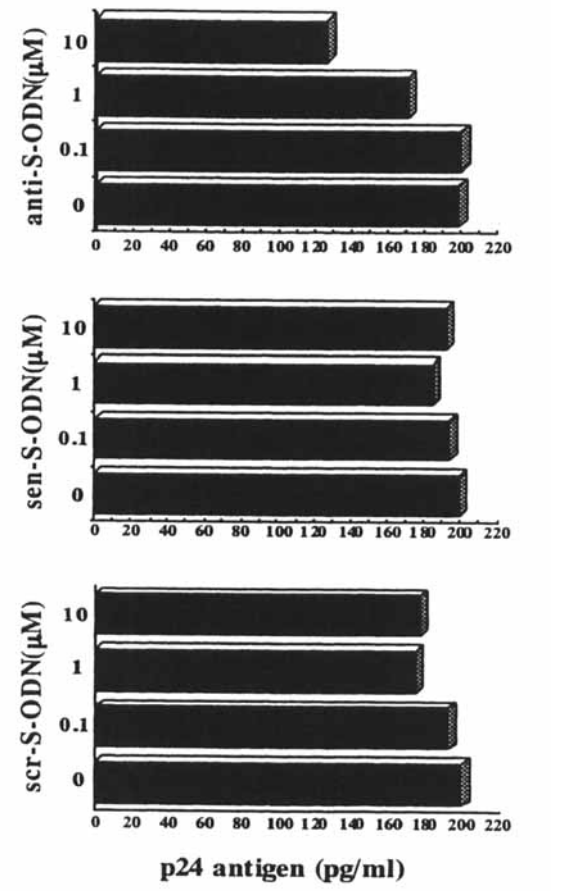

FIG. 1. Sequence-specific inhibition of HIV-1 infection in HeLa-CD4 cells by naked oligodeoxyribonucleotides (anti-, sen-, and scr-S-ODN). HeLa-CD4 cells were infected with HIV-1 NL432 in the presence or absence of different concentrations of naked oligodeoxyribonucleotides (anti-, sen- and scr-S-ODNs). Aliquots of the culture supernatant were assayed for HIV-1 gag-p24 content by ELISA. The experiment reported is representative of a set of three different experiments.

S-ODNs may inhibit a CXCR4 expression by a mechanism of antisense competitive hybridization to the target CXCR4 mRNA.

In order to clarify the inhibition of CXCR4 expression with the anti-S-ODN, the surface CXCR4 expression on HeLa-CD4 cells was detected by flow cytometry using the monoclonal anti-CXCR4 antibody (12G5 Mouse IgG2a). As shown in Figure 2, significant inhibition of CXCR4 expression on the cell surface was observed in cells treated with the anti-S-ODN. In these experiments, the surface CXCR4 levels were reduced up to 50% as compared with the controls when a high concentration of anti-S-

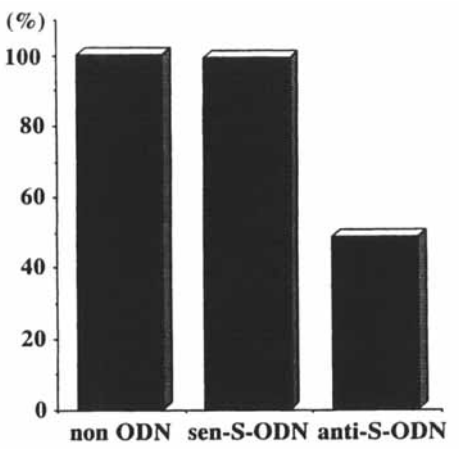

FIG. 2. Inhibition of CXCR4 expression in HeLa-CD4 cells by naked anti-S-ODN. HeLa-CD4 cells were incubated with 10 μM of naked oligodeoxyribonucleotides (anti- and sen-S-ODNs). The experiment reported is representative of a set of three different experiments.

ODN was used. In contrast, almost no inhibition was observed when the inhibition reaction was carried out using the control ODN, sen-S-ODN, instead of the anti-S-ODN. These results also suggest that the down-regulation of CXCR4 expression by the anti-S-ODN may block HIV-1 entry into human cells.

Localization of encapsulated and naked FITC-S-ODNs in HeLa-CD4 cells.

The *in vitro* and *in vivo* applications of antisense ODNs are hampered by several limitations. Factors such as ODN stability, cellular uptake, subcellular viability, and other pharmacokinetic parameters lead to relatively poor delivery to the targeted molecular sites, and make the treatment cost prohibitive.[21-23] Thus, transport and intracellular delivery are important and fundamental considerations when developing an effective ODN-based therapy. The delivery system is one technique that addresses these concerns. Attempts to use liposomes in the delivery of antisense ODNs have been reported.[24-28]

Efficient cell penetration by antisense ODNs is a critical step in their molecular targeting. First, we analyzed the efficiency of the delivery of the encapsulated FITC-labeled-anti-S-ODN with a basic peptide transfection reagent, nucleosomal histone proteins (RNP)[20,21], and a cationic liposome (DMRIE-C reagent)[29] into HeLa-CD4 cells using the FACSCalibur flow cytometer. The nucleosomal histone proteins, which are known as basic proteins, possess nuclear localization signals and helical domains. The RNP was prepared as indicated in the Experimental. As shown in Figure 3, the amount of

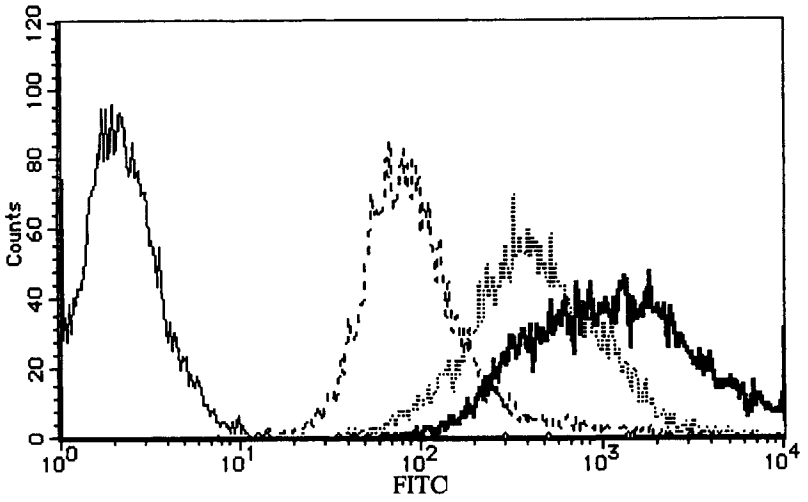

FIG. 3. Cellular uptake of FITC-labeled-anti-S-ODN. HeLa-CD4 cells were incubated with FITC-labeled-anti-S-ODN for 4 h and then were analyzed by the FACSCalibur cytometer and the CellQuest software. The thin-line histograms with shading represent the control HeLa-CD4 cells in the absence of the FITC-labeled-ODN. Comparison of the uptake of the naked FITC-labeled-anti-S-ODN and the FITC-labeled-anti-S-ODN encapsulated with the transfection reagents, RNP and DMRIE-C. The thin-line histograms represent the naked FITC-labeled-anti-S-ODN treatment of HeLa-CD4 cells. The dotted-line histograms represent the encapsulated FITC-labeled-anti-S-ODN with RNP treatment of HeLa-CD4 cells. The broken-line histograms represent the encapsulated FITC-labeled-anti-S-ODN with DMRIE-C treatment of HeLa-CD4 cells.

the encapsulated FITC-labeled-anti-S-ODN with RNP was relatively high as compared with the naked FITC-labeled-anti-S-ODN and the encapsulated FITC-labeled-anti-S-ODN with DMRIE-C in HeLa-CD4 cells.

However, these flow cytometric experiments do not distinguish intracellular from surface-bound material; even though the cells were washed, some material may adhere to the external surface of the cell. Therefore, the distribution of the encapsulated FITC-labeled-anti-S-ODN with RNP in HeLa-CD4 cells was investigated using laser-assisted confocal microscopy. The results showed that the fluorescent signals were apparent in the nucleus (Figure 4B). In contrast to this observation, fluorescent signals were weakly observed in the endosomes and cytoplasms of Hela-CD4 cells treated with the naked FITC-labeled-anti-S-ODN (Figure 4A). Thus, in the presence of the RNP transfection reagent, the antisense oligodeoxyribonucleotides first enter the cytoplasm and then quickly accumulate in the nucleus. Therefore, our data also suggest that the encapsulated FITC-labeled-anti-S-ODN was located in the nucleus and the cytoplasm of HeLa-CD4 cells.

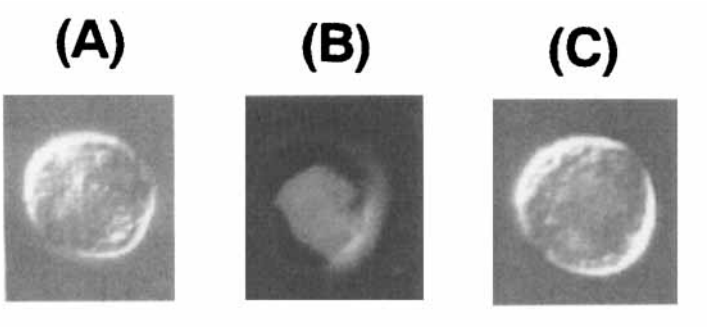

FIG. 4. Intracellular distribution of FITC-labeled-anti-S-ODN. HeLa-CD4 cells were incubated with the naked FITC-labeled-anti-S-ODN (A), the FITC-labeled-anti-S-ODN encapsulated with RNP (B), and without the FITC-labeled-anti-S-ODN (C). The fluorescence was observed with a scanning confocal microscope.

Ideally, the FITC-labeled-anti-S-ODN encapsulated with RNP would penetrate the HeLa-CD4 cells and the anti-S-ODN would hybridize to the target sequence in the mRNA.

Inhibition of the chemokine receptor CXCR4 function by the encapsulated anti-S-ODN in HeLa-CD4 cells.

Antisense oligonucleotides with phosphorothioate backbones exhibit several advantages over the other forms, including relatively high nuclease resistance and the capacity to induce the degradation of the target sequence by RNase H. However, a problem in the use of anti-S-ODNs is their inefficient cellular uptake. They are mainly found in endosomes and lysosomes. We investigated the inhibition of the functional expression of the CXCR4 receptor gene by the anti-S-ODN encapsulated with a basic peptide transfection reagent, nucleosomal histone proteins (RNP).[20,21] The RNP was prepared as indicated in the Experimental.

The anti-S-ODN encapsulated with RNP showed 70% inhibition of p24 antigen expression at a low concentration of 1 μM, in a sequence-specific manner. For the control sequence, the sen-S-ODN, we could not detect any inhibitory effects at a concentration of 1 μM. These results suggest that the encapsulated anti-S-ODN conferred sequence specific inhibition (Figure 5). Furthermore, we examined the inhibition of HIV-1 replication by the anti-S-ODN encapsulated within cationic liposomes (DMRIE-C reagent)[29] in HeLa-CD4 cells. The anti-S-ODN encapsulated with RNP showed higher inhibitory effects than the anti-S-ODN encapsulated with cationic liposomes (Figure 5). The RNP transfection reagent has a significant advantage over the use of the anti-S-ODN

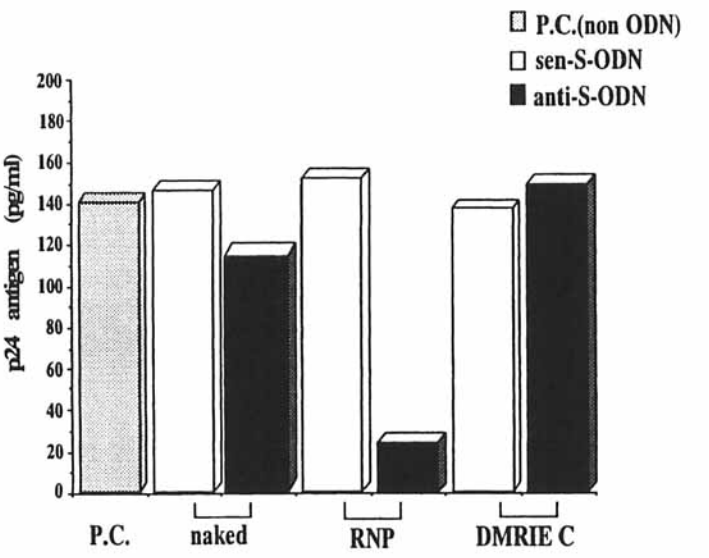

FIG. 5. Inhibition of HIV-1 infection in HeLa-CD4 cells by anti-S-ODN encapsulated with either the transfection reagent, RNP, or liposomes (DMRIE-C). HeLa-CD4 cells were infected with HIV-1 NL432 in the presence of 1 μM concentrations of the naked and encapsulated oligodeoxyribonucleotides (anti- and sen-S-ODNs). Aliquots of the culture supernatant were assayed for HIV-1 gag-p24 content by ELISA. The experiment reported is representative of a set of three different experiments.

alone for the delivery of the anti-S-ODN into HeLa-CD4 cells. These results suggest that a basic peptide transfection reagent, nucleosomal histone proteins, (RNP), enhanced the antisense activities. In addition, these preliminary data suggest that the encapsulated antisense S-ODN could be used to block HIV-1 entry into human cells.

EXPERIMENTAL

Cells and viruses. The HeLa-CD4 cell line (human cervical carcinoma) expressing CXCR4 was grown in RPMI 1640 media supplemented with 10% Fetal Bovine Serum (FBS) (United Biotechnology), 50 units/ml penicillin, and 50 units/ml streptomycin at 37°C in a 5% CO_2 - gassed incubator.

HIV-1 NL432 was produced by transfection of the proviral DNA clone, pNL432 into COS cells. The supernatants were clarified by filtration, and the levels of p24 antigen, the core antigen of HIV-1, were determined by ELISA. The amounts of viruses were expressed as the concentrations of p24 antigen. The virus stocks were determined for their p24 antigen levels and were stored at –80°C until use.

Oligodeoxyribonucleotides (ODNs) and transfection reagents. The antisense sequence, including the initiation region of the CXCR4 mRNA (MEDLINE, Human mRNA for HM89) and its sense and scramble sequence, were tested in the present study. The nucleotides within the initiation codon are underlined. S-ODNs and FITC-labeled-S-ODN (5`-labeled) were purchased from KURABO Biomedical Co. (Japan). They were the antisense-S-ODN [5'-GATCCCCTCCATGGTAACCG-3' (anti-S-ODN)], the sense-S-ODN [5'-CGGTTACCATGGAGGGGATC-3' (sen-S-ODN)], corresponding to the sequence of bases 69 to 88 of the human CXCR4 mRNA, and the scramble-S-ODN [5'-ATCGCCTAGCTACCTACGCG-3' (scr-S-ODN)] with the same base composition as the antisense 1 target (69-88 bases).

The sequence of the FITC-labeled-anti-S-ODN was the same as that of the antisense.

Our originally constructed peptide-transfectam (named RNP: Reconstituted Nucleosomal Protein) was prepared with the nucleosormal histone subunits: H2A, A2B, H3, H4. Histone subunits were purchased from Boehringer Mannheim (GmbH. Germany). The optimal proportions for ionic complex formation between the peptide and the ODNs were determined using a 20% denatured poly-acrylamide gel electrophoresis (PAGE) retardation system. It was necessary to mix H2A (0.004 μg) and H2B (0.004 μg) in 20mM phosphate buffer (pH 7.2) before any other subunits. Next, the antisense ODN (1 μg) was added to the solution. Then H3 (0.0047 μg) was added, followed by H4 (0.0034 μg). This mixture was added to the culture medium for the transfection of H eLa-CD4 cells with the S-ODNs by the use of RNP.

The liposomally encapsulated anti-S-ODN was prepared according to the manufacturer's recommendations. Briefly, the antisense ODN 6.6 μg (1μM) was mixed with 6.6 μg of the DMRIE-C reagent (Gibco-BRL). After a 15 min incubation at room temperature, this mixture was added to the cells, which were at a concentration of 1×10^5 cells/ml/well in 24-well plates.

Anti-HIV-1 activity of anti-S-ODN. Several concentrations of the oligonucleotide phosphorothioate (S-ODNs), as an anti-HIV drug, were added to cultured HeLa-CD4 cells for 24 h in 24-well plates (1×10^5 cells/ml/well). After 24 h of culture, the HeLa-CD4 cells were washed twice with fresh medium, and then were infected with the HIV-1 NL432 (MOI: multiplicity of infection = 0.1) in 1 ml of medium at 37°C and with 5% CO_2 for 3 h. These cells were washed twice with fresh medium to remove the residual virions, gently resuspended in fresh medium with 10% FBS, and cultured for 24 h. Anti-HIV activity was determined by measuring the amount of p24 antigen in the supernatant, using an HIV-1 p24 ELISA.

Cellular uptake of FITC-labeled-anti-S-ODN into HeLa-CD4 cells (Flow cytometry). HeLa-CD4 cells were incubated in RPMI 1640 at 37°C and in a 5% CO_2 atmosphere for 24 hours. The cultures, in 6-well plates (1×10^6 cells/2ml/well), were washed twice with PBS, and then 5 μM FITC-labeled-anti-S-ODN was added and the plates were incubated for 4 h as above. The cultured cells were removed by scraping and were washed twice with PBS. The washed cells were incubated sequentially at room temperature for 10 min in the dark, each in FACS Lysing Solution and FACS Permeabilizing Solution (Becton Dickinson Immunocytometry Systems) for permeabilization, and then were washed with PBS and resuspended in 0.5% HCHO/PBS. The FITC-labeled-anti-S-ODN uptake in HeLa-CD4 cells was analyzed by flow cytometry.

Intracellular distribution of FITC-labeled-anti-S-ODN in HeLa-CD4 cells (Laser-assisted confocal microscopy). HeLa-CD4 cells were incubated in RPMI 1640 at 37°C and in a 5% CO_2 atmosphere for 24 hours. The cultures, in 6-well plates (1×10^6 cells/2ml/well), were washed twice with PBS, and then 5 μM FITC-labeled-anti-S-ODN was added and the cultures were incubated for 4 h as above. The scraped cells were washed twice with PBS, scraped off, resuspended in 0.5% HCHO/PBS and 0.5% glycerol/PBS, and observed by laser-assisted confocal microscopy (Molecular Dynamics, MultiProbe 2001).

Staining of treated HeLa-CD4 cells. To down-regulate CXCR4 expression, a 10μM concentration of anti-S-ODN, was incubated for 24 h with washed HeLa-CD4 cells (1×10^6 cells/2ml/well) that had been inoculated for 24 h previously and maintained as described above. After the ODN treatment, the cells were scraped, washed twice in cold PBS, and resuspended in PBS. After centrifugation for 2 min at 2,000 rpm, the cells were stained with monoclonal antibodies specific for human CXCR4 (12G5 Mouse IgG2a) (Pharmingen), and then incubated with secondary FITC-labeled antibodies (Rat anti Mouse IgG2a: FITC) (Serotec). As a control (data not shown), the cells were stained with monoclonal antibodies specific for CD4 (conjugated to phycoerythrin, PE) (Dako Japan Co., Ltd.). After staining, the cells were washed, and resuspended in 0.5% HCHO/PBS.

Flow cytometry and analysis. CXCR4 expression in stained cells and FITC-labeled-anti-S-ODN uptake in HeLa-CD4 cells were analyzed by flow cytometry with a FACSCalibur (Becton Dickinson) flow cytometer and Cell Quest software (Becton Dickinson). For each sample, 30,000 total events were analyzed, with sequential gating of HeLa-CD4 cells.

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REFERENCES

1. Deng, H.; Liu, R.; Ellmeier, W.; Choe, S.; Unutmaz, D.; Burkhart, M.; Di Marzio, P.; Marmon, S.; Sutton, R. E.; Hill, C. M.; Davis, C. B.; Peiper, S. C.; Schall, T. J.; Littman, D. R.; Landau, N. R. *Nature* **1966**, 381, 661-666.
2. Choe, H.; Farzan, M.; Sun, Y.; Sullivan, N.; Rollins, B.; Ponath, P.D.; Wu, L.; Mackay, C. R.; LaRosa, G.; Newman, W.; Gerard, N.; Gerard, C.; Sodroski, J. *Cell* **1996**, 85, 1135-1148.
3. Dragic, T.; Litwin, V.; Allaway, G P.; Martin, S. R.; Huang, Y.; Nagashima, K. A.; Cayanan, C.; Maddon, P. J.; Koup, R. A.; Moore, J. P.; Paxton. W. A. *Nature* **1996**, 381:667-673.
4. Feng, Y.; Broder, C. C.; Kennedy, P. E.; Berger, P. E. *Science* **1996**, 278, 872 -877.
5. Doranz, B.J.; Rucker, J.; Yi, Y.; Smyth, R. J.; Samson, M.; Peiper, S. C.; Parmentier, M.; Collman, R. G.; Doms, R. W. *Cell.* **1996**, 85, 1149-1158.
6. Alkhatib, G.; Combadiere, C.; Broder, C. C.; Feng, Y.; Kennedy, P. E.; Murphy, P. M.; Berger, E. A. *Science* **1996**, 272, 1955-1958.
7. Cocchi, F.; DeVico, A. L.; Garzino-Demo, A.; Arya, S. K.; Gallo, R. C.; Lusso, P. *Science* **1995**, 270, 1811-1815.
8. Bleul, C. C.; Farzan, M.; Choe, H.; Parolin, C.; Clark-Lewis, I.; Sodroski, J.; Springer, T.A. *Nature* **1996**, 382, 829-833.
9. Oberline, E.; Amara, A.; Bachelerie, F.; Bessia, C.; Virelizier, J. L.; Arenzana-Seisdedos, F.; Schwartz, O.; Heard, J.M.; Clark-Lewis, I.; Legler, D. F.; Loetscher, M.; Baggiolini, M.; Moser, B. *Nature* **1996**, 382, 833-835.
10. Proudfoot, A. E., Power, C. A., Hoogewerf, A. J., Montjovent, M. O., Borlat, F., Offord, R. E., Wells, T. N. *J.Biol.Chem.* **1996**, 271, 2599-2603.
11. Simmons, G.; Clapham, P. R.; Picard, L.; Offord, R. E.; Rosenkilde, M. M.; Schwartz, T. W.; Buser, R.; Wells, T. N. C.; Proudfoot, A. E. *Science* **1997**, 276, 276-279.
12. Murakami, T.; Nakajima, T.; Koyanagi, Y.; Tachibana, K.; Fujii, N.; Tamamura, H.; Yoshida, N.; Waki, M.; Matsumoto, A.; Yoshie, O.; Kishimoto, T.; Yamamoto, N.; Nagasawa, T. *J. Exp. Med.* **1997**, 186, 1389-1393.
13. Baba, M.; Nishimura, O.; Kanzaki, N.; Okamoto, M.; Sawada, H.; Iizawa, Y.; Shiraishi, M.; Aramaki, Y.; Okonogi, K.; Ogawa, Y.; Meguro, K.; Fujino, M. *Proc. Natl. Acad. Sci. USA* **1999**, 96, 5698-5703.
14. Wagner, R. W. *Nature* **1994**, 372, 333-335.
15. Giles, R.V; Spiller, D. G.; Tidd, D. M. *Antisense Res. Dev.* **1995**, 5, 23-31.
16. Galderisi, U.; Cipollaro, M.; Melone, M. A.; Iacomino, G.; Di Bernardo, G.; Galano, G.; Contrufo, R.; Zappia, V.; Cascino, A. *Biochem. Biophys. Res. Commun.* **1996**, 221, 750-754.
17. Matsukura, M.; Shinozuka, K.; Zon, G.; Mitsuya, H.; Reitz, M.; Cohen, J. C.; Broder, S. *Proc Natl Acad Sci USA* **1987**, 84, 7706-7710.
18. Zhang, R.; Yan, J.; Shahinian, H.; Amin, G.; Lu, Z.; Liu, T.; Saag, M. S.; Jiang, Z.; Temsamani, J.; Martin, R. R.; Schechter, P. J.; Agrawal, S.; Diasio, R. B. *Clin. Pharmaco. Exp. Ther.* **1995**, 49, 929-939.
19. Kuwasaki, T.; Hosono, K.; Takai, K.; Ushijima, K.; Nakashima, H.; Saito, T.; Yamamoto, N.; Takaku, H. *Biochem. Biophys. Res. Commun.* **1996**, 228, 623-631.

20. Fritz, J. D.; Herweijer, H.; Zhang, G.; Wolff, J. A. *Hum. Gene Ther.* **1996**, 7, 1395-1404.
21. Budker, V.; Hagstrom, J. E.; Lapina, O.; Eifrig, D.; Fritz, J.; Wolff, J. A. *Biotechniques.* **1997**, 23, 142-147.
22. Leserman, L.; Machy, P.; Leonetti, J. P.; Milhaud, P. G.; Degols, S. G.; Lebleu, B. *Prog. clin. Biol. Res.* **1990**, 343, 95-102.
23. Akhtar, S.; Juliano, R.L. *Trends Cell Biol.* **1992**, 2, 139-144.
24. Lasic, D. D.; Papahadjopoulos, D. *Science.* **1995**, 267, 1275-1276.
25. Bennett, C. F.; Chiang, M. Y.; Chang, H.; Shoemaker, J. E.; Mirabelli, C. K. *Mol. Pharmacol.* **1992**, 41, 1023-1033.
26. Zhou, X.; Huang, L. *Biochim. Biophys. Acta.* **1994**, 1189, 195-203.
27. Felgner, J. H.; Kumar, R.; Sridhar, C. N.; Wheeler, C. J.; Tsai, Y. J.; Border, R.; Ramsey, P.; Martin, M.; Felgner, P. L. *J. Biol. Chem.* **1994**, 269, 2550-2561.
28. Lewis, J. G.; Lin, K. Y.; Kothavale, A.; Flanagan, W. M.; Matteucci, M. D.; DePrince, R. B.; Mook, R. A. jr; Hendren, R. W.; Wagner, R. W. *Proc. Natl. Acad. Sci. USA* **1996**, 93, 3176-3181.
29. Konopka, K.; Pretzer, E.; Felgner, P. L.; Duzgunes, N. *Biochem. Biophys. Acta* **1996**, 1312, 186-196.