JOURNAL OF VIROLOGY, Jan. 1991, p. 468–472
0022-538X/91/010468-05$02.00/0

Vol. 65, No. 1

Inhibition of Human Immunodeficiency Virus Type 1 Replication in Human T Cells Stably Expressing Antisense RNA

GEORG SCZAKIEL* AND MICHAEL PAWLITA

Institut für Virusforschung, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany

Received 10 July 1990/Accepted 20 September 1990

Human T-lymphoid cell lines with constitutive intracellular expression of antisense RNA complementary to a 407-bp sequence of the 5′ leader-*gag* region of human immunodeficiency virus type 1 were established by using a nonretroviral expression vector. In cell lines with antisense RNA expression detectable by Northern (RNA) hybridization, human immunodeficiency virus type 1 replication was inhibited to 88% 10 days postinfection and this inhibition lasted 3 weeks postinfection.

Antisense nucleic acids have been used successfully to inhibit specifically the expression of cellular genes (for reviews, see references 22, 24, and 25). Viral gene expression or other essential steps in the viral life cycle can also be affected by antisense nucleic acids (20, 28). This has been demonstrated for procaryotic viruses (7) as well as for eucaryotic viruses including retroviruses. Thus, replication of the Rous sarcoma virus was partially suppressed by antisense RNA expressed from eucaryotic vectors (6, 23), and the replication of human immunodeficiency virus type 1 (HIV-1) could be reduced by antisense oligodeoxyribonucleotides (1, 9), chemically modified antisense oligodeoxyribonucleotides (5, 10, 21, 27), and chemically modified antisense oligoribonucleotides (19) added to the culture medium.

In contrast to antisense oligonucleotides being applied extracellularly, intracellularly expressed antisense RNA is a potential antiviral agent which might be used for the development of "intracellular immunization" against virus infection (2). This is also indicated by the finding that HIV-1 replication can be inhibited temporarily when infectious proviral HIV-1 DNA is comicroinjected with an HIV-1 antisense RNA expression plasmid into human cells (18).

In this work, we show that HIV-1 replication is reduced in human T-cell lines constitutively expressing HIV-1 antisense RNA targeted against 407 bp of the 5′ leader-*gag* region. This result indicates that intracellularly expressed antisense RNA is a potential anti-HIV-1 agent.

Expression vectors. The expression plasmid pKEX-1, as well as the plasmid vectors p2as and p2s designed for the expression of HIV-1 antisense and sense RNA, respectively (Fig. 1A) have been described elsewhere (18). Briefly, constitutive transcription is directed by the human cytomegalovirus immediate-early promoter-enhancer element (4). The HIV-1 fragment expressed in p2as and p2s was derived from the HIV-1 proviral clone pBH10 (14) as a 407-bp *Sac*I-*Hin*dIII fragment (positions 222 to 629) covering 112 nucleotides of the 5′ leader and 295 nucleotides coding for the *gag* 5′ region. This sequence also includes the first splice donor, which is used in all known spliced HIV-1 transcripts (11, 17). The resulting transcripts are terminated by 3′-processing signals from the simian virus 40 early region (12) and have a total expected length of 1,120 nucleotides. Additionally, the hygromycin B resistance gene (3), in *cis* on all plasmids, serves as a dominant selection marker.

Cell lines with constitutive antisense-sense RNA expression. The human $CD4^+$ T-lymphoid cell line Jurkat (16, 26) was used as a tissue culture model system for measuring the effects of intracellular HIV-1 antisense RNA expression on HIV-1 replication. For the generation of stably transfected clones, Jurkat cells were electroporated in the presence of one of the plasmids pKEX-1, p2as, and p2s under conditions essentially as described previously (8) except for the initial voltage (250 V). Under hygromycin B selection (500 μg/ml) in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO_2, resistant clones arose from total viable transfected cells with a frequency in the range of 5×10^{-6}. The morphology of the stable Jurkat clones was indistinguishable from that of the parental cells, and all cell lines exhibited the same proliferation rate. The expression levels of the T-cell-specific surface antigens OKT4a, OKT4, and OKT8 as measured by radioimmunoassay also were indistinguishable for the parental cell line Jurkat, one representative (i.e., inhibitory) p2as clone, and one p2s clone.

The Jurkat clones were analyzed for the status of the transfected recombinant plasmid DNAs by restriction of chromosomal DNA and subsequent Southern hybridization with ^{32}P-labeled plasmid DNA. Approximately 90% (25 of 28) transfectants analyzed integrated the plasmid DNA into their chromosome as a single copy. In most cases, this occurred without rearrangements in regard to the expression cassette for antisense and sense transcription, which is shown for four representative p2as and p2s clones in Fig. 1B. Northern (RNA) hybridization of total cytoplasmic RNAs from 14 independent clones which had been positive for integrated plasmid DNA showed detectable amounts of HIV-1 sequence containing transcripts in 9 cases (64%). The apparent size of p2as-derived HIV-1 antisense transcripts on Northern blots (Fig. 1C) was approximately 50 nucleotides greater than that of the sense RNA transcribed from p2s, and also, the antisense transcripts appeared to be less abundant.

HIV-1 replication in antisense RNA-expressing cells. Since clonal variation was expected in the course of HIV-1 infection of different cell clones, to exclude clonal differences unrelated to the antisense RNA expression, HIV-1 infection was investigated in a total number of 10 independent stable p2as transfectants and was compared with infection in 10

* Corresponding author.

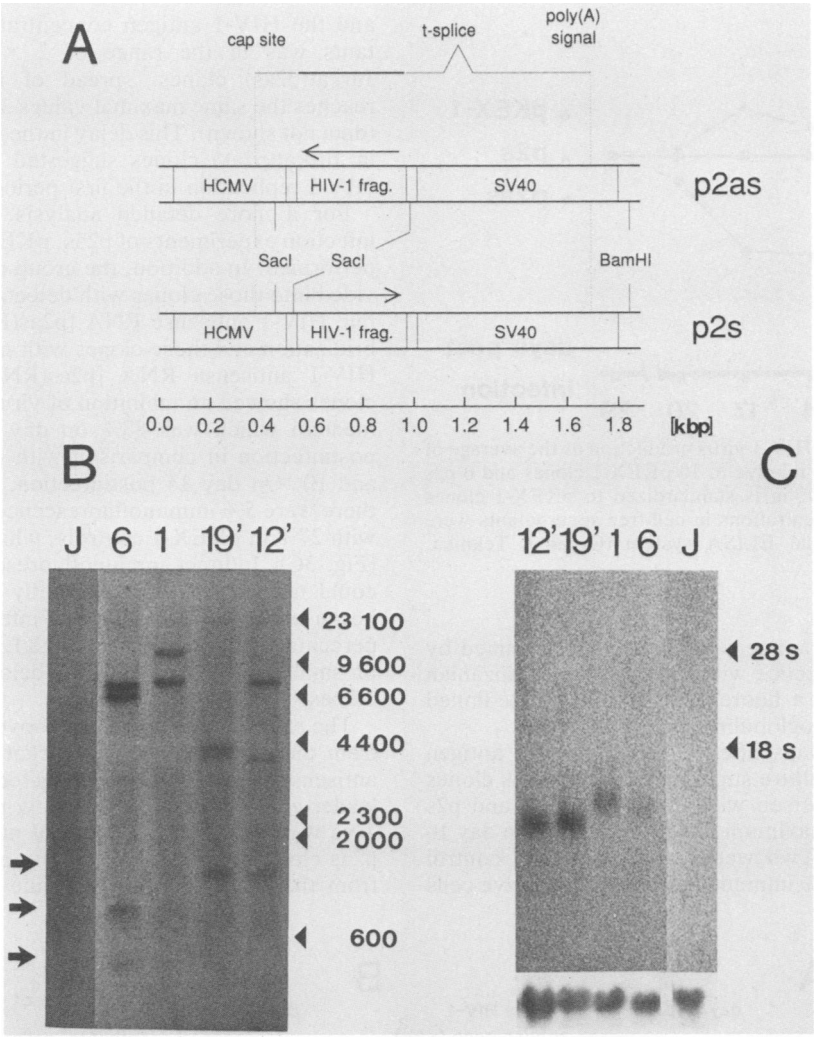

FIG. 1. Chromosomal integration and expression of HIV-1 antisense RNA and sense RNA in stably transfected Jurkat clones. (A) Schematic representation of the expression cassettes for antisense RNA (p2as) and sense RNA (p2s) transcription. The derived transcripts (above) contain 54 bp of the human cytomegalovirus (HCMV) immediate-early promoter-enhancer fragment downstream from the transcriptional start site followed by 407 bp of HIV-1 5′ leader-*gag* sequence and the t-splice and polyadenylation signals from simian virus 40 (SV40). The approximate distances between the restriction sites used in Southern analysis (*Sac*I and *Bam*HI) can be seen with the scale indicated below. (B) Autoradiogram from Southern hybridization of *Sac*I-*Bam*HI-restricted chromosomal DNA (10 μg) of two p2as clones (lanes 1 and 6), two p2s clones (lanes 12′ and 19′), and the parental cell line Jurkat (lane J). DNAs were separated on 0.8% agarose gel, blotted onto Gene Screen Plus membrane, and hybridized with ^{32}P-labeled plasmid p2s under stringent conditions (hybridization, 50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 42°C; washing, 2× SSC, 0.1% sodium dodecyl sulfate, three 10-min washes, 68°C). In addition to two flanks from the single-copy integration in each clone, the relatively small internal *Bam*HI-*Sac*I fragments indicate the difference between p2as and p2s clones. The arrows on the left indicate positions of the 1,200-bp *Bam*HI-*Sac*I fragment from p2s clones and the 800- and 400-bp fragments, respectively, in p2as clones. (C) Autoradiogram from Northern hybridization of total cytoplasmic RNA (10 μg) isolated from p2as clones (lanes 1 and 6), p2s clones (lanes 12′ and 19′), and parental Jurkat cells (lane J). RNAs were separated by formaldehyde-agarose gel electrophoresis, blotted onto Gene Screen Plus membrane, and hybridized with ^{32}P-labeled plasmid p2s under stringent conditions (hybridization and washing as described above). Hybridization signals at the same positions as shown here were seen with ^{32}P-labeled strand-specific riboprobes, additionally distinguishing between the antisense and sense transcripts. As an internal probe for the relative amounts of RNA in each sample, the blot was hybridized with a human β-actin cDNA probe (bottom part of the figure).

independent Jurkat(pKEX-1) clones as well as eight independent Jurkat(p2s) clones.

Jurkat clones were infected with cell-free supernatants from HIV-1-infected human T-cell lines (H9 or Jurkat; HIV-1 strain HTLV-IIIB [13]). Since the spread of HIV-1 in human T cells was to be measured, rather than the initial infection efficiency immediately after the addition of infectious virus to the cells, a low dose of infectivity was used. The initial infectious dose generated approximately 1% HIV-1 antigen immunofluorescence-positive cells 10 days postinfection in pKEX-1 and p2s clones as well as in the parental cell line Jurkat. The replication of HIV-1 in Jurkat clones was monitored by two assays: enzyme-linked immunosorbent assay (ELISA) measurements of HIV-1-specific antigens released into the culture supernatants, indicating production of free virus particles; and the percentage of

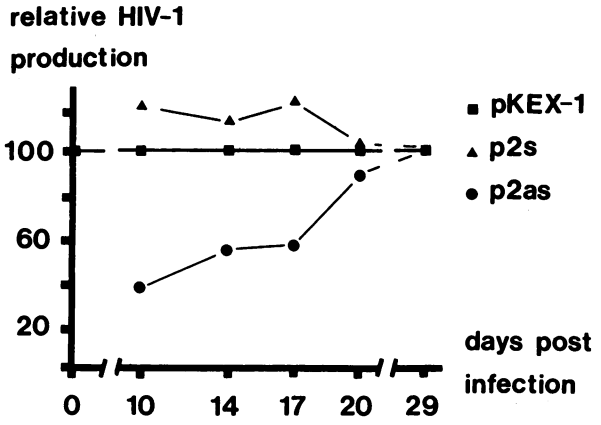

FIG. 2. Time course of HIV-1 virus production as the average of 9 independent p2as clones relative to 10 pKEX-1 clones and 6 p2s clones. The HIV-1 replication is standardized to pKEX-1 clones (100). HIV-1 antigen concentrations in cell-free supernatants were measured by a commercial ELISA system (Organon Teknika, Boxtel, The Netherlands).

cells expressing HIV-1 antigens, which was determined by indirect immunofluorescence with serum from a Tanzanian patient with AIDS and a fluorescein isothiocyanate-linked goat anti-human immunoglobulin antiserum.

During the first 14 days after infection, HIV-1 antigen concentrations in the culture supernatants from p2as clones were reduced in comparison with those of pKEX and p2s control clones, with a maximum of 65% reduction on day 10 postinfection (Fig. 2). Two weeks after infection, control clones showed 50 to 80% immunofluorescence-positive cells and the HIV-1 antigen concentration in cell-free supernatants was in the range of 2×10^4 units (Fig. 3). In Jurkat(p2as) clones, spread of infection is slower and reaches the same maximal values 3 to 4 weeks postinfection (data not shown). This delay in the spread of HIV-1 infection in Jurkat(p2as) clones suggested a transient inhibition of HIV-1 replication in the first period of infection (2 weeks).

For a more detailed analysis, more individual HIV-1 infection experiments of p2as, pKEX-1, and p2s clones were performed. In addition, the group of p2as clones was subdivided into those clones with detectable amounts of cytoplasmic HIV-1 antisense RNA [p2as(RNA$^+$)] by Northern hybridization and those clones with no detectable cytoplasmic HIV-1 antisense RNA [p2as(RNA$^-$)]. The p2as(RNA$^+$) clones showed an inhibition of virus release into the culture medium which was 88% on day 10 and 68% on day 14 postinfection in comparison with pKEX-1 clones (Fig. 3A and B). On day 14 postinfection, in p2as(RNA$^+$) cultures there were 5% immunofluorescence-positive cells compared with 27% in pKEX-1 controls, which is a fivefold reduction (Fig. 3C). Indirect immunofluorescence in this experiment could not be quantitated exactly on day 10 postinfection because of the low numbers of infected cells. However, the percentage of positive cells in pKEX-1 controls was equal to or smaller than 1% and was below 0.1% in p2as(RNA$^+$) clones.

The experiments described above showed that CD4$^+$ cells from the human T-cell line Jurkat with constitutive HIV-1 antisense RNA expression targeted against 407 bp of the 5' leader-*gag* region of HIV-1 show reduced viral replication. This was measured with a total number of 10 independent p2as clones in comparison with control clones, i.e., clones from the same human T-cell line which have been stably

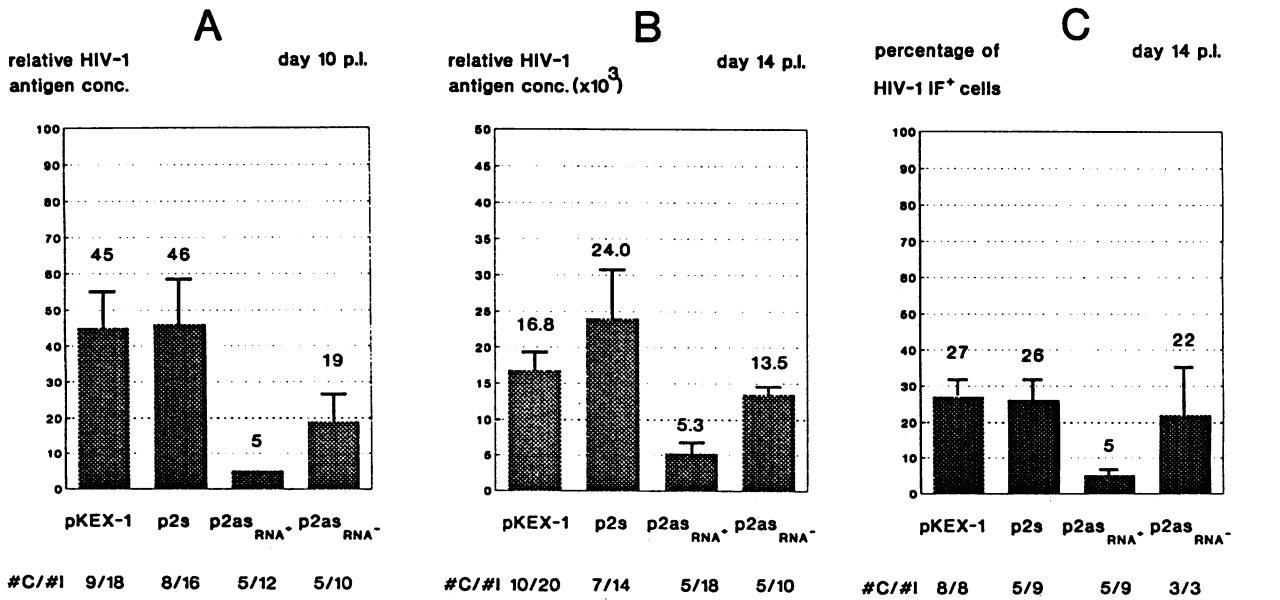

FIG. 3. HIV-1 infection in pKEX-1, p2s, and p2as clones. The p2as group was subdivided into clones with antisense RNA expression [p2as(RNA$^+$)] and clones without detectable antisense RNA expression [p2as(RNA$^-$)] as measured by Northern hybridization. Numbers below the bars of each group indicate the number of individual clones (#C) and the number of independent infection experiments within the group (#I). The error bars indicate the standard error of the mean. (A and B) Comparison of the release of cell-free virus, i.e., relative antigen concentrations in HIV-1-infected pKEX-1, p2s, p2as(RNA$^+$), and p2as(RNA$^-$) clones measured by ELISA on days 10 and 14 postinfection. There is no error bar for p2as(RNA$^+$) in panel A since the relative antigen concentration of 5 is the greatest estimate for this value, i.e., the true value is equal to or smaller than 5. (C) Percentage of HIV-1-infected cells on day 14 postinfection as measured by indirect immunofluorescence.

transfected with the original expression plasmid pKEX-1 or the corresponding sense RNA expression vector p2s by using the same transfection and selection conditions. This indirect evidence for the involvement of antisense RNA in the antiviral effect is further supported by the observation that p2as clones with undetectable antisense RNA expression in Northern analysis [p2as(RNA$^-$) clones] show reduction of virus replication to a clearly smaller extent in comparison with p2as(RNA$^+$) clones (Fig. 3).

The inhibition of HIV-1 replication in human CD4$^+$ cells by intracellular antisense RNA expression, although incomplete and with variations among different clones, suggests that antisense RNA could be used as an antiretroviral agent. Inhibition of HIV-1 replication had also been observed previously in a transient comicroinjection assay with the same antisense RNA expression plasmid p2as (18).

The reason why HIV-1 antisense RNA-expressing cells are protected in early stages of HIV-1 infection (i.e., delay in spread of HIV-1 infection) but become infected in later stages with similar virus proliferation rates as in pKEX-1 clones is unknown. However, the fact that finally (2 weeks postinfection) p2as(RNA$^+$) cultures were also infected was not due to reduced antisense RNA expression. Even after 3 weeks of HIV-1 infection, p2as(RNA$^+$) cells (40 to 70% HIV-1 antigen immunofluorescence-positive cells) did not show a detectable difference in antisense RNA expression levels as measured by Northern analysis (data not shown).

The sense RNA expression vector (p2s) in stably transfected cells did not have an inhibitory effect on HIV-1 replication, whereas it inhibited HIV-1 replication to the same extent as did p2as when comicroinjected together with infectious proviral HIV-1 DNA into human cells (18). This differential behavior of p2s might be due to differences of both test systems. In the transient comicroinjection assay with human epithelioid cells, probably other steps in the replication cycle of HIV-1 are affected, as in T cells with constitutive RNA expression, in which all steps of the viral life cycle involving single-stranded viral nucleic acids are potential targets. Recently, intracellular expression of ribozyme-containing RNAs has been used to inhibit HIV-1 replication in stably transfected CD4$^+$ HeLa cells (15). The inhibitory effect in this case (95 to 97.5%) was measured 7 days after HIV-1 infection and thus seems to be in a range of inhibition similar to that measured in this work for antisense RNA.

Future improvements in the antiviral activity of intracellularly expressed antisense RNA will involve prolongation of the inhibitory effect as well as increase of the antisense effect. The latter could be achieved by the use of more effective HIV-1 target regions and might be supported by ribozyme sequences inserted into antisense transcripts.

We thank H. zur Hausen for continuous encouragement, G. Moldenhauer for examining cell surface antigen expression, R. C. Gallo for HIV-1 clone BH10, and K. Rittner for expression vector pKEX-1. We also thank V. Bosch, H.-G. Kräusslich, and L. Gissmann for critically reading this manuscript.

This work was supported by BMFT grant FKZ II-083-89.

ADDENDUM IN PROOF

After submission of this note an article was published (A. Rhodes and W. James, J. Gen. Virol. 71:1865–1974, 1990) in which similar results were obtained with Jurkat cells and a retroviral vector system expressing HIV-1 *tat* antisense RNA.

REFERENCES

1. **Agrawal, S., T. Ikeuchi, D. Sun, P. S. Sarin, A. Konopka, J. Maizel, and P. C. Zamecnik.** 1989. Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogs. Proc. Natl. Acad. Sci. USA **86**:7790–7794.
2. **Baltimore, D.** 1988. Intracellular immunization. Nature (London) **335**:395–396.
3. **Bernard, H.-U., G. Krämmer, and W. G. Röwekamp.** 1985. Construction of a fusion gene that confers resistance against hygromycin B to mammalian cells in culture. Exp. Cell Res. **158**:868–871.
4. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Häsler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41**:521–530.
5. **Buck, H. M., L. H. Koole, M. H. P. van Genderen, L. Smit, J. L. M. C. Geelen, S. Jurriaans, and J. Goudsmit.** 1990. Phosphate methylated DNA aimed at HIV-1 RNA loops and integrated DNA inhibits viral infectivity. Science **248**:208–212.
6. **Chang, L.-J. R., and C. M. Stoltzfus.** 1985. Gene expression from both intronless and intron-containing Rous sarcoma virus clones is specifically inhibited by antisense RNA. J. Virol. **61**:921–924.
7. **Coleman, J., A. Hirashima, Y. Inokushi, P. J. Green, and M. Inouye.** 1985. A novel immune system against bacteriophage infection using complementary RNA (mic RNA). Nature (London) **315**:601–603.
8. **Döffinger, R., M. Pawlita, and G. Sczakiel.** 1988. Electrotransfection of human lymphoid and myeloid cell lines. Nucleic Acids Res. **16**:11840.
9. **Goodchild, J. S., S. Agrawal, M.-P. Civeira, P. S. Sarin, D. Sun, and P. C. Zamecnik.** 1988. Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. USA **85**:5507–5511.
10. **Matsukura, M., K. Shinozuka, G. Zon, H. Mitsuya, M. Reitz, J. S. Cohen, and S. Broder.** 1987. Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **84**:7706–7710.
11. **Muesing, M. A., D. H. Smith, C. D. Cabradilla, C. V. Benton, L. A. Lasky, and D. J. Capon.** 1985. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature (London) **313**:450–458.
12. **Mulligan, R. C., and P. Berg.** 1981. Selection of animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**: 2072–2076.
13. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224**:497–500.
14. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV III. Nature (London) **313**:277–283.
15. **Sarver, N., E. M. Cantin, P. S. Chang, J. A. Zaia, P. A. Ladne, D. A. Stephens, and J. J. Rossi.** 1990. Ribozymes as potential anti-HIV-1 therapeutic agents. Science **247**:1222–1225.
16. **Schneider, U., H.-U. Schwenk, and G. Bornkamm.** 1977. Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int. J. Cancer **19**:621–630.
17. **Schwartz, S., B. K. Felber, D. M. Benko, E.-M. Fenyö, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64**:2519–2529.
18. **Sczakiel, G., M. Pawlita, and A. Kleinheinz.** 1990. Specific inhibition of human immunodeficiency virus type 1 replication by RNA transcribed in sense and antisense orientation from the

5′-leader/gag region. Biochem. Biophys. Res. Commun. **169:** 643–651.

19. **Shibahara, S., S. Mukai, H. Morisawa, H. Nakashima, S. Kobayashi, and N. Yamamoto.** 1989. Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives. Nucleic Acids Res. **17:**239–252.
20. **Stephenson, M. L., and P. C. Zamecnik.** 1978. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. Proc. Natl. Acad. Sci. USA **75:**285–288.
21. **Stevenson, M., and P. L. Iversen.** 1989. Inhibition of human immunodeficiency virus type 1-mediated cytopathic effects by poly(L-lysine)-conjugated synthetic antisense oligodeoxynucleotides. J. Gen. Virol. **70:**2673–2682.
22. **Stout, T., and C. T. Caskey.** 1987. Antisense RNA inhibition of endogenous genes. Methods Enzymol. **151:**519–530.
23. **To, R. Y., S. C. Booth, and P. Neiman.** 1986. Inhibition of retroviral replication by antisense RNA. Mol. Cell. Biol. **6:**4758–4762.
24. **Walder, J.** 1988. Antisense DNA and RNA: progress and prospects. Genes Dev. **2:**502–504.
25. **Weintraub, H. M.** 1990. Antisense RNA and DNA. Sci. Am. **262:**34–40.
26. **Wendler, J., K. D. Jentsch, J. Schneider, and G. Hunsmann.** 1987. Efficient replication of HTLV-III and STLV-IIImac in human Jurkat cells. Med. Microbiol. Immunol. **176:**273–280.
27. **Zamecnik, P. C., J. Goodchild, Y. Taguchi, and P. S. Sarin.** 1986. Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA. Proc. Natl. Acad. Sci. USA **83:**4143–4146.
28. **Zamecnik, P. C., and M. L. Stephenson.** 1978. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc. Natl. Acad. Sci. USA **75:**280–284.