JOURNAL OF VIROLOGY, Sept. 1992, p. 5576–5581
0022-538X/92/095576-06$02.00/0

Vol. 66, No. 9

NOTES

Tat- and Rev-Directed Antisense RNA Expression Inhibits and Abolishes Replication of Human Immunodeficiency Virus Type 1: a Temporal Analysis

GEORG SCZAKIEL,* MONIKA OPPENLÄNDER, KAROLA RITTNER, AND MICHAEL PAWLITA

Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-6900 Heidelberg, Germany

Received 2 March 1992/Accepted 22 May 1992

Replication of human immunodeficiency virus type 1 (HIV-1) was inhibited by stable intracellular expression of antisense RNA in the human T-lymphoid cell line Jurkat. When the viral subregion encoding the HIV-1 activator proteins was targeted, the extent of antisense RNA-mediated inhibition was greater than 97% during the first 2 weeks postinfection. Later in the time course, productive HIV-1 infection broke through at high initial infective doses. However, at initial multiplicities of infection equal to or smaller than 0.1, HIV-1 production was not detectable during the 5 weeks of observation. The results underline the effectiveness of stable intracellular antisense RNA expression in inhibiting HIV-1 replication.

Chemotherapy and vaccination are classical antiviral principles. Recently, methods of molecular and cellular biology allowed a consideration of alternative approaches which may be summarized by the term "(somatic) gene therapy" (for reviews, see references 9, 42, and 43). The intracellular expression of an antivirally active gene may lead to resistance of the expressing cell against virus infection or to inhibition of virus replication. These alternatives might become relevant for the treatment of the disease associated with infection by human immunodeficiency virus type 1 (HIV-1), i.e., AIDS. Tools for this approach include inhibitory (i.e., antivirally active) proteins and nucleic acids. It was shown in tissue culture cells that the intracellular expression of mutated HIV-1 Gag and Rev proteins, dominant negative interfering proteins (19, 40), led to a significant reduction of HIV-1 replication. Similar negative effects on HIV-1 replication were exerted by intracellular expression of negatively interfering HIV-1 sense RNAs (30, 38) as well as by antisense RNAs (summarized in reference 13). Antisense nucleic acids have been used successfully as inhibitors of gene expression (for reviews, see references 12, 13, 41, and 45) and viral replication in both procaryotic (3) and eucaryotic (2, 13, 32, 39) cells. The inhibition of HIV-1 by antisense nucleic acids has been shown for antisense oligonucleotides (1, 10, 21, 33, 36, 48), although complete inhibition of the virus could not be achieved. So far, intracellularly expressed antisense RNA has shown significant but temporary inhibitory effects; i.e., 2 to 3 weeks postinfection, HIV-1 replication broke through (14, 23, 32). Antiviral effects were also observed with HIV-1-directed hammerhead ribozymes (5, 26, 44). However, it cannot be determined whether inhibition of HIV-1 in these cases was due to ribozyme activity, to classical antisense effects, or to a mixture of both.

In this work, we studied the time course of inhibition of HIV-1 replication in the human T-lymphoid cell line Jurkat over a time period of 5 weeks with various initial infective HIV-1 doses. We report that stable intracellular antisense RNA expression directed against the region of the viral activator/regulator proteins, including Tat and Rev, led to complete inhibition of HIV-1 replication when an infective dose of 0.1 infectious HIV-1 particle per cell or smaller was used.

The strongest inhibitory antisense RNAs are directed against the genes coding for the viral activator proteins. It is plausible and has been shown experimentally that the extent of antisense nucleic acid-mediated inhibition of gene expression is influenced by the biological functions of the subgenic target sequences and by structural properties of the involved RNAs (8, 18, 23, 24, 47). The same was also shown for inhibition of HIV-1 replication with antisense oligonucleotides (1, 10, 21, 48) and with antisense RNA transcribed from microinjected expression plasmids (24). In human T-cell lines with stable antisense RNA expression directed against the 5′ leader/*gag* region, it was shown earlier that a significant but limited extent of inhibition of HIV-1 replication could be achieved (29). By transient microinjection experiments, the extents of HIV-1 inhibition of a series of different HIV-1-targeted antisense RNAs were compared (24). The results indicated that the most effective viral target region for antisense RNA contained coding exons of the regulator proteins, including Tat and Rev. Therefore, we analyzed whether stable intracellular expression of Tat- and Rev-directed antisense RNA in human T cells also had enhanced antiviral effects. It should be mentioned that this viral subregion also codes for a large number of other viral factors (28).

The human T-lymphoid cell line Jurkat (27, 46) used in this study does not show a significant short-term HIV-1-induced cytotoxic effect. The course of HIV-1 infection can be monitored for several weeks, thus enabling one to test whether antiviral effects are temporary or can last over a

* Corresponding author.

TABLE 1. Expression of CD4 surface antigen and HIV-1 replication in parental Jurkat cells and Jurkat clones stably transfected with the antisense RNA expression plasmid pAR6 or the control plasmid pCAT[a]

Clone	% of CD4+ cells	Relative HIV-1 antigen concn in culture supernatants 10 days postinfection
Parental Jurkat cells	37	10,000
pCAT(J) clone:		
1	55	11,000
2	94	20,000
3	90	16,000
4	25	4,000
5	52	16,000
6	52	20,000
pAR6(J) clone:		
3	40	4,200
5	43	<500
7	53	2,800
8	51	1,700
11	25	4,500

[a] Expression of CD4 antigen was measured by fluorescence-activated cell scanning, using monoclonal anti-CD4 antibodies (HP2/6).

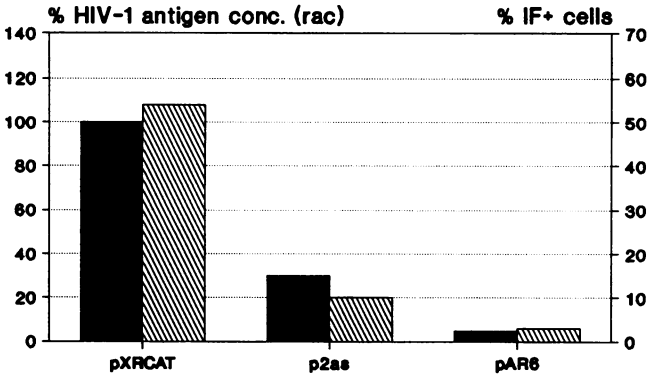

FIG. 1. HIV-1 production in Jurkat clones stably transfected with plasmid pXRCAT, p2as, or pAR6 on day 10 postinfection at an initial infective dose of 10 infective particles (MOI_{MT-4}) per cell. The HIV-1 antigen concentrations in cell-free culture supernatants were measured by a commercial HIV-1 antigen ELISA recognizing p24. Indirect immunofluorescence staining of infected cells was performed with an AIDS patient serum. The columns give the mean of six clones in three series of infection. rac, relative antigen concentration (■); IF^+, immunofluorescence positive (▨).

longer period of time. The plasmid for expression of antisense RNA against the activator genes of HIV-1 (pAR6 [24]) which had led to the strongest inhibition of HIV-1 replication in the transient assay was chosen to analyze the antiviral effects of stably expressed antisense RNA in the human T-lymphoid cell line Jurkat. Antisense RNA expression was driven by the immediate-early promoter/enhancer element of human cytomegalovirus (HCMV) and terminated by the small-t-antigen splice and polyadenylation signal of simian virus 40. The HIV-1 sequence in pAR6 was derived from the proviral clone pBH10 (*SalI* [position 5366]-*KpnI* [position 5928] [22]). Stably pAR6-transfected Jurkat clones were generated by electroporation and hygromycin B selection as described previously (29). The presence of the antisense RNA expression cassette was confirmed by the polymerase chain reaction (PCR) essentially as described previously (35) with a 5′ primer (20-mer; 5′-CACGCTGTTT TGACCTCCAT-3′) located on HCMV sequences and a 3′ primer (20-mer; 5′-CCAATTATGTCACACCACAG-3′) derived from simian virus 40 sequences (25). Seven of eight generated clones were positive for antisense RNA expression, as shown by Northern (RNA) analysis (data not shown). The concentration of infective particles (3×10^9 ml^{-1}) was determined by infecting MT-4 cells (11) with serial dilutions of the thawed HIV-1 supernatants. In the text that follows, the number of infectious HIV-1 particles per infected MT-4 cells will be designated MOI (multiplicity of infection)$_{MT-4}$. However, virus stocks apparently have a higher titer on MT-4 cells than on Jurkat cells. Although this finding indicates that the MOI_{MT-4} used in the infection experiments leads to an overestimated MOI for Jurkat cells, the inoculum was sufficient to infect control Jurkat clones reproducibly. In the course of infection, cell cultures were not grown in the presence of hygromycin B.

Under established experimental conditions (29, 32), i.e., at high initial infective doses ($MOI_{MT4} > 10$), the mean inhibition in pAR6-derived clones versus control clones was 81% 10 days postinfection (Table 1). To determine the extent of clonal variability in the course of HIV-1 infection, five pAR6 clones were compared with six control Jurkat clones generated with the chloramphenicol acetyltransferase (CAT) expression plasmid pKEX-2-XR-CAT (pCAT) (25). CAT activity had been detected in pCAT clones over a time period of 8 weeks, indicating the continuous intracellular presence of plasmid DNA and of CAT mRNA (data not shown). It should be noted that there was no significant difference in proliferation rates (data not shown) and CD4 expression (Table 1) in pAR6 and control clones used in HIV-1 infection experiments. Thus, it was concluded that the difference in HIV-1 infection (Table 1) between the group of antisense RNA-producing pAR6 clones and the group of control clones reflect the influence of intracellular antisense RNA expression on HIV-1 replication.

Inhibition of HIV-1 replication in six pAR6-derived Jurkat clones was also compared with that of six Jurkat clones expressing antisense RNA against the 5′ leader/*gag* region of HIV-1 (p2as Jurkat clones [29]) at 10 infective HIV-1 particles per cell. In this case, the percentage of infected cells and the concentration of virus antigen in supernatants were reduced by a factor of approximately 6 in pAR6 clones as compared with p2as clones (Fig. 1). This difference in antiviral efficacy is significant because the two antisense RNA expression plasmids (p2as and pAR6) were assayed in the same system. However, it is difficult to directly compare these results with published data derived from other experimental systems.

Temporal analysis of HIV-1 inhibition. For testing inhibition of HIV-1 replication in a short time range, i.e., within the first 50 h postinfection, we used high infective doses ($MOI_{MT-4} \sim 100$). Fifty hours is the time period which had been found to cover two viral replication cycles in a related cell line (15). HIV-1 replication in pAR6(J) clone 3 and pCAT(J) clone 3, which had shown average HIV-1 replication in their respective groups of clones, was monitored by measuring relative viral antigen concentrations in culture supernatants (Fig. 2A). The extent of inhibition in pAR6(J) clone 3 during this initial phase of infection was approximately 80%. In the following days, HIV-1 production was inhibited almost completely. Later in the time course, HIV-1 infection broke through and virus was produced and released with the same kinetics as had been observed several days earlier in unprotected control clones (Fig. 2C). The latter

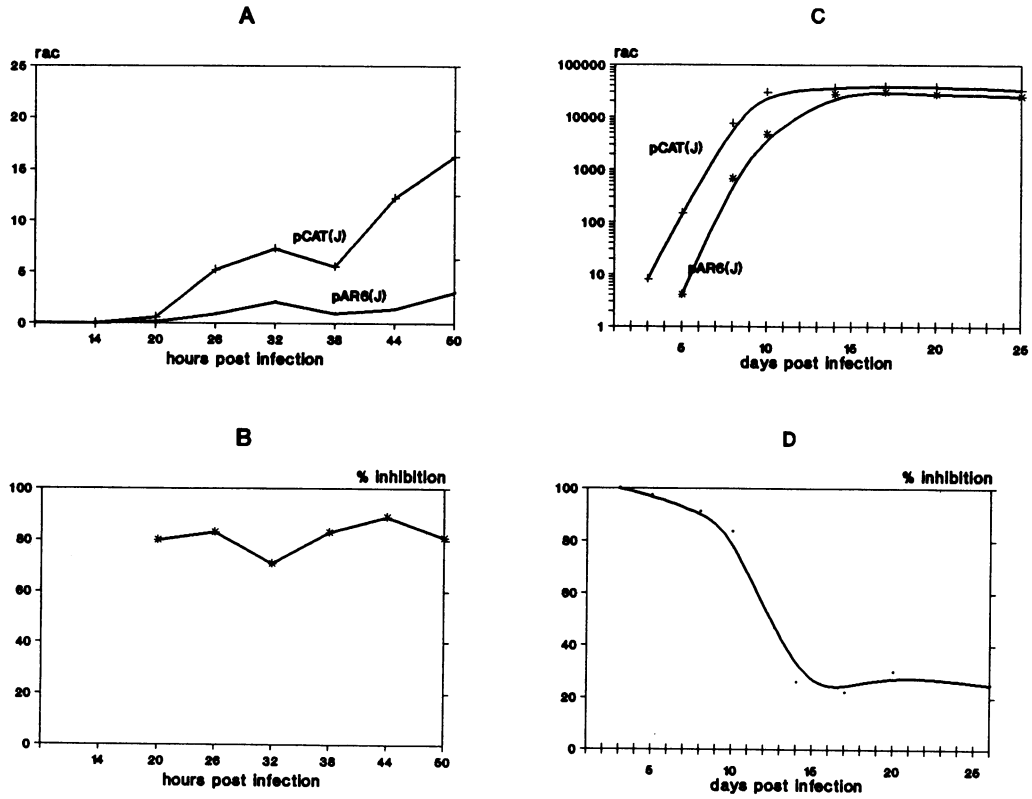

FIG. 2. Time course of HIV-1 production (A) and extent of inhibition in the initial phase of infection (B) of a representative pAR6 clone (clone 3) and a representative control clone [pCAT(J) clone 3]. Infection was monitored by measuring the concentrations of HIV-1-specific antigens in the culture supernatant by an HIV-1 antigen ELISA. The MOI_{MT-4} of approximately 10^3 used in this experiment was the highest which could be used experimentally. (C) Long-term inhibition of HIV-1 replication in eight pAR6 clones versus six pCAT control clones. Production of HIV-1 was monitored as for panel A. The MOI_{MT-4} was 10. (D) Time-dependent extent of inhibition. rac, relative antigen concentration.

observation is surprising, since at the time when pAR6 clones started to produce HIV-1 with the same increase as in control cultures (day 5; Fig. 2C), only approximately 0.1% of the cells expressed HIV-1 antigens as measured by indirect immunofluorescence. Even 3 days later (day 8; Fig. 2C), which corresponds to two to three viral replication cycles, the percentage of HIV-1-positive cells was in the range of 5%. The reasons for breakthrough of infection are not known. However, selection for antisense RNA-resistant virus types does not seem to explain the breakthrough of HIV-1 infection, since reinfection of pAR6(J) clones with HIV-1 taken from productively infected pAR6(J) cells again led to the same extent of HIV-1 inhibition. We can also exclude the possibility that the HCMV immediate-early promoter/enhancer element is down-regulated by HIV-1. This effect was shown earlier for antisense RNA transcription and was also indicated by nonaltered CAT expression levels in Jurkat clones with stable HCMV-driven CAT expression during the course of HIV-1 infection (data not shown).

Transient antisense RNA-mediated inhibition of HIV-1 replication. In stable pAR6(J) clones at high initial infective HIV-1 doses, the extent of inhibition measured in the first hours postinfection (Fig. 2A) was 80%. Extrapolation of this 80% inhibition for each viral replication cycle for the first four viral replication cycles would result in more than 99% inhibition. However, in contrast to results for low initial infective doses (Fig. 3), for high infective doses the extent of inhibition was high initially but decreased with time (Fig. 2C and D). The ability to inhibit HIV-1 replication at the single-cell level might be lost at later stages of infection, since there is a high rate of virus spread following the breakthrough of infection. Since at the time at which breakthrough initiates, only 0.1% of cells are positive for HIV-1 antigens, it might be possible that signals from the few infected cells contribute to a switch-off of antisense RNA-mediated inhibition of HIV-1 replication in other cells in the same culture. So far, we have no experimental evidence for

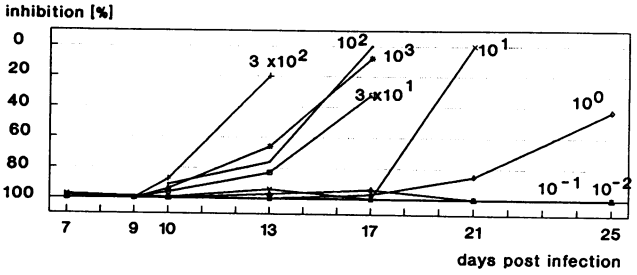

FIG. 3. Time course of inhibition of HIV-1 replication at various initial infective doses calculated with one representative antisense RNA expressing pAR6 clone 3 versus one control clone [pCAT(J) clone 3]. The ratio of infectious virus particles (MOI_{MT-4}) and cells is noted above each curve.

TABLE 2. HIV-1 production in Jurkat clones 35 days after exposure to 0.1 infectious virus particle per cell

Clone	Infectious particle/cell	Days until breakthrough of HIV-1
pCAT(J) clone:		
1	0.1	8 - 11
2	0.1	8 - 11
3	0.1	11 - 15
4	0.1	8 - 11
6	0.1	11 - 15
pAR6(J) clone:		
3	0.1	—[a]
5	0.1	—
7	0.1	—
8	0.1	—
11	0.1	—
p2as(J) clone 1	0.1	—
	0.3	—

[a] —, No breakthrough of HIV-1 production up to 36 days postinfection, measured by ELISA with undiluted culture supernatants (definition of breakthrough, relative antigen concentration of >1,000) and by indirect immunofluorescence with an AIDS patient serum.

the existence and nature of such hypothetical viral or virus-induced factors. However, in tissue culture cells, the release of Tat from infected cells (6) and the uptake of Tat from the culture medium (7, 20) is known. From our data and further published data on antisense RNA- or ribozyme-mediated inhibition of HIV-1 replication, we conclude that the inhibition of viral replication was transient (23, 29). In some published reports, transiency of inhibition has not been excluded, and thus further conclusions cannot be drawn (14, 26, 44).

Breakthrough of HIV-1 infection in Jurkat cells 5 to 10 days after infection was also observed during chronic treatment with the reference anti-HIV-1 compound 3'-azido-3'-deoxythymidine (zidovudine) (AZT; 20 and 200 nM [data not shown]). This observation could be explained by the finding that in vivo and in vitro, certain mutated positions within the *pol* coding sequence led to the expression of AZT-resistant reverse transcriptase (16, 17). In this work on antisense RNA-mediated inhibition, we excluded experimentally the possibility that breakthrough of infection is caused by the selection of antisense RNA-resistant HIV-1 subtypes.

Long-term suppression of HIV-1 replication after low-dose infection. An excess of more than 10 infectious virus particles per cell might not reflect the situation in vivo. For this reason, we tested the influence of low initial infective doses on the course and the time point of breakthrough of HIV-1 infection. Breakthrough of HIV-1 production in pAR(J) clone 3 versus the control, pCAT(J) clone 3, was delayed at least up to 25 days postinfection at an MOI_{MT-4} equal to or smaller than 0.1 (Fig. 3). No virus replication was also observed with a larger number of individual pAR6(J) clones at least for the observation period of 35 days postinfection (Table 2). Three of these long-term-protected Jurkat cultures were tested for the presence of proviral HIV-1 DNA by PCR analysis with cellular chromosomal DNA isolated 35 days postinfection. HIV-1-specific PCR primer sequences were derived from the *env* gene; i.e., they were not contained on pAR6. In one of the three samples, HIV-1 DNA was detected. To test whether there is continuous low-level production of HIV-1 in protected antisense clones, we performed the following experiment in duplicate. A total of 5×10^5 cells of five independent pAR6(J) clone 3 cultures which did not show any HIV-1 antigen production up to 59 days postinfection were cocultivated with 2×10^5 MT-4 cells. After 4 days, an HIV-1 antigen enzyme-linked immunosorbent assay (ELISA) with coculture supernatants did not indicate any HIV-1 antigen production, i.e., HIV-1 replication.

The experiments performed in this work at high initial infective doses demonstrate a stronger antiviral effect for pAR6 than for p2as. At low infective doses, however, complete inhibition of productive HIV-1 infection was also observed with one p2as(J) clone (clone 1), indicating for p2as an antiviral potential similar to that for pAR6 at low HIV-1 concentrations.

Parameters affecting the extent of inhibition: further improvements. The extent of inhibition of HIV-1 replication in tissue culture cells can be increased by using more effective subgenomic and subgenic target sequences (23, 24). It was shown that above certain threshold levels, there is no additional effect of increased intracellular concentrations of antisense RNA (23, 29) on the replication of HIV-1 (32). A similar phenomenon was also observed in divergent biological systems, e.g., in transgenic potato plants in which antisense RNA-mediated inhibitory effects were not dependent on the intracellular antisense RNA concentration (37). These data suggest that further improvements of intracellular steady-state levels of antisense RNA, either by transcription from stronger promoters or by increasing the half-lives of the transcripts, may not necessarily increase antiviral effects.

On the other hand, one of the major parameters for the successful application of antisense RNA probably is the local target region which is linked with biological functions and structural properties of the target transcripts. Analysis of the latter could be the basis for the design of antisense RNAs with structural modifications, possibly leading to improved RNA-RNA interactions. For example, local structural elements such as stem-loops of the antisense RNA and the target RNA may have a great influence on RNA double-strand formation, as was shown in naturally occurring antisense RNA-regulated systems (34). The identification of such structural elements might be supported by decreasing the length of the antisense RNAs. For example, in vitro hybridization studies (31) as well as HIV-1 inhibition studies showed that a 69-nucleotide transcript was at least as effective as the 562-nucleotide pAR6-derived progenitor antisense RNA (12a).

Another promising approach appears to be insertion of ribozyme sequences into antisense RNA transcripts, although it is technically difficult to demonstrate ribozyme-mediated cleavage reactions in living cells. Ribozyme-mediated inhibition may consist of at least two inhibitory reactions which cannot be distinguished easily, namely, the recognition of the target by antisense interactions and the cleavage reaction. Since the contribution of the two steps to the overall inhibition is not known, the question remains open as to whether ribozymes have a greater antiviral potential than does antisense RNA. This view is supported by the finding of Cotten et al. (4), who investigated inhibition of U7 small nuclear ribonucleoprotein-mediated histone pre-mRNA processing in vitro and found that antisense RNA-mediated inhibition took place at an even lower molar excess of the antisense RNA than did inhibition at comparable extents mediated by ribozymes. However, irreversible destruction of target RNAs made theoretically possible by ribozymes or by other mechanisms (e.g., unwinding and modifying activity) might become a powerful tool for antivi-

ral therapy in the future. Also, improved antisense constructs may contribute to specific and complete shutoff of undesired cellular or viral functions.

We thank H. zur Hausen for continuous support, M. Knobloch and G. Moldenhauer for help with CD4 antigen determination, and J. ter Meulen for initial PCR experiments. We also thank R. S. Goody for providing AZT, H. Steuler for PCR primers, and L. Gissmann, V. Bosch, and M. Homann for critically reading the manuscript.

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

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. **Chang, L.-J. I., 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.
3. **Coleman, J., A. Hirashima, Y. Inokuchi, P. J. Green, and M. Inouye.** 1985. A novel immune system against bacteriophage infection using complementary RNA (mic RNA). Nature (London) **315:**601–603.
4. **Cotten, M., D. Schaffner, and M. L. Birnstiel.** 1989. Ribozyme, antisense RNA, antisense DNA inhibition of U7 small nuclear ribonucleoprotein-mediated histone pre-mRNA processing in vitro. Mol. Cell. Biol. **9:**4479–4487.
5. **Dropulic, B., N. H. Lin, M. A. Martin, and K.-T. Jeang.** 1992. Functional characterization of a U5 ribozyme: intracellular suppression of human immunodeficiency virus type 1 expression. J. Virol. **66:**1432–1441.
6. **Ensoli, B., G. Barrillari, S. Z. Salahuddin, R. C. Gallo, and F. Wong-Staal.** 1990. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature (London) **345:**84–86.
7. **Frankel, A. D., and C. O. Pabo.** 1989. Cellular uptake of the tat protein from human immunodeficiency virus. Cell **55:**1189–1193.
8. **Freier, S. M., R. Kierzek, J. A. Jaeger, N. Sugimoto, M. H. Caruthers, T. Neilson, and T. H. Turner.** 1986. Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. USA **83:**9373–9377.
9. **Friedmann, T.** 1989. Progress towards human gene therapy. Science **244:**1275–1281.
10. **Goodchild, J., 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.
11. **Harada, S., Y. Koyanagi, and N. Yamamoto.** 1985. Infection of HTLV-III/LAV in HTLV-I carrying cells MT-2 and MT-4 and application in a plaque assay. Science **229:**563–566.
12. **Hélène, C., and J. J. Toulmé.** 1990. Specific regulation of gene expression by antisense, sense and antigene nucleic acids. Biochim. Biophys. Acta **1049:**99–125.

12a. **Homann, M., and K. Rittner.** Unpublished data.

13. **James, W.** 1991. Towards gene inhibition therapy: a review of progress and prospects in the field of antiviral antisense nucleic acids and ribozymes. Antiviral Chem. Chemother. **2:**191–214.
14. **Joshi, S., A. van Brunschot, S. Asad, I. van der Elst, S. E. Read, and A. Bernstein.** 1991. Inhibition of human immunodeficiency virus type 1 multiplication by antisense and sense RNA expression. J. Virol. **65:**5524–5530.
15. **Kim, S., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**3708–3713.
16. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731–1734.
17. **Larder, B. A., and S. D. Kemp.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high level resistance to zidovudine (AZT). Science **246:**1155–1158.
18. **Maher, L. J., III, and B. J. Dolnik.** 1988. Comparative hybrid arrest by tandem antisense oligodeoxyribonucleotides or oligodeoxyribonucleoside methylphosphonates in a cell free system. Nucleic Acids Res. **16:**3341–3358.
19. **Malim, M. H., S. Böhnlein, J. Hauber, and B. R. Cullen.** 1989. Functional dissection of the HIV-1 rev transactivator—derivation of a trans dominant repressor of rev function. Cell **58:**205–215.
20. **Mann, D. A., and A. D. Frankel.** 1991. Endocytosis and targeting of exogenous HIV-1 Tat protein. EMBO J. **10:**1733–1739.
21. **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.
22. **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, HTLVIII. Nature (London) **313:**277–283.
23. **Rhodes, A., and W. James.** 1990. Inhibition of human immunodeficiency virus replication by endogenously synthesized antisense RNA. J. Gen. Virol. **71:**1965–1974.
24. **Rittner, K., and G. Sczakiel.** 1991. Identification and analysis of antisense RNA target regions of the human immunodeficiency virus type 1. Nucleic Acids Res. **19:**1421–1426.
25. **Rittner, K., H. Stöppler, M. Pawlita, and G. Sczakiel.** 1991. Versatile eucaryotic vectors for strong and constitutive transient and stable gene expression. Methods Mol. Cell. Biol. **2:**176–181.
26. **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.
27. **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.
28. **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.
29. **Sczakiel, G., and M. Pawlita.** 1991. Inhibition of human immunodeficiency virus type 1 replication in human T-cells stably expressing antisense RNA. J. Virol. **65:**468–472.
30. **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.
31. **Sczakiel, G., M. Pawlita, K. Rittner, and M. Homann.** Antisense RNA-mediated inhibition of the replication of the human immunodeficiency virus type 1: discussion of critical parameters. Ann. N.Y. Acad. Sci., in press.
32. **Sczakiel, G., K. Rittner, and M. Pawlita.** 1991. Human immunodeficiency virus type 1 replication is reduced by intracellular antisense RNA expression, p. 179–193. *In* E. Wickstrom (ed.), Prospects for antisense nucleic acid therapy of cancer and AIDS. Wiley-Liss, New York.
33. **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.
34. **Simons, R. W.** 1988. Naturally occurring antisense RNA control—a brief review. Gene **72:**35–44.
35. **Steuler, H., B. Storch-Hagenlocher, and B. Wildemann.** 1992. Distinct populations of human immunodeficiency virus type 1 in blood and cerebrospinal fluid. AIDS Res. Hum. Retroviruses **8:**53–59.
36. **Stevenson, M., and P. C. 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.
37. **Stockhaus, J., M. Höfer, G. Renger, P. Westhoff, T. Wydrzynski, and L. Willmitzer.** 1990. Antisense RNA efficiently inhibits formation of the 10kd polypeptide of photosystem II in transgenic potato plants: analysis of the role of the 10 kd protein. EMBO J. **9**:3013–3021.
38. **Sullenger, B. A., H. F. Gallardo, G. E. Ungers, and E. Gilboa.** 1990. Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. Cell **63**:601–608.
39. **To, R. Y.-L., S. C. Booth, and P. E. Neiman.** 1986. Inhibition of retroviral replication by antisense RNA. Mol. Cell. Biol. **6**:4758–4762.
40. **Trono, D., M. B. Feinberg, and D. Baltimore.** 1989. HIV-1 gag mutants can dominantly interfere with the replication of the wild type virus. Cell **59**:113–120.
41. **Walder, J.** 1988. Antisense DNA and RNA: progress and prospects. Genes Dev. **2**:502–504.
42. **Weatherall, D. J.** 1988. The slow road to gene therapy. Nature (London) **331**:13–14.
43. **Weatherall, D. J.** 1991. Gene therapy in perspective. Nature (London) **349**:275–276.
44. **Weerasinghe, M., S. E. Liem, S. Asad, S. E. Read, and S. Joshi.** 1991. Resistance to human immunodeficiency virus type 1 (HIV-1) infection in human $CD4^+$ lymphocyte-derived cell lines conferred by using retroviral vectors expressing an HIV-1 RNA-specific ribozyme. J. Virol. **65**:5531–5534.
45. **Weintraub, H. B.** 1990. Antisense RNA and DNA. Sci. Am. **262**:34–40.
46. **Wendler, J., K. D. Jentsch, J. Schneider, and G. Hunsmann.** 1987. Efficient replication of HTLV-III and SIV-IIImac in human Jurkat cells. Med. Microbiol. Immunol. **176**:273–280.
47. **Wickstrom, E., W. S. Simonet, K. Medlock, and I. Ruiz-Robles.** 1986. Complementary oligonucleotide probe of vesicular stomatitis virus matrix protein mRNA translation. Biophys. J. **49**:15–17.
48. **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.