Journal of General Virology (1990), **71**, 1965–1974. *Printed in Great Britain*

Inhibition of human immunodeficiency virus replication in cell culture by endogenously synthesized antisense RNA

Andrew Rhodes† and William James*

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K.

Antisense RNA, which has a sequence complementary to mRNA, may provide the basis for antiviral therapies of high selectivity. We have explored the inhibitory effect of six antisense RNAs upon the replication of human immunodeficiency virus (HIV) in cell culture. We chose regions of the HIV genome to test whether sequences required for splicing or for translation initiation were more susceptible to antisense RNA interference. Our results suggest that inhibitory antisense RNAs contain sequences complementary to the AUG initiation codon of the *tat* gene and have a comparatively low tendency to form intramolecular base pairs which would interfere with intermolecular duplex formation. Inhibition can be substantial (over 70%) but is transient. Transience does not result from mutation of the input virus. Inhibition was not a consequence of the induction of interferon by antisense RNA–mRNA duplex formation. Our results suggest that at least part of the inhibitory effect is at the post-transcriptional level.

Introduction

Regulation of gene expression by antisense RNA occurs naturally in prokaryotes (Simons & Kleckner, 1988) and eukaryotes (Gordon *et al.*, 1988; Khochbin & Lawrence, 1989; Kimelman & Kirschner, 1989; Volk *et al.*, 1989). It has been used experimentally to modulate gene activity *in vivo* in plants (Rodermel *et al.*, 1988; Rogers, 1988; Smith *et al.*, 1988; Cuozzo *et al.*, 1988; Hemenway *et al.*, 1988) and animals (Giebelhaus *et al.*, 1988). The mechanism of inhibition may include interference with RNA processing or inhibition of the initiation of translation (Munroe, 1988; Walder, 1988; Cotten *et al.*, 1989). For this reason, there is a possibility that one could exploit antisense nucleic acid to inhibit the replication of viruses *in vivo* without affecting the expression of host genes. HIV is a good system for investigating this approach because it is a pathogen of great and growing significance, it is relatively well characterized at the molecular level, and it is not as yet amenable to immunoprophylaxis or curative chemotherapy.

There are two major methods of introducing antisense nucleic acid into cells. Firstly, one can bathe susceptible cells in preformed oligonucleotides (Zamecnik *et al.*, 1986; Matsukura *et al.*, 1987; Agrawal *et al.*, 1988; Goodchild *et al.*, 1988; Sarin *et al.*, 1988; Zaia *et al.*, 1988; Shibahara *et al.*, 1989). Although oligonucleotides have proved to be reasonably effective against HIV, they appear to exert their effects in a largely sequence non-specific manner and to be variably cytotoxic. Alternatively, one can introduce a gene into susceptible cells which is transcribed to produce antisense RNA. This approach has been used for other viruses (Chang & Stoltzfus, 1987; von Ruden & Gilboa, 1989; Cuozzo *et al.*, 1988; Hemenway *et al.*, 1988). We chose to use retroviral vectors rather than transfection to introduce the antisense genes into cells since we find that transfected cells frequently show abnormalities in their cell surface markers, rates of growth and susceptibilty to HIV. In this paper we report that some antisense RNAs can substantially inhibit HIV replication, albeit transiently, when expressed from a provirus. We investigate the features of an antisense RNA which determine its inhibitory potential, whether it induces interferon, the effect it has on HIV mRNA levels and the reasons for the transience of inhibition.

Methods

Virus strains. HIV-1 strain HTLV-IIIB (HIV-1_{IIIB}) (Ratner *et al.*, 1985) was obtained from the NIH AIDS Research and Reference Program and was propagated by acute infection of CEM cells. Virus-containing, cell-free tissue culture supernatant was stored at −85 °C. Cocal virus strain TR40233, a member of the genus *Vesiculovirus*, was obtained from the Yale Arbovirus Research Unit, and was maintained by passage in BHK cells.

Cell lines. CEM cells (Foley *et al.*, 1965) were grown in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS; Gibco). Jurkat clone E6-1 cells (Weiss *et al.*, 1984) were grown in RPMI 1640 medium supplemented with 10% FCS and 2-mercaptoethanol

† Present address: Xenova Ltd, Slough SL1 4EQ, U.K.

(5×10^{-7} M). NIH/3T3 cells (Jainchill *et al.*, 1969), PA317 cells (Miller & Buttimore, 1986) and Psi-2 cells (Mann *et al.*, 1983) were grown in Dulbecco's modified Eagle's medium (Northumbria Biotechnology) supplemented with 10% FCS. Cells were routinely screened for mycoplasma contamination and were cultured with neomycin, streptomycin and penicillin (Sigma) at 37 °C in an atmosphere of 5% CO_2.

Construction of antisense RNA-encoding retroviral vectors. The antisense genes were constructed from the proviral clones of HIV-1_{IIIB}, pBH10 and pHXB-2D (Hahn *et al.*, 1984; Fisher *et al.*, 1985; Fig. 1*b* and *c*). To construct the antisense genes AR5, AR6 and AR10, *Sac*I restriction sites were introduced at positions 5438 and 5582 by oligonucleotide-directed mutagenesis (Taylor *et al.*, 1985; Nakamaye & Eckstein, 1986). The retroviral vector, IRV.Neo.SV (Fig. 1*a*), was donated by Jay Morgenstern, Imperial Cancer Research Fund Laboratories, London, U.K.; of four variant vectors provided, this provided the highest levels of expression in cells of human leukocyte origin and a high titre of transducing particles. The antisense genes were subcloned in the antisense orientation using conventional methods (Ausubel *et al.*, 1987) into the retroviral vector.

Introduction of antisense RNA-encoding proviruses into HIV-susceptible cells. Psi-2 cells were transfected with the retroviral constructs by calcium phosphate coprecipitation (Brown *et al.*, 1987), and the transfectants were selected using G418 (800 μg/ml; Gibco). PA317 cells were transduced with the ecotropic retrovirus-containing supernatants of the transfected Psi-2 cells (Brown *et al.*, 1987). G418-resistant transduced PA317 cell lines were cloned and analysed individually for high level production of amphotropic G418-resistant colony-forming units. Jurkat cells (4×10^5) were exposed to 6×10^5 c.f.u. of amphotropic retrovirus for 24 h, after which G418 (800 μg/ml) was added. The resistant cells grew to a density of 10^5 cells/ml after 10 to 14 days, suggesting that the transduced cultures contained approximately 10^4 clones of transduced cells. The name of each transduced cell line is derived from the parental cell and the antisense gene. Jurkat.AR0 was transduced with the retroviral vector alone.

Analysis of surface-expressed CD4 using flow cytometry. One-million cells were resuspended in PBS/BSA [Dulbecco's phosphate-buffered saline with calcium or magnesium, supplemented with bovine serum albumin (1%, w/v)] containing the monoclonal antibody, MT151 (anti-CD4; 5 μg/ml; code no. 508 in McMichael, 1987) or OX21 (negative control; 5 μg/ml) and incubated at 4 °C for 2 h. The cells were washed twice in PBS/BSA, then resuspended in fluorescein isothiocyanate-conjugated, rabbit anti-mouse Ig (200 μg/ml, 50 μl) and incubated for 1 h at 4 °C. The cells were diluted to 1 ml with PBS/BSA and analysed in a Becton-Dickinson FACSCAN.

Detection of sense and antisense RNA in cells. Total cellular RNA was prepared and analysed by Northern blotting as described by Ausubel *et al.* (1987). GeneScreen-Plus (DuPont) was used as the transfer membrane. Two plasmids were constructed for the synthesis of riboprobes: the first, pSPT19.AR2, was constructed by cloning the *Eco*RI (5325)–*Kpn*I (5926) fragment of pBH10 (see above) into the transcription vector, pSPT19 (Pharmacia); the second, pSPT19.NeoSV, was constructed by cloning the *Kpn*I (365)–*Sac*I(3615) fragment of the retroviral vector, IRV.Neo.SV, into pSPT19. Both were transcribed with SP6 polymerase and [^{32}P]CTP (Ausubel *et al.*, 1987) to generate radiolabelled probes that hybridized to antisense RNA derived from sequences within the AR2 region of HIV (see above), or to all RNAs transcribed from the retroviral vector, respectively. Hybridization was done using 10^5 c.p.m./ml of riboprobe in a sealed plastic bag at 60 °C overnight. The hybridization solution contained 50% formamide, 1% SDS, 5 × SSC, 8 × Denhardt's solution, 250 μg/ml sheared salmon sperm DNA and 300 μg/ml yeast RNA. The filter was washed twice in 2 × SSC at room temperature, once in 2 × SSC, 1% SDS (30 min, 65 °C), and then exposed to X-ray film (Kodak X-omat S) overnight at −70 °C.

Growth and detection of HIV. The $TCID_{50}$ of HIV stocks was determined in Jurkat cells. The growth of HIV was measured using the DuPont p24 core antigen ELISA kit. Jurkat cells and their derivatives were infected with HIV at an m.o.i. of 0.01 and incubated overnight. Input virus was removed by washing the cells in growth medium 16 h post-infection (p.i.). Cell-free samples of culture supernatant (0·5 ml) were taken at intervals and stored at −85 °C. Since we had observed that the rate of HIV replication in Jurkat cells depended upon the growth rate of the cells, we ensured that, in any comparison between transduced Jurkat cells, all cells were growing at the same, exponential rate during the 3 days preceding challenge with HIV.

Prediction of RNA secondary structure. The predicted pattern of folding of antisense RNAs was generated using the FOLD-VAX RNA secondary structure prediction program of Michael Zuker, VAX/VMS version, 1986. We used Turner's energy rules (Freier *et al.*, 1986) which are optimized for 37 °C and have been adjusted in the light of experimental observations (see Zuker, 1989). The predicted structures were substantially similar when Salser's energy rules were used (Salser, 1978). The prediction of the secondary structure of HIV mRNA in the region discussed in the text was done on the 1121 nucleotides beginning at nucleotide position 5000 relative to the start of transcription. The secondary structure predictions of each of the antisense RNAs was done on the antisense RNA and 225 bases of vector sequence flanking it to the 3′ and 5′ ends. The total length of RNA folded in each case was: AR2, 1067 bases; AR5, 569 bases; AR6, 502 bases; AR9, 626 bases; AR10, 608 bases; AR11, 474 bases. None of the values or structures referred to in the text were substantially altered by including 50 bases more or less in the flanking regions for computer analysis.

Detection of interferon in cell culture supernatants. We used an assay that depended upon the reduction of Cocal virus plaque numbers in HeLa cells preincubated with interferon-containing medium. HeLa cells ($2{\cdot}5 \times 10^5$) were added to each well of a 24-well plate (Costar). Samples (0·5 ml) of the culture supernatant to be tested were added to each well and the plate was incubated at 37 °C, 5% CO_2 for 24 h. Cocal virus (50 or 500 p.f.u. in 0·1 ml) was added to each well under test and incubated at 37 °C for 30 min. A semi-solid overlay consisting of growth medium containing 1·5% carboxymethyl cellulose (0·5 ml) was added to each well and the plate was incubated for 1 to 2 days at 37 °C, 5% CO_2. The plate was then washed with normal saline and stained using amido black. A reduction in the apparent titre of Cocal virus indicated the presence of interferon. A standard preparation of natural, human α-interferon (Wellferon, a generous gift of the Wellcome Foundation) was used to establish the sensitivity of the assay. In our hands, one unit of Wellferon produced a 50% reduction in plaque numbers, in agreement with the suppliers' assay.

Results

Construction of antisense genes and their transfer into Jurkat cells

We constructed antisense RNA-encoding genes aimed at two regions of HIV transcripts. Within the central region of the HIV genome, these correspond to the *tat*, *rev* and *vpu* splice acceptor sites, the *tat*/*rev* splice donor site and the initiation codons for translation of tat, rev, vpu and env proteins (Fig. 1*b* and Methods). The antisense gene AR9 corresponds to an RNA sequence in the 5′ non-

coding region common to all HIV mRNAs (Fig. 1*c* and Methods). These constructs were designed not only to identify effective antisense genes but also to shed some light on the mechanism of antisense RNA action.

We transduced Jurkat cells with the antisense genes using a retrovirus vector. The polyclonal nature of the transduced lines ensured that observed differences between antisense genes would not depend upon the site of integration of retrovirus in particular transduced clones. Since we had observed that Jurkat cells occasionally lost CD4 expression during culture, we checked that the levels of surface expression of CD4 were comparable in each cell line using flow cytometry (results not shown).

To check that the transduced cells were producing antisense RNA, we probed total cellular RNA prepared from each Jurkat cell line with strand-specific RNA

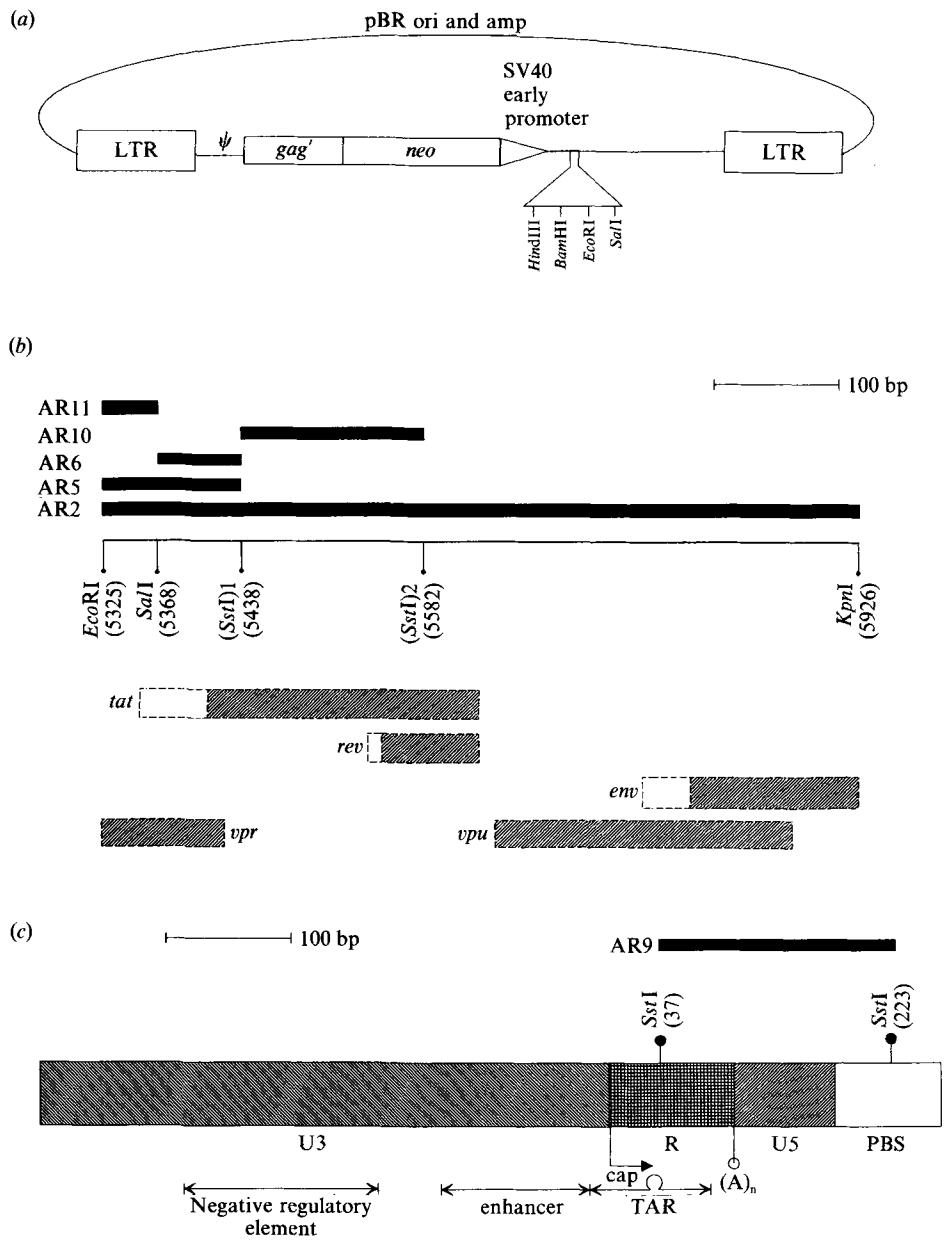


Fig. 1. Construction of the antisense gene transducing particles. (*a*) Structure of the vector, pIRV. Neo. SV (6·5 kb) in which the cloning sites lie 3′ to an internal simian virus 40 (SV40) early promoter. The symbol (ψ) indicates the packaging signal. (*b*) Antisense genes derived from the central region of the HIV genome. Dashed boxes represent exons, hatched areas represent open reading frames and solid bars represent antisense genes. Restriction enzyme sites used in the construction of the antisense genes are indicated and those introduced by site-directed mutagenesis are in parentheses. Numbers indicate nucleotide position relative to the start of transcription. (*c*) An antisense gene in the 5′ non-coding region common to all HIV mRNAs.

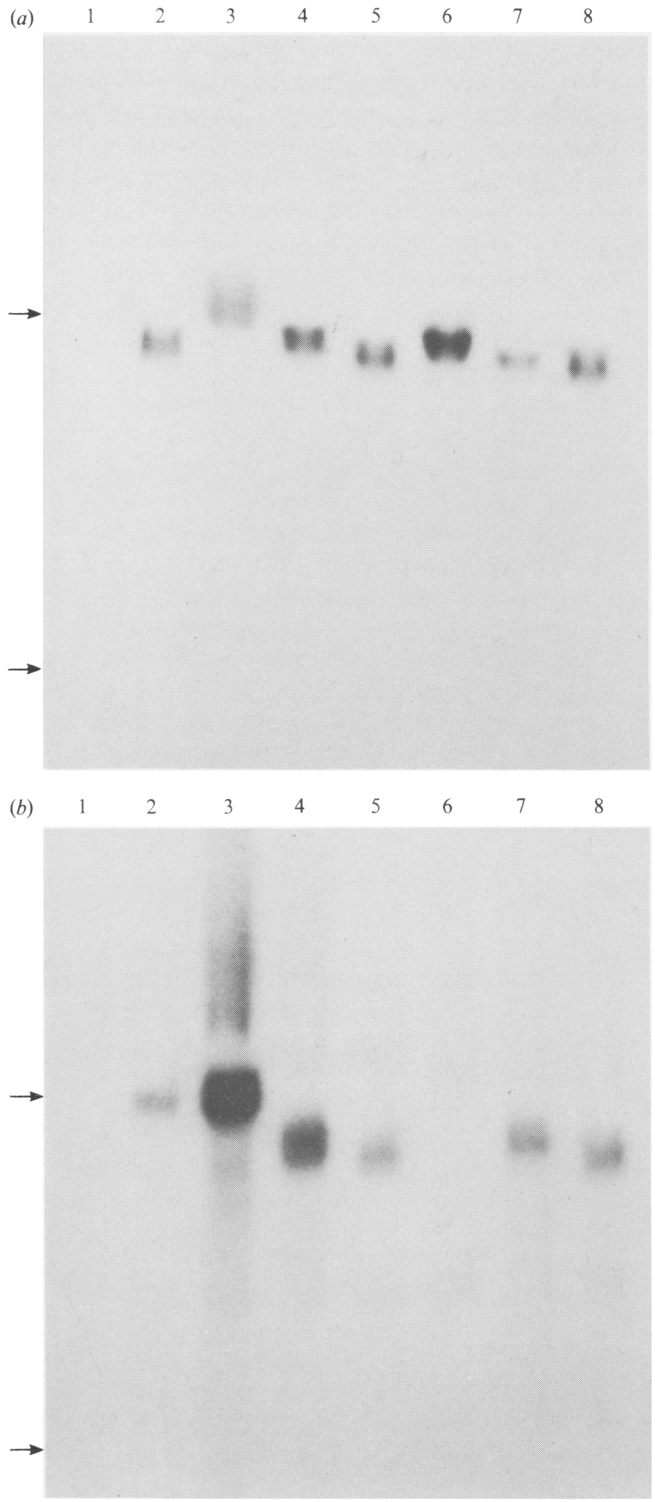


Fig. 2. Detection of HIV antisense RNA in Jurkat cells. Total cellular RNA was extracted from Jurkat cells and from antisense RNA-transduced polyclonal Jurkat cell lines. The cell lines are as follows: Jurkat.AR0, Jurkat cells transduced with the retroviral vector alone; Jurkat.AR2, transduced with the vector containing the antisense gene AR2 (see Fig. 1*b*), etc. Ten μg (except where noted) of RNA was loaded per lane on a denaturing agarose gel, electrophoresed and blotted onto GeneScreen-Plus filters. (*a*) The filter was probed with a riboprobe complementary to a Moloney murine leukaemia virus LTR sequence common to all the antisense constructs. Lanes: 1, Jurkat control; 2, Jurkat.AR0; 3, Jurkat.AR2; 4, Jurkat.AR5; 5, Jurkat.AR6; 6, Jurkat.AR9; 7, Jurkat.AR10; 8, Jurkat.AR11. (*b*) The filter was probed with a riboprobe complementary to the sequence of the AR2 antisense RNA. Lanes: 1, Jurkat.AR0; 2, Jurkat.AR2 (1 μg); 3, Jurkat.AR2 (10 μg); 4, Jurkat.AR5; 5, Jurkat.AR6; 6, Jurkat.AR9; 7, Jurkat.AR10; 8, Jurkat.AR11. The arrows correspond to the migration position of 28S and 18S rRNA (4·8 kb and 1·9 kb), respectively.

probes (Fig. 2). The relative abundance of antisense RNAs transcribed from the vectors can be determined from the autoradiogram (Fig. 2*a*), since the probe used hybridizes to a sequence common to all of the RNAs transcribed from the retroviral vector. The values, relative to that transcribed from the vector alone, are: AR2, 1·47; AR5, 1·66; AR6, 1·52; AR9, 3·66; AR10, 0·54; AR11, 1·22. Fig. 2 (*b*) shows a similar blot probed with an AR2 antisense RNA-specific probe. As expected, no hybridization is detected to RNA transcribed from the vector alone nor from the vector containing AR9, which was derived from the HIV long terminal repeat (LTR). The relative intensities of these bands are affected by the length of the complementarity between the probe and the antisense RNA.

The effect of antisense RNA upon HIV replication

It was possible that the retroviral vector *per se* might alter the rate of HIV replication in transduced Jurkat cells. The polyclonal line of Jurkat cells carrying the vector sequence alone (Jurkat.AR0) grew at the same rate in medium containing G418 as did the parental Jurkat cells grown in G418-free medium (results not shown). We challenged both Jurkat.AR0 and control Jurkat cells with HIV and measured the production of HIV in each case (Fig. 3*a*). The results show that the retroviral vector *per se* does not affect the replication rate of HIV.

We challenged the antisense RNA-transduced cells with HIV and then monitored the replication of HIV by assaying for the core antigen, p24 (Fig. 3*b*). Our results show that the replication of HIV in Jurkat cells can be inhibited by endogenously synthesized antisense RNA. Only two of our antisense constructs (AR2 and AR6) give consistently high levels of inhibition of HIV. The other antisense genes give negligible inhibition. There was no correlation between the abundance of the antisense RNA in the cell and the degree of inhibition.

Secondary structure predictions

The differences observed in the inhibitory potential of each antisense RNA might result from their relative

tendency to form intramolecular base pairs that would preclude hybridization to HIV transcripts. Accordingly, we used the Fold-Vax program (Zuker, 1989) to predict the secondary structure adopted by the HIV primary transcript in the region of the antisense RNAs (Fig. 4). One measure of the tendency of each antisense RNA to form intramolecular base pairs is the predicted change in free energy per nucleotide resulting from folding. The least tightly folded is AR2, −1·00 kJ/mmol, followed by AR6 (−1·05), AR11 (−1·05), AR10 (−1·09), AR9 (−1·09) and AR5 (−1·13). These results suggest that AR5 might be non-inhibitory because it is tightly folded. However if this were the whole explanation, one would expect AR11 to be inhibitory. Two reasonable explanations for this are that the target of AR11 (the splice acceptor site at the 5′ end of *tat* exon 2) is not susceptible to antisense inhibition, or that the length of the antisense–sense RNA duplex formed by AR11 (43 bp) is not sufficiently stable to be inhibitory. These possibilities cannot be distinguished by our results. One might have expected that the local secondary structure in the region of a particular feature such as an anti-initiation codon would have been important. However this does not seem to be the case. For example, the predicted secondary structure in the region around the anti-tat initiation codon is identical in AR5 and AR6 (Fig. 4). Inhibitory antisense RNAs, AR2 and AR6, have a combination of two properties not found in the non-inhibitory antisense RNAs: they have only a low tendency to form intramolecular base pairs and they possess a region complementary to the tat initiation codon.

Transcription of HIV after infection of antisense RNA-transduced cells.

To investigate whether antisense RNA inhibition intervenes before or after the formation of spliced HIV

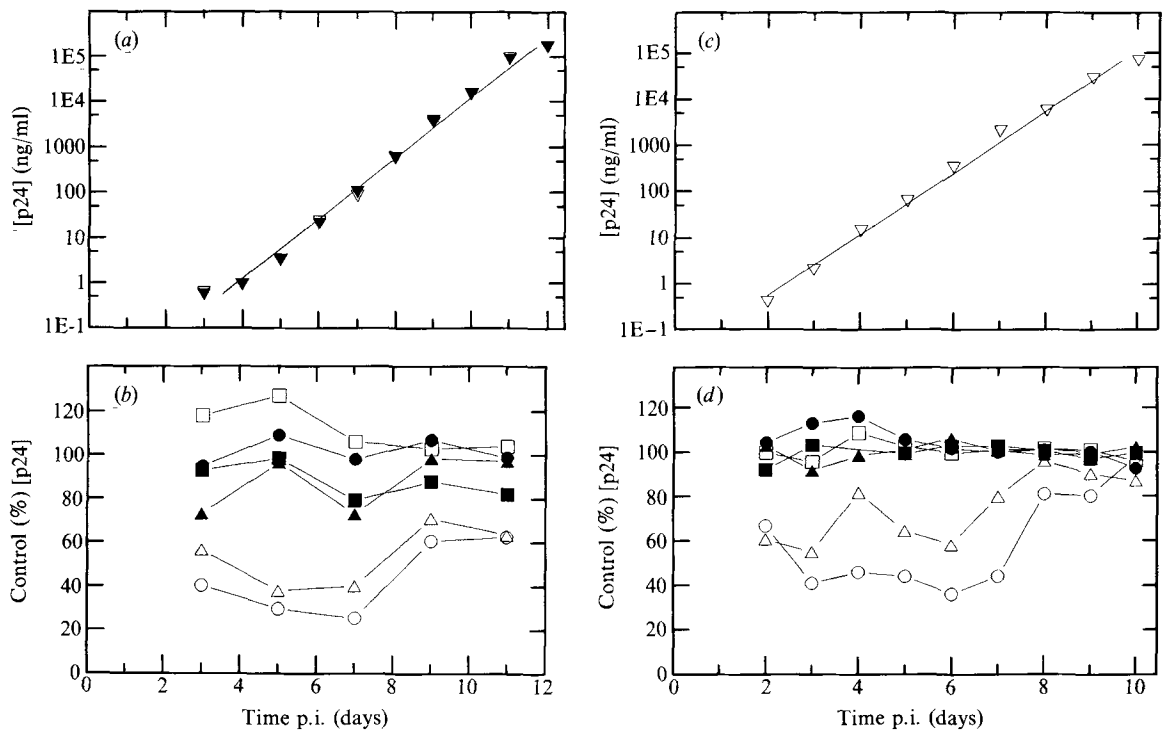


Fig. 3. The effect of the retroviral vector and of the antisense genes upon HIV replication in Jurkat cells. The Jurkat and antisense RNA-transduced Jurkat cell lines were exposed to HIV strain IIIB at an m.o.i. of approximately 0·01. Replication of HIV was detected by measuring extracellular levels of p24 core antigen. The results are adjusted to take account of the volume of medium added at each time of cell feeding (every 3 or 4 days). (*a*) The exponential accumulation of p24 antigen in Jurkat and Jurkat.AR0 following infection with HIV. (*b*) The equivalent p24 titres of the supernatants of the HIV-challenged, antisense RNA-transduced Jurkat cells, expressed as a percentage of the corresponding titre in the culture of the challenged, Jurkat.AR0 cells. (*c* and *d*) Virus was harvested from Jurkat.AR2 cells 12 days after challenge with HIV by which time virus replication had substantially escaped antisense RNA inhibition (see Fig. 4). This antisense-escape virus was used to challenge the retrovirus-transduced Jurkat lines as before. (*c*) The accumulated concentration of HIV p24 antigen, assayed by ELISA, in the culture of Jurkat.AR0 cells challenged with antisense-escape virus. (*d*) The p24 titres of the challenged, antisense-transduced Jurkat lines, expressed as a percentage of the corresponding titre of the Jurkat.AR0 cell supernatant symbols: (▼), Jurkat (control); (▽), Jurkat.AR0; (○), Jurkat.AR2; (●), Jurkat.AR5; (△), Jurkat.AR6; (▲), Jurkat.AR9; (□), Jurkat.AR10; (■), Jurkat.AR11.

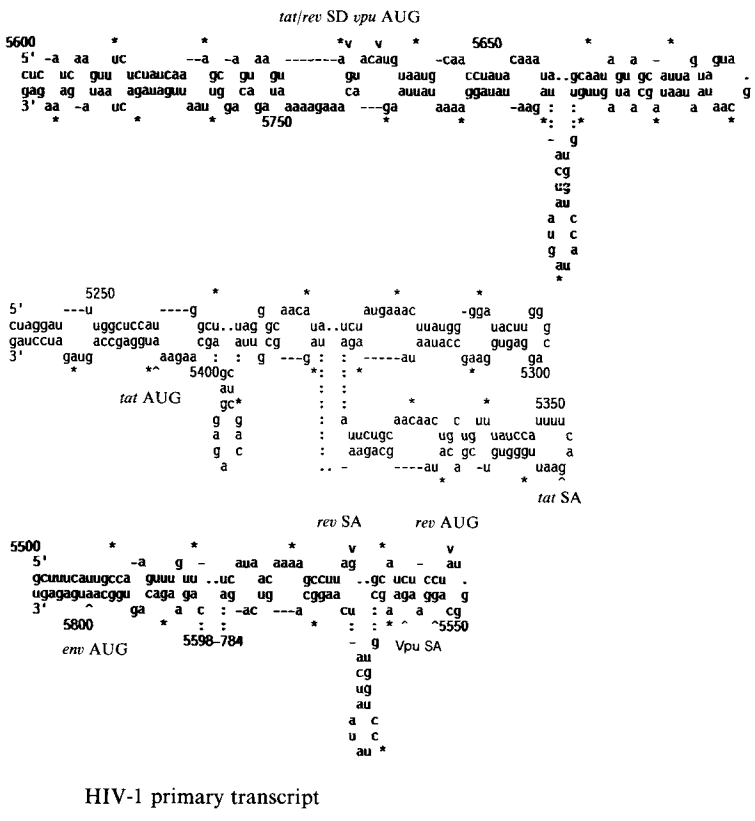
mRNAs, we measured the abundance of HIV mRNAs in antisense RNA-transduced and control Jurkat cells 7 days after challenge with HIV. Total cellular RNA was probed with a riboprobe complementary to the AR9 region contained in all HIV mRNAs. Three bands are identified in such experiments. These correspond to a full-length transcript of the HIV-1 genome, approximately 9·2 kb, a spliced transcript of about 4·3 kb which contains the *env* gene and a set of transcripts of about 2 kb which encode the small regulatory proteins of HIV-1 such as rev and tat (Fig. 5). The antisense gene AR2 gives an approximately 50% reduction in all three HIV mRNA species. The abundance of the HIV mRNAs was not substantially altered in any of the other transduced cells. Since, of the two inhibitory antisense genes, AR2 produces a significant reduction in HIV mRNA levels and AR6 does not, it is tempting to conclude that the inhibition caused by AR6 occurs at the translational level whereas that by AR2 is partly caused by a reduction in the production of HIV mRNAs.

Is antisense inhibition the result of interferon induction?

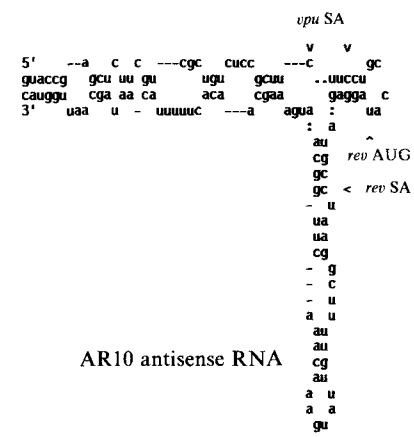
It was possible that the inhibition of HIV replication observed in antisense RNA-expressing cells might be the result of the induction of interferon by antisense–sense RNA duplex formation. Inhibition of HIV replication of the magnitude shown in Fig. 3 could be caused by α-interferon at concentrations of 10 to 100 units/ml (Yamada *et al.*, 1988; Bednarik *et al.*, 1989; Crowe *et al.*, 1989). We developed an assay that was capable of detecting less than 1 unit of α-interferon per ml (see Methods). We assayed culture supernatants from Jurkat cells expressing each antisense RNA 7 days following challenge with HIV in an experiment similar to that described above. In no culture was interferon activity detected (results not shown).

The transience of antisense RNA inhibition

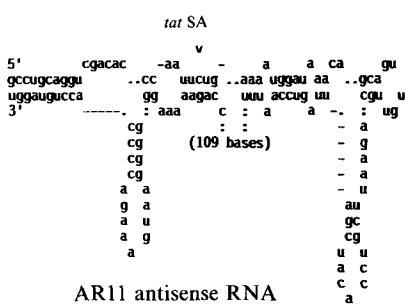
The greatest degree of inhibition occurs about 1 week p.i. (Fig. 3*b*) after which there appears to be a significant and reproducible, though incomplete, escape from inhibition. One explanation for these results is that escape from inhibition represents the outgrowth of sequence variants of HIV which would be at a selective advantage because antisense RNA would not bind to their transcripts. If the emergence of antisense-escape mutants accounted fully for the transience of inhibition, one would then expect that the virus, harvested from a culture of antisense RNA-expressing cells in the second week following challenge with HIV, would be largely resistant to



HIV-1 primary transcript



AR10 antisense RNA



AR11 antisense RNA

antisense inhibition if used to challenge fresh, antisense-transduced cells. Conversely, if this population of virus were still as susceptible to antisense inhibition as was the wild-type, one might then conclude that escape from inhibition represented regulatory changes in the expression of HIV within the challenged cells. Accordingly, we harvested HIV from a culture of HIV-infected Jurkat.AR2 cells 12 days p.i., and used this in a challenge experiment. In this experiment, the virus was still inhibited by both AR2 and AR6. The degree of inhibition was slightly less than that shown in Fig. 3(*b*) and the escape from inhibition was slightly advanced. These small differences, however, are not sufficient to explain the degree of escape observed in the previous experiment and might be due to the higher dose (approximately twofold) of input virus used in the latter experiment.

Consequently, we believe that the reason for escape from antisense RNA inhibition is not mutational but regulatory; that is, it lies in the relative strengths of the regulatory elements governing transcription of the antisense and HIV RNAs. Whereas the antisense RNAs are expressed at a relatively constant level, transcription of HIV rises from an initially low level on entry into the cell to a much higher level once tat is expressed. One explanation is that the relatively constant amount of antisense RNA, transcribed from the retroviral vector, is sufficient to inactivate most of the RNA transcribed from the non-activated HIV LTR but is insufficient to match the transcripts from the tat-activated LTR. Any HIV *tat* mRNA that escapes antisense inhibition at the early stage will be translated to produce tat which in turn will stimulate the production of greater quantities of *tat* mRNA. There are additional mechanisms whereby escape of HIV from antisense inhibition in one cell in the population might induce HIV replication in neighbouring cells. Unconfirmed reports suggest that tat can be released from HIV-infected cells and it is known that tat can be taken up by nearby cells resulting in transactivation of resident HIV proviruses (Frankel & Pabo, 1988)

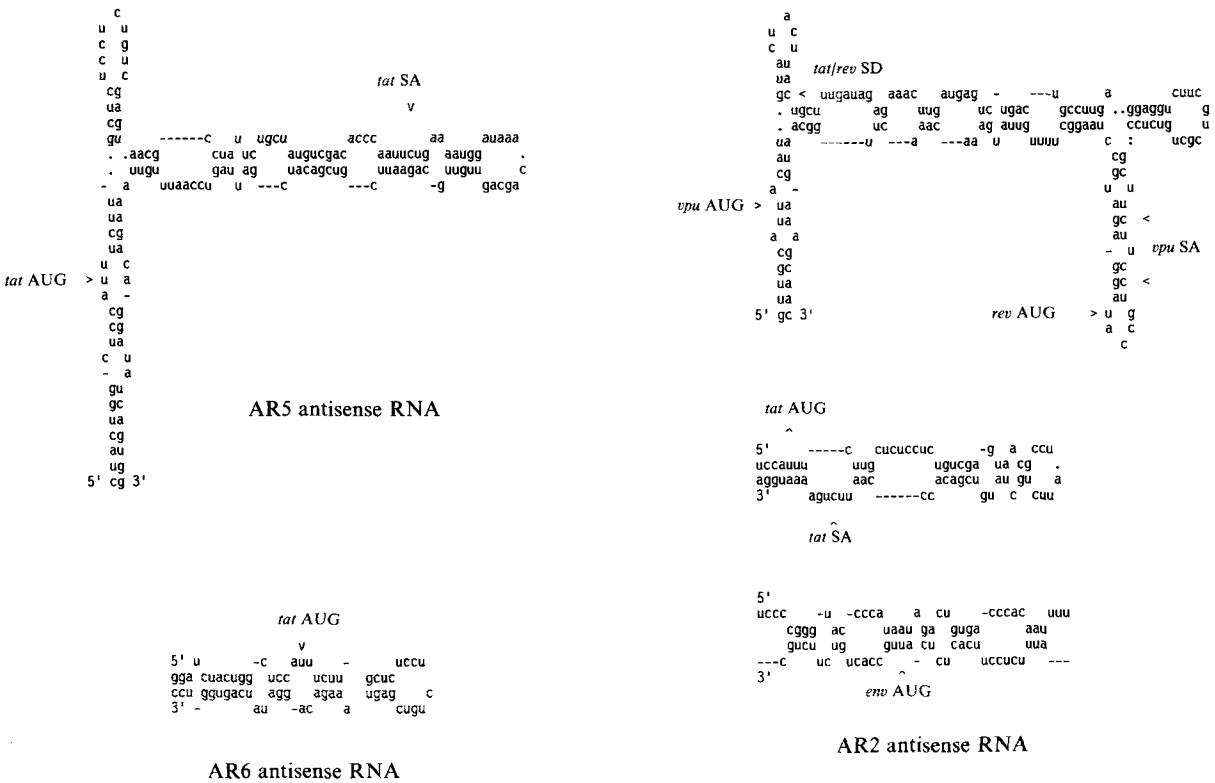


Fig. 4. Predicted secondary structures of HIV and antisense RNAs. The FOLD-VAX program of Zuker (1989) was used to predict the minimum energy structures of the HIV primary transcript and each antisense RNA. Small portions of the predicted structures, containing particular features, are illustrated. In each portion, the RNA strand generally runs clockwise with the 5′ position marked. Hyphens indicate asymmetric bulges and dots connect otherwise adjacent bases. In the HIV transcript, bases are numbered from the start of transcription and asterisks mark every tenth base. The arrowheads mark the first or last base of a particular intron or the A residue of an initiation codon. In the antisense RNAs, the base marked is complementary to the feature indicated. AUG refers to an initiation codon, SA to a splice acceptor site and SD to a splice donor site.

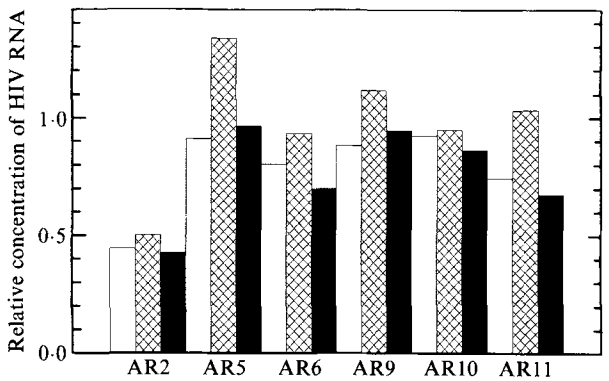


Fig. 5. Abundance of HIV mRNAs in infected, antisense RNA-expressing Jurkat cells. The latter, transduced with antisense genes using the retroviral vector, IRV.Neo.SV, were infected with HIV-1_{IIIB} as before. At 7 days p.i., total cellular RNA was extracted and 10 μg samples were analysed by Northern blotting using a riboprobe complementary to a region common to all HIV mRNAs. The autoradiogram was scanned and the activity of each of the HIV mRNA species was determined. In the histogram, the open bars represent the full-length mRNA (9·2 kb), the cross-hatched bars, the *env* mRNA (4·3 kb) and the solid bars, the *tat/rev* mRNAs (2 kb). The results (mean of two experiments), are expressed as the ratio of concentration of the RNA in question to that of the equivalent RNA observed in Jurkat cells transduced with the vector alone.

and that tat can induce increased expression of CD4 (Koka *et al.*, 1988) which may in turn increase virus production. This means that even if one HIV-infected cell were to escape from antisense RNA inhibition, the effect would spread to the rest of the population.

Discussion

It is somewhat difficult to compare our results with those of other workers using different viruses. In the most comparable case, using an antisense RNA-encoding retrovirus against human T cell lymphoma-leukaemia virus type I (von Ruden & Gilboa, 1989), the effect of the virus on the proliferation of T cells rather than on the production of virus itself was measured. In another study in which a 10-fold molar excess of antisense RNA-encoding plasmid was cotransfected with Rous sarcoma proviral clones (Chang & Stoltzfus, 1987), levels of inhibition of the production of infectious virus were between 23% and 82% depending on the construct. In our system, there is likely to be one copy of the antisense gene per cell. Cases in which absolute abolition of gene expression have been noted involve the introduction of a large excess of the antisense gene into the cell (e.g. about 10^7 microinjected copies per cell; Giebelhaus *et al.*, 1988). However, in one case a 1000-fold excess of antisense RNA failed to inhibit the translation of chloramphenicol acetyltransferase mRNA (Kerr *et al.*, 1988). Judging from a number of positive results which have appeared in the literature, the levels of inhibition that we have demonstrated seem to be within the range that one would expect from an unmodified antisense system (Izant & Weintraub, 1984; Crowley *et al.*, 1985; Kim & Wold, 1985; McGarry & Lindquist, 1986; Sandri-Goldin *et al.*, 1987; Jennings & Molloy, 1987; Hambor *et al.*, 1988).

Can one conclude anything from our results concerning the mechanism of antisense RNA inhibition that would help one to design inhibitory antisense genes? Our results suggest that an antisense RNA must not have a strong tendency to fold upon itself; otherwise it cannot hybridize to the target RNA. The prediction of RNA secondary structure is not perfect although we have taken a number of precautions to maximize its reliability. In addition, it appears that initiation codons of regulatory genes are particularly good targets for antisense inhibition and our results are consistent with translational arrest being a component of the inhibitory mechanism. Finally we have shown that the induction of interferon by antisense–sense RNA duplexes is not the mechanism of inhibition.

A number of complementary approaches might be taken to increase the effectiveness of antisense RNA inhibition. One might seek further to attenuate the residual level of target mRNA translation so that the breakthrough concentration of tat is never attained. This might be achieved by employing stronger expression vectors or by developing vectors which could provide coordinately regulated synthesis of antisense RNA to counteract any rise in target mRNA expression. Secondly one might develop more effective antisense genes, and in the process test our proposals for the requirements of inhibitory antisense genes, outlined above. Finally one might attempt to produce antisense ribozyme genes by incorporation of catalytic sequences into the inhibitory antisense genes.

We thank Neal Nathanson for helpful discussions. A.R. is the holder of an MRC (non-ADP) research studentship and W.J. is a Monsanto Senior Research Fellow, Exeter College, Oxford. The work was supported by the EPA Research Fund, Monsanto Research Inc. and the MRC AIDS-Directed Programme.

References

AGRAWAL, S., GOODCHILD, J., CIVEIRA, M. P., THORNTON, A. H. SARIN, P. S. & ZAMECNIK, P. C. (1988). Oligodeoxynucleoside phosphoramidates and phosphorothioates as inhibitors of HIV. *Proceedings of the National Academy of Sciences, U.S.A.* **85**, 7079–7083.

AUSUBEL, F. M., BRENT, R., KINGSTON, R. E., MOORE, D. D., SEIDMAN, J. G., SMITH, J. A. & STRUHL, K. (1987). *Current Protocols in Molecular Biology*. New York: John Wiley.

BEDNARIK, D. P., MOSCA, J. D., RAJ, N. B. & PITHA, P. M. (1989). Inhibition of human immunodeficiency virus (HIV) replication by HIV-trans-activated alpha 2-interferon. *Proceedings of the National Academy of Sciences, U.S.A.* **86**, 4958–4962.

BROWN, A. M. C., SCOTT, M. R. D. & GLOVER, E. M. (1987). Retroviral Vectors. In *DNA Cloning: A Practical Approach*, vol. III, pp. 189–212. Edited by D. M. Glover. Oxford: IRL Press.

CHANG, L. J. & STOLTZFUS, C. M. (1987). Inhibition of Rous sarcoma virus replication by antisense RNA. *Journal of Virology* **61**, 921–924.

COTTEN, M., SCHAFFNER, G. & BIRNSTIEL, M. L. (1989). Ribozyme, antisense RNA, and antisense DNA inhibition of U7 small nuclear ribonucleoprotein-mediated histone pre-messenger RNA processing *in vitro*. *Molecular and Cellular Biology* **9**, 4479–4487.

CROWE, S. M., MCGRATH, M. S., ELBEIK, T., KIRIHARA, J. & MILLS, J. (1989). Comparative assessment of anti-retrovirals in human monocyte-macrophages and lymphoid cell lines acutely and chronically infected with the human immunodeficiency virus. *Journal of Medical Virology* **29**, 176–180.

CROWLEY, T. E., NELLEN, W., GOMER, R. H. & FIRTEL, R. A. (1985). Phenocopy of discoidin I-minus mutants by antisense transformation in *Dictyostelium*. *Cell* **43**, 633–641.

CUOZZO, M., O'CONNELL, K. M., KANIEWSKI, W., FANG, R. X., CHUA, N. H. & TUMER, N. E. (1988). Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. *Bio/Technology* **6**, 549.

FISHER, A. G., COLLALTI, E., RATNER, L., GALLO, R. C. & WONG-STAAL, F. (1985). A molecular clone of HTLV-III with biological activity. *Nature, London* **316**, 262–265.

FOLEY, G. E., LAZARUS, H., FARBER, S., UZMAN, B. G. BOONE, B. A. & MCCARTHY, R. E. (1965). Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* **18**, 522–529.

FRANKEL, A. D. & PABO, C. O. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55**, 1189–1193.

FREIER, S. M., KIERZEK, R., JAEGER, J. A., SUGIMOTO, N., CARUTHERS, M. H., NEILSON, T. & TURNER, D. H. (1986). Improved free-energy parameters for predictions of RNA duplex stability. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 9373–9377.

GIEBELHAUS, D. H., EIB, D. W. & MOON, R. T. (1988). Antisense RNA inhibits expression of membrane skeleton protein 4.1 during embryonic development of *Xenopus*. *Cell* **53**, 601–615.

GOODCHILD, J., AGRAWAL, S., CIVEIRA, M. P., SARIN, P. S., SUN, D. & ZAMECNIK, P. C. (1988). Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides. *Proceedings of the National Academy of Sciences, U.S.A.* **85**, 5507–5551.

GORDON, Y. J., JOHNSON, B., ROMANOWSKI, E. & ARAULLOCRUZ, T. (1988). RNA complementary to herpes simplex virus type-1 ICP0 gene demonstrated in neurons of human trigeminal ganglia. *Journal of Virology* **62**, 1832–1835.

HAHN, B. H., SHAW, G. M., ARYA, S. K., POPOVIC, M., GALLO, R. C. & WONG-STAAL, F. (1984). Molecular cloning and characterization of the HTLV-III virus associated with AIDS. *Nature, London* **312**, 166–169.

HAMBOR, J. E., HAUER, C. A., SHU, H.-K., GROGER, R. K. & KAPLAN, D. R. (1988). Use of an Epstein–Barr virus episomal replicon for anti-sense RNA-mediated gene inhibition in a human cytotoxic T-cell clone. *Proceedings of the National Academy of Sciences, U.S.A.* **85**, 4010–4014.

HEMENWAY, C., FANG, R. X., KANIEWSKI, W. K., CHUA, N. H. & TUMER, N. E. (1988). Analysis of the mechanism of protection in transgenic plants expressing the potato virus-X coat protein or its antisense RNA. *EMBO Journal* **7**, 1273–1280.

IZANT, J. G. & WEINTRAUB, H. (1984). Inhibition of thymidine kinase gene expression by anti-sense RNA: a molecular approach to genetic analysis. *Cell* **36**, 1007–1015.

JAINCHILL, J., AARONSON, S. A. & TODARO, G. J. (1969). Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. *Journal of Virology* **4**, 549–553.

JENNINGS, P. A. & MOLLOY, P. L. (1987). Inhibition of SV40 replicon function by engineered antisense RNA transcribed by RNA polymerase III. *EMBO Journal* **6**, 3043–3047.

KERR, S. M., STARK, G. R. & KERR, I. M. (1988). Excess antisense RNA from infectious recombinant SV40 fails to inhibit expression of a transfected, interferon-inducible gene. *European Journal of Biochemistry* **175**, 65–73.

KHOCHBIN, S. & LAWRENCE, J.-J. (1989). An antisense RNA involved in p53 mRNA maturation in murine erythroleukemia cells induced to differentiate. *EMBO Journal* **8**, 4107–4114.

KIM, S. K. & WOLD, B. J. (1985). Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA. *Cell* **42**, 129–138.

KIMELMAN, D. & KIRSCHNER, M. W. (1989). An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in *Xenopus* oocytes. *Cell* **59**, 687–696.

KOKA, P., YUNIS, J., PASSARELLI, A. L., DUBEY, D. P., FALLER, D. V. & YUNIS, E. J. (1988). Increased expression of CD4 molecules on Jurkat cells mediated by human immunodeficiency virus Tat protein. *Journal of Virology* **62**, 4353–4357.

MCGARRY, T. J. & LINDQUIST, S. (1986). Inhibition of heat shock protein synthesis by heat-inducible antisense RNA. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 399–403.

MCMICHAEL, A. (1987). *Leukocyte Typing*, vol. 3. Oxford: Oxford University Press.

MANN, R., MULLIGAN, R. C. & BALTIMORE, D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**, 153–159.

MATSUKURA, M., SHINOZUKA, K., ZON, G., MITSUYA, H., REITZ, M., COHEN, J. S. & BRODER, S. (1987). Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 7706–7710.

MILLER, A. D. & BUTTIMORE, C. (1986). Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Molecular and Cellular Biology* **6**, 2895–2902.

MUNROE, S. H. (1988). Antisense RNA inhibits splicing of pre-mRNA *in vitro*. *EMBO Journal* **7**, 2523–2532.

NAKAMAYE, K. & ECKSTEIN, F. (1986). Inhibition of restriction endonuclease *Nci*I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. *Nucleic Acids Research* **14**, 9679–9698.

RATNER, L., HASELTINE, W., PATARCA, R., LIVAK, K. J., STARCICH, B., JOSEPHS, S. F., DORAN, E. R., RAFALSKI, J. A., WHITEHORN, E. A., BAUMEISTER, K., IVANOFF, L., PETTEWAY, S. R., PEARSON, M. L., LAUTENBERGER, J. A., PAPAS, T. S., GHRAYEB, J., CHANG, N. T., GALLO, R. C. & WONG-STAAL, F. (1985). Complete nucleotide sequence of the AIDS virus. *Nature, London* **313**, 277–284.

RODERMEL, S. R., ABBOTT, M. S. & BOGORAD, L. (1988). Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. *Cell* **55**, 673–681.

ROGERS, J. C. (1988). RNA complementary to alpha-amylase mRNA in barley. *Plant Molecular Biology* **11**, 125–138.

SALSER, W. (1978). Globin mRNA sequences: analysis of base pairing and evolutionary implications. *Cold Spring Harbor Symposia on Quantitative Biology* **42**, 985–1002.

SANDRI-GOLDIN, R. M., SEKULOVICH, R. E. & LEARY, K. (1987). The alpha protein, ICP0, does not appear to play a major role in the regulation of herpes simplex virus gene expression during infection in tissue culture. *Nucleic Acids Research* **15**, 905–919.

SARIN, P., AGRAWAL, S., CIVEIRA, M. P., GOODCHILD, J., IKEUCHI, T. & ZAMECNIK, P. C. (1988). Inhibition of AIDS virus by oligodeoxynucleoside methyl phosphonates. *Proceedings of the National Academy of Sciences, U.S.A.* **85**, 7448–7451.

SHIBAHARA, S., MUKAI, S., MORISAWA, H., NAKASHIMA, H., KOBAYASHI, S. & YAMAMOTO, N. (1989). Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives. *Nucleic Acids Research* **17**, 239–252.

SIMONS, R. W. & KLECKNER, N. (1988). Biological regulation by antisense RNA in prokaryotes. *Annual Review of Genetics* **22**, 567–601.

SMITH, C. J. S., WATSON, C. F., RAY, J., BIRD, C. R., MORRIS, P. C., SCHUCH, W. & GRIERSON, D. (1988). Antisense RNA inhibition of

polygalacturonase gene expression in transgenic tomatoes. *Nature, London* **334**, 724.

TAYLOR, J. W., OTT, J. & ECKSTEIN, F. (1985). The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. *Nucleic Acids Research* **13**, 8764–8785.

VOLK, R., KOSTER, M., POTING, A., HARTMANN, L. & KNOCHEL, W. (1989). An antisense transcript from the *Xenopus laevis* bFGF gene coding for an evolutionarily conserved 24kd protein. *EMBO Journal* **8**, 2983–2988.

VON RUDEN, T. & GILBOA, E. (1989). Inhibition of human T-cell leukaemia virus type 1 replication in primary human T cells that express antisense RNA. *Journal of Virology* **63**, 677–682.

WALDER, J. (1988). Antisense DNA and RNA: progress and prospects. *Genes and Development* **2**, 502–504.

WEISS, A., WISKOCIL, R. L. & STOBO, J. D. (1984). The role of T3 surface molecules in the activation of human T cells: a two stimulus requirement for IL-2 production reflects events occurring at a pre-translational level. *Journal of Immunology* **133**, 123–128.

YAMADA, O., HATTORI, N., KURIMURA, T., KITA, M. & KISHIDA, T. (1988). Inhibition of growth of HIV by human natural interferon *in vitro*. *AIDS Research and Human Retroviruses* **4**, 287–294.

ZAIA, J. A., ROSSI, J. J., MURAKAWA, G. J., SPALLONE, P. A., STEPHENS, D. A., KAPLAN, B. E., ERITJA, R., WALLACE, R. B. & CANTIN, E. M. (1988). Inhibition of human immunodeficiency virus by using an oligonucleoside methyl-phosphonate targeted to the tat-3 gene. *Journal of Virology* **62**, 3914–3917.

ZAMECNIK, P. C., GOODCHILD, J., TGUCHI, Y. & SARIN, P. (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. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 4143-4146.

ZUKER, M. (1989). Computer prediction of RNA structure. *Methods in Enzymology* **180**, 262–288.

(Received 22 December 1989; Accepted 24 May 1990)