MOLECULAR AND CELLULAR BIOLOGY, Dec. 1986, p. 4758–4762
0270-7306/86/124758-05$02.00/0

Vol. 6, No. 12

Inhibition of Retroviral Replication by Anti-Sense RNA

RICHARD Y.-L. TO,* SHANE C. BOOTH, AND PAUL E. NEIMAN

Division of Basic Sciences, Fred Hutchinson Cancer Center, Seattle, Washington 98104

Received 3 April 1986/Accepted 8 September 1986

We tested the effect of anti-sense RNA on the replication of avian retroviruses in cultured cells. The replication of a recombinant retrovirus carrying a neomycin resistance gene (*neo*^{r}) in the anti-sense orientation was blocked when the cells expressed high steady-state levels of RNA molecules with *neo*^{r} sequences in the sense orientation, i.e., complementary to the viral sequence. Viral DNA bearing *neo*^{r} sequences was not detected specifically in host cells where this anti-sense RNA inhibition of viral replication occurred. These observations suggest that anti-sense RNA inhibition may be a useful strategy for the inhibition of retroviral infections.

The anti-sense RNA-mediated inhibition of gene expression (5) has potential as a tool for genetic analysis (1, 8, 10, 11) and has also been suggested as a novel approach to inhibiting viral infections (5; E. C. M. Mariman, Letter, Nature (London) **318**:414, 1985; R. Tellier and J. M. Weber, Letter, Nature (London) **318**:414, 1985). We carried out experiments which addressed the question of whether anti-sense RNA can inhibit retroviral replication. We constructed a recombinant, replication-competent avian retrovirus which can express anti-sense RNA molecules complementary to abundant RNA transcripts from a particular cellular gene. We then examined the consequences of infection of host cells on the expression of the target host cell gene and on the replication of the retrovirus bearing the complementary RNA sequence.

The target sense and anti-sense sequences for these experiments were derived from the *neo*^{r} gene of the bacterial transposable element Tn*5*. This gene confers resistance to the cytotoxic effects of the neomycin analog G418 in eucaryotic cells (12) and therefore can serve as a convenient marker for gene transfer. Furthermore, the *neo*^{r} gene is of a size appropriate for packaging within a nondefective retroviral vector system, 779NCTAQ26, originally developed from cloned Rous sarcoma virus (RSV) genomes by Hughes and Kosik (4). This vector was engineered to maximize the stability of genes inserted in place of the RSV *src* gene by deletion of one of the flanking direct repeats. A *neo*^{r}-containing 2.14 -kilobase (kb) *Hin*dIII-*Hpa*I fragment from pSV2-neo (Fig. 1) was inserted into the *Cla*I site of the 779NCTAQ26 vector in either the sense (N-10) or the anti-sense (αN-10) orientation with respect to the viral genome. The constructs were digested with *Sal*I to remove the bacterial sequences and religated prior to transfection into chicken embryo fibroblasts by the calcium phosphate precipitation technique (2). Chicken embryo fibroblasts were propagated at 37°C in F10 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% (vol/vol) tryptose phosphate broth, 5% calf serum, and 0.05% (wt/vol) sodium bicarbonate in the presence of penicillin, streptomycin, and nystatin (Mycostatin; E. R. Squibb & Sons, Princeton, N.J.). Progeny viruses were recovered from supernatant fluids. Cells infected with N-10 virus grew normally in the presence of 200 μg of G418 per ml, while those infected with αN-10 virus and uninfected control cells were killed completely in 5 to 7 days by the same concentration of G418.

The *neo*^{r} sequences of N-10 and αN-10 virion RNAs were detected selectively with ^{32}P-labeled single-stranded *neo*^{r} RNA probes complementary to sense and anti-sense *neo*^{r} sequences, respectively, prepared by using an SP6-T7 plasmid system (pGEM4; Promega Biotec, Madison, Wis.). Both sense and anti-sense *neo*^{r} inserts were equally stable in viral RNA upon chronic passage in both chicken embryo fibroblasts and quail cells. Figure 2, panel I, shows a dot blot analysis of *neo*^{r} sequences in N-10 and αN-10 viral stocks grown on quail cells. The level of *neo*^{r} sequences relative to total viral RNA was comparable in both stocks. RNA blot hybridization analysis with a viral sequence probe of N-10 and αN-10 viral stocks from chronic passages also showed the accumulation of virions which had deleted *neo*^{r} inserts. These deleted genomes never amounted to more than 50 to 65% of the total viral stock (Fig. 2, panel III), in comparison with the 10-fold or greater excess of analogous transformation-defective deletions common in wild-type stocks of chronically passaged RSV (7).

The host cell system we used to test possible mutual anti-sense effects on both viral replication and target host cell gene expression was derived from a chemically transformed Japanese quail cell line, QT35 (9), which had been made G418 resistant by transfection with a defective retroviral vector carrying the *neo*^{r} gene. QT35 cells and *neo*^{r} derivatives were propagated under the same conditions as those described earlier for chicken embryo fibroblasts, except that the medium was supplemented with 1% dimethyl sulfoxide. Figure 3 shows a slot blot analysis of virion RNA produced by cultures of three clones of G418-resistant QT35 derivatives infected with either N-10 or αN-10. The three clones were derived from three independent transfections of QT35 with constructs that express *neo*^{r} from an avian retroviral promoter. The steady-state levels of *neo*^{r} transcripts in each of the clones was determined by *neo*^{r} hybridization by slot blot analysis of total cellular RNA in comparison with the signal obtained from authentic *neo*^{r} RNA prepared in vitro and blotted on the same membrane. These levels varied from 0.001 to 0.04% of total cellular RNA (Fig. 3). *neo*^{r}-containing viral RNA was detected in the supernatants from all three N-10-infected clones. However, we did not detect *neo*^{r}-containing viral RNA in the supernatants from two of the clones (GG and A1) infected with αN-10 and detected only very low levels of this RNA in the supernatant from the αN-10-infected C1-2 clone. This clone had the lowest level of *neo*^{r} transcripts.

* Corresponding author.

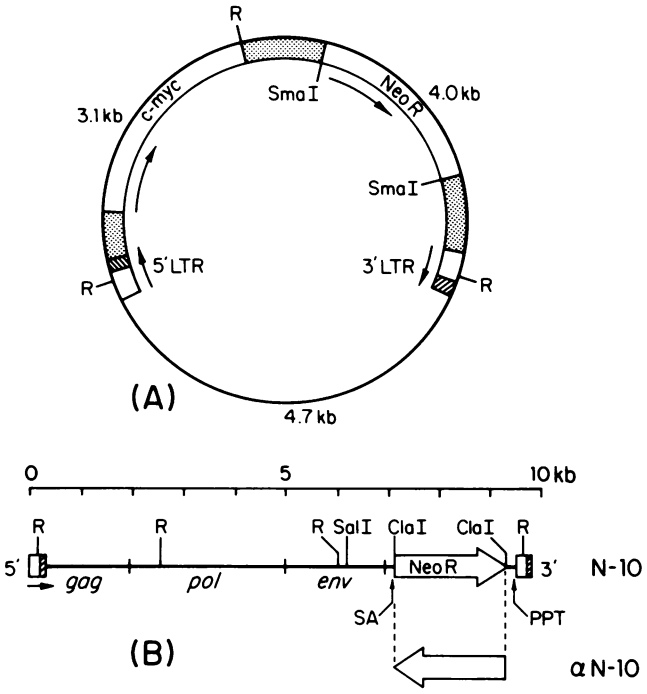

FIG. 1. Retroviral vectors for transferring *neo*^{r} sequences. (A) Plasmid used to transfer G418 resistance to A1 cells by transfection. The solid line denotes bacterial plasmid pBR322 sequences; dotted segments represent retroviral sequences; and hatched segments represent LTRs. The *neo*^{r} insert (Neo R) and a region containing a c-*myc* gene (c-myc) are indicated. (B) Structures of the DNA forms of N-10 and αN-10 retroviruses. Hatched segments are U_5 regions of viral LTRs; PPT is a polypurine tract; *gag* is the viral gene for internal structural proteins; *pol* is the viral gene for reverse transcriptase; *env* is the viral envelope gene; R denotes *Eco*RI sites.

This initial analysis suggested a specific inhibition of the replication of αN-10, perhaps related to the level of *neo*^{r} transcripts in the cells. Further analysis was performed with A1 cells. The construct used for transfection during the isolation of this G418-resistant clone is shown in Fig. 1. A 2.14-kb *Hin*dIII-*Hpa*I fragment of pSV2-neo (12) containing the complete *neo*^{r} gene was inserted in the sense orientation (indicated by arrows in Fig. 1) at the *Sma*I site of a replication-defective retroviral vector that contained a 5′ long terminal repeat (LTR) from cloned Schmidt-Ruppin RSV subgroup A and a 3′ LTR from a subgroup B helper virus. *neo*^{r} transcripts, which represented about 0.003% of A1 total cellular RNA (Fig. 3), were detected by blot hybridization analysis (Fig. 2, panel IIA).

Table 1 shows an analysis of the effect of infection with a retrovirus expressing anti-sense *neo*^{r} RNA (αN-10) on the ability of A1 cells to grow in the presence of G418. A1 cells were infected with αN-10 virus and with a virus derived from a retroviral vector without any insert. Viral stocks were titrated by comparison of reverse transcriptase levels with those of control stocks of wild-type RSV. Infections were carried out at a multiplicity of infection of 0.1 to 1.0. Uninfected and infected cells were passaged three times in the absence of G418 and seeded onto culture dishes. Assays were performed in the presence of 200 μg of G418 per ml by direct cell counting in standard cultures and by colony formation on plastic dishes and in soft agar. Uninfected QT35 cells were also assayed as a control for the toxic effect of G418 on parental cells. For direct cell counting in standard

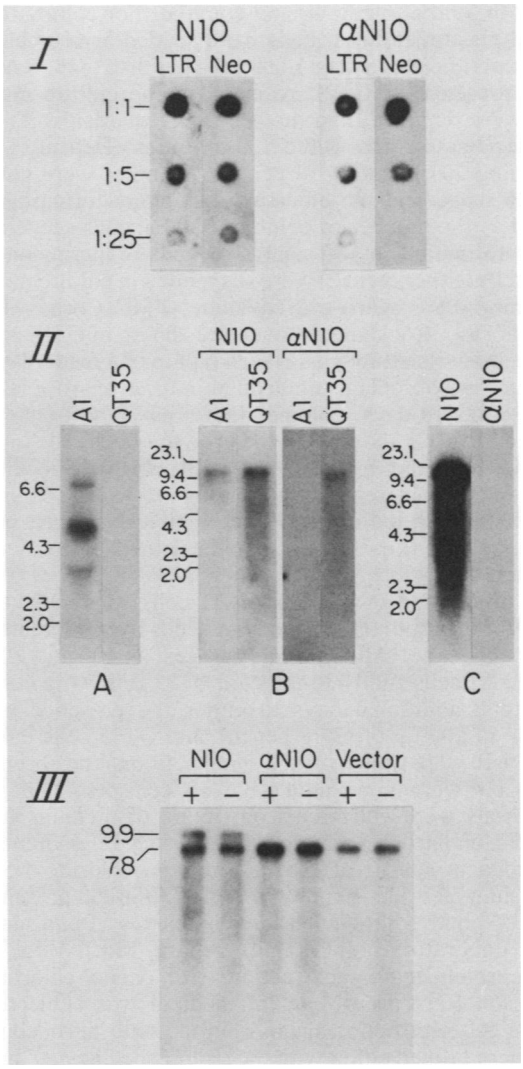

FIG. 2. Analysis of viral and cellular RNAs. (Panel I) Dot blot analysis of virion RNAs from stocks of N-10 and αN-10 grown on QT35 cells. Virion RNA representing 4.0 ml of culture fluid and serial fivefold dilutions were bound to nitrocellulose membranes and probed with either a ^{32}P-labeled 0.75-kb *Pvu*II-*Sst*I fragment of Schmidt-Ruppin RSV subgroup A DNA spanning the LTR or strand-specific *neo*^{r} RNA probes prepared as described in the text. (Panel II) Blot hybridization analysis of *neo*^{r} RNA molecules in cells and viruses. (A) *neo*^{r} transcripts in A1 cells. About 5 μg of total cytoplasmic RNA was separated by electrophoresis on a 1% agarose gel in 2.2 M formaldehyde, blotted onto nitrocellulose, and hybridized to a ^{32}P-labeled 2.14-kb *Hin*dIII-*Hpa*I *neo*^{r} DNA fragment of pSV2-neo. (B) Virion RNA from N-10- and αN-10-infected QT35 and A1 cells probed with strand-specific ^{32}P-labeled *neo*^{r} RNA probes. (C) Virion RNA from N-10- and αN-10-infected A1 cells in a separate experiment from that shown in panel B. Autoradiograms were overexposed for increased sensitivity. Numbers at left are kilobases. (Panel III) Viral RNA from supernatants of A1 cells infected with N-10, αN-10, or vector viruses. Following electrophoresis and transfer to nitrocellulose membranes, viral RNA was detected with a nick-translated probe for viral LTR sequences. Numbers at left are kilobases.

cultures, cells were seeded at 5×10^5 cells per dish, and G418 was added. After 5 days (experiment 1) and 8 days (experiment 2) of culturing, the cells were trypsinized, diluted in 3 ml of medium, and counted. For colony formation on plastic surfaces, cells were seeded at 100 cells per dish (experiment 1) and at 1,000 cells per dish (experiment 2) in the presence of G418. After 24 h, the culture medium fluids were removed, and the cells were overlaid with 0.6% agarose (Bacto-Agar; Difco Laboratories, Detroit, Mich.) containing 200 μg of G418 per ml. Colonies were counted after 16 days, and the efficiency of colony formation was defined as the number of colonies per 100 cells seeded. For colony formation in soft agar, 100 cells (experiment 1) or 1,000 cells (experiment 2) were suspended in culture medium containing 0.5% agarose (SeaPlaque; FMC Corp., Marine Colloids Div., Rocklane, Maine) and 200 μg of G418 per ml. Each cell-agar mixture was placed on top of a feeder layer of quail embryo fibroblasts embedded in 0.7% agarose (Bacto-Agar). After 10 days, colonies that grew in soft agar were counted. Table 1 shows that the growth of A1 cells in G418 was not inhibited by infection with αN-10. Specifically, infection with a retrovirus bearing anti-sense *neo*[5] sequences, αN-10, did not appear to inhibit the expression of *neo*[r] in A1 cells (i.e., it did not increase the sensitivity of A1 cells to G418).

The production of virus from A1 cells was analyzed by blot hybridization. QT35 and A1 cells were infected as described above. After three passages, N-10- and αN-10-infected A1 cells and N-10-infected QT35 cells were cultured for 7 to 8 additional days in either the presence or the absence of 200 μg of G418 per ml, and QT35 cells infected with αN-10 were grown for the same period in the absence of G418. Cells were then plated at 3×10^6 cells per 10-cm plate, and virions were collected from 35 ml of medium after 2 days. Virion particles were purified in sucrose gradients and suspended in 400 μl of 150 mM sodium chloride–1.5 mM magnesium chloride–10 mM Tris (pH 8.0) in the presence of 10 mM vanadyl-ribonucleoside complexes (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Viral RNA was deproteinized by extraction with phenol-chloroform, and virion RNA from 4 ml of medium was subjected to agarose gel electrophoresis and blotted onto nitrocellulose. Blots were probed either with the strand-specific *neo*[r] probes or with a viral LTR probe. Hybridization conditions were as previously described (6). Figure 2, panels IIB and IIC, shows that the sense *neo*[r] sequence in full-length virion RNA (9.8-kb band) was easily detected in progeny viruses from N-10-infected A1 cells. However, when αN-10 was used to infect A1 cells in the presence of G418, we did not detect any virus that carried the anti-sense *neo*[r] sequence in supernatant culture fluid. This block to αN-10 was not due to an unknown effect of G418 because the result was not influenced by the presence or absence of G418. This result is shown in Fig. 2, panel III, in which virion RNAs from A1 cells infected with N-10, αN-10, or vector viruses were detected with a probe for viral sequences. The 9.8-kb RNA species (which contains *neo*[r], Fig. 2, panel II) was only detected in supernatants of N-10-infected A1 cells. The 7.8-kb RNA (which lacks *neo*[r]) was produced when cells were infected with N-10, αN-10, or vector viruses. Since αN-10 virus replicated in parental QT35 cells and since viruses either containing *neo*[r] sequences in the sense orientation (N-10, Fig. 2, panel IIB) or lacking *neo*[r] sequences altogether (vector, Fig. 2, panel III) replicated in A1 cells, we conclude that the *neo*[r] RNA transcripts in A1 cells (which were complementary to the anti-sense *neo*[r] sequences in αN-10) inhibited the replication of αN-10.

TABLE 1. Effect of G418 on A1 cells infected with anti-sense construct viruses

Cells and virus	Standard culture cell count (10^5 cells/ml) in expt:		Efficiency of colony formation (colonies/100 cells seeded) in indicated expt:			
			Plastic under agar		Soft agar suspension	
	1	2	1	2	1	2
QT35, no virus	0.0	0.0	0.0	0.0	ND[a]	ND
A1, no virus	4.5	ND	9.0	4.8	14	12.4
A1, vector virus	ND	2.0	8.0	4.1	9	6.3
A1, αN-10 virus	2.8	5.0	9.0	4.3	15	8.4

[a] ND, Not done.

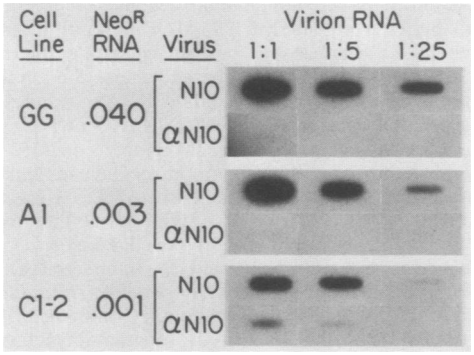

FIG. 3. Slot blot analysis of virion RNAs produced following infection of three G418-resistant clones with N-10 and αN-10. Levels of *neo*[r] transcripts in individual clones were determined as described in the text and are presented as percentages of total cellular RNA. Virion RNA representing 1.5 ml of culture fluid and two fivefold dilutions thereof were bound to the membrane in each case and hybridized to strand-specific ^{32}P-labeled *neo*[r] RNA probes.

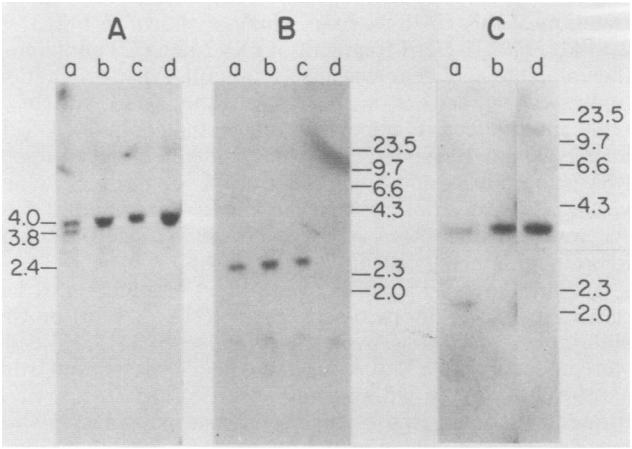

FIG. 4. Viral DNA sequences detected in N-10- and αN-10-infected A1 cells. DNA was extracted from isolated nuclei and digested with *Eco*RI (A and B) or *Eco*RI and *Cla*I (C). Fragments were separated by elecrophoresis on a 1% agarose gel and blotted onto nitrocellulose. The membrane was hybridized with ^{32}P-labeled probes prepared by nick translation of a 1.1-kb *Hin*dIII-*Eco*RI *neo*[r] fragment (A and C) and a 1.7-kb *Eco*RI-*Bgl*I fragment of the viral LTR and *gag* region (B). Lanes: a, N-10 virus; b, αN-10 virus; c, vector virus; d, control. Numbers are in kilobases.

We attempted to detect the formation of viral DNA in infected A1 cells. Figure 4A shows a Southern blot hybridization analysis with probes for *neo*^{r} sequences in *Eco*RI-digested genomic DNAs from A1 cells infected with N-10 and αN-10. The restriction map of the *neo*^{r} construct used to transfect A1 cells (Fig. 1) indicated that uninfected A1 cells contained *neo*^{r} sequences on a 4.0-kb *Eco*RI DNA fragment (Fig. 4A, lane d). A1 cells infected with N-10 contained an additional 3.8-kb *neo*^{r}-containing DNA fragment (Fig. 4A, lane a) attributable to N-10 proviral DNA and consistent with the *Eco*RI restriction map (Fig. 1). In contrast, A1 cells infected with αN-10 did not contain additional *Eco*RI DNA fragments, a result which would have been diagnostic of αN-10 proviral DNA (Fig. 4A, lane b). These data revealed that, while *neo*^{r}-bearing N-10 viral DNA was present in infected A1 cells, analogous αN-10 viral DNA carrying *neo*^{r} sequences was not detectable. Figure 4B shows that when the same blot was probed with viral sequences other than *neo*^{r} (viral *gag* gene sequences), viral DNA was detected in αN-10- as well as N-10-infected A1 cells. These proviruses in αN-10-infected A1 cells were probably derived from the viruses lacking *neo*^{r} inserts in the infecting viral stocks (as mentioned above), although the deletion of the anti-sense *neo*^{r} sequence could also have been induced by an anti-sense effect of the *neo*^{r} RNA in A1 cells. Figure 4C shows a blot of DNA digested with both *Eco*RI and *Cla*I and probed for *neo*^{r} sequences. This double digest yielded the same 4.0-kb endogenous *neo*^{r} fragment from A1 cells as that shown in Fig. 4A, since there were no *Cla*I sites within this sequence. However, the *neo*^{r} sequences of N-10 and αN-10 are bound by *Cla*I sites (Fig. 1), so DNA from cells infected with either virus should contain a 2.14-kb *neo*^{r} fragment. As expected, such a fragment was found in DNA from A1 cells infected with N-10 but not αN-10, confirming the data presented in Fig. 4A.

Our results suggest that the introduction of anti-sense RNA by retroviral vectors may fail to inhibit the expression of a target host cell gene to a functionally significant level when that gene generates a certain level of steady-state RNA transcripts. The cellular RNA apparently exerts an antiviral effect on the infecting retrovirus even though, in this experiment, the complementary target sequences were not a part of the viral genes essential for normal viral replication. Retroviruses replicate in the host cell by reverse transcription of genomic RNA molecules, leading to the formation of double-stranded DNA molecules. Circular forms of these viral DNAs are then integrated into the host genome as a provirus, from which viral genes are transcribed into progeny genomic RNAs and mRNAs (13). Failure to detect the presence of αN-10 proviruses bearing *neo*^{r} sequences in A1 cells (Fig. 4) is consistent with an anti-sense effect early in the replication cycle, although later steps could also be susceptible to inhibition. It is unlikely that the inhibition involves unintegrated viral DNA molecules, since the cellular *neo*^{r} RNA transcripts in A1 cells should always be anti-sense with respect to one strand of the viral double-stranded DNA molecule generated from N-10 infection and since N-10 replicated normally. A plausible mechanism might be the hybridization of the cellular *neo*^{r} RNA sequence with the complementary sequence in the αN-10 viral genomic RNA early in the viral infection, leading to the degradation of viral RNA templates (3) or otherwise blocking the process of reverse transcription replication. An alternative, more complicated mechanism would be for reverse transcription to bypass the double-stranded RNA region, resulting in a deletion of the viral genome. If the deleted region were nonessential for viral replication (as was the case in this experiment with anti-sense *neo*^{r} sequences), the resultant virus could continue to replicate. Further experiments are required to determine more precisely the stage(s) of viral replication susceptible to inhibition by anti-sense RNA.

Although not fully obvious from these experiments, it is reasonable to speculate that the relative levels of complementary cellular RNA and infecting viral DNA determine the effectiveness of viral inhibition (and the potential inhibition of cellular gene expression). Further experiments are also required to determine the levels required for the maximum effectiveness of these anti-sense RNA effects. Regardless of the mechanism, our results suggest a useful strategy for inhibiting retroviral infections. In this report, we have demonstrated the feasibility of this novel approach to inhibiting retroviruses. Our data are consistent with a previous report of the inhibition of retroviral replication by an oligodeoxynucleotide complementary to LTR sequences of RSV (14, 15). The suggestion that anti-sense RNA inhibition can act at specific stages of viral replication (in addition to potentially inhibiting viral gene expression) encourages further exploration of this approach.

We thank G. Loring for technical help and M. Groudine, H. Weintraub, J. Izant, D. Miller, and C. Thompson for helpful comments.

This work was supported by Public Health Service grants CA28151 and CA20068 from the National Institutes of Health. R.Y.-L.T. and S.C.B. are fellows of the Leukemia Society of America and the Damon Runyon Walter Winchell Cancer Fund, respectively.

LITERATURE CITED

1. **Chang, L.-J., and M. Stolzfas.** 1985. Gene expression from both intronless and intron-containing Rous sarcoma virus clones is specifically inhibited by anti-sense RNA. Mol. Cell. Biol. **5**:2341–2348.
2. **Cooper, G. M., S. Okenquist, and L. Silverman.** 1980. Transforming activity of DNA of chemically transformed and normal cells. Nature (London) **284**:418–421.
3. **Crowley, T. E., W. Nellen, R. H. Gamer, and R. A. Firtel.** 1985. Phenocopy of discoidin 1-minus mutants by antisense transformation in dictyostelium. Cell **43**:633–641.
4. **Hughes, S., and E. Kosik.** 1984. Mutagenesis of the region between *env* and *src* of the SR-A strain of Rous sarcoma virus for the purpose of constructing helper independent vectors. Virology **136**:89–99.
5. **Izant, J. G., and H. Weintraub.** 1985. Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA. Science **299**:345–352.
6. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Martin, A. S., and P. H. Duesberg.** 1972. The α subunit in the RNA of transforming avian tumor viruses: I. Occurrence in different virus strains. II. Spontaneous loss resulting in nontransforming variants. Virology **47**:494–497.
8. **Melton, D. A.** 1985. Injected anti-sense RNAs specifically block messenger RNA translation in vivo. Proc. Natl. Acad. Sci. USA **82**:144–148.
9. **Moscovici, C., M. G. Moscovici, H. Jiminez, M. M. C. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell **11**:95–103.
10. **Pestka, S., B. L. Dougherty, V. Jung, K. Holta, and R. K. Pestka.** 1984. Anti-mRNA: specific inhibition of translation of single mRNA molecules. Proc. Natl. Acad. Sci. USA **81**:7525–7528.
11. **Rosenberg, U. B., A. Preiss, E. Seifert, H. Jackle, and D. C.**

Knipple. 1985. Production of phenocopies by *Kruppel* anti-sense RNA injection into *Drosophila* embryos. Nature (London) **313**:703–706.

12. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
13. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1984. RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Zamecnik, P. C., J. Goodchild, Y. Taguchi, and P. S. Savin.** 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.
15. **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.