Inhibition of Rous Sarcoma Virus Replication by Antisense RNA

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Previous results have indicated that Rous sarcoma virus *env* gene expression is specifically inhibited by antisense RNA (L.-J. Chang and C. M. Stoltzfus, Mol. Cell. Biol. 5:2341-2348, 1985). In this study, we compare the extents of inhibition by antisense RNA derived from different parts of the Rous sarcoma virus genome, and we show that antisense constructs containing the 3'-end noncoding region inhibit *env* expression to a similar extent as those containing the 5'-end noncoding region or coding region. Furthermore, we show that antisense RNA inhibits virus replication at other levels in addition to translation.

Antisense RNA (cRNA or mRNA-interfering cRNA) has been used in a number of systems and has been proposed as a possible genetic approach for the prevention and treatment of diseases (1, 2, 4, 6, 7, 11). The idea of using antisense RNA to regulate gene function has been developed based on previous findings that in procaryotes some naturally transcribed RNA species regulate DNA replication or gene expression by hybridizing to their complementary target sequences (12, 15, 16). The mechanisms by which antisense RNA can specifically inhibit its sense target are still not well understood. Experiments with antisense genes to repress the expression of endogenous eucaryotic cellular genes have been successful (3, 10, 13). In those examples, antisense RNA was either microinjected at a specific developmental stage (13) or expressed from an established chromosomal gene (3, 10). In this study, we constructed a series of antisense RNA clones in which different parts of the Rous sarcoma virus (RSV) genome were inverted relative to the viral promoter. These antisense clones were cotransfected with the RSV *env* gene expression clone or the wild-type RSV genomic clone, and the effect on *env* gene expression was studied. The expression of the *env* gene from an intronless *env*-expressing clone, pLC32, was detected by the rescue of *env*-defective Bryan high-titer RSV (BH-RSV) from an infected quail cell line, R(-)Q cells (9). We also observed a specific effect on virus production by investigating the progeny viruses released from the transfected R(-)Q cells.

A series of antisense plasmids were constructed to further characterize the extent and efficiency of inhibition by antisense RNAs which were derived from different parts of the viral genome (Fig. 1). In addition to the plasmids that we have previously studied (1), four more antisense plasmids were constructed. Plasmid pSL1 contains an inverted viral sequence inside the *env* intron region. pSS5, pAS6, and pAS32 contain different lengths of inverted sequences from downstream of the *env*-coding region (note that sometimes the 5'-leader region is also inverted in pAS32). The genetic organizations of these plasmids are shown in Fig. 1. Also shown is the genetic map of the endogenous BH-RSV genome deleted in the *env* region (9). These antisense plasmids were cotransfected with pLC32 at a 10-to-1 molar ratio by using previously described techniques (1). Standard deviation (σ) was determined by calculating four to six sets

of duplicates, and the Student *t* test was done to determine the *P* values compared with those of the control. The amount of infectious virus released was determined as focus-forming units (FFU) and was compared with the amount of infectious virus produced by pLC32 alone (Fig. 1). As we showed previously, pLC25 (antisense to the *env* coding region), and pLC55 and pLC58 (antisense to the 5' leader) exhibited efficient inhibition effects. To our surprise, the other four antisense clones also exhibited significant inhibition of infectious virus production. The most efficient clone was pAS6, which contained a 1,702-base inversion in the 3'-*src* region and which inhibited infectious virus production up to 82%. The shorter antisense clone pSS5, with 285 bases inverted in the 3'-*src* region, was less inhibitory (43%) than the others were. Although antisense RNA from these plasmids (pAS6 and pSS5) would be expected to hybridize to the BH-RSV genome, a similar-sized antisense clone, pSL1, which only hybridizes to the BH-RSV genome, only inhibited infectious virus production approximately 25%. We thus attribute most of the inhibition effect of pAS6 and pSS5 to the inhibition of *env* expression from pLC32. These results suggest that antisense RNA from the 3'-noncoding region as well as that from the 5' region can block gene expression. The results also suggest that transiently expressed antisense RNA does not affect the resident viral gene expression to the same extent as it does the exogenous gene (*env*) in this type of assay.

To determine the effects of antisense RNA on virus replication, we transfected R(-)Q cells with the wild-type Prague A (PrA)-RSV clone pJD100 or with pJD100 plus antisense clones (Fig. 2). Culture media were harvested in the next 20 days and were assayed for FFU production on chicken embryo fibroblasts. Cells transfected with the wild type alone, after a transient expression at approximately 2 days after transfection, began to gradually produce an increasing amount of infectious virions at 4 to 6 days (Fig. 2). In the presence of the antisense clones, however, this increase was delayed for at least another 10 days. The inhibition efficiencies of pLC25, pLC55, and pLC58 were approximately the same. There was a dose-dependent inhibition effect as shown by pLC25, which was also true for all other antisense inhibition experiments. The sample with a 15-to-1 molar ratio (pLC25 versus pJD100) revealed no breakthrough of infectious virus even after 20 days. Because transient *env* expression (i.e., after 2 days) was only inhibited by 70 to 80% under these conditions (Fig. 1), it was surprising that there would be such a dramatic effect on the spread of infectious virus derived from pJD100. It suggested

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FIG. 1. Genetic maps representing the endogenous genome of BH-RSV and different plasmid constructs and the FFU analysis for antisense inhibition. The nucleotide number of each cloning site is noted. The numbering system is based on the complete nucleotide sequence of the Prague C RSV genomic RNA (14). The viral long terminal repeats are indicated by the open boxes at each end. The deletion boundary of the BH-RSV *env* gene is as described by Lerner and Hanafusa (9). pLC32, pLC25, pLC55, and pLC58 were constructed as described previously (1). pSL1, pSS5, pAS6, and pAS32 were constructed with an RSV-long terminal repeat vector, pLTR1, which was derived from pJD100 with a deletion from the *Pst*I site at nucleotide 263 to the *Pst*I site at nucleotide 862. To construct these antisense clones, each inverted fragment (as indicated by inverted bars) was isolated, blunt-ended, and ligated to the *Sac*I linker before insertion into pLTR1 at the *Sac*I site (nucleotide 255). M, *Sma*I; P, *Sph*I; A, *Sac*I. Locations of viral genes *gag*, *pol*, *env*, and *src* are indicated on the maps of BH-RSV and pLC32. Sequences not present in the construct are shown by dashed lines. The splice sites are indicated by arrows: S.D., splice donor site; S.A., splice acceptor site. Inverted regions of antisense constructs are shown by the solid arrows with length of base pairs (bp). The percent inhibition and standard deviations (σ) were determined as described in the text, and the Student *t* test was done to determine the *P* values. Culture media were harvested 42 h after transfection and were assayed for FFU as described previously (1).

that there might be additional effects of antisense RNA on virus replication.

If such an effect were operating, it would be expected that the ratio of the virions obtained under conditions of *env*-antisense-RNA inhibition would be altered in favor of the endogenous BH-RSV, because BH-RSV genome RNA is not capable of hybridizing to antisense RNA directed specifi-

cally to *env* gene sequences. Therefore, we examined the individual foci which contained viruses generated from transfected plates. The chicken embryo fibroblast foci that were formed by the rescued viruses were picked and cocultured with fresh chicken embryo fibroblasts. In 5 to 7 days, usually after the plate was split once, transformation of the whole plate of chicken embryo fibroblasts was observed

FIG. 2. Time course study of antisense inhibition. pJD100 plasmid DNA (obtained from J. T. Parsons, University of Virginia, Charlottesville) was used as wild-type (W.T.) RSV (PrA strain) in the transfection assay. Antisense clones pLC25, pLC55, and pLC58 were cotransfected with pJD100 in the molar ratios indicated in parentheses. Medium harvest and FFU analysis are as described in the text and in the legend to Fig. 1.

if the isolated focus contained wild-type virus. Instead, if the isolated focus was formed by rescued defective BH-RSV (*env*⁻), no overall transformation could occur. By this assay, we studied the ratio of PrA-RSV genome and BH-RSV genome generated from the transfected R(-)Q cells.

When R(-)Q cells were transfected with pJD100, both BH-RSV and PrA-RSV genomes could be packaged into virus particles and detected by FFU assay. More than 100 single foci were picked and studied in repeated experiments. The results in Table 1 indicate that, when R(-)Q cells were transfected with pJD100, the ratio of PrA-RSV versus BH-RSV from the rescued progeny virions was about 1.3 to 1. No PrA-RSV was produced when R(-)Q cells were transfected with pLC32 alone or with pLC32 plus the defective virus clone pJDΔ*Sal*I (1). However, when pJD100 was cotransfected with an antisense plasmid (pLC25) in which *env* expression was inhibited up to 80%, the ratio of PrA-RSV versus BH-RSV was reduced almost 10-fold, suggest-

ing that production of infectious virus derived from pJD100 was specifically blocked by antisense RNA.

Our preliminary results indicate that when antisense clones complementary to both the PrA- and BH-RSV genomes (e.g., pLC55 and pLC58) were used, the ratio of PrA- to BH-RSV was reduced to a smaller extent. We concluded, therefore, that there are additional effects by antisense RNA on the replication of PrA virus derived from pJD100. Taken together, our data suggest that the mechanism by which antisense RNA inhibits RSV expression is not restricted to translation inhibition (5, 11).

We have shown here that the antisense RNA complementary to the 3'-end noncoding region inhibits gene expression to the same extent as that of the 5' regions, supporting the possibility that multiple inhibitory mechanisms are operating. According to the results presented here, the efficiency of antisense inhibition is more length dependent when 3'-end sequences are involved. This is in contrast to the results of antisense RNA inhibition studied in the *Escherichia coli* system in which the longer is less efficient than shorter antisense RNA (8). Besides inhibiting *env* gene expression, our data suggest antisense RNA also inhibits virus production, possibly by blocking viral RNA packaging or transport from nucleus to cytoplasm, an effect of antisense RNA which has previously been demonstrated in other systems (7).

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LITERATURE CITED

1. Chang, L.-J., and C. M. Stoltzfus. 1985. Gene expression from both intronless and intron-containing Rous sarcoma virus clones

TABLE 1. Effect of *env* gene antisense RNA on virus production

Transfected plasmid(s) ^a	No. of foci studied	Ratio ^b (PrA-RSV vs BH-RSV)
pJD100	53	1.32
pJD100 (1), pLC25 (10)	25	0.16
pJD100 (1), pLC25 (7)	3	0
pLC32	16	0
pLC32 (1), pJDΔ <i>Sal</i> I ^c (10)	4	0

^a Numbers in parentheses are the molar ratios of cotransfected plasmid DNAs.

^b Ratio of two different progeny viruses, PrA-RSV and BH-RSV, rescued from transfected R(-)Q cells.

^c pJDΔ*Sal*I is a plasmid derived from pJD100 with deletion in the *env*-coding region as described previously (1).

- is specifically inhibited by anti-sense RNA. *Mol. Cell. Biol.* **5**:2341-2348.
2. Coleman, J., A. Hirashima, Y. Inokuchi, P. T. Green, and M. Inouye. 1985. A novel immune system against bacteriophage infection using complementary RNA (micRNA). *Nature (London)* **315**:601-603.
 3. Crowley, T. E., W. Nellew, R. H. Gomer, and R. A. Firtel. 1985. Phenocopy of discoidin I-minus mutants by antisense transformation in *Dictyostelium*. *Cell* **43**:633-641.
 4. Green, P. J., O. Pines, and M. Inouye. 1986. The role of antisense RNA in gene regulation. *Annu. Rev. Biochem.* **55**:569-597.
 5. Harland, R., and H. Weintraub. 1985. Translation of mRNA injected into *Xenopus* oocytes is specifically inhibited by antisense RNA. *J. Cell Biol.* **101**:1094-1099.
 6. Izant, J. G., and H. Weintraub. 1984. Inhibition of thymidine kinase gene expression by antisense RNA: a molecular approach to genetic analysis. *Cell* **36**:1007-1015.
 7. Kim, S. K., and B. J. Wold. 1985. Stable reduction of thymidine kinase activity in cells expressing high levels of antisense RNA. *Cell* **42**:129-138.
 8. Klausner, A. 1985. Turning off unwanted genes with anti-RNA. *Bio/Technology* **3**:763-764.
 9. Lerner, T. L., and H. Hanafusa. 1984. DNA sequence of the Bryan high-titer strain of Rous sarcoma virus: extent of *env* deletion and possible genealogical relationship with other viral strains. *J. Virol.* **49**:549-556.
 10. McGarry, T. J., and S. Lindquist. 1986. Inhibition of heat shock protein synthesis by heat-inducible antisense RNA. *Proc. Natl. Acad. Sci. USA* **83**:399-403.
 11. Melton, D. A. 1985. Injected antisense RNAs specifically block messenger RNA translation *in vivo*. *Proc. Natl. Acad. Sci. USA* **82**:144-148.
 12. Mizuno, T., M.-Y. Chou, and M. Inouye. 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc. Natl. Acad. Sci. USA* **81**:1966-1970.
 13. Rosenberg, U. B., A. Preiss, E. Seifert, H. Jackle, and D. C. Knipple. 1985. Production of phenocopies by kruppel antisense RNA injection into *Drosophila* embryos. *Nature (London)* **313**:703-706.
 14. Schwartz, D. E., R. Tizard, and W. Gilbert. 1983. Nucleotide sequence of Rous sarcoma virus. *Cell* **32**:853-869.
 15. Simons, R. W., and N. Kleckner. 1983. Translational control of IS 10 transposition. *Cell* **34**:683-691.
 16. Tomizawa, J., T. Itoh, G. Selzer, and T. Som. 1981. Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc. Natl. Acad. Sci. USA* **78**:1421-1425.