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Gene Expression from Both Intronless and Intron-Containing Rous Sarcoma Virus Clones Is Specifically Inhibited by Anti-Sense RNA

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To distinguish the inhibitory effect of anti-sense RNA on translation from the effect on splicing, a plasmid (pLC32) was constructed from a cDNA clone of the Rous sarcoma virus (RSV) envelope gene (*env*) mRNA. Transcription of this plasmid results in the synthesis of RNA identical to the RSV *env* gene mRNA which does not require splicing to be expressed. Plasmids derived from pLC32 were also constructed in which the *env* gene coding sequence and 5′ noncoding leader sequences were inserted in the opposite orientation relative to the RSV long terminal repeats (LTRs). pLC32 DNA transfected by the calcium phosphate coprecipitation technique efficiently rescued infectious virus from quail cells infected with an RSV mutant deleted in the *env* gene [R(−)Q cells], indicating that the intron sequences are dispensable in *env* gene expression. When the inverted constructs were cotransfected with pLC32, significantly less infectious virus was produced. The extent of the inhibition depended upon the concentration ratio of the two plasmids. The maximum inhibition (80%) occurred when the ratio of inverted constructs to pLC32 was 12:1. The inhibition is specific for the inverted orientation since cotransfection of pLC32 with several other plasmids containing viral LTRs and defective *src* and *env* genes at similar concentrations did not inhibit the production of infectious virus. In addition, the inverted constructs did not interfere with the expression of an LTR-driven chloramphenicol acetyltransferase gene. When cotransfected with a wild-type Prague A RSV DNA plasmid (pJD100), the inverted constructs also greatly inhibited expression and replication of virus in R(−)Q quail cells. These data suggest that the specific inhibition is caused by hybridization of complementary RNA transcribed from the inverted constructs to the *env* mRNA, thereby blocking its expression. The fact that expression of both intron-containing and intronless clones are inhibited to the same extent suggests that inhibition by anti-sense RNA from the *env* exon regions does not act at the level of RNA splicing.

Recently several procaryotic systems have been described in which the production of RNA complementary to mRNA (anti-sense RNA) plays a role in the regulation of gene expression (18, 24). In another recent set of reports, artificial regulation of gene expression in cells with DNA constructs which are transcribed into anti-sense RNAs has been described (2, 12, 20). This regulation was accomplished by inverting the gene sequences relative to the promoter sequence. Coleman et al. have described a system whereby genes encoding a number of *Escherichia coli* outer membrane proteins were placed in an inverted orientation relative to a strong inducible promoter (2). These plasmid constructs were then used to transform bacteria. Under these conditions, the synthesis of the appropriate gene product was specifically inhibited. It was also shown in this study that RNAs complementary either to the initiation site for protein synthesis or to the coding region were able to inhibit expression efficiently. Similar experiments were carried out by Pestka et al. (20), who showed that the presence of RNA complementary to β-galactosidase mRNA inhibited the synthesis of the enzyme by 98%. Izant and Weintraub have used this approach to inhibit gene expression in eukaryotic cells (12). When the herpes simplex virus thymidine kinase (TK) gene constructs were microinjected into TK^- L cells together with an inverted construct at a 1:100 ratio, there was a four- to fivefold reduction in the transient expression of TK. The inhibition was specific for the inverted orientation of the TK gene relative to the normal TK promoter or to a mouse sarcoma virus long terminal repeat (LTR) promoter.

The hypothesis that anti-sense RNA hybridizes to mRNA and blocks its translation has recently been directly tested (17). RNA complementary to globin mRNA was synthesized in vitro and microinjected into *Xenopus* oocytes. The presence of a 50-fold excess of the anti-sense RNA specifically inhibited the synthesis of globin from the microinjected β-globin mRNA. Some of the β-globin RNA from the microinjected embryos became resistant to digestion with RNases A and T1, strongly suggesting that the inhibition was caused by RNA-RNA hybridization. Anti-sense RNA complementary to the 5′ noncoding sequence effectively blocked β-globin translation. In contrast, anti-sense RNA complementary to only the 3′ coding sequence did not block translation (17).

Retroviruses such as Rous sarcoma virus (RSV) provide unique model systems for investigating the interactions of anti-sense and sense RNA in eucaryotic cells. The RSV proviral DNA is transcribed into a 9.3-kilobase (kb) RNA and used as full-length mRNA or genomic RNA (29). Two spliced mRNAs are formed from this 9.3-kb RNA in which a common 5′ donor site is spliced to two alternative 3′ acceptor sites to form the 4.6-kb *env* gene and the 2.7-kb *src* gene mRNAs (1, 6, 27, 28). The expression of these genes can be biologically detected either by cell transformation in the case of the *src* gene or by a complementation assay to be described below in the case of the *env* gene. The unspliced 9.3-kb RNA serves as mRNA for *gag* gene structural proteins and is also packaged into virus particles. By assaying for the expression of the appropriate viral genes, it is possible to elucidate whether RNAs complementary to dif-

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ferent regions of the genome can inhibit RNA synthesis, splicing, translation, or packaging.

To avoid possible complications due to inhibition of splicing and to specifically study the effect of anti-sense RNA on translation, we have constructed an expression plasmid of Prague A (PrA) RSV in which the intron sequences for the envelope protein gene (*env*) were precisely deleted. The *env* RNA transcribed from this plasmid does not need splicing to be expressed. We have used this plasmid to rescue infectious virus from a continuous line of quail cells [R(−)Q cells] infected and transformed by the Bryan high-titer RSV (BH-RSV), which is deleted in the entire *env* gene (14). R(−)Q cells produce noninfectious BH-RSV virus particles which can be rescued by the intracellular production of *env* gene protein (11); the number of infectious particles produced by these cells is proportional to the amount of *env* mRNA present in the cells (25). It has been shown that complementation of *env* can occur when R(−)Q cells are transfected with plasmid DNA containing the *env* gene with an LTR promoter (4). Also, expression of *env* gene activity by this assay paralleled the amount of synthesis of insulin mRNA driven by the same LTR element (3). We report in this paper, first, that the *env* gene intron sequences are not required for expression of the *env* gene, and second, that the expression of the *env* gene is specifically inhibited by the introduction of plasmids capable of being transcribed to anti-sense RNAs which are complementary to the *env* gene coding region or to the 5′ noncoding region. The inhibition is specific for the expression of the *env* gene from either the intronless *env* gene plasmid or the plasmid coding for infectious virus, suggesting that the inhibition occurs by a mechanism not involving RNA splicing, probably at the level of translation.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from Bethesda Research Laboratories and New England BioLabs, Inc. T4 DNA ligase was purchased from New England BioLabs. Mung bean nuclease and acetyl coenzyme A were obtained from P-L Biochemicals, Inc. [^{14}C]chloramphenicol (54 mCi/mmol) was purchased from Amersham Corp. Other materials and bacterial strains were obtained as described previously (1).

Construction of recombinant DNAs. We have previously described the construction of cDNA clones of both RSV *env* and *src* mRNAs and determined the nucleotide sequences across their splice junctions (1). RSV genomic clone pJD100 was obtained from J. T. Parsons, University of Virginia. pRSV-CAT (7) was obtained from L. Turek, University of Iowa. pLC32, pLC25, pLC55, and pLC58 were constructed as shown in Fig. 1. All constructs were characterized by appropriate restriction enzyme digests. The conditions for restriction enzyme digestions were carried out as recommended by the suppliers. Other recombinant DNA techniques were carried out as described previously (16). The construction of the *env* gene cDNA clone pENV73 is described elsewhere (1). It was used to obtain a DNA fragment (*Sac*I-*Xho*I) which contains the sequence spanning the *env* mRNA splice junction. This fragment was inserted into the *Xho*I-*Sac*I-digested pJD100 to construct pLC2. pLC2 contains only the 5′ part of the *env* gene without the intron sequences and was used to construct the four plasmids studied in this paper (pLC32, pLC25, pLC58, and pLC55). A fragment (*Xho*I-*Xho*I) was obtained from pJD100 and inserted into pLC2 to obtain pLC32, in which the complete *env* gene was reconstituted, and pLC25, in which this fragment was inverted. pLC58 and pLC55 were obtained from pLC2 as shown in Fig. 1. For the construction of pLC58, an *Xho*I-*Bst*EII fragment (503 base pairs [bp]) was isolated from pLC2. Both the *Xho*I-*Bst*EII fragment and the *Xho*I-*Bst*EII-cut pLC2 were blunted by Klenow enzyme fill-in. Blunt-end ligation was performed thereafter. A clone with the 503-bp fragment reinserted in the opposite orientation was selected. This clone contains two reconstructed *Xho*I restriction sites. For the construction of pLC55, the same *Xho*I-*Bst*EII fragment was isolated. This fragment was further digested with restriction enzyme *Sau*3AI, and three subfragments were obtained. These fragments were ligated to the *Xho*I-*Bst*EII-cut pLC2, and a clone with an inverted *Sau*3AI fragment (216 bp) was selected. pLC58 has an inversion (503 bp) which includes the initiation codon and 5′ noncoding region of the *env* gene, whereas pLC55 has an inversion (216 bp) only in the 5′ noncoding region.

Cell transfection and focus-forming unit (FFU) assay. Chicken embryo fibroblasts (CEF) were propagated and used in focus assays as described previously (30). The quail cell line R(−)Q (31) was obtained from L. Turek. This cell line was transformed by the replication-defective *env*$^-$ BH-RSV strain (14). R(−)Q cells were propagated at 37°C in medium 199 (GIBCO Laboratories) supplemented with 10% (vol/vol) tryptose phosphate broth, 0.1% (wt/vol) sodium bicarbonate, 5% calf serum, 1% heat-inactivated chick serum, and 1% dimethylsulfoxide. Calcium phosphate-mediated DNA transfection (8) was performed as described by Sealy et al. (23), with modifications as noted below. Transfections were performed with mixtures of plasmid DNAs and sheared salmon sperm DNA. The DNA concentrations used in each experiment are given below. DNA concentration was determined by measuring optical density at 260 nm and confirmed by ethidium bromide staining of appropriate samples on agarose gels. Cell culture plates (60 mm) were seeded with 3×10^6 R(−)Q cells 1 day before transfection. The DNA samples were coprecipitated with calcium phosphate and applied to R(−)Q cells without removal of the medium. After being exposed to DNA for 4 to 6 h at 37°C, the cells were treated for 3 min with 2 ml of fresh medium containing 30% dimethylsulfoxide, washed carefully with 3 ml of medium, and fed with 5 ml of medium. Medium from these cultures was harvested at various times as described below. Focus assays for each sample were carried out in duplicate, and the two values for each sample were averaged.

CAT assay. Transfection of DNAs into the R(−)Q cell line was performed as described above. After incubation at 37°C for 42 h, cell lysates were prepared and assayed for chloramphenicol acetyltransferase (CAT) activity by previously described methods (15). In brief, 40 μl of the supernatants was mixed with 85 μl of 0.25 M Tris hydrochloride (pH 7.8), 20 μl of 4 mM acetyl coenzyme A, and 0.5 μCi of [^{14}C)]chloramphenicol. Incubation at 37°C for 1 h was followed by the extraction of chloramphenicol and its acetylated derivatives with 1 ml of ethyl acetate. The organic phase was evaporated to dryness and analyzed by thin-layer chromatography (silica gel IB2; J. T. Baker Chemical Co.). The enzyme activity was expressed as the percentage of conversion of the chloramphenicol to the 1-acetyl, 3-acetyl, and 1,3-diacetyl forms.

RESULTS

Expression of pLC32 in BH-RSV-infected quail cells. We have constructed an expression plasmid, pLC32, containing

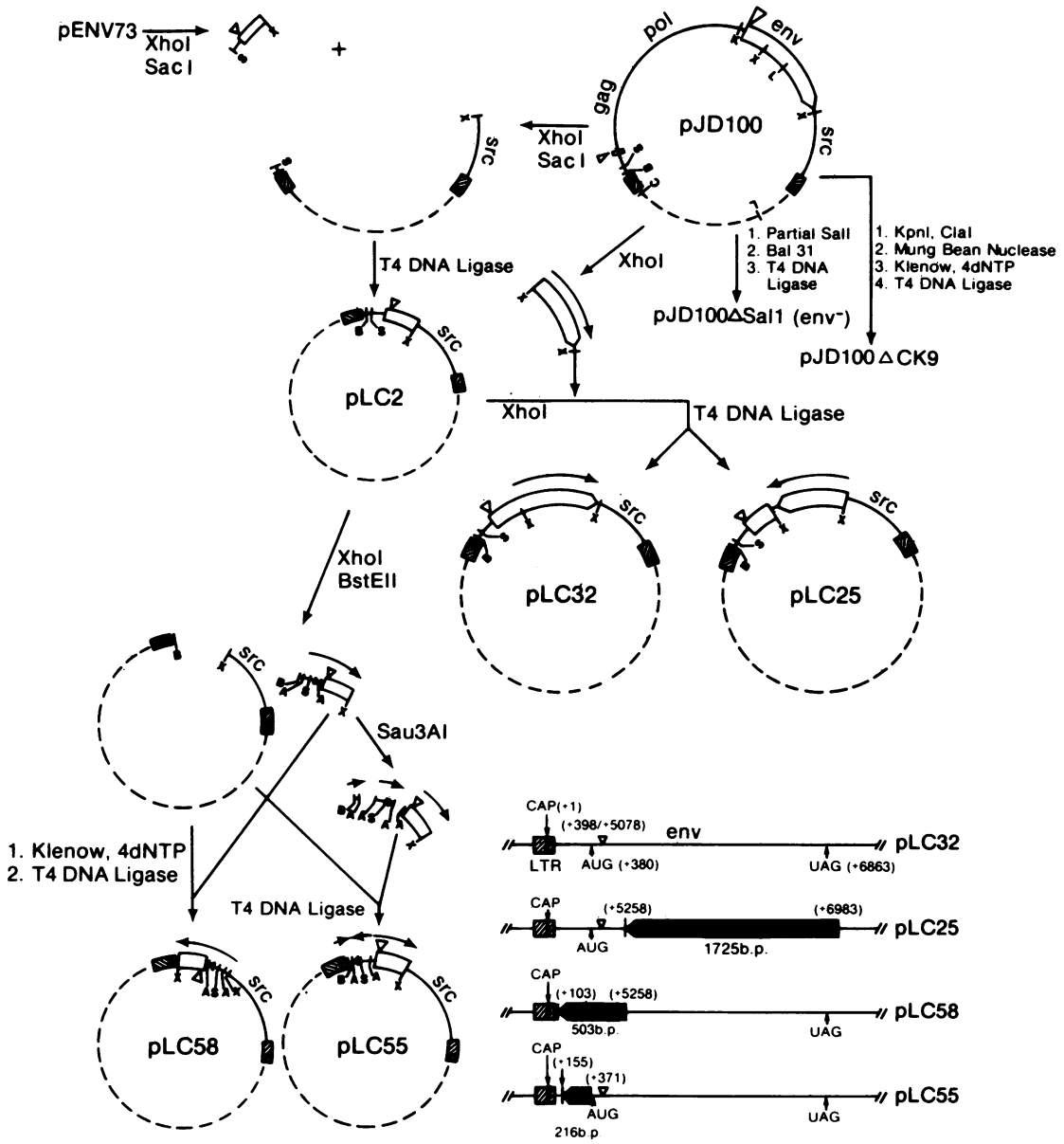

FIG. 1. Construction of RSV *env* gene plasmids. Details of the methodology are given in Materials and Methods. The features of the four constructs are shown at bottom right. The closed arrows refer to the inverted regions. The initiation and termination codons for the *env* protein as well as the initiation site for RNA synthesis are noted. pBR322 sequences are shown as a broken line, and inserts are shown as solid lines. The coding regions are shown as a double line. Two deletion clones were also constructed. pJD100Δ*SalI* has a small deletion (<100 bp) in the *env* gene. pJD100ΔCK9 has a large deletion from the 5′ LTR (*Cla*I site in the pBR322 sequences) to the end of the *pol* gene (*Kpn*I site). ▽, Splice junction; X, *Xho*I; S, *Sac*I; L, *Sal*I; A, *Sau*3AI; B, *Bst*EII; C, *Cla*I; K, *Kpn*I. DNA sequences are numbered according to Schwartz et al. (22).

cDNA sequences of the envelope protein gene (*env*) mRNA by using the protocols outlined in Fig. 1. The final constructions are given in the bottom right portion of the figure. To test for its expression, plasmid pLC32 was introduced by calcium phosphate-mediated transfection into R(−)Q cells. This transformed cell line contains defective RSV which lacks *env* gene sequences and thus requires the expression of exogenous *env* mRNA to produce infectious FFU (11). Virus harvests were obtained from cells transfected with various amounts of pLC32 DNA after 42 h. The results shown in Table 1 indicate that approximately 1 μg of DNA per culture was sufficient to obtain maximum *env* expression. To determine the time of maximum infectious-particle production, the BH-RSV-infected R(−)Q cells were transfected with 1 μg of pLC32 DNA, and virus was harvested from the medium at 12- to 24-h intervals thereafter (Fig. 2A). The expression reached a maximum level at 24 to 36 h, followed by a steady decline in the release of FFU. Transfected plates which were maintained for periods of up to 3 months did not release nondefective virus (data not shown). These results indicate that pLC32 is capable of providing a transient expression of envelope protein. Twelve individual foci

TABLE 1. Rescue of BH-RSV by transfection[a]

Transfected DNA	Amt of DNA (μg) per culture	FFU
pLC2	1	0
pLC-src1[b]	1	0
pJD100Δ*Sal*I	1	0
pJD100ΔCK9	1	0
pLC25	1	0
pLC55	1	0
pLC58	1	0
pLC32	0.1	420
pLC32	0.5	940
pLC32	1	1,730
pLC32	3	900
pLC32	5	1,210
pJD100	0.5	960
pJD100	1	1,360

[a] R(−)Q cells were transfected with DNA by the procedures described in the text. Carrier (sheared salmon sperm DNA; 5 μg) was used for each assay. Released virus was quantitated by the focus-forming assay. Values are the mean of duplicate determinations.

[b] pLC-src1 is an intronless *src* construct analogous to pLC32 with a 128-bp deletion within the *src* gene coding region.

formed by the progeny virions were tested for the production of virus capable of transforming CEF, and all foci were found to be negative, a result consistent with infection by BH-RSV alone. These results indicate that the rescued virus is not derived from recombination of the plasmid with the resident BH-RSV genomes, since such recombinants would be expected to be nondefective. We conclude that pLC32 DNA is transcribed into *env* mRNA, which is then translated to the envelope protein required for the production of infectious BH-RSV.

The expression at 42 h after transfection with pLC32 is similar to that seen with plasmid pJD100, which contains the entire genome of the PrA strain of RSV (Table 1). RNA splicing is required for the expression of *env* mRNA from pJD100, and therefore we concluded that the presence of the *env* intron sequence is not required for the production of functional *env* mRNA molecules. This result is in contrast to results with simian virus 40 late mRNA (9) and globin mRNA (10), in which the intron sequences are required for the transport and production of stable cytoplasmic mRNA. Clones pLC25, pLC55, and pLC58 containing inverted *env* sequences, clone pJD100Δ*Sal*I defective in the *env* gene, and a deleted *src* gene cDNA clone, pLC-src1 (Table 1), all have no activity in the virus rescue assay. A construction (pJD100ΔCK9) which contains an intact *env* gene but is deleted in the upstream LTR, *gag*, and *pol* sequences also has no activity in the rescue assay.

Cotransfection of pLC32 with other plasmids. In the next set of experiments, pLC32 was cotransfected with other *env*-containing plasmids. Various amounts of pJD100Δ*Sal*I, pLC-src1, or pLC25 were cotransfected with 1 μg of pLC32 DNA into R(−)Q cells. After 40 h, the amount of infectious virus produced by these cells was measured as FFU on CEF (Fig. 3). Little or no change in the level of expression occurred when pLC32 was transfected with increasing amounts of pJD100Δ*Sal*I or pLC-src1. In contrast, the cotransfection of pLC32 with the inverted *env* construct pLC25 caused a significant decrease in the amount of FFU production. The extent of inhibition increased when greater amounts of pLC25 DNA were added. In this experiment, the inhibition of FFU production was approximately 80% when 12 μg of pLC25 DNA was used. To test whether pLC25 inhibits expression of pLC32 even after a longer time, we transfected 1 μg of pLC32 DNA either by itself or with 12 μg of pLC25 DNA into R(−)Q cells. Culture medium was harvested at 12- to 24-h intervals for 8 days and assayed on CEF (Fig. 2A). The inhibition of FFU production was still apparent even after 5 days of transfection. A similar experiment shown in Fig. 2A was carried out with 1 μg of the infectious virus pJD100 DNA (Fig. 2B). In contrast to the kinetics of pLC32 expression, where the FFU production reached a peak at 24 to 36 h and then steadily decreased over a period of 2 weeks (Fig. 2A), the expression of pJD100 followed biphasic kinetics. Initially, the FFU production increased to a maximum at 1 day and then decreased. However, after 3 days, the virus production again increased and continued to rise steadily for up to 8 days, when the measurements were discontinued. The first burst of virus production presumably results from the transient expression of *env* mRNA in the initially transfected cells, and this burst follows the same kinetics as the expression of pLC32. We interpret the second rise in virus production to be due to the secondary spread of the progeny nondefective PrA virions to the nontransfected R(−)Q cells. If this explanation is the case, we would expect the progeny virus arising from the pJD100 transfections to be a mixture of infectious BH-RSV and PrA RSV. Indeed, when progeny virus arising from 22 individual foci of transformed cells were examined, 12 foci produced virus that was capable of transforming CEF and showed spreading of viruses. Presumably these virions were nondefective infectious PrA RSV.

The cotransfection of pJD100 and pLC25 inhibited FFU production by 70 to 80% during both the first and second phases of expression (Fig. 2B). This result suggests that the synthesis of anti-*env* RNA by pLC25 inhibits not only the rescue of BH-RSV but also the replication and subsequent release of nondefective infectious PrA RSV from the transfected cells. Thus, subsequent infection of surrounding cells would also be inhibited.

We next investigated whether or not the synthesis of RNA complementary to 216 bases of the 5′ noncoding leader sequence of the *env* mRNA (pLC55) or to 503 bases of the sequences which include both the 5′ noncoding leader and the sequences corresponding to the initiation site for *env* protein synthesis (pLC58) would also inhibit the expression of *env* mRNA from both pLC32 and pJD100 (Table 2). Both inverted constructs inhibit *env* expression from pLC32 by 70 to 80% at a ratio of 8:1, a level of inhibition similar to that observed with cotransfection with pLC25. However, neither pLC55 nor pLC58 showed an obvious dose effect for inhibition of pLC32 expression. pLC58 inhibited the expression of *env* from pJD100 to approximately the same extent (Table 2). In these experiments, the inhibition obtained by cotransfection of pJD100 and pLC55 (44%) was less than that observed when pLC32 and pLC55 were cotransfected (74%). The significance of this difference is currently under investigation. It should be noted that pLC58 and pLC55 express anti-sense RNA that would hybridize to both the resident BH-RSV RNA as well as to transcripts from the introduced plasmids. In contrast, pLC25 expresses RNA that hybridizes only to the introduced plasmids.

Effect of inverted plasmids on expression of pRSV-CAT. We next investigated whether an excess of the inverted constructs would inhibit the transcription or translation of all LTR-driven genes and by this mechanism inhibit *env* expression of pLC32 or pJD100. To test for this possibility, we cotransfected increasing amounts of the inverted construct

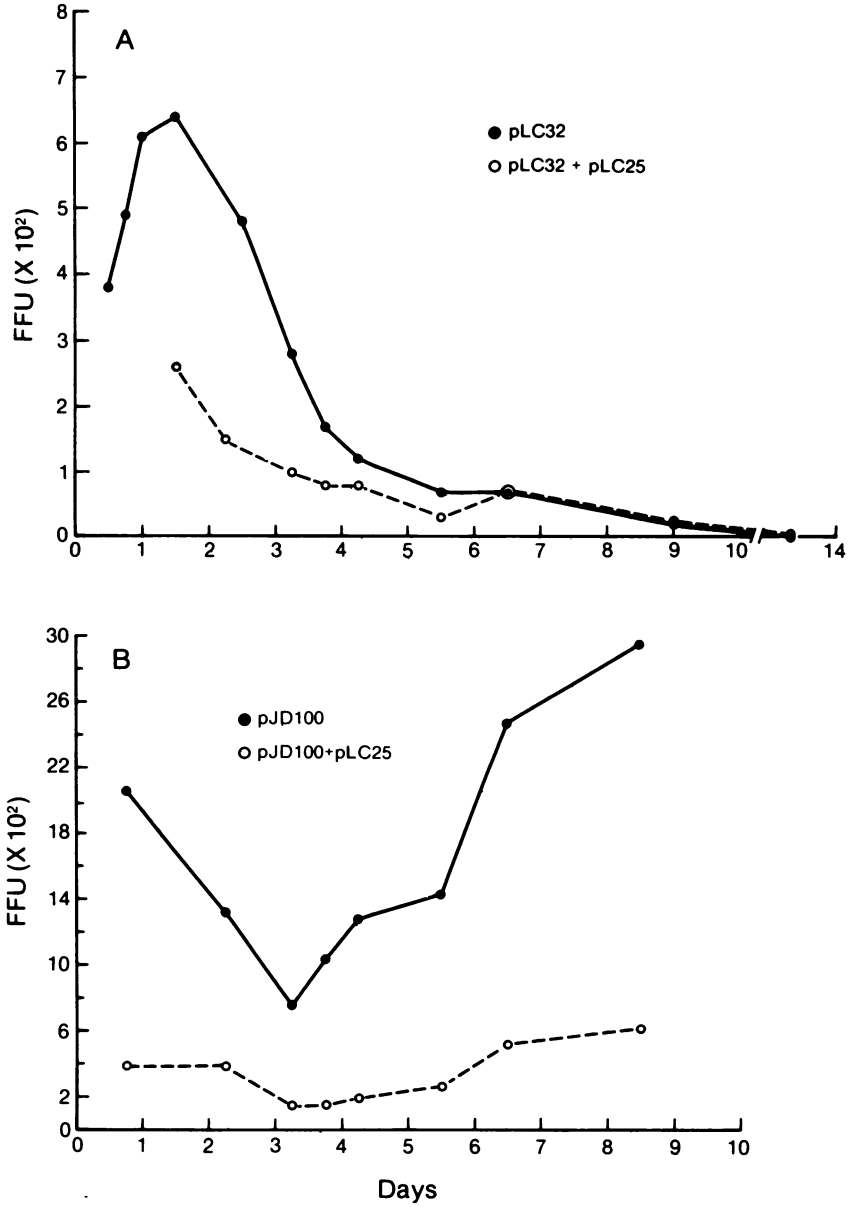

FIG. 2. Time course for production of transforming virus from transfected cells. (A) R(−)Q cells were transfected with 1 μg of pLC32 DNA (———) or 1 μg of pLC32 DNA plus 12 μg of pLC25 DNA (– – – – –). Salmon sperm carrier DNA was added such that the final amount of DNA added was 15 μg per plate. Culture fluid containing rescued transforming virus was harvested at the indicated times after the transfection procedure. The culture fluid was analyzed for infectious virus by the focus-forming assay. Cells were passaged (1:2) at 60-h intervals. (B) R(−)Q cells were transfected with 1 μg of pJD100 DNA (———) or with 1 μg of pJD100 DNA plus 10 μg of pLC25 DNA (– – – – –).

pLC25 with constant amounts of pLC32 and pRSV-CAT into R(−)Q cells. pRSV-CAT contains the CAT gene from Tn*9* inserted downstream from the RSV promoter, and its expression is easily assayed by measuring the acetylation of [^{14}C]chloramphenicol in extracts from transfected cells (15). If the inverted construct causes a general inhibition of gene expression from the RSV promoter, it would be expected to inhibit the expression of the CAT gene as well as the *env* gene. The mRNA transcribed from pRSV-CAT has no sequences complementary to pLC25-generated RNAs, since only 32 bases of RSV sequences from the 5′ end are present in the chimeric CAT mRNA (7). Therefore, if the inhibition is due to the formation of specific anti-sense RNA, no reduction of CAT activity would be expected under conditions by which *env* expression is inhibited. The results of these experiments are given in Fig. 4. Little or no inhibition of CAT activity was observed even when a 10-fold excess of pLC25 DNA was present. As expected from the results given above, the relative amount of *env* expression as determined by the production of infectious BH-RSV was inhibited. Similar results were obtained when pRSV-CAT and the infectious virus plasmid pJD100 were cotransfected with increasing amounts of pLC25 DNA (data not shown). We conclude from these results that the presence of pLC25 DNA does not cause a general inhibition of gene expression from LTR-driven transcripts

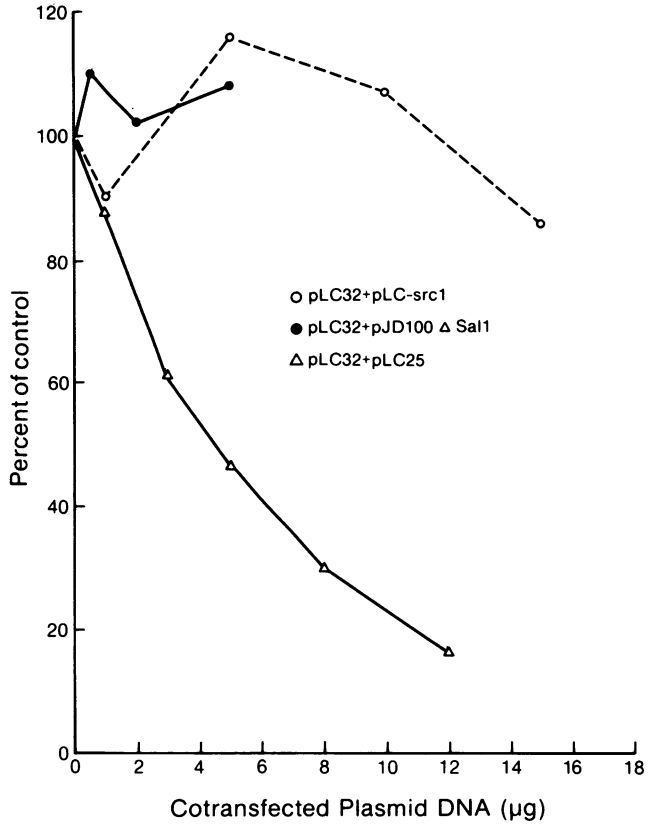

FIG. 3. Dose effect for inhibition of *env* gene expression. Various amounts of pLC25, pJD100Δ*Sal*I, or pLC-src1 DNA were cotransfected with 1 μg of pLC32 DNA. Salmon sperm carrier DNA was added as described in the legend to Fig. 2. Culture fluid was harvested at 40 h after transfection and assayed for infectious virus. The results are expressed as the percentage of inhibition based on a control which was transfected with 1 μg of pLC32 alone.

DISCUSSION

We have shown that cotransfection of an RSV *env* gene expression plasmid pLC32 with plasmids in which the *env* gene coding sequences or 5′ leader sequences were placed in the opposite transcriptional orientation relative to the LTRs results in an inhibition of *env* gene expression. This inhibition is specific, since cotransfection of pLC32 with plasmids pJD100Δ*Sal*I, which contains a small deletion in the *env* gene, and pLC-src1, which contains a deletion in the *src* gene, did not inhibit expression. Since these two plasmids contain *env* and *src* sequences as well as the same LTR structure as pLC25, we conclude that the inhibition is specific for the inverted orientation of the *env* sequence in pLC25. We also showed that the inverted constructs inhibited expression of both pLC32, where RNA splicing is not required, as well as the wild-type PrA RSV clone pJD100, where splicing of transcripts is required for *env* mRNA expression. Inhibition of *env* gene expression also resulted in the decrease of infectious PrA RSV virus production in the assay of pJD100 transfection. Finally, we have shown that pLC25 does not generally inhibit all LTR-driven transcription, since the expression of pRSV-CAT was not affected (Fig. 4).

A moderate excess of the inverted constructs was required to obtain a significant inhibition in FFU production. Only a small (less than 20%) inhibition effect was obtained when equal amounts of pLC25 and pLC32 were added (Fig. 3). The presence of 5- to 10-fold excesses of pLC25 was required to obtain the maximum inhibition of 80% (Fig. 3, Table 2).

TABLE 2. Effect of cotransfection on rescue of BH-RSV[a]

Cotransfected DNAs (μg)	FFU	Control units[b]	% Inhibition
pLC32 (1), pJD100Δ*Sal*I (5)	1,846	1,720	
pLC32 (1), pLC-src1 (5)	1,990	1,980	
pLC32 (1), pLC25 (1)	1,475	1,690	13
pLC32 (1), pLC25 (3)	1,035	1,690	39
pLC32 (1), pLC25 (5)	785	1,690	54
pLC32 (1), pLC25 (8)	505	1,690	70
pLC32 (1), pLC25 (12)	275	1,690	84
pLC32 (1), pLC55 (4)	625	2,705	77
pLC32 (1), pLC55 (8)	720	2,705	74
pLC32 (1), pLC58 (4)	585	2,705	78
pLC32 (1), pLC58 (8)	600	2,705	78
pJD100 (1), pLC25 (5)	800	1,945	59
pJD100 (1), pLC25 (10)	380	1,945	80
pJD100 (1), pLC55 (4)	700	1,170	40
pJD100 (1), pLC55 (8)	650	1,170	44
pJD100 (1), pLC58 (4)	800	1,170	32
pJD100 (1), pLC58 (8)	215	1,170	82

[a] R(−)Q cells were cotransfected by procedures described in the text. Appropriate amounts of carrier DNA were used to make the final amount of DNA 15 μg in each assay. Released virus was quantitated by the focus-forming assay. Values are the mean of duplicate determinations.

[b] Control refers to FFU released by transfection with pLC32 or pJD100 alone in the appropirate experiments.

The mechanism by which inhibition by the inverted con-

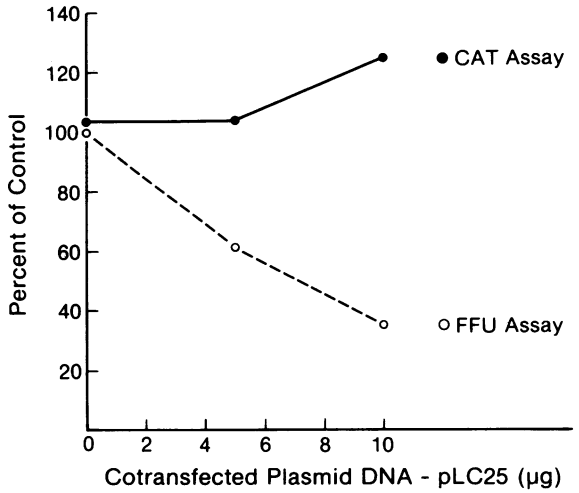

FIG. 4. Cotransfection of *env* gene constructs with pRSV-CAT. Various amounts of pLC25 were cotransfected with 1 μg each of pLC32 and pRSV-CAT DNA into R(−)Q cells. Sheared low-molecular-weight salmon sperm carrier DNA was added such that the final plasmid-size-DNA concentration was 12 μg per plate. In addition, 5 μg of high-molecular-weight salmon sperm DNA was added. After 42 h, the medium was harvested and assayed for infectious virus. Cell lysates were prepared, and CAT activity was determined as described in Materials and Methods. The control plate for the CAT assay was transfected by 1 μg of pRSV-CAT only, whereas for the FFU measurements, the control was transfected with 1 μg of pLC32 plus 1 μg of pRSV-CAT. The control CAT activity was 20.1% conversion of chloramphenicol to its acetylated derivatives.

structs occurs has not yet been clarified. It seems unlikely that the inhibition was caused by competition for RNA polymerase or transcriptional factors, since plasmids with identical LTR sequences did not cause detectable inhibition when present at the same concentrations. Furthermore, expression of the CAT gene from the pRSV-CAT construct was not inhibited even when a 10-fold excess of the inverted construct pLC25 was present. This result is in contrast to results with DNA constructs containing the simian virus 40 72-bp repeat enhancer sequences, which compete for limiting cellular factors when cotransfected into CV-1 cells (21). It is unlikely that the inhibition is simply a result of an interference with splicing, since expression of both pLC32, which does not require splicing, and pJD100, which does require splicing, were inhibited.

It has been proposed in procaryotic systems that cRNA inhibits gene expression at the level of translation (2, 18, 20, 24). This proposal appears to be the most reasonable explanation for the results presented here, since anti-sense RNA could anneal to the authentic mRNA and block translation. Indeed, it has recently been shown that, after microinjection of anti-sense RNA into *Xenopus* oocytes, the translation of microinjected β-globin mRNA was specifically inhibited (17). This inhibition was concomitant with the formation of RNA-RNA hybrids containing the β-globin mRNA sequences. We have shown above that inhibition of *env* gene expression occurs in the presence of anti-sense constructs with inverted sequences either from the *env* coding region (pLC25) or the 5′ noncoding region (pLC55 and pLC58). The hybridization of RNA to the 5′ noncoding region would be expected to block the initiation of translation, since the current models for eucaryotic protein synthesis involve a mechanism whereby the ribosome binds to the 5′ end of the mRNA and "scans" for the initiator AUG codon (13). Even if scanning is not used, hybridization of complementary RNA to the 5′ leader might disrupt secondary structure required for the initiation of the correct polypeptide synthesis (5). Earlier studies by Zamecnik and Stephenson (32) showed that synthetic oligonucleotides complementary to sequences at the 5′ terminus of RSV RNA inhibit RSV infection. Stephenson and Zamecnik (26) correlated these results with the inhibition of in vitro translation of RSV genome RNA by the presence of the oligonucleotide. Hybridization of anti-sense RNA to the coding region of the mRNA would also be expected to inhibit protein synthesis if elongation were blocked by the presence of RNA-RNA hybrid structures. It has been demonstrated that elongation is arrested by the in vitro formation of DNA-RNA hybrids (19). In vivo, *E. coli* outer membrane protein synthesis was inhibited equally well by anti-sense RNAs complementary either to the coding region or to the initiation site for protein synthesis (2). The experiments of Melton (17), on the other hand, suggest that anti-sense RNA which hybridizes to only the 3′ half of the globin-coding region does not affect globin mRNA translation. This apparent discrepancy will need to be resolved. The stability of the RNA-RNA hybrid is related to the hybrid length, which may account for the different extents of interference of anti-sense RNA on elongation of protein synthesis. We also cannot eliminate the possibility that the formation of RNA duplexes may affect other processes besides translation, including the transport of mRNA from nucleus to cytoplasm and RNA turnover.

It is surprising that the inhibition of the expression of the wild-type clone pJD100 by pLC25 persisted for as long as 8 days (Fig. 2B). The expression of the intronless clone pLC32 peaked at 24 to 36 h and then decreased to a low level (Fig. 2A). The expression of the antisense constructs would be expected to follow similar kinetics and to decay over the 8 days. We have interpreted the rise in infectious virus observed in pJD100 transfections to be due to spreading of progeny PrA virions. If the production of PrA virions was inhibited by 80% (the level of inhibition of *env* expression observed with pLC32 transfections), the amount of residual virus should have been sufficient to fully infect all the cells within the time of the experiment. This result suggests that there may be additional selective effects of anti-sense RNA on the replication and spread of PrA virus. This possibility is currently under investigation.

Inhibition of specific viral and cellular gene functions by the production of the appropriate anti-sense RNAs has been proposed as an alternative approach to genetic analysis in both procaryotes and eucaryotes (2, 12). We have shown in this paper that such an approach may indeed be feasible for retroviral genes and for other eucaryotic genes. However, our results suggest that for such a system to be effective, an excess of anti-sense RNA relative to sense mRNA must be present. This requirement also is characteristic of other systems for which inhibition by cRNA transcripts has been described (2, 12, 17, 18, 20, 24). A likely explanation of these results is that an excess of anti-sense RNA is required to increase the concentration of anti-sense RNA and favor the hybridization kinetics of duplex formation within the infected cell. If this possibility is the case, to achieve a sufficient inhibition effect, either the transcription of anti-sense RNA must be driven by a strong promoter such as a retroviral LTR, or the DNA encoding the anti-sense RNA must be present in multiple copies within the cell.

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