Insertion of Several Different DNAs in Reticuloendotheliosis Virus Strain T Suppresses Transformation by Reducing the Amount of Subgenomic mRNA

CYNTHIA K. MILLER AND HOWARD M. TEMIN*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received 25 September 1985/Accepted 13 December 1985

The highly oncogenic retrovirus reticuloendotheliosis virus (Rev) strain T (Rev-T) has, relative to its helper virus Rev strain A, a substitution of the oncogene *v-rel* for most of the *env* gene and a large deletion of *gag* and *pol* sequences. When the helper virus sequences that are deleted in Rev-T are replaced, the recombinant virus is nontransforming (I. S. Y. Chen and H. M. Temin, Cell 31:111-120, 1982). We show that suppression of transformation occurs when several different DNA sequences are inserted in Rev-T and that suppression is correlated with a reduction in the amount of *v-rel* mRNA and *v-rel* protein in infected cells. The reduced amount of *v-rel* protein is insufficient for transformation.

Highly oncogenic retroviruses efficiently cause cancer in vivo and transform cells in vitro. These viruses carry viral oncogenes, sequences which are related to normal cellular genes (proto-oncogenes) but, unlike normal cellular genes, are able to transform cells. Most highly oncogenic retroviruses are replication defective and are found to be associated with replication-competent helper viruses which supply the *trans*-acting factors which are required for replication.

During retrovirus replication, virion RNA is reverse transcribed to form a DNA copy of the genome, which is then integrated into the cellular DNA. Viral RNA is transcribed from this integrated viral DNA (21). The *gag* and *pol* genes are expressed from unspliced RNA, while the *env* gene is expressed from spliced RNA. The unspliced RNA also functions as virion RNA and is packaged into virus particles. Thus, the level of splicing must be modulated both so that all three genes are expressed at appropriate levels and so that there is an appropriate amount of unspliced RNA to be packaged into virions for additional replication cycles. The relative amounts of spliced and unspliced viral RNAs in infected cells have been reported to be approximately equal for both avian and murine leukemia viruses (10, 13, 16).

Highly oncogenic retroviruses arise by transduction of proto-oncogenes into retrovirus genomes. The evolution of a highly oncogenic retrovirus, however, includes not only the acquisition of an oncogene by a progenitor virus but also changes both in the oncogene and in the viral sequences. Viral oncogenes and cellular proto-oncogenes are homologous, but there are usually qualitative differences between them. Viral oncogenes may contain only part of the homologous cellular oncogene, may be expressed as fusion products with viral sequences, and may have nucleotide differences from the cellular sequences (2). Almost all highly oncogenic retroviruses also have large deletions of sequences found in homologous replication-competent retroviruses (2).

We have been studying the evolution of reticuloendotheliosis virus strain T (Rev-T). Rev-T is a highly oncogenic avian retrovirus that transforms stem cells in spleen and bone marrow in vitro (8) and causes a rapid, lethal lymphoma in vivo (20). Rev-T is replication defective

and is associated with a replication-competent helper virus (reticuloendotheliosis virus strain A; Rev-A) which provides the *trans*-acting factors necessary for replication of Rev-T. The genome of Rev-T contains a substitution of the oncogene *v-rel* for the *env* gene found in the helper virus Rev-A and also contains a large deletion of helper virus-related *gag* and *pol* sequences (3, 9, 17). Because *v-rel* is expressed from a spliced mRNA, this deletion is located in the intron of the *v-rel* mRNA. When these helper virus-related sequences are replaced in Rev-T, transformation is suppressed (4).

We present evidence that this suppression of transformation is caused by insertion of several different DNA sequences in the *v-rel* mRNA intron. This suppression is correlated with a reduction in the amount of subgenomic *v-rel* mRNA resulting in a reduction in the amount of *v-rel* protein that is produced. Modulation of splicing by intron sequences is an essential feature of replication-competent retroviruses, and therefore, we propose that the evolution of Rev-T included deletion of these suppressing sequences to increase the expression of the *rel* oncogene.

MATERIALS AND METHODS

Nomenclature. Plasmid pCM103, for example, indicates the plasmid from which virus CM103 was made.

Recovery of virus. Virus was recovered after transfection of chicken embryo fibroblasts with plasmid DNAs. Plasmids that contain a permuted copy of the virus genome were first digested with *SalI* to linearize the viral DNA and then were ligated with T4 DNA ligase to form concatamers. Plasmids that contained a nonpermuted copy of the viral DNA were not treated before transfection. Chicken embryo fibroblasts in 60-mm-diameter plates were cotransfected by the calcium phosphate precipitation procedure described by Graham and van der Eb (6) with 1 to 2 μ g of viral DNA, 100 ng of Rev-A helper virus DNA, and 10 μ g of salmon sperm DNA per ml in a volume of 0.5 ml. Virus was harvested 5 days after transfection. Recovery of virus was variable with different DNA samples and for different experiments; however, the average recovery was the same for all viruses. Because of this variability, in all experiments the amounts of transforming virus were standardized as follows. Chicken embryo fibroblasts were infected with each virus stock, and the

* Corresponding author.

unintegrated viral DNA was prepared by the method of Hirt (7) 3 days later. The DNA was electrophoresed in an agarose gel, transferred to nitrocellulose, and hybridized to a middle *v-rel* probe (3) as previously described (4). Preparation of hybridization probes was as described by Rigby et al. (18). Relative amounts of hybridization were determined by densitometry of autoradiographs to determine the relative amounts of unintegrated DNA of the *rel*-containing virus in infected chicken embryo fibroblasts. Relative virus production was defined as the amount of recombinant virus determined by hybridization relative to the amount of Rev-T determined by hybridization. The production of virions from a provirus requires transcription, and thus the measure of relative virus production was also a measure of transcription and RNA stability.

Cells and transformation assay. Chicken embryo fibroblasts (from SPAFAS chickens) were grown in Temin-modified Eagle minimal essential medium with 20% tryptose phosphate broth–2% fetal bovine serum–2% calf serum. Transformed spleen cell lines were grown as described previously (3). Transforming virus was assayed in vitro by infection of chicken spleen cells as described by Hoelzer et al. (8). The transforming titer of wild-type Rev-T ranged from 20 to 500 CFU/ml in different experiments. Because of this variability, wild-type Rev-T was used as a standard in all infections, and all transforming titers were expressed relative to Rev-T. Because the recovery of virus after transfection was variable, transforming titers were normalized to the relative virus production (as described above). This double normalization procedure was necessitated by the experimental variability in transfection and in transformation. However, the normalized results were consistent in repeated experiments.

S1 nuclease analysis. S1 nuclease analysis was done essentially as described previously (23). Total cell RNA from infected chicken embryo fibroblasts was prepared using guanidine thiocyanate (15). The DNA probe was prepared from pKW104, a subclone of Rev-T that contains sequences from the *Bam*HI site at 1.9 kilobase pairs (kbp) to the *Bam*HI site at 4.1 kbp (25). The 1.9-kbp *Sal*I to *Eco*RI fragment from pKW104 was isolated from an agarose gel and end-labeled with T4 polynucleotide kinase. Hybridization of 50 µg of RNA with 100 ng of labeled DNA probe (approximately 20,000 cpm) was done at 55°C for 12 h, and the S1 digestion was done at 37°C as described previously (23). S1 nuclease-resistant fragments were analyzed by electrophoresis in a gel containing 4% polyacrylamide and 8.3 M urea. The gel was electrophoresed until the xylene cyanol dye had migrated 50 cm. The gel was exposed to film, and relative amounts of hybridization were determined by densitometry of autoradiographs.

Immunoprecipitation. Chicken embryo fibroblasts were infected or transfected with *rel*-containing viruses or plasmids. After 4 days, the cells were transferred into 35-mm-diameter dishes. Two days later, cells were labeled with [³⁵S]methionine (100 µCi/ml) for 5 h and were lysed with 20 mM Tris hydrochloride (pH 7.4)–5 mM MgCl₂–0.1 M NaCl–1% Nonidet P-40–0.5% sodium dodecyl sulfate–1% aprotinin (lysis buffer). A total of 1.5×10^7 trichloroacetic acid-precipitable counts were immunoprecipitated with excess rabbit *rel* antiserum (generously provided by T. Gilmore) for 1 h and then were complexed with 10% Staph A (IgGSorb; Enzyme Center). The complexes were washed three times with lysis buffer, suspended in sodium dodecyl sulfate sample buffer, and subjected to electrophoresis in an 8% sodium dodecyl sulfate-polyacrylamide gel. The gels

were impregnated with En³Hance (New England Nuclear Corp.), dried, and exposed to film. Relative amounts of hybridization were determined by densitometry of autoradiographs. The relative amount of p59^{v-rel} for each sample was defined as the amount of hybridization of p59^{v-rel} for that sample divided by the amount of hybridization of p59^{v-rel} for Rev-T. The normalized amount of p59^{v-rel} was defined as the relative amount of p59^{v-rel} divided by the relative amount of virus production. This double normalization procedure was necessitated by the experimental variability in transfection and in transformation. However, the normalized results were consistent in repeated experiments.

Construction of recombinant DNAs. Construction of recombinant DNAs was done by standard techniques (12). Figure 1 shows the structure of the DNA genome of each recombinant virus.

RESULTS AND DISCUSSION

Helper virus-related sequences suppress transformation. The deletion of helper virus-related sequences in Rev-T is required for transformation. Results of previous work in which cotransfection of subgenomic fragments of Rev-A and Rev-T was used show that helper virus sequences suppress transformation by Rev-T (4). Because recombination occurs at high levels during transfection of retrovirus subgenomic DNA fragments (1, 14), it is possible that these results reflect some peculiarity caused by recombination. Therefore, we repeated these experiments by using molecular clones constructed in vitro. Three recombinant virus clones were constructed containing the *gag* and *pol* sequences from the helper virus Rev-A (or the closely related spleen necrosis virus) and the sequences normally found in Rev-T. These viruses (CM104, CM105, CM106) contain Rev-A sequences from within the 5' long terminal repeat (LTR) to the *Hind*III site at 5.6 kbp and Rev-T sequences from the equivalent *Hind*III site to sequences within the 3' LTR (Fig. 1A). CM104 contains a single base pair mutation that changes the *gag* ATG codon to ATA.

Virus stocks were prepared by cotransfection of chicken embryo fibroblasts with the recombinant molecular clones and a molecular clone of the helper virus Rev-A. In agreement with results of the previous study in which cotransfection of subgenomic fragments was used (4), the recombinant viruses were able to transform spleen cells at a very low level. Two of these recombinant viruses (CM105, CM106) each generated only one transformed colony (which failed to grow beyond 50 to 100 cells), while Rev-T generated 1,500 colonies (which proliferated indefinitely). The other of these recombinant viruses (CM104) had a transforming titer that was approximately 2% that of Rev-T. Some of the rare colonies that arose after infection of chicken spleen cells with CM104 were expanded into cell lines. In each case the virus produced was a variant, rather than the input virus (data not shown). These variant viruses ranged from as large as the input virus to as small as 4.5 kbp. Each variant virus was able to transform spleen cells with a characteristic titer, ranging from 10 to 100% that of Rev-T (rCM104, Table 1; data not shown).

Level of *rel* protein correlates with transformation. To determine whether there is a correlation between transformation and expression of *rel* protein in infected cells, we analyzed the levels of *rel* protein by immunoprecipitation with *rel* antiserum in cells that were either infected or transfected with *rel*-containing viruses. Because non-transformed spleen cells cannot grow in culture, it is not possible to label metabolically spleen cells infected with

FIG. 1. Structure of recombinant viruses derived from Rev-T and Rev-A. The structures of the DNA genomes of different recombinant viruses are shown. The open boxes represent the viral LTRs. The heavy lines represent sequences derived from Rev-A, and the lighter lines represent sequences derived from Rev-T. The open rectangles represent *v-rel* sequences. The extent of deletions is indicated by dotted lines, with the numbers at the end indicating map position (in kilobase pairs) of Rev-A. (A) Recombinant viruses derived from Rev-T and Rev-A. H3, the *Hind*III site at 5.6 kbp; X in the viral sequence, a base mutation in the *gag* initiation codon; d and a, the splice donor and splice acceptor sites at 0.84 and 5.7 kbp, respectively. The genome of Rev-A is 8.3 kbp, while the genome of Rev-T is only 5.6 kbp. Coordinates are given using the Rev-A genome as standard, and the coordinates from Rev-T are given here in parentheses when they differ from those of Rev-A (all sites beyond 2.2 kbp). pCM104 contains spleen necrosis virus (a virus with 98% homology with Rev-A) sequences from the LTR to the *Hind*III site at 5.6 kbp, with ATA substituted for the normal *gag* ATG, and Rev-T sequences from the *Hind*III site at 5.6 (2.9) kbp through the LTR continuing to the *Sall* site at 0.9 kbp. pCM105 contains flanking cellular sequences and Rev-A sequences from the 5' LTR to the *Hind*III site at 5.6 kbp and Rev-T sequences from the *Hind*III site at 5.6 (2.9) kbp through the LTR continuing to the *Sall* site at 0.9 kbp. pCM106 contains SNV sequences of a noninfectious SNV clone with multiple lesions from the LTR through the *Hind*III site at 5.6 kbp and Rev-T sequences from the *Hind*III site at 5.6 (2.9) kbp through the LTR continuing to the *Sall* site at 0.9 kbp. The viruses produced from these clones contain LTRs with U5 from Rev-A and U3 from Rev-T. (B) Recombinant viruses containing

TABLE 1. Relative virus production, transformation, and $p59^{v-rel}$

Virus	Virus production ^a	CFU ^b	$p59^{v-rel}$ ^c
Rev-A	10		
		1.0	1.0
CM104	1.0	0.02	0.008
CM105	1.0	<0.0007	0.003
CM106	1.0	<0.0007	ND ^d
rCM104	1.0	1.0	1.0
ME106	1.0	0.05	0.006
CM108	1.0	0.02	0.004
CM109	1.0	0.05	0.003
CM125	1.0	ND	0.04
CM122	1.0	ND	0.04
CM124	1.0	ND	0.04

^a Virus production is the relative amount of virus produced after transfection (determined as described in the text). Values are averages of at least three experiments.

^b CFU are normalized to the amount of virus production and are expressed relative to Rev-T (as described in the text). Values are averages of at least three experiments.

^c The amount of $p59^{v-rel}$ is normalized to the amount of virus production and is expressed relative to the amount of $p59^{v-rel}$ in cells infected with Rev-T (as described in the text). Values are averages of at least two experiments.

^d ND, Not determined.

nontransforming viruses. For this reason, the immunoprecipitation analyses were performed on chicken embryo fibroblasts. The *v-rel* protein has an apparent molecular mass of 59,000 daltons (T. Gilmore and H. M. Temin, Cell, in press). The amount of $p59^{v-rel}$ is high and is approximately equal in spleen cells transformed by Rev-T and in fibroblasts infected with Rev-T (data not shown; Gilmore and Temin, in press). The amount of $p59^{v-rel}$ in cells that were either infected or transfected with any particular *rel*-containing virus was comparable (data not shown). Fibroblasts infected with CM104 expressed very low levels of $p59^{v-rel}$ (less than 5% of that observed in Rev-T-infected cells; Table 1). Fibroblasts infected with a strongly transforming revertant of CM104 (rCM104) and spleen cells transformed by this virus expressed approximately the same amount of $p59^{v-rel}$ as Rev-T-infected cells (Table 1; data not shown). Cells infected with viruses which were able to transform spleen cells expressed higher levels of $p59^{v-rel}$ (the same as that observed in Rev-T-infected cells) than cells infected with viruses that were unable to transform spleen cells (less than 5% compared with Rev-T-infected cells).

insertions in Rev-T. The hatched rectangles represent inserted DNA. The numbers represent the map position of Rev-T at which the additional DNA is inserted. pCM108 contains a 1.0-kbp *Bam*HI fragment containing the *neo* gene from pSV2neo (19) inserted at the 2.0-kbp *Bgl*II site of Rev-T in the same orientation. pCM109 contains a 1.0-kbp *Bam*HI fragment containing the *neo* gene from pSV2neo inserted at the 2.0-kbp *Bgl*II site of Rev-T in the opposite orientation. pCM122 contains a 2.0-kbp *Hind*III fragment containing *c-rel* intron sequences inserted at the 2.9-kbp *Hind*III site of Rev-T in the opposite orientation. pCM124 contains a 2.3-kbp *Hind*III fragment containing *c-rel* intron sequences and the *neo* gene (from pSV2neo) inserted at the 2.9-kbp *Hind*III site of Rev-T in the same orientation. pCM125 contains a 2.0-kbp *Hind*III fragment containing *c-rel* intron sequences inserted at the 2.9-kbp *Hind*III site of Rev-T in the same orientation. pME106 contains a 2.0-kbp *Bam*HI fragment containing the herpes simplex virus thymidine kinase gene inserted in the 2.0-kbp *Bgl*II site of Rev-T in the same orientation (generously provided by M. Emerman).

FIG. 2. S1 nuclease analysis of viral RNAs in infected fibroblasts. S1 nuclease-resistant fragments were analyzed as described in the text. The two sets of autoradiograms are from different experiments; CM108 was analyzed in parallel with Rev-T (as a positive control) and no RNA (as a negative control; data not shown). Molecular size markers were prepared by digesting lambda DNA with *Hind*III and *Eco*RI and end-labeling. Size markers are indicated on the side of the autoradiograms. The line to the right indicates the position of spliced *rel* mRNA. In the Rev-T and CM108 lanes, a minor band of 1,430 bases is seen. There are two possible explanations for this band. One possibility is that recombination occurred between Rev-T and the helper virus Rev-A, resulting in viral RNA that was homologous to the probe only to the deletion endpoint (see discussion of CM104 in text). An alternative explanation is that a cryptic splice acceptor located at this site was used at a low frequency. In the lanes labeled CM104 and rCM104, the faint bands between 1,350 and 800 bases are probably the result of mismatch of the probe with the RNA, because Rev-T and Rev-A have some nucleotide sequence differences. The structure of Rev-T

Relative levels of spliced and unspliced viral RNA. Because p59^{v-rel} is expressed from a spliced mRNA in CM104, CM105, and CM106, reduced levels of p59^{v-rel} from these viruses might be caused by reduced levels of subgenomic mRNA. Splicing in replication-competent retroviruses such as Rev-A must be regulated so that enough unspliced RNA is available for translation of *gag* and *pol* proteins and for packaging into virions. However, the splicing in Rev-T could be altered in its regulation, resulting in increased expression of *rel*.

Support for this hypothesis comes from the observation that Rev-T was rapidly lost from viral stocks on repeated passage in chicken embryo fibroblasts; that is, the amount of Rev-T relative to that of Rev-A decreased (data not shown) (26). This decrease would occur if Rev-T RNA were normally spliced more efficiently than Rev-A RNA, because there would be less unspliced Rev-T RNA available for packaging in virions. Sequences in Rev-A that modulate the level of splicing could be responsible for the suppression of transformation in CM105. These sequences do not include the actual splice acceptor, because all of the recombinant viruses contain the Rev-T splice acceptor.

To test the hypothesis that levels of subgenomic mRNA are reduced in those recombinant viruses that are unable to transform spleen cells, we used S1 nuclease mapping to compare the relative amounts of spliced and unspliced viral RNAs in chicken embryo fibroblasts infected with *rel*-containing viruses (Fig. 2). (Chicken embryo fibroblasts were used because chicken spleen cells infected with nontransforming viruses cannot be propagated.) The DNA probe, from pKW104, was end-labeled at the *Eco*RI site at 3.6 kbp within *rel* sequences so that only *rel*-containing RNA was detected, to avoid complications due to the presence of helper virus RNA. Cells infected with Rev-T contained about two-thirds unspliced viral RNA and one-third spliced viral RNA. (The same relative amounts of spliced and unspliced viral RNAs were seen in Rev-T-transformed spleen cells [data not shown].)

Cells infected with CM104 (which transforms spleen cells only rarely) had the same amount of viral RNA as cells infected with Rev-T (Fig. 2). Considerable amounts of unspliced RNA were visible, but no spliced RNA was detectable (less than 1% of total viral RNA; Fig. 2). The size of the unspliced RNA was slightly smaller than that of Rev-T. The reason for this size difference is that the probe was derived from Rev-T, which has a large deletion relative to Rev-A, and the probe overlapped the junction sequences in Rev-T which spanned the ends of the deletion. CM104 RNA was homologous only to those regions of the probe that were shared by Rev-T and Rev-A. Thus, hybridization of CM104 RNA and probe results in molecules which have a large region of unhybridized, single-stranded nucleic acid

is shown in the lower part of the figure. The open boxes represent the viral LTRs, and the open rectangle represents *v-rel* sequences. The small inverted triangle represents the large deletion of helper virus-related sequences in Rev-T. The DNA probe was prepared by digesting pKW104 (a subclone of Rev-T that contains sequences from the *Bam*HI site at 1.9 kbp to the *Bam*HI site at 4.1 kbp) with *Sal*I and *Eco*RI and end-labeling with T4 polynucleotide kinase. The *Eco*RI site is located at 3.6 kbp within *rel* sequences, and the *Sal*I site is located within pBR322 sequences. The structure of the DNA probe is shown with Rev-T sequences as a straight line and pBR322 sequences as a jagged line. The X indicates the site of the label. The expected protected fragments are shown, and their sizes (in bases) are indicated.

which is sensitive to S1 nuclease digestion. Because the probe spans the Rev-T junction sequences at the ends of the large deletion, the end of the probe was subjected to digestion by S1 nuclease, and the protected fragment was slightly smaller. The protected fragment of approximately 500 bases was seen because Rev-A contains a deletion of 8 of 10 bases relative to Rev-T 500 bases upstream of the site at which the probe is end-labeled (24). This region of RNA could not hybridize to the probe, and thus this region of mismatch was partially sensitive to S1 nuclease.

It is surprising that cells infected with CM104 contained no detectable spliced viral RNA, because CM104 is a recombinant between Rev-A and Rev-T and cells infected with either Rev-T or Rev-A contained both spliced and unspliced viral RNAs. The amount of splicing of these two viral RNAs differed; approximately one-third of Rev-T RNA was spliced, and only approximately one-tenth of Rev-A RNA was spliced (data not shown). The lack of splicing of CM104 RNA was probably not a consequence of the mixing of the splice donor from Rev-A and the splice acceptor from Rev-T, because viruses that are identical to CM104 but that contain both splice donor and splice acceptor from Rev-T have the same transforming titer as CM104 (4) and, thus, presumably the same amount of splicing of viral RNA. We believe that the different relative amounts of spliced and unspliced viral RNAs are due to differences in splicing. We consider it less likely that changes in the relative amounts of spliced and unspliced viral RNAs are the result of changes in the relative stability of the RNAs, because virus production was the same for all constructs.

Cells infected with rCM104, which is identical to CM104 by restriction endonuclease analysis and transforms spleen cells as efficiently as Rev-T, contained both unspliced viral RNA and spliced viral RNA, in approximately the two-to-one ratio seen in cells infected with Rev-T (Fig. 2). Therefore, the suppression of transformation by helper virus-related sequences in full-length recombinant viruses (CM104, CM105, CM106) is correlated with a reduction in the amount of subgenomic mRNA.

Different splice acceptor sites are used by rCM104 and Rev-T. The size of the spliced viral RNA was slightly smaller for rCM104 than for Rev-T. The splice acceptor sites which were used by these two viruses were different: rCM104 used the *env* splice acceptor of Rev-A, while Rev-T used a different splice acceptor site located 34 bases upstream of the *env* splice acceptor site. (The two splice sites were mapped more precisely with smaller S1 nuclease probes. The splice acceptor site of Rev-T was located at nucleotide 2919, while the splice acceptor site of rCM104 was located at nucleotide 5698 [the equivalent of nucleotide 2953 of Rev-T; data not shown].) This difference is surprising because the sequences around the splice acceptor in CM104 were derived from Rev-T. Furthermore, both viruses contained both splice acceptor sites, and the nucleotide sequences of the two viruses in this region were nearly identical (24). (Rev-A contained an additional splice acceptor consensus sequence which was mutated in Rev-T [24], but this splice site was not used in Rev-A.) The significance of the use of two different splice acceptor sites is unclear, but this usage may reflect different interactions of different intron nucleotide sequences in Rev-A and Rev-T. Both splice acceptor sites were located upstream of the *rel* translation initiation codon, and the use of different splice acceptor sites did not appear to have any effect on the amount of splicing, the amount of p59^{v-rel} expressed, or the transforming titer of the viruses.

Insertion of several DNA sequences in the *rel* intron sup-

presses transformation and reduces levels of *rel* protein and *rel* mRNA. To test whether the suppression of transformation is a result of specific helper virus sequences or an effect of other DNA sequences inserted in the *rel* intron, a series of molecular clones with the Rev-T splice donor and splice acceptor and different intron sequences were constructed (Fig. 1B). The inserted DNAs were from the herpes simplex virus thymidine kinase gene (viral coding) sequences, the neomycin resistance gene from Tn5 (bacterial coding) sequences, the neomycin resistance gene in the opposite orientation (bacterial noncoding) sequences, or part of a *c-rel* intron (eucaryotic intron). All of these viruses transformed spleen cells; however, the relative transforming titers were less than or equal to 5% that of Rev-T (Table 1). Some transformed colonies were expanded into cell lines, and the virus that was produced was examined. Colonies derived from spleen cells infected with ME106 produced viruses that were smaller than the input virus and that had relative transforming titers as high as that of Rev-T (data not shown). Therefore, the input viruses contain sequences that suppress transformation; mutation, including deletion, of these sequences allows transformation to occur.

The level of p59^{v-rel} in chicken embryo fibroblasts infected with these viruses was analyzed (Table 1). Cells infected with each of these viruses expressed very little p59^{v-rel} (less than 5% that of cells infected with Rev-T; Table 1). These results indicate that insertion of several types of DNA into the intron of the *rel* mRNA suppresses transformation and *rel* expression and that there is a correlation between *rel* expression and transformation.

To test whether the reduced expression of p59^{v-rel} in these constructs was the result of reduced amounts of subgenomic mRNA, the relative amounts of spliced and unspliced RNA were determined for one construct using S1 nuclease mapping. Cells infected with CM108 contained unspliced RNA but no detectable spliced RNA (Fig. 2).

Other workers have observed an apparently similar phenomenon. Insertion of the herpes simplex virus thymidine kinase gene in a derivative of Friend spleen focus-forming virus suppressed expression of gp55, which is expressed from a subgenomic mRNA (11). Insertion of fragments from phage lambda in the *env* mRNA intron of avian leukosis virus reduced, but did not abolish, *env* expression (5). Insertion of a murine leukemia virus provirus into a Rous sarcoma virus provirus abolished expression of *src* (22). These results indicate that splicing is greatly reduced by insertion of certain DNAs into retrovirus introns, although it is not necessarily completely abolished.

Splicing of retrovirus RNA must be modulated so that adequate amounts of both unspliced and spliced RNA are present for packaging into virus particles and as translational templates for viral proteins. This modulation must involve some interactions of nucleotide sequences in the viral intron. The observation that insertion of different DNAs in the viral intron reduces splicing indicates that these interactions can be disrupted by the presence of additional sequences. This result has relevance to construction of retrovirus vectors and expression of viral oncogenes in highly oncogenic retroviruses.

ACKNOWLEDGMENTS

We thank S. Hellenbrand and A. Troup for technical assistance; T. Gilmore for the rabbit *rel* antiserum and help with immunoprecipitation; and J. Embretson, M. Emerman, C. Gelinas, T. Gilmore, J. Mertz, N. Panganiban, B. Sugden, and B. Sylla for helpful comments on the manuscript.

This research was supported by Public Health Service research grants CA-22443 and CA-07175 from the National Institutes of Health. C.K.M. is supported by Public Health Service training grant CA-09135 from the National Institutes of Health. H.M.T. is an American Cancer Society Research Professor.

LITERATURE CITED

1. Bandyopadhyay, P. K., S. Watanabe, and H. M. Temin. 1984. Recombination of transfected DNAs in vertebrate cells in culture. *Proc. Natl. Acad. Sci. USA* **81**:3476-3480.
2. Bishop, J. M. 1983. Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* **52**:501-554.
3. Chen, I. S. Y., T. W. Mak, J. J. O'Rear, and H. M. Temin. 1981. Characterization of reticuloendotheliosis virus strain T DNA and isolation of a novel variant of reticuloendotheliosis virus strain T by molecular cloning. *J. Virol.* **40**:800-811.
4. Chen, I. S. Y., and H. M. Temin. 1982. Substitution of 5' helper virus sequences into non-*rel* portion of reticuloendotheliosis virus strain T suppresses transformation of chicken spleen cells. *Cell* **31**:111-120.
5. Cullen, B. R., J. J. Kopchick, and D. W. Stacey. 1982. Effect of intron size on splicing efficiency in retroviral transcripts. *Nucleic Acids Res.* **10**:6177-6190.
6. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.
7. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
8. Hoelzer, J. D., R. B. Lewis, C. R. Wasmuth, and H. R. Bose, Jr. 1980. Hematopoietic cell transformation by reticuloendotheliosis virus: characterization of the genetic defect. *Virology* **100**:462-474.
9. Hu, S. S. F., M. M. C. Lai, T. C. Wong, R. S. Cohen, and M. Sevoian. 1981. Avian reticuloendotheliosis virus: characterization of a genome structure by heteroduplex mapping. *J. Virol.* **37**:899-907.
10. Hwang, L.-H. S., J. Park, and E. Gilboa. 1984. Role of intron-contained sequences in formation of Moloney murine leukemia virus *env* mRNA. *Mol. Cell. Biol.* **4**:2289-2297.
11. Joyner, A. L., and A. Bernstein. 1983. Retrovirus transduction: segregation of the viral transforming function and the herpes simplex virus *tk* gene in infectious Friend spleen focus-forming virus thymidine kinase vectors. *Mol. Cell. Biol.* **3**:2191-2202.
12. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Mann, R., and D. Baltimore. 1985. Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. *J. Virol.* **54**:401-407.
14. Miller, C. K., and H. M. Temin. 1983. High-efficiency ligation and recombination of DNA fragments by vertebrate cells. *Science* **220**:606-609.
15. Müller, R., D. J. Slamon, E. D. Adamson, J. M. Tremblay, D. Müller, M. J. Cline, and I. M. Verma. 1983. Transcription of *c-onc* genes *c-ras*^{K1} and *c-fms* during mouse development. *Mol. Cell. Biol.* **3**:1062-1069.
16. Quintrell, N., S. H. Hughes, H. E. Varmus, and J. M. Bishop. 1980. Structure of viral DNA and RNA in mammalian cells infected with avian sarcoma virus. *J. Mol. Biol.* **143**:363-393.
17. Rice, N. R., R. R. Hiebsch, M. A. Gonda, H. R. Bose, Jr., and R. V. Gilden. 1982. Genome of reticuloendotheliosis virus: characterization by use of cloned proviral DNA. *J. Virol.* **42**:237-252.
18. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**:237-251.
19. 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.
20. Theilen, G. H., R. F. Ziegel, and M. J. Twiehaus. 1966. Biological studies with RE virus (strain T) that induces reticuloendotheliosis in turkeys, chickens, and Japanese quail. *J. Natl. Cancer Inst.* **37**:731-743.
21. Varmus, H., and R. Swanstrom. 1982. Replication of retroviruses, p. 369-512. *In* R. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), *The molecular biology of RNA tumor viruses*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. Varmus, H. E., N. Quintrell, and S. Ortiz. 1981. Retroviruses as mutagens: insertion and excision of a nontransforming provirus alter expression of a resident transforming provirus. *Cell* **25**:23-36.
23. Watanabe, S., and H. M. Temin. 1983. Construction of a helper cell line for avian reticuloendotheliosis virus cloning vectors. *Mol. Cell. Biol.* **3**:2241-2249.
24. Wilhelmssen, K. C., K. Eggleton, and H. M. Temin. 1984. Nucleic acid sequences of the oncogene *v-rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene *c-rel*. *J. Virol.* **52**:172-182.
25. Wilhelmssen, K. C., and H. M. Temin. 1984. Structure and dimorphism of *c-rel* (turkey), the cellular homolog to the oncogene of reticuloendotheliosis virus strain T. *J. Virol.* **49**:521-529.
26. Witter, R. L., H. G. Purchase, and G. H. Burgoyne. 1970. Peripheral nerve lesions similar to those of Marek's disease in chickens inoculated with reticuloendotheliosis virus. *J. Natl. Cancer Inst.* **45**:567-577.