Transforming Viruses Spontaneously Arise from Nontransforming Reticuloendotheliosis Virus Strain T-Derived Viruses as a Result of Increased Accumulation of Spliced Viral RNA

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The highly oncogenic avian retrovirus reticuloendotheliosis virus strain T (Rev-T) contains a substitution of the oncogene *v-rel* for much of *env* and a deletion of *gag* and *pol* relative to the helper virus Rev-A. Replacement of *gag* and *pol* sequences in Rev-T suppresses transformation by reducing the accumulation of spliced viral mRNA and *v-rel* protein in infected cells (C. K. Miller and H. M. Temin, J. Virol. 58:75-80, 1986). After infection of spleen cells with viruses containing *gag* and *pol* sequences, revertant viruses that are strongly transforming were found. Approximately three-fourths of the revertant viruses appeared structurally the same as the parental virus, and approximately one-fourth of the revertant viruses had large deletions (similar in size and location to the deletion in Rev-T). Two revertant viruses that appeared structurally the same as the parental virus were molecularly cloned. The regions sufficient to change the parental virus to a strongly transforming virus were determined by construction of recombinant viruses. In one revertant virus, the region sufficient for transformation contained a 327-base-pair insertion 5' of the 3' splice site used by Rev-T. In the other revertant virus, the region sufficient for transformation contained a 1-base-pair transition and a deletion of one copy of a 9-base-pair direct repeat, both 3' of the 3' splice site used by Rev-T. These differences resulted in the accumulation of increased levels of subgenomic *v-rel* mRNA and protein, ultimately leading to transformation.

Highly oncogenic retroviruses arose by transduction of cellular proto-oncogenes by replication-competent retroviruses. The formation of a highly oncogenic retrovirus often requires not only the actual transduction event but also multiple changes in both the oncogene and viral sequences. Viral oncogenes usually differ from the homologous proto-oncogenes by base changes, loss of part of the cellular gene sequences, or fusion to viral coding sequences to create a chimeric protein (1). In addition to changes in the oncogene itself, there are usually changes in the viral sequences in highly oncogenic retroviruses. For example, most highly oncogenic retroviruses have large deletions relative to their helper viruses (1).

We are studying the evolution of one highly oncogenic retrovirus, reticuloendotheliosis virus strain T (Rev-T). Rev-T is an avian retrovirus that transforms spleen and bone marrow stem cells in vitro (7) and causes a rapid lethal lymphoma in vivo (19). Rev-T is replication defective and is found associated with its helper virus, Rev-A. Rev-T contains a substitution of the oncogene *v-rel* for much of *env* and a large deletion of *gag* and *pol* sequences relative to Rev-A (2, 9, 16). The *v-rel* protein is translated from a spliced mRNA (21); therefore, the *gag-pol* deletion is in the *v-rel* intron.

We have been studying recombinant viruses that are derived from Rev-T but contain the *gag* and *pol* sequences. When the *gag* and *pol* sequences are replaced in Rev-T, transformation is suppressed (3, 14). This suppression is correlated with a reduction in the steady-state amount of subgenomic *v-rel* mRNA, resulting in a reduction in the amount of *v-rel* protein produced (14). We isolated revertant

viruses that did not contain large deletions and still transformed cells in vitro as efficiently as did Rev-T, and we molecularly cloned two of them. By making recombinants between these revertants and the parental virus, we showed that a small region of each revertant virus is sufficient to convert the parental nontransforming virus to a transforming phenotype. The changes in these small regions include a 9-base-pair (bp) deletion 3' of the 3' splice site used by Rev-T for one virus and a 327-bp insertion 5' of the 3' splice site used by Rev-T for the other virus. These changes resulted in increased levels of subgenomic *v-rel* mRNA and protein, ultimately leading to transformation.

MATERIALS AND METHODS

Nomenclature. Plasmids have small p's before their names (e.g., pCM104), whereas viruses made from those plasmids do not (e.g., CM104).

Recovery of virus. Virus was recovered after transfection of chicken embryo fibroblasts (CEF) with plasmid DNAs. CEF in 60-mm-diameter plates were cotransfected by the dimethyl sulfoxide-Polybrene procedure (10) with 5 µg of *rel*-containing viral DNA and 0.1 µg of pSW253 (Rev-A helper virus DNA). 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 by determining the relative amount of unintegrated viral DNA for each virus as previously described (14).

Cells and transformation assay. CEF (from SPAFAS chickens) and transformed spleen cell lines were grown as previously described (3, 14). Virus was assayed for transformation in vitro by infection of chicken spleen cells as

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described previously (7). The transforming titer of wild-type Rev-T ranged from 400 to 2,500 CFU/ml in different experiments. Because of this variability, wild-type Rev-T was used as a standard for all infections, and all transforming titers are expressed relative to that of Rev-T. Because the recovery of virus after transfection was also variable, transforming titers were normalized to the relative virus production as described previously (14). This double normalization procedure was necessary because of the experimental variability in transfection and transformation. However, the normalized results were consistent in repeated experiments.

Physical analysis of recombinant viruses. The physical structures of virus genomes were determined by Southern blot analysis of unintegrated viral DNA as previously described (14) except for the preparation of hybridization probes. Hybridization probes were prepared by labeling DNA restriction endonuclease fragments by using random primers as described previously (6). The unintegrated viral DNA was isolated from CEF infected with virus harvested from each transformed spleen cell line. Southern blot analysis was done both with undigested DNA and with DNA digested with various restriction endonucleases.

Analysis of viral RNA. Total cellular RNA from infected CEF was prepared by using guanidine thiocyanate (4). For Northern (RNA) blot analyses, RNA was denatured with glyoxal as described previously (13). Denatured RNA was subjected to electrophoresis in 1.2% agarose gels, transferred to PALL membranes (Biohyne), and subjected to hybridization as described previously (20).

For S1 nuclease analyses, 20 to 40 μ g of RNA from infected CEF was hybridized with ca. 60,000 cpm of 5'-end-labeled probe overnight at 50°C. S1 digestion conditions were as previously described (5, 14). S1-resistant products were separated on a 3% polyacrylamide-8.3 M urea gel and were visualized by autoradiography with an intensifying screen. The 5'-end-labeled probe for the analysis of the 3' splice site(s) used in CM174 was made by digesting pCM174 with *EcoRI*, treating the ends with calf intestinal phosphatase (Boehringer Mannheim Biochemicals), and then digesting with *XhoI*. After gel purification, the appropriate fragment was end labeled by using T4 polynucleotide kinase.

Primer extension analyses were done as previously described (5). Briefly, 10⁶ cpm of a 5'-end-labeled primer (Pr5; University of Wisconsin Biotechnology Center) homologous to nucleotides 3256 to 3275 in *v-rel* (3) was hybridized with 20 to 40 μ g of RNA from infected CEF. After the addition of avian myeloblastosis virus reverse transcriptase, the extension products were separated on a 3% polyacrylamide-8.3 M urea gel.

Immunoprecipitation. CEF were infected or transfected with *rel*-containing viruses, and immunoprecipitations were done as previously described (14). Relative and normalized amounts of p59^{v-rel} were determined as previously described (14). The normalized results were consistent in repeated experiments.

Molecular cloning and construction of recombinant DNAs. High-molecular-weight DNA was isolated from transformed spleen cells as previously described (2). The DNA was partially digested with *EcoRI*. Isolation of DNA of 10 to 25 kbp was done essentially as described previously (12). The DNA was ligated with EMBL4 DNA (Promega Biotec) that had been digested with *EcoRI*, and the ligated DNA was packaged into bacteriophage particles by using the Packagene in vitro packaging mix (Promega Biotec) as recommended by the manufacturer. The phages were grown on *Escherichia coli* DP50 *supF*, and DNA was prepared as

FIG. 1. Structures and biological activities of recombinant viruses derived from Rev-T and Rev-A. The structures of the molecular clones of different recombinant viruses are shown. The open boxes represent the viral LTRs. The thin lines represent sequences derived from Rev-A, and the thick lines represent sequences derived from Rev-T. The open rectangles represent *v-rel* sequences. The extent of the deletion in Rev-T is indicated by a broken line, and the coordinates at the ends of the deletions are given in kilobase pairs. H, *HindIII* site at 5.6 kbp; S, *SacII* site at 5.9 kbp; 5s, the 5' splice site; 3s, the 3' splice site. The X in the CM104 sequence represents a mutation in the *gag* initiation codon. The genome of Rev-A is 8.3 kbp, and the genome of Rev-T is 5.6 kbp. Coordinates are given with the Rev-A genome as a standard. The amount of *rel* protein expressed in CEF transfected with each viral construct (relative protein) was determined by immunoprecipitation and is expressed relative to that observed with Rev-T. The transforming titer of each virus (relative CFU) was determined by infection of chicken spleen cells and is expressed relative to that of Rev-T. The DNA genomes produced by virus recovered from those clones with LTRs from two sources contained LTRs with U5 sequences derived from the 5' LTR and U3 sequences derived from the 3' LTR.

previously described (15). The phages were screened by hybridization with both a middle *v-rel* probe (3) and a *gag-pol* probe (p63Sal-HindIII; 3).

Subcloning into pBR322 and construction of recombinant DNAs were done by standard techniques (11). Details of the constructions are available upon request.

DNA sequencing. Subcloning into M13 mp18 and mp19 and dideoxy sequencing were done as described by Sanger et al. (17), as modified by Horowitz and Risser (8). Both strands of the *HindIII* (5.653 kbp)-to-*XbaI* (5.938 kbp) fragment were sequenced for each virus. Both strands of the *HindIII* 330-bp insertion were sequenced for CM136. In addition, one strand of the *SmaI* (5.578 kbp)-to-*XbaI* (5.938 kbp) fragment was sequenced for CM104 and CM136.

RESULTS

Previously, we demonstrated that insertion of *gag* and *pol* helper virus sequences into Rev-T suppresses transformation of spleen cells (3, 14). Three such viruses (CM104, CM105, and CM106) expressed low levels of p59^{v-rel} in infected cells (10% of that in Rev-T-infected cells; Fig. 1). Infection with two of these recombinant viruses (CM105 and CM106) resulted in only a single transformed colony each (which failed to grow beyond 50 to 100 cells). At the same time, Rev-T infection resulted in 1,500 colonies, which were capable of indefinite growth. A third virus (CM104) transformed cells at a low frequency, with a transforming titer approximately 2% that of Rev-T (Fig. 1). Each of these recombinant viruses contained the 5' splice site of Rev-A

and the 3' splice site of Rev-T. CM105 and CM106 contained the *gag* initiation codon, whereas CM104 contained a mutation in the *gag* initiation codon (14).

To test whether the presence of splice sites from both Rev-A and Rev-T contributed to the suppression of transformation, we made an additional virus, CM137, which was similar to CM105. CM137 contained Rev-A sequences from the 5' end of the virus to the *Sac*II site at 5.9 kbp and Rev-T sequences from the equivalent *Sac*II site to the 3' end of the virus, whereas CM105 contained Rev-A sequences only to the *Hind*III site at 5.6 kbp (Fig. 1). Thus, the only difference between CM105 and CM137 was the region between the *Hind*III site and the *Sac*II site, which includes the 3' splice site, with this region derived from Rev-T in CM105 and Rev-A in CM137. Comparison of DNA sequences of these regions from Rev-A and Rev-T indicated the following differences in Rev-A relative to Rev-T: ten 1-bp changes, one 1-bp insertion, and three deletions of 2 to 6 bp (21). CM137 was able to transform spleen cells at low efficiency (5% compared with Rev-T) and expressed slightly more *p59^{v-rel}* than did CM105 (20 versus 10% relative to Rev-T). Thus, the origin of the sequences between the *Hind*III site at 5.6 kbp and the *Sac*II site at 5.9 kbp in CM105 and CM137 only slightly affected the biological activity of these viruses. This result indicates that the inability of CM104 to transform spleen cells efficiently is not the result of the mutation in the *gag* initiation codon or the incompatibility of the 5' splice site of Rev-A with the 3' splice site of Rev-T.

Revertant viruses. To determine whether the spleen colonies transformed by CM104 contained variant viruses, some transformed colonies were expanded into cell lines and the virus produced by each of them was analyzed for its ability to transform spleen cells (Fig. 2). In each instance a variant virus was produced which differed from the parental virus by being strongly transforming. The transforming titers of all the revertant viruses except one (rev2) were the same as that of Rev-T. rev2 had a transforming titer 10% that of Rev-T.

By restriction enzyme mapping the unintegrated linear viral DNA of these revertant viruses, we found that five of seven of these viruses were the same size as CM104 and were indistinguishable from it by restriction enzyme analysis (rev1 to rev5; C. K. Miller, Ph.D. thesis, University of Wisconsin-Madison, 1986; Fig. 2). Two of these seven revertant viruses had large deletions (2.7 to 2.8 kbp) in *gag* and *pol* sequences (rev6 and rev7; Miller, Ph.D. thesis; Fig. 2). These deletions were approximately the same size as that found in Rev-T and removed much (but not all) of the same sequences. rev6 retained the *Bam*HI site at 1.9 kbp and the *Xho*I site at 4.8 kbp; rev7 retained the *Bam*HI site at 1.9 kbp but was missing the *Xho*I site at 4.8 kbp. One of the revertant viruses, rev8, was first designated SNV(63)-rel-3a (3). The virus used to transform spleen cells in that experiment was similar to CM104, but it was recovered after transfection of a mixture of subgenomic DNA clones containing spleen necrosis virus sequences from *Sal*I (0.86 kbp) to *Hind*III (5.6 kbp) and Rev-T sequences from the equivalent *Hind*III site through the long terminal repeat (LTR) to *Sal*I (0.86 kbp). These subgenomic DNA clones were digested with *Hind*III and *Sal*I and subjected to ligation. rev8 contained a 0.33-kbp insertion at the *Hind*III site at 5.6 kbp, probably introduced during the ligation of subgenomic DNA clones before transfection (see below). rev8 was approximately the same size as CM104, but it was distinguishable by restriction enzyme mapping of its unintegrated viral DNA (Miller, Ph.D. thesis; Fig. 2).

Molecular cloning of revertant viruses. To analyze in more

FIG. 2. Structures and biological activities of revertant viruses spontaneously arising after infection of chicken spleen cells with CM104. The structures of the molecular clones of different revertant viruses are shown. The thin lines represent parental CM104 sequences, and the thick lines represent revertant virus sequences. Symbols are defined in the legend to Fig. 1. The exact endpoints of the deletions in rev6 and rev7 are not known. The small triangle in rev8 represents a 327-bp insertion in the virus. Restriction endonuclease cleavage sites are indicated as follows: B, *Bam*HI; X, *Xho*I; H, *Hind*III; R, *Eco*RI. The *Eco*RI cleavage sites are indicated only for CM104. The transforming titer of each virus (relative CFU) was determined by infection of chicken spleen cells and is expressed relative to that of Rev-T (Rev-T titer = 100). rev8 was previously designated SNV(63)-rel-3a (3).

detail the changes in some of the nondeleted revertant viruses, two revertant viruses were molecularly cloned from proviruses in the transformed spleen cell lines. Thus, the initial revertant viruses were cloned rather than viruses generated from these initial revertant viruses which might have undergone additional mutations during subsequent rounds of viral replication. Genomic spleen cell DNA was partially digested with *Eco*RI, and 10- to 25-kbp molecules were selected. *Eco*RI digests the viruses twice in *v-rel* sequences (Fig. 2), but it cleaves nowhere else in the viral sequences. Phage plaques were screened with two probes, one specific for *v-rel* sequences located between the *Eco*RI sites and the other specific for *gag-pol* sequences. Only those plaques which hybridized to both probes were analyzed. In this way, any recombinant phage clone was guaranteed to contain at least 85% of the viral genome: the 5' sequences through most of *v-rel*.

Two libraries each gave rise to a single positive clone (Miller, Ph.D. thesis). These clones were mapped with restriction enzymes (Miller, Ph.D. thesis). In both instances, an incomplete provirus was obtained; each contained the 5' part of the virus through most of the *v-rel* sequences (Miller, Ph.D. thesis). The 3' sequences (the 3' end of *v-rel*, a small untranslated region, and the 3' LTR) were not obtained.

FIG. 3. Structures and biological activities of recombinant viruses derived from a molecular clone of rev1 (CM135) and from CM104. The structures of the molecular clones of different recombinant viruses are shown. Symbols are defined in the legend to Fig. 1, except that the thick lines represent cloned revertant virus sequences and the thin lines represent CM104 sequences. The coordinates indicate the junctions between the parental and revertant sequences. The amount of *rel* protein expressed in CEF transfected with each virus (relative protein) was determined by immunoprecipitation and is expressed relative to that observed with Rev-T. The transforming titer of each virus (relative CFU) was determined by infection of chicken spleen cells and is expressed relative to that of Rev-T (Rev-T titer = 100). ND, Not determined.

These clones were subcloned into pBR322 for further analysis. First, complete provirus clones were generated by inserting the 3' virus sequences from the parental virus CM104. These clones were then tested to verify that they were able to transform spleen cells and to produce high levels of p59^{v-rel} in transfected CEF. (Previous work showed that there is a correlation between the ability to transform spleen cells and the ability to produce high levels of p59^{v-rel} in transfected fibroblasts [14].) The DNA clones were also subjected to extensive restriction endonuclease mapping to determine whether there were any physical differences between them and the parental clone CM104.

pCM135 (Fig. 3), a molecular clone of rev1, did not differ from pCM104 by restriction endonuclease mapping. However, its biological properties were very different. CM135 (and the virus it was cloned from, rev1) transformed spleen cells as efficiently as did Rev-T and produced as much p59^{v-rel} in infected or transfected cells as did Rev-T (Fig. 3). Northern blot analysis of RNA from CM135-, rev1-, and Rev-T-infected fibroblasts showed that there were similar amounts of unspliced viral RNAs in fibroblasts infected with these viruses (Fig. 4). As expected, there was no detectable spliced viral RNA in CM104-infected cells. The smaller (spliced) *rel*-specific RNA in CM135-infected cells was the same size as the spliced RNA used to express *v-rel* in Rev-T-infected cells.

pCM136, a molecular clone of rev8, contained four differences from pCM104 detectable by restriction endonuclease mapping (Fig. 5). It had a 0.2-kbp deletion in *gag* sequences,

it was missing a *Bst*EII site at 4.7 kbp on the virus map, it had a 0.33-kbp insertion at the *Hind*III site at 5.6 kbp, and it had 30 bp deleted within the *v-rel* coding sequences. DNA sequence analysis showed that there were two deletions within the *v-rel* coding sequences (T. Gilmore, personal communication). These deletions included nucleotides 3401 to 3414 and 3436 to 3451 (Rev-T coordinates of Wilhelmsen et al. [21]). Biologically, CM136 also differed from CM104. CM136 (and the virus it was cloned from, rev8) produced as much p59^{v-rel} in infected or transfected cells as did Rev-T. Fibroblasts infected with CM136, rev8, or Rev-T contained approximately equal amounts of spliced viral RNAs, and the sizes of the spliced RNAs in CM136- and rev8-infected cells were the same as that in Rev-T-infected cells (Fig. 4).

The *rel* protein produced by rev8-infected cells was the same size as that produced by Rev-T-infected cells, whereas that produced by CM136-infected cells was slightly smaller (Miller, Ph.D. thesis). In addition, the transforming titer of rev8 was as high as that of Rev-T, but CM136 was unable to transform spleen cells, presumably because of the small deletion in *v-rel*. Further restriction enzyme analysis of the rev8 provirus in transformed spleen cells showed that there was no deletion in *v-rel* of rev8, indicating that the deletion in *v-rel* in CM136 arose during cloning (data not shown).

Mapping the changes in rev8 required for its transforming ability. To localize the changes in these revertant viruses that were sufficient for the altered biological activity, recombinants between each of the cloned revertant viruses and their parent, CM104, were constructed (Fig. 3 and 5). These recombinant viruses were tested for their ability to produce

FIG. 4. RNA transcripts of *rel*-containing viruses. Total RNA was extracted from cells 3 days after infection with each of the indicated viruses or from transformed spleen cells. RNA from transformed spleen cells is marked with an asterisk. The RNA (5 μ g per lane) was denatured by glyoxal and subjected to electrophoresis through a 1.2% agarose gel and Northern blotting as described in the text. The filter was hybridized to a *rel*-specific probe. The sizes on the right correspond to those of unspliced CM104 RNA (7.7 kb), unspliced Rev-T RNA (5.0 kb), and spliced *rel* RNA (2.9 kb). The size markers on the left correspond to rRNA. Transformed spleen cells and cells infected with each virus except CM104 contained approximately equal amounts of unspliced and spliced viral RNAs. In different experiments, the values for the relative amounts of spliced viral RNA (compared with total viral RNA) ranged from 25 to 75% of that of unspliced viral RNA. In cells infected with CM104, spliced viral RNA was never more than 5% of the total viral RNA. Each virus was tested at least four times. RNA isolated from cells infected with Rev-A alone was analyzed in parallel as a negative control; no bands were seen (data not shown).

that the mutation arose during that process and was not spontaneous. However, it was absolutely required for the transforming phenotype of rev8.

To determine whether the 3' splice site(s) used to express *v-rel* in CM174 was different from that used by Rev-T, we used S1 nuclease analysis. The probe, derived from pCM174, was end labeled at the *EcoRI* site in *v-rel* (Fig. 7). Using this probe, we found the expected-size S1-resistant fragments of 650 bases (genomic-length RNA) and 620 bases (spliced RNA) for Rev-T, and a band corresponding to unspliced RNA (650 bases) for CM104. There were three S1-resistant fragments for CM174, one of which (620 bases) corresponded to that seen for the spliced mRNA of Rev-T. The other two were larger. (In all three samples, the 527-base S1-resistant band was the result of mispairing between a 10-base direct repeat 90 bases 3' to the *v-rel* 3' splice site.)

To determine whether all three CM174 S1-nuclease-resistant products could be detected by another method, we used primer extension (Fig. 8). The primer was homologous to 20 nucleotides near the 5' end of *v-rel*. As expected, there was only a single major extension product from RNA of Rev-T-infected fibroblasts, corresponding within 10 bases to the splice site mapped by S1 nuclease analysis, and no detectable *rel*-specific extension product from CM104-infected cells. There was only a single *rel*-specific extension product in CM174-infected cells, and it was somewhat larger than the Rev-T band. Thus, the increased levels of *v-rel* protein in CM174 (rev8) appeared to be the result of the use of a splice site 5' to that used by Rev-T.

Mapping the changes in rev1 required for its transforming ability. The results of experiments to map the changes in CM135 are shown in Fig. 3. Different regions from CM135 were substituted in CM104, and the resulting viruses were tested. CM179 contained revertant sequences from 5.6 to 5.9 kbp, including the splice acceptor sequence. CM179 expressed as much p59^{v-rel} in transfected cells as did Rev-T and had a transforming titer comparable to that of Rev-T. Fibroblasts infected with CM179 contained approximately equal amounts of unspliced and spliced viral RNAs (Fig. 4). Thus, this 285-bp region from the revertant virus contained sequences sufficient to restore a transforming phenotype to CM104.

One virus, CM177, with CM135 sequences from 4.8 to 5.6 kbp, had an intermediate phenotype, producing slightly higher levels of p59^{v-rel} in transfected cells than did CM104, although considerably lower than those produced by CM135. CM177 was able to transform spleen cells at a low efficiency (approximately 10% compared with Rev-T), but still at a higher efficiency than CM104. Therefore, the sequences from 4.8 to 5.6 kbp in the revertant genome probably had some change related to the increased expression of *v-rel* but not sufficient for a fully transforming phenotype.

To determine exactly what changes occurred in the 285-bp *HindIII* (5.6 kbp)-to-*XbaI* (5.9 kbp) fragment in CM135, these fragments from CM104 and CM135 were sequenced and compared (Fig. 6A). The sequences were nearly identical, with only two changes. The first change was a T-to-C transition, and the second change was the deletion in CM135 of one copy of a 9-bp direct repeat in CM104. Both of these changes were 3' of the splice acceptor used by Rev-T (14).

We were unable upon repeated attempts to determine the 3' splice site(s) of CM179 by S1 nuclease analysis. Primer extension analyses, analogous to those used for CM174, indicated that the splice sites used for *v-rel* expression were different than those used by Rev-T (data not shown).

These results indicate that the changes within the insert-

FIG. 7. S1 nuclease analysis of the 3' splice site(s) in CM174. RNA (20 µg) from CEF infected with Rev-T, CM104, or CM174 was hybridized with a 5'-end-labeled pCM174 probe. The plasmid from which the probe was made (pCM174) contains a 327-bp insertion (relative to pCM104), which is shown as a thick line. The asterisk denotes DNAs with a ³²P 5'-end label. The schematic diagram shows the size of the probe and the sizes expected for S1-resistant products for each of the viruses. b, Bases.

FIG. 8. Primer extension analysis of *rel*-specific RNAs. RNA (20 to 40 μ g) from infected CEF was hybridized with 10^6 cpm of a 5'-end-labeled primer (shown as a small closed box with an asterisk at the right end) homologous to the first 20 unique nucleotides of *v-rel*. The extension products were separated on a 3% denaturing polyacrylamide gel. The extension products expected for Rev-T and CM104, based on S1 nuclease data (Fig. 7), and the product observed from CM174-infected cells are shown schematically below the autoradiogram. The 5' splice site used to express *v-rel* in CM174 is assumed to be the one used to express *env* (i.e., the 5' splice site is located 260 bases [b] from the 5' end of the RNA [5]).

ed 285-bp region allowed the accumulation of sufficient amounts of *v-rel* mRNA and p59^{*v-rel*} for transformation.

DISCUSSION

We showed that infection of spleen cells with a *v-rel*-containing virus that mediates transformation at only a low frequency (CM104) resulted in the appearance of some transformed colonies producing revertant viruses able to transform spleen cells at a high frequency. The low efficiency of obtaining a transformed colony after infection of spleen cells with CM104 was not the result of a mutation in the *gag* ATG or the use of a splice donor from Rev-A and a splice acceptor from Rev-T (Fig. 1). However, multiple types of mutations (e.g., large deletions, small deletions, and insertions) were able to create strongly transforming viruses from the nontransforming parental virus.

Analysis of revertant viruses recovered after infection of spleen cells with CM104 showed that there were two major classes of revertants. Approximately three-fourths of the revertant viruses were grossly the same as the parental virus, as determined by restriction endonuclease mapping. One-fourth of the revertant viruses contained large deletions (approximately the same sizes and locations as those of the deletion found in Rev-T).

We concentrated our analysis on two revertant viruses that were structurally similar to the parental virus. The DNA genomes of these viruses were molecularly cloned. We showed that a 285-bp fragment from *rev1* (CM135), when substituted for the same fragment in CM104, was sufficient for biological activity comparable to that of Rev-T. The only nucleotide sequence differences in this region are a T-to-C transition and the deletion in *rev1* (CM135) of one copy of a 9-bp direct repeat present in CM104. The deletion of one copy of this direct repeat was probably a result of slippage of reverse transcriptase during the RNA-to-DNA phase of the retrovirus life cycle or of recombination during transfection. The changes within the 285-bp region were sufficient to increase the level of steady-state subgenomic RNA and *rel* protein.

A small region from *rev8* (CM136) containing a 0.33-kbp insertion, when substituted for the analogous region in CM104, was sufficient for biological activity comparable to that of Rev-T. The insertion itself was absolutely essential for this ability. The insertion was a *HindIII* fragment at a *HindIII* restriction endonuclease cleavage site, probably introduced during the ligation of subgenomic clones before transfection. This insertion contains no homology to any sequence in the GenBank data base and was probably derived from bacterial DNA contaminating the plasmid clone DNA. However, the presence of this insertion increased both the stable subgenomic mRNA and *rel* protein in viruses which contained it.

Thus, a transition and deletion in CM135 3' of the 5' splice site used by Rev-T and an insertion in CM136 5' of the 3' splice site used by Rev-T were sufficient to convert a nontransforming virus (CM104) into a strongly transforming virus (CM179 and CM174, respectively). Each change increased the amount of subgenomic viral RNA present in infected cells compared with that in cells infected by the parental CM104. The transition and deletion in CM135 are located between the 3' splice site used by Rev-T and the *v-rel* ATG codon, and the insertion in CM136 is 5' to the 3' splice site normally used by Rev-T. The changes in both of the revertants analyzed correlated with the use of different 3' splice sites from Rev-T and the accumulation of subgenomic

viral RNAs not present in cells infected with the parental nontransforming viruses.

Different strains of Rous sarcoma virus can cause different morphologies of transformed fibroblasts as a result of differences in the amount of *src* expression caused by differences in the relative amounts of unspliced and spliced RNAs (18). These differences have been shown to be the result of a difference in splicing rather than a difference in the relative stabilities of the RNAs. Differences in splicing in different cloned isolates of Rous sarcoma viruses have been mapped to a region that contains only four base-pair changes between the two isolates: two in the intron and two in the exon (18). These results and the present ones show that even very small sequence changes can have dramatic effects on the amount of subgenomic retrovirus RNA.

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