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The Putative *trans*-Activator in the MA^{gag} Region of Rous Sarcoma Virus Is Not Required for Cell Transformation

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A stop codon created by oligonucleotide-directed mutagenesis in the proposed transcriptional *trans*-activator of Rous sarcoma virus (S. Broome and W. Gilbert, Cell 40:537–546, 1985) which truncated the *trans*-activator by half did not alter the transforming activity or the replication of the virus in primary chicken embryo fibroblasts. This result proves that this *trans*-activator is not essential for transformation of primary cells by Rous sarcoma virus.

Transformation of primary chicken embryo fibroblasts (CEF) by avian sarcoma viruses has been studied extensively as a model of "carcinogenesis in vitro." However, unlike the transformation of primary rat embryo fibroblasts by transfection of plasmids expressing various oncogenes (8), transformation of CEF by viral infection appears to require only one oncogene, pp60$^{v\text{-}src}$. Broome and Gilbert (2) reported that there is a transcriptional *trans*-activator encoded in the MA protein (formerly known as p19^{gag} [9])-encoding region of the Prague-C strain of Rous sarcoma virus (RSV) which produces a 10-fold stimulation of expression from the preproinsulin enhancer and a 2-fold stimulation from the RSV long terminal repeat (LTR). The putative *trans*-activator was proposed to be coded for by an open reading frame which overlaps with, but is different from, the MA-coding reading frame. They also provided evidence for the existence of an alternately spliced mRNA that could allow for the expression of the *trans*-activator. The presence of a virally coded *trans*-activator which has a pronounced effect on at least one cellular promoter opened up the possibility that expression (or repression) of one or more cellular genes by the *trans*-activator contributes to the rapid and efficient transformation of CEF by RSV. This possibility is all the more compelling in view of recent reports of the cooperation of virally encoded nuclear proteins, such as v-*fos* or adenovirus E1A, and cellular genes, such as c-*jun* or the retinoblastoma gene product (13, 17). It is worth noting that both v-*fos* and E1A have been widely reported to have transcriptional *trans*-activating functions.

Examination of the sequence of the Schmidt-Ruppin strain (SR-A) of RSV (16) revealed that the *trans*-activator open reading frame was conserved, prompting us to test whether it was required for transformation of CEF by RSV. Our aim was to create a point mutation by oligonucleotide-directed mutagenesis such that the *trans*-activator would be truncated, leaving the *gag* reading frame intact, and to simultaneously mark the mutation with a restriction site polymorphism so that we could check the proviral DNA for the persistence of the mutation. To facilitate the construction of the mutant, we isolated the 1.7-kilobase-pair (kb) *Xho*I-630-*Eco*RI-2319 fragment from the *gag-pol* region of SR-A RSV (6) and subcloned it into a pBR322-based plasmid and into the replicative form of M13mp18 by standard procedures (10, 11). Since the entire nucleotide sequence of SR-A RSV is not known, we have used the numbering system of Prague-C RSV (15) for the sake of convenience. A deoxyoligonucleotide, CACCTAAAACCCTAGGCACATCC, was synthesized such that it was identical to the SR-A RSV sequence from nucleotides 815 to 837 except for two mismatches at 826 and 828 (Fig. 1). This oligonucleotide was used to produce a mutant phage by the two-primer method of Zoller and Smith (18). Once a pure stock of mutant M13 phage was available, single-stranded DNA was prepared and sequenced by standard techniques (11, 14) to confirm the creation of the double point mutant without any other changes. Replicative form plasmid was made from the mutant phage, and the mutant 1.7-kb fragment was manipulated through a secondary plasmid to eventually produce the plasmid pM-REP. pM-REP, like pSR-REP (4), contains the 5′ LTR, *gag*, *pol*, and part of the *env* genes of SR-A RSV on a pBR322 backbone, except that it now carries the oligonucleotide-directed mutation at nucleotides 826 and 828 (Fig. 1).

To make the complete RSV provirus, we followed previously described procedures (4). Samples (1 μg each) of pM-REP (or pSR-REP) and pSR-XD2 (which provides the rest of *env*, v-*src*, and the 3′ LTR on a *Sal*I fragment) were cut with *Sal*I, and the REP and XD2 fragments were ligated as previously described (4). It has been shown that the ligation products from such a reaction contain enough copies of the complete RSV provirus for infectious virus to be recovered following transfection of the products into CEF (4).

The mutant RSV NY MXD2 (M-REP + SR-XD2) would differ from the wild-type virus NY SRXD2 (SR-REP + SR-XD2) because it would have truncated the 124-amino-acid *trans*-activator at proline 55 (2) (Fig. 1). NY MXD2 would also carry a valine-to-leucine mutation in MA^{gag} as shown in Fig. 1.

In several transfections of CEF from several different embryos, the wild-type virus NY SRXD2 and the mutant virus NY MXD2 produced confluent transformation about 12 to 14 days after transfection. The morphology of the cells transformed by NY MXD2 was indistinguishable from that of the cells transformed by NY SRXD2 (not shown).

To ensure that the virus had not regenerated the open reading frame by a point mutation or a major recombination, we recovered virus from the transfected cultures and infected several plates of CEF. We planned to examine the proviral DNA from NY MXD2-infected cells on a Southern blot for the persistence of the *Avr*II site marking the mutation. When the plates were fully transformed, total cellular

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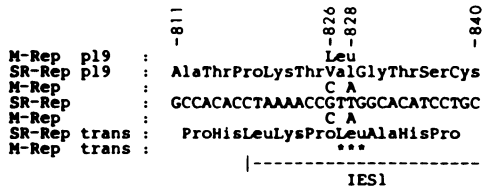

FIG. 1. Nucleic acid sequence of pSR-REP and mutations. The pSR-REP (wild-type SR-A RSV) sequence is shown in the middle line, and the mutations produced in pM-REP are shown above and below. The predicted amino acid sequences of $p19^{gag}$) (MA^{gag}) and of the putative transcriptional *trans*-activator are indicated above and below the base sequences, respectively. For M-REP, only those positions that differ from SR-REP are shown. Symbols: ***, termination codon; – – –, 5′ extent of the footprint of nuclear protein factors on the internal enhancer sequence 1 (IESl) previously described (3, 7). The numbers at the top correspond to the previously reported base sequence (15). Note that the mutation in pM-REP creates an *Avr*II site (CCTAGG).

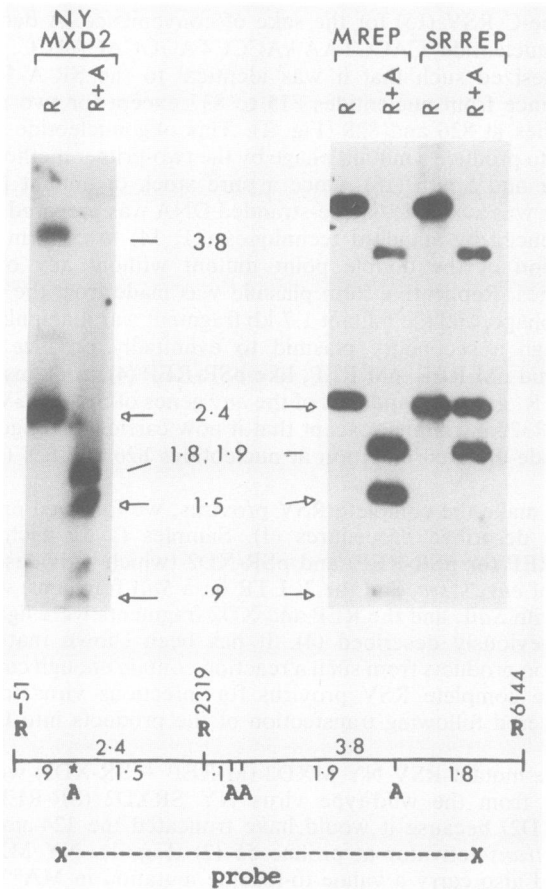

FIG. 2. Southern blot analysis of proviral DNA from cells infected with NY MXD2. Total cellular DNA from infected cells is analyzed in the first two lanes, and plasmid DNA is analyzed in the remaining lanes. The sources of the DNA and the enzymes used are indicated above the lanes (R, *Eco*RI; A, *Avr*II). The sizes of the fragments are shown in kilobase pairs. The arrows mark the fragments that are changed by the mutation in NY MXD2. The schematic restriction map of proviral DNA of NY MXD2 at the bottom extends from the *Eco*RI site in the U3 segment of the LTR (position −51) to the *Eco*RI site in *env* (position 6144) and shows the sizes of fragments (in kilobase pairs) expected to hybridize with the *Xho*I (X) 4.6-kb fragment used as the probe (– – –). The sizes above the solid line are for an *Eco*RI digest, and those below are for an *Eco*RI-*Avr*II digest. The new *Avr*II site that marks the mutation is shown (*).

DNA was purified, digested to completion with *Eco*RI or *Eco*RI and *Avr*II, electrophoresed on a 0.8% agarose gel along with the corresponding digests of pSR-REP and pM-REP, transferred to a nitrocellulose membrane, and probed with a nick-translated *Xho*I 4.6-kb fragment of pSR-REP by using standard techniques (10). The Southern blot in Fig. 2 shows that the NY MXD2 proviral DNA has the same restriction map in the *gag-pol* region as pM-REP and has not undergone any major recombination in the area. Further, the cleavage of the 2.4-kb *Eco*RI fragment from NY MXD2 into 1.5- and 0.9-kb fragments by *Avr*II shows that the *Avr*II site introduced by the mutation (and not present in the wild-type plasmid pSR-REP) persists in the transforming virus. The 0.9-kb fragment appears lighter than the other fragments because the probe overlaps it only by about 150 base pairs.

Since the *trans*-activator was reported to stimulate expression from the RSV LTR (2) and since the point mutations were introduced in the area of the proviral genome that is covered by the footprint of the transcription factor EBP-20 on the internal enhancer sequence (1, 3, 7), it was necessary to see if the replication of the mutant virus was affected. At 18 days after transfection, virus was harvested from the transformed CEF and assayed on fresh CEF. The titers of NY MXD2 (25.8×10^9 focus forming units per ml) and NY SRXD2 (13.9×10^9 focus forming units per ml) were comparable. We also examined the viral RNA in NY MXD2- and NY SRXD2-infected cells to see if there were any differences in the amounts of RNA produced by the two viruses. The Northern blot (RNA blot) in Fig. 3 shows the results from two independent experiments on fibroblasts from two different embryos. It can be seen that the amounts of genomic and subgenomic messages between the two viruses were roughly comparable, suggesting that the mutations did not dramatically affect viral RNA transcription, stability, or splicing.

Since a frameshift mutation at the *Nar*I site that disrupted the *trans*-activator polypeptide at amino acid 45 was reported to have lost its *trans*-activating function (2), we expect that the 55-residue polypeptide coded for by NY MXD2 is inactive. It is theoretically possible that the extra 10 amino acids restores some of the *trans*-activating function, but we think it is extremely unlikely, particularly in view of the inability to detect transcriptional *trans*-activation activity in a Rous-associated virus-1 infected quail cell line

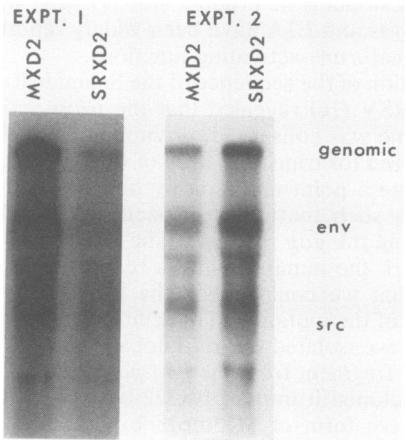

FIG. 3. Total cellular RNA (10 μg) from CEF infected with NY MXD2 and NY SRXD2 were probed on a Northern blot with a nick-translated probe specific for v-*src* (0.8-kb *Pvu*II fragment of pTT107).

(5), in RSV-infected turkey embryo fibroblasts (12), and in td107-infected CEF (unpublished results) on the preproinsulin promoter (5) and the RSV LTR (12; unpublished results). Therefore, the totally normal replication and transforming activity of NY MXD2 suggest that the putative *trans*-activator in the MA^{gag} region of RSV does not play a role in the replication of RSV or the transformation of primary CEF by RSV.

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