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Isolation of a Transformation-Defective Deletion Mutant of Moloney Murine Sarcoma Virus

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A transformation-defective (td) deletion mutant of Moloney murine sarcoma virus (td Mo-MSV) and a transforming component termed Mo-MSV 3 were cloned from a stock of clone 3 Mo-MSV. To define the defect of the transforming function, the RNA of td Mo-MSV was compared with those of Mo-MSV 3 and of another transforming variant termed Mo-MSV 124 and with helper Moloney murine leukemia virus (Mo-MuLV). The RNA monomers of td Mo-MSV and Mo-MSV 3 comigrated on polyacrylamide gels and were estimated to be 4.8 kilobases (kb) in length. In agreement with previous analyses, the RNA of Mo-MSV 124 measured 5.5 kb and that of Mo-MuLV measured 8.5 kb. The interrelationships among the viral RNAs were studied by fingerprinting and mapping of RNase T_1-resistant oligonucleotides (T_1-oligonucleotides) and by identification of T_1-oligonucleotides present in hybrids formed by a given viral RNA with cDNA's made from another virus. The nontransforming td Mo-MSV RNA lacked most of the Mo-MSV-specific sequence, i.e., the four 3'-proximal T_1-oligonucleotides of the six T_1-oligonucleotides that are shared by the Mo-MSV-specific sequences of Mo-MSV 3 and Mo-MSV 124. The remaining two Mo-MSV-specific oligonucleotides identified td Mo-MSV as a deletion mutant of MSV rather than a deletion mutant of Mo-MuLV. td Mo-MSV and Mo-MSV 124 exhibited similar deletions of *gag*, *pol*, and *env* sequences which were less extensive than those of Mo-MSV 3. Hence, td Mo-MSV is not simply a deletion mutant of Mo-MSV 3. In addition to their MSV-specific sequences, all three MSV variants, including td Mo-MSV, shared the terminal sequences probably encoding the proviral long terminal repeat, which differed from their counterpart in Mo-MuLV. This may indirectly contribute to the oncogenic potential of MSV. A comparison of td Mo-MSV sequences with either Mo-MSV 124 or Mo-MSV 3 indicated directly, in a fashion similar to the deletion analyses which defined the *src* gene of avian sarcoma viruses, that Mo-MuLV-unrelated sequences of Mo-MSV are necessary for transformation. A definition of transformation-specific sequences of Mo-MSV by deletion analysis confirmed and extended previous analyses which have identified Mo-MuLV-unrelated sequences in Mo-MSV RNA and other studies which have described transformation of mouse 3T3 fibroblasts upon transfection with DNAs containing the Mo-MSV-specific sequence.

Several substrains of Moloney murine sarcoma virus (Mo-MSV) have been derived from the original isolate (3, 11, 23, 26–28). Mo-MSV clone 124 (Mo-MSV 124) was the first substrain to be characterized extensively in terms of genome identification and biochemical comparisons of its RNA with that of nondefective helper virus (9, 21). The RNA genome of replication-defective Mo-MSV 124 is about 5.5 kilobases (kb) in length (1, 19, 21) and contains two classes of sequences (9). One class is closely related to the helper Moloney murine leukemia virus (Mo-MuLV) RNA, and the other is specific to Mo-MSV. Heteroduplex analyses of Mo-MSV 124 RNA, as well as of the RNAs of other Mo-MSV strains, have further indicated that the Mo-MSV genome contains terminal regions closely related to Mo-MuLV and an internal specific region unrelated to Mo-MuLV extending from about 1 to 2.5 kb from the 3' end (1, 6, 11, 19). A comparative study of many Mo-MSV strains showed that this specific, Mo-MuLV-unrelated sequence is conserved together with some terminal Mo-MuLV-related sequences in all Mo-MSV strains investigated (11). Therefore, the Mo-MSV-specific sequence is believed to be necessary for transformation. Transfection studies with proviral DNA have shown that subgenomic DNAs containing the Mo-MSV-specific se-

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quence, in conjunction with 3'- and 5'-terminal Mo-MSV sequences, termed LTR (long terminal repeat) are sufficient for fibroblast transformation (4, 28). However, since no transformation-specific gene product has been identified in cells transformed by Mo-MSV-specific DNA, it is uncertain whether such transformation is a complete and authentic measure of natural transformation by Mo-MSV.

Here we describe the isolation of a transformation-defective (td) Mo-MSV from a stock of virus termed clone 3 Mo-MSV, studied previously in this and other laboratories (7, 21, 22). The td mutant allowed a direct identification of transformation-specific sequences by deletion analysis, analogous to the definition of the *src* gene of Rous sarcoma virus.

Cells and viruses. NIH 3T3 cells were supplied by S. A. Aaronson of the National Cancer Institute. Rat XC cells were obtained from the American Type Culture Collection. A clonal cell line of Fischer rat embryo cells (FRE Cl.2) was supplied by E. M. Scolnick of the National Cancer Institute. Clone 3 Mo-MSV was derived in this laboratory as an Mo-MSV-producing cell line from a focus of transformed NRK cells after infection with an uncloned stock of Mo-MSV (21, 22). A mouse TB cell line producing Mo-MSV (Mo-MSV 124) was originally obtained from J. Ball (2) and has been maintained in this laboratory for a number of years. Mo-MuLV- and Friend MuLV (Fr-MuLV)-propagating FRE cells were supplied by E. Scolnick and were obtained by transfection of cells with a molecular clone of Mo-MuLV (clone 1387) or a molecular clone of Fr-MuLV (clone 57) (24). All cells were grown in Eagle minimum essential medium supplemented with 10% calf serum and changed at 2- to 3-day intervals.

Subcloning of clone 3 Mo-MSV. Viral components present in clone 3 Mo-MSV were subcloned in FRE cells. FRE cells (2×10^5 cells per 100-mm tissue culture dish) were infected with dilutions of membrane (Millipore Corp.)-filtered (0.22 μm) medium from the clone 3 Mo-MSV NRK cells. After 10 h, the infected cells were trypsinized, diluted, and plated onto 100-mm tissue culture dishes (50 cells per dish). After 6 h, the medium was replaced by 5 ml of 0.7% agar (Difco Laboratories) in tissue culture medium, and the cultures were maintained by weekly additions of fresh agar until cell colonies were visible (approximately 3 weeks). The colonies were individually trypsinized and propagated to establish clonal cell lines. Cell lines were tested for MuLV production by labeling with [^{3}H]uridine as described previously (21) and by syncytium formation on XC cells. XC cells were seeded on 60-mm tissue culture dishes (10^5 cells per dish) and after 12 h were treated for 1 h with medium containing 4 μg of Polybrene per ml. The treated cells were infected with undiluted medium harvested from cell lines after 24 h of logarithmic growth. The cells were grown to confluency and examined for syncytium formation 6 to 8 days after infection. All media containing detectable virion-associated RNA were positive for syncytium formation on XC cells. MSV production was assayed on NIH 3T3 cells. A total of 2×10^5 cells were seeded on 100-mm dishes and infected as described above for XC cells. The cultures were examined for transformation after 6 to 8 days and thereafter for periods of up to 3 weeks. Cell lines negative for MuLV and MSV production were infected with Fr-MuLV to rescue replication-defective components. After infection, the cells were transferred at least three times at a 1:10 dilution and assayed for the release of MSV activity as described above and for the release of small virion RNAs typical of defective viral genomes by electrophoresis of ^{3}H-labeled virion RNA on 2.1% polyacrylamide gels as described previously (14).

Purification of viral RNAs and T_1-oligonucleotide fingerprinting. ^{32}P labeling and purification of viral RNAs have been described previously (22, 29). RNase T_1-resistant oligonucleotide (T_1-oligonucleotide) fingerprinting on cellulose acetate at pH 2.5 followed by homochromatography was performed according to recently published procedures (15). T_1-oligonucleotides were ordered relative to the 3'-polyadenylic acid [poly(A)] end of the viral RNAs by procedures described previously (29).

Synthesis of cDNA and hybridization reactions. The syntheses of virus-specific cDNA's were performed by an endogenous RNA-dependent DNA polymerase reaction as described elsewhere (17, 25). Mo-MSV 124-specific, td Mo-MSV-specific, and Mo-MSV 3-specific cDNA's were prepared by hybridizing cDNA of the respective virus complexes with a 30-fold excess of heat-denatured 70S Mo-MuLV RNA. Typically, 1 μg of cDNA was hybridized to 30 μg of Mo-MuLV RNA in a reaction volume of 10 μl for 24 h. The hybridization mixture was used directly as a specific cDNA (15, 16). Identification of T_1-oligonucleotides in RNA-cDNA hybrids has been described in detail previously (15, 16).

Isolation of a nontransforming component from clone 3 Mo-MSV. Our stock of clone 3 Mo-MSV contains two major size classes of RNA components at a molar ratio of 1:5, the 8.5-kb component of Mo-MuLV and a 4.8-kb component thought to be the RNA of the replication-defective Mo-MSV (22).

Heteroduplex analysis carried out with Mo-MuLV cDNA and RNA from clone 3 Mo-MSV

has indicated the presence of a 4.8-kb RNA species that lacks a specific sequence (7). However, a 4.8-kb RNA species containing the intact Mo-MSV-specific region was detectable by heteroduplex analysis of intracellular RNA (D. Donoghue and R. Weinberg, personal communication). This result suggested that sequences representing the Mo-MSV-specific region were present in our stock of clone 3 Mo-MSV. Such sequences could be contained in a subspecies of 4.8-kb Mo-MSV RNA. In this case, the 4.8-kb RNA of clone 3 Mo-MSV may be heterogeneous, including species with, as well as species without, Mo-MSV-specific sequences. Alternatively, the Mo-MSV-specific sequences of clone 3 Mo-MSV may be contained in a less prevalent, as yet physically undetected RNA component. To identify the transforming genome and any nontransforming components which may be present in clone 3 Mo-MSV, we employed the following strategy. FRE cells were infected with clone 3 Mo-MSV at low multiplicities of infection (MOIs) with respect to the transformation titer, and clonal cell sublines were obtained as described above. Each subline was tested for MuLV production by syncytium formation on XC cells and by release of virions containing ^{3}H-labeled 50 to 70S RNA. Morphological transformation of our FRE cells in colonies producing both Mo-MuLV and Mo-MSV is difficult to discern because of extensive syncytium formation in colonies producing MuLV. Thus, transformation of NIH 3T3 cells by progeny virus from clonal cell sublines was the criterion used to define productive Mo-MSV infection. High-molecular-weight virion RNAs were evaluated for 8.5-kb helper RNA and smaller RNA monomers typical of defective retroviruses by polyacrylamide gel electrophoresis after denaturation of 50 to 70S RNA (12). Sublines negative for virus production were infected with Fr-MuLV at an MOI of approximately 0.2 and evaluated after three to four transfers for the release of MSV activity and for virions containing RNA monomers smaller than the 8.5-kb Fr-MuLV RNA monomer.

Table 1 shows the screening results of clonal sublines obtained from FRE cells infected with clone 3 Mo-MSV at an MOI of 0.3 focus-forming unit per cell. Approximately two thirds (9 of 15) of the sublines were positive for MuLV release. Of these MuLV-producing sublines, three were also positive for the release of MSV; however, 4.8-kb RNA monomers were detectable in only two of the three MSV-producing clonal cell lines. This result presumably reflects the large degree of variation observed in the ratios of defective to helper virus components in individual virus pseudotypes released by infected cells (10, 12). Two clonal sublines, no. 4 and 5 (Table 1), were positive for MuLV production and negative for the release of MSV activity, but virions released by these lines contained 4.8-kb RNA monomers in addition to 8.5-kb helper MuLV RNA monomers. Six clonal sublines (no. 10 to 15) failed to release virus particles; however, upon superinfection with MuLV, virus was rescued from two of these lines, no. 10 and 11 (Table 1), that contained 4.8-kb RNA monomers. One of these, no. 10 (Table 1), was positive for MSV activity. These results indicate that at least two species of 4.8-kb RNA monomers exist in our clone 3 Mo-MSV stock, one which is associated with MSV activity and one which is nontransforming. At a further dilution of the clone 3 Mo-MSV stock (MOI, ≅0.1), 2 of 15 sublines were positive for MuLV and negative for both MSV and 4.8-kb RNA monomers (data not shown). Of the 13 sublines which failed to release virions, 1 was positive for the release of MSV activity upon superinfection with Fr-MuLV, and a 4.8-kb RNA monomer was rescued. In another subline, a 4.8-kb RNA was rescued by superinfection but failed to transform fibroblasts. Since only 1 of 15 clonal sublines obtained after infection at an MOI of 0.1 was found to contain MSV, it is quite likely that this subline was infected with a single virus particle

TABLE 1. Biological activity and RNA monomers released from clonal cell sublines after infection with clone 3 Mo-MSV[a]

Clone no.	MuLV[b]	MSV[c]	30S RNA[d]
1	+	+	+
2	+	+	+
3	+	+	−
4	+	−	+
5	+	−	+
6	+	−	−
7	+	−	−
8	+	−	−
9	+	−	−
10	−	+[e]	+[e]
11	−	−	+[e]
12	−	−	−
13	−	−	−
14	−	−	−
15	−	−	−

[a] Infection of FRE cells at an MOI of ~0.3 MSV focus-forming unit per cell.

[b] Sublines positive for MuLV in the XC assay and releasing virions containing 50 to 70S RNA monomers.

[c] Sublines releasing MSV activity into the medium.

[d] Sublines were tested for the release of virions containing 30S RNA subunits by polyacrylamide gel electrophoresis.

[e] Sublines negative for MuLV production were tested for the release of MSV activity and virions containing 30S RNA monomers after superinfection by Fr-MuLV.

corresponding to the transforming component of clone 3 Mo-MSV. We have designated this 4.8-kb RNA component Mo-MSV 3 to distinguish it from the nontransforming 4.8-kb RNA species of clone 3 Mo-MSV. Likewise, since only 1 of 15 sublines was found to contain a 4.8-kb RNA in the absence of MSV activity, it is likely that this subline was infected with a single virus particle containing a replication-defective, nontransforming component of clone 3 Mo-MSV.

Defining the genetic structure of td Mo-MSV by comparisons with Mo-MSV strains and Mo-MuLV. (i) Experimental plan. The identification of a replication-defective, nontransforming component in clone 3 Mo-MSV does not establish the existence of a td Mo-MSV. Such a component may correspond to a deletion mutant of MuLV or to another defective, retrovirus-like RNA such as the endogenous rat or mouse 30S components detected in some virus stocks and cell lines (13, 18). Since a td Mo-MSV would contain no biological markers (i.e., defective in *gag*, *pol*, *env*, and *onc*), it is necessary to rely on structural markers for its identification. Our approach to the structural analysis of td Mo-MSV RNA has been first to identify and compare with each other the large T_1-oligonucleotides of the RNAs of td Mo-MSV, Mo-MSV 3 and 124, and MuLV and then to determine, by fingerprinting RNA from RNase T_1-resistant RNA-cDNA hybrids, oligonucleotides representing specific and common sequences (15). Subsequently, genetic structures of viral RNAs have been deduced by mapping T_1-oligonucleotides of each viral RNA relative to the 3′-poly(A) coordinate (29).

(ii) T_1-oligonucleotides of Mo-MuLV RNA sequences related and unrelated to MSV RNA. Fifty-eight T_1-oligonucleotides of Mo-MuLV were identified, and their compositions in terms of RNase A-resistant fragments are given in Table 2. The compositions of some T_1-oligonucleotides which comigrated with others as single chromatographic spots when total RNA was analyzed were identified in fingerprints of RNA fragments prepared to map T_1-oligonucleotides (e.g., T_1-oligonucleotide 16 [see Fig. 1A]) or in the less complex fingerprint patterns of RNA-cDNA hybrids.

For identification of Mo-MuLV T_1-oligonucleotides which have Mo-MSV 124 homologs, ^{32}P-labeled Mo-MuLV RNA was hybridized to Mo-MSV 124 cDNA and the unhybridized RNA was digested with RNase T_1. The hybrid was separated from the digested RNA and denatured, and the RNA released from the hybrid was fingerprinted. Since the ratio of the replication-defective MSV component to the helper Mo-MuLV component is high (>30:1) in our Mo-MSV 124 stock, Mo-MuLV cDNA present in Mo-MSV 124 cDNA is negligible under our conditions of hybridization (cDNA/RNA ratio, <10) (9). Thus, Mo-MSV 124 cDNA synthesized in the endogenous reaction was used directly as a source of Mo-MSV 124 cDNA without further fractionation. Twenty-five Mo-MuLV T_1-oligonucleotides related to Mo-MSV 124 were identified, 22 of which were mapped on the Mo-MuLV RNA and are shown in Fig. 1. These included 15 T_1-oligonucleotides identically shared with Mo-MSV 124 and 10 T_1-oligonucleotides which have related, but not identical, counterparts in Mo-MuLV 124. The latter group likely represents Mo-MuLV sequences which have diverged slightly from allelic sequences in Mo-MSV 124.

(iii) T_1-oligonucleotides of Mo-MSV 124-specific sequences. Twenty-eight large, Mo-MSV 124 T_1-oligonucleotides were resolved in this analysis and were further characterized by RNase A digestion (Table 2). Approximately one half (15 of 28) of the Mo-MSV 124 T_1-oligonucleotides were identical to Mo-MuLV T_1-oligonucleotides and were numbered as their Mo-MuLV counterparts (Table 2). For identification of Mo-MSV 124 T_1-oligonucleotides which were not identically shared with Mo-MuLV (no. 101 to 113) but were closely related to Mo-MuLV sequences, ^{32}P-labeled Mo-MSV 124 RNA was hybridized to Mo-MuLV cDNA and the T_1-oligonucleotides contained in hybridized RNA sequences were determined. Of the 28 Mo-MSV 124 T_1-oligonucleotides, 22 were found to be closely related to Mo-MuLV RNA sequences. These included the 15 T_1-oligonucleotides shared with MuLV and 7 of the 13 Mo-MuLV RNA. Thus, the remaining six Mo-MSV 124-specific T_1-oligonucleotides represented sequences unrelated to Mo-MuLV (Fig. 1).

For direct identification of Mo-MSV 124-specific T_1-oligonucleotides, T_1-oligonucleotides present in a hybrid of ^{32}P-labeled Mo-MSV 124 RNA with Mo-MSV 124-specific cDNA were analyzed. In this experiment, Mo-MSV 124 cDNA was first hybridized with an excess of Mo-MuLV RNA and then annealed to ^{32}P-labeled Mo-MSV 124 RNA. Unhybridized RNA was digested with RNase T_1, and the RNA-cDNA hybrid was isolated and then fingerprinted. Seven Mo-MSV 124 T_1-oligonucleotides (no. 102, 103, 105, 107, 108, 109, and 113) were recovered. These included the six Mo-MSV 124-specific T_1-oligonucleotides identified above as unrelated to Mo-MuLV and one T_1-oligonucleotide (no. 107) which was found above to be Mo-MuLV related (Fig. 1). T_1-oligonucleotide 107, which mapped near the 3′ terminus (Fig. 1), likely resides in the terminally redundant sequences of Mo-MSV 124. T_1-oligonucleotide 107 was also present in Mo-MSV 3 and td Mo-MSV

TABLE 2. T_1-oligonucleotides of Mo-MuLV, Mo-MSV 124, Mo-MSV, and td Mo-MSV

Oligonucleotide no.	RNase A digestion products	Oligonucleotide no.	RNase A digestion products
1[a]	8U, 10C, G, AU, A_2U	41[a]	2C, AU, A_3G, A_5N
2[a]	6U, 12C, G, 3AC, A_2C	42[a]	U, 2C, AC, AU, AG, A_4N
3[a,b,c,d]	6U, 3C, G, A_2C, A_2U, A_4N	43[a]	6U, 5C, AU, A_2G
4[a]	4U, 6C, A_2G, $2A_4N$	44[a]	7U, 6C, G, AC, AU
5[a]	3U, 6C, 3AC, AU, AG, A_4N	45[a]	5U, 3C, AC, AU, AG
6[a]	7U, 11C, G, A_2U	46[a]	6U, C, AU, A_4N
7[a]	3U, 4C, AG, A_2C, A_2U, A_5N	47[a]	U, 5C, AC, A_2C, A_2G
8[a]	6C, 10C, G, AC, $2A_2C$	48[a,c,d]	2U, 2C, AG, A_2C, A_4N
9[a]	U, 5C, G, AC, 2AU, A_2U, A_4N	49[a,b,c,d]	C, AC, A_2C, A_2U, A_2G
10[a]	5C, 6C, G, 3AC, AU, A_2C	50[a,c]	AU, $2A_4N$
11[a]	2U, 3C, G, AC, A_2C, A_4N	51[a]	6U, 3C, 2AU, AG
12[a]	3U, 4C, G, AC, A_2C, A_2U, A_2G	52[a]	2C, AC, A_2G, A_4N
13[a]	U, 3C, G, AC, 2AU, A_2C, A_3N	53[a,b,d]	3C, AC, $2A_2C$, A_4N
14[a]	2U, 10C, 3AC, 2AU, AG	54[a]	4U, 2AC, AU, AG
15[a,b,c,d]	5U, C, G, AC, $2A_2C$, A_3N	55[a]	7U, 3C, G, AC, 2AU
16[a]	4U, 5C, G, AU, A_2U, A_3C	56[a,b,c,d]	U, G, A_2C, A_6N
17[a,b,c,d]	2U, 5C, A_2G, $2A_3C$	57[a]	U, 4C, G, AC, A_2C
18[a,b,c,d]	U, 4C, AC, AU, AG, A_2C, A_2U	58[a,b,d]	5U, 2C, G, 2AC, A_2C
19[a]	4U, 7C, 3AC, AU, AG	101[b]	6U, 11C, AU, AG, A_2U
20[a]	4U, 5C, G, AC, A_2C	102[b,e]	6U, 5C, 2AC, A_3G
21[a,b,c,d]	2U, 8C, G, 2AC, A_2C	103[b,c,e]	8U, 7C, AU, AG, A_2U
22[a]	5U, 4C, 2AU, AG, A_3C	104[b]	6U, 11C, G, AU
23[a]	3U, 8C, AC, 2AU, AG	105[b,c,e]	3U, 10C, 2AC, 2AU, AG
24[a,c,d]	5U, 11C, G, AU	106[b]	7U, 7C, G, AC
25[a,c,d]	4U, 7C, 2–3AU, AG	107[b,c,d]	2U, 5C, AU, A_2U, A_4N
26[a]	U, 9C, 2AC, AG, A_3C	108[b,c,e]	6U, 2C, G, 2AC, AU
27[a]	2U, 7C, G, 2AC, 2AU	109[b,c,d,e]	4U, 7C, G, A_3C
28[a]	4U, 7C, G, 2AC, A_7N	110[b]	3U, C, $2A_6N$
29[a]	5U, 8C, AC, AG	111[b]	4U, 7C, AG, A_5N
30[a]	3U, 4C, G, AC, 2AU, A_2U	112[b,c,d]	4U, 4C, G, A_2C
31[a,c]	2U, 2C, G, AC, 4AU	113[b,c,e]	U, 3C, G, AC, 3AU, A_2U
32[a,b,d]	3U, 2C, G, AU, A_2U, A_3C	201[c,e]	10U, 5C, G, 2AC, AU, $2A_4N$
33[a,b,c,d]	5U, 4C, 2AC, A_2G	202[c]	4U, 7C, AC, AG
34[a]	2U, 7C, AC, AG, A_2C	203[c]	U, 13C, AG, 2–3AU, G
35[a,b,d]	U, 5C, G, A_2C, A_3C	204[c]	4U, 2C, AU, A_2U, A_2G
36[a,b,c,d]	2U, G, AC, AU, A_2C, A_6N	205[c]	2U, 4C, AG, AU, A_2U
37[a,b,d]	2U, 8C, AC, 2AU, AG	206[c]	C, A_2G, A_6N
38[a,b,c,d]	5U, 7C, AC, AG	207[c]	4U, 6C, G, 2AC, AU, A_2C
39[a]	3U, 7C, 2AC, AU, AG, A_6N	301[d,e]	8U, 4C, AC, AU, AG
40	2U, C, AU, A_2G, A_5N	302[d,e]	4U, 2C, G, 2AC, AU

[a] T_1-oligonucleotides contained in Mo-MuLV RNA.
[b] T_1-oligonucleotides contained in Mo-MSV 124 RNA.
[c] T_1-oligonucleotides contained in Mo-MSV 3 RNA.
[d] T_1-oligonucleotides contained in td Mo-MSV RNA.
[e] Mo-MSV-specific oligonucleotides defined as unrelated to Mo-MuLV and related to Mo-MSV 124.

and was retained in RNA-cDNA hybrids formed with specific cDNA's of these isolates (Fig. 1).

From these analyses we conclude that 6 of the 28 T_1-oligonucleotides of Mo-MSV 124 represent specific sequences, defined as being unrelated to Mo-MuLV sequences. These sequences are closely related to sequences contained in the transforming component of clone 3 Mo-MSV (Mo-MSV 3) (see below). Of the remaining 22 oligonucleotides, 15 represent sequences identically shared with Mo-MuLV and 7 represent sequences which are distinct from Mo-MuLV but have nevertheless closely related Mo-MuLV sequence counterparts from which they probably differ through point mutations.

(iv) T_1-oligonucleotides of Mo-MSV 3-specific sequences. Of the 29 Mo-MSV 3 T_1-oligonucleotides, 17 were identically shared with Mo-MSV 124 (Table 2). These included 10 of the 15 T_1-oligonucleotides shared between Mo-MSV 124 and Mo-MuLV RNAs and 7 T_1-oligonucleotides found in Mo-MSV 124 RNA but not in Mo-MuLV RNA. Five additional Mo-MSV 3 T_1-oligonucleotides were shared with Mo-MuLV but were not present in Mo-MSV 124. The remaining seven Mo-MSV 3 T_1-oligonucleotides were not found in either Mo-MuLV or Mo-MSV 124 RNA and were designated by numbers of the 200 series (no. 201 to 207 [Table 2]).

Mo-MSV 3 RNA contained five of the six T_1-

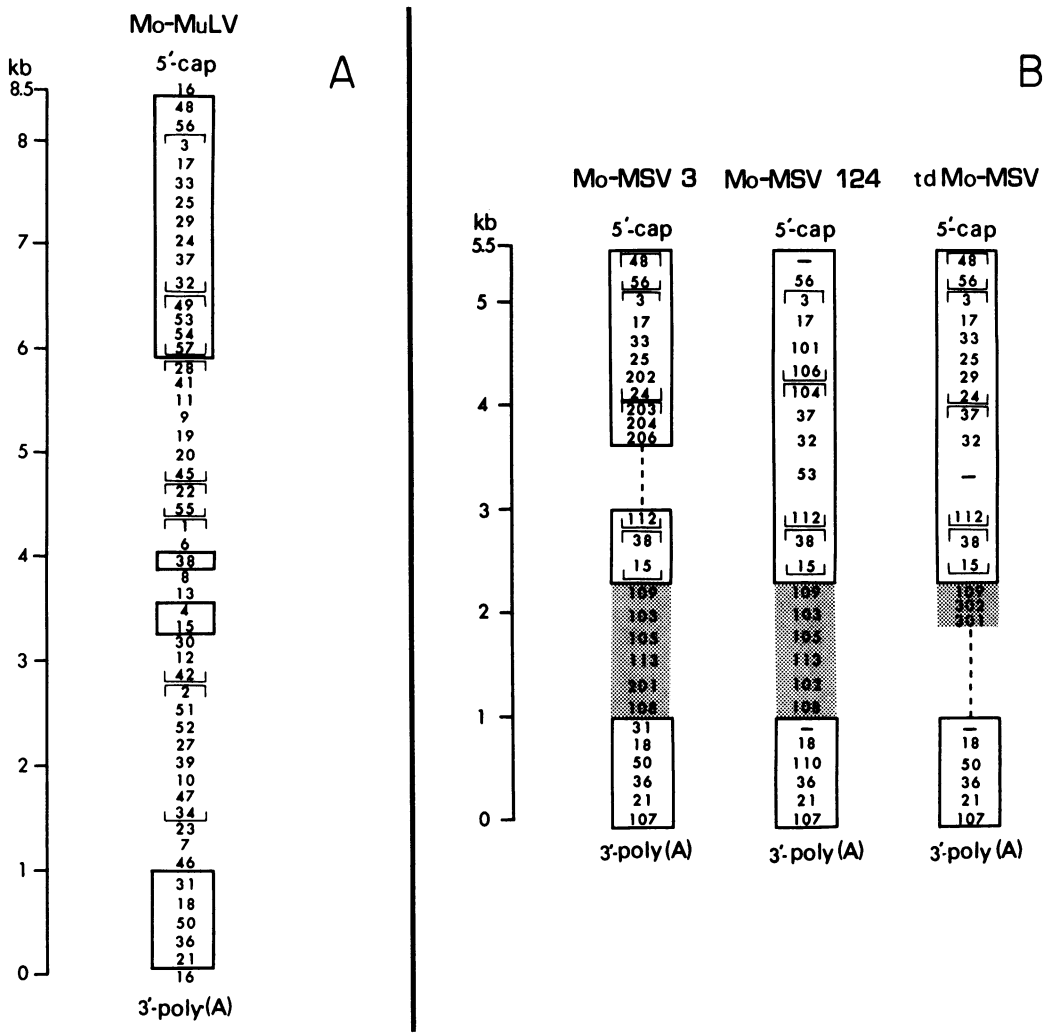

FIG. 1. T_1-oligonucleotide maps of Mo-MuLV, Mo-MSV 3, Mo-MSV 124, and td Mo-MSV RNAs. The large T_1-oligonucleotides of each RNA were located relative to the 3'-poly(A) end based on the size of the smallest poly(A)-containing RNA fragment from which they could be isolated (15, 29). (A) T_1-oligonucleotide map of Mo-MuLV RNA. T_1-oligonucleotides are numbered as in Table 2. Mo-MSV 124-related T_1-oligonucleotides obtained from Mo-MuLV DNA–Mo-MSV 124 RNA hybrids are enclosed in solid lines. (B) T_1-oligonucleotide maps of Mo-MSV 3, Mo-MSV 124, and td Mo-MSV. T_1-oligonucleotides are numbered as in Table 2. Mo-MuLV-related T_1-oligonucleotides obtained from RNA-DNA hybrids are enclosed in solid lines, and Mo-MSV-specific T_1-oligonucleotides are in shaded regions of the maps. Vertical dashed lines in the maps of Mo-MSV 3 and td Mo-MSV indicate RNA sequences which are deleted from these RNAs when compared with Mo-MSV 124. The relative order of T_1-oligonucleotides within the vertical brackets on the maps is uncertain.

oligonucleotides identified in Mo-MSV 124 as Mo-MuLV unrelated or MSV specific, thus indicating a close relationship between the specific sequences of the two Mo-MSV isolates. To determine whether Mo-MSV 3 contains additional T_1-oligonucleotides unrelated to Mo-MuLV, we analyzed a hybrid between ^{32}P-labeled Mo-MSV 3 RNA and Mo-MuLV cDNA. Included in this fingerprint were the 15 Mo-MSV 3 T_1-oligonucleotides shared with Mo-MuLV and 8 additional T_1-oligonucleotides which were related but not identical to Mo-MuLV sequences. Six Mo-MSV 3-specific T_1-oligonucleotides were missing from this fingerprint and therefore are unrelated to Mo-MuLV. These included the five MSV-specific T_1-oligonucleotides shared with Mo-MSV 124 and one T_1-oligonucleotide (no. 201) not shared with Mo-MSV 124 (Fig. 1).

As indicated earlier, all Mo-MSV 124-specific T_1-oligonucleotides were found to be related to Mo-MSV 3 sequences. To determine whether

the converse is true or whether T_1-oligonucleotide 201 represents an additional Mo-MuLV-unrelated sequence of Mo-MSV 3 not found in Mo-MSV 124, we analyzed a hybrid of Mo-MSV 3 RNA and Mo-MSV 124-specific cDNA. It contained all Mo-MSV 3-specific T_1-oligonucleotides defined as unrelated to Mo-MuLV, including no. 201. Thus, in terms of large T_1-oligonucleotides, the specific sequences of Mo-MSV 3 and Mo-MSV 124 are allelic (Fig. 1).

Mo-MSV 124 and Mo-MSV 3 each contain several T_1-oligonucleotides shared with Mo-MuLV but not with each other. In addition, each Mo-MSV RNA contains several T_1-oligonucleotides which are unique with respect to each other and to Mo-MuLV RNA, but are Mo-MuLV related (see below; Fig. 1). These T_1-oligonucleotides may represent different sets of Mo-MuLV sequences in the two Mo-MSV RNAs or may represent allelic, but nonidentical, sequences of RNAs. To determine whether Mo-MSV 3 contains Mo-MuLV-related sequences not present in Mo-MSV 124, Mo-MSV 3 RNA was hybridized to unfractionated Mo-MSV 124 cDNA, and the T_1-oligonucleotides retained in the hybrid were analyzed. All Mo-MSV 3-specific T_1-oligonucleotides were identified in this hybrid (data not shown), indicating that the smaller Mo-MSV 3 RNA is a subset of the larger Mo-MSV 124 RNA and that all Mo-MSV 3 sequences have allelic counterparts in Mo-MSV 124.

(v) MSV-specific and MuLV-related sequences of td Mo-MSV. A T_1-oligonucleotide fingerprint of 4.8-kb td Mo-MSV RNA from 8.5-kb Mo-MuLV RNA (15, 16) resolved 25 large T_1-oligonucleotides (Fig. 1). Only two of these T_1-oligonucleotides (no. 301 and 302) were unique to this RNA. The remainder were shared with one or more of the RNA components of Mo-MuLV, Mo-MSV 124, and Mo-MSV 3 examined above.

Only one T_1-oligonucleotide (no. 109) of td Mo-MSV corresponded to a Mo-MSV-specific T_1-oligonucleotide defined above (Table 2). To determine whether td Mo-MSV 3 contained additional Mo-MSV-specific sequences, we analyzed Mo-MSV 3 and 124 RNA–td Mo-MSV 3 cDNA hybrids for Mo-MSV-specific oligonucleotides. A hybrid of ^{32}P-labeled Mo-MSV 124 RNA with td Mo-MSV-specific cDNA contained two (i.e., no. 103 and 109) of the six Mo-MSV 124-specific T_1-oligonucleotides, indicating that only a portion of the Mo-MSV 124-specific sequences is contained in td Mo-MSV (Fig. 1).

An experiment in which Mo-MSV 3 RNA was hybridized to td Mo-MSV-specific cDNA rendered specific by prehybridization with Mo-MuLV RNA identified two Mo-MSV 3-specific T_1-oligonucleotides (no. 103 and 109). From these results and from the extent of quantitative hybridization of Mo-MSV 124 or Mo-MSV 3 RNA to td Mo-MSV-specific cDNA (about 5%) compared with Mo-MSV 124-specific cDNA (20 to 25%) (data not shown), we estimate that td Mo-MSV is lacking 70 to 80% of the Mo-MSV-specific sequences.

Figure 1 shows in boxes most of the T_1-oligonucleotides of td Mo-MSV RNA present in an RNase T_1-resistant hybrid formed with Mo-MuLV cDNA. Of 25 td Mo-MSV T_1-oligonucleotides, 22 were recovered. Thus, 22 of 25 td Mo-MSV-specific T_1-oligonucleotides were from sequences related to Mo-MuLV, and 3 were from Mo-MuLV-unrelated sequences (no. 109, 301, and 302). These T_1-oligonucleotides were shown to be related to MSV-specific sequences by their identification in a hybrid formed with Mo-MSV 124-specific cDNA (shaded regions in Fig. 1).

Further experiments showed that all td Mo-MSV-specific T_1-oligonucleotides are retained in hybrids of td Mo-MSV RNA with Mo-MSV 124 cDNA (data not shown). Thus, td Mo-MSV (like Mo-MSV 3) is comprised entirely of sequences closely related to Mo-MSV 124.

From these analyses we conclude that td Mo-MSV indeed corresponds to a td Mo-MSV rather than to a defective Mo-MuLV. This distinction rests primarily on the finding that some, but not all, of the Mo-MSV-specific sequences were contained in td Mo-MSV and that td Mo-MSV was a subset of Mo-MSV 124-related RNA sequences.

Further, we conclude that the residual MSV-specific sequences of td MSV are allelic but not identical to the MSV-specific sequences of Mo-MSV 3 and 124, because the td MSV cDNA hybridized oligonucleotides 103 and 109 from both MSVs although td MSV did not contain oligonucleotide 103 (Fig. 1). Conversely, MSV cDNA hybridized td Mo-MSV oligonucleotides 109, 301, and 302 as representatives of the residual MSV-specific sequence of td Mo-MSV although MSV did not contain oligonucleotides 301 and 302 (Fig. 1).

(vi) Comparison of T_1-oligonucleotide maps of Mo-MuLV, Mo-MSV 124, Mo-MSV 3, and td Mo-MSV. For location of specific and common regions in the RNA genomes of the respective viruses, most of their T_1-oligonucleotides were mapped relative to the 3′-poly(A) coordinate of viral RNA (29) (Fig. 1). RNA sequences of Mo-MuLV related to Mo-MSV 124 are enclosed in solid lines and defined large contiguous regions near the 3′ and 5′ ends of Mo-MuLV RNA. At the 3′ end, five T_1-oligonucleotides define a region extending from approximately 0.1 to 1.2 kb on the Mo-MuLV map. The 3′-terminal T_1-

oligonucleotide (no. 16), which has been shown to be a terminally redundant T_1-oligonucleotide of Mo-MuLV (8), was not retained in Mo-MSV 124 cDNA hybrids under our hybridization conditions. Near the 5′ end, a region defined by 14 large Mo-MSV 124-related T_1-oligonucleotides extends from approximately 6.0 to 8.5 kb on the Mo-MuLV map. Three additional T_1-oligonucleotides related to Mo-MSV 124 are located near the middle of the Mo-MuLV T_1-oligonucleotide map. Heteroduplex analyses of Mo-MSV 124 and Mo-MuLV (1, 19) have indicated that two small internal sequences of Mo-MuLV are homologous to Mo-MSV 124; however, because of uncertainties in the exact map positions of these three T_1-oligonucleotides (no. 4, 15, and 38), it is unclear whether they represent a contiguous sequence of Mo-MuLV RNA or whether they represent two noncontiguous segments as shown in Fig. 1A.

The T_1-oligonucleotide maps of Mo-MSV 3, Mo-MSV 124, and td Mo-MSV are shown in Fig. 1B. Mo-MuLV-related regions are enclosed in a solid line and comprise two large contiguous regions which flank the specific T_1-oligonucleotides, indicated by shaded regions in each T_1-oligonucleotide map. Shared T_1-oligonucleotides in the maps of the three isolates have been juxtaposed, revealing the deleted Mo-MSV-specific sequences of td Mo-MSV (vertical dashed line) and the probable location of a deletion of Mo-MuLV-related sequences from Mo-MSV 3 when compared with Mo-MSV 124 or td Mo-MSV. td Mo-MSV lacked the four 3′-proximal T_1-oligonucleotides of the specific sequences of Mo-MSV 3 and Mo-MSV 124. In other respects, td Mo-MSV and Mo-MSV 124 were quite similar, probably differing only by minor base changes. The deletion indicated in the Mo-MSV 3 map (vertical dashed line) is postulated to account for the smaller size of Mo-MSV 3 compared with Mo-MSV 124. Hence, this region is apparently not essential for transformation and virus replication, a conclusion which confirms the appearance of spontaneous deletions not affecting replicative or transforming functions in this part of the genome noted by others (5). Its location, which must be considered tentative, was based on the close correspondence of T_1-oligonucleotides of Mo-MSV 3 with homologous regions of Mo-MSV 124. From these analyses we conclude that Mo-MSV 124 and Mo-MSV 3 have similar structures. Each contains a nearly identical Mo-MuLV-unrelated sequence flanked by Mo-MuLV-related sequences. Similar size heterogeneities among Mo-MSV strains have been described and for the most part reflect a variation in the number of Mo-MuLV-related sequences in different isolates (11, 21).

The genetic structure of td Mo-MSV was essentially congenic with Mo-MSV 124 in the helper virus-related terminal sequences. However, td Mo-MSV lacked a substantial portion of the Mo-MSV-specific region. Moreover, td Mo-MSV contained some helper virus-related sequences not present in Mo-MSV 3. Thus, it cannot be considered a direct deletion mutant of Mo-MSV 3. These comparisons of td Mo-MSV with wild-type Mo-MSV directly define *onc* gene-specific elements of Mo-MSV by deletion analysis in a manner similar to the analyses which defined the *src* gene of avian sarcoma viruses (20, 29). td Mo-MSV is the first example of an *onc* gene deletion mutant of any transforming retrovirus other than avian sarcoma viruses.

In addition, the T_1-oligonucleotide analyses presented here reveal heterogeneity in homologous regions of MuLV-related and MSV-specific regions of Mo-MSV 3, Mo-MSV 124, td Mo-MSV, and Mo-MuLV at the sequence level. Thus, although Mo-MSV 3 and td Mo-MSV are subsets of Mo-MSV 124 in terms of homologous sequences, several base differences resulting in altered T_1-oligonucleotides exist among the isolates. Likewise, Mo-MSV 124 sequences differ from homologous sequences of Mo-MuLV in several T_1-oligonucleotides. Only two Mo-MuLV-related T_1-oligonucleotides (no. 112 and 107), which have no identical counterparts in Mo-MuLV RNA, are found in all three of the replication-defective genomes. (i) T_1-oligonucleotide no. 112, which maps near the 5′ end of the MSV-specific sequence, is present in Mo-MSV 124, Mo-MSV 3, and td Mo-MSV but not in Mo-MuLV. Although the significance of this difference between Mo-MuLV and Mo-MSV RNAs is unclear, it is of interest that transformation by subgenomic fragments of Mo-MSV-specific DNA has not been demonstrated in the absence of a short Mo-MuLV-related segment flanking the 5′ side of the specific sequence (28). (ii) A second Mo-MuLV-related T_1-oligonucleotide which is common to all three replication-defective isolates but absent from Mo-MuLV is no. 107. This T_1-oligonucleotide maps nearest the 3′ terminus of all three isolates and likely resides in their terminal redundancies. Since it is hybridized by Mo-MuLV cDNA, it represents a distinct, MSV-specific marker of an allelic element shared by MSV and MuLV. Moreover, the terminally redundant T_1-oligonucleotide of Mo-MuLV, no. 16 (8), was not hybridized by cDNA of Mo-MSV 124 under our conditions of hybridization. Hence, this may signal the presence of nonallelic elements that set apart the terminal sequences of MSVs and MuLV. It is likely that these oligonucleotides belong to the terminally redundant sequences of proviral DNA termed LTR, which are thought to have a promoter function (4, 28). These observations are compat-

ible with a minor nonhomology detected previously between Mo-MuLV and Mo-MSV 124 near the 5′ terminus by heteroduplex analyses (7) and more recently by complete sequence analyses of proviral DNAs (24a). Since the terminal sequence of Mo-MSV has been shown to be essential for efficient transformation of 3T3 mouse cells by DNA (4, 28) and since our results indicate that the terminal sequences of Mo-MSV and Mo-MuLV are not identical, it would be of interest to examine the efficiency of transformation by viral Mo-MSV-specific DNA and its cellular homolog coupled with Mo-MuLV terminal sequences.

The availability of a td Mo-MSV capable of replication in the presence of helper virus should allow by comparison with Mo-MSV a direct identification of transformation-specific mRNA and protein.

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