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Base Sequence Differences Between the RNA Components of Harvey Sarcoma Virus

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The 50 to 70*S* RNA of the Harvey sarcoma-Moloney leukemia virus (MLV) complex consists of 30 to 40*S* RNA subunits of two different size classes and contains sequences homologous to Moloney mouse leukemia virus and to information contained in a C-type rat virus, termed NRK virus. We have isolated by preparative gel electrophoresis the large (component 1) and the small (component 2) 30 to 40*S* RNA species from the Harvey sarcoma-MLV complex. Harvey RNA component 1 was completely complementary to DNA transcribed from MLV RNA and showed no homology to DNA transcribed from NRK virus when annealed under conditions of DNA excess. Harvey RNA component 2 was about 65% complementary to MLV DNA and about 33% complementary to NRK virus DNA. Approximately 60 to 80% of the MLV-specific sequences in RNA component 2 is either a distinct molecular species or is part of a hydrid molecule including NRK virus- and MLV-specific sequences. The rest of the MLV sequences in component 2 could be accounted for by degraded component 1 co-purifying with component 2. The possible role of these sequences in the ability of the virus to transform cells is discussed.

The Harvey murine sarcoma virus (H-MSV) was first isolated from rats inoculated with Moloney mouse leukemia virus (M-MLV) (6). Like other murine sarcoma viruses, H-MSV is replication defective, replicating only in the presence of helper leukemia virus (5, 7, 12). Sarcoma virus preparations therefore consist of a mixture of leukemia virus particles and defective sarcoma virus in the envelope of leukemia virus, termed pseudotypes (12) and abbreviated H-MSV(MLV).

The 30 to 40*S* RNA species of H-MSV consists of two electrophoretically distinct components. The larger one, termed here component 1, has the same electrophoretic mobility as that of the helper M-MLV. The smaller one, termed here component 2, is thought to be specific for the defective sarcoma virus (8). Further, the virus contains two distinguishable sets of nucleic acid sequences: one is homologous to the RNA of M-MLV; the other is homologous to RNA found in a C-type virus released spontaneously by a rat kidney cell line (NRK line) (9-11). The RNAs of M-MLV and of the rat virus, termed NRK virus, are unrelated (2, 4, 10, 11, 14). M-MLV contains only one 30 to 40*S* RNA component. By contrast, the NRK virus was found to contain two electrophoretically distinct 30 to 40*S* RNA components which have about the same size as those found in Harvey virus (8, 13).

It is the purpose of this paper to determine how the M-MLV-specific and NRK virus-specific sequences are distributed between the two RNA components of Harvey virus. The most complex possibility assumes that each RNA species of Harvey virus includes NRK virus-specific and MLV-specific components. The simplest possibility assumes that Harvey RNA component 1 consists of MLV RNA and that component 2 consists of the small RNA component of NRK virus.

We have used hybridization of MLV and NRK virus DNA with electrophoretically purified RNA components of Harvey virus to distinguish between such alternatives. It was found that Harvey RNA component 1 consists only of MLV-specific sequences. Harvey RNA component 2 contains 65% MLV- and 35% NRK virus-specific sequences. It appears that 60 to 80% of the MLV-specific sequences in RNA component 2 is either a distinct molecular species or is part of a hybrid molecule including NRK virus- and MLV-specific sequences. The rest of the MLV-specific sequences in RNA component 2 can be accounted for by degraded component 1 co-purifying with component 2.

The following methodology was used. H-

MSV(MLV) was propagated in a chronically infected line of NIH-3T3 mouse cells as described (10, 11). The NRK cell line spontaneously producing NRK virus and the NIH-3T3 mouse cell line chronically infected with MLV have been described (9, 10).

Preparation of viral [^{3}H]RNA components followed procedures described earlier (8). In short, infected cells were labeled with 200 μCi of [^{3}H]uridine (30 to 40 Ci/mmol, New England Nuclear Corp.) per 6 ml of medium in a 10-cm petri dish. Virus was harvested at 4- to 6-h intervals and purified as described (8). The 50 to 70*S* RNA was prepared by sucrose gradient sedimentation. After ethanol precipitation, the 50 to 70*S* RNA was heated (100 C, 45 s at low ionic strength) and subjected to electrophoresis in 2% polyacrylamide gels (8). After electrophoresis the gel was sliced into 1-mm sections, and the RNA of each section was eluted by mildly shaking each slice for 24 h at room temperature in 1.5 ml of buffer containing 0.4 M LiCl, 0.01 M Tris (pH 7.4), 1 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS). In later experiments the gel slices were crushed by squeezing them through a 26-gauge syringe needle prior to elution. Radioactivity in each slice was determined from an aliquot of the eluate in toluene-based scintillation fluid containing 10% Nuclear-Chicago solvent. The eluate of appropriate fractions of each RNA component was pooled, centrifuged for 5 min at 30,000 $\times$ *g* to pellet contaminating polyacrylamide particles, and precipitated by addition of 2 volumes of 95% ethanol. The precipitate containing RNA and water-soluble polyacrylamide was then re-dissolved in 0.4 ml of standard buffer (0.1 M NaCl, 0.01 M Tris, pH 7.4, 1 mM EDTA, 0.1% SDS) and sedimented in a 15 to 30% glycerol gradient in the same buffer to separate the viral [^{3}H]RNA from the soluble polyacrylamide. Gradient fractions containing viral [^{3}H]RNA were then ethanol precipitated in the presence of 12 μg of carrier tobacco mosaic virus RNA.

DNAs complementary to about 90% of the viral RNAs (cDNA) were made during 1-h endogenous reactions using detergent-disrupted purified viruses. The procedure is a modification of one described earlier (1). Reactions of 30 to 60 ml were incubated at 37 C and contained: 100 μg of actinomycin D per ml, 0.02 M Tris-hydrochloride (pH 7.8), 0.06 M KCl, 2 mM dithiothreitol, 0.02% Triton X-100, 6 mM magnesium acetate, and 5×10^{-4} M each of TTP, dATP, dGTP, and dCTP. Parallel reactions with [^{3}H]dCTP of known specific radioactivity were used to estimate the yield of DNA made. To prepare DNA, SDS was added to 1% to the reaction mixture, and the solution was extracted with an equal volume of a mixture consisting of 48% phenol, 48% $CHCl_3$, and 4% isoamyl alcohol. The aqueous phase was then dialyzed for 36 h against H_2O (3 $\times$ 2 *l*, changed at 12-h intervals), adjusted to 0.2 N NaOH and incubated for 60 min at 37 C. The solution was then re-dialyzed and lyophilized. DNA made in this fashion was about 80 to 90% complementary to 60 to 70*S* viral RNA and < 10% was double stranded (1, 9–11).

RNA-DNA hybridization was a modification of a procedure described earlier (1, 10). Hybridizations were at 66 C for 30 h in 0.1 ml containing 0.02 M Tris-hydrochloride (pH 7.2), 0.4 M NaCl, 0.5 mM EDTA, 0.1% SDS, 2 μg of calf thymus DNA, 50 μg of yeast RNA, 100 ng of viral DNA, and 1 to 10 ng of viral [^{3}H]RNA (approximate specific activity, 10^5 to 5×10^5 counts/min per μg). RNA·DNA hybrids were assayed with S1 nuclease (1). Background of 40 counts/min was subtracted from each sample.

Preparation of the two 30 to 40*S* RNA components of 50 to 70*S* H-MSV(MLV) RNA was as follows. The 50 to 70*S* RNA species of the Kirsten and Moloney sarcoma-leukemia virus complexes were shown to form a broader and more slowly sedimenting peak in sucrose gradients than the 60 to 70*S* RNA species of M-MLV, suggesting greater RNA size heterogeneity in the sarcoma virus preparation (3, 8, 14). A similar observation was made when the 50 to 70*S* RNA complex of Harvey sarcoma-leukemia virus was compared to that of M-MLV (Fig. 1). The basis for the heterogeneity of the sarcoma-leukemia virus RNAs has been shown to be the presence of two 30 to 40*S* subunit classes of different size in the 50 to 70*S* RNA species of these sarcoma-leukemia virus complexes (8). Given that the two different subunit species of the Harvey sarcoma-leukemia virus complex are present at roughly equimolar concentrations (reference 8 and Fig. 2) and that the virus complex also contains about equal concentrations of helper leukemia virus (assayed by the XC test [7a]; sarcoma virus assayed by focus formation [Edward Scolnick, unpublished data]), the viral RNA complex may have either one of the following two structures. The 50 to 70*S* RNA complex could consist of two subclasses each containing identical large or small subunits, or it could consist of one class containing different subunits associated within the same complex. The broad pattern shown in Fig. 1 is compatible with the view that the 50 to 70*S* RNAs of H-MSV includes complexes of only large and only small subunits, though the existence of some complexes of large and small

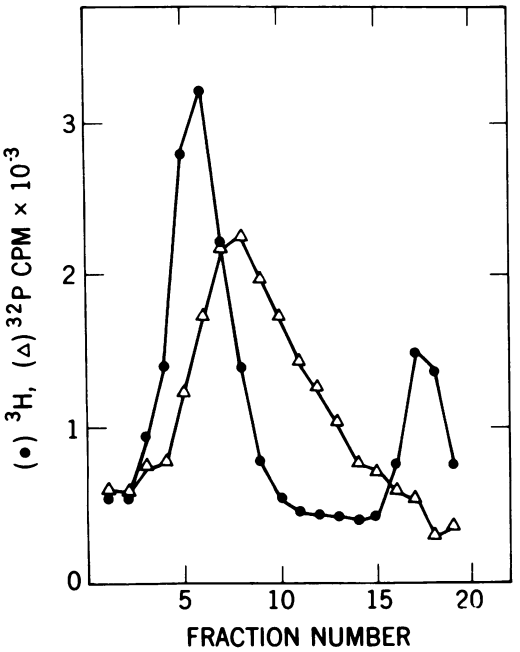

FIG. 1. *Sedimentation analyses of the 50 to 70S RNAs of H-MSV(MLV) and of M-MLV. Purification of the viruses and extraction of their RNAs are described in the text. Centrifugation of the RNAs was in a sucrose gradient containing 0.1 M NaCl, 0.01 M Tris (pH 7.4), 1 mM EDTA, and 0.1% SDS for 45 min at 65,000 rpm in a Spinco SW65 rotor at 20 C. Radioactivity of each fraction was determined in a Tri-Carb liquid scintillation counter from an aliquot of each fraction dissolved in toluene-based scintillation fluid containing 10% Nuclear-Chicago solvent.*

subunits cannot be excluded.

The two 30 to 40S RNA subunit species of Harvey virus were isolated by preparative electrophoresis of heat-dissociated 50 to 70S RNA (Fig. 2). After elution from the gel, each RNA component was freed of buffer-soluble polyacrylamide by glycerol gradient sedimentation (Fig. 2B, C). RNA component 1, which is thought to be at least in part the RNA of the helper MLV, is probably completely free of component 2 if prepared by this method. RNA component 2 is expected to include 20 to 40% partially degraded component 1 RNA, which migrates with component 2 RNA. The extent of contamination of RNA component 2 by degraded component 1 RNA can be roughly estimated. An upper limit of the amount of randomly degraded component 1 contaminating component 2 can be obtained by connecting the valley between the two RNA components with the background of degraded RNA migrating toward the cathode of the gel (dashed line, Fig. 2A). More than one-half of the radioactivity below this line in RNA component 2 is considered to be degraded component 1 RNA.

Given the presence of M-MLV- and NRK virus-specific sequences in the RNA of Harvey virus, the question was asked how these sequences are distributed between the two RNA components of H-MSV(MLV). To answer this question RNA components 1 and 2 were hybridized in separate experiments to saturating amounts of DNA (4a) complementary to M-MLV RNA and to DNA complementary to NRK virus RNA. All hybridizations were carried out at a 10- to 100-fold excess of the respective DNA over each viral RNA component (Table 1). It can be seen in Table 1 that Harvey RNA component 1 is 83 to 90% homologous to M-MLV DNA but only 2 to 3% homologous to NRK virus DNA if compared by this method. However, Harvey RNA component 2 is 65 to 67% homologous to MLV DNA and 31 to 35% homologous to NRK virus DNA.

Hybridization with saturating concentrations of DNAs complementary to M-MLV and NRK virus RNAs rendered 91% of RNA component 2 resistant to nuclease S1 (Table 1). Thus, at least 91% of the component 2 sequences can be accounted for by sequences present in M-MLV DNA and NRK virus DNA. The extent of homology between RNA component 2 and the mixture of MLV and NRK virus DNA may be even higher than 91%, because some (~10%) [^{3}H]-RNA is rendered soluble during the hybridization reaction. The exact extent of thermal degradation cannot be determined because a single-stranded RNA control is degraded faster than RNA·DNA hybrids formed in the presence of complementary DNA.

These results indicate that only Harvey component 2 RNA contains RNA sequences homologous to two different RNA tumor viruses, M-MLV and NRK virus. Harvey RNA component 1 appears to represent entirely M-MLV-specific sequences. Component 2, which is thought to be specific for the defective sarcoma virus (8), appears to contain 66% M-MLV-related and 33% NRK virus-related sequences. About 60 to 80% of the MLV-specific sequences in RNA component 2 are either a distinct molecular species of component 2 or part of a hybrid molecule including NRK virus- and MLV-specific sequences. Although our experiments cannot distinguish directly between these alternatives, the second alternative accounts more readily for the presence of two different sets of viral sequences in an electrophoretically homogenous component. The rest of the MLV-specific sequences in RNA compo-

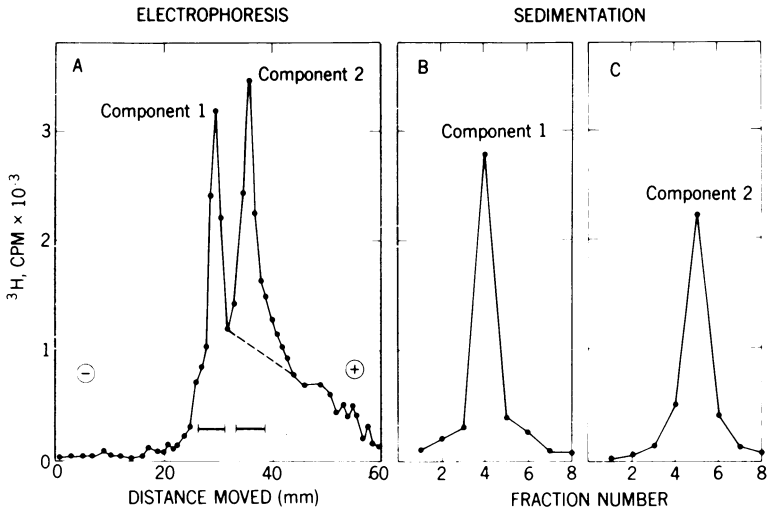

FIG. 2. *Preparation of the 30 to 40S RNA components 1 and 2 of H-MSV(MLV). (A) The 50 to 70S [^{3}H]RNA of H-MSV(MLV) was prepared as described for Fig. 1. After ethanol precipitation the [^{3}H]RNA was heated at 100 C for 45 s in 30 to 50 μl of 4 mM Tris, 2 mM sodium acetate (pH 7.2), 0.2% SDS, 10% glycerol and subjected to electrophoresis in 2% polyacrylamide (8). Elution of the RNA from gel sections and determination of its radioactivity are described in the text. The horizontal bars indicate the electrophoretic fractions pooled to prepare RNA components 1 and 2. After ethanol precipitation RNA components 1 (B) and 2 (C) were freed of soluble polyacrylamide by glycerol gradient (15 to 30%, vol/vol) sedimentation for 90 min and otherwise as described in Fig. 1.*

TABLE 1. *Hybridization results*[a]

Expt	Harvey RNA component	Input RNA[b]	RNA hybridized[b] to	
			M-MLV DNA (counts/min)	NRK virus DNA (counts/min)
1	1	325	299 (91%)	5 (<2%)
	2	334	210 (66%)	105 (31%)
2	1	360	297 (83%)	4 (<2%)
	2	256	162 (65%)	89 (35%)
3	1	1,200	1,085 (91%)	41 (3%)
	2	848	573 (67%)	228 (27%)
	2	310	202 (65%)	108 (35%)
4	2	1,101	1,010 (91%)[c]	

[a] Each hybridization was carried out at 66 C for 30 h and contained in 0.1 ml: 0.02 M Tris-hydrochloride (pH 7.2), 0.4 M NaCl, 5 × 10^{-4} M EDTA, 0.1% SDS, 2 μg of calf thymus DNA, 50 μg of yeast RNA, and 100 ng of cDNA probe (saturation).

[b] Numbers represent [^{3}H]radioactivity of RNA, precipitated by 5% trichloroacetic acid and determined on dried membrane filters (Millipore Corp.) in toluene-based scintillation fluid in a Tri-Carb spectrometer. RNA components of Harvey sarcoma virus had specific activities of approximately 10^5 to 5 × 10^5 counts/min per μg. About 1 to 10 ng of RNA was used per experiment. The reactions were assayed for hybridization with S1 nuclease. Background of 40 counts/min has been subtracted from each value. Levels of S1 nuclease-resistant counts per minute in the absence of cDNA were less than 10 counts/min above the background. The input radioactivities of [^{3}H]RNAs were checked for each experiment at zero time before incubating at 66 C.

[c] M-MLV plus NRK virus DNA.

nent 2 probably represents degraded component 1 comigrating with component 2. In addition, the presence of some sequences other than those from MLV and NRK virus in component 2 cannot be excluded because about 9% of the RNA did not hybridize to a mixture of MLV and NRK virus DNAs.

Our finding of MLV- and NRK virus-specific sequences in the sarcoma-specific component 2 of Harvey virus is consistent with the notion that sequences homologous to both viruses are present in Harvey virus-transformed nonproducer cells (11), though the size of the MLV-like and NRK virus-like RNA components in these

cells was not determined. Analogous studies on the two RNA components of Kirsten sarcoma virus agree with our results in that Kirsten RNA component 1 was said to be helper MLV RNA; however, Kirsten RNA component 2 was suggested to contain only NRK virus-specific sequences, which is at variance with our findings (13, 14).

Two hypotheses which are similar to those discussed earlier (8–11) could explain the origin of both NRK virus sequences and M-MLV RNA sequences in RNA component 2 of Harvey sarcoma virus. (i) RNA component 2 could have been generated by recombination of M-MLV, when it was first injected into rats, with a rat virus, such as the NRK virus. Since neither the NRK virus nor the M-MLV seems to transform rat or mouse cells, transforming ability of Harvey virus could be the result of a particular combination of NRK virus-specific and MLV-specific sequences, perhaps including some additional ($<10\%$) sequences of unknown origin in the same RNA component 2 of Harvey virus. (ii) Alternatively, M-MLV could have rescued from rat cells a defective sarcoma virus which shares sequences both with NRK virus and with M-MLV.

A definitive answer to the question of whether the MLV- and NRK virus-specific sequences of Harvey RNA component 2 are part of the same molecular species is expected from further studies using either Harvey virus pseudotyped by a helper virus unrelated to M-MLV or by studying the RNAs of Harvey virus containing component 2 at great excess over component 1, to minimize contamination of component 2 by degraded component 1.

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