Biological and Molecular Biological Characterization of the Virus Progeny from Transformed Clones MuSV–124 and MuSV–349: Evidence for MuLV-specific Nucleotide Sequences in the MoMuSV Size Class of RNA from MoMuSV–124

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SUMMARY

The genetic information of MoMuSV–349 and MoMuSV–124, two clones of productively transformed TB cells, was distributed between two size classes of RNA (mol. wt. 2.9 x 10^6 and 1.9 x 10^6) in the proportions of 5:1. Some preparations of MoMuSV–124 lacked the large RNA. The virions produced by both clones also contained all the nucleotide sequences of Moloney leukaemia virus and the ratio of MuSV:MuLV produced by the two clones differed markedly.

The distribution of the sequences specific for Moloney murine leukaemia virus (MoMuLV) between the two size classes of RNA was studied using molecular hybridization to DNA probes complementary to and representative of: (i) the Moloney murine sarcoma virus (MoMuSV) RNA genome (mol. wt. 1.9 x 10^6); (ii) those nucleotide sequences shared by MoMuSV and MoMuLV; (iii) nucleotide sequences specific for MoMuSV; (iv) nucleotide sequences specific for MoMuLV. The only detectable Moloney leukaemia virus-specific nucleotide sequences present in MoMuSV–124 virions were in the RNA of mol. wt. 1.9 x 10^6, whereas these sequences were detected in the RNA of mol. wt. 2.9 x 10^6 isolated from MoMuSV–349 virions. The biological properties of the replicating information in MoMuSV–124 suggest that, consistent with the small size of RNA, it is defective, whereas MoMuSV–349 produces virions containing an intact MoMuLV genome, competent for replication.

INTRODUCTION

Most, if not all, isolates of murine sarcoma viruses (MuSV) have the ability to infect and morphologically transform cells but are defective for replication (Aaronson & Rowe, 1970; Aaronson & Weaver, 1971; Harvey & East, 1971). It has been proposed that these viruses arose by recombination between non-defective murine leukaemia viruses and certain cellular sequences present in uninfected mouse cells (Scolnick et al. 1975; Hu et al. 1977). In order to replicate, MuSV requires additional information which can be provided by helper leukaemia viruses (MuLV) which provide virus envelope glycoproteins and reverse transcriptase (Barbacid et al. 1976; Parks et al. 1976; Peebles et al. 1976). Available data suggest that there can be considerable heterogeneity with regard to the extent of expression of proteins coded for by the replication defective MuSV genome in transformed, non-producer cells (Stephenson et al. 1978).

In 1973 we reported the isolation of a clone of mouse cells transformed by Moloney
murine sarcoma virus (clone MoMuSV-124). These original cells were producing a high titre of MuSV but virus activity characteristic of MuLV could not be detected (Ball et al. 1973). The original MoMuSV-124 cells were XC- and the MoMuSV produced titrated with one-hit kinetics (under conditions where focus formation was dependent on virus spread).

Furthermore, electrophoretic analysis in polyacrylamide-agarose and formamide gels indicated that both the native and denatured RNA from MoMuSV-124 were larger in size than the corresponding RNA of MoMuLV (Lo & Ball, 1974). After storage as frozen stocks and subsequent passage the properties of MuSV-124 cells were found to be markedly altered. First, 5 to 10% of the MoMuSV-124 cells induced XC syncytia that were very much smaller than those induced by MoMuLV producing cells. Both XC- and XC+ subclones on passage resulted in approximately the same proportion of XC- to XC+ clones. Second, it was shown that the virus progeny from MoMuSV-124 cells consisted of two components: (i) MoMuSV [titre \(6 \times 10^8\) infectious units (i.u.) per ml] which infected and transformed cells but which appeared to require a helper in order to replicate and (ii) an aberrant replicating activity. The aberrant replication properties were manifest primarily by the induction of very small XC syncytia and the inability of the virus to spread widely in monolayers of cells which functioned as indicators for MuLV and/or MuSV infection. Further, we (unpublished data) and others (Dina et al. 1976; Maisel et al. 1977) showed that in gel electrophoresis analyses the major species of RNA isolated from MoMuSV-124 cells had a mol. wt. of \(1.9 \times 10^6\).

Although we do not know the reason for this change, changes in the biological properties of viruses on passage of chronically-infected cells are not unique to MoMuSV-124 (Bilello et al. 1977; Wainberg et al. 1978; Shields et al. 1979). In any event MoMuSV-124 because it produces MoMuSV in excess of MoMuLV has proved to be useful in numerous investigations of the genome of MuSV (Canaani et al. 1977; Hu et al. 1977; Deng & Wimmer, 1978; Dina 1978; Dina & Penhoet, 1978; Fan & Verma, 1978; Maisel et al. 1978; Philipson et al. 1978; Benz & Dina, 1979; Donoghue et al. 1979).

The virus progeny of MoMuSV-124 have been further characterized in molecular hybridization experiments using specific virus probes and separated RNA corresponding to the two size classes of virus RNA. The results obtained have been contrasted with those found for a second clone of MoMuSV-producing cells, MoMuSV-349. This latter clone was derived from MoMuSV-124 virus progeny (Ball et al. 1973).

The virions produced by both clones contained all the information of MoMuLV grown on TB cells (Ball et al. 1973) but this information was differently distributed between the size classes of RNA of MoMuSV-124 and MoMuSV-349. The biological properties of the replicating information in MoMuSV-124 suggest that, consistent with the small size of the RNA, it is defective, whereas in MoMuSV-349 it is not.

**METHODS**

**Cells and culture conditions.** All cell lines were grown in Eagle’s minimal essential medium supplemented with 10% heat-inactivated foetal calf serum (Gibco) and antibiotics (Ball et al. 1973).

TB cells are an established cell line derived from a mixed culture of foetal thymus and bone marrow cells from CFW/D mice (Ball et al. 1964) established using the procedures of Wright et al. (1967). JLS-V11 cells (Wright et al. 1967) chronically infected with MoMuLV were kindly provided by Dr K. Manley. Details concerning the isolation and biological properties of clone MoMuSV-124 and MoMuSV-349 cells have been reported (Ball et al. 1973). Neither of these clones produced detectable levels of xenotropic virus [assessed using
the mink S+L- cell assay of Peebles (1975)] nor was mink cell focus forming virus detected (Hartley et al. 1977) showing that the helper for both viruses is not xenotropic or amphotropic.

The SMB7 cell line is an established tissue culture line of rat myoblasts which are fusion resistant. They were kindly provided by Dr B. Sanwal, Department of Biochemistry, University of Western Ontario. These cells support the growth of MoMuLV almost as well as mouse cells and produce less reverse transcriptase activity than do NRK (normal rat kidney) cells.

**Biological assays.** Titres of MoMuSV were determined using the standard focus assay of Hartley & Rowe (1966) and TB cells, or by the infectious centres assay for MoMuSV and MoMuLV described by Ball et al. (1973). Titres of MoMuLV were determined using 15F cells (McCarter, 1977) and by the use of virus cloning techniques in which TB cells, in suspension, were infected with MoMuSV-124 at a m.o.i. of 0.5 and individual cells plated at 50 cells/60 mm culture dish. When cells became attached the medium was replaced with medium containing 0.7% agar. After growth for 3 to 4 days individual clones were picked and the cells screened for MoMuLV production using XC cells. From Poisson's distribution the proportion of cells not infected = $e^{-x}$ where $x = m.o.i$. The titre = m.o.i x the number of cells exposed to virus x the dilution factor.

Productive infection by MoMuLV was also assessed using the rat cell line SMB7. The cells were infected in suspension with a wide range of dilutions of MoMuSV-124 virus, plated as single cells at low numbers (200) in 60 mm plastic tissue culture dishes. Following growth at 37 °C for 3 days, the medium was removed and each plate seeded with $2 \times 10^5$ 15F cells which served as the indicator cells for the replication of MoMuLV. Following incubation for 3 days, the medium was removed and the plates fixed and stained. The plates were scored for the proportion of replication-positive SMB7 clones (detected by the presence of a halo of transformed 15F cells). The titre, based on the proportion of cells not infected, was determined as above.

**Gel electrophoresis procedures.** Radioactive labelling of virus and preparation of RNA for gel electrophoresis were carried out as previously described (Lo & Ball, 1974; Ball et al. 1979).

**Preparation of virus RNA.** The vRNA for gel electrophoresis, hybridization studies and the preparation of cDNA probes was isolated from virus harvested at 4 to 6 h intervals as described (Ball et al. 1979).

**Separation of vRNA species.** The vRNA species were separated using a modification of the method of Howk et al. (1978) in which the virus RNA was extracted with phenol:chloroform (1:1). Fractions (0.4 ml) from the sucrose gradient were collected and their absorbance determined. Peak fractions corresponding to each of the size classes of RNA were pooled, precipitated in ethanol, resuspended in 2 x SSC and stored at $-70 \, ^\circ C$.

**Selection of poly(A) containing vRNA.** The vRNA species were separated using the method of Howk et al. (1978) as previously described (Ball et al. 1979).

**Source of virus nucleotide sequences to prepare the specific cDNA probes.** The MoMuLV used in this work was isolated from the same MoMuSV (MuLV) stock as were MoMuSV-124 and MoMuSV-349. This MoMuLV, grown on either TB (TBMoMuLV) or NIH/3T3 cells was completely homologous with MoMuLV released from JLS-V11 cells (Wright et al. 1967) or MoMuLV released from NIH/3T3 cells infected with JLS-V11 MoMuLV (Ball et al. 1979).

The source of RNA used in the preparation of the specific probes was the separated RNA of MoMuSV-349 of mol. wt. 1.9 x 10^6.

**Synthesis of virus cDNA probes.** The virus cDNA probe corresponding to the MoMuLV isolate (TBMoMuLV) was synthesized using detergent-disrupted virions as described
(Rothenberg & Baltimore, 1976) in the presence of added calf thymus primer DNA. All other virus specific probes were synthesized by using purified virus RNA and avian myeloblastosis virus reverse transcriptase (a gift from Life Science Inc., St. Petersburg, Fla., U.S.A.) according to the method of Taylor et al. (1976) and processed as described (Ball et al. 1979). The cDNA probes had a specific activity of $1 \times 10^7$ to $4 \times 10^7$ ct/min/µg. The representative nature of each of the cDNA probes was verified by showing that when polyadenylated virus RNA [nicked by treatment with alkali (Wang et al. 1975)] was fractionated according to mol. wt. in a sucrose gradient each of the RNA-containing fractions shared nucleotide sequence homology with the corresponding cDNA probe (data not shown).

Selection of cDNA COMM virus probe. To prepare a probe representing the sequences common to MoMuSV and MoMuLV a hybridization reaction mixture consisting of $5.3 \times 10^6$ ct/min (0.17 µg) $^3$H-TBMoMuLV cDNA, 15 µg separated MoMuSV RNA of size $1.9 \times 10^6$ and 7 ml of $2 \times$ SSC (0.3 M-NaCl-0.02 M-sodium citrate, pH 7.0) plus 0.1% SDS was incubated at 68 °C for 24 h. The double-stranded homologous hybrid was separated from the single-stranded cDNA using HAP chromatography (Stehelin et al. 1976).

The double-stranded hybrid was digested with S1 nuclease and RNA was removed by treatment with NaOH (2 h at 37 °C). Following neutralization with HCl the cDNA was precipitated with ethanol.

Preparation of MoMuLV-specific cDNA. This cDNA was also isolated from the hybridization reaction mixture containing MoMuLV cDNA and separated MoMuSV-349 RNA of mol. wt. $1.9 \times 10^6$. The MoMuLV-specific cDNA was separated from double-stranded hybrids by HAP chromatography and desalted by passage through a Sephadex G50 column using 2 mM-EDTA. The fractions containing the cDNA were treated with NaOH (2 h at 37 °C), neutralized with HCl and precipitated with ethanol.

Preparation of polyadenylated, size-selected MoMuLV fragments. These fragments were generated by alkali treatment (Wang et al. 1975) and from these, polyadenylated fragments were selected on oligo(dT)-cellulose. The polyadenylated fragments were further selected for those corresponding in size to 60% or less of the MoMuLV genome by centrifugation in a sucrose gradient. These fragments were used to locate the nucleotide sequences present in each of the virus-specific cDNA probes with respect to the 3′ end of the MoMuLV genome.

Preparation of MuSV-specific cDNA. A hybridization mixture of $1 \times 10^7$ to $1.5 \times 10^7$ ct/min MuSV-349 $^3$H-cDNA plus TBMoMuLV RNA at a DNA:RNA ratio of 1:10 in 0.22 M-phosphate buffer, pH 6.8 plus 2 mM-EDTA was incubated at 68 °C for 20 h. The single-stranded DNA was separated on a HAP column and this process was repeated up to five times. The extent of protection of the probe by TBMoMuLV RNA became constant after the third cycle and could not be reduced to less than 15%. After the final hybridization cycle the single-stranded DNA was separated by HAP chromatography, desalted on a Sephadex G50 column and eluted with 2 mM-EDTA. The eluted material was treated with 3 M-NaOH (2 h at 37 °C), neutralized with HCl and precipitated with ethanol. This single-stranded material was protected 100% by MuSV-349 DNA.

Preparation of a cDNA probe to virus-like 30 SRNA. A cDNA probe was prepared from virus induced from TB cells with 5-iodo-2′-deoxyuridine (Sigma Chemical Co., St. Louis, Mo., U.S.A.) according to the method of Besmer et al. (1979). As reported by Besmer et al. (1979) this RNA consisted of two species: a minor one of mol. wt. $3.0 \times 10^6$ and a major one (93% of the total RNA) of mol. wt. $1.6 \times 10^6$. The cDNA probe represented primarily the nucleotide sequences corresponding to the virus-like 30 SRNA (Ball et al. 1979) and could be used to assess the level of homologous sequences present in the RNA preparations used in this study.

Nucleic acid hybridizations. Each virus $^3$H-cDNA (approx. 1000 ct/min per hybridization) was hybridized to excess vRNA in $2 \times$ SSC plus 0.1% SDS, 1 mg/ml yeast RNA and 100
Nucleotide sequence heterogeneity in MuSV RNA

\[ \mu g/ml \text{ calf thymus DNA. All kinetic hybridization curves except where noted were carried out to a } C_t \text{ value of } 2.3 \text{ mol s/l at } 68 \, ^\circ \text{C. The extent of hybridization was assessed by measuring resistance to } S_1 \text{nuclease as described (Leong et al. 1972).} \]

RESULTS

Biological characteristics of virions produced by MoMuSV clones 124 and 349

MoMuSV-124

Titres of MoMuSV were determined using the method of Hartley & Rowe (1966) which detects transformed foci produced by spread of virus infection in monolayers of murine cells. For defective MoMuSV, the production of foci is dependent on simultaneous infection by helper MuLV and the kinetics of infection are therefore two-hit. As shown in Fig. 1, the kinetics of focus formation by MoMuSV-124 in TB cells were two-hit. This observation implies infection by two virus particles, one being transformed and the other being a helper. It was also observed that the foci produced by MoMuSV-124 were very small as compared with those produced by a mixture of competent MoMuLV and MoMuSV.

MoMuSV titres were also determined by the use of the infectious centres assay for MoMuSV and MoMuLV described by Ball et al. (1973). In this assay, cells infected by MoMuSV are detected by their transformed morphology. The titre of MoMuSV found in MoMuSV-124 using this assay was \( 6 \times 10^3 \text{ i.u./ml} \) and the kinetics of transformation were one-hit over a wide range of virus dilutions. The titre of MoMuLV in MoMuSV-124 could not be determined using this assay because of the limited extent to which fusion of the indicator (XC) cells occurred. The XC syncytia formed consisted of only 2 to 3 nuclei per syncytium.

To determine the MoMuLV titre in MoMuSV-124 virions the 15F assay described by McCarter (1977) was used. In this assay the 15F indicator cells used carry the MuSV genome but the cells are not morphologically transformed. Infection with MuLV produces a focus of transformed cells and the kinetics of focus-formation are one-hit. The titre of MoMuLV in virus produced by MoMuSV-124 cells was \( 2.5 \times 10^6 \text{ i.u./ml} \). The MoMuLV titres determined using virus cloning techniques were \( 2 \times 10^7 \text{ to } 5 \times 10^7 \text{ i.u./ml} \). The discrepancy in the titres found using the two different assays is not peculiar to MoMuSV-124 virus. For unknown reasons titres of all MoMuLV isolates tested are about 10-fold lower in 15F assays as compared to those obtained using the XC assay. The foci formed in the 15F cell assay were small compared with those produced by MoMuLV. The small size of the XC syncytia and the foci in TB and 15F cells suggested that the helper might be defective and unable to spread as effectively as competent MoMuLV.

This possibility was examined by infecting SMB7 rat cells with MoMuSV-124 virus and looking for virus production using 15F cells as outlined in Methods. These cells would be transformed only if replicating virus was produced by the SMB7 cells. If the replicating activity produced by MuSV-124 virions were defective, productive infection could be established only when a SMB7 cell was infected by complementing defective particles and the kinetics of replication would become two-hit at that virus dilution where the level of one of the defective particles would become limiting. The data in Table 1 indicate that the productive infection of SMB7 cells by MoMuSV-124 virions was one-hit initially but as the virus was diluted the kinetics changed to two-hit. Furthermore, those clones of rat cells productively infected with the MoMuSV-124 virus were characterized by a very narrow halo (1 to 2 cells thick) of transformed indicator cells (15F) indicative of very limited virus spread. This extent of spreading was in marked contrast to the very wide (100 plus cells) halo of transformed 15F cells found in control experiments utilizing clones of rat cells productively infected with replication competent MoMuLV.
Fig. 1. Effect of virus dilution on the titre of MuSV in different tissue culture harvests of MoMuLV-124 harvested at different passage levels of the chronically infected cells. Each set of symbols represents a different MuSV-124 harvest assayed in individual experiments. ●—●, Average titre for each virus dilution; ---, theoretical two-hit titration. Numbers in parentheses indicate total number of assay dishes screened at each virus dilution.

Table 1. Titration kinetics for replicating virus produced by MoMuSV-124 in the non-infected rat cell line SMB7

<table>
<thead>
<tr>
<th>Virus dilution</th>
<th>No. of MuLV-producing colonies</th>
<th>Total no. colonies screened</th>
<th>Titre* (×10^-x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/40</td>
<td>601</td>
<td>11987</td>
<td>2.0</td>
</tr>
<tr>
<td>1/80</td>
<td>416</td>
<td>18667</td>
<td>1.7</td>
</tr>
<tr>
<td>1/160</td>
<td>297</td>
<td>20347</td>
<td>2.3</td>
</tr>
<tr>
<td>1/320</td>
<td>90</td>
<td>19012</td>
<td>1.5</td>
</tr>
<tr>
<td>1/640</td>
<td>6</td>
<td>8661</td>
<td>0.48†</td>
</tr>
</tbody>
</table>

* Titre, % colonies not infected = e^-x where x = m.o.i x virus dilution x number of cells exposed to virus. For 1/40 dilution = 0.95 = 0.05 x 40 x 10^6 = 2 x 10^6 i.u./ml (based on the average of six separate experiments).
† Based on the average of two separate experiments and calculated as above.

MoMuSV-349

The titre of MoMuSV in virus harvests from MoMuSV-349 cells was 2 x 10^8 i.u./ml as determined in the infectious centres assay (Ball et al. 1973). The titre of MoMuLV in the virus progeny from MoMuSV-349 cells was 1 x 10^4 to 3 x 10^4 i.u./ml as determined in the 15F assay (McCarter, 1977). The foci produced in this assay were as large as those produced by competent MoMuLV.

Based on the maximum titres for the replication activity we estimate that the ratio of MuSV:MuLV in MoMuSV-124 was 6.1 x 10^8:0.2 x 10^8 to 0.5 x 10^8 (that is a 12- to 30-fold excess of MuSV) and in MoMuSV-349 virions 2.0 x 10^8:1 x 10^8 to 3 x 10^4 which represents an approx. 10000- to 20000-fold excess of MuSV over MuLV.
Fig. 2. (a) Co-electrophoresis in denaturing agarose gels of $^{32}$P-MoMuSV-349 (○—○) RNA and $^3$H-MoMuSV-124 (■—■) RNA. (b) Electrophoresis in a denaturing agarose gel of $^3$H-RNA from MoMuSV-124. Both electrophoresis gel runs were for 3 h at 160 V, 4 °C. Mol wt. estimations were based on the selection of standard RNA markers described in Methods.

Analysis of RNA from MoMuSV-124 and MoMuSV-349

Co-electrophoresis profiles of the denatured RNA from the virus harvests of MoMuSV-124 and MoMuSV-349 in methylmercury agarose gels are shown in Fig. 2(a). The RNA from MoMuSV-349 virus contained two species of RNA, a minor species of mol. wt. $2.9 \times 10^6$ corresponding in size to that of the MoMuLV genome (designated 349-LRNA) and a major species of mol. wt. $1.9 \times 10^6$ corresponding in size to that of the MoMuSV genome (349-SRNA; Bondurant et al. 1979). This latter size class of RNA was the major species present in MoMuSV-124 virions (124-SRNA). In some RNA isolates from MoMuSV-124 virions (Fig. 2b) a low level of RNA of mol. wt. $2.9 \times 10^6$ was also present (124-LRNA).

Characterization of virus cDNA probes

**MoMuSV-349 RNA of mol. wt. $1.9 \times 10^6$**

This separated RNA resulted in 48% protection of the MoMuLV cDNA probe at a $C_t = 1.5$ mol s/l (Fig. 3) and this value agrees very well with that (43%) found in the studies of Hu et al. (1977) for the protection of full-length cDNA of another isolate of MoMuLV by RNA of MuSV-124.

**MoMuSV-MoMuLV cDNA COMM virus probe**

The selected polyadenylated RNA fragments were able to protect this cDNA COMM virus probe to the extent of 51% (Table 2) in close agreement with the value predicted (45%) from the data of Hu et al. (1977).
Fig. 3. Kinetic analysis of the nucleotide sequences present in MoMuSV-349 RNA. Unfractionated MoMuSV-349 was hybridized to the \(^3\)H-cDNA probe representative of the nucleotide sequences of the MoMuLV genome (●—●) and to a \(^3\)H-cDNA probe corresponding to the MoMuLV-specific region of the MoMuLV genome (○—○) to the indicated C\(_t\) values. Also the purified 349-SRNA was hybridized to both the \(^3\)H-cDNA probe corresponding to the MoMuLV genome (△—△) as well as to the \(^3\)H-cDNA probe corresponding to the MoMuLV-specific region of the MoMuLV genome (□—□) to the indicated C\(_t\) values.

Table 2. Molecular hybridization of cDNA probes representing the MuLV-specific sequences and those common to the MuSV and MuLV genomes

<table>
<thead>
<tr>
<th>Virus RNA</th>
<th>(^3)H-cDNA COMM</th>
<th>(^3)H-cDNA MuLV-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBMoMuLV (mol. wt. 2.8 × 10^6)</td>
<td>92.7</td>
<td>86.1</td>
</tr>
<tr>
<td>MoMuSV (mol. wt. 1.9 × 10^6)</td>
<td>95.3</td>
<td>9.1</td>
</tr>
<tr>
<td>Polyadenylated 60% molecules†</td>
<td>51.0</td>
<td>66.0</td>
</tr>
</tbody>
</table>

* At a RNA:DNA ratio of 400:1 to a C\(_t\) of 2.3.
† Molecules corresponding in size to 60% or less of the MoMuLV genome (see Methods).

MoMuLV-specific cDNA virus probe

This cDNA virus probe, representing those nucleotide sequences not shared by MoMuSV and MoMuLV (Table 2) and specific for MoMuLV should represent, according to heteroduplex mapping studies (Hu et al. 1977), 59% of all the sequences of MoMuLV and of these, 69% should be located in that half of the genome proximal to the 3' end. To check the location of the nucleotide sequences present in this MoMuLV-specific probe with respect to the 3' end of the MoMuLV genome, polyadenylated MoMuLV RNA molecules of 60% genome size or smaller were prepared as described and shown to protect the MoMuLV-specific cDNA 66% (Table 2) in close agreement with the value calculated from the data of Hu et al. (1977).
Nucleotide sequence heterogeneity in MuSV RNA

Fig. 4. Kinetic analysis of the nucleotide sequences present in MoMuSV-124 virions. Unfractionated MoMuSV-124 RNA was hybridized to a \(^3\)H-cDNA probe representative of the nucleotide sequences of the MoMuLV genome (●—●) to the indicated \(C_r\) value. Hybridizations to a similar \(C_r\) value were carried out using the purified 124-SRNA and \(^3\)H-cDNA virus probes representative of the nucleotide sequences corresponding to the following virus RNA species: MoMuLV (▲—▲); common region (■—■) and MoMuLV-specific region (□—□).

TBMoMuLV cDNA

In our study MoMuLV grown in TB cells is completely homologous with MoMuLV released from JLS-V11 cells (Wright et al. 1967) and MoMuLV released from NIH/3T3 cells infected with JLS-V11 MoMuLV (J. K. Ball et al. unpublished data). The heteroduplex data of Hu et al. (1977) were obtained using MoMuLV produced by NIH/3T3 cells infected with JLS-V11 MoMuLV (Fan & Baltimore, 1973). This fact, as well as the close agreement between our data described above and values found by Hu et al. (1977) or predicted from their data, suggest that the findings are comparable and that our cDNA probes probably represent those regions of MoMuLV and MoMuSV seen in the heteroduplex studies.

Molecular hybridization

Total unfractionated RNA from MoMuSV-124 and MoMuSV-349

A cDNA probe representative of the virus sequences present in MoMuSV-124 virions was completely protected by MoMuSV-124 RNA (100% at \(C_r = 2\cdot3 \text{ mol s/l}\)) and by MoMuSV-349 RNA (97.8% at \(C_r = 2\cdot3 \text{ mol s/l}\)). A cDNA probe representative of the sequences in MoMuSV-349 virions was completely protected by MoMuSV-349 RNA (100% at \(C_r = 2\cdot3 \text{ mol s/l}\)) and by MoMuSV-124 RNA (100% at \(C_r = 2\cdot3 \text{ mol s/l}\)). The two unfractionated virus RNAs appear, therefore, to be completely homologous.

Virus RNA of MoMuSV-124

(i) Total virus RNA. The total virus RNA from MoMuSV-124 virions (124-L+SRNA) appeared to contain an almost complete copy of the total nucleotide sequences present in MoMuLV (Fig. 4) at \(C_r\) of 9·7 mol s/l. Furthermore, two distinct sets of nucleotide sequences were detected in the total MoMuSV-124 RNA using a cDNA virus probe
(ii) 124-SRNA. The kinetics of protection of a cDNA virus probe representative of the nucleotide sequences present in MoMuLV by both the total unfractionated RNA from MoMuSV-124 virions and the 124-SRNA were virtually identical. At a Ct of 5.7 mol s/l 124-SRNA was approx. 80% homologous with this cDNA probe and contained both an abundant set of nucleotide sequences (set A) and a less abundant set (set B). Furthermore, the Ct values for each of these two sets of nucleotide sequences in 124-SRNA did not differ significantly from those values found for the two sets of nucleotide sequences present in 124-L+SRNA.

Identification of the sets of nucleotide sequences present in 124-SRNA preparations isolated from MoMuSV-124 was made based on the Ct values obtained using cDNA probes specific for defined regions of the MoMuLV genome. The virus cDNA probe representing those nucleotide sequences common to MoMuSV and MoMuLV detected the most abundant species of nucleotide sequences present (Ct = 1.9 x 10^-2 mol s/l). The virus cDNA probe specific for MoMuLV detected low concentrations of homologous sequences in 124-L+SRNA (Ct = 1.2 mol s/l) and in 124-SRNA (Ct = 1.3 mol s/l). Identical kinetics of protection by 124-L+SRNA or 124-SRNA were found whether a mixture of these two specific virus probes (COMM + MoMuLV specific) or a cDNA probe representative of the MoMuLV genome were used (Fig. 5). The level of non-homologous virus-like 30 SRNA present in the 124-SRNA was estimated from Ct analyses to be > 2.0%.

(iii) 124-LRNA. In order to get sufficient RNA it was necessary to use a large quantity
Nucleotide sequence heterogeneity in MuSV RNA

Table 3. \( C_{t/2} \) values* for the protection of cDNA virus probes representing MoMuLV nucleotide sequences by fractionated and unfractionated RNA isolated from MoMuSV-349 and MoMuSV-124 virions

<table>
<thead>
<tr>
<th>Source of virus cDNA</th>
<th>TBMuLV (total)</th>
<th>MoMuLV-specific</th>
<th>'SRC'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of vRNA</td>
<td>Species A</td>
<td>Species B</td>
<td>COMM</td>
</tr>
<tr>
<td>MuSV-349 (L+S)</td>
<td>( 1.2 \times 10^{-2} )</td>
<td>( 0.8-1.2 )</td>
<td>ND†</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MuSV-349 (L)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MuSV-349 (S)</td>
<td>( 1.2 \times 10^{-1} )</td>
<td>Absent</td>
<td>ND</td>
</tr>
<tr>
<td>MuSV-124 (L+S)</td>
<td>( 2.2 \times 10^{-2} )</td>
<td>1.0</td>
<td>ND</td>
</tr>
<tr>
<td>MuSV-124 (L)</td>
<td>( 8.0 \times 10^{-1} )</td>
<td>Absent</td>
<td>( 7.3 \times 10^{-1} )</td>
</tr>
<tr>
<td>MuSV-124 (S)</td>
<td>( 2.2 \times 10^{-3} )</td>
<td>1.7</td>
<td>( 1.9 \times 10^{-2} )</td>
</tr>
</tbody>
</table>

* \( C_{t/2} \) values expressed in mol s/l.
† ND, Not determined.
‡ 42% hybridization at \( C_t = 7.8 \) mol s/l.

(1401) of virus harvest and to select rigorously for this size class of RNA using sucrose gradient centrifugation procedures.

The data (Table 3 and Fig. 6) clearly show that 124-LRNA does contain those nucleotide sequences corresponding to the MoMuSV genome, that is MoMuSV:MoMuLV COMM and MoMuSV. The origin of these nucleotide sequences in 124-LRNA most likely results from the inability of the techniques used to completely separate the 124-LRNA from the 124-SRNA which is present in large excess. However, no MoMuLV-specific sequences were detected in 124-LRNA when assessed to a \( C_t \) of 6 mol s/l.

The absence of the MoMuLV-specific nucleotide sequences in 124-LRNA was confirmed using 124-LRNA isolated from three different batches of virus harvest from MoMuSV-124 cells. The only variable found between the RNA preparations was the extent of contamination by 124-SRNA (data not shown).

The data in Table 3 show that the ratio of MoMuLV-specific sequences present in the RNA of a size class corresponding to that of the MoMuSV genome (and which must be responsible for the replicating activity present in MoMuSV-124 virions) to sarcoma-specific sequences was 1:39, in close agreement with that found in the biological experiments (1:10 to 30). Further, the \( C_{t/2} \) (1·3 mol s/l) for the MoMuLV-specific sequences in MoMuSV-124-SRNA was in close agreement with the value calculated from the \( C_{t/2} \) for the hybridization of the cDNA of MoMuLV and its homologous RNA. The \( C_{t/2} \) value found for the latter homologous hybridization was \( 4.2 \times 10^{-2} \) mol s/l, at an RNA concentration of 4 μg/ml (data not shown). At the same concentration of 124-SRNA the MoMuLV-specific sequences represented approx 1/30th of the RNA. Therefore, to achieve equivalent hybridization (to \( C_{t/2} \)) of the MoMuLV sequences in MoMuSV-124 would require a \( C_{t/2} \) 30-fold higher than that used for MoMuLV (i.e. \( C_{t/2} = 1.26 \) mol s/l; found \( C_{t/2} = 1.3 \) mol s/l).

Virus RNA of MoMuSV-349

(i) Total RNA. The total RNA extracted from MoMuSV-349 virions (349-L+SRNA) was able to protect a cDNA virus probe representative of all the nucleotide sequences present in MoMuLV grown in TB cells (Fig. 3). Furthermore, using this same virus probe two distinct sets of nucleotide sequences were detected, one abundant set (A) characterized by a \( C_{t/2} \) of \( 1.2 \times 10^{-2} \) mol s/l and reaching a plateau at approx. 50% and a second set (B) with an apparent \( C_{t/2} \) of \( 8.0 \times 10^{-1} \) mol s/l. In repeated experiments using different prepar-
Fig. 6. Kinetic analysis of the nucleotide sequences present in 124-LRNA. RNA of this size class was hybridized to the COMM (O—O), MoMuLV-specific (Δ—Δ) and MuSV-specific (■—■) virus cDNA probes to the indicated Cₜ values. Reference hybridizations show the kinetics of protection of each of the COMM (●—●) and MoMuLV-specific (▲—▲) cDNA probes by MoMuLV RNA to the same Cₜ values.

ations of MoMuSV-349 virions, the maximum extent of hybridization varied between 85 and 95%. When the ³H-cDNA MoMuLV-specific probe was used with MoMuSV-349-L+SRNA, only the less abundant set of nucleotide sequences (set B) was hybridized with a Crₜ₂ = 1·2 mol s/l. These less abundant nucleotide sequences were therefore identified as MoMuLV specific while the most abundant set of nucleotide sequences was confirmed as those shared by MoMuLV and MoMuSV. It was noted, however, that the ratio of the Crₜ₂ for these two sets of sequences (approx. 100-fold) did not agree with the ratio of the titres of MoMuSV-MoMuLV determined by biological assays (10 000- to 20 000-fold) nor with the ratio of the amounts of the two species of RNA separated by gel electrophoresis (five-fold).

(ii) 349-SRNA. The kinetic analyses of the protection of defined cDNA virus probes by the isolated 349-SRNA are shown in Fig. 3. This RNA was found to be homologous to 48% of the MoMuLV genome with a Crₜ₂ of 1·2 × 10⁻² mol s/l. This level of shared nucleotide sequences agreed very closely with the extent of shared sequences (43%) found using heteroduplex mapping techniques (Hu et al. 1977; Donoghue et al. 1979). The 349-SRNA also contained detectable levels of nucleotide sequences homologous with the MoMuLV-specific cDNA probe (26% hybridization at a Crₜ = 8·3 mol s/l). The non-homologous virus-like 30 SRNA sequences represented < 1% of the 349-SRNA (determined from Crₜ₂ values).

(iii) 349-LRNA. The separated MoMuSV-349-LRNA contained a low concentration (42% hybridization at a Crₜ = 7·8 mol s/l) of MoMuLV-specific sequences (data not shown).
DISCUSSION

Virus particles produced by MoMuSV-349 cells contain two size classes of RNA of mol. wt. $2.9 \times 10^6$ and $1.9 \times 10^6$ in the proportions of 1:5. Most preparations of RNA from MoMuSV-124 virions are similar to those from MoMuSV-349 but some lacked a peak of RNA of mol. wt. $2.9 \times 10^6$ even though these same virus harvests had a replicating titre in the order of $2 \times 10^7$ i.u./ml. The virions produced by MoMuSV-124 and MoMuSV-349 contained the same nucleotide sequences. Therefore, molecular hybridization studies were undertaken to study the distribution of virus-specific nucleotide sequences in the two size classes of RNA from each of the clones. Molecular hybridization was used in the present studies because using appropriate cDNA virus probes it is possible to detect and quantify minor species of virus nucleotide sequences.

In studies of the protection of a cDNA representing the entire genome of MoMuLV by unfractionated RNA of MoMuSV-349 and MoMuSV-124, the kinetics of molecular hybridization were virtually identical and at a C$_{t}$ = 8 mol s/l the protection of the MoMuLV cDNA was almost complete. Therefore, the vRNA from each of the two MoMuSV clones appears to contain a complete set of MoMuLV nucleotide sequences. The MoMuLV-specific sequences were, however, distributed differently between the two size class of RNA isolated from virions produced by the two clones.

For MoMuSV-124 RNA, in that portion of a sucrose gradient encompassing the RNA of mol. wt. $2.9 \times 10^6$, which should have contained the MoMuLV genome subunits had they been present, MoMuLV-specific nucleotide sequences were not detected at a C$_{t}$ = 6 mol s/l.

The absence of an intact MoMuLV genome in MoMuSV-124 has also been suggested by the studies of Andersson et al. (1979) and Benz & Dina (1979) using detergent permeabilized MoMuSV-124 virions to synthesize long cDNA virus transcripts. These authors observed extensive synthesis of MoMuSV cDNA of 5.8 kilobases (kb) but did not detect synthesis of 9 kb cDNA of helper virus. Even though the concentration of MoMuLV was low, a cDNA of this size should have been detected if the corresponding RNA had been present.

The statement (Dina et al. 1976; Maisel et al. 1977; Donoghue et al. 1979) that the helper virus for the defective MuSV in MoMuSV-124 is present as an intact MoMuLV genome with a genome size of mol. wt. $3.0 \times 10^6$ appears to have been based on incomplete evidence. Dina et al. (1976) showed that the unfractionated RNA of virus particles produced by MoMuSV-124 cells was homologous with a cDNA probe prepared from MoMuLV RNA at RNA/DNA ratios of 900 to 2000, thus implying that the complete genome of MoMuLV was present. However, fractionated 30 SRNA (corresponding in size to our MoMuSV-t24 SRNA) only protected the same cDNA probe 50 to 55% at a RNA/DNA ratio of 500. Higher ratios were not studied. These results were interpreted to indicate that those sequences characteristic of the MoMuLV genome were present in the RNA of mol. wt. $3 \times 10^6$.

In contrast to the results reported by Dina et al. (1976) we find MoMuLV grown on TB cells to be completely homologous in reciprocal hybridizations with the total virus RNA of MoMuSV-124 virions and MoMuLV produced by NIH/3T3 cells infected with MoMuLV from JLS-V11 cells. The discrepancy may arise because procedures used to prepare the cDNA probes and the conditions used for hybridization were quite different in the two studies.

We found that the cDNA probe prepared using MoMuLV grown on TB cells was protected to the extent of 50% by fractionated MoMuSV-124 SRNA at a C$_{t}$ of approx. 1. However, at C$_{t}$ values greater than 3 mol s/l the probe was virtually completely protected by this RNA. Therefore, we conclude that the full complement of MoMuLV genetic in-
formation detected by Dina et al. (1976) is contained within RNA of mol. wt. $1.9 \times 10^6$. However, it cannot be determined from the present data how these nucleotide sequences are organized with respect to the subunits which comprise the RNA of mol. wt. $1.9 \times 10^6$.

The MoMuSV-124-LRNA was found to contain nucleotide sequences characteristic of MuSV but a comparison of the C$_r$ values for the cDNA probes used to detect these sequences (cDNA COMM and MoMuSV-specific cDNA) suggests that these two sets of nucleotide sequences were derived by contamination from the RNA of mol. wt. $1.9 \times 10^6$ which is present in excess. It may be argued that for the same reason MoMuLV-specific sequences should have been detected in the MuSV-124-LRNA. However, to detect these sequences C$_r$ values much higher than those used (C$_r$ = 6 mol s/l) would have been necessary because these sequences represent only 2 to 3% of the total RNA of MoMuSV-124-SRNA.

The defective nature of the replicating activity of MuSV-124 was also suggested from the results of our biological experiments. This was manifest by the apparent inability of the virus to spread to the same extent as does competent MoMuLV or mixtures of MoMuSV and competent MoMuLV in infected monolayers of mouse cells (TB and 15F) and by the production of very small XC syncytia. Further, the kinetics of replicating activity of MoMuSV-124 virions were found to be two-hit. Our data do not permit us to determine if the complementing defective particle is the defective MoMuSV genome or a second defective MoMuLV genome.

For MoMuSV-349 a low level of MoMuLV-specific sequences was detected in MoMuSV-349-LRNA. A very low level of these same sequences was also detected in MoMuSV-349-SRNA (26% hybridization at C$_r$ = 8 mol s/l). However, the significance of this result is unclear because we cannot rule out the possibility that these sequences were derived by degradation from the MoMuLV present in MoMuSV-349-LRNA.

For MoMuSV-124 virions our data show a very good correlation for the ratios of MuSV:MuLV as assessed in biological assays (10 to 30:1) with those found using the techniques of molecular hybridization (30 to 40:1). However, for MoMuSV-349 the ratio of MuSV:MuLV activities found using biological assays (10,000 to 20,000:1) differed significantly from the ratio as determined by molecular hybridization (100:1) suggesting that a large fraction of the MuLV-specific nucleotide sequences was derived from virions which were inactive in the biological assays. As far as the distribution of MuLV-specific sequences is concerned, the data seem certain: they are present in 124-SRNA but not in 124-LRNA; they are also present in 349-LRNA and probably in 349-SRNA. The question arises as to whether these MoMuLV-specific sequences are the only ones responsible for the helper function for the defective MoMuSV. While the titres of replicating activity are only a fraction of the titres of MoMuSV in both MoMuSV-349 and MoMuSV-124 there could be low levels of RNA sequences present in both viruses that remain unidentified in the present analysis. However if these have a helper function then they must be ecotropic because the MoMuSV and the replicating activity registered in murine cells and not in mink cells.

We have considered how to account for the presence of an intact helper in MoMuSV-349 when it is lacking from MoMuSV-124 from which it was derived. MoMuSV-349 cells were cloned when MoMuSV-124 from an early passage in TB cells was used to infect TB cells. Both viruses have been carried as chronically-infected cell lines. MoMuSV-124 virions appear to be comprised very largely of defectives as do those of MuSV-349 except for a minor component of intact MoMuLV. Shields et al. (1979) reported that lymphoid cells transformed with Abelson murine leukaemia virus may cease production of infectious virus but release defective virions that are non-infectious and lack 70 SRNA. We have shown (Ball et al. 1979) that TB cells (and JLS-V9 cells which are also lymphoid-derived) infected
at limiting dilution with MoMuLV produce 9 kb RNA for the first passage, but thereafter begin to produce a mixture of 9 kb and 6 kb size classes of RNA. The 6 kb RNA is polyadenylated and contains all the nucleotide sequences present in the 9 kb RNA. The polyadenylation of all the sequences indicates that trivial degradation is not responsible for the production of 6 kb RNA. We speculate that the cells comprising MoMuSV-124 and MoMuSV-349 process the virus information that they contain in a manner similar to that in which they process the RNA of MoMuLV, but to different extents. Some of the virus sequences in the virions may be derived from endogenous virus sequences, but, if so, they do not register in the biological assays that we employed.

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