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Molecular Cloning and Characterization of a Leukemia-Inducing Myeloproliferative Sarcoma Virus and Two of Its Temperature-Sensitive Mutants

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The myeloproliferative sarcoma virus (MPSV) induces extensive hematopoietic changes, including spleen foci in adult mice, and transforms fibroblasts in vitro. NRK nonproducer cell lines of MPSV and its temperature-sensitive mutants were analyzed by restriction enzyme digestion and Southern blotting. *Eco*RI fragments containing the proviral DNAs of MPSV and two temperature-sensitive mutants and rat cellular sequences homologous to c-*mos* were molecularly cloned. By comparing restriction enzyme cleavage sites, it was shown that the MPSV genome consists only of sequences related either to Moloney murine leukemia virus or to the c-*mos* mouse oncogenic sequences. Two regions of fragment heterogeneity were observed: (i) in the defective *pol* gene, where MPSV and the two cloned temperature-sensitive mutants were different from Moloney murine sarcoma virus and from each other, although MPSV wild-type retained more of the *pol* gene than any of the Moloney murine sarcoma virus isolates; (ii) in the area 3′ to the *mos* gene, which was identical in MPSV and its temperature-sensitive mutants but different from other Moloney murine sarcoma virus variants. Transfection of cloned MPSV DNA in RAT4 cells and virus rescue on infection with Friend murine leukemia virus yielded MPSV which transformed fibroblasts in vitro and also induced spleen foci in adult mice, thus proving that both properties are coded by the same viral genome.

The induction of hematopoietic changes is a complex process of interacting factors and is still poorly understood. Several viruses interfere with normal hematopoietic development in mice. Friend murine leukemia virus (F-MuLV) and Rauscher murine leukemia virus (9, 23, 30) induce spleen foci and erythroleukemia in adult mice but do not induce sarcomas. Kirsten sarcoma virus and Harvey sarcoma virus cause hematopoietic changes in newborn and, to a lesser extent, in adult animals (26). They also transform fibroblasts in vitro and induce sarcomas in mice (1). Myeloproliferative sarcoma virus (MPSV) is similar in that it causes erythroid, stem cell, and myeloid leukemia and spleen focus formation as well as myelofibrosis in adult mice (15, 19).

The MPSV complex was isolated originally by Chirigos et al. (6) from plasma of BALB/c after serial passage in adult mice of a tumor induced in newborn mice by uncloned Moloney murine sarcoma virus (Mo-MuSV). It consists of a replication-independent helper virus, Moloney murine leukemia virus (Mo-MuLV), and a defective component. The isolation of MPSV-transformed NRK nonproducer cells at endpoint dilution of the virus indicated that the genetic information responsible for induction of spleen focus formation and fibroblast transformation is coded by the same defective subunit (19). The molecular relationship of MPSV to its progenitors was demonstrated by hybridization analysis with specific cDNA probes (22). It was shown that MPSV is a modified sarcoma virus consisting of only Mo-MuLV- and Mo-MuSV-related sequences. No newly acquired cellular components could be detected within the limitations of this method. It was suggested that MPSV, despite limited changes in the genome as compared with Mo-MuSV, had acquired a broader range of target cell specificity than the standard Mo-MuSV (22). To examine whether the same viral gene function is involved in both sarcoma and hematopoietic transformation, temperature-sensitive mutants for the fibroblast-transforming gene (v-*mos*) were isolated (18). It was shown that the temperature-sensitive viruses are deficient not only for fibroblast transformation at the restrictive temperature but also for spleen focus formation in mice.

In this paper, we describe the molecular cloning of the MPSV genome and two independently isolated temperature-sensitive mutants. Detailed restriction enzyme analysis was used to confirm the structural relationship between MPSV and Mo-MuSV and to define the differences between these two viruses on one side and MPSV and its temperature-sensitive mutants on the other. The molecularly cloned virus has the same target specificity as the original MPSV, thus proving that the dual target cell specificity of MPSV is a property of the defective MPSV genome.

MATERIALS AND METHODS

Cells and viruses. All cells were grown in modified Eagle medium supplemented with 10% fetal calf serum. Infectious virus was obtained from tissue culture supernatants after 10- to 100-fold concentration. The concentrates are usually 30 to 45% lower in relative biological activity than virus used before concentrating the supernatant, measured by focus-forming units (FFU) in fibroblasts (19). Thymidine kinase-deficient (TK^-) normal rat cells (RAT4) were obtained from W. C. Topp (31).

Transfection. DNA transfection of NIH 3T3 (TK^-) and RAT4 (TK^-) cells was performed by modifications of established procedures (39). Plasmid py747LTK$^+$ containing the herpes simplex gene for thymidine kinase (obtained from M.

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Yaniv) was used for cotransfection at a ratio of 10:1 with cloned viral DNA. TK^+ or morphologically transformed cells were selected from individual foci by single-cell cloning in microwell tissue culture dishes.

Biological activity. Spleen focus formation and fibroblast focus formation were assayed as described by Ostertag et al. (18, 19). Macrophage-granulocyte colony assays were done by the method of Klein et al. (14).

DNA analysis. Cell lines were grown in roller bottles at 37°C, lysed with sodium dodecyl sulfate (0.5%), and treated with proteinase K (Boehringer-Mannheim) (200 μg/ml). Protein was removed by phenol-chloroform extraction, and the high-molecular-weight DNA was spooled out after isopropanol addition (50% [vol/vol]), washed in 96% ethanol, and suspended in TE buffer (10 mM Tris-hydrochloride, pH 8, 1 mM EDTA).

DNA samples were digested with restriction endonucleases (BRL; Boehringer-Mannheim) under the conditions recommended by the supplier and separated on 1-cm-thick 0.8% agarose gel in TEA buffer (40 mM Tris-OH, 20 mM sodium acetate, 20 mM NaCl, 2 mM EDTA, pH 8.1). Gels were soaked twice for 10 min each time in 0.25 N HCl for partial depurination of the DNA. After denaturation (0.5 M NaOH, 1 M NaCl) and neutralization of the gel (0.5 M Tris-hydrochloride, pH 7.4, 3 M NaCl), the DNA was transferred to nitrocellulose filters (28). Filters were hybridized (12) to a nick-translated c-*mos*-containing plasmid (pMS1) or alternatively a plasmid containing the Mo-MuSV M1 genome (pm1). Both plasmids were kindly provided by G. F. Vande Woude (4, 17, 36). Nick translation was performed as described by Rigby et al. (25).

Molecular cloning of integrated proviral DNA. Cellular DNA was digested to completion with *Eco*RI and enriched for sequences containing proviral integrated DNA by centrifugation in a 5 to 20% NaCl gradient (10 mM Tris-hydrochloride, pH 8.1, 5 mM EDTA). Similarly, λ Charon 4A DNA was digested with *Eco*RI, and the arms were separated in a 50 to 20% NaCl gradient and further treated with calf intestine phosphatase (Boehringer-Mannheim) as described by the supplier. The enriched cellular DNA and the dephosphorylated λ 4A arms were ligated in approximately equal molar ratios overnight with T4 DNA ligase (New England Biolabs) under the recommended conditions. The ligated DNA was subsequently packaged in vitro into phage particles obtained from sonicated extracts of BHB2690 (prehead donor) and a freeze-thaw lysate of BHB2688 (packaging protein donor) as described by D. Ruegg and B. Hohn (personal communication). The packaging efficiency for the recombinant DNA obtained was $\sim 6 \times 10^5$ PFU/μg of DNA.

The recombinant phages were plated onto *Escherichia coli* DP-50 *supF* in petri dishes (20 by 20 cm) (Nunc) at approximately 10^5 PFU per plate. Blots of the plates were made with nitrocellulose filters by the method of Benton and Davis (2) and hybridized to the nick-translated c-*mos* probe as described above. Minilysates were prepared from unpurified plaques hybridizing positively. Plaques were selected that contained *Sst*I fragments corresponding in size to restricted integrated proviral DNA and subjected to further cycles of purification until more than 95% of the phage showed positive hybridization. Bulk preparation of recombinant phage DNA was performed as described by Yamamoto et al. (40).

Cloning into pBR322. Recombinant phage DNA was cut with *Eco*RI, and the proviral DNA insert was isolated and subcloned into *Eco*RI-digested pBR322.

Restriction endonuclease mapping of cloned plasmids. Large-scale isolation of plasmid DNA was performed as described by Godson and Vapnek (11). Single and double digestions with various restriction endonucleases were run on 0.6 to 1.4% agarose gels in TEA buffer (as above), stained with ethidium bromide, and photographed. Fragment sizes were determined by comparison of marker λ DNA digested with *Hin*dIII and pBR322 DNA digested with *Alu*I. Plasmid DNA was linearized with one of the single cutters or with *Eco*RI and then subjected to *Bal*31 digestion (Bethesda Research Laboratories) under conditions described by the manufacturer to remove 180 base pairs (bp)/min. Enzyme was inactivated by the addition of EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid) (10 mM), and samples were phenol-chloroform extracted twice, ethanol precipitated, and subjected to further restriction endonuclease degradation analysis.

RESULTS

Characterization of MPSV and its temperature-sensitive mutants by Southern blot analysis of cellular DNA. To understand the biological background of the molecular study presented here, it is necessary to introduce cell lines and viruses briefly. 6-6#3 is a transformed nonproducer cell clone which was obtained after infection of NRK cells with diluted supernatants from spleens of MPSV-infected DBA/2J mice. It is presumed to contain only one copy of MPSV proviral DNA (19).

The provirus was rescued by superinfection of F-MuLV helper, and the released virus was used for mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. Four cell clones infected with this mutagenized MPSV and expressing a transformed phenotype at 32°C were chosen for further examination: ts 124, ts 143, ts 159, and ts 259. Selection and detailed characterization of these isolates are published in parallel (18).

ts 124, ts 143, ts 159, and ts 259 are characterized by the following criteria: (i) clonability in methylcellulose at the permissive temperature (32°C), (ii) fibroblast-like morphology at the nonpermissive temperature (39.5°C), (iii) release of comparable level of virus at both temperatures, and (iv) high number of FFU at 32 versus 39.5°C.

259 RAG is a revertant of the temperature-sensitive mutant ts 259. 259 RAG was isolated by plating the ts 259 virus on NRK cells. Cells of individual foci obtained at the nonpermissive temperature were isolated and regrown. Virus released by the 259 RAG cell clone was tested for focus formation on fibroblasts at 32 and 39.5°C. The ratio of FFU at 32°C to FFU at 39.5°C was 500 for 259 (18) and 2.5 for 259 RAG.

Nonproducer NRK cell lines infected at end-point dilution with temperature-sensitive MPSV mutants were isolated, and their specific phenotype at the permissive (transformed phenotype) and the nonpermissive (normal NRK phenotype) temperatures was verified. Two clones of the mutants ts 124 (ts 124/1 and ts 124/2) and ts 143 (ts 143/1/1 and ts 143/4/1) were established in parallel, as was one mutant of ts 159 (ts 159/5/1). No nonproducer clones could be isolated from ts 259.

(i) Southern blot analysis of 6-6#3 and the temperature-sensitive cell clones. The DNA of the cell clones was isolated and subjected to Southern blot analysis. The blots were hybridized with c-*mos* (17) (Fig. 1). The restriction endonuclease *Sst*I (Fig. 1A) is known to cut within the long terminal repeat (LTR) sequence of Mo-MuLV, the different Mo-MuSV isolates, and many other retroviruses. The *Sst*I fragments generated in the DNA of 6-6#3 and the tempera-

ture-sensitive mutants should therefore most likely contain viral sequences which are located within the two LTRs. The cell lines transformed by the wild-type MPSV (nonproducer 6-6#3) and by the temperature-sensitive mutants before isolation of nonproducer cell lines (see above) showed newly acquired restriction fragments in addition to the two rat c-*mos* endogenous fragments present in the NRK uninfected control which hybridized to the c-*mos* probe. The size of the MPSV-specific fragment (6.6 kilobase pairs [kbp]) was altered in ts 259 (3.7 kbp) and in the cell clone 259 RAG (5.2 kbp), which was infected by a revertant virus of ts 259, but not altered in ts 124 and ts 159. In addition, a slightly smaller fragment (6.4 kbp) was observed in ts 124. Since the probe hybridizes only to the viral *mos* gene and not to other regions of the proviral genome, it could not be excluded that MPSV contains additional internal *Sst*I cleavage sites.

When cleaved with *Eco*RI (Fig. 1B), the MPSV-specific fragment in 6-6#3 had a similar size as the endogenous c-*mos*-related fragment of NRK cells. ts 124, ts 159, ts 259, and ts 259 RAG also showed additional fragments hybridizing to the c-*mos* probe.

In the *Eco*RV digest (Fig. 1C) the c-*mos*-related fragments of ts 124, ts 143, and ts 159 had the same size as the specific fragment of 6-6#3 (3.2 kbp). ts 259 and 259 RAG both contain larger *Eco*RV fragments (3.4 and 4.1 kbp, respectively) than the one generated by this enzyme in wild-type MPSV nonproducer clone 6-6#3.

The results of the restriction enzyme analysis show some heterogeneity in the size of the specific fragments among the temperature-sensitive cell clones as compared with parental line 6-6#3. Compared to the intensity of the signal of the c-*mos* rat endogenous fragment (two copies per diploid genome), it was determined that the number of integrated provirus is different in the individual cell clones, ranging from about 10 to 12 copies in ts 159, about 4 in ts 124, and 1 in 6-6#3 ts 259 and 259 RAG per diploid genome.

(ii) Southern blot analysis of nonproducer cell lines infected with temperature-sensitive virus. Five nonproducer temperature-sensitive cell lines were examined for their c-*mos*-related sequences. The DNA was extracted, digested with restriction enzymes, and analyzed by agarose gel electrophoresis and Southern blotting. Figure 2A to E shows the radioautographs, and Table 1 summarizes the size of the c-*mos* homologous fragments occurring in addition to the rat endogenous sequences. Based on the single copy appearance of the MPSV-specific fragments compared with the two copies of rat endogenous c-*mos* sequences per diploid genome, it was concluded that the clones indeed arose by infection through a single virus particle.

The size of the *Sst*I (Fig. 2C) and *Eco*RV (Fig. 2E) fragments of ts 124/1 and ts 159/5/1 did not correspond to the

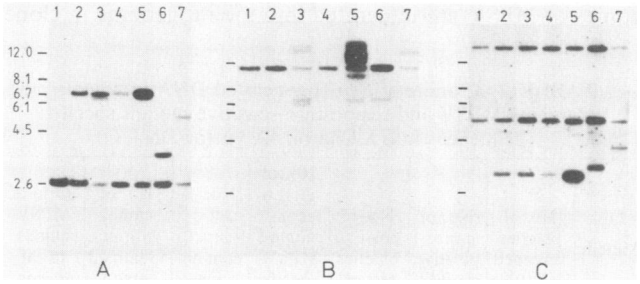

FIG. 1. Southern blot analysis of 6-6#3 and the temperature-sensitive cell clones. DNAs were digested with (A) *Sst*I, (B) *Eco*RI, and (C) *Eco*RV and fractionated by electrophoresis through 0.8% agarose gels. The DNA was transferred to nitrocellulose filters and hybridized to a ^{32}P-labeled c-*mos* probe. Lane 1, NRK; lane 2, 6-6#3; lane 3, ts 124; lane 4, ts 143; lane 5, ts 159; lane 6, ts 259; lane 7, 259 RAG. A plasmid containing Friend spleen focus-forming virus (41), digested separately with different restriction enzymes, was used as a marker. The lengths of the fragments were determined by comparison with *Hin*dIII- and *Eco*RI-digested λ DNA and are given in kbp.

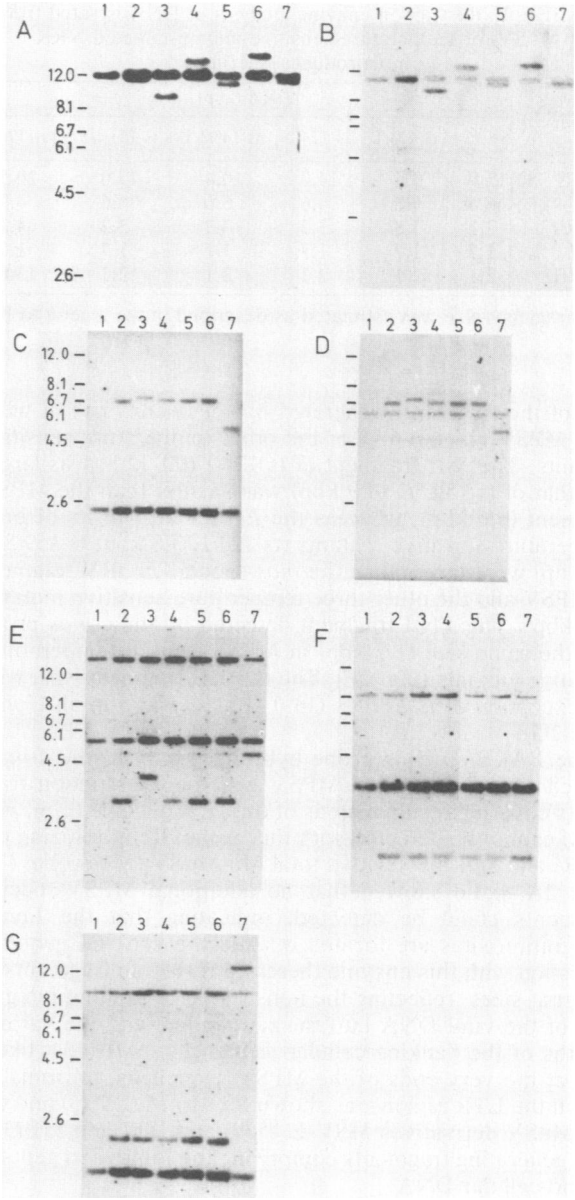

FIG. 2. Analysis of nonproducer cell lines. DNAs were extracted, digested with restriction endonucleases, separated on 0.8% agarose gels, and transferred to nitrocellulose filters, which were then hybridized to a ^{32}P-labeled probe. (A) and (B), *Eco*RI; (C) and (D), *Sst*I; (E) *Eco*RV; (F) *Xba*I; (G) *Hin*dIII. (A), (C), (E), (F), and (G) were hybridized to a ^{32}P-labeled c-*mos* probe, and (B) and (D) were hybridized to a ^{32}P-labeled M1 viral probe. Lane 1, NRK; lane 2, 6-6#3; lane 3, ts 124/1; lane 4, ts 124/2; lane 5, ts 143/1/1; lane 6, ts 143/4/1; lane 7, ts 159/5/1. The marker was the same as in Fig. 1.

TABLE 1. Restriction enzyme analysis of DNA isolated from MPSV- or temperature-sensitive-mutant-infected NRK nonproducer cell lines

Restriction enzyme	DNA (kbp)					
	6-6#3	ts 124/1	ts 124/2	ts 143/1/1	ts 143/4/1	ts 159/5/1
*Eco*RI	11.0	9.4	14.0	10.4	15.0	10.5
*Sst*I	6.6	6.8	6.6	6.6	6.6	4.9
*Eco*RV	3.2	3.8	3.2	3.2	3.2	4.7
*Xba*I	1.7	1.7	1.7	1.7	1.7	1.7
*Hin*dIII	2.1	2.1	2.1	2.1	2.1	13.0

[a] Fragment size was estimated as described in the legend to Fig. 1.

size of the fragments generated by these enzymes in wild-type MPSV clone 6-6#3 or the other temperature-sensitive mutants. The *Sst*I fragment of ts 124/1 (6.8 kbp) was larger and that of ts 159/5/1 (4.9 kbp) was smaller than the MPSV fragment (6.6 kbp), whereas the *Eco*RV fragments of both temperature-sensitive mutants (ts 124/1, 3.8 kbp; ts 159/5/1, 4.7 kbp) were larger than the *mos*-specific *Eco*RV fragment of MPSV and the other three temperature-sensitive mutants (3.2 kbp). The *Xba*I fragment detected by the c-*mos* probe had the same size (1.7 kbp) in MPSV and all temperature-sensitive mutants (Fig. 2F). The *Hin*dIII fragment was, with the exception of ts 159/5/1 (13.0 kbp), 2.1 kbp in all clones (Fig. 2G).

The BALB/c c-*mos* probe hybridizes only to the c-*mos*-related sequences of the MPSV genome. Restriction fragments covering residual areas of the viral genome (*gag*, *pol*, LTR) cannot be detected with this probe. Rehybridizing the *Eco*RI and *Sst*I blots with a total Mo-MuSV (M1 probe) (36) (Fig. 2A to D) showed that no additional MPSV-specific fragments could be detected, indicating that the *Eco*RI recognition sites are located outside the proviral genome. Digestion with this enzyme therefore results in fragments of different sizes, reflecting the nonspecific or random integration of the viral DNA into the rat genome and the various lengths of the flanking cellular sequences. *Sst*I most likely cuts at the very ends of the MPSV sequences, presumably within the LTR regions, as shown for Mo-MuLV (3) and the Mo-MuSV derivatives MSV-124 (37) and HT1 and M1 (36), thus generating fragments comprising the total virus genome but no cellular DNA.

Cloning of MPSV and temperature-sensitive mutant proviral DNAs. DNA of the nonproducer cell lines 6-6#3, ts 124/1, ts 143/1/1, and ts 159/5/1 was digested with *Eco*RI and cloned into λ Charon 4A vector arms (Table 2). Along with the MPSV proviral DNAs, sequences were cloned which comprise the rat gene homologous to c-*mos*. This gene is located on an *Eco*RI fragment of 11.5 kbp (Fig. 2A). The λ clone containing this fragment was purified and designated λ rat-*mos*, but it was not examined further. The resulting MPSV clones were named p18-663 (MPSV), p19-124 (ts 124/1), and p20-159 (ts 159/5/1). No molecular clone could be isolated with DNA from ts 143/1/1. Although the one λ clone which was obtained during the cloning experiment (Table 2) was plaque purified several times, MPSV-specific sequences were lost with a very high frequency.

Restriction enzyme analysis of plasmid clones. The restriction maps are presented in Fig. 3. The region of MPSV covering the *mos* gene as shown by hybridization to c-*mos* (Fig. 2) is the 2.1-kbp *Hin*dIII, the 1.7-kbp *Xba*I, or the 3.2-kbp *Eco*RV fragment, respectively. Assuming that the genetic organization of MPSV and its temperature-sensitive derivatives is similar to that of other retroviruses, especially the Mo-MuSV variants, this region is localized at the 3′ end of the viral genome. This assumption is strongly supported by the homologous arrangement of several other restriction enzyme sites in the presumed 5′ area compared with Mo-MuLV or Mo-MuSV. The sequential appearance of *Eco*RV, *Xba*I, *Sst*I, *Sma*I, and *Kpn*I cleavage sites indicates the location of the LTR sequences.

Based on the restriction enzyme data, there are two areas in which differences between MPSV and MSV-124 (24, 32, 33, 35, 37) and other Mo-MuSV variants such as M1 and HT1 (16, 36) can be observed: (i) The region between the *Xho*I (bp 2050) site and *Xba*I (bp 5050) site covering the 3′ end of the *gag* and all of the *pol* region (MPSV contains more of this region than any of the other Mo-MuSV isolates, which results in a larger size of its genome) and (ii) the very 3′ end of the genome around the recombination site of the *mos* gene with Mo-MuLV helper virus sequences (8, 33). A schematic comparison of these differences is shown in Fig. 4. The first area comprising mainly the defective gene for the reverse transcriptase shows extensive differences between MPSV and the Mo-MuSV isolates as well as among the latter ones. Differences in the *pol* gene region are also seen among the cloned temperature-sensitive mutants of MPSV. The second region of heterogeneity, the 3′ end of the virus (*mos-env* junction), was not altered in the temperature-sensitive mutants as compared with MPSV.

Biological activity of cloned MPSV DNA. Molecularly cloned wild-type MPSV DNA was used to transfect RAT4TK$^-$ fibroblasts. The MPSV DNA was mixed with the DNA of the herpes simplex thymidine kinase-carrying plasmid py747L in a molar ratio of 10:1 and transfected into RAT4TK$^-$ cells. Cell clones growing in medium supplemented with 0.1 mM hypoxanthine–0.4 μM aminopterin–16 μM thymidine (HAT) were characterized as outlined in Table 3. Six cell clones were taken for further examination: RAT4 1, RAT4 4, RAT4 5, RAT4 10, RAT4 13, and RAT4 14. RAT4 5 and RAT4 14 are HAT resistant but do not express a transformed phenotype, whereas a large proportion of the cells of the other four HAT-resistant clones have the typical appearance of transformed cells.

DNA of the MPSV-transfected RAT4 clones and of two F-MuLV-superinfected cell clones (1 and 3) was extracted and subjected to restriction endonuclease digestion and Southern blot analysis. Figure 5A (*Eco*RI digest) shows that several copies of MPSV are integrated into the rat genome in clone

TABLE 2. Cloning of *Eco*RI-generated DNA fragments containing MPSV and temperture-sensitive mutant-specific sequences into λ Charon 4A vector DNA

Mutant	No. of plaques screened ($\times 10^5$)	No. of c-*mos*-positive plaques[a]	No. of positive plaques tested[b]	No. of clones containing MPSV-specific fragments	No. of rat c-*mos*-containing clones	No. of clones with unidentified fragments	Ratio of MPSV clones to rat c-*mos* clones
6-6#3	3	13	13	3	9	1	0.33
124/1	6	19	19	3	9	5	0.33
143/1/1	6	23	23	1	20	2	0.05
159/5/1	3	10	10	2	6	2	0.33

[a] Plasmid pms1 (17) containing BALB/c mouse c-*mos* sequences was used as a probe.

[b] Restriction enzyme digest (*Sst*I) and agarose gel analysis of DNA isolated from small-scale phage lysates.

4. Clones 1, 10, and 13 also contain additional c-*mos* homologous sequences besides the rat endogeneous *Eco*RI fragment. The *Sst*I digest (Fig. 5B) also shows diverse MPSV-specific bands in clones 1, 4, 10, and 13. The size of the MPSV proviral genome was frequently altered; multiple copies of different lengths occurred in clone 4. A homogeneous population of shorter, MPSV-specific fragments is characteristic for clone 13. This clone generated only very few transforming viral copies after superinfection with F-MuLV helper virus (Table 3). MPSV-related sequences were not detected in clones 5 and 14. This is in agreement with the HAT resistance but untransformed phenotype of these two cell lines.

The tissue culture supernatant of F-MuLV-superinfected RAT4 1 cells was taken and inoculated intravenously into mice. FFU were tested in parallel on NRK fibroblasts. RAT4 10 cells were also superinfected with F-MuLV. The virus-containing tissue culture supernatant was used to infect NRK cells. Transformed, virus-releasing NRK cells (designated NRK 10) were utilized to obtain high titers of transforming virus. The supernatant of phenotypically transformed NRK 10 cells was concentrated, and FFU were tested in parallel on NRK fibroblasts and on infection of mice. Six mice for each serial dilution were infected with the virus-containing supernatant of either RAT4 1 or NRK 10 cells; three were killed on day 10 and three on day 16, and their spleens were examined for foci. The number of foci was low (1 to 10) after 10 days but increased dramatically until day 16 (15 to 150). Spleen weight also increased rapidly in a manner which is typical for the MPSV disease: from a normal 0.12 g to about 0.36 g at day 16 and 0.4 to 2 g 4 weeks after injection. The ratio of spleen FFU to fibroblast FFU observed in these experiments was 0.05 to 0.15 (about 50 to 150 spleen FFU per 10^3 fibroblast FFU), indicating that the cloned DNA carries all the information necessary for the characteristic biological activity of MPSV (Table 4).

Four weeks after injection of virus originating from molecularly cloned MPSV, six mice with large spleens were checked for histopathology. All of the spleens showed extensive hyperplasia of the red pulp, and most spleens showed evidence of macrophage/granulocyte expansion as described previously for uncloned and biologically cloned MPSV (14). Six mice were examined in detail. A proportion of the macrophage/granulocyte colonies did not require added colony-stimulating factor to proliferate. Most of the mice showed severe anemia. The hematocrit was 35, as compared with 44 to 49 in uninfected animals. Induction of tumor nodules as described for uncloned MPSV (13) was detected in mice 4 to 5 weeks after injection of the molecularly cloned virus.

Spleen focus formation induced by molecularly cloned MPSV was compared with the disease generated by two

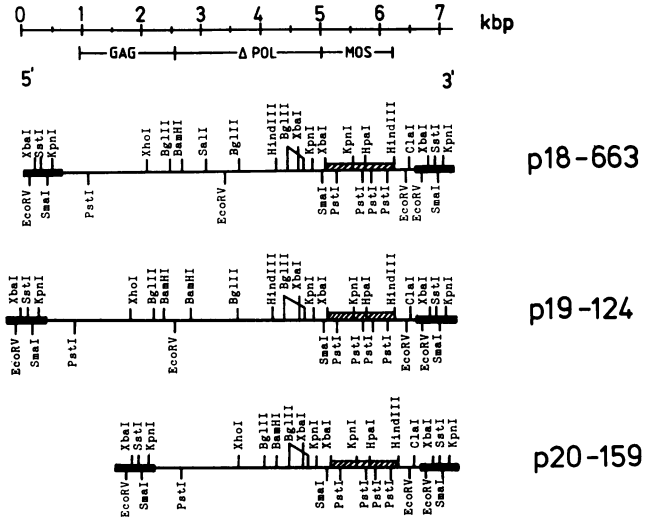

FIG. 3. Physical maps of the cloned MPSV and temperature-sensitive mutant DNAs. Restriction enzyme cleavage sites were localized by single and double digestions and by the *Bal*31 procedure. The heavy lines represent the LTR sequences, which are defined by the sequential appearance of five restriction enzyme sites. The *mos* gene (cross-hatched bar) was defined by hybridization of restriction fragments (Fig. 1 and 2) to the c-*mos* probe.

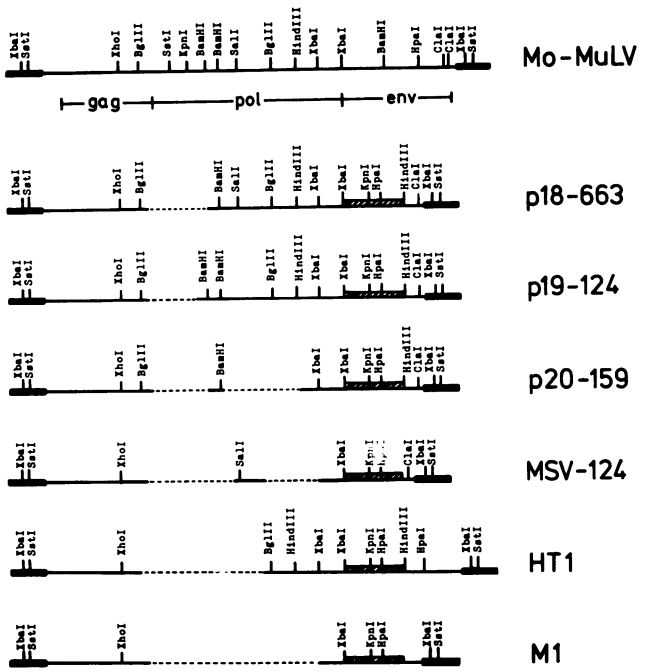

FIG. 4. Comparison of MPSV and its temperature-sensitive mutants to Mo-MuLV and the Mo-MuSV variants. The maps of Mo-MuLV (3, 10, 27, 33, 38), MSV-124 (24, 32, 33, 35), M1, and HT1 (16, 36) were drawn by the use of published results. Only the restriction enzyme cleavage sites which are significant for the comparative presentation of the genomes are shown. Heavy lines, LTR; cross-hatched bar, v-*mos*; dashed lines, deletions.

TABLE 3. Isolation of transformed RAT4 cell clones transfected with cloned MPSV (λ 18-663) and py747L (TK^+) DNA

RAT4 clone	Transformed phenotype	Thymidine kinase	Reverse transcriptase[a]	Focus formation[a,b]
1	+	+	+	+
4	+	+	+	+
5	−	+	+	−
10	+	+	+	+
13	++	+	+	−, −, (+)[c]
14	−	+	+	−

[a] After superinfection with F-MuLV helper virus. Supernatants were used as described in the text.

[b] By concentrated tissue culture supernatant on NRK cells.

[c] Supernatant of RAT4 13 yielded a low level of FFU on NRK cells only once in three independent rescue experiments.

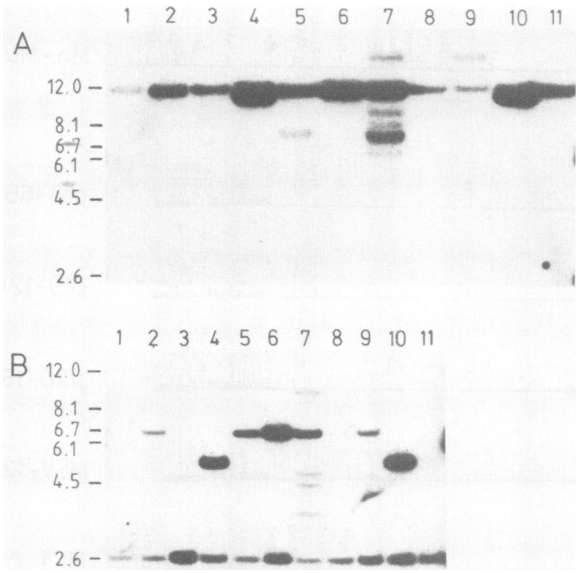

FIG. 5. Transfection of RAT4 cells by MPSV DNA. Restriction enzyme analysis of six independently isolated cell clones with (A) *Eco*RI and (B) *Sst*I. The blots were hybridized to a ^{32}P-labeled c-*mos* probe. The marker was the same as in Fig. 1. Lane 1, NRK; lane 2, 6-6#3; lane 3, RAT4; lane 4, RAT4 13 superinfected with Mo-MuLV; lane 5, RAT4 1 superinfected with Mo-MuLV; lane 6, RAT4 1; lane 7, RAT4 4; lane 8, RAT4 5; lane 9, RAT4 10; lane 10, RAT4 13; lane 11, RAT4 14.

variants of Mo-MuSV, MSV-124 and M1 (Table 4). Two different pseudotypes of all viruses were tested in mice (18). Neither MSV-124 rescued by either Mo-MuLV or F-MuLV nor M1 rescued by Mo-MuLV induced spleen foci at all. A very low incidence of spleen focus induction was observed upon infection of mice with an M1/F-MuLV complex. However, the ratio was 10^{-4} times lower than activity of MPSV.

These results confirm that MPSV differs from the other Mo-MuSV in that it induces spleen foci in mice with a very high incidence, compared with none or a low incidence of induction in Mo-MuSV. The induction of spleen foci mediated by MPSV does not depend on the nature of the helper virus used.

Cloned DNA of ts 159/5/1 (p20-159) was also transfected into NRK cells. Virus-containing clones grown at 32°C expressed a transformed morphology, which reverted to almost normal NRK fibroblast morphology after a temperature shift to 39.5°C (J. Friel, personal communication). This indicates that the expression of the temperature-sensitive phenotype is an intrinsic property of the ts 159/5/1 genome. A detailed characterization of the molecularly cloned mutants and the lesion leading to the temperature-sensitive phenotype will be published elsewhere.

DISCUSSION

Detailed restriction enzyme analysis of MPSV showed that the molecular structure of this virus is strikingly similar to that of Mo-MuSV. All restriction enzymes tested cleaved within the viral sequences at comparable positions. Most of the *gag* region, comprising the sequences between about 1,000 and 2,500 bp on the MPSV genomic map (Fig. 3) (27, 35), seems to be retained if one considers the *Bgl*II site at the 5′ end of the *gag* gene within the coding sequence for p30 (27). It cannot be excluded, however, that some of the information responsible for p10 is included in a deletion which covers the 5′ half of the *pol* gene adjacent to *gag*.

MPSV does not produce an active reverse transcriptase (19). The molecular reason for this defect can now be localized as a deletion between the *Bgl*II site (2,450 bp) and the *Bam*HI site (2,650 bp) (Fig. 3), because the *Sst*I and *Kpn*I cleavage sites, characteristic of the 5′ end of the *pol* gene in Mo-MuLV (27), are missing in MPSV. The deletion comprises about 1.1 to 1.3 kbp at maximum. Compared with other mouse sarcoma viruses generated through recombination between Mo-MuLV and the cellular *mos* gene, MPSV carries the smallest deletion in the *gag-pol* area; MSV-124 suffered a deletion of 2.8 kbp (24, 32, 35, 36), HT1 of 2.3 kbp, and M1 of 3.1 kbp (16, 36). The length and position of the deleted sequences were confirmed by the heteroduplex analysis presented in an accompanying paper (29), in which a deletion of about 1 kbp in this region is described.

One difference which was observed in the *gag* region of MPSV compared with Mo-MuLV (27) was the lack of a second *Pst*I cleavage site in a distance of about 150 bp 3′ to the one indicated at 1,100 bp. This *Pst*I site is also missing in MSV-124 (37).

The v-*mos* gene of MPSV, defined as the sequence between the *Xba*I site at 5,050 bp and the *Hin*dIII site at 6,200 bp (Fig. 3), contains all cleavage sites which are characteristic for the *mos* gene of Mo-MuSV (7, 17, 33). The region 3′ to the gene (between *mos* and LTR) is different in MSV-124 (35), HT1, and M1 (36) compared with MPSV (Fig. 4). Whether these differences can be directly related to the characteristic biological activity of spleen focus induction, which is expressed by MPSV but not by M1 or MSV-124, is unclear.

Donoghue and Hunter (8) have shown that the Mo-MuSV isolates MSV-124, M1, HT1, and Gazdar have an identical 5′ junction between the Mo-MuLV *env* gene and the v-*mos* gene, but that the 3′ junctions between v-*mos* and Mo-MuLV *env* differ in the individual isolates. The restriction enzyme data of MPSV already indicate clearly that this virus

TABLE 4. Biological activity of MPSV and Mo-MuSV[a]

Transforming virus	Transforming variant	Helper virus	Ratio of spleen FFU to fibroblast FFU		FFU tested in mice
			Average	Range	
Mo-MuSV	MSV-124	Mo-MuLV	0	0	2×10^6
	MSV-124	F-MuLV	0	0	5×10^7
	M1	Mo-MuLV	0	0	5×10^7
	M1	F-MuLV	2×10^{-5}	$0–7.2 \times 10^{-5}$	8×10^7
MPSV	6-6#3	Mo-MuLV	0.11	0.08–0.25	10^4
	6-6#3	F-MuLV	0.12	0.07–0.30	4×10^4
p18-663	RAT4 1	F-MuLV	0.10	0.07–0.11	1.1×10^4
	NRK 10	F-MuLV	0.12	0.12	4.3×10^4

[a] Spleen focus formation induced by pseudotypes of MPSV and Mo-MuSV. Virus was concentrated and tested for reverse transcriptase activity (not shown) and for fibroblast FFU and spleen FFU 16 days postinfection of DBA/2J mice. MSV-124 is the Bal1 virus, whereas M1 is the virus which was recovered from NRK cells after transfection with molecularly cloned M1 DNA (36). Range is the average of at least three experiments. Each experiment was done with a minimum of six mice per serial fivefold dilution of the virus concentrate. Numbers higher than 150 spleen foci per mouse were not included in the calculation.

exhibits similar features, although the fine structure of the junction points needs to be analyzed by other methods (29).

The only significant difference in the molecular structure which could be found in the two temperature-sensitive mutants compared with MPSV was a rearrangement around the *Sal*I site in the *pol* gene of ts 124/1 (3,050 bp on the genomic map of MPSV) (Fig. 3), leading to a final addition of about 150 bp of unknown origin and the generation of a second *Bam*HI site. ts 159/5/1 has lost, in addition to the sequences already missing in MPSV, about 1.6 kbp of the *pol* gene comprising most of the sequence between the *Bam*HI site and the *Bgl*II site (2,650 and 4,400 bp on the MPSV map [Fig. 3], respectively). ts 124/1 and ts 159/5/1 are the only temperature-sensitive mutants of MPSV cloned in nonproducer cells and characterized so far which contain alterations in the *pol* gene. The uncloned precursors ts 124 and ts 159 do not show these size alterations, except that ts 124 contains among multiple proviral copies of normal size (Fig. 1A) one copy of reduced size. Since they exhibit the same biological properties as the cloned viruses, the alterations seem to be of secondary origin, occurring during the biological cloning procedure, and are unlikely to be connected with the lesion leading to the temperature-sensitive phenotype. Other temperature-sensitive mutants have been characterized (ts 124/2, ts 143/1/1, and ts 143/4/1 [Fig. 3]) (19) which do not contain these structural changes. It is possible that these mutants have minor alterations as compared with the wild-type MPSV which would only be detectable by detailed restriction enzyme analysis or sequencing. These alterations may interfere with the expression of the *mos* gene, as shown in the case of Mo-MuSV ts 110 (5, 20, 21). Deletions in the viral genomes were also observed after transfection of molecularly cloned MPSV into RAT4 cells. These deletions did not interfere with the transforming capacity of the virus, except that RAT4 13 had apparently lost its potential to be rescued by the helper virus (Table 3). One possible interpretation of this phenomenon is that a function required for *trans*-complemented replication, e.g., a primer binding site, is affected by the extensive deletion of viral sequences.

The biological activity of molecularly cloned, biologically cloned, and uncloned MPSV was compared. All three wild-type isolates induce spleen enlargement in adult DBA/2 mice. The degree of spleen focus to fibroblast focus formation in adult DBA/2 mice was almost identical in all three virus isolates (Table 4) (18), and they all induced an expansion of the red pulp of the spleen and myelofibrosis. All of the isolates altered the proliferation of macrophage/granulocyte colony cells and rendered a fraction independent of colony-stimulating factor for colony formation (14). It was shown that the ability of MPSV to induce spleen foci at a very high frequency is not dependent on the nature of the helper virus and is unique in comparison with Mo-MuSV. These data prove that these biological properties are directly associated with, and characteristic of, MSPV.

Two questions remain to be answered. The first concerns the location of the mutation leading to the temperature-sensitive phenotype, and the second concerns the dual transformation capacity of MPSV for fibroblasts and hematopoietic cells. Recombinants between the temperature-sensitive mutants and MPSV, as well as consequent sequencing of the viral gene responsible for the temperature-sensitive effect, should be helpful in answering the first question. The answer to the second question may be more difficult to obtain. The specific transforming potential of MPSV is determined by either the *mos* gene or the additional residual Mo-MuLV sequences, since no further cellular sequences have been acquired by MPSV (22, 29) which could account for the acquisition of the capacity to transform hematopoietic cells. Experiments are in progress to determine which of the two possibilities is correct.

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LITERATURE CITED

1. **Bassin R. H., P. J. Simons, F. C. Chesterman, and J. J. Harvey.** 1968. Murine sarcoma virus (Harvey): characteristics of focus formation in mouse embryo cell cultures, and virus production by hamster tumor cells. Int. J. Cancer **3**:265–272.
2. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. Science **196**:180–182.
3. **Berns, A. J. M., M. H. T. Lai, R. A Bosselman, M.A. McKennett, L. T. Bacheler, H. Fan, E. C. Robanus Maandag, H. v.d. Putten, and I. M. Verma.** 1980. Molecular cloning of unintegrated and a portion of integrated Moloney murine leukemia viral DNA in bacteriophage lambda. J. Virol. **36**:254–263.
4. **Blair, D. G., W. L. McClements, M. K. Oskarsson, P. J. Fischinger, and G. F. Vande Woude.** 1980. Biological activity of cloned Moloney sarcoma virus DNA: terminally redundant sequences may enhance transformation efficiency. Proc. Natl. Acad. Sci. U.S.A. **77**:3504–3508.
5. **Brown, R., I. P. Horn, L. Wieble, R. B. Arlinghaus, and B. R. Brinkley.** 1981. Analysis of the sequence of events in the transformation process in cells infected with a ts transformation mutant of Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **78**:5593–5597.
6. **Chirigos, M. A., W. Scott, W. Turner, and K. Perk.** 1968. Biological, pathological and physical characteristics of a possible variant of a murine sarcoma virus (Moloney). Int. J. Cancer **3**:223–237.
7. **Donoghue, D. J.** 1982. Demonstration of biological activity and nucleotide sequence of an in vitro synthesized clone of the Moloney murine sarcoma virus *mos* gene. J. Virol. **42**:538–546.
8. **Donoghue, D. J., and T. Hunter.** 1983. Recombinational junctions of variants of Moloney murine sarcoma virus: generation and divergence of a mammalian transforming gene. J. Virol. **45**:607–617.
9. **Friend, C.** 1957. Cell free transmission in adult Swiss mice of a disease having the character of a leukemia. J. Exp. Med. **105**:307–318.
10. **Gilboa, E., S. Goff, A. Shields, F. Yoshimura, S. Mitra, and D. Baltimore.** 1979. In vitro synthesis of a 9 Kb terminally redundant DNA carrying the infectivity of Moloney murine leukemia virus. Cell **16**:863–874.
11. **Godson, G. N., and D. Vapnek.** 1973. A simple method of preparing large amounts of ϕX174RF supercoiled DNA. Biochim. Biophys. Acta **299**:516–523.
12. **Jähner, D., H. Stuhlmann, and R. Jaenisch.** 1980. Conformation of free and of integrated Moloney leukemia virus proviral DNA in preleukemic and leukemic BALB/Mo mice. Virology **101**:111–123.
13. **Jasmin, C., M. C. Le Bousse-Kerdiles, B. Klein, F. Smadja-Joffe, K. J. Mori, B. Fagg, W. Ostertag, K. Vehmeyer, and I. B. Pragnell.** 1980. The physiopathology of the disease induced in DBA/2 by MPSV, p. 183–192. *In* G. B. Rossi (ed.), In vivo and in vitro erythropoiesis. Elsevier/North Holland, Amsterdam.
14. **Klein, B., M. C. Le Bousse, B. Fagg, F. Smadja-Joffe, K. Vehmeyer, J. Mori, C. Jasmin, and W. Ostertag.** 1981. Effects of myeloproliferative sarcoma virus on the pluripotent stem cell and granulocyte precursor cell populations of DBA/2 mice. J.

Natl. Cancer Inst. **66**:935–944.
15. **Le Bousse-Kerdiles, M. C., F. Smadja-Joffe, B. Klein, B. Caillon, and C. Jasmin.** 1980. Study of a virus-induced myeloproliferative syndrome associated with tumor formation in mice. Eur. J. Cancer **16**:43–51.
16. **McClements, W. L., L. W. Enquist, M. Oskarsson, M. Sullivan, and G. F. Vande Woude.** 1980. Frequent site-specific deletion of coliphage λ murine sarcoma virus recombinants and its use in the identification of a retrovirus integration site. J. Virol. **35**:488–497.
17. **Oskarsson, M., W. L. McClements, D. G. Blair, J. V. Maizel, and G. Vande Woude.** 1980. Properties of a normal mouse cell DNA sequence (*sarc*) homologous to the *src* sequence of Moloney sarcoma virus. Science **207**:1222–1224.
18. **Ostertag, W., M. Freshney, K. Vehmeyer, C. Jasmin, and G. Rutter.** 1984. Action of temperature-sensitive mutants of myeloproliferative sarcoma virus suggests that fibroblast transforming and hematopoietic transforming viral properties are related. J. Virol. **49**:173–181.
19. **Ostertag, W., K. Vehmeyer, B. Fagg, I. B. Pragnell, W. Paetz, M. C. Le Bousse, F. Smadja-Joffe, B. Klein, C. Jasmin, and H. Eisen.** 1980. Myeloproliferative virus, a cloned murine sarcoma virus with spleen focus-forming properties in adult mice. J. Virol. **33**:573–582.
20. **Peltier-Horn, J., T. G. Wood, D. G. Blair, and R. Arlinghaus.** 1980. Partial characterization of a Moloney murine sarcoma virus 85,000 dalton polypeptide whose expression correlates with the transformed phenotype in cells infected with a temperature-sensitive mutant virus. Virology **105**:516–525.
21. **Peltier-Horn, J., T. G. Wood, E. C. Murphy, Jr., D. G. Blair, and R. B. Arlinghaus.** 1981. A selective temperature sensitive defect in viral RNA expression in cells infected with a ts transformation mutant of murine sarcoma virus. Cell **15**:37–46.
22. **Pragnell, I. B., A. Fusco, C. Arbuthnott, F. Smadja-Joffe, B. Klein, C. Jasmin, and W. Ostertag.** 1981. Analysis of the myeloproliferative sarcoma virus genome: limited changes in the prototype lead to altered target cell specificity. J. Virol. **38**:952–957.
23. **Rauscher, F.** 1962. A virus induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J. Natl. Cancer Inst. **29**:515–543.
24. **Reddy, E. P., M. J. Smith, and S. A. Aaronson.** 1981. Complete nucleotide sequence and organization of the Moloney murine sarcoma virus genome. Science **214**:445–450.
25. **Rigby, P. W. J., M. Diekmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
26. **Scher, C. D., E. M. Scolnick, and R. Siegler.** 1975. Induction of erythroid leukemia by Harvey and Kirsten sarcoma viruses. Nature (London) **256**:225–227.
27. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukemia virus. Nature (London) **293**:543–548.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–513.
29. **Stacey, A., C. Arbuthnott, R. Kollek, L. Coggins, and W. Ostertag.** 1984. Comparison of myeloproliferative sarcoma virus with Moloney murine sarcoma virus variants by nucleotide sequencing and heteroduplex analysis. J. Virol. **50**:725–732.
30. **Steeves, R. A.** 1975. Spleen focus forming virus in Friend and Rauscher leukemia virus preparations. J. Natl. Cancer Inst. **54**:289–297.
31. **Topp, W. C.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113**:408–411.
32. **Tronick, S. R., K. C. Robbins, E. Canaani, S. G. Devare, P. R. Andersen, and S. A. Aaronson.** 1979. Molecular cloning of murine sarcoma virus: arrangement of virus-related sequences within the normal mouse genome. Proc. Natl. Acad. Sci. U.S.A. **76**:6314–6318.
33. **Van Beveren, C., J. A. Galleshaw, V. Jonas, A. J. M. Berns, R. F. Doolittle, D. J. Donoghue, and I. M. Verma.** 1981. Nucleotide sequence and formation of the transforming gene of a mouse sarcoma virus. Nature (London) **289**:258–262.
34. **Van Beveren, C., J. G. Goddard, A. J. M. Berns, and I. M. Verma.** 1980. Structure of Moloney murine leukemia viral DNA: nucleotide sequence of the 5′ long terminal repeat and adjacent cellular sequences. Proc. Natl. Acad. Sci. U.S.A. **77**:3307–3311.
35. **Van Beveren, C., F. van Straaten, J. A. Galleshaw, and I. M. Verma.** 1981. Nucleotide sequence of the genome of a murine sarcoma virus. Cell **27**:97–108.
36. **Vande Woude, G. F., M. Oskarsson, L. W. Enquist, S. Nomura, M. Sullivan, and P. J. Fischinger.** 1979. Cloning of integrated Moloney sarcoma proviral DNA sequences in bacteriophage lambda. Proc. Natl. Acad. Sci. U.S.A. **76**:4464–4468.
37. **Verma, I. M., M.-H. T. Lai, R. A. Bosselman, M. A. McKennett, H. Fan, and A. Berns.** 1980. Molecular cloning of unintegrated Moloney mouse sarcoma virus DNA in bacteriophage λ. Proc. Natl. Acad. Sci. U.S.A. **77**:1773–1777.
38. **Verma, I. M., and M. A. McKennett.** 1978. Genome organization of RNA tumor viruses. II. Physical maps of in vitro synthesized Moloney murine leukemia virus double stranded DNA by restriction endonuclease. J. Virol. **26**:630–645.
39. **Wigler, M., S. Silverstein, L.-S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.
40. **Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734–745.
41. **Yamamoto, Y., C. L. Gamble, S. P. Clark, A. Joyner, T. Shibuya, M. E. McDonald, D. Mager, A. Bernstein, and T. W. Mak.** 1981. Clonal analysis of early and late stages of erythroleukemia induced by molecular clones of integrated spleen focus-forming virus. Proc. Natl. Acad. Sci. U.S.A. **78**:6893–6897.