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Analysis of the Myeloproliferative Sarcoma Virus Genome: Limited Changes in the Prototype Lead to Altered Target Cell Specificity

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The myeloproliferative sarcoma virus (MPSV) derived from Moloney sarcoma virus (MSV-Mol) is a unique sarcoma virus which causes expansion of the hematopoietic stem cell compartment as well as the erythroid and myeloid cell lineages. MPSV also induces spleen focus formation in adult mice as do Friend and Rauscher viruses. Analysis of the MPSV genome on methyl mercury gels showed that the genome size is 7.0 kilobases, which is larger than the defective genome of any known MSV-Mol isolate. Hybridization analysis with specific cDNA probes showed that MPSV is a modified sarcoma virus with no sequences in the unique region of the defective sarcoma genome related to unique Friend virus sequences. The only viral sequences in the defective genome other than helper virus-related sequences are derived from the Moloney sarcoma virus genome with no new cellular sequences added. There was no evidence for induction of xenotropic virus sequences in MPSV-infected spleens of DBA/2J mice, indicating that spleen focus formation can be obtained by different mechanisms.

We have described a myeloproliferative sarcoma virus [MPSV or MPV(MSV)] (15), derived from a Moloney sarcoma virus preparation (3). This virus induces spleen foci, erythroblastosis, and myelofibrosis in adult mice (11, 15) while retaining the sarcoma virus property of transforming fibroblasts in vitro. We have shown that MPSV consists of two separable entities which have been cloned (15). The MPSV complex consists of a replication-competent helper virus, murine leukemia virus (MuLV), and a defective sarcoma virus. The fibroblast-transforming and spleen focus-forming properties reside in the defective virus genome (15).

There is considerable evidence which suggests that transforming retroviruses contain different specific sequences which are related to genes in the normal cellular DNA (5-7, 18, 19, 22-25). The ancestral virus of MPSV derived from Moloney sarcoma virus, MSV-Mol, has been demonstrated to contain sequences (*src*) homologous to cellular DNA (6), and these sequences are presumably involved in both the transformation of fibroblasts in vitro and the induction of sarcoma tumors in newborn mice. MSV-Mol was itself derived from MuLV-Mol. Similarly, Kirsten sarcoma virus (KiSV) and Harvey sarcoma virus (HaSV) have been derived by passage of murine erythroblastosis virus in rats (10). It has been demonstrated that the HaSV and KiSV sequences are derived partly from rat cellular sequences and partly from helper virus sequences (21). Some of the rat cellular sequences are required for transforming potential (29).

Since MPSV both transforms fibroblasts in vitro and affects the hematopoietic compartment in vivo, we analyzed the MPSV genome in some detail, to delineate the specific parts of the genome and their homology to other known transforming viruses. The molecular analysis suggests a hypothesis to explain the complex nature of the disease induced by MPSV in susceptible mice.

MATERIALS AND METHODS

Cell culture and viruses. All cell lines were grown in modified Eagle medium supplemented with 10% fetal calf serum (15). The cell lines used in these studies and referred to in the figures and text are as described below. Unless stated otherwise we have used the same nomenclature for both the cell line and the virus released by that particular cell line.

MPSV has been cloned on normal rat kidney (NRK) cells (15). We have used two clones of MPSV in these studies, 6-6#3+F (cloned once) and p5-8#1+F (cloned twice) (15). Clone 6-6#3+F is released by 6-6#3+F producer cells which were obtained by

rescue of transformed cloned 6-6#3 nonproducer NRK fibroblasts with Friend helper virus (MuLV-F). The helper virus used to rescue MPSV was 643/22N (15). Similarly, clone p5-8#1+F is virus released by p5-8#1+F producer cells which were obtained by rescue of cloned p5-8#1 nonproducer NRK cells with helper virus 643/22N. Friend virus (17) was cloned on SC1 cells to give an SFFV-nonproducer cell line, SC204. Cloned Friend virus SC204+F was rescued from SC204 cells with helper virus 643/22N. Cloned MuLV-Mol was released by cell line a2, an NIH/3T3 cell line infected with MuLV-Mol. The murine sarcoma virus MSV-Mol used was the Ball variant virus released by the murine TB cell line (G8-124) of CFW1D mice coinfected with MSV-Mol and MuLV-Mol. The Abelson virus used here was an uncloned isolate which was released by transformed murine B cells (Steinheider and Ostertag, unpublished data). The xenotropic virus-infected cell lines were provided by J. Bilello, Hamburg (NZB xenotropic virus) and N. Teich, London (BALB-2 xenotropic virus). The KiSV used was that released by a rat NRK cell line which contains both Friend helper virus (643/22N) and KiSV (obtained from J. Bilello, Hamburg). MSV-Harvey was the Harvey strain of murine sarcoma virus, with 643/22N as helper virus.

Isolation of viral and cytoplasmic RNA. Viral RNA was extracted from virions purified on sucrose gradients. Total virion RNA was precipitated with ethanol and desalted by dialysis through Sartorius Collodion dialysis bags. RNA (50 to 70S) was isolated from the various virion preparations as described (14). Cytoplasmic RNA was isolated from cells in culture by methods described previously (2). Total cellular RNA from normal DBA/2J and virus-infected mouse spleen cells was extracted by the guanidinium thiocyanate-cesium chloride method (28).

Molecular size determination. Genomic viral RNA (50 to 70S) was isolated from the supernatant of cells labeled with sodium [^{32}P]phosphate as described previously (14). Electrophoretic analysis of 30 to 35S subunit RNAs was carried out on 5 mM CH_3HgOH–1.5% agarose gels (4). Gels were run and dried, and autoradiography was performed with X-ray intensifying screens.

Synthesis of cDNA. (i) Total representative cDNA for the viruses used in this study was synthesized with the endogenous enzyme in a lysed virion incubation, in all cases with calf thymus DNA hydrolysate as primer (16). The probes were shown to be uniformly representative of the MPSV genome by the fact that 65% of the ^{32}P-labeled MPSV genome is protected at a cDNA-RNA ratio of 2:1 (14). (ii) Total representative MPSV-specific cDNA from the two independently cloned isolates 6-6#3+F and p5-8#1+F was hybridized to excess viral RNA isolated from the cloned helper virus 643/22N (see above), and the nonhybridized material was separated on hydroxylapatite (16). (iii) Total representative MSV-Mol cDNA was prepared. MSV-specific cDNA was isolated by subtractive hybridization as described above, with MuLV-Mol viral RNA as the source of helper virus RNA. (iv) SFFV-specific cDNA was isolated from total representative cDNA by using F4-6 virus. The source of helper virus RNA was virus clone 643/22N. This SFFV-specific cDNA contains xenotropic virus-related sequences, in agreement with data published by Troxler et al. (26). In contrast, when Friend helper virus clone 643/22F or a mixture of 643/22N and 643/22F is used, as in previous studies (16), then the SFFV-specific cDNA does not contain xenotropic virus-related sequences, since they are removed during the subtractive hybridization to viral RNA from clone 643/22F, which contains recombinant sequences.

Hybridization analysis with specific cDNA probes. (i) For measurement of expression of specific sequences, cytoplasmic or total cellular RNA was mixed with 0.2 ng (2,200 cpm) of specific cDNA at a cDNA-RNA ratio of 1:20,000 and incubated in 0.36 M NaCl–0.025 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.0)–0.005 M EDTA–0.1% sodium dodecyl sulfate at 60°C for various times. The amount of cDNA hybridized was determined by S1 nuclease digestion (17). The percentage of virus-specific RNA in cellular RNAs was calculated on the basis of the Cr_0t of a standard curve, made with specific cDNA and purified genomic 50 to 70S RNA. The rate of reaction was 2.3-fold faster than the standard conditions with 0.18 M Na^+ and was therefore normalized. (ii) For measurement of genome relatedness, specific cDNA probes were hybridized to various preparations of viral RNA in conditions of large RNA excess (500:1 and 1,000:1 RNA-cDNA ratios in molar amounts). The values obtained represent saturation values and are the means of the two ratios in a number of experiments. There was no more than a 5% variation in the values obtained in these experiments, which is normal for this type of plateau hybridization analysis.

RESULTS

Molecular size of the MPSV genome. Our previous studies have shown that MPSV contains a defective transforming virus and a replication-competent helper virus (15). The properties of fibroblast transformation in vitro and spleen focus formation in vivo have been shown to reside in the same defective viral genome subunit. Since MPSV was derived from MSV-Mol, an analysis of the genome size and its relatedness to MSV-Mol and other defective transforming viruses was carried out. ^{32}P-labeled genomic RNA was analyzed on methyl mercury gels (Fig. 1). Lane 1 shows cytoplasmic RNA extracted from G8-124 cells used as a marker in this experiment. Lane 2 shows genomic RNA from MSV-Mol virus; the size of the major genomic RNA species is 30S (6.0 kilobases [kb]), in agreement with data published previously (12). There is another band at 5.6 kb which may represent a deletion in the 6.0-kb genome. Lane 3 shows that the 6-6#3+F virus has a major band at 7.0 kb (33S RNA) and a minor band at 8.5 kb (35S RNA). The other MPSV clone, p5-8#1+F, also displays bands at 33 and 35S (lane 4). We can assign the 8.5-kb subunit to the helper virus, since cloned helper virus genomic RNA has a major band at 8.5 kb (lane 5). We

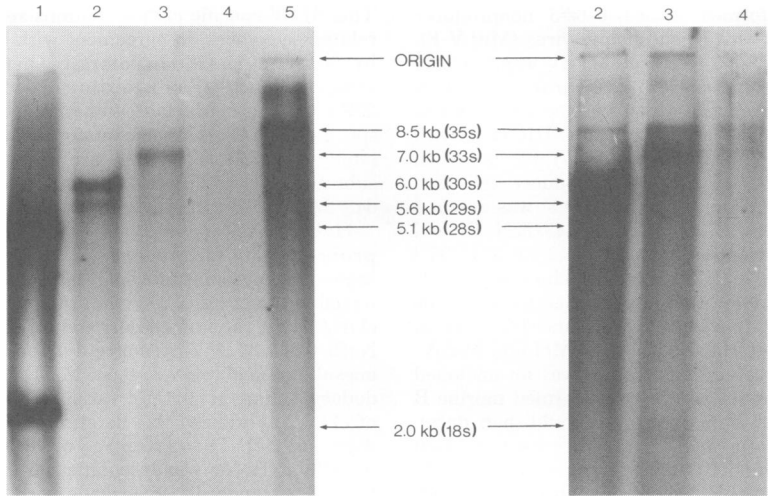

FIG. 1. *Analysis of genomic RNA on methyl mercury gels. ^{32}P-labeled genomic RNAs were run on methyl mercury gels as described in the text. Lane 1, Cytoplasmic RNA from G8-124 cells used as a marker. Lanes 2 to 5 contain genomic viral RNA. Lane 2, MSV-Mol (Ball variant). Lane 3, MPSV clone 6-6#3+F. Lane 4, MPSV clone p5-8#1+F. Lane 5, MuLV-F clone 643/22N. A longer exposure of lanes 2, 3, and 4 is shown on the right, to enhance the MPSV defective genome and helper virus genome of MPSV clone p5-8#1+F. Lengths were calculated from a semilogarithmic plot of the distance of each band from the origin of the gel, using the ribosomal 28S (5.1 kb) and 18S (2.0 kb) RNAs as markers. The plot is linear in the range 0.85 to 7.05 kb.*

therefore conclude that the MPSV genome is about 7.0 kb. There is an excess of genomic RNA from defective virus over helper virus RNA in both clones. This ratio varied with each infection of the nonproducer cells.

Characterization of MPSV-specific cDNA. We isolated specific cDNA probes and determined their relatedness to the viral genomes of other transforming viruses. A specific cDNA probe containing only nonhelper virus-related sequences was prepared from MPSV clone p5-8#1. Saturation of MPSV representative cDNA with excess helper virus RNA gave a plateau hybridization value of 70% compared with 100% for the template viral RNA. Thus, approximately one-third of the MPSV genome is MPSV specific. The specificity of the probes was shown by hybridization to genomic MPSV viral RNA which completely protected the cDNA's and to helper virus RNA (both MuLV-F and MuLV-Mol) which did not show any significant hybridization (Table 1). Thus, the specific cDNA does not contain any helper virus-related sequences, but does contain the specific sequences of MPSV clones p5-8#1+F and 6-6#3+F.

Relatedness of MPSV to MSV-Mol. Since MPSV was derived from MSV-Mol it was important to determine, first, whether the unique (nonhelper-related) part of the MSV genome had been retained and, second, whether there were any sequences present in MPSV not related to MSV-Mol. MSV-Mol-specific cDNA was completely protected by MSV-Mol viral RNA, whereas hybridization to both MuLV-Mol and MuLV-F (clone 643/22N) showed that there were no helper virus-related sequences in this probe (Table 1).

The MSV-Mol-specific cDNA was completely protected with excess MPSV viral RNA (Table

TABLE 1. *Relatedness of MPSV to other viral genomes*[a]

Viral RNA	% Specific cDNA hybridized		
	MPSV p5-8#1	MSV	SFFV
MuLV-F	9	4	7
MuLV-Mol	4	8	10
MPSV p 5-8#1+F	75	89	11
6-6#3+F	71	85	7
MSV-Mol	70	93	NT
KiSV	4	NT	NT
HaSV	6	NT	NT
A-MuLV	6	NT	NT
F4-6	10	NT	79
SC204+F	5	NT	81
NZB-X	6	NT	57
BALB-2-X	8	NT	54

[a] Specific cDNA (0.2 ng, 2,200 cpm) was hybridized with a large excess of viral RNA (see text) in 1 μl of hybridization buffer at 60°C for 4 days. The values shown represent saturation values and are the means of the two ratios (see text) in a number of experiments. NT, Not tested.

1). Thus, the complete unique part of the MSV-Mol genome is retained in both clones of MPSV. It can be seen that most or all of the MPSV-specific cDNA of clone p5-8#1+F could be protected with excess MSV-Mol viral RNA (Table 1), indicating that most or all of the specific part of the MPSV genome (p5-8#1) is MSV-Mol derived.

Relatedness of MPSV to other viral genomes. KiSV and HaSV induce splenomegaly and erythroblastosis in newborn mice, but no KiSV- or HaSV-related sequences could be found in p5-8#1 specific cDNA (Table 1), and very few contaminating NRK cytoplasmic RNA sequences were found in this probe (data not shown).

A-MuLV is a defective retrovirus which, like MPSV, transforms fibroblasts in vitro (19), but in contrast to MPSV, A-MuLV transforms B-type lymphoid cells (24). There was no significant relatedness of A-MuLV to the specific part of the MPSV genome (Table 1). These results confirm that sarcoma virus-specific sequences are not present in the A-MuLV genome (20).

Both MPSV and SFFV induce spleen foci and erythroblastosis in susceptible mice. Both viruses are subject to restriction by the *Fv-2* gene (13). Hybridization of MPSV-specific cDNA to excess Friend virus RNA (F4-6 virus, see above) did not result in any significant hybrid formation (Table 1). In a reciprocal experiment, SFFV-specific cDNA was not protected by MPSV viral RNA (Table 1). There was no significant hybridization of MPSV-specific cDNA to RNA of two isolates of xenotropic virus NZB and BALB-2. In the same experiment the presence of xenotropic virus sequences was detected in SFFV-specific cDNA (Table 1, see above). The SFFV-specific cDNA could be completely protected by viral RNA from two FV isolates (F4-6 and SC204+F) and contained no helper virus-related sequences (Table 1). We conclude that there is no detectable homology between the non-helper virus-related sequences of MPSV and SFFV.

The expression of MPSV in transformed nonproducer cells and in infected spleens in vivo. We have measured the levels of MPSV-specific RNA in two clones of MPSV nonproducer-transformed fibroblasts (Table 1). The levels detected are somewhat lower than those found in infected spleen cells in vivo.

To test whether MPSV induces SFFV-related sequences during infection of the spleen cells in vivo, we measured MPSV- and SFFV-related expression in both MPSV- and SFFV-infected spleens (Table 2) with specific cDNA. The relative amount of MPSV RNA was similar to that detected in SFFV-infected spleens with SFFV-specific cDNA. We did not find any increase in SFFV-related expression in MPSV-infected spleens over the levels detected in normal spleen cells with SFFV-specific cDNA. We conclude that there is not detectable induction of xenotropic virus or SFFV-related sequences in MPSV-infected spleens.

DISCUSSION

Apart from induction of myeloproliferation in susceptible DBA/2J and BALB/c mice, MPSV also induces spleen focus formation and erythroblastosis (15). MPSV, like SFFV, does not induce spleen foci in C57BL mice, and increased expression of SFFV-related sequences in normal DBA/2J mice has been linked to the susceptibility of these mice to SFFV infection (1). SFFV has also been shown to be a recombinant between an ecotropic murine provirus and the *env* gene region of a xenotropic virus (27). We did not find any evidence for the presence of xeno-

TABLE 2. *Expression of MPSV- and SFFV-specific sequences*[a]

RNA	MPSV expression (%)		SFFV expression (%)
	p5-8#1	6-6#3	
Normal spleen	<0.0001	NT	0.0012 ± 0.0004
MPSV-infected spleen	0.036 ± 0.01	NT	0.0018 ± 0.0006
SFFV-infected spleen	0.0001	NT	0.089 ± 0.03
p5-8 1 nonproducer cells	0.011 ± 0.005	0.0045	<0.0001
6-6 3 nonproducer cells	0.005 ± 0.002	0.012	NT

[a] Infected DBA/2J mice were sacrificed 9 days after injection of 10^3 spleen focus-forming units of SFFV or 2 × 10^3 spleen focus-forming units of MPSV into the tail vein, at which time there was marked splenomegaly. The spleen weights in both cases were about 1 g. Injection of higher dilutions of SFFV or MPSV gave rise to spleen focus formation in these conditions. Total cellular RNA and cytoplasmic RNA were extracted from normal and infected spleens as described in the text. Cytoplasmic RNA was extracted from MPSV nonproducer NRK fibroblasts as described in the text. MPSV- and SFFV-specific cDNA's were used to determine the percentage of the RNA which is virus related as described in the text. Except in cases where values of <0.0001% were obtained, the specific cDNA probes were completely protected (70%) at high Cr_0t values. The values shown constitute the means of a number of experiments. NT, Not tested.

tropic virus recombinant sequences in the MPSV genome (Table 1). We conclude from our measurements of MPSV and SFFV expression in susceptible mice (Table 2) that although there may be a relationship between susceptibility to SFFV and expression of endogenous SFFV-related RNA in normal, susceptible mice (1), there is no such link in the case of MPSV. Our results show in addition that xenotropic virus recombinant sequences are not induced in MPSV-infected spleens. The mechanism of transformation by MPSV is therefore likely to be mediated in a manner different from that by SFFV.

Molecular size analysis of the MPSV genome has shown it to be 7.0 kb (this paper), in contrast to the 6.0-kb size of the MSV-Mol genome (12 and this paper). Thus, the MPSV genome is larger than the MSV-Mol sarcoma virus genome. It is therefore possible that sequences have been added to the original MSV-Mol genome to give rise to the different biological activity. However, since we do not have the ancestral MSV-Mol available, it is equally possible that MPSV is derived from an MSV-Mol prototype with a larger genome, or even from MuLV-Mol itself. The original experiments by Chirigos et al. (3) employed MSV with MuLV-Mol as helper virus.

It is clear that most, if not all, of the non-helper virus-related part of the defective MSV-Mol genome is present in the MPSV since all of the MSV-specific cDNA is protected by viral RNA from both MPSV clones. Since most of clone MPSV p5–8≠1-specific cDNA can be protected by MSV-Mol viral RNA, we conclude that the specific part of the MPSV genome in clone p5–8≠1 is almost wholly MSV-Mol derived. This shows that MPSV is not a new virus generated fortuitously during infection of mice with MSV-Mol, but must be derived from MSV-Mol or MuLV-Mol by some kind of genomic alteration.

We found no rat cellular RNA-, KiSV-, or HaSV-related sequences in clone p5–8≠1-specific cDNA (Table 1). We did, however, detect NRK RNA-related sequences in the specific cDNA prepared from MPSV clone 6–6≠3+N (data not shown). Since both virus clones have similar biological activities, we assume that the rat cellular RNA sequnces are not present in the genome of MPSV clone 6–6≠3+F, but are fortuitously included in virus particles during virus release and therefore have no significance regarding the pathology of MPSV.

Preliminary evidence from oligonucleotide fingerprint data (Pragnell, Nunn, and Duesberg, unpublished data) suggests that all of the sequences in the defective genomes of both MPSV clones are related to MSV-Mol and helper virus (MuLV-Mol) sequences. Thus, we can conclude that the MPSV genome does not contain a large section of sequences which are not related to the MSV-Mol genome. It is likely, therefore, that the larger size of the MPSV genome in comparison to MSV-Mol is afforded by MuLV-Mol-derived sequences. We cannot exclude the attractive hypothesis that the additional 1.0-kb sequences are located adjacent to the MSV-Mol-specific sequences in MPSV, assuming in fact that these sequences are related to MuLV-Mol.

The disease caused by MPSV in adult mice is complex and involves a number of different cell types in the hemopoietic compartment (9, 11), whereas MSV-Mol does not have this biological activity. The molecular analysis of the MPSV genome and its expression in spleen cells leads us to propose a molecular basis for the induction of myeloproliferative disease by MPSV. We suggest that MPSV, by virtue of limited changes in the MSV-Mol genome involving no new cellular sequences or non-MuLV-Mol viral sequences, has become a potent sarcoma virus which will transform a wide range of cells. The broad range of target cell specificity could be investigated using an in vitro transformation system (8).

In summary, MPSV is the first murine retrovirus not containing xenotropic virus recombinant sequences which induces spleen foci in mice. Spleen focus formation can thus be obtained in adult mice by two different mechanisms. It has been observed that avian and murine retroviruses can act to give rather similar disease patterns, yet have different transforming genes (5). MPSV is also the first sarcoma virus which has gained an altered target cell specificity without the addition of new cellular sequences or non-helper virus-related sequences. This indicates a mechanism whereby a virus with multiple target cell specificity may develop.

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