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The Malignant Histiocytosis Sarcoma Virus, a Recombinant of Harvey Murine Sarcoma Virus and Friend Mink Cell Focus-Forming Virus, Has Acquired Myeloid Transformation Specificity by Alterations in the Long Terminal Repeat

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The malignant histiocytosis sarcoma virus (MHSV), in contrast to other viruses with the *ras* oncogene, induces acute histiocytosis in newborn and adult mice. Molecular structure and function studies were initiated to determine the basis of its unique macrophage-transforming potential. Characterization of the genomic structure showed that the virus evolved by recombination of the Harvey murine sarcoma virus (Ha-MuSV) and a virus of the Friend-mink cell focus-forming virus family. Structural analysis of MHSV showed two regions of the genome that are basically different from the Ha-MuSV: (i) the *ras* gene, which is altered by a point mutation in codon 181 leading to a Cys → Ser substitution of the p21 protein, and (ii) the U3 region of the long terminal repeat, which is largely derived from F-MCFV and contains a deletion of one direct repeat as well as a duplication of an altered enhancer-like region. Biological studies of Ha-MuSV, MHSV, and recombinants between the two viruses show that the U3 region of the MHSV long terminal repeat is essential for the malignancy and specificity of the disease. A contributing role of the *ras* point mutation in determining macrophage specificity, however, cannot be excluded.

Strong tissue-specific enhancer elements have been shown to determine the oncogenic potential of slowly transforming retroviruses (2, 4, 7). A high replication rate, via efficient transcription, in a permissive cellular background promotes virus spread and thus increases the probability of an integration event that activates a transformation-sensitive locus.

In acutely transforming retroviruses that carry a transduced oncogene in their genome, pathogenicity can be determined solely by the transforming protein. Indeed, chimeric viruses obtained either by the exchange of oncogenes encoding protein-tyrosine kinases or by substitution of the long terminal repeat (LTR) have shown that disease specificity is a function of the oncogene (15, 17, 27, 28, 34). However, there are also examples in which a unique transformation property is not determined by the oncogene but primarily by specific sequences of the viral enhancer (40, 41), a feature shared with slowly transforming viruses. Hence, in these cases, target cell specificity is established by a match of *cis*-acting elements in the LTR and their cognate proteins expressed in a particular cell. As a practical application, viral enhancers that confer a restrictive target cell selection may be useful probes for the detection of cell-specific transcription factors.

We have described a murine retrovirus carrying a c-Ha-*ras*-related oncogene that was isolated after passage of a cloned Friend helper virus through newborn mice. The virus induced splenomegaly and severe anemia within a few days after inoculation of adult mice. Histological examination of the spleens and bone marrow of infected animals showed that they were composed almost exclusively of histiocytic tumor cells, which led to the designation malignant histiocytosis sarcoma virus (MHSV) (12, 25). As the pathogenesis induced by MHSV resembles that of the malignant histiocytosis found in humans, the virus may provide a suitable animal model for the human disease.

On infection of single isolated myeloid precursor cells, MHSV, unlike other acutely transforming murine retroviruses, induces growth factor-independent proliferation and differentiation at a high frequency (16, 18). A minority of these cells can acquire immortality after prolonged in vitro culture. These cell lines retain most of the basic physiological functions of macrophages, such as synthesis of lysozyme (19, 25) or enzymes regulating eicosanoid synthesis (K. Wessel, V. Kaever, W. Ostertag, and K. Resch, J. Leukocyte Biol., in press). MHSV can also convert immature macrophage precursor cells in murine osteopetrosis to functional macrophages and can thus partially correct the deficiency in this disease (W. W. Jedrzejczak and W. Ostertag, unpublished observations).

Macrophage target cell selectivity and transformation potential of MHSV are unique compared with those of other transforming viruses containing *ras*-related sequences, such as the Harvey murine sarcoma virus (Ha-MuSV) or the newly isolated NS.C58 murine sarcoma virus type 1. The latter viruses elicit a pronounced erythroid hyperplasia in newborn mice (13) and either no disease or only a transient erythroid hyperplasia and benign proliferation of myeloid precursor cells in sensitive adult mice (12).

To understand which sequences of MHSV determine cell tropism and leukemogenic potential, the proviral DNA was molecularly cloned and its structure and biological activity were characterized. The construction of a recombinant virus

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showed that the LTR is a major determinant of disease specificity.

MATERIALS AND METHODS

Cell lines, transfections, and viruses. The cell line 643/22F is an SC1 mouse fibroblast cell line that was established after endpoint dilution of a biologically cloned Friend murine leukemia virus (F-MuLV) (32). Analysis has shown that, in addition to the F-MuLV genome, this cell line releases a replication-defective cytopathic Friend-mink cell focus-forming virus (F-MCFV), which presumably arises through recombination during establishment of the virus-producing cell. A BALB/c mouse infected with this virus complex developed splenomegaly and sarcoma. Two nonproducer NRK cell lines, 19T clone 3 and 24T clone 3, were established after endpoint dilution of the virus complex obtained from the spleen supernatant of the infected mouse. Superinfection of these cell lines with cloned F-MuLV (643/22N; 30) results in release of high titers of transforming virus, which induces a rapid malignant histiocytosis in infected adult animals. Southern blot analysis of genomic DNA determined that both 19T clone 3 and 24T clone 3 contain a single proviral genome which harbors a c-Ha-*ras*-related oncogene (12).

RAT1 cells (44) were used for transfection/infection and titration experiments. DNA transfections were carried out by modifications of established procedures (40). Plasmid DNA (5 μg) was cotransfected with plasmid pM5neo (23) at a molar ratio of 10:1, and medium supplemented with the amino-glycoside analog G418 (GIBCO Laboratories) at a concentration of 400 μg/ml was used for selection of neomycin-resistant clones.

To obtain infectious pseudotypes, RAT1 clones were superinfected with cloned F-MuLV helper virus (30). The titer of infectious virus was determined by measuring reverse transcriptase activity (33) and transformation of RAT1 fibroblasts. Titers of transforming virus are expressed as fibroblast focus-forming units (FFU) per milliliter.

All cells were grown in modified Eagle medium (MEM; GIBCO) supplemented with 10% fetal calf serum (30).

Nucleic acid analysis. Cellular DNAs were prepared as described previously (26). Restriction enzymes (Bethesda Research Laboratories, Inc.) were used as suggested by the manufacturer. DNA fragments were separated in 0.8% agarose gels and blotted onto GeneScreen Plus filters (Dupont, NEN Research Products) by the method of Southern (39). Filters were hybridized to ^{32}P-labeled DNA probes (11).

DNA sequence analyses were performed by the chemical degradation (29) or dideoxynucleotide (38) methods. Sequencing was primed with the 17-base-pair M13 primer (Bethesda). DNA restriction fragments were subcloned in M13-derived vectors mp18 and mp19. Both strands were sequenced.

Molecular cloning of proviral DNA. DNA prepared from nonproducer NRK cell line 24T clone 3 was digested with *Xho*I. Size selection was performed by sucrose gradient fractionation (26). Genomic DNA in the size range of 7 to 15 kilobases (kb) was ligated with *Xho*I-digested lambda L47.1 purified bacteriophage arms, packaged in vitro (Amersham Corp.), and plated with *Escherichia coli* LE 392 host cells. Recombinant phages were screened with a ^{32}P-labeled Ha-*ras* BS-9 probe (10).

MHSV-specific sequences were lost with high frequency from recombinant phages, presumably because of recombination between the LTRs (45). Thus, DNA from recombinant phages was isolated from pooled minilysates and cut with *Xho*I and *Bam*HI, resulting in a 1-kb fragment containing the 5′ LTR and an 11.7-kb fragment encompassing the remaining portion of the proviral genome. The 5′ LTR fragment was subcloned into a pUC18 vector modified by ligation of *Xho*I linkers at the *Sma*I site. The 11.7-kb fragment was first subcloned in a *Bam*HI-digested EMBL 3A vector and subsequently in *Bam*HI-digested pBR322. Both fragments were combined in a *Sal*I-*Bam*HI-digested pBR322 vector to yield a full-length copy of the MHSV genome with 5′ and 3′ LTRs.

Spleen focus-forming assay. Twelve-week-old female BALB/c or strain 129 mice were injected intravenously with 0.5 ml of fivefold serial dilutions of virus-containing cellular supernatant. Three mice were injected for each dilution of virus. Fourteen days postinfection, spleens were removed, weighed, and fixed in 70% ethanol, the foci were counted, and the titer in terms of spleen focus formation (spleen focus-forming units [SFFU]) was determined (30).

Hematopoietic colony assays. Hematopoietic precursor cells from the spleens of infected and uninfected mice were assayed by colony formation in vitro, and their requirements for stimulating factors was tested. Twelve-week-old 129 mice were injected with either 1×10^2 FFU of molecularly cloned MHSV or 5×10^4 FFU of Ha-MuSV or the MHSV-H1 U3 recombinant virus. Fourteen days postinfection, mice were killed and the wet weights of the spleens were recorded. Single-cell suspensions of 10^5 spleen cells were plated in 35-mm-diameter petri dishes in Iscove modified Dulbecco medium (Boehringer GmbH, Mannheim, Federal Republic of Germany) 0.8% methylcellulose containing 300 μg of human transferrin (Boehringer Mannheim) per ml, and 10% fetal calf serum in the absence or presence of hematopoietic factors. Concentrated supernatants from the cell line IW 32 (42) or WEHI-3B (49) were used as a source of erythropoietin and multi-colony stimulating factor (Multi-CSF), respectively, at concentrations that gave maximum stimulation of hematopoietic colony formation.

Cultures were incubated at 37°C and 5% CO_2 in a humidified atmosphere. BFU-E and CFU-C colonies derived from early erythroid and myeloid progenitor cells, respectively, were counted after 9 days in culture. The type of colony was determined by morphology and verified by routine histochemical staining procedures.

RESULTS

Molecular cloning and characterization of MHSV. To elucidate which part of the MHSV genome confers the altered disease specificity compared with Ha-MuSV, similarities and differences of the molecular structure between the two proviruses were determined. To this end, the MHSV provirus was cloned as an *Xho*I fragment from NRK 24T clone 3 DNA containing the complete retrovirus and associated cellular sequences in *Sal*I-cleaved lambda L47.1 phage DNA as described in Materials and Methods.

Ha-MuSV contains sequences derived from both termini of Moloney-MuLV (Mo-MuLV), rat-derived VL-30 sequences, and the rat c-*ras* gene (3). Hybridization analysis between the cloned MHSV proviral DNA and that of Ha-MuSV clone H-1 showed that these viruses are composed of sequences from identical or similar origins (data not shown). A detailed restriction endonuclease analysis of the recombinant MHSV DNA, however, showed several differences between the two genomes (Fig. 1). First, the MHSV U3 region of the LTRs contains a different restriction site

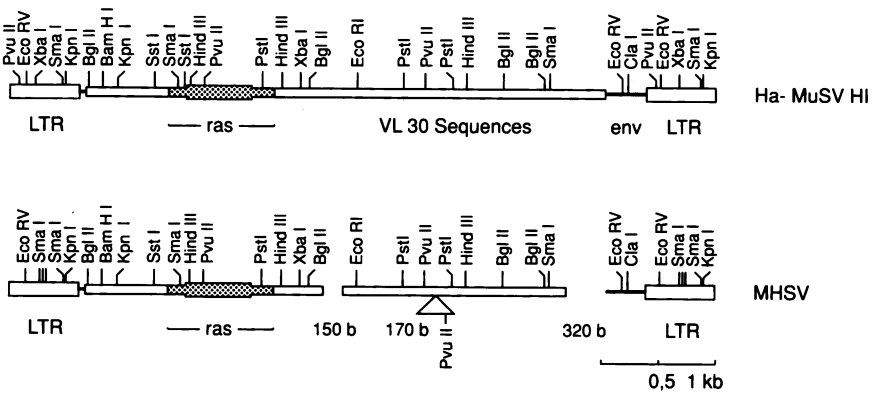

FIG. 1. A comparison of the restriction maps of the molecularly cloned MHSV and Ha-MuSV. Sequences derived from rat VL-30 and c-*ras* sequences are indicated (10). Interruptions in open bars represent deletions of VL-30 sequences in the MHSV genome.

pattern from that found in Ha-MuSV. Second, an approximate 300-base-pair deletion and an altered restriction pattern downstream of the deletion was observed at the VL-30–*env* junction. Last, an insertion and a deletion of approximately similar size (150 base pairs) was detected between the two genomes within the VL-30 sequences.

DNA sequence analysis of the *ras* gene and flanking sequences. The striking similarity based on restriction analysis between the MHSV and Ha-MuSV provirus prompted a more detailed analysis of the origin of the MHSV virus. Although this virus was originally believed to be the result of a recombination event between mouse endogenous sequences and F-MuLV (or an F-MCFV generated after recombination between F-MuLV and xenotropic viral sequences), the possibility that a recombination event had occurred with Ha-MuSV could not be excluded. Sequence analysis of the *ras* gene and the crossover points with the acquired VL-30 sequences would indicate whether or not the MHSV was derived from Ha-MuSV. In addition, such analysis would reveal any point mutations or alterations in the *ras* gene that may explain the altered target specificity of the virus.

The nucleotide sequence was determined and compared with known Harvey *ras* sequences (Fig. 2). The sequence data predict that the MHSV *ras* gene encodes a protein of the same size as the viral Ha-*ras* protein (189 amino acids) and shows a greater degree of homology with the transduced gene in Ha-MuSV than with the murine Ha-*ras* (*bas*) sequence of BALB/c-MuSV or NS.C58 MuSV in both the coding and flanking sequences. MHSV and Ha-MuSV differ by only 6 nucleotides within the sequences of c-*ras* origin.

Of the six base pairs that differ between MHSV and Ha-MuSV within the transduced *ras*, three were found in the coding sequences. Two of the changes were silent transitions (corresponding to amino acid positions 40 and 152), whereas the third was a point mutation, which replaced the UGC codon at amino acid position 181 with an AGC codon, resulting in an amino acid exchange from cysteine in v-Ha-*ras* to serine in MHSV *ras*. No changes were found between MHSV *ras* and v-Ha-*ras* in the codons for amino acids 12 and 59, which have been implicated in playing a critical role in the activation of the *ras* proto-oncogene (43). A single nucleotide exchange resulting in the deletion of the *Sst*I restriction endonuclease recognition site was found in the noncoding region.

The comparison also showed that both the VL-30–exon−1 junction, the exon+4–VL-30 junction, and the junctions between the VL-30 sequences and Mo-MuLV-derived sequences (data not shown) were identical to that of Ha-MuSV.

It is uncertain whether the deletions and insertion in the VL-30 sequences of MHSV, compared with those in Ha-MuSV, are involved in the altered pathogenicity. The VL-30 sequences do not code for a protein within the Ha-MuSV genome, and several studies have shown that the transforming function is not dependent on these sequences (5, 6, 10, 14; unpublished results). However, recent work by Velu et al. (46) has defined a putative enhancer element downstream of the *ras* oncogene in the VL-30 sequence whose deletion impairs the transformation activity of Ha-MuSV. Although we cannot exclude the possibility that this enhancer element is deleted in MHSV, we find this unlikely, as the fibroblast transformation capacity of MHSV is not reduced compared with that of Ha-MuSV.

Part of the 3′ *env* gene homologous region found in both MHSV and Ha-MuSV was also sequenced to establish whether the regions were identical in the two viruses (Fig. 3). This region shows close homology to Ha-MuSV-derived *env* sequences until position 4836, and from position 4837, it is similar to published F-MCFV sequences (Fig. 3). The sequences just upstream of position 4836 are thus the point where recombination between Ha-MuSV and a second virus, presumably F-MuLV (or F-MCFV), must have occurred.

DNA sequence analysis of the MHSV LTR. Restriction analysis and the sequence data presented above suggest that MHSV was the result of a recombination event at the 3′ end. Such a recombination would result in chimeric LTRs. The entire MHSV 5′ LTR was sequenced and compared with that of Ha-MuSV (Mo-MuLV), as well as with that of known F-MuLV and F-MCFV clones (Fig. 4).

Apart from the alteration in the enhancer region (see below), the MHSV LTR U3 region shows more than 50 base substitutions compared with the same region in Ha-MuSV, as opposed to only eight nucleotide exchanges when compared with F-MuLV. Thus, the U3 region of the MHSV LTR resembles closely the Friend group of LTRs, whereas the R and U5 regions show more homology with the Moloney family of LTRs.

The most striking difference found in the MHSV LTR sequence is a triplicate sequence element which, by homology with other retroviruses, can be regarded as an enhancer. The comparable region in F-MuLV and Mo-MuLV contains tandemly repeated sequence motives experimentally corre-

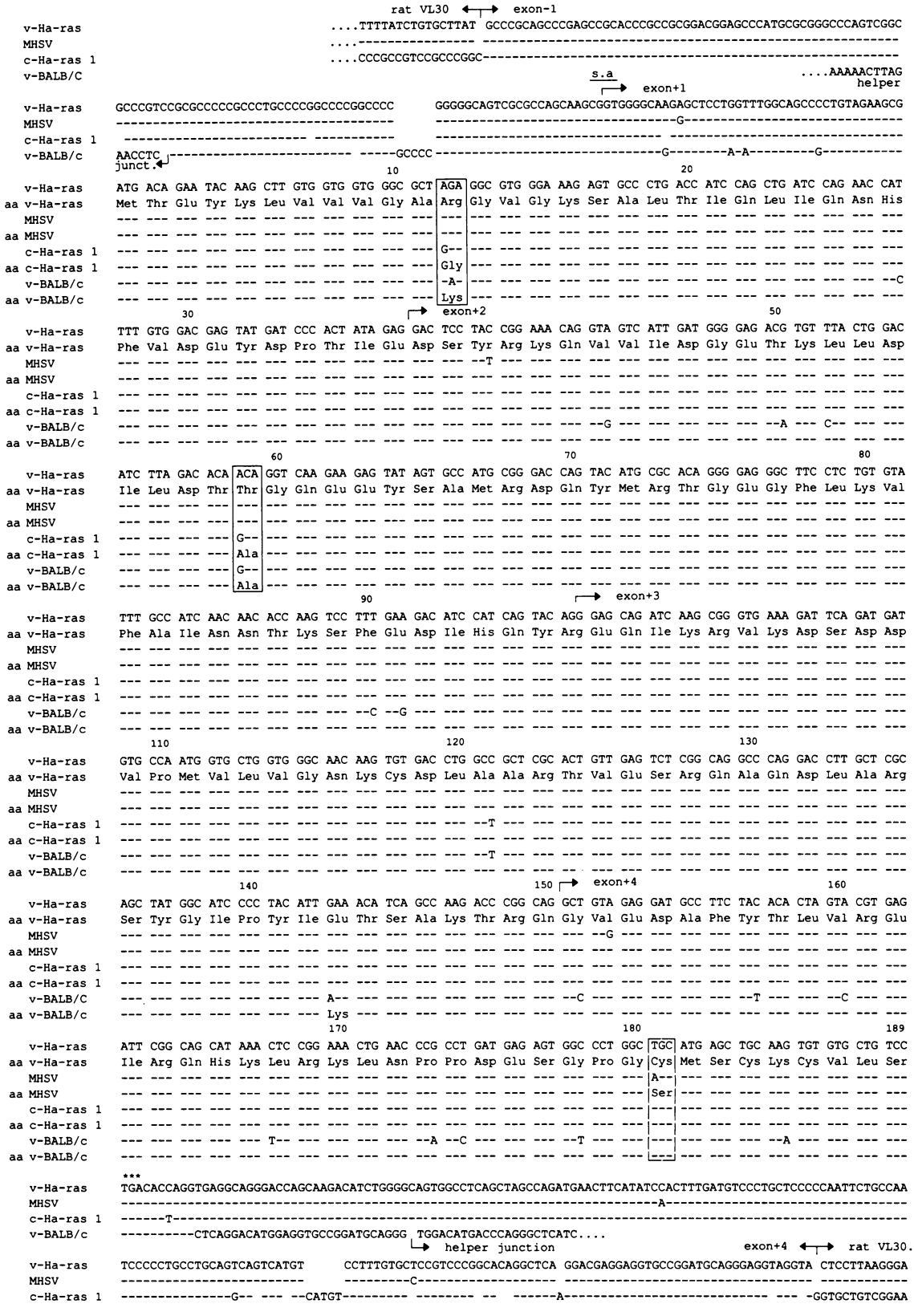

FIG. 2. Comparison of *ras* DNA and protein sequences of MHSV with other members of the Ha-MuSV family. Residues identical to v-Ha-*ras* are designated by dashes. The exon boundaries of c-Ha-*ras* are indicated by arrows. Substitutions in codon 12 and 59 found in retroviral *ras* oncogenes are boxed. The Cys (TGC) → Ser (TAC) alteration in codon 181 of MHSV is marked by a dashed box. Sequence data are those of Dhar et al. (8) (v-Ha-*ras*), Ruta et al. (37) (c-Ha-*ras*), and Reddy et al. (35) (v-BALB/c-*ras*).

FIG. 3. Analysis of the *env* nucleotide sequence of MHSV compared with that of F-MCFV and Ha-MuSV H1 indicates that MHSV is a single recombinant between Ha-MuSV and F-MCFV in the region indicated by a horizontal bar. Dashes indicate sequences identical to those of Ha-MuSV H1. Sequence data are those of Koch et al. (20) (F-MuLV clone 57 and F-MCFV 54B), Adachi et al. (1) (F-MCFV Nx), and Soeda and Yasuda cited in Weiss et al. (47) (Ha-MuSV H1).

lated with the enhancer function (22, 24). Several Friend-spleen focus-forming virus and F-MCFV clones have only a single copy of this sequence. The MHSV triplicate repeat shares strong homology with the direct repeat of the Nx clone of F-MCFV, and the repeated motif is analogous to the 3′ half of the F-MuLV direct repeats.

Malignancy of the disease induced by MHSV is determined by properties of the U3 region of the LTR. Since enhancer elements in the U3 region are the most important components of the LTR for regulating transcription in a tissue-specific manner (for a review, see reference 6), a molecular recombinant was constructed that exchanged the U3 region of the MHSV LTR with that of Ha-MuSV (MHSV-H1 U3) to determine whether the pathogenicity of MHSV was influenced by its unique LTR (Fig. 5).

Nonproducer cell lines containing a single copy of the cloned MHSV provirus and the LTR of the recombinant provirus were established and superinfected with F-MuLV to obtain infectious pseudotypes. Presence of a single unrearranged copy was verified by Southern blot analysis (Fig. 6). Adult strain 129 mice were inoculated intravenously with serial dilutions of the parental virus stock or with supernatant from cell lines producing the molecularly cloned MHSV, Ha-MuSV, or the recombinant MHSV-H1 U3, as described in Materials and Methods. FFU were assayed in parallel in RAT1 fibroblasts. At day 14 postinfection, spleens were removed and spleen foci (SFFU) were examined (Table 1). No difference was observed between the original biological MHSV clone and the molecular MHSV clone (12). An almost equivalent number of SFFUs and FFUs were obtained with either virus. The spleen size of MHSV-infected mice was enlarged by approximately 20-fold. In contrast, no visible spleen foci and no spleen enlargement was observed with the same titer of either Ha-MuSV or the MHSV recombinant with the U3 region of Ha-MuSV. Injection of 100-fold-higher titers, as measured by fibroblast transformation, resulted in only a fourfold enlargement of spleen size and the formation of a few visible spleen foci. The relative spleen focus-forming titer of the MHSV recombinant virus was reduced by more than 3 orders of magnitude as compared with that of the wild-type or molecularly cloned MHSV (Table 1).

Specificity of the disease induced by MHSV is in part determined by properties of the U3 region of the LTR. Biologically cloned MHSV, in contrast to Ha-MuSV, induces a disease in adult mice which is characterized by a huge and preferential increase of macrophages and macrophage precursor cells. Although some macrophage precursors infected with Ha-MuSV do not require CSFs for proliferation and colony formation, this loss of CSF requirement is much more pronounced in macrophage precursors infected with MHSV (12, 18). To test whether these properties were unaltered in the molecularly cloned MHSV and whether they were influenced by the type of LTR, spleen cells from mice infected with MHSV, Ha-MuSV, and MHSV-H1 U3 were analyzed by hematopoietic colony assays. This assay can be used to show the potential of the virus to induce growth factor-independent proliferation and differentiation into specific lineages (Table 2). Five-hundred-fold-higher titers of Ha-MuSV or of the recombinant MHSV-H1 U3, compared with those of the molecularly cloned MHSV, were used for injections into adult mice so that comparable increases in spleen size were obtained. The results of colony assays of infected animals are shown in Table 2. As addition of erythropoietin allows optimal growth of erythroid colonies and multi-CSF is required for growth and differentiation of both myeloid (granulocyte and macrophage) and erythroid colonies, these two growth factors were added or omitted to determine the type of colony which could proliferate in response to virus infection.

Ha-MuSV evoked a mild proliferative response but did not change the proportion of colonies within the myeloid and erythroid lineages. CSF-independent growth was observed in 40% of all myeloid colonies (CFU-C). MHSV infection resulted in a dramatic increase in colony formation of splenic cells, and 90% of these colonies grew independently of growth factors. Furthermore, the differentiation pattern showed a preponderance of macrophage (>90%) and mixed granulocyte-macrophage colonies. The number of granulocyte colonies per number of spleen cells plated was also increased. Similar to Ha-MuSV, MHSV H1 U3 induced only a mild proliferative response, and some of the myeloid colonies did not require exogenous CSF for proliferation. The growth factor-independent colonies induced by this virus, unlike those obtained from the spleens of Ha-MuSV-infected mice, however, showed a somewhat higher proportion of macrophage colonies. Indeed, a slight increase in the proportion of macrophage colonies in the total myeloid

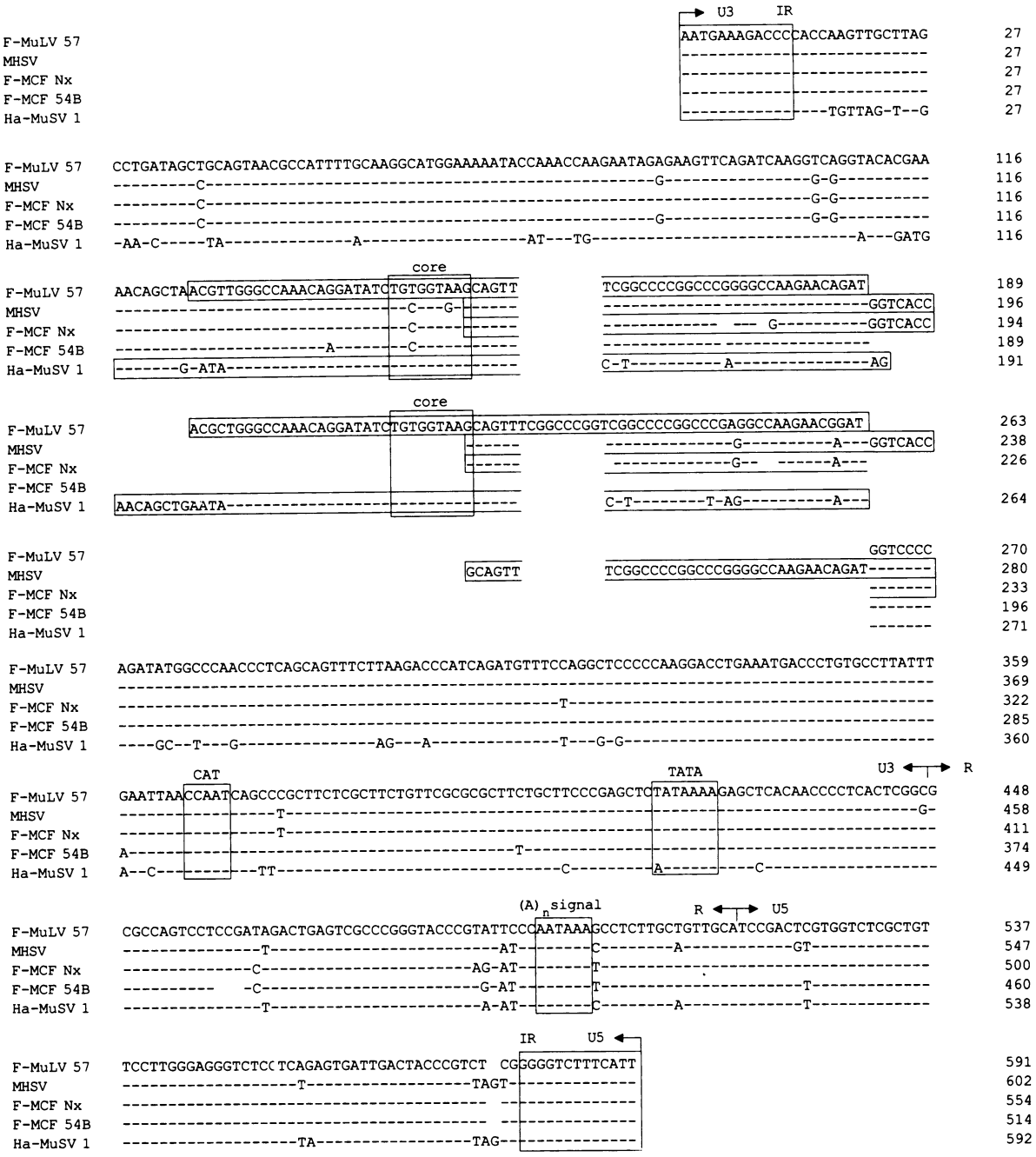

FIG. 4. Comparison of the LTR nucleotide sequence of MHSV with those of the F-MCFV family and of Mo-MuLV/Ha-MuSV. Nucleotides identical to F-MuLV clone 57 are indicated by dashes. Regulatory sequences are enclosed in large boxes. Repetitive sequences in the enhancer domain are demarcated by flat boxes. Inverted repeats (IR) are found at the ends of the LTR. Sequence data are those of Koch et al. (20) (F-MuLV clone 57 and F-MCFV 54B), Adachi et al. (1) (F-MCFV Nx), and Soeda and Yasuda cited in Weiss et al. (47) (Ha-MuSV H1).

colonies (from 20 to 25% to ~40%) was also observed in the presence of multi-CSF (plus or minus erythropoietin) in MHSV H1 U3-infected mice, compared with that in uninfected or Ha-MuSV-infected mice. These results were confirmed in several subsequent experiments (data not included) and may indicate that the MHSV *ras* gene contributes to the disease specificity. However, MHSV with its own LTR induced a much higher preponderance (75 to 80%) of macrophage colonies, supporting the conclusion that the U3 region of MHSV is necessary not only for the malignancy but also for the macrophage lineage specificity of the virus-induced disease.

DISCUSSION

The molecular analysis of the MHSV genome provided strong evidence that the virus is the result of a recombination event between Ha-MuSV and the F-MCFV component of

TABLE 1. Malignancies of the molecularly cloned MHSV, MHSV H1 U3, and Ha-MuSV as shown by splenomegaly and spleen focus formation[a]

Virus	FFU injected	Inoculation period (days)	Spleen wt (mg [mean ± SD]) (*n*)	SFFU	Ratio (SFFU/FFU)
None	0		49 ± 7 (5)	0	
MHSV	2.5×10^3	14	1,380 ± 212 (4)	ND[b]	
	5×10^2	14	775 ± 89 (4)	ND	
	2.5×10^2	14	555 ± 147 (4)	ND	
	5×10^1	14	215 ± 58 (4)	41 ± 9	0.8
	2.5×10^1	14	157 ± 21 (4)	19 ± 3	0.8
	5×10^2	18	765 ± 21 (2)	ND	
	2.5×10^2	18	430 ± 85 (2)	ND	
	5×10^1	18	180 ± 20 (2)	30 ± 6	0.6
	2.5×10^1	18	145 ± 18 (2)	20 ± 3	0.8
MHSV H1 U3	1×10^5	14	301 ± 26 (4)	22 ± 7	5×10^{-3}
	5×10^4	14	123 ± 23 (4)	14 ± 4	3×10^{-4}
	1×10^4	14	67 ± 10 (4)	2 ± 1	2×10^{-4}
	1×10^5	18	240 ± 40 (2)	17 ± 2	2×10^{-4}
	5×10^4	18	110 ± 20 (2)	11 ± 7	2×10^{-4}
	1×10^4	18	78 ± 18 (2)	2 ± 1	2×10^{-4}
Ha-MuSV	1×10^5	14	289 ± 41 (4)	ND	
	5×10^4	14	144 ± 31 (4)	9 ± 3	2×10^{-4}
	1×10^4	14	80 ± 5 (4)	0	0
	1×10^5	18	1,280 ± 200 (2)	ND	
	5×10^4	18	415 ± 20 (2)	13 ± 13	3×10^{-4}
	1×10^4	18	155 ± 15 (2)	12 ± 7	1×10^{-3}

[a] Virus pseudotyped with F-MuLV was injected into adult strain 129 mice. The mice were sacrificed after different periods of inoculation with virus, and spleen weight and focus formation (SFFU) were determined. In parallel experiments, the fibroblast focus-forming activity (FFU) of the same viral supernatant was determined and the ratio of SFFU to FFU was calculated.

[b] ND, Not determined (too many SFFU per spleen).

TABLE 2. Stimulation of colony-forming cells in mice by MHSV, MHSV H1 U3, and Ha-MuSV[a]

Virus	FFU injected	Spleen wt (mg)	SFFU/ml	Growth factor		No. and type of colonies/10^5 cells						% of CFU-C		
						CFU-C			BFU-E	Mixed	Σ			
				Epo	multi-CSF	M	G	GM				M	G	GM
None	0	35	0	−	−	0	2 ± 2	0	0	0	2	0	0	0
				+	−	0	1 ± 0	0	0	0	1	0	0	0
				−	+	18 ± 3	39 ± 2	7 ± 1	0	1 ± 0	65	28	61	11
				+	+	6 ± 2	26 ± 1	3 ± 1	1 ± 1	1 ± 1	37	17	74	9
MHSV	1×10^2	400	1×10^2	−	−	1,218 ± 79	66 ± 7	49 ± 14	0	13 ± 1	1,346	91	5	4
				+	−	1,079 ± 131	55 ± 21	51 ± 3	0	6 ± 2	1,191	91	5	4
				−	+	1,109 ± 65	265 ± 26	108 ± 3	0	13 ± 3	1,495	75	18	7
				+	+	1,203 ± 169	155 ± 35	102 ± 6	0	13 ± 2	1,473	82	11	7
MHSV H1 U3	5×10^4	120	15	−	−	37 ± 6	29 ± 3	5 ± 2	0	0	71	52	41	7
				+	−	29 ± 5	20 ± 6	5 ± 1	0	0	54	54	37	9
				−	+	86 ± 14	120 ± 31	20 ± 5	7 ± 2	2 ± 2	235	38	53	9
				+	+	95 ± 15	118 ± 22	22 ± 5	9 ± 4	10 ± 3	254	40	50	9
Ha-MuSV	5×10^4	100	5	−	−	26 ± 6	50 ± 4	13 ± 2	2 ± 1	2 ± 2	93	29	57	15
				+	−	38 ± 6	38 ± 4	8 ± 3	3 ± 1	2 ± 1	89	45	45	10
				−	+	53 ± 23	129 ± 17	23 ± 9	0	5 ± 3	210	26	63	11
				+	+	58 ± 14	149 ± 11	15 ± 5	0	3 ± 1	217	23	70	7

[a] Experimental details are as described in Materials and Methods. For each virus concentration, three strain 129 mice were injected with the indicated amount of virus particles measured by fibroblast focus-forming activity (FFU). The spleen weight and focus formation (SFFU) were determined 10 days after intravenous injection of virus. Single-cell suspensions of spleen cells were tested in the presence or absence of erythropoietin (Epo) or multi-CSF, and all colonies were counted (Σ, total number of all types of colonies) and analyzed as myeloid (CFU-C), early erythroid (BFU-E) or mixed colonies 9 days after the initial plating of cells. The proportions (%) of granulocyte (G), macrophage (M), and granulocyte and macrophage (GM) colonies within the CFU-C category were also determined. Results are the average of two independent experiments.

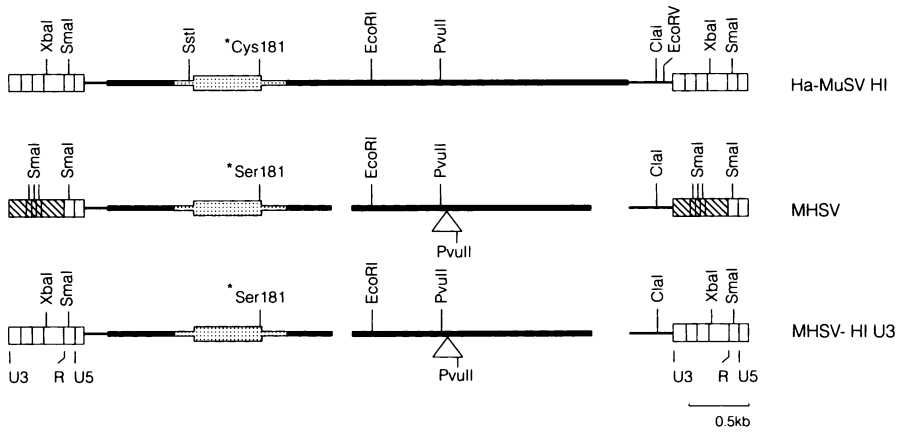

FIG. 5. Schematic structure of MHSV, Ha-MuSV H1, and MHSV H1 U3. Only restriction enzyme cleavage sites that are significant for this comparison are shown. Symbols: *, point mutation in the *ras* gene of MHSV that leads to a substitution of Ser (MHSV) for Cys (Ha-MuSV); □, LTRs of the Ha-MuSV; ▨, U3 region of MHSV; ▩, *ras* sequences.

the 643/22F helper cell line. Our structural data on the molecular clone of MHSV indicate that recombination between Ha-MuSV and F-MCFV occurred upstream of the 3′ LTR within the *env* region of F-MCFV resulting in a viral genome with the 5′ end derived from Ha-MuSV and the 3′ end from F-MCFV (Fig. 7). How this recombinant has been generated is unknown. One would have to assume multiple events if recombination originated during reverse transcription as a result of heterodimer formation of Ha-MuSV and F-MCFV. Analysis of the murine retroviral genomes of viral particles by electron microscopy has not yet shown such heterodimers in other viral complexes (9). Only a single breakage-reunion event, however, is required if recombination occurs during direct pairing of homologous regions of the two DNA genomes and subsequent crossover (Fig. 3 and 7). The resultant virus may have been further modified by structural mutations in the LTR or, alternatively, the peculiar features of the MHSV LTR may have already been present in the F-MCFV of 643/22F.

Despite their similar structures, MHSV and Ha-MuSV induce quite different diseases in adult animals. Unlike Ha-MuSV, MHSV induces a very rapid malignant histiocytosis in adult mice (25) and allows the establishment of growth factor-independent macrophage cell lines from infected spleens (12, 19). In vitro infection of single granulocyte-macrophage or macrophage precursor cells results in immediate factor-independent growth and, more rarely, can be adapted to permanent growth in vitro (16, 18). In contrast, Ha-MuSV causes only a transient increase of macrophage colony forming cells in the spleen (25), and in vitro bone marrow infection results in transformed macrophage cells that are neither immortal nor factor independent (31, 36).

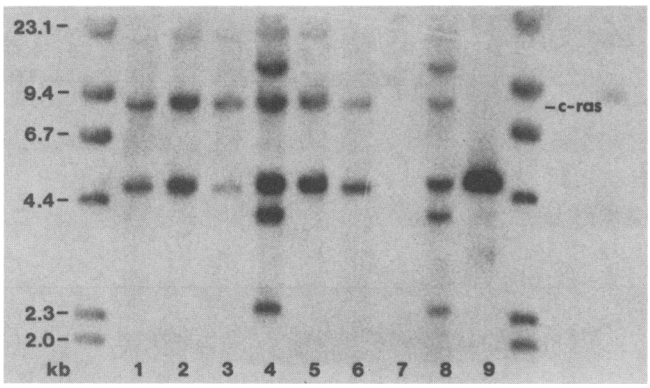

FIG. 6. Southern blot analysis of MHSV-H1 U3 virus-producing clones used for infections. Cellular DNA was digested with *Eco*RV, separated by agarose gel electrophoresis, and transferred to GeneScreen Plus. Blots were hybridized with the BS-9 *ras* probe (10). Lanes 1 through 3, 5, and 6, Five independent RAT1 clones infected with supernatant containing recombinant MHSV H1 U3 from RAT1 clone 5; lanes 4 and 8, RAT1 cellular clone 5 transfected with MHSV H1 U3 DNA and infected with F-MuLV; lane 9, plasmid DNA of MHSV H1 U3 (70 pg).

The detailed molecular analysis of MHSV has identified two regions of the genome which may play a decisive role in determining its unique pathogenicity, the MHSV *ras* sequences that encode a unique p21 protein and the MHSV LTR that is more closely related to the Friend-MCFV family than to the Moloney group.

The importance of the unique LTR of MHSV and other sequences within the MHSV genome was analyzed by comparing the malignancies (Table 1) and the specificities (Table 2) of the diseases induced by MHSV, Ha-MuSV, and MHSV H1 U3. The results were unambiguous and do not require much discussion. The generation of spleen foci in infected mice, used as a criterion for disease malignancy, was reduced by 3 to 4 orders of magnitude in both Ha-MuSV and the recombinant MHSV H1 U3 compared with MHSV. Similarly, the disease specificity of the recombinant MHSV H1 U3 was drastically altered compared with wild-type MHSV, resembling more closely that of Ha-MuSV. Thus, substitution of the U3 region of the MHSV LTR for the Moloney-based Ha-MuSV LTR in an MHSV recombinant virus resulted in a drastic change in pathogenicity of the virus. These results implicate the involvement of transcriptional control elements in determining disease specificity and malignancy. Indeed, in vitro analysis of the enhancer region of MHSV showed a unique protein-binding domain that bound preferentially to a protein(s) found in abundance in the macrophage lineage (Nowock et al., unpublished data).

It cannot be excluded that the altered *ras* oncogene or some other part of the MHSV genome also contributes to the MHSV pathology. Although Ha-MuSV infection does not shift the differentiation equilibrium within the myeloid lineage, the chimeric virus induced a slight but reproducible increase in the fraction of macrophages which also showed a higher incidence of factor-independent growth (Table 2). On

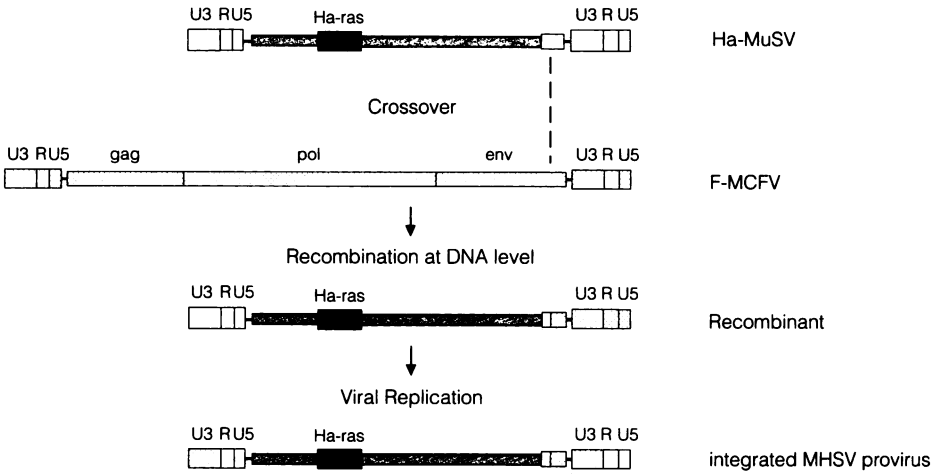

FIG. 7. Predicted sequence of recombinational events that result in the MHSV genome starting with F-MCFV and Ha-MuSV (see also Fig. 3 and 4). ▒, sequences derived from rat VL-30 sequences; □, sequences derived from Ha-MuSV; ▩, sequences derived from F-MCFV.

the basis of sequence analysis, the MHSV *ras* gene encodes a protein that carries a serine instead of a cysteine at position 181. Such an alteration could change the specificity of transformation. The amino acid sequences of Ha-*ras*, Ki-*ras* and N-*ras* are highly conserved, except for the hypervariable region at the carboxy terminus which may impart cellular specificity to the various members of the *ras* family. Although this domain is dispensable for the known biochemical properties of *ras* p21 (21, 48), this segment has been maintained within the *ras* gene family throughout evolution and may serve an important function. The analysis of further recombinants will be necessary to determine whether the altered *ras* protein plays a significant role in the unique transformation capacity of MHSV.

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