Sequence Analysis of Amphotropic and 10A1 Murine Leukemia Viruses: Close Relationship to Mink Cell Focus-Inducing Viruses

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Viral interference studies have demonstrated the existence of four distinct murine leukemia virus (MuLV) receptors on NIH 3T3 mouse cells. The four viral interference groups are ecotropic MuLV; mink cell focus inducing virus (MCF); amphotropic MuLV; and 10A1, a recombinant derivative of amphotropic MuLV that uses a unique receptor but also retains affinity for the amphotropic MuLV receptor. We report here that 10A1 infects rat and hamster cells, unlike its amphotropic parent. We isolated an infectious molecular clone of 10A1 and present here the sequences of the env genes and enhancer regions of amphotropic MuLV and 10A1. The deduced amino acid sequences of amphotropic MuLV and 10A1 gp70^{su} are remarkably similar to those of MCF and xenotropic MuLV (for which mouse cells lack receptors), with 64% amino acids identical in the four groups. We generated a consensus from these comparisons. Further, the differences are largely localized to a few discrete regions: (i) amphotropic MuLV has two short insertions relative to MCF, at residues 87 to 92 and 163 to 169, and (ii) amphotropic MuLV and MCF are totally different in a hypervariable region, which is >30% proline, at residues ~253 to 304. 10A1 closely resembles amphotropic MuLV in its N terminus but contains an MCF-type hypervariable region. These results suggest the possibility that receptor specificity is localized in these short variable regions and further that the unique receptor specificity of 10A1 is due to the novel combination of amphotropic MuLV and MCF sequences rather than to the presence of any novel sequences. The Env proteins of ecotropic MuLVs are far more distantly related to those of the other four groups than the latter are to each other. We also found that the enhancer regions of amphotropic MuLV and 10A1 are nearly identical, although 10A1 is far more leukemogenic than amphotropic MuLV.

The *env* gene of murine leukemia viruses (MuLVs) encodes an external glycoprotein, $gp70^{su}$, which interacts with a receptor molecule on the surface of a host cell in the initial stage of infection. Viral interference studies have shown that MuLVs can be divided into four groups, using four distinct receptors on mouse cells (20, 21) plus a fifth group, xenotropic MuLVs, for which mouse cells lack receptors (3).

One approach to understanding the gp70-receptor interaction in molecular terms is to compare gp70 sequences from different viral groups. Several examples from two of these groups, the ecotropic and mink cell focus-inducing viruses (MCFs), have been sequenced. The gp70s of these groups are quite different in their N-terminal two-thirds; the Cterminal regions, on the other hand, are nearly identical. Thus, receptor specificity is apparently determined by the sequences found in the N-terminal two-thirds of the molecule.

MCFs arise in mice infected with ecotropic MuLVs. The MCF env gene is generated by recombination between the ecotropic MuLV and env sequences of endogenous MuLVs present in the mouse genome; this recombinational event substitutes the endogenous N-terminal env region for the ecotropic env sequences. The endogenous MuLV genomes which participate in this recombination have been carefully characterized by Stoye and Coffin (28). One such genome, termed MX27, has been sequenced and appears to be a prototype of the endogenous sequences involved in the creation of MCFs. It seems likely that these endogenous sequences encode gp70s which carry the specificity for interaction with the MCF cell surface receptor, since the gp70s of some MCFs are composed almost entirely of endogenous sequences.

The other two classes of MuLV which can infect mouse cells are amphotropic MuLV and 10A1. The relationship between these two classes appears analogous to that between ecotropic MuLVs and MCFs, since 10A1 was isolated from a mouse infected with amphotropic MuLV (19). In addition, RNase T1 fingerprint analysis of 10A1 has shown that it contains an MCF-like (or xenotropic MuLV-like) substitution near the *pol-env* junction (15). Another parallel with the ecotropic MuLVs and MCFs is that 10A1 is strikingly more leukemogenic than its amphotropic parent, just as many MCFs are more leukemogenic than their ecotropic parents. Nevertheless, 10A1 has unique biological properties; i.e., it interacts with a unique receptor on mouse cells, which is not shared with any other known MuLV, and also appears to interact with the amphotropic receptor. This is the only known case of dual mouse cell receptor specificity among the MuLVs.

We now present the nucleotide sequences of the *env* genes and enhancer regions of amphotropic MuLV and 10A1. The *env* sequence of amphotropic MuLV is remarkably close to that of the MX27 class of endogenous MuLVs, except in three localized regions. In turn, 10A1 is very closely related to both amphotropic and MX27 sequences; inspection of these three divergent regions showed that 10A1 is a recombinant between amphotropic MuLV and MX27-related endogenous sequences. These findings appear to have important implications for the relationship between gp70 structure and receptor specificity.

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MATERIALS AND METHODS

Cell culture. NIH 3T3, Rat-2 (a gift of W. Vass, National Cancer Institute, Bethesda, Md.), and CHO cell lines were maintained in Dulbecco Modified Eagle medium supplemented with 10% calf serum, Dulbecco Modified Eagle medium supplemented with 10% fetal bovine serum, and alpha modified minimal essential medium supplemented with 10% fetal bovine serum, respectively. Viral infections were performed with 20 μ g of DEAE-dextran per ml. Infectivity was assayed by plating cells in an infectious-center assay on mouse S⁺ L⁻ indicator cells (1).

Hirt DNA isolation and analysis. Unintegrated 10A1 proviral DNA was prepared after cocultivation of a 10A1-producing CHO cell line with CHO cells in 2 µg of Polybrene per ml by using a ratio of 1:4 with a total cell density of 7 \times 10^4 /cm². After 2 days of cocultivation, DNA was isolated by the method of Hirt (11). 10A1 Hirt supernatant DNA was then digested with diagnostic restriction enzymes and analyzed by hybridization (25) to retroviral plasmid clone p4070A (5), which had been labeled with ³²P by nick translation (22). Hybridization conditions were 10% dextran sulfate-5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) $-2 \times$ Denhardt solution, 0.2% sodium dodecyl sulfate-50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid; pH 6.8)-40% formamide-10 µg of poly(rA) per ml-50 µg of sheared salmon sperm DNA per ml-1 \times 10⁶ to 5 \times 10⁶ cpm of ³²P-labeled probe per ml. Hybridizations were performed for at least 18 h at 42°C. After hybridization, filters were washed in $0.1 \times$ SSC-0.1% sodium dodecyl sulfate at 65°C for 2 to 4 h with changes of wash solution every 30 min.

Cloning and subcloning. Hirt supernatant DNA was digested with SalI to linearize circular 10A1 proviral DNA. The DNA was fractionated on an agarose gel, and 7.5- to 9.5-kilobase (kb) fragments were purified by electroelution with a UEA electroeluter (International Biotechnologies, Inc., New Haven, Conn.). EMBL3 SalI-digested bacteriophage arms were prepared by double digestion of the phage with SalI and BamHI. Following ligation, packaging (Stratagene Cloning Systems, La Jolla, Calif.), and plating of a mixture of insert and vector, screening was accomplished by probing with ³²P-labeled p4070A by standard methods (2). DNAs from candidate phage clones were mapped with several restriction enzymes; clones with restriction maps similar to that of amphotropic MuLV (5) were tested for infectivity as follows. The DNAs were digested with SalI to release the circularly permuted viral DNA. These DNAs were then transfected without carrier DNA into CHO cells. The cells were passaged for 2 weeks and then plated as infectious centers in the $S^+ L^-$ focus assay. *env* and long terminal repeat (LTR) regions from both the infectious 10A1 clone and the p4070A clone (5; a gift of Allen Oliff) were subcloned for sequence analysis. 10A1 fragments were subcloned into either a Bluescript KS⁺ or KS⁻ plasmid, while 4070A DNA fragments were subcloned into either M13 phage mp 12 or mp 13.

Sequencing. Single-stranded DNAs from either M13 cultures or helper virus-rescued Bluescript cultures were sequenced by using the dideoxynucleotide chain termination method of Sanger et al. (23) with either DNA *PolI* Klenow fragment or the Sequenase system (U.S. Biochemicals, Cleveland, Ohio). In all cases, both DNA strands were sequenced. Sequences were assembled and analyzed by using the Stephens software package (27).

Sequence alignment. Nucleotide sequence alignments were

TABLE 1. Host ranges of amphotropic and 10A1 MuLVs^a

	No. of infectious centers/2 \times 10 ⁴ cells					
MULV	NIH 3T3	Rat-2	СНО			
1504A	9,800	1	0			
10A1	14,500	900	6,000			

^{*a*} Flasks were seeded with 3×10^5 NIH 3T3, Rat-2, or CHO cells. On the following day, they were infected with amphotropic MuLV (isolate 1504A, a kind gift of Janet Hartley, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) or 10A1. Two days later, the cells were trypsinized and plated as infectious centers on S⁺ L⁻ mouse indicator cells (1). Results similar to those shown for 1504A were observed with 4070A MuLV (data not shown).

made with the NUCALN program (29) by using the following parameters: K-tuple size, 3; window size, 20; gap penalty, 7. Sequence translations and alignments were achieved by using the GAP program (7) with a gap penalty of 5 and a length weight of 0.3 and allowing only identical matches. Other MuLV sequences were obtained from Genbank.

RESULTS

Host range of 10A1 MuLV. We previously reported (21) that 10A1, a recombinant derived from amphotropic MuLV, uses a receptor different from that of amphotropic MuLV on NIH 3T3 mouse cells. It seemed possible that the 10A1 *env* gene product could also interact with receptors present on cells lacking the amphotropic MuLV receptor. We therefore tested the ability of 10A1 to productively infect CHO cells and Rat-2 cells, which are both almost totally nonpermissive for amphotropic MuLV. 10A1 infected both of these cell lines with nearly the same efficiency as it infected NIH 3T3 cells (Table 1). This expanded host range of 10A1 is fully consistent with its distinct receptor specificity on mouse cells.

Molecular cloning and partial sequence analysis of 10A1 MuLV. Eight molecular clones of 10A1 proviral DNA were isolated from Hirt supernatant DNA and tested as described in Materials and Methods. One of these clones was found to be infectious. The MuLV produced after transfection of this clone appeared to be a faithful replica of 10A1, since it had an expanded host range and the expected interference properties on NIH 3T3 cells (data not shown). It also reproduced the biological properties of 10A1 by inducing lymphomas within 3 months of intraperitoneal inoculation into newborn NIH Swiss mice (data not shown).

Comparison of amphotropic and 10A1 MuLV genomes. As a first step in characterizing the genome of 10A1 MuLV, we generated a restriction map of the 10A1 clone. This map is shown in Fig. 1 along with the map of the 4070A amphotropic MuLV clone (5). It is evident that the two genomes are quite similar, although they show a cluster of restriction site differences at 5 to 6 kb pairs from the 5' end. The presence of a novel BglII site at 5.25 kb, together with several restriction site differences immediately downstream, suggested that a recombinational event had occurred 5' of this BglII site. This is consistent with the RNase T1 fingerprint analysis of Lai et al. (15). The 3' limit of this recombination appeared to lie between the 10A1-specific BamHI site at 6.0 kb and the common *XhoI* site at 6.9 kb. The regions which we sequenced are also indicated in Fig. 1.

Sequence analysis of amphotropic and 10A1 env genes. To examine the structure of amphotropic env and to compare the 10A1 genome with that of amphotropic MuLV, we sequenced the two genomes from just 5' of the beginning of



FIG. 1. Restriction enzyme maps of 10A1 and 4070A retroviruses. The two viruses are drawn as complete linear genomes for convenience, although the clones themselves are circularly permuted, single LTR genomes: 10A1 cloned at the SalI site and 4070A cloned at the EcoRI site. The regions sequenced are indicated by arrowed brackets. Abbreviations: B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; H, HindIII; Ps, PstI; Pv, PvuII; S, SalI; Ss, SstI; Xb, XbaI; Xh, XhoI.

the env gene to within the LTR (Fig. 1). The nucleotide sequences of the env regions of the 4070A and 10A1 clones are shown in Fig. 2.

1 Kb

Comparison of the two sequences showed them to be very similar, except for two stretches of nucleotide sequence differences. Remarkably, the 10A1 sequences that diverged from those of 4070A corresponded almost exactly to the endogenous polytropic sequence MX27 (Fig. 2) (28). Thus, 10A1 apparently contains a stretch of polytropic sequences beginning to the 5' side of env, within the presumed pol region, and extending to nucleotides 67 to 87 in the env leader region (because of the close homology among the three sequences in this region, the exact crossover point could not be determined). A second pair of crossover events, one at nucleotides 724 to 731 and the other at nucleotide 959, contributed a short polytropic MuLV nucleotide sequence within the gp70-coding region. (It should be noted that there are several distinct isolates of amphotropic MuLV and that the source of 10A1 was a mouse injected with amphotropic MuLV isolate 1504A rather than 4070A [19]. However, the sequence relationship between 4070A and 10A1 is so close, outside of these divergent regions [Fig. 2], that we used 4070A as a prototype amphotropic MuLV for the sequence comparisons described here.)

Deduced amino acid sequences of amphotropic MuLV and 10A1 Env proteins: comparison with other Env proteins. Figure 3A presents the amino acid sequences of the 4070A and 10A1 env genes, as deduced from the nucleotide sequences shown in Fig. 2. For comparison, we also show the deduced sequences of MX27 and a xenotropic MuLV derived from an NZB mouse (17). Under the four sequences, we present a polytropic MuLV-related MuLV (PRM) consensus sequence, showing all of the identical amino acids in the four sequences and also indicating positions at which only conservative changes are found. The remaining class of MuLVs is the ecotropic group. We selected two ecotropic MuLVs, one exogenous (Moloney MuLV) and one endogenous (Akv), for comparison with the PRM sequences. Figure 3B presents an alignment of the Moloney and Akv env sequences with the PRM consensus. Finally, we compiled an all-MuLV consensus sequence, shown in the last row of Fig. 3B. The implications of these comparisons will be considered in the Discussion.

Partial sequence analysis of amphotropic MuLV and 10A1 LTRs. We also sequenced the 3'-noncoding regions of am-

photropic MuLV and 10A1. These sequences are compared in Fig. 4. The 4070A sequence contains two inverted repeats, as found in all MuLV LTRs, and a CCAAT box, a TATAA box, and a poly(A) additional signal. We compared the enhancer regions of 4070A and 10A1 with that of Moloney MuLV, which has been analyzed in detail by Speck and Baltimore (26). Both 4070A and 10A1 contain a sequence, AGAACAGAA, which resembles the canonical glucocorticoid-responsive element sequence AGAACAGATG; a sequence, AAACAG, like the LVa factor-binding sequence GAACAG; sequences like the consensus binding sites for NF-1 (TGA[N₆₋₇]GCCA) and LVb; the core enhancer element TGTGGTAA; and a second glucocorticoid-responsive element sequence (Fig. 4). Both viruses lack the LVc sequence. They differ in that 4070A may have a second NF-1 site (CGGN₆GCCA), while the 10A1 sequence diverges slightly from 4070A in this region. There is no duplication of the enhancer region in either 4070A or 10A1. A second difference is that 10A1 contains the negative regulatory consensus sequence, CGCCATTTT, described by Flanagan et al. (9), while in amphotropic MuLV, the sequence is disrupted by a 4-nucleotide duplication, i.e., CGCCATTTA TTTT. Aside from this difference, the two sequences are extremely similar (95% identity).

DISCUSSION

We report here the nucleotide sequences of the 3' ends of the amphotropic MuLV and 10A1 genomes. Translation of these sequences allowed us, for the first time, to compare the Env sequences of all of the known MuLV receptor specificity groups.

The amino acid sequences from these groups are aligned in Fig. 3. One striking conclusion which emerges from this alignment is the remarkable degree of conservation in these sequences. In particular, four of these groups are extremely similar: amphotropic MuLV, 10A1, endogenous polytropic MuLV, and xenotropic MuLV (Fig. 3A). We will refer to these four groups as the PRM family for simplicity here. We found that the mature gp70s of these four MuLV groups are identical at 296 positions or approximately 64% of the total. Isolates from the fifth group, the ecotropic MuLVs, showed far less homology with PRMs (only 40% identity with the PRM consensus sequence) than the latter do with each other (Fig. 3B); there is also considerably more diversity within

	Initiator						I					
4070A			GGCCGAC	10 ACCCAGAGTG	20 GACCATCCTO	30 TGGACGGAC	40 ATGGCGCGTT	50 CAACGCTCTC	60 AAAACCCCCT	↓ 70 CAAGATAAG	80 ATTAACCCGTG	J 90 GAAGCC
10A1	GGATCCACGCCGCTCA	CGTAAAGGC	GGCGACAACC	CCTCCGGCCG	GAACAGCATO	AGGACCGAC	ATGGAAGGTC	CAGCGTTCTC	AAAACCCTT	AAAGATAAG	ATTAACCCGTG	GAAGTC
MX27	GGATCCACGCCGCTCA	CGTAAAAGC	GGCGACAACC	CCTCCGGCCG	GAACAGCATO	AGGACCGAC	ATAGAAGGTC	CAGCGTTCT	AAAACCCCTT	AAAGATAAG	ATTAACCCGTG	: GGGCCC
	10	20	30	40 gp70	50	60	70	80	90	100	110	1
40704		110 GTCCTGTTA	120 GGAGTAGGGA	130 TGGCAGAG	AGO	140 CCCCCATCAG	150 GTCTTTAATG	160 TAACCTGGAG	170 GAGTCACCAAC	180 CTGATGACT	190 GGGCGTACCGC	200 CAATGC
1041		GTCTATTTA	AGAGTAGGGA	TGGCAGAG	:: AG6	CCCCATCAG	GTCTTTAATG	TAACCTGGA	AGTCACCAAC	CTGATGACT	GGGCGTACCGC	CAATGC
MY27				TATCAGTACA	ACATGACAG	CCTCATCAG	GTCTTCAATG	TTACTTGGA	GAGTTACCAAC	TTAATGACA	GGACAAACAGC	TAATGC
MAZ7	20 130	140	150	160	170	180	190	200	210	220	230	2
	210	220	230	240	250	260	270	280	290	300 GAACCGTAT	310 GTCGGGTATGG	320 CTGCAA
4070A			CATCOCTTCC	::::::::::::::::::::::::::::::::::::::	TTTGATCT	ATGTGATCTG	GTCGGAGAAG	AGTGGGACC	TTCAGACCAC	GAACCATAT	GTCGGGTATGG	CTGCAA
10A1			CATGCCTTCC				ATACCCCACC	ACTOGOATO		CACTOGOGT	GTCGCACT	
MX27	40 250	260	270	280	290	300	310	320	RUACITI	330	340	
	330	340	350	360	370	380	390	400	410	420	430	440 TGAAAC
4070A	GTACCCCGCAGGGAGA	CAGCGGACC	CGGACITIIG				AAGTCGGGG					
10A1	ATACCCCGGAGGGAGA	AAGCGGACC	CGGACTTTTG	ACTTTTACGT	GTGCCCTGG	GCATACCGTA	AAATCGGGGGI				GAATGGGGTTG	1 GAAAC
MX27	CCCGGGGGAAGA 350	AAAAGGGCA 360	AGAACATTTG 370	ACTTCTATGT 380	TTGCCCCGG 390	GCATACTGTA 400	CCAACAGGGT 410	GTGGAGGGC 420	CGAGAGAGGGG 430	440	450	1GAGAC 460
	450	460	470	480	490	500	510	520	530	540	550	560
4070A	CACCGGACAGGCTTAC	TGGAAGCCC	ACATCATCGT	GGGACCTAAT	CTCCCTTAA	GCGCGGTAAC	ACCCCCTGGG		GCTCTAAAGT	GCCTGTGGC	CCCTGCTACGA	
10A1	CACCGGACAGGCTTAC	TGGAAGCCO	ACATCATCAT	GGGACCTAAT	CTCCCTTAA	GCGCGGTAAC	ACCCCCTGGG	ACACGGGAT	GCTCCAAAAT	GCTTGTGGC	CCCTGCTACGA	:::
MX27	CACTGGACAGGCATAG 470	CTGGAAGCCA 480	ATCATCATCAT 490	IGGGACCTAAT 500	TTCCCTTAA 510	GCGAGGAAAC 520	ACCCCTCGGA 530	ATCAGGG 540		C	CCCTGTTATGA 550	56
	570	580	590	600	610	620	630	640	650	660	670	680
4070A	CAAAGTATCCAATTC	CTTCCAAGG	GCTACTCGAG	GGGGGCAGATO	CAACCCTCT	AGTCCTAGAA	TTCACTGATO	GCAGGAAAAA	AGGCTAACTG	GGACGGGCCC		ACTGAG
10A1	CAAAGTATCCAATTC	CTTCCAAGGO	GCTACTCGAG	GGGGGCAGATO	CAACCCTCT	AGTCCTAGAA	TTCACTGAT	CAGGAAAAA	AGGCTAATTG	GGACGGGCCC	AAATCGTGGGG	ACTGAG
MX27	AGCGGTCTCCAGTGA	CATCAAGGGG 580	GCCACACCGG 590	GGGGGTCGATC 600	CAATCCCCT	AGTCCTGGAA 620	TTCACTGACO	GCGGGCAAAA 640	AGGCCAGCTG 650	GGATGGCCCC 660	AAAGTATGGGG 670	ACTAAG 68
	690	700	710	720 🗸	730	740	750	760	770	780	790	8
4070A	ACTGTACCGGAC	AGGAACAGA	ICCTATTACCA	ATGTTCTCCCI	GACCCGGCA	GGTCCTTAAT	GTGGGACCCO	GAGTCCCCA	TAGGGCCCAA	CCCAGTATTA		ACTCCC
10A1	ACTGTACCGGAC	AGGAACAGA	ICCTATTACCA	ATGTTCTCCCI	GACCCGCCA	GGTCCTCAA	ATAGGGCCCC	GCATCCCCA	TTGGGCCTAA	TCCCGTGATC	ACTGGTCAACT	
MX27	ACTGTACCGATCCAC	AGGGACCGA	CCCGGTGACCO 710	CGGTTCTCTT1 720	GACCCGCCA 730	GGTCCTCAA1 740	ATAGGGCCCC 750	CGCGTCCCCA 760	TTGGGCCTAA 770	TCCCGTGATC 780	ACTGACCAGT1 790	80
	00 810	820		830	840	1	150 1	360	870	880	890 9	000
4070A	TTCCTCACCAATAGA	GATTGTACC	GGCT(CCACAGCCACO	TAGCCC	CCTC/	ATACCAGTT	ACCCCCCTTC	CACTACCAGT	ACACCCTCAA	CCTCCCCTAC	AGTCCA
10A1	CTCCCGACCCGTGCA	GATCAGGCT	CCCAGGCCT	CCTCAGCCTCO	TCCTACAGG	CGCAGCCTC	ATAGTCCCT	GAGACTGCCC	CACCTTCTCA	ACAACCTGGG	ACG	
MX27	CTCCCGACCCGTGCA	GATCATGCT	CCCCAGGCCT	CCTCAGCCTCO 840	TCCTCCAGG 850	CGCAGCCTC 860	ATAGTCCCT	GAGACTGCCC 880	CACCTTCTCA 890	ACAACCTGGO 900	ACG	
	910 92	0 9:	30 94	40 9!	ии 50 До	60	70	980	990	1000	1010 1	1020
4070 <i>4</i>	AGTGTCCCACAGCCA	CCCCCAGGA	ACTGGAGATA	GACTACTAGC	TCTAGTCAAA	GGAGCCTAT	AGGCGCTTA	ACCTCACCAA	TCCCGACAAG	ACCCAAGAAT	GTTGGCTGTG	CTTAGTG
10 A 1			GGAGACA	GGCTGCTAAA	CTGGTAGAA	GGAGCCTAT	AGGCGCTTA	ACCTCACCAA	TCCCGACAAG	ACCCAAGAAT	GTTGGCTGTGG	CTTAGTG
MX27			GGAGACA0 910	GGCTGCTAAA 920	CTGGTAGAT 930	GGAGCCTAC	CAAGCTCTCA 950	ACCTCACCAG 960	TCCTGACAAA 970	ACCCAAGAG1 980	IGCTGGTTGTGI 990	ICTGGTA
	1030 10	40 1	050 10	060 10	070 I	.080	1090	1100	1110	1120	1130	1140
40704	TCGGGACCTCCTTAT	TACGAAGGA	GTAGCGGTCG	TGGGCACTTA	FACCAATCAT	TCCACCGCT	CGGCCAACT	GTACGGCCAC	TTCCCAACAT	AAGCTTACCO	TATCTGAAGT	GACAGGA
10A1	TCGGGACCTCCTTAT	TACGAAGGA	GTAGCGGTCG	TGGGCACTTA	FACCAATCAT	TCTACCGCC	CGGCCAGCT	GTACGGCCAC	TTCCCAACAT	AAGCTTACCO	TATCTGAAGT	GACAGGA
MX27	GCGGGACCCCCCTAC	TACGAAGGG 1020	GTTGCCGTCC 1030	TAGGTACTTA 1040	TTCCAACCAT	ACCTCTGCC 1060	CAGCTAACT	GCTCCGTGGC 1080	CTCCCAACAC	AAGCTGACCO 1100	TGTCCGAAGT	GACCGGA 1
	1150 11	6 0 1	170 1	180 1	190 1	200	1210	1220	1230	1240	1250	1260
4070/	A CAGGGCCTATGCATG	GGGGCAGTA	CCTAAAACTC	ACCAGGCCTT	ATGTAACACO	ACCCAAAGC	GCCGGCTCAG	GATCCTACTA	CCTTGCAGCA	CCCGCCGGA	ACAATGTGGGC	TTGCAGC
10A1	CAGGGCCTATGCATG	GGAGCACTA	CCTAAAACTC	ACCAGGCCTT	ATGTAACACO	ACCCAAAGT	GCCGGCTCAG	GATCCTACTA	CCTTGCAGCA	CCCGCTGGA	ACAATGTGGGC	TTGTAGC
MX27	CAGGGACTCTGCGTA 120 1130	GGAGCAGTT 1140	CCCAAAACCC	ATCAGGCCCT 1160	GTGTAATACO 1170	ACCCAGAAG	ACGAGCAACG 1190	GGTCCTACTA 1200	TCTGGCTGCT 1210	CCCGCCGGG	ACCATTTGGGC D 1230	TTGCAAC

FIG. 2. Nucleotide alignments of 4070A, 10A1, and MX27 *env* genes. The start of the *env* precursor and the inferred boundaries of the mature processed proteins are indicated above the aligned sequences. Additionally, the three areas of probable recombination between amphotropic and polytropic MuLV sequences in 10A1 are denoted by roman numerals. 10A1 is most similar to MX27 to the 5' side of I, to 4070A between I and II, to MX27 between II and III, and to 4070A on the 3' side of III. The MX27 sequence is from Stoye and Coffin (28).



the ecotropic MuLV group (13) than within any of the PRM groups.

The close alignment of the PRM Env sequences allowed us to derive a consensus PRM Env sequence (Fig. 3A). The degree of identity found among these MuLV Env sequences suggests that all PRM Env proteins share a common overall framework, while the biological differences in receptor specificity and host range among the viruses must result from the minor differences in the Env proteins.

The close resemblance among the different gp70s also raises the possibility that the different receptors are similar to each other, just as a series of similar keys would require a series of similar locks. In this connection, it is interesting to note the findings of Chesebro and Wehrly (6). These investigators made the remarkable observation that some MuLV isolates fall into different interference classes in tests on Mus dunni cells than they do on NIH 3T3 M. musculus cells. The fact that differences were observed between M. musculus and M. dunni cells indicates that the gp70-binding sites of some *M*. dunni receptors are somewhat different from those of their M. musculus homologs. The fact that some MuLV isolates which use different receptors in one host nevertheless share a common receptor in the other implies that the receptor-binding regions of their gp70s are similar to each other. In turn, since each gp70 must fit its receptor, this conclusion suggests that the gp70-binding regions of some distinct receptors in a given host also resemble each other.

Figure 3A also shows that the differences among the env sequences within the PRM group are predominantly localized in a few short regions, as if receptor specificity were carried in these regions of the molecule. In particular, the amphotropic MuLV and 10A1 Env sequences contain two short stretches, at residues 87 to 92 and 163 to 169 (numbering from the initiator methionine of the consensus sequence), which are not present in MX27. Xenotropic MuLV contains a partial copy of the first of these regions but lacks the second. The other region which shows striking diversity among different viruses is the "hypervariable region" (14). This region comprises residues 253 to 304 of the PRM consensus. All MuLV Env proteins have a sequence stretch in this region of the molecule which is >30% proline, and this region has been noted to show significant variation, even within the ecotropic group of MuLVs (13). The sequences immediately flanking this hypervariable region are completely conserved in all MuLVs, and indeed there is very little variation seen to the C-terminal side of this region or in the p15E portion of the Env polyprotein (Fig. 3B). These observations are summarized in Table 2, which indicates the degree of amino acid identity found at different regions of the Env protein. Since the p15Es of all MuLVs are very similar to each other, it seems likely that these transmembrane

Α	Env Leader I gp70
4070A	MARSTLSKPPODKINPWKPLIVMGVLLGVGMAESPHQVFNVTWRVTNLMTGRTANATSLLGTVQDAFPKLYF
MX27	IEGPAF+++LK+++++G++++L+I+IRA+VSVQHD+++++++++++++++Q+++++++MT++++++
NZB-9-1	+EG+AF+++LK+++++G+++++I+VRA+ASVQRD++++I+ <u>+++++++++++Q++++++++MT</u> +T++++++
PRM Consensus:	MSKPDKINPWL*V*G*-*G*SPHQ*F <u>INVT</u> WRVTNLMTG-TA <u>NAT</u> SLLGT*-D-FP*LYF
40704	
4070A 10A1	
MX27	+++++ I+DD++ET+L++RT+G++K+A+++++++++PT+++++R+++++++++++++++++++++
NZB-9-1 PRM Consensus:	++++++DY+++P++DI+D++RT+G++R+++LY+++++PI+++++PI+++++++++++++++++++++
	160 170 180 190 200 210 220
407UA 10A1	MULISLKKGNIPMUIGCSKVACGPCTULSKVSNSFQGAIKGGKCNPLVLEFIDAGKKANMUGPKSMGLKLTK.IG
MX27	+++++++++++S+A++SDIK+++P+++++++++++++++++++++++++++++++++
NZB-9-1 PRM Consensus:	++++++++++K+Q+++++.+S++SGV++++P++++++++++++++++++++++++++++
	II Hypervariable Region
	230 240 250 260 270 280 290 300
4070A	TDP ITMFSLTRQVLNVGPRVPIGPNPVLPDQRLPSSPIEIVPAPQPPSPLNTSYPPSTTSTPSTSPSVPQPP
MX27	+++V+R++++++I++++++++++++++1T++LP++R+VQIMLPRP+QPPPGAASIVPETAP+SQQ
NZB-9-1	A++V+R+++++++++++++++++++++++++++++++++
PRM Consensus:	-DP*T-FSLTRQVLN*GPR*PIGPNPVQPSPPPPPPP
	310 320 330 340 350 360 370
4070A	PGTGDRLLALVKGAYQALNLTNPDKTQECWLCLVSGPPYYEGVAVVGTYTNHSTAPANCTATSQHKLTLSEVTGQ
MX27	+++++N++D++++++S++++++S++++++++++A++++++++++
NZB-9-1	++++++N++E++++++SVA++++++S+++++++++++++++++++++
PRM Consensus:	PGTGDRLL-LV-GAYQAUNLTJ-PDKTQECWLCLV-GPPYYEGVAV*GTY*NH*J*APA-C*SQHKLTLSEVTGQ
	380 390 400 410 420 430 440 450
4070A	GLCMGAVPKTHQALCNTTQSAGSGSYYLAAPAGTMWACSTGLTPCLSTTVLNLTTDYCVLVELWPRVIYHSPDYM
10A1	++++++L+++++++++++++++++++++++++++++++
NZB-9-1	+++V++++++++++KTSD++++++KTSD+++++++
PRM Consensus:	GLC*GA*PKTHQALCNTTPGSYYLAAPAGT*WAC-TGLTPCLSTT*L*LTTDYCVLVELWP**-YHSP-Y*
	p15E 450 470 480 480 500 510 520
4070A	YGQLEQRTKYKREPVSLTLALLLGGLTMGGIAAGIGTGTTALIKTQQFEQLHAAIQTDLNEVEKSITNLEKSLTS
10A1	******
MX27 N78-9-1	+++F+RK+R++++++++++++++++++++++++++++++
PRM Consensus:	YGQ-E-*T*YKREPVSLTLALLLGGLTMGGIAAG*GTGTTAL*-T-QFEQL-AAI-TDL*-KS**-L-KSLTS
	530 540 550 560 570 580 590 600
4070A	LSEVVLQNRRGLDLLFLKEGGLCAALKEECCFYADHTGLVRDSMAKLRERLNQRQKLFETGQGWFEGLFNRSPWF
10A1 MX27	++++++++++++++++++++++++++++++++++++++
NZB-9-1	++++++++++++++++++++++++++++++++++++++
PRM Consensus:	LSEVVLQNRRGLDLLFLKEGGLCAALKEECCFYADHTG*VRDSMAKLRERLNQRQKLF-*GQGWFEG-F <mark>NRS</mark> PWF
	p2E
	610 620 630 640 650 660
4070A	TTLISTIMGPLIVLLLILLFGPCILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP
MX27	++++++++++S+DP+EVESRE
NZB-9-1	++++++++++++++++++++++++++++++++++++++

NZB-9-1 PRM Consensus: TTLISTIMGPLI*LLIIL-GPCILNRLVQFVKDRISVVQAL*LTQQYHQLK-I*-E------FIG. 3. Amino acid alignment of the PRM groups and all MuLVs. (A) Amino acid sequence of 4070A Env. Periods indicate gaps introduced for maximal alignment. The other sequences, 10A1, MX27, and NZB-9-1 (17), are presented below that of 4070A, and identical amino acids are denoted by +. A PRM consensus is presented below the alignments; identities are shown by letters, and mismatches are indicated by dashes. Conservative amino acid changes (A,G; S,T; F,W,Y; I,L,M,V; K,H,R; and D,E,N,Q) are indicated by asterisks. Recombination points and boundaries of Env proteins are shown above the alignments, as in Fig. 2. Inferred elycosylation sites are boxed

Recombination points and boundaries of Env proteins are shown above the alignments, as in Fig. 2. Inferred glycosylation sites are boxed on the consensus, and missing or novel sites are underlined. (B) Amino acid sequences of two ecotropic MuLV Env proteins, that of Moloney MuLV (24) and that of Akv (10). Residues in Akv identical to those of Moloney MuLV are indicated by a +. Also shown is the PRM consensus from panel A. Residues which are identical in the PRM consensus, Moloney, and Akv sequences are shown as an MuLV consensus in the bottom line.

proteins interact with the conserved, C-terminal portion of gp70 in the mature Env complex.

Since 10A1 uses a unique receptor on NIH 3T3 cells, we previously suggested (21) that it had acquired unique env sequences from an endogenous env gene. The distinctive

nature of the $10A1 \ env$ gene is further highlighted by the ability of this virus to infect CHO cells (Table 1). However, our sequence analysis shows that the $10A1 \ env$ gene contains almost no novel coding sequences. Rather, it appears to be a unique combination of env sequences which are also present

В	
	Env Leader gp70
	1 10 20 30 40 50 60 70
PRM Consensus:	MSKPDKINPWL*V*G*-*G*SPHQ*FNVTWRVTNLMTG-TANATSLLGT*-D-FP*L
Moloney	MARSTLSKPLKNKVNPRGPLIPLILLMLRGVSTASPGSSPHQVYNITWEVTN.GDRETVWATSGNHPLWTWWPDL
AKV	+EST+++++F++Q+++W+++++V+LI+GGVNPV+L+N+++++F++L+++++.+++++++IT+++++++IT+++++++
MuLV Consensus:	MSKPI-NPL*-*-*SPHQ**N*-W-VINI-A-*P-L
	80 90 100 110
PRM Consensus:	YFDLCDL*G*-WDG-GC*-P*GR-R-R-*D.
Molonev	TPDLCMLAHHGPSYWGLEYOSPFSSPPGPPCCSGGSSPGCSRDCEEPLTSLTPRCNTAWNRLKLDOTTHKSNE
AKV	++++++L++++++RA+++P++++++S+DST+++++++Y+++++++SKV++AH+G
MuLV Consensus:	DLC-L
PRM Consensus:	120 130 140 150 160 170 180 .FYVCPG.HTVGCGGP-EGYCG-WGCETTGQAYWKP*SSWDLISLKRGNTPGPCYD-S-VS
Moloney	GFYVCPGPHRPRESKSCGGPDSFYCAYWGCETTGRAYWKPSSSWDFITVNNNLTSD
AKV	+++++++++WAR+++++E+++++S++++++S++++++Y+++S++++++Y+++S++++++
MuLV Consensus:	-FYVCPG-HCGGPY-*-WGCETTG-A-WKP*SSWD-I** <u>-T-</u> DD
	190 200 210 220 230 240 250
PRM Consensus:	GAT-GGRCNPLVLEFTDAG*K.A-WD*PK-WGLRLYR-TG-DP*T-FSLTRQVLN*GPR*PIGPNPVQ
Moleney	
AKV	++TP+++G+E+++S+T++++SF+KOA+++V+++W++++++++++++++++++++++++++++++
MuLV Consensus:	CN-L-*-FTG*WWGLRLY*G-DPF-**-GPR*PIGPNPV*
	Hypervariable Region
PRM Consensus:	260 270 280 290 300 ¥ 310 320
Moloney	QPLSKPKP.VKSPSVTKPPSGTPLSPTQLPPAGTENRLLNLVDGAYQALNLTSPDKTQEC
MuLV Consensus:	RRPPSRPRP1R++PPSNS1P1E1PL1L+.EP++++V+++++++++++++++++++++++++++++++
PRM Consensus:	330 340 350 360 370 380 390 400 WLCLV-GPPYYEGVAV*GTY*NH**APA-C*SQHKLTLSEVTGQGLC*GA*PKTHQALCNTTQGSYYLA
Moloney	WLCLVAGPPYYEGVAVLGTYSNHTSAPANCSVASQHKLTLSEVTGQGLCIGAVPKTHQALCNTTQTSSRGSYYLV
AKV	+++++S++++++++++++++++++++++++++++++++
MuLV Consensus:	WLCLV-GPPYYEGVAV*GTY NHI *APA-C* SQHKLTLSEVTGQGLC*GA*PKTHQ-LONTTQGSYYL-
	410 420 430 440 450 460 470
PRM Consensus:	APAGT*WAC-TGLTPCLSTT*L*LTTDYCVLVELWP**-YHSP-Y*YGQ-E-*T*YKREPVSLTLALLLGGLTMG
Moloney	
AKV	++++++T+++++++++++++++++++++++++++++++
MuLV Consensus:	AP-GT-WAC-TGLTPC*STT*L <u>*LTTDYCVLVELWP**-YHSP-Y*YE*-KREPVSLTLALLLGGLTMG</u>
	480 490 500 510 520 530 540 550
PRM Consensus:	GIAAG*GTGTTAL*-T-QFEQL-AAI-TDL*-KS**-L-KSLTSLSEVVLQNRRGLDLLFLKEGGLCAALKEE
Malaa	
AKV	GIAAGIGIGIGIALMAIQQFQQLQAAVQDDLREVERSISNLERSLISLSEVVLQNRRGLDLLFLREGGLCAALREE
MuLV Consensus:	GIAAG*GTGTTAL*-T-QF*QL-AA*DL*-KS**-L-KSLTSLSEVVLQNRRGLDLLFLKEGGLCAALKEE
PRM Consensus:	560 570 580 590 600 610 620 CCEYADHTG*VRDSMAKI RERI NOROKI F. *GOGWEFG-ENRSPWETTI ISTIMGPI I*III TII -GPCII NRI V
i illi consensus.	
Moloney	CCFYADHTGLVRDSMAKLRERLNQRQKLFESTQGWFEGLFNRSPWFTTLISTIMGPLIVLLMILLFGPCILNRLV
AKV Mul V Consensus	++++++++++++++++++++++++++++++++++++++
	(p2E
PRM Consensus	630 640 650 660 OEVKORISVOAL *I TOOYHOI K . I * . E
i nivi Gonaenaus.	ALAURISINAUF FIAALUAFU.I -E
Moloney	QFVKDRISVVQALVLTQQYHQLKPIEYEP
AKV Mul V Consensus	++1+++++++++++++++++++++++++++++++++++
MULT CONSTINUS:	
	FIG. 3—Continued

separately in other MuLVs. Thus, 10A1 contains the two N-terminal insertions noted above as characteristic of amphotropic MuLV, while its hypervariable region is nearly identical to that of MX27 and the polytropic MuLV-derived (28) MCFs. The results suggest that receptor specificity is determined combinatorially, since substitution of an MX27like hypervariable region in an otherwise amphotropic gp70 has created an Env molecule with a third receptor specificity. The recombinant nature of the 10A1 gp70 is indicated schematically in Fig. 5.

Our previous biological studies indicated that in addition to its affinity for a unique receptor, the 10A1 Env protein also interacts with the amphotropic MuLV receptor on NIH 3T3 cells (21). Our results with 10A1 show that the presence of the amphotropic MuLV hypervariable region is not necessary for amphotropic MuLV receptor specificity. One possibility is that one or both of the N-terminal insertions determine amphotropic MuLV receptor specificity independently of the hypervariable region, while the second, unique receptor specificity of 10A1 reflects the "combinatorial" interaction between these regions of gp70. It seems possible that the dual receptor specificity contributes to the leukemogenicity of 10A1. The ability of an Env protein to interact with more than one receptor may also be important in



FIG. 4. 4070A LTR sequence and alignment with 10A1 transcriptional signals. The sequences start immediately after the *env* termination codon. Boxes indicate the structural elements of the 4070A LTR and transcriptional regulatory sequences (9, 26).

understanding the pathogenicity of human immunodeficiency virus, since this virus is known to infect $CD4^+$ cells by interaction with CD4 but is also found in a number of $CD4^-$ cell types.

It is also possible that receptor specificity is not entirely determined by the variable-sequence blocks in gp70, since amphotropic MuLV and 10A1 also differ at 15 individual positions within the more highly conserved regions of the molecule. Experiments are under way to establish the role of the variable regions in determining receptor specificity.

Several of our conclusions on receptor specificity in MuLVs are similar to those reached in previous studies on avian retroviruses. Thus, gp85^{su} sequences of avian subgroups A, B, C, and E were aligned by Bova et al. (4), who noted that the differences among these Env molecules with different receptor specificities were confined to discrete regions of the molecule. Dorner and Coffin (8) analyzed a series of recombinants between subgroups B, C, and E viruses and showed that receptor specificity was determined principally by two sequence blocks. The relative positions of these blocks correspond rather closely to those we have described in the murine system; they are found at 36 and 59% of the distance from the N terminus to the C terminus of gp85, while the two short insertions in amphotropic MuLV are at 12 and 30% and the hypervariable region covers 51 to 63%. Avian viruses also show somewhat different interference patterns on chicken and turkey cells. Finally, Dorner and Coffin showed directly that receptor specificity is not always determined in a single region of gp85 but is sometimes a result of the combination of sequences present in a chimeric gp85 molecule. This finding is similar to what we suggest here on the basis of our comparison of the 10A1 sequence with those of 4070A and MX27, although in avian retroviruses, as far as is known, a novel sequence combination does not generate a completely novel receptor specificity or extended host range. The analogies noted here strongly suggest that similar structure-function relationships exist in murine and avian surface glycoproteins and in their interactions with their respective receptors.

Comparison of the 10A1 nucleotide sequence with those of amphotropic MuLV and MX27 also allowed us to determine the sites of recombination involved in the generation of 10A1. As indicated in Results, 10A1 is nearly identical to MX27 over two limited regions: (i) from a site in the *pol* gene to nucleotides 67 to 87 in the *env* leader region and (ii) 724-731 to 959, which encodes the hypervariable region plus 24 to 27 additional amino acids. With the exception of these two regions, the 10A1 *env* gene shows over 96% nucleotide

TABLE 2. Amino acid conservation in Env

Consensus		% Identical amino acids at the indicated amino acid position ^{a} in:									
		NH ₂	one-third f gp70		Middle of g	one-third gp70	C-terminal one- third of gp70	p15E + p2E			
	32-86	87–94	95–162	163-172	173-252	253-304	305-462	(403-003)			
PRM All MuLV ^b	76 32	0 0	79 26	0 0	78 35	21 6	80 75	85 81			

^a Counting from the initiator methionine of the consensus.

^b PRM plus Moloney MuLV and Akv.



FIG. 5. Schematic of amphotropic MuLV, 10A1, and MCF mature gp70 amino acid sequence comparisons. Black lines indicate regions of all three viruses that are >75% identical. Amphotropic sequence insertions relative to MCF are indicated. MCF hypervariable proline-rich regions are represented by black boxes, and the amphotropic region is shown as a white box.

sequence identity with amphotropic MuLV. Thus, 10A1 appears to have arisen by two substitutions of MX27-related sequences into the parental amphotropic MuLV *env* gene. (Additional recombinations may, of course, have occurred in the *gag* or *pol* region.)

The limited extent of the substitutions that generated 10A1 is in sharp contrast to the recombinations which give rise to MCFs. As far as is known, all viable recombinants between ecotropic MuLVs and endogenous *env* genes use the MCF receptor. In all MCFs which have been analyzed, at least the entire N-terminal approximately two-thirds of the ecotropic gp70-coding region, from the N terminus through the hypervariable region, has been replaced with MX27 (or MX33 [28])-related sequences. The consistency of these findings strongly suggests that if other types of recombinants occur, such as crossovers within the N-terminal two-thirds of gp70, they are not selected for outgrowth in viremic mice. It would be interesting to test the viability and receptor specificity of such chimeras generated in vitro.

O'Neill et al. (18) have subcloned a 133-base-pair fragment of the amphotropic MuLV genome and have demonstrated its utility as an amphotropic MuLV-specific probe. Using this subclone, they showed that this sequence is not present in the DNA of uninfected animals; they concluded that amphotropic MuLV is only transmitted horizontally. This subclone represents nucleotides 191 to 324 of the amphotropic MuLV sequence (Fig. 2), which encode residues 53 to 96 of the Env protein (Fig. 3). It therefore spans the first of the two N-terminal insertions in the amphotropic MuLV protein sequence.

We also present here U3 sequences of amphotropic MuLV and 10A1. The two sequences are extremely similar to each other (94.9% identity) (Fig. 4). Many studies of MuLVs have pointed to the U3 regulatory sequences as major determinants of pathogenicity. On the other hand, the MCF *env* gene has also been shown to contribute to the pathogenicity of Akv- and F-MuLV-derived MCFs (12, 16). It is striking that the two MuLVs analyzed in this study have similar LTRs but differ enormously in leukemogenicity. In light of this difference, it seems possible that the 10A1 *env* gene plays a key role in the pathogenicity of 10A1.

The mechanism by which the MCF env gene contributes to the pathogenicity of a recombinant MuLV remains a major unsolved problem in understanding leukemia induction by nonacutely transforming retroviruses. While the sequences presented here suggest the possibility that the 10A1 env gene is important in the pathogenicity of this virus, it will be of great importance to test this hypothesis empirically. Experiments testing the leukemogenic potential of chimeric MuLVs are under way.

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