A Replication-Competent, Endogenous Retrovirus from an Aged DBA/2 Mouse Contains the Complete *env* from Emv-3 and a Novel *gag* Partially Related to AKT-8

THOMAS BARTMAN,^{1,2} DONNA M. MURASKO,³ AND KENNETH J. BLANK^{2*}

Graduate Program in Molecular Biology and Genetics, Temple University School of Medicine, Philadelphia, Pennsylvania 19140¹; Department of Pathology and Laboratory Medicine, Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19102²; and Department of Microbiology and Immunology, Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19129³

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We previously described an endogenous murine retrovirus, rv-DBA/2_{aged}, isolated from an aged DBA/2 mouse. The previous report showed that a recombination which resulted in the replacement of Emv-3 *gag* sequences with *gag* sequences homologous to those found in the AKT-8 virus had taken place. This recombination allowed production of a competent virus from the defective *Emv-3* locus. However, the extent of replacement of Emv-3 *gag* was not known. We report here the entire sequence for the *gag* gene of rv-DBA/2_{aged} as well as the previously unsequenced 3' end of the Emv-3 *gag* gene. These data demonstrate that while sequences homologous to the entire *gag* gene fragment found in AKT-8 are represented in rv-DBA/2_{aged}, the remainder of rv-DBA/2_{aged} *gag* is not derived from Emv-3 but is a unique *gag* sequence. Furthermore, a complete comparison of *env* sequences shows that the *env* of rv-DBA/2_{aged} is derived entirely from Emv-3. Additional data suggest that the recombination which led to production of the rv-DBA/2_{aged} virus may be a common event in aging DBA/2 mice. Finally, comparison of the new sequences of Emv-3 with those of the Akv virus (also designated AKR-623 and Emv-11) and Emv-1 shows that this endogenous virus locus is very closely related to the other *Emv* loci at the nucleotide sequence level.

The genomes of virtually all strains of laboratory mice have been shown to contain endogenous retrovirus sequences (17). In many cases, these sequences are fragments of retrovirus genomes, but in several mouse strains, such as BALB/c, C57BL/6, DBA/2, and AKR, complete endogenous ecotropic proviruses have been identified (6, 16, 21, 29). The endogenous ecotropic retroviruses found in these and other strains were identified by Southern analysis of genomic DNA, and retrovirus sequences were grouped and classified according to the chromosomal location of each provirus (17). For example, both BALB/c and CBA/J mice have a complete virus sequence, Emv-1 (endogenous murine virus-1), integrated at the same genomic location. C57BL/6 and DBA/2 contain Emv-2 and Emv-3, respectively, while AKR mice contain Emv-11, -12, -13, and -14. Interrelatedness among these provirus loci has been studied most often at the gross molecular level by mapping restriction enzyme recognition sites. This work has shown that these viruses seem to be highly related (17), although only a small amount of sequence data exists to completely substantiate this possibility (15, 20). To date, the only endogenous ecotropic virus to be completely sequenced is the Akv-1 virus derived from one of the endogenous ecotropic loci (*Emv-11*) of AKR mice (12). Other than this sequence, the only *Emv* sequences found in the GenBank database are the *pol* and *env* genes of Emv-1 (28) and a portion of the *gag* gene of Emv-3 (7).

While it has been known for some time that the expression of *Emv* loci is responsible for infectious ecotropic virus production in young AKR mice (25), it has recently been deter-

mined that the presence of *Emv* loci also contributes to endogenous virus production in aged mice of strains which produce no infectious virus early in life (1, 33). Although the *Emv* loci encode complete endogenous virus genomes, most *Emv* loci are defective in the production of replication-competent virus as a result of relatively minor defects in their virus sequences. Thus, the ability to produce infectious virus depends on repair of the particular defect in the genome of the endogenous ecotropic virus. For example, the *Emv-1* locus contains a defect at the junction of gp70 and p15e which prevents the cleavage of these two molecules from the precursor, Pr85 (10). The Emv-2 endogenous ecotropic sequence has been found to have a defect in the *pol* region (19). The Emv-3 sequence, found in DBA/2 mice, has been shown to have a single base defect in the third codon of *gag* (4). This base change in Emv-3 results in the presence of a proline residue at the site where a glutamine residue is found in replication-competent viruses. This amino acid difference interferes with the requisite myristylation of the adjacent amino acid (18). Both site-directed mutagenesis and chemical-mediated mutagenesis, resulting in a proline-to-glutamine change at this amino acid, have been shown to be permissive for Emv-3-encoded virus replication (18, 22). However, we have recently demonstrated that expression of endogenous ecotropic Emv-3 virus occurs as a result of recombination in the *gag* gene which results in a virus with an Emv-3 *env* sequence but with *gag* sequences similar to those of the AKT-8 *gag* gene fragment (1). This recombination results in the production of a replication-competent virus which we have called rv-DBA/2_{aged}.

In this study, we have sequenced the entire *gag* and *env* genes of rv-DBA/2_{aged} as well as the 3' half of Emv-3 *gag* and the entire *env* of Emv-3, neither of which had previously been

* Corresponding author. Phone: (215) 762-7175. Fax: (215) 762-3274. Internet: blankk@hal.hahnemann.edu.

sequenced. Our results allowed us to draw further conclusions about the origins of rv-DBA/2_{aged} as well as to compare the *Emv-3* locus with the other sequenced *Emv* loci (*Emv-11* and *Emv-1*). The results demonstrate that the replication-competent rv-DBA/2_{aged} virus contains a complete *gag* gene whose 5' portion is identical to the partial *gag* sequence found in AKT-8. The 3' portion of the rv-DBA/2_{aged} *gag* gene (i.e., that part not found in AKT-8) is related to but clearly unique from previously sequenced ecotropic murine leukemia viruses. The *env* genes of rv-DBA/2_{aged} and *Emv-3* were found to be 99.9% identical, indicating very few differences between the *env* genes of these two viruses. Since this work also involved complete sequencing of the *env* and *gag* genes of *Emv-3*, we were able to show this locus to be highly related to other *Emv* loci at the sequence level. Finally, this study also presents data for seven additional viruses from aged DBA/2 mice which indicate that the recombination event which led to production of the rv-DBA/2_{aged} virus may be a common event in aged DBA/2 mice.

Sequence analysis of *Emv-3*. Previous sequencing of the first half of *Emv-3 gag* showed this locus to be similar to the sequence for the homologous region of AKR-623 (also designated Akv and *Emv-11*) (7). However, since most of the *Emv-3* locus had not been sequenced, it was necessary to sequence the complete *gag* and *env* genes for us to determine whether the virus expressed in aged DBA/2 mice was derived from *Emv-3*. Previously, an 18-kb fragment (pEMV-3, pDB.1) that contained the entire *Emv-3* locus was cloned from genomic DNA (5). We subcloned the *gag* and *env* portions of this clone (kindly provided by N. Jenkins) by performing double digestion with *Pst*I and *Xho*I, resulting in fragments of 4.6 and 4.1 kb that contained the 5' and 3' portions of the *Emv-3* genome, respectively (*Pst*I cuts the virus only in the long terminal repeats, and *Xho*I makes one cut in the *pol* gene). These fragments were cloned unidirectionally into both pBluescript SK- and pBluescript SK+ (Stratagene) which had been double digested with *Pst*I and *Xho*I. Single-stranded DNA was obtained from each construct by phage rescue with VCS-M13 and sequenced by using the Sequenase version 2 kit (U.S. Biochemicals). The primers used for sequencing the *Emv-3* genes were designed from the AKR-623 sequence (12) and are indicated along with the sequence of *Emv-3 gag* in Fig. 1. The sequences from *Emv-3 gag* were entered into a computer and aligned with each other until both strands were sequenced over the entire gene-coding region and all bases on both strands were correlated. All data analysis was performed by using the University of Wisconsin Genetics Computer Group suite of computer programs (9). The 5' 762 bases of *Emv-3 gag*, which had been previously sequenced by Copeland et al. (7), were not resequenced except for a suitable overlap to ensure a continuous *gag* sequence. Comparison of the complete sequence of *Emv-3 gag* (Fig. 1) with other *gag* sequences (Tables 1 and 2) shows that it is highly related to AKR-623 (encoded by *Emv-11*) at both sequence and amino acid levels over the length of the coding region. Other than the two amino acid differences previously reported between *Emv-3* and AKR-623 (proline for glutamine at position 3 and proline for lysine at position 169, the former of which is responsible for the lack of myristylation of *Emv-3 gag* and failure to produce retrovirus particles [7, 18]), the only other amino acid change in *gag* is an aspartic acid-to-asparagine change in the p30 molecule. We also sequenced the entire *Emv-3 env* gene and compared it with the *env* genes of AKR-623 and *Emv-1* (Table 3). This analysis showed that the *env* gene of *Emv-3* is virtually identical to the *env* genes of AKR-623 and *Emv-1* at both nucleotide and amino acid sequence levels. One amino acid difference between *Emv-1* and *Emv-3* is at the junction of gp70 and p15e:

* The sequences of *Emv-3* and rv-DBA/2_{aged} *gag* not present in these references are being reported here for the first time.

FIG. 1. Comparison of the *gag* genes of AKR-623, *Emv-3*, rv-DBA/2_{aged}, and AKT-8. The entire sequence of rv-DBA/2_{aged} is shown from the start codon to the stop codon. Only bases which differ from those in rv-DBA/2_{aged} are shown for other sequences. The locations and directions of internal primers used in sequencing are shown below the sequence line as dashed arrows. A primer upstream of the coding region had the sequence 5'-TCTGTCTCAGTATTGT TTTG-3', and a primer downstream of the coding region had the sequence 5'-ACAGTGAGGGTTATCCTGGG-3'.

Code	Sequence Description	GenBank sequence ID	Bases	Reference
Akr	Sequence of AKR-623 (<i>Emv-11</i>)	Mlccg.gb_vi	639-2252	(12)
Emv	Endogenous <i>Emv-3</i> sequence	Musmlv.gab_ro Mlvgap.gb_ro	637-1398 1-1614	(7)*
Aged	Sequence of rv-DBA/2 _{aged} virus	Mlvager.gb_vi Mlvpgag.gb_vi	1-777 1-1614	(1)*
AKT8	Portion of <i>gag</i> found in AKT-8 virus	Aktakta.gb_vi	324-1109	(2)

TABLE 1. Numerical relatedness among the 5' ends of *gag* genes

Source	% Identity ^a			
	AKR-623	Emv-3	AKT-8	rv-DBA/2 _{aged}
AKR-623		99.5 (4)	96.7 (26)	96.6 (27)
Emv-3	99.2 (2)		96.7 (26)	96.6 (27)
AKT-8	96.9 (8)	96.9 (8)		99.9 (1)
rv-DBA/2 _{aged}	96.6 (9)	96.6 (9)	99.6 (1)	

^a Over the 5' portion of the *gag* gene (786 bases or 262 residues). The percent identity between the indicated pair of genes for nucleotide (upper right of diagonal) or amino acid (lower left of diagonal) sequences is given, with the number of nucleotide or amino acid differences noted within parentheses. Data for genes that are greater than 99% identical are in boldface.

the presence of lysine in Emv-1 prevents the cleavage of Pr85 *env* into these two components, thus preventing infectious retrovirus particle formation (10).

These sequence data indicate that *Emv-1*, *Emv-3*, and *Emv-11* (AKR-623), although located at different chromosomal positions, are extremely similar at both nucleotide and amino acid levels. This observation is consistent with the possibility that all endogenous ecotropic virus genomes have been derived from a common progenitor and remain virtually identical. However, in animals with a defective *Emv* locus (such as *Emv-1* or *Emv-3*), either point mutations of small single-gene defects or recombination with other endogenous retrovirus sequences (e.g., retrovirus gene fragments) is required for production of replication-competent retrovirus.

Sequence analysis of rv-DBA/2_{aged}. In a previous study, we demonstrated that an aged DBA/2 mouse expressed a replication-competent endogenous retrovirus as the result of a recombination which formed a virus that included at least portions of both a *gag* gene homologous to the partial *gag* gene found in AKT-8 and the *Emv-3 env* gene (1). At that time, however, sequencing rv-DBA/2_{aged} failed to reach the putative recombination breakpoint and the end of the *gag* sequence homologous to the AKT-8 *gag* sequence. Therefore, it was not known at exactly what point the crossover had occurred. Furthermore, it was not known if the entire *Emv-3 env* gene had been encoded by rv-DBA/2_{aged} or further recombinations with other endogenous virus loci had occurred in this region. To address these issues, we completed the sequencing of rv-DBA/2_{aged gag} and *env* genes.

The original rv-DBA/2_{aged} virus was obtained from an aged DBA/2 mouse as previously described (1). The *env* and *gag* genes were molecularly cloned from PCRs on Hirt lysates of virus-infected SC-1 cells as previously described (13, 26). The primers used for PCRs in subcloning rv-DBA/2_{aged} genes were derived from the AKR-623 sequence. These same primers were used for sequencing the resulting *gag* and *env* genes. Double-stranded DNA was sequenced by the dideoxy termination method as previously described (27). Briefly, double-stranded DNA was denatured by melting 3 µg of DNA and 200 ng of primer at 85°C for 5 min in the presence of 0.2 N NaOH–0.2 mM EDTA. Then DNA was precipitated with ethanol and dried. After resuspension in enzyme reaction buffer, double-stranded DNA was sequenced by using Sequenase version 2.0 (U.S. Biochemicals). The results of this sequencing (Fig. 1) show that the entire *gag* gene fragment in AKT-8 is represented in the *gag* gene of rv-DBA/2_{aged}. The *gag* gene of rv-DBA/2_{aged} extends beyond this point but does not demonstrate high sequence homology with the equivalent *Emv-3 gag* sequence. In fact, while the AKT-8 portion (i.e., the first 786 bases) of the rv-DBA/2_{aged gag} gene is 96.6% homologous with the *gag* gene of *Emv-3* (Table 1), the non-AKT-8 portion (i.e.,

TABLE 2. Numerical relatedness over the entire coding regions for *gag* genes

Source	% Identity ^a		
	AKR-623	Emv-3	rv-DBA/2 _{aged}
AKR-623		99.5 (8)	92.4 (123)
Emv-3	99.4 (3)		92.4 (123)
rv-DBA/2 _{aged}	96.1 (21)	95.9 (22)	

^a Over the entire coding region (1,614 bases or 537 residues). The percent identity between the indicated pair of genes for nucleotide (upper right of diagonal) or amino acid (lower left of diagonal) sequences is given, with the number of nucleotide or amino acid differences noted within parentheses. Data for genes that are greater than 99% identical are in boldface.

the last 828 bases) of the rv-DBA/2_{aged gag} gene is only 88.4% homologous with *Emv-3 gag*. Overall, the homology of rv-DBA/2_{aged gag} with *Emv-3 gag* at the nucleotide level is only 92.4%, although these two *gag* genes are 95.9% identical at the amino acid level, with many conservative amino acid changes (Table 2). Analysis of the sequence of the rv-DBA/2_{aged env} gene (Table 3) indicates that it is virtually identical (99.9%) to the *env* of *Emv-3*, demonstrating that no recombinations occurred in the *env* gene.

Since the 3' 828 bases of the rv-DBA/2_{aged gag} gene did not match those of *Emv-3*, it became necessary to try to identify the source of the remainder of the rv-DBA/2_{aged gag} gene by performing a GenBank search. This search indicated that although the 5' portion of rv-DBA/2_{aged} is almost identical (99.9% homologous) to the *gag* gene of AKT-8 and is 96 to 98% identical to other *gag* genes in the database (including those of AKR-623 and *Emv-3*), the 3' portion of the rv-DBA/2_{aged gag} gene is at most 90% identical at the nucleotide level to any other *gag* sequence in the GenBank database. All of the sequences which best match the 3' portion of rv-DBA/2_{aged} are ecotropic retroviruses, indicating that the source of this novel *gag* gene may be an endogenous ecotropic retrovirus locus. The single best match (90.0% homologous) is the sequence for the ecotropic component of LP-BM5, the virus complex responsible for murine AIDS (3). However, the relatively low level of homology to known sequences indicates that the 3' part of rv-DBA/2_{aged gag} is a unique *gag* sequence.

Despite the fact that the DBA/2 mouse genome contains a complete endogenous retrovirus sequence, *Emv-3*, only sporadic endogenous ecotropic virus production is found in young DBA/2 mice (23). The inability of endogenous ecotropic virus to be produced from the *Emv-3* genome in young DBA/2 mice is a result of the lack of myristylation of the *gag* protein precursor, which prevents production of replication-competent virus (18). This failure to produce an infectious ecotropic virus has been localized to one base in the *Emv-3 gag* sequence, since site-directed mutagenesis of the third codon of *gag* which results in an amino acid change from proline to glutamine has been found to result in virus production (18). Therefore, in aged mice which spontaneously begin to produce infectious endogenous retrovirus, this change might occur spontaneously by point mutation or recombination with other endogenous retrovirus sequences. This possibility was suggested by previous studies in which aged BALB/c mice were found to produce B-tropic endogenous ecotropic retrovirus despite the fact that the *Emv-1* locus encoded an N-tropic endogenous retrovirus (24). This finding indicated that changes must have occurred in the *Emv-1 gag* sequence that encodes the p30 molecule which determines the tropism of the virus (8).

Our previous report indicated that, in fact, the change in the *gag* gene required for production of replication-competent,

TABLE 3. Relationships among the coding regions of four *env* genes

Source ^a	GenBank sequence identification	GenBank sequence bases	Reference	% Identity ^b			
				AKR	Emv-1	Emv-3	Aged
AKR	Mlocg.gb_vi	5783-7792	12		99.7 (7)	99.6 (8)	99.5 (10)
Emv-1	Musmulvea.gb_ro	2064-4073	28	99.6 (3)		99.8 (5)	99.7 (7)
Emv-3	Mlvgpenvpa.gb_ro	1-2010		99.6 (3)	99.6 (3)		99.9 (2)
Aged	Mlvgpenvpb.gb_vi	1-2010		99.3 (5)	99.3 (5)	99.7 (2)	

^a AKR, AKR-623 (Emv-11); Emv-1, endogenous Emv-1 sequence; Emv-3, endogenous Emv-3 sequence; Aged, rv-DBA/2_{aged} virus.

^b The percent identity (over 2,010 bases or 669 residues) between the indicated pair of genes for nucleotide (upper right of diagonal) or amino acid (lower left of diagonal) sequences is given, with the number of nucleotide or amino acid differences noted within parentheses.

infectious endogenous retrovirus had occurred by recombination with an endogenous *gag* sequence related to the AKT-8 retrovirus (1). AKT-8 contains the 5' portion of a *gag* gene fused to the oncogene *v-akt* (2). The exact origin of the *gag* sequence in this virus is unknown, and a search of the Gen Bank database indicates that no other *gag* sequence matches the *gag* sequences of rv-DBA/2_{aged} and AKT-8. In contrast to the AKT-8 virus, which contains only a partial *gag* sequence, rv-DBA/2_{aged} encodes a complete *gag* sequence. Therefore, although we suspected that the remainder of the *gag* gene in rv-DBA/2_{aged} may have been derived from the *Emv-3* locus, this could not be demonstrated until the region was sequenced and the sequence was compared with the 3' end of *Emv-3 gag*. This report shows that the 3' end of rv-DBA/2_{aged} *gag* is not derived from *Emv-3* but is a unique *gag* sequence.

Analysis of additional viruses from aged DBA/2 animals.

The studies described so far examined only one virus isolate from one aged animal. Therefore, we wished to determine if the recombination event which led to the creation of the rv-DBA/2_{aged} virus was a common event in aged DBA/2 mice. For this purpose, seven additional virus isolates, each from a different DBA/2 mouse between the ages of 24 and 30 months, were obtained. Hirt DNAs from these viruses were prepared, and PCRs which amplified the entire coding region of the *gag* gene except for the 5' 430 bases were performed. PCR products were cloned, and two molecular clones of each isolate were partially sequenced (except in one case in which only one clone was obtained). Sequencing was performed with the M13 forward and reverse primers, which bind to cloning vector sequences. This protocol allowed us to obtain the sequences of the 5' and 3' ends of these PCR products. The results from sequencing demonstrate that the first 100 bases at either the 5' end or 3' end were identical to those of either *Emv-3* or rv-DBA/2_{aged} and indicate that five of the seven clones are related to *Emv-3 gag*, one clone is related to rv-DBA/2_{aged} *gag*, and one clone is related to both the *Emv-3* and the rv-DBA/

2_{aged} *gag* genes (Table 4). Therefore, three of eight viruses isolated from aged DBA/2 mice thus far demonstrate recombination between *Emv-3* and the endogenous *gag* sequence homologous to AKT-8. These data, however, do not preclude the possibility that recombination between endogenous virus sequences occurred in the other isolates since the entire *gag* genes of the seven new virus isolates have not been sequenced.

Preliminary data indicate that the recombination which led to production of the rv-DBA/2_{aged} virus was not an isolated event in one DBA/2 animal but may be a common event during production of endogenous retrovirus in aging DBA/2 mice. The results suggest that ecotropic endogenous virus production in aged DBA/2 mice arises as a result of either recombination or point mutation that involves the third codon of the *gag* sequence. However, since the 5' 430 bases of the *gag* region of the new virus isolates have not been sequenced, the possibility that recombinations occurred in this region cannot be ruled out at this time. Alternatively, it is possible that virus encoded by *Emv-3* becomes replication competent as a result of point mutation and subsequently undergoes recombination with other endogenous virus sequences.

This work has characterized an endogenous virus expressed in aged DBA/2 mice. This mouse strain has been defined as having a low incidence of leukemia because few if any leukemias are found during the first year of life. High-leukemic strains (e.g., AKR) produce endogenous ecotropic virus early in life, whereas low-leukemic strains do not (11, 24, 25, 31). However, recently mice from various strains that are considered to have a low incidence of leukemia were monitored over their entire life span and were shown to eventually develop a high incidence of lymphoma (>60%) late in life (33). Our findings that DBA/2 mice begin to produce endogenous virus late in life and that virus production is the result of a recombination between *Emv-3* virus sequences and a novel *gag* sequence related to the *gag* gene fragment in AKT-8 suggest that lymphoma development in aged mice may be directly related to endogenous ecotropic virus production late in life. In addition, the observation that this novel *gag* sequence is found in endogenous virus isolated from at least three mice suggests that expression of this sequence may confer some selective advantage to virus growth. At this time, it is not known if rv-DBA/2_{aged} is leukemogenic. However, it is possible that this virus is involved in further recombinations with other endogenous virus sequences, which may generate a leukemogenic virus in a manner that is similar to the sequence of events which occurs in AKR mice (14, 30, 32).

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TABLE 4. Similarities of the *gag* genes of seven other viruses from aged DBA/2 animals to either *Emv-3* or rv-DBA/2_{aged} *gag*

Clone ^a	Similarity at end of clone	
	5'	3'
AL2	Emv3	Emv3
AL8	Emv3	Emv3
CR3	Emv3	Emv3
CR4	Emv3	Emv3
CR5	Emv3	Emv3
CR2	rv-DBA/2 _{aged}	rv-DBA/2 _{aged}
AL4	Emv3	rv-DBA/2 _{aged}

^a Duplicate clones were sequenced in all cases except for AL2, for which only one clone was obtained.

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