

MOUSE MAMMARY TUMOR VIRUS IN HYBRIDS FROM  
STRAINS C57BL AND GR:  
BREEDING TEST OF BACKCROSS SEGREGANTS

BY W. E. HESTON, B. SMITH, AND W. P. PARKS

*(From the Laboratory of Biology and Laboratory of Tumor Virus Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014)*

Using the European GR strain of mice, Bentvelzen in 1968 first postulated that mammary tumor virus (MTV)<sup>1</sup> viral information could be transmitted as a chromosomally integrated provirus under the control of a regulator gene (1, 2). From segregation ratios measured by the development of mammary tumors in outcrosses of GR males, he concluded that the ability of GR male to transmit the chromosomally integrated mouse MTV (MMTV) was due to a single dominant gene and further that transmission from males to offspring was also controlled by a single dominant gene, both probably being the same gene. Subsequent tests of the first backcross segregants appeared to support the 50-50 segregation ratio of the first backcross, but the number of animals studied was too small for valid statistical analysis. Although confirmation by similar segregation ratios has been reported (3), no evidence of linkage has been found (J. Hilgers, personal communication). Also, studies by Nandi and Helmich (4) using GR and BALB/c mice were interpreted as indicating a three to one ratio in the first backcross and led those authors to conclude that two genes regulate mammary tumor genesis in this cross.

From the early studies of Wright (5) on polydactyly in guinea pigs it has been evident that there are multifactorial traits whose segregation in the F<sub>2</sub> and first backcross generations mimic single gene segregation. However, breeding tests of these backcross segregants to observe segregation in their second backcross progeny have often uncovered greater complexity than originally appreciated; thus, to prove a single gene analysis of second backcross segregants is necessary.

It is now clear from recent molecular studies that the genetic information for MMTV viral structural genes is stably inherited as multiple gene copies in the DNA of all mice examined (6, 7). Further it appears that regulation of the expression of these inherited sequences is the major determinant of virus expression and perhaps mammary tumor genesis (8-10). Thus, to define the genetics of MMTV it is important to study viral expression independent of milk virus transmission and further to measure virus expression directly and not indirectly as measured by tumor development.

Because of the primary importance of establishing whether or not transmission of GR MMTV is controlled by a single dominant gene, we undertook an extensive study of hybrids between the strains GR and C57BL with special emphasis on first and second backcross segregants in order to test the single gene hypothesis. Both virus expression in milk and tumor development were measured and this report concerns segregation analysis of virus expression. A

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<sup>1</sup> Abbreviations used in this paper: BC<sub>1</sub>, first backcross; MMTV, mouse MTV; MTV, mammary tumor virus.

subsequent paper will report the genetic analysis of tumor segregation and virus expression. The results suggest that contrary to a single gene, multiple genetic influences result in a wide spectrum of virus expression in the backcross segregants. Further, the hormonal and multiple genetic influences suggest that the genesis of mammary tumors in mice is largely controlled by the regulation of chromosomally integrated and inherited viral genes, rather than by horizontal transmission of virus.

### Materials and Methods

*Strains Used.* The GR strain was obtained in 1970 through the courtesy of the Netherlands Cancer Institute, Amsterdam, and thus the animals we used were closely related to those used by the Dutch workers (11). The strain has been maintained similar to other strains we have previously reported (12-14). Briefly, mice are produced by brother-sister matings and because of the relatively poor lactation and early tumor age of GR females only first or second parity offspring were used for these studies.

The C57BL strain was that which we had maintained for over 30 yr in our laboratory (12). Mammary tumors have been observed in untreated females of this strain at a frequency of less than 1% in mice living to an age of 18 mo or longer. Throughout the study all mice have been provided with open formula diet (15) and tap water; they have been bedded on a 3:1 mixture of pine and cedar shavings.

*Matings.* In most of the crosses, matings were made so that transmission of MMTV would be through the male. To produce the  $F_1$  hybrids C57BL (hereinafter designated as B) females were mated to GR males. The  $(B \times GR)F_1$  hybrids were mated to produce a  $(B \times GR)F_2$  generation. In designating hybrids the strain of mother is given first. Since expression of MMTV was a dominant trait, all  $F_1$  females tested had high levels of MMTV expression in first parity milk, thus milk transmission of MMTV was possible.  $(B \times GR)F_1$  males were backcrossed to virgin C57BL females to produce the  $[B \times (B \times GR)F_1]$  first backcross ( $BC_1$ ) generation. In this cross there should have been no milk transmission of MMTV. In later experiments to measure putative milk virus transmission, comparable numbers of milk MMTV-positive  $(B \times GR)F_1$  females whose mothers were milk MMTV negative were mated with C57BL males to produce  $[(B \times GR)F_1 \times B]BC_1$  females.

For the crucial breeding test of the backcross segregants, 25  $[B \times (B \times GR)F_1]BC_1$  females were mated with C57BL males and each female was permitted to produce as many  $[B \times (B \times GR)F_1]BC_1 \times B-BC_2$  females as possible. Also, to avoid maternal effects, 25  $[B \times (B \times GR)F_1]BC_1$  males were mated with C57BL females, each male being allowed to produce approximately 25  $[B \times (B \times GR)F_1]BC_2$  females.

*Milking Procedures.* Essentially the procedure of Feller and Boretos was employed (16). Between 7 and 14 days postparturition, nursing mothers were removed from their offspring and 3-4 h later were inoculated subcutaneously with 0.1 U of oxytocin and all eight mammary glands were aspirated using a hand-held apparatus with a soft rubber cup attached to a low pressure vacuum line. The gland area was first moistened with 0.14 M NaCl containing 0.01 M potassium phosphate buffer, pH 7.2, to increase the effectiveness of the vacuum. Approximately 0.3-0.6 ml of milk/mouse was removed and kept refrigerated at 4°C until frozen at -70°C. The milk was thawed and diluted for immunoassay determination of MMTV concentration.

*Immunologic Measurement of MMTV.* Immunoassays for viral antigen in milk were performed. Briefly, double antibody radioimmunoassays for the major MMTV viral glycoprotein, gp52, or a lower molecular weight internal viral polypeptide, p14, were used and have been described in detail (17, 18). Milk was diluted to 1:40 and 1:400 with a solution containing 0.01 M Tris-HCl, pH 7.6; 0.1 M NaCl; and 0.1% Triton X-100 with 1% rabbit serum as carrier. These dilutions were routinely tested by radioimmunoassay since preliminary experiments revealed that over 95% of GR females had milk titers greater than 1:400. The lower level was arbitrarily selected to reflect a 10-fold lower level of viral antigen. Animals whose milks were positive for MMTV antigen at both the 1:40 and 1:400 dilutions were classified as ++ and animals positive at 1:40 but not 1:400 were +-. C57BL mice were negative for antigen even when tested at a 1:10 dilution of milk. Standard curves of purified MMTV gp52 or p14 were included in each assay to ensure

TABLE I  
*MMTV in Females of Strains GR and C57BL and Their Hybrids at Early Lactations*

Strain or hybrid*	Designation	Total No.	MMTV positive		MMTV Negative	
			No.	%	No.	%
GR	Parental	114	114	(100)	0	(0)
C57BL	Parental	117	0	(0)	117	(100)
(B × GR)F <sub>1</sub>	F <sub>1</sub> hybrid	73	73	(100)	0	(0)
(B × GR)F <sub>2</sub>	F <sub>2</sub> hybrid	104	92	(88)	12	(12)
[B × (B × GR)F <sub>1</sub> ]BC <sub>1</sub>	First backcross	106	64	(60)	42	(40)
[(B × GR)F <sub>1</sub> × B]BC <sub>1</sub>	First backcross	100	88	(88)	12	(12)

\* In all crosses the female is listed first.

comparable assay sensitivity throughout the 2-yr period of these experiments. Periodically, milks were retested to verify past results and all immunoassays were performed on coded materials. Preliminary results indicate that the levels of viral antigen in mouse milk correlate well with the likelihood of mammary tumor development in the mouse strains examined so far.<sup>2</sup> Further, the viral antigen present in milk was shown to band predominantly in the 1.16-1.18 g/cc region of sucrose density gradient characteristic of intact MMTV (19). Thus, the antigens measured probably reflect intact virus.

## Results

*Segregation of Presence of MMTV.* The presence of MMTV in strains GR and C57BL and the segregation of MMTV milk expression in various hybrids are shown in Table I along with the strain and hybrid designations. MMTV was measured in the milk collected from first or second lactation females. All 114 GR females tested were highly positive for MMTV and 117 C57BL females tested were negative. Three MMTV-positive C57BL mice were noted, but two had been mated with GR males and the third was mated to a backcross male that undoubtedly had virus, since he had positive female offspring from matings with negative C57BL females. Thus, the most likely interpretation for these few MMTV-positive C57BL females is that they were generally infected from the GR and backcross males with which they were mated.

All of the 73 (B × GR)F<sub>1</sub> females tested had high levels of MMTV which is comparable with all earlier observations of such F<sub>1</sub> hybrids (2, 3). This suggests dominance of MMTV expression, however, since many genes may be involved, the observations could also represent an average result of multiple genes instead of dominance of a single gene. Of relevance to this point, all 73 F<sub>1</sub>'s tested had MMTV levels of 1:400 and thus resembled more closely the GR phenotype.

Of 106 [B × (B × GR)F<sub>1</sub>]BC<sub>1</sub> females tested at early lactations (first or second and occasionally third) 64 (60%) were positive and 42 were negative. This backcross segregation frequency is not significantly beyond the bounds of the 50% expectancy from segregation of a single dominant gene trait ( $P > 0.05$ ) nor is this significantly different than the 75% ratio which would have been expected from a two-gene segregation pattern ( $P > 0.05$ ).

Nongenetic transmission of virus, such as through milk, would obviously affect the frequency of positive results and influence any analysis. For example,

<sup>2</sup> Heston, W. E., and W. P. Parks. Manuscript in preparation.

of 104 (B × GR) $F_2$  females, 92 or 88% had virus and 12 or 12% were negative. The number of positive females is significantly higher ( $X^2 = 6.74$ ;  $P < 0.01$ ) than the 75% expected if the presence of virus were due to a single dominant gene. However, since milk transmission of the virus may have occurred in this cross, no meaningful analysis of these results in genetic terms is possible. The effect of milk transmission on the observed ratios was further examined by analysis of MMTV levels in milk from 100 backcross females tested from the reciprocal cross of the highly milk virus-positive (B × GR) $F_1$  females to C57BL males, [(B × GR) $F_1$  × B]BC $_1$ . 88% were virus positive, a significantly higher proportion ( $X^2 = 19.609$ ;  $P < 0.01$ ) than the 60% in the reciprocal cross. 88% positive is also a significantly higher proportion than would be expected if transmission of the virus were due to a single dominant gene alone, but again the probable effect of milk virus transmission complicates any purely genetic conclusion. Obviously, a maternal influence can be demonstrated when females of high MMTV milk titer are used in matings. Therefore, only matings to the low virus strain, C57BL were used for genetic studies of the backcross segregants.

*Virus in BC $_1$  Females at Late Lactations.* Of the 25 [B × (B × GR) $F_1$ ]BC $_1$  females selected for second backcross testing, 12 were positive and 13 were negative at first and second lactations. When testing BC $_2$  progeny, however, some positive BC $_2$  females were found among the progeny of these negative females, which prompted retesting of the BC $_1$  mothers at later lactations. Results of these tests (Table II) indicated that most backcross females although negative at early lactations have virus at late lactations (fourth or later), albeit in lower concentration indicated as +−, positive at 1:40 dilution and negative at 1:400. Of the 13 BC $_1$  females that were negative for virus at early lactations (p1–3), 9 had virus at later lactations, 2 were still negative at the sixth lactation, and 2 were not tested later. Four females that were positive at early lactations were tested again at the fourth or fifth lactations and again observed to be positive.

The occurrence of viral antigen in the later parity lactations could be due to horizontal transmission, to lactation, and/or age activation of C57BL or GR sequences in the hybrid. Since all BC $_1$  females were mated to C57BL males which have never clearly transmitted MMTV to other strains, horizontal transmission is unlikely. Also C57BL females housed with both positive hybrids and GR mice have remained virus and tumor free (W. E. Heston, unpublished observation). The possibility of activation of C57BL MMTV is more difficult to exclude. In three C57BL female mice bred only to C57BL males and tested through five or six lactations, MMTV was not detected. Thus, we favor the third possibility that late lactation-positive mice reflect a net result of genetic influences from both GR and C57BL parental backgrounds.

*Breeding Test of [B × (B × GR) $F_1$ ]BC $_1$  Female Segregants.* The presence of MMTV at early lactations in BC $_2$  female progeny of the 25 BC $_1$  females that were tested is shown in Table III. Some of these BC $_1$  females had relatively few BC $_2$  female offspring and that reduces the significance of any analysis of individual families, so totals are also given. The 12 BC $_1$  females that were positive at early lactation had a total of 116 BC $_2$  female offspring of which 96 or 83% had detectable milk virus. Of these 96, 65 had virus titers that resemble GR levels.

All MMTV-“negative” BC $_1$  females ultimately produced progeny that were

TABLE II  
Effect of Parity on MMTV Antigen Expression in  $[B \times (B \times GR)F_1]BC_1$  Females

	No. positive* p1-3			Number negative† p1-3			Positive/total		
	GR, 114	BC <sub>1</sub> , 12	C57BL, 0	GR, 0	BC <sub>1</sub> , 13	C57BL, 117	GR, 114/114	BC <sub>1</sub> , 12/25	C57BL, 0/117
No. positive p4-8	NT	4	0	0	9	0	—	21/25	0/3
No. negative p4-8	NT	0§	0	0	2‡	5	—	—	—

\* Measured by Measurement of MMTV antigen expression in milk.

‡ Two remaining negative mice at p1-3 were not tested at later lactations because they did not deliver additional litters.

§ Eight remaining mice not tested at p4-8.

TABLE III  
MMTV in  $[B \times (B \times GR)F_1]BC \times B = BC_2$  ♀♀ Progeny of  $[B \times (B \times GR)F_1]BC_1$  ♀♀

BC <sub>1</sub> mother: early lacta- tion positive	No. BC <sub>2</sub> female progeny with MMTV expression			BC <sub>1</sub> mother: early lacta- tion nega- tive	No. BC <sub>2</sub> female progeny with MMTV expression		
	++	+-	--		++	+-	--
1852	7*	3	4	1854	2	3	9
1853	2	—	2	1866	—	3	5
1855	3	5	4	1867	3	2	1
1856	2	—	1	1888	—	3	11
1857	11	2	1	1940	1	—	4
1886	2	7	2	1978	—	11	9
1981	6	3	1	1979	—	4	18
2060	7	—	—	1980	—	2	8
2209	9	3	1	2114	—	2	14
2256	4	3	—	2115	—	4	14
2261	7	2	1	2159	3	5	8
2262	5	3	3	2160	2	3	6
				2257	—	2	12
Total	65	31	20	Total	11	44	119

\* BC<sub>2</sub> females tested at first or occasionally second lactation for MMTV.

positive. The percentage of positive females in this group was significantly less than in the offspring of positive BC<sub>1</sub> females ( $P > 0.05$ ). Of the 174 BC<sub>2</sub> female progeny of these negative BC<sub>1</sub> females, 55 or 32% had virus but only 11 had high levels of MMTV characteristic of the GR phenotype.

*Breeding Tests of  $[B \times (B \times GR)F_1]BC_1$  Male Segregants.* The presence of MMTV at early lactations in BC<sub>2</sub> female progeny of the 25 tested BC<sub>1</sub> males is shown in Table IV. Since more female offspring were obtained from each BC<sub>1</sub> male than from each BC<sub>1</sub> female, the analysis of individual families of the BC<sub>1</sub> males is more significant than any analysis of individual families of the BC<sub>1</sub> females would have been. In only one family of the BC<sub>1</sub> males were all the females negative. All 24 other families had positive females with incidences ranging from 5 to 100%. There was no readily discernable grouping of these positive families with respect to percentage of high MMTV-positive females (Fig. 1). Further, the proportion of high MMTV expressors increased proportionately with the percentage of positive offspring in each family. This finding in the first or occasionally second lactation, supports the interpretation that late



lactation positively is a threshold expression of multifactorial genetic inheritance from the parental genotypes.

### Discussion

The concept of a single mouse gene regulating MMTV transmission was initially proposed in 1945 by Heston and co-workers (12); however, in a series of more detailed investigations by the same group (13, 14), it was finally concluded that multiple genes regulate transmission. Thus, the report of Bentvelzen in 1968 (1) of a single gene in the GR strain regulating transmission was of special interest. Since 1967 in GR hybrids no specific linkage assignment has been found, and adequate studies of critical backcross segregants have not been done. In order to resolve this basic issue and assess its relevance to the natural history of MMTV, we have carried out a more detailed analysis of the GR  $\times$  C57BL crosses and backcrosses. Although our F<sub>1</sub> and first backcross data resembled closely a "dominant" transmission of MMTV expression, the ratios were not significantly different from either a single gene hypothesis as proposed by Bentvelzen (1) or a two gene model as described by Nandi and Helmich (4).

In our analysis there were two major independent results that appear to exclude the single gene hypothesis. First, when first backcross females were tested at later lactations (p4 or greater) many mice scored as negative at early lactation were found to be virus positive. In many of these cases, the MMTV milk titer was intermediate. Late appearing virus in these females could not have come as a venereal infection from the males to which they were mated, since all were mated to C57BL males. Nor could horizontal transmission account for the results since C57BL females at comparable parities and ages in the same laboratory have consistently been negative. In our opinion, the most likely explanation of this lactation-associated (hormonal) activation is that the virus observed in these females at late lactations is the result of age or parity related activation of chromosomally integrated provirus. Of interest is the observation that these putative activating factors do not appear to influence the ability of these females to transmit MMTV through their milk. There was no increase in detectable virus in the BC<sub>2</sub> females born from p4 or later litters (28 vs. 32% positive) suggesting the virus detected in their milk was primarily genetically transmitted.

The second major observation that obviates an interpretation of single dominant gene segregation derives from tests of first backcross segregants. With single dominant gene inheritance, one would expect half of the female offspring of each family of the positive BC<sub>1</sub> females to have virus and the other half to be virus negative. Of the 12 families from positive BC<sub>1</sub> mothers, 100% had positive offspring and 83% of all individual mice had virus, a figure significantly greater than the 50% expectancy. This increase, however, cannot be evaluated genetically because of the possibility of milk transmission of virus by these females. If a single gene determined MMTV expression, all offspring in families of early lactation-negative BC<sub>1</sub> females should be virus free. Exactly the opposite occurred. All families had one or more offspring with virus, and 32% of offspring from all families had virus. The fact that there were fewer females with virus among the BC<sub>2</sub> offspring of initially negative BC<sub>1</sub> females than among the BC<sub>2</sub> progeny of initially positive BC<sub>1</sub> females indicated the effect of segregation of

genes, but the segregation ratios in the two groups of families from BC<sub>1</sub> females did not support an interpretation of single gene segregation. Similarly, analysis of females from the BC<sub>2</sub> families of BC<sub>1</sub> males, half of which would be expected to have virus and half virus free if the virus expression were due to a single dominant gene, revealed only 1 family of 25 that was virus free. The incidence of positive females in the other 24 families varied from 5 to 100% with no evidence of grouping. Thus, segregation of genetic influences is possible when it was possible to type the parents (females) but not when a convenient assay is not available as in the case of the BC<sub>1</sub> males.

The effect of parity (Table II), the intermediate levels of virus expression (Tables III and IV), and the relationship of virus expression to tumorigenesis (unpublished data) suggest that there are other genetic influences whose effect on MMTV expression remains to be determined. Obviously, the number of genes that are major determinants of MMTV expression is not clear from this work, however, the observation that early lactation MMTV-negative mothers did show segregation towards lower levels of expression justifies the use of other genetic techniques to attempt delineation of the major genetic loci affecting mammary tumor genesis. With approaches such as recombinant-inbred lines and the assays now available for detecting virus it may ultimately be possible to establish the major determinants that govern MMTV expression and mammary tumorigenesis.

### Summary

F<sub>1</sub> and F<sub>2</sub> and first backcross hybrids and second backcross families of the high mammary tumor incidence strain GR and the low incidence strain C57BL were examined for the segregation of mouse mammary tumor viral (MMTV) expression. Although GR has been reported to transmit MMTV as a single dominant gene, several lines of evidence suggest there are multiple genetic factors that influence MMTV expression. MMTV expression as measured by double antibody radioimmunoassay for MMTV p14 segregated in 106 first backcross progeny at a 60:40 ratio, intermediate between what would be expected for either a single or two gene hypothesis. In female second backcross progeny of either male or female first backcross, a heterogeneous pattern of expression was noted that does not fit any simple Mendelian pattern. From an analysis of serial lactations of first backcross and second backcross families, it appears that all hybrid females contain MMTV proviral information that may be expressed either at late lactations or in a variable proportion of progeny mice. These combined results are most consistent with a vertically transmitted genome regulated by multiple factors in these crosses.

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