CELLULAR GENES IN THE MOUSE REGULATE *IN TRANS* THE EXPRESSION OF ENDOGENOUS MOUSE MAMMARY TUMOR VIRUSES

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Manuscript received June 11, 1984
Revised copy accepted July 22, 1985

ABSTRACT

The transcriptional activities of the eleven mouse mammary tumor virus (MMTV) proviruses endogenous to two sets of recombinant inbred (RI) mouse strains, BXD and BXH, were characterized. Comparison of the levels of virus-specific RNA quantitated in each strain showed no direct relationship between the presence of a particular endogenous provirus or with increasing numbers of proviruses. Association of specific genetic markers with the level of MMTV-specific RNA was examined by using multiple regression analysis. Several cellular loci as well as proviral loci were identified that were significantly associated with viral expression. Importantly, these cellular loci associated with MMTV expression segregated independently of viral sequences.

MAMMARY carcinomas in the mouse are associated with infection by a retrovirus, mouse mammary tumor virus (MMTV), which synthesizes a double-stranded DNA intermediate and integrates into the host genome (NANDI and MCGRATH 1973; MOORE 1975; BENTVELZEN and HILGERS 1980; WEISS *et al.* 1982). Infection of germline cells and subsequent recombination results in the presence of MMTV genomic sequences, or proviruses, at various sites in the genome (COHEN, MAJORS and VARMUS 1979; COHEN and VARMUS 1979; HYNES *et al.* 1979; GRONER and HYNES 1980). Many of the integration sites appear identical in the common American inbred mouse strains (TRAINA-DORGE and COHEN 1983), although differential expression of MMTV-specific loci, as well as differential incidence of tumor formation, is observed among these strains (NANDI and MCGRATH 1973; BENTVELZEN and HILGERS 1980). Nonviral loci that act *in trans* to either repress or enhance expression of polymorphic *cis* elements in the mouse may be responsible for variable expression among the inbred strains of mice.

Recombinant inbred (RI) strains were employed in a previous study to evaluate MMTV provirus locations in mouse chromosomes (TRAINA, TAYLOR and COHEN 1981). These strains were developed by systematic inbreeding, begin-

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ning with the F_2 generation of a cross between two preexisting progenitor mouse strains (TAYLOR 1978). The BXD and BXH RI strains were derived from crosses of the C57BL/6J with strains DBA/2J and C3H/HeJ, respectively (TAYLOR, BEDIGIAN and MEIER 1977; TAYLOR 1978). Importantly, in these strains many genetic loci have been identified and mapped to specific chromsomes (TAYLOR 1978). Cosegregation analysis of the eleven MMTV proviruses identified in the BXD and BXH RI strains with the genetic markers established chromosomal linkage at four specific loci (TRAINA, TAYLOR and COHEN 1981). Two proviruses, *Mtv-7* and *Mtv-10*, were shown to be independently linked on chromosome *1*. Another provirus, *Mtv-1*, was mapped to chromosome 7, which is presumably identical to the previously defined genetic locus *Mtv-1* (VAN NIE and VERSTRAETEN 1975; VERSTRAETEN and VAN NIE 1978). The subgenomic *Mtv-14* provirus was tentatively assigned to chromosome *6*. Linkage of *Mtv-12* with the chromosome *14* markers was later established (TRAINA, TAYLOR and COHEN unpublished results).

Having characterized the endogenous proviruses in the RI strains, their transcriptional activity was evaluated to identify the endogenous viral sequences expressed in normal tissues. Virus-specific RNA was analyzed quantitatively as a percentage of the total cellular levels. Virus-infected animals showed the greatest levels of viral-specific RNA and protein in the mammary glands (NANDI and MCGRATH 1973; VARMUS *et al.* 1973; MICHALIDES *et al.* 1978; MARCUS, SMITH and SARKAR 1981; WEISS *et al.* 1982). Therefore, the mammary gland was the tissue of choice for these studies. All sequences necessary for virus expression reside within the provirus. Thus only the presence of the provirus should be necessary for transcriptional activity. However, the results indicated a more complex system. Multigene interactions between nonviral gene products with one or more viral loci may be required for viral expression. Statistical analyses were used to identify possible loci involved in the regulation of MMTV transcription.

MATERIALS AND METHODS

Mice: Inbred mouse strains C3H/HeJ, C57BL/6J, DBA/2J, DBAfB/2J and C3HfB/HeN were provided by the Jackson Laboratory and Charles Rivers Laboratory. RI strains of mice, BXD (C57BL/6J × DBA/2J) and BXH (C57BL/6J × C3H/HeJ), were both developed and obtained from Jackson Laboratory, Bar Harbor, Maine.

When possible, several mice of the same strain were bred to allow for different environmental parameters that may influence endogenous virus expression. Sacrifice of female mice for lactating mammary glands was at four distinct periods, based on parity and lactation. The females were either early (5–7 days postpartum) or late (10–14 days postpartum). The age range of the females tested was from 3–9 months. Sacrifice of adult mice was expeditiously performed by cervical dislocation after ether anesthesia.

Lipopolysaccharide (LPS, *E. coli* strain 0127;B8) was obtained from Difco. Primiparous C3HfB/HeN females were injected on their 12th day of lactation with LPS at a concentration of 2 mg/kg body weight in 0.1 ml sterile saline. Control mice were injected with an equal volume of sterile saline. Mice were sacrificed 6 hr subsequent to injection, and lactating mammary glands were removed and processed for RNA (as described below).

RNA isolation: Mouse mammary tumor virus, strain MMTV(C3H), was obtained from the Mm5mt/Ci cell line provided by the National Cancer Institute. Viral genomic RNA was purified using a modification of a method originally described by ROBINSON, PITKANEA and RUBIN (1965).

Whole cell RNA from normal tissue and lactating mammary glands was prepared by the guanidinium thiocyanate procedure of CHIRGWIN *et al.* (1979). Excised tissues were immediately homogenized with a mechanical homogenizer in 4 M guanidinium thiocyanate, 0.05% sodium *N*-lauroyl sarcosine (sarkosyl), 25 mM sodium citrate (pH 7.0), and 0.1 M 2-mercaptoethanol (BME). Cellular RNA was isolated by a 12–16 hr centrifugation at 150,000 × *g* through a 5.7 M CsCl cushion buffered with 25 mM sodium citrate, pH 5.0, (GLISIN, CRKVENJAKOV and BYUS 1974). The RNA pellet was dissolved in the guanidinium thiocyanate solution, extracted once with an equal volume of chloroform:1-butanol (4:1) and the final organic phase extracted with an equal volume of 10 mM Tris-HCl, pH 7.4, 5 mM EDTA and 0.1% SDS. Aqueous phases were combined and RNA was precipitated with ethyl alcohol. RNA samples were stored in 70% ethyl alcohol at −20°.

RNA dot blot: Total mammary gland cellular RNA was analyzed for the proportion of virus-specific transcripts present by spot hybridization on treated nitrocellulose. For the multiple RNA samples, initial suspensions at equal concentrations (1 mg/ml) were made, and serial dilutions of each sample were prepared with a yeast RNA diluent, also 1 mg/ml. By starting at a fixed initial concentration for each sample, direct comparison of sequence concentrations in different samples was possible. Aliquots of each of four 1:2 dilutions prepared on every sample were spotted onto treated nitrocellulose sheets (Schleicher and Schuell) in dots of uniform diameter. Serial dilutions of RNA extracted from purified MMTV virions were used as standards and were included on each filter.

The dot blot hybridization procedure was based on the method of THOMAS (1980). Nitrocellulose was prepared by first wetting with distilled water and then soaking the filter with 3 M NaCl; 0.3 M sodium citrate (20× SSC). Once each filter was dry, aliquots of each dilution (1–5 μl), including standard and unknown samples, were dispensed onto the filter and were washed once with an equal volume of the yeast RNA diluent. The nitrocellulose was removed, placed on Whatman 3 mm filter paper and dried with a heat lamp. The filters were then heated in a vacuum oven at 80° for 2 hr to immobilize the RNA and were stored under vacuum dessication until hybridization.

Hybridization and autoradiography: The p4.2A recombinant plasmid (generously provided by J. MAJORS, University of California, San Francisco) containing the entire genomic MMTV(C3H) DNA sequence was used as a template to prepare a nick-translated MMTV radiolabeled probe (RIGBY *et al.* 1977).

Filter hybridization of RNA bound to nitrocellulose paper was performed by a modification of the method of THOMAS (1980). Briefly, filters were annealed for 24 hr at 41° in annealing buffer that included 50% formamide, Denhardt's buffer (0.02% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll; DENHARDT 1966), 3× SSC, 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.0), 200 μg/ml yeast RNA and 50 μg/ml alkali sheared and denatured salmon sperm DNA (prepared by treating with 0.3 N NaOH, 80°, 3 hr). Subsequently, filters were incubated for 48 hr with annealing buffer supplemented with 10^6 cpm/ml ^{32}P-labeled DNA. After incubation, filters were washed in 2× SSC for 1 hr at room temperature, incubated in 0.1× SSC, and 1% SDS at 50° with agitation, rinsed twice with 0.1× SSC and 0.1% SDS and five times with 0.1× SSC at room temperature. Filters were air-dried and exposed at −70° to Kodak XAR-5 film, using Cronex "Lightening Plus" intensifying screens (SWANSTROM and SHANK 1978), for a period of 48 hr to 7 days.

Regression analysis: Due to the large size of the data base (about 130 animals), the use of a statistical approach to the genetic correlation of MMTV-specific RNA expression was indicated. The computer program for the regression analysis used was provided by the BMDP Biomedical computer program (DIXON 1981). The mean level of MMTV RNA was first expressed as a percentage of the total cellular RNA. Two sets of analyses were then performed. In the first set, this percentage (after adding 1% to avoid having any value of zero) was used as the dependent variable. In the second set, a transformation of the data was made utilizing the natural logarithm of this percentage as the dependent variable in order to obtain approximate homoscedasticity. This transformation was necessary because, when heteroscedasticity prevails, the estimates of the regression coefficients are no longer minimum variance, unbiased estimators. Age (in days), parity (primiparous or multiparous), length of lactation (5–9 days, early; or 12–16 days, late) and two

sets of genetic markers that differed in the progenitor strains were the independent variables. Each marker present in a mouse was given the value 0 if it was the same as that present in the C57BL/6J progenitor, and the value 1 if otherwise. The chromosomal location of the majority of these polymorphic genetic markers is known, and the markers have been described previously (O'BRIEN 1980). All analyses were performed separately for the BXD and BXH mice.

Because the number of markers is larger than the number of RI strains available, the markers were initially screened by performing a series of individual multiple linear regression analyses. In each analysis, only a single marker locus, together with age, parity and lactation, was included as an independent variable. The program estimated the coefficients of the regression model and performed a *t*-test for each coefficient. Let *bi* be the estimate of the coefficient for variable *i* and *s* (*bi*) be the standard error of the coefficient. Then the test statistic is $bi/s(bi)$. The associated two-tailed probabilities of the *t*-test for each coefficient are calculated and given as a final result by the program. Those coefficients significant at the 0.05 level were determined.

The data were then analyzed using an all possible subsets regression program. The independent variables used in these analyses were chromosome genetic markers that demonstrated significance in the first analysis, the individual proviruses present in the BXH and BXD mice, along with parity, lactation and age. The program calculated regression models for all possible subsets of the specified independent variables and then chose the "best" of these subsets of predictor variables on the basis of either of two different criteria: the adjusted R^2 and Mallow's Cp. Let R^2 be the squared multiple correlation between the dependent variable y and the predicted value y from the regression equation. Let N be the number of cases, p be the number of independent parameters in the subset (not including the y-intercept), and p' equal $p + 1$. Then the adjusted R^2 criterion is to maximize $R^2 - p(1 - R^2)(N - p')$ (THEIL 1971). Let *RSS* be the residual sum of squares based on the selected independent variables in the subset and S^2 be the residual mean square based on the regression using all independent variables in the model. Then Mallow's Cp criterion is to maximize $RSS/S^2 - (N - 2p')$ (MALLOWS 1973).

RESULTS

Quantitation of MMTV-specific RNA in the mammary glands of RI strains: The percentage of total RNA specific for MMTV in the lactating mammary gland RNA preparations was determined in each mouse by the dot blot techniques (Figure 1; THOMAS 1980). MMTV-specific RNA in the lactating mammary glands from each of 90 BXD and 40 BXH mice was quantitated by multiple dot blot analyses on each sample; numerical strain means and standard deviations over all assays were calculated and the data are presented in Tables 1 and 2.

Virus-specific RNA expression differed among the strains. Mean RNA levels in the BXD strains ranged from zero in the primiparous BXD-14 strain to 0.032% in the multiparous BXD-1 strain (Table 1). Interestingly, the range of values seen with the BXD RI strains is much greater than the values observed in the progenitor strains. The mean for the C57BL/6 parent was 0.027 ± 0.009% and 0.024 ± 0.009% for primiparous and multiparous animals, respectively, while the mean for the DBAfB/2J parent, which was foster-nursed and therefore was free from exogenous virus infection was 0.016% for the primiparous female tested. As with the BXD strains, the RNA levels differed among the BXH strains (Table 2); however, some of the differences were quite dramatic. BXH-7 failed to demonstrate any hybridizable RNA even in multiparous animals. Of all the strains, BXH as well as the BXD, this was the only viral RNA-negative strain. For the other strains, the proportion of total RNA that was virus-specific ranged up to 0.24 ± 0.09%. Much greater differences

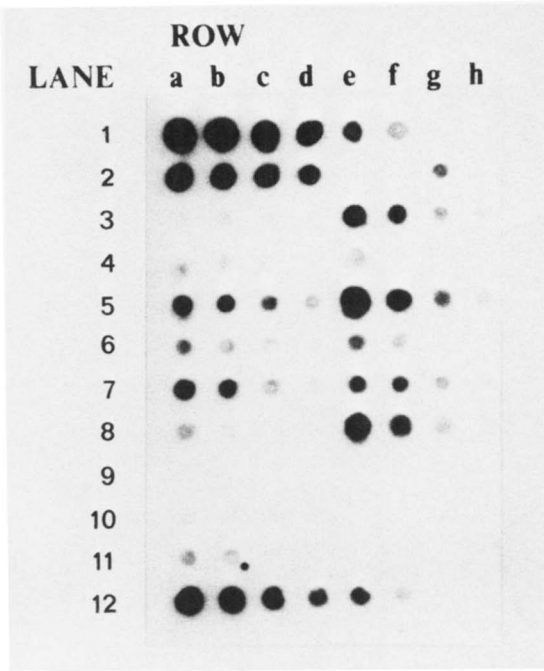

FIGURE 1.—Autoradiograph of dot blot assay with RI mouse RNA. Total cellular LMG RNA was prepared with four serial twofold dilutions in a yeast RNA diluent and was applied to 20× SSC-treated nitrocellulose in a series of horizontal spots. The RNA was then hybridized with cloned MMTV genomic sequences (p4.2A) labeled to a high specific activity by nick translation. MMTV viral RNA standards (13.2 ng-0.04 ng) were applied, lane 1 and lane 12, rows a–h. Lane 2, rows a–d, primary mammary tumor RNA; row e, yeast RNA diluent; row f, BALB/c liver RNA; row g, plasmid DNA p4.2A. Lanes 3–11 represent BXD RI mouse LMG RNA, rows a–d, BXD-21(M), BXD-24(P), BXD-24(M), BXD-23(P), BXD-25(P), BXD-28(M), BXD-14(P), BXD-22(P), BXD-22(M), respectively. Lanes 3–8, rows e–h: BXD-32(M), BXD-2(P), BXD-11(M), BXD-11(P), BXD-12(M), BXD-29(M), respectively. (P indicates a primiparous animal; M indicates a multiparous animal.) The standard value equivalent for each unknown, when both values are in a range of linearity, is divided by the total RNA applied to the spot divided by 100, to yield a value of % MMTV-specific RNA for each animal.

were seen with RNA from BXH strains than was seen with the BXD strain. When a comparison of the levels of RNA present in each mouse (BXD and BXH) with the total number of MMTV loci present in the genome of the strain was made, Figure 2, no relationship between expression and increasing numbers of proviruses was apparent.

To evaluate any relationship of parity with viral expression, mammary glands were obtained from either primiparous mice nursing their first litter or from multiparous mice nursing the second or greater litter (Figure 3). An overall increase in the mean of the distribution of virus-specific RNA in both BXD and BXH with increased parity is observed. For the BXD strains, the mean value is increased from 0.027% to 0.045% (Figure 3). Regression analysis indicates the change in the BXD strains to be statistically significant at a level of $P < 0.05$ (Table 3). The association of parity with the BXH strains was not

TABLE 1

Parity distribution of virus-specific RNA in BXD mouse strains

	% Virus-specific RNA			
	Primiparous		Multiparous	
Strain	Mean	SD	Mean	SD
BXD-1	0.0216	±0.0070	0.0320	0
-2	0.0062	±0.0029	0.0077	±0.0029
-5	0.0230	±0.0100	0.0280	±0.0068
-6	0.0192	±0.0066	0.0212	±0.0091
-8	0.0138	±0.0100	0.0161	0
-9	0.0064	±0.0021	ND	ND
-11	0.0141	±0.0040	0.0213	±0.0083
-12	0.0120	±0.0041	0.0213	±0.0083
-13	0.0149	±0.0013	0.0240	±0.0112
-14	0	0	0.0034	±0.0021
-15	0.0134	±0.0076	ND	ND
-16	0.0067	±0.0018	0.0252	±0.0081
-18	0.0118	±0.0058	0.0161	0
-19	0.0198	±0.0067	0.0214	±0.0067
-21	0.0062	±0.0019	0.0064	±0.0019
-22	0.0030	±0.0011	0.0160	0
-23	0.0160	±0.0001	0.0133	±0.0040
-24	0.0107	±0.0051	0.0240	±0.0072
-25	0.0268	±0.0072	ND	ND
-28	0.0077	±0.0037	0.0184	±0.0051
-29	0.0198	±0.0067	0.0230	±0.0076
-30	0.0190	±0.0084	0.0160	0
-31	0.0267	±0.0092	0.0160	0
-32	0.0240	±0.0092	0.0310	±0.0190
C57BL/6J	0.0265	±0.0091	0.0240	±0.0090
DBAfB/2J	0.0160	±0.0001	ND	ND

SD = standard deviation; ND = not determined.

shown to be statistically significant; this may reflect the smaller sample size and the larger variance for these strains.

Linkage of MMTV expression with chromosomal markers: One advantage of the RI strains is that many genetic loci have been mapped to particular chromosomes. In the BXD and BXH mouse strains, 74 and 55 polymorphic genetic loci, respectively, had been mapped to 12 of 20 chromosomes at the time the analysis was carried out. Therefore, these genetic markers provide a systematic way to analyze not only the linkage of proviruses (TRAINA, TAYLOR and COHEN 1981) but also their association with virus expression.

Multiple linear regression analyses were used to determine the association between each single chromosomal marker and the level of MMTV RNA when the environmental effects of parity, lactation and age were taken into account. In these analyses, the level of virus expression was related to the presence or absence of particular alleles to determine linkage relationships between viral RNA transcription and the loci segregating among the RI populations.

TABLE 2

Parity distribution of virus-specific RNA in BXH mouse strains

	% Virus Specific RNA			
	Primiparous		Multiparous	
Strain	Mean	SD	Mean	SD
BXH-2	0.0073	±0.0013	ND	ND
-3	0.0152	±0.0068	0.0320	0
-4	0.0267	±0.0090	0.0360	±0.0200
-6	0.0077	±0.0043	0.0064	±0.0022
-7	0	0	0	0
-8	0.0013	±0.0016	0.0240	±0.0112
-9	0.0240	±0.0086	0.0320	0
-10	0.0061	±0.0019	0.2400	±0.0970
-11	0.0120	±0.0056	0.0086	±0.0048
-12	0.0048	±0.0028	0.0067	±0.0024
-14	0.0960	±0.0387	0.0600	±0.0410
-19	0.0320	±0.0230	0.0746	±0.0261
C57BL/6J	0.0265	±0.0091	0.0240	±0.0090
C3Hf/HeJ	0.0017	±0.0004	ND	ND

SD = standard deviation; ND = not determined.

In the BXD strains, five chromosomal loci were found to be significant predictors of MMTV RNA expression when analyzed singly (Table 3). Two closely linked cellular loci on chromosome *1* (*Pep-3* and *Sas-1*, $P = 0.04$ and 0.03, respectively; WILSON *et al.* 1978); the endogenous C57BL/6J ecotropic murine leukemia virus, *Emv-2* ($P = 0.02$) on chromosome *8* (JENKINS *et al.* 1981, 1982); a chromosome *9* locus, "*F*" ($P = 0.03$; RACINE and LANGLEY 1980); and *Mtv-11* ($P = 0.04$), a subgenomic MMTV provirus whose chromosome location is not known (TRAINA, TAYLOR and COHEN 1981); as well as the condition of increased parity, displayed a positive relationship with MMTV-specific RNA in the mammary gland.

In the BXH mouse strains, five independently segregating loci were found to be significantly correlated with expression at a level $P < 0.05$ (Table 4). These associated loci were *Mup-1* and *Lps*, both located on chromosome *4* (WATSON, RIBLET and TAYLOR 1977), *Sep-1* and *Lap-1*, both located on chromosome *9* (EICHER *et al.* 1979), and *H-2*, located on chromosome *17* (WATSON, RIBLET and TAYLOR 1977). The possible involvement of either or both of two tightly linked loci on chromosome *4*, as well as two linked loci on chromosome *9* were determined in separate analyses. In contrast to the BXD strains, the only environmental parameter significantly and positively associated with expression in the individual analyses was age.

The results of these single locus analyses suggested the involvement of one or more nonviral cellular alleles in the expression of MMTV proviral genes. However, because the assay detected MMTV-specific expression, the positive requirements for MMTV proviral alleles seemed obvious. In the BXH strains

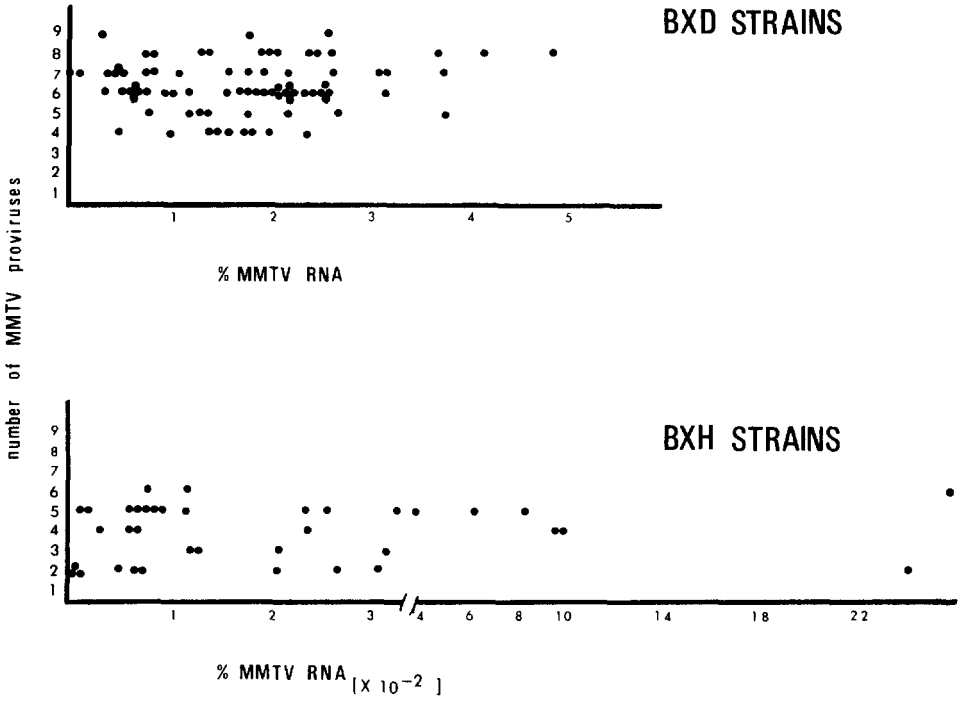

FIGURE 2.—Comparison of the MMTV-specific RNA level with the total number of endogenous MMTV proviruses in the BXD and BXH RI strains. MMTV-specific RNA quantitated in individual mice by dot blot analysis (see MATERIALS AND METHODS) is indicated as a percentage of total cellular RNA and is graphed against the corresponding number of endogenous MMTV proviruses. The upper panel depicts the comparison in the BXD strains; the lower panel compares the BXH strains.

the analysis failed to strongly associate any MMTV proviruses with the effect (Table 4). Two retroviral loci, *Emv-2*, and *Mtv-11*, were associated with viral RNA expression in the BXD strains; however, each was a negative association. In other words, as indicated in Table 3, the progenitor strain that contributed the allele responsible for increased expression lacked the viral sequences. More specifically, *Emv-2*, the ecoptropic murine leukemia virus structural provirus present on chromosome *8*, was present only in C57BL/6J. However, the DBA/2 parental genotype, which lacks the *Emv* proviral sequences (*Emv-2*$^-$), was responsible for the positive effect on MMTV expression. Similarily, the C57BL/6J parental genotype, which contains the *Mtv-11*$^-$ allele lacking proviral sequences, was associated with increased expression.

A complex genetic system for the phenotype of viral RNA expression was indicated. If this were so, the involved combination of alleles could be more significantly associated with RNA expression than the individual alleles. Therefore, a subsets regression analysis was conducted on all the significant genetic markers obtained by single-locus regression analyses, as well as on all the MMTV proviral loci present in each set of strains. This phase of the analysis determined the best of all possible subsets of input variables, including parity,

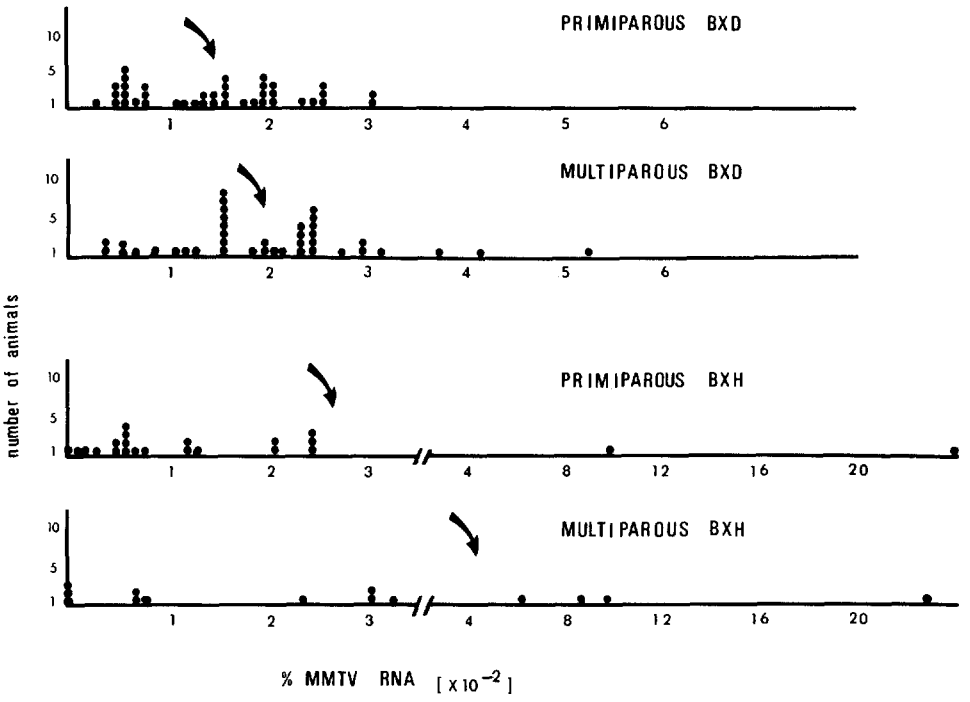

FIGURE 3.—The relationship of MMTV RNA expression and parity. Quantitative levels of MMTV-RNA in either the BXD strains (A and B) or the BXH strains (C and D) are separately graphed according to the number of pregnancies of the mouse (see MATERIALS AND METHODS). The mean value calculated for each segregated set of animals is indicated with an arrow (see text).

lactation and age. A multiple linear regression equation was solved on every possible combination of variables, and the ability of the subset to significantly predict the effect (level of viral RNA) was assessed by either of two values, the adjusted R^2 or Mallow's Cp (see MATERIALS AND METHODS), and the best combination was identified that best explained the phenotype of viral expression. The best model in the BXD system when assessed by both criteria included *Emv-2*, *Mtv-1*, *Mtv-11*, *Mtv-12*, *Mtv-14* and multiparity (Table 3). The model correlated with MMTV expression at a level of $P < 0.001$.

Theoretically, when multiple analyses are conducted on the same data set and the significance level chosen is 0.05, five analyses out of 100 will show significance due to chance alone. To compensate for these multiple analyses, it was reasonable to set one's limit lower for statistical significance. For this reason, in this second phase of the analysis, only the identified loci with P values <0.01 (less than 1 in 100-chance occurrence) were considered significant. At this level, then, the model identified that best explained all of the data included the *Emv-2* locus plus the influence of multiparity (Table 3).

Best subsets regression analysis was similarly performed analyzing those significant marker loci identified in the BXH strains, the MMTV proviral loci present in these strains and the environmental parameters. The results were also the same on the basis of either the adjusted R^2 or Mallow's Cp criteria.

TABLE 3

Regression analysis of MMTV expression in BXD mouse strains .

Individual analysis				
Independent variable	Chromosome	Level of significance		Parental correlation[c]
		Raw[a]	Log[b]	
Pep-3	1	0.044	0.044	D
Sas-1	1	0.029	0.014	D
Bcg	1		0.016	D
Lsh	1		0.019	D
Mtv-1	7		0.026	B
Emv-2	8	0.021	0.026	D
F	9	0.032	0.071	B
Mtv-9			0.028	D
Mtv-11		0.047	0.046	B
Multiparity		0.020	0.036	

Best fit model subsets regression analysis		
Independent variables available for model-building	Variables picked for best-fit models with their levels of significance	
	Raw	Log
Pep-3/Sas-1		
Bcg/Lsh		
Cdh		
Mtv-1		0.001
Emv-2	0.006	0.003
Mtv-7		
Mtv-9		0.045
Mtv-11	0.050	0.105
Mtv-12	0.020	0.035
Mtv-13		
Mtv-14	0.090	0.050
Multiparity	0.004	0.004
Age		
Lactation		

[a] Significance levels calculated from untransformed data.
[b] Significance levels calculated from log-transformed data.
[c] B = C57BL/6J; D = DBA/2J.

In both cases, the same subset, inclusive of one locus, was the chromosome *4* locus defined by *Mup-1* or *Lps* (Table 4). The allele at this locus, when contributed by the C57BL/6J parent, associated positively with expression and best predicted the results of viral RNA expression. This association was calculated to be significant at a level of $P < 0.001$.

Due to the statistical basis of this analysis, one must control for the presence of unequal variances in the MMTV expression of the different strains. A standard numerical log transformation of the MMTV expression (see MATERIALS AND METHODS) was performed and the analysis repeated. In all cases, the original significantly associated loci remained significant; however, several

TABLE 4

Regression analysis of MMTV expression in BXH mouse strains

Individual analysis

Independent variable	Chromosome	Level of significance		Parental correlation[c]
		Raw[a]	Log[b]	
Akp-1	1		0.029	H
Mup-1	4	0.012	0.015	B
Lps	4	0.012	0.015	B
Pgm-1	5		0.008	H
Gus	5		0.004	H
Mtv-14	6		0.001	B
Mtv-1	7		0.009	H
Mod-2	7		0.002	H
Sep-1	9	0.050	0.007	H
Lap-1	9	0.018	0.003	H
Mod-1	9		0.042	H
Es-10	14		0.029	H
For-5	14		0.029	H
H-2	17	0.035	0.006	B
Mtv-9			0.001	B
Mtv-11			0.024	H
Age		0.020	0.050	

Best fit model subsets regression analysis

Independent variables available for model-building	Variables picked for best-fit models with their levels of significance	
	Raw	Log
Akp-1	<0.001	
Lps	<0.001	<0.001
Pgm-1		
Mtv-14		
Mtv-1		
Lap-1		
For-5		
H-2		
Mtv-9		<0.001
Mtv-6		<0.001
Mtv-11		
Multiparity		
Age		
Lactation		

[a] Significance levels calculated from untransformed data.
[b] Significance levels calculated from log-transformed data.
[c] B = C57BL/6J; H = C3H/HeJ.

more loci were shown to be significant. The unequal variance in the untransformed data obscured the association of four additional loci in the BXD strains (Table 3) and eleven additional loci in the BXH strains (Table 4).

Best subsets analysis results of the data subsequent to log transformation

changed the identified genetic loci only slightly. In the BXD animals, a negative association with the MMTV provirus *Mtv-1* was significant ($P < 0.001$) along with the previously identified *Emv-2* provirus and multiparity (Table 3). In the BXH animals, the locus defined by *Lps* and *Mup-1* remained very significant ($P < 0.001$). However, the model also now included the positive association of two MMTV proviral loci, *Mtv-6* and *Mtv-9*, and the chromosome *1* locus *Akp-1* (all significant at $P < 0.001$, Table 4).

The statistical correlation of genetic markers with viral RNA levels in the lactating mammary glands was to identify chromosomal regions associated with MMTV-specific RNA expression. The correlation of the locus defined by *Mup-1/Lps*, however, strongly suggested that the cellular gene, not just the marker chromosomal region directly involved in the regulation of MMTV proviral genes, was identified. The positive influence of the $Mup\text{-}1^b/Lps^n$ alleles was observed in the BXH RI strain, but not in the BXD RI strains, yet the common C57BL/6J progenitor was the contributor of these alleles in both BXH and BXD strains. Thus, the same chromosomal region was not identified as significantly associated with viral expression in both sets of RI strains. Examination of the genotype in this region revealed that the *Mup-1* gene was polymorphic in both the BXD and BXH strains, whereas the *Lps* gene was not. When MMTV expression was examined in the BXD strains (*Mup-1* was polymorphic, *Lps* was not), no significant effect ($P > 0.05$) was observed. However, in the BXH strains (*Mup-1* and *Lps* were both polymorphic), a significant increase ($P < 0.001$) in MMTV expression was correlated with the presence of the Lps^n allele. These genes are tightly linked on chromosome *4*, suggesting the direct involvement of the Lps^n allele in the positive regulation of MMTV expression and not the linkage of a regulatory locus to this marker.

Lipopolysaccharide induction of MMTV expression: The *Lps* locus defines the host response to bacterial lipopolysaccharide (LPS; GALANOS *et al.* 1977). The two alleles Lps^n and Lps^d govern the ability of the host to either respond or not respond to LPS, respectively. Therefore, proof of whether this significantly identified cellular gene was integral in the expression of endogenous MMTV proviruses in the BXH strains was directly testable. LPS-responder, lactating female mice (C3HfB/HeN) were injected with either bacterial LPS or a control saline solution. Six hours after injection, the mammary glands were harvested and monitored for virus-specific RNA. Figure 4 demonstrates at least a four-fold increase in MMTV-specific transcription (lane b) over that of the control animal (lane a), following the injection of LPS or saline, respectively. The MMTV response to LPS was not seen in Lps^d, C3Hf/HeJ mice (CARR, TRAINA-DORGE and COHEN 1985). These biochemical results directly confirmed the positive association of the Lps^n allele originally identified by statistical analysis. Confirmation of the other significantly associated alleles may require more extensive genetic testing.

DISCUSSION

Genetic techniques, in combination with molecular hybridization studies, were used to define the cellular loci required for transcription of endogenous

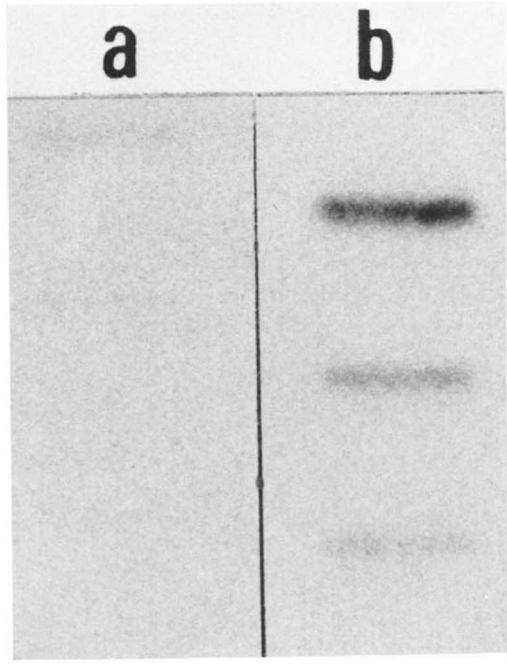

FIGURE 4.—Autoradiograph of MMTV-specific RNA in LPS-responder mice following injection of LPS. Lactating mammary gland RNA from primiparous LPS-responder (*Lps*^{n}) mice (C3HfB/HeN) following injection of either saline (lane a) or 2 mg LPS per kilogram body weight in sterile saline (lane b) were spotted onto treated nitrocellulose (see MATERIALS AND METHODS). The filter was hybridized with radiolabeled genomic MMTV sequences (specific activity of 10^8 cpm/μg DNA).

MMTV proviruses in the BXD and BXH RI mouse strains, as well as in their progenitor strains. The relative levels of progenitor strain MMTV-specific RNA expression were consistent with the results of others (Tables 1 and 2; VARMUS *et al.* 1973; MICHALIDES *et al.* 1978; VAIDYA *et al.* 1983). Levels of expression of the progeny RI strains were highly variable. Strain comparison of virus-specific expression with individual endogenous proviruses showed no direct correlation (Tables 1, 2, 5 and 6). There was also no correlation observed when increasing numbers of proviruses were compared with expression (Figure 2). These results suggest the involvement of cellular genes in the regulation of MMTV expression.

Regression analysis was utilized to evaluate the effect of environmental parameters in this system. Both the BXD and BXH RI strains showed an overall positive correlation of virus-specific RNA expression with increased parity. The effect was most consistent in the BXD strains, and confirmation of the response was provided by its overall significance level as determined by the multiple regression. This is not a surprising finding; the hormone responsiveness of MMTV has long been recognized. Multiparous females of inbred mice demonstrate higher levels of viral antigens, and virions, as well as tumor incidence, than do virgin or primiparous animals, suggesting the role of hormones *in vivo* (NANDI and MCGRATH 1973; VAN NIE and VERSTRAETEN 1975). Glucocorti-

TABLE 5

Distribution of MMTV proviruses in BXD mouse strains

Strain	Mtv- loci										Total
	1	7	8	9	10	11	12	13	14	15	
BXD-1	−	−	+	+	−	+	−	+	+	±	6
-2	+	−	+	+	+	+	+	−	−	±	7
-5	−	+	+	+	+	+	−	+	−	±	7
-6	−	−	+	+	−	+	+	−	+	±	6
-8	−	+	+	−	+	+	+	−	−	±	6
-9	−	+	+	+	−	+	+	+	−	±	7
-11	−	+	+	+	+	+	+	−	+	±	8
-12	+	−	+	−	−	+	−	+	−	+	5
-13	−	−	+	+	−	−	−	−	+	±	4
-14	+	−	+	+	−	+	+	+	−	+	7
-15	+	−	+	−	−	+	+	+	−	±	6
-16	−	−	+	+	−	+	+	+	−	+	6
-18	−	−	+	−	−	+	−	+	−	+	4
-19	−	−	+	−	−	+	+	+	+	+	6
-21	+	−	+	+	−	+	−	+	−	±	6
-22	+	−	+	+	+	+	+	+	+	±	9
-23	−	−	+	−	−	+	−	+	−	+	4
-24	−	+	+	+	+	−	+	−	−	±	6
-25	+	+	+	−	+	+	+	+	+	±	9
-28	−	+	+	−	+	+	−	+	−	±	6
-29	−	+	+	+	+	+	+	+	−	±	8
-30	−	+	+	+	+	+	−	+	−	±	7
-31	+	−	+	−	−	+	+	−	−	+	5
-32	+	+	+	−	+	+	+	+	−	±	8
C57BL/6J	−	−	+	+	−	−	−	−	−	−	2
DBAfB/2J	+	+	+	−	+	+	+	+	+	±	9

coids, a major lactogenic hormone in the mouse, has been found to activate MMTV expression *in vitro* (WEISS *et al.* 1982; BUETTI and DIGGLEMANN 1981).

Independent linear regression analysis identified the segregation of chromosomal markers with increases in virus-specific RNA levels. Independently segregating loci in both the BXD and BXH sets of mice were identified as significantly correlated with expression. Best subsets regression analysis subsequent to the numerical log transformation of the data indicated that the *Emv-2*$^-$ and *Mtv-1*$^-$ alleles in the BXD strains and the *Mup-1*^{b}, *Lps*^{n} and *Akp-1*^{b} alleles and *Mtv-9* and *Mtv-6* proviral loci in the BXH strains were significantly associated with MMTV expression at a level of confidence of $P < 0.01$ and $P < 0.001$, respectively (Tables 3 and 4). This means that the *Emv-2*$^-$ and *Mtv-1*$^-$ alleles in combination with multiparity best explained any increase in expression observed in the BXD strains. The negative association of both the *Emv-2* and the *Mtv-1* proviruses indicates an inverse correlation of the viral sequences with expression. Although these results are difficult to interpret, one explanation is the insertional inactivation of a *trans*-acting gene required for MMTV expression. Insertional inactivation by retrovirus integration has been

TABLE 6

Distribution of MMTV proviruses in BXH mouse strains

Strain	Mtv- loci						Total
	1	6	8	9	11	14	
BXH-2	+	+	+	+	+	−	5
-3	+	−	+	+	−	−	3
-4	+	+	+	+	+	−	5
-6	+	+	+	+	−	+	5
-7	−	−	+	−	−	+	2
-8	+	+	+	+	−	+	5
-9	−	−	+	+	−	−	2
-10	+	−	+	−	−	−	2
-11	+	+	+	+	+	+	6
-12	+	+	+	−	+	−	4
-14	+	+	+	−	+	−	4
-19	+	+	+	+	+	−	5
C57BL/6J	−	−	+	+	−	−	2
C3Hf/HeJ	+	+	+	−	+	+	5

described in several systems (JENKINS *et al.* 1981; JAENISCH *et al.* 1983; SCHNIEKE, HARBERS and JAENISCH 1983). The most probable alleles responsible for MMTV expression in the BXH strains were *Mup-1*^{b}, *Lps*^{n} and *Akp-1*^{b}, as well as the *Mtv-9*$^+$ and *Mtv-6*$^+$ proviral alleles.

Biochemical testing proved not only the positive association of the *Mup-1*^{b}/*Lps*^{n} markers but also the specific involvement of the *Lps*^{n} allele in MMTV proviral expression. Although one cannot exclude the possibility that any of the other identified loci are involved, either singly or in combination, or that loci linked to those genetic markers are involved, the loci identified are the most likely genetic elements responsible for the characteristic endogenous viral expression of each mouse strain.

An inherent limitation in the system was the number of RI strains available for testing. Because the basis of analysis was cosegregation, one would need all possible combinations of the viral loci for conclusive results. At this point, any one of the several identified cellular loci that were significantly associated with expression could play a role in regulation of MMTV expression. However, the most likely alleles responsible for the phenotype were *Emv-2*$^-$ and *Mtv-1*$^-$ in the BXD strains and *Lps*^{n}, *Akp-1*^{b}, *Mtv-9*$^+$ and *Mtv-6*$^+$ in the BXH strains.

The results of regression analysis demonstrated a positive and significant association of the genome length *Mtv-9* as well as the subgenomic *Mtv-6* proviruses with virus expression in the BXH strains. These results strongly suggest that certain of the endogenous proviruses in the BXH genomes are more transcriptionally active than others. The regression analysis, however, failed to implicate any specific genomic length structural viral locus positively cosegregating with expression in the BXD strains. Production of detectable MMTV-specific RNA necessitates transcription from one or several endogenous proviruses. If there existed a generalized cellular regulatory influence of MMTV

viral genes involving multiple proviruses, the effect calculated in the regression equation would be diluted for any one of the viral elements alone. This situation is more pronounced with the presence of ten proviruses in the BXD strains *vs.* six in the BXH strains. The transcriptional activity of particular proviruses in the BXD strains may be confounded by a variety of other transcriptionally active proviruses and involved cellular loci. The results for both BXH and BXD, however, are consistent with a multigenic effect by independently segregating loci in the control of endogenous MMTV gene expression. A similar suggestion of *trans* regulation was made in a study by VAIDYA *et al.* (1983), in which C57BL, BALB/c and their backcross progeny were used.

In the experimental design, chromosomal regions associated with MMTV RNA expression would be identified by the positive association of particular marker genes. The differences in significant association of two tightly linked genetic markers in either the BXD and BXH strains suggested the direct involvement of the Lps^n allele in the positive regulation of MMTV expression and not the linkage of a regulatory locus to these markers.

The Lps^n gene elicits diverse biological effects in the mouse, most notably affecting the lymphoreticular response to bacterial LPS. This then activates a multitude of cellular responses, such as immunostimulation, immunosuppression, and increases in intracellular cAMP metabolism (GALANOS *et al.* 1977). In addition, B lymphocytes show direct induction of an endogenous ecotropic murine leukemia virus upon stimulation with LPS (STOYE and MORONI 1983). Their normal expression in differentiated lymphocytes is thought to be heightened due to the mitogenic effects of LPS. The corresponding Lps^d allele from the C3H/HeJ parent is deficient in the LPS response and, therefore, fails to demonstrate these same effects. The pleiotropic effects observed in the LPS response suggest a common mechanism of activation likely shared in many pathways. Therefore, the Lps^n gene may encode a protein product (*e.g.*, cell surface receptor) able to activate many genes, probably including the opportunistic MMTV genes, *in trans*.

The LPS nonresponder strain, BXH-7, contains only two endogenous proviruses (Table 6) and failed to demonstrate any detectable MMTV-specific RNA in the lactating mammary glands of both primiparous and multiparous females (Table 2). The BXH-12 and BXH-14 strains, although they harbor the same MMTV proviruses, differed in their *Lps* alleles. The presence of the Lps^d allele in the nonexpressor strain BXH-7 and the low expressor strain BXH-12, and the presence of the Lps^n allele in the high expressor strain BXH-14, are consistent with regulation *in trans* of transcription-competent viral genes by the Lps^n cellular product.

Due to the function of the *Lps* gene in regulating the response of the host to LPS, direct biochemical testing of the association of the Lps^n allele with viral expression was possible. Intravenous injection of LPS into the responder Lps^n inbred mouse strain C3HfB/HeN resulted in a substantial increase in MMTV RNA expression over that of control animals in as short a time period as 6 hr (Figure 4). These results confirm the statistically significant association of the Lps^n allele with viral expression in the BXH mouse strains. Preliminary

evidence demonstrates that LPS induction results in the increase of a properly sized MMTV-specific transcript (CARR, TRAINA-DORGE and COHEN 1985). With the mapping data, it is also certain that the *Lps* locus segregates independent of any MMTV proviruses (TRAINA, TAYLOR and COHEN 1981) and, therefore, must act *in trans* to stimulate MMTV specific transcription.

OUTZEN (1980) compared the mammary tumor incidence and the time of occurrence of mammary tumors in three C3H substrains, C3H/An, C3H/HeJ and C3H/OuJ. The C3H/An and C3H/HeJ were previously shown (COHEN and VARMUS 1979) to harbor the same MMTV proviruses. The C3H/OuJ substrain was separated from C3H/HeJ mice in 1952 to propagate the W^x mutation. This substrain also harbors the Lps^n allele, not the Lps^d mutation that subsequently became fixed in the C3H/HeJ substrain. Comparison of the mean tumor latency period among these strains revealed that LPS responder strains, C3H/An and C3H/OuJ, developed mammary tumors earlier (at 39 and 33 wk, respectively) than did the LPS nonresponder strain, C3H/HeJ (58 wk). Because the MMTV-specific sequences are identical in all these strains, these data suggest that the presence of the Lps^n allele may also facilitate mammary tumorigenesis.

This work was supported, in part, by Public Health Service grants R01-CA-34823 (JCC), R01-CA-28198 (RE), R01-GM-18684 (BT) and R01-CA-33093 (BT) from the National Institutes of Health. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care. JCC is supported by the Optimist Leukemia Foundation of Louisiana, Inc.

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Communicating editor: R. E. GANSCHOW