LOCALIZATION OF A GENE FOR EXPRESSION OF MOUSE MAMMARY TUMOR VIRUS ANTIGENS IN THE GR/Mtv-2⁻ MOUSE STRAIN*

By R. NUSSE,‡ J. DE MOES, J. HILKENS, AND R. VAN NIE

From the Division of Genetics, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Analysis of the inheritance of mouse mammary tumor virus (MMTV)[1] expression in mice has originated the concept that this virus can be genetically transmitted, as a chromosomal provirus (1).

In the mouse strain GR, a single dominant gene was responsible for a high expression of MMTV in the milk, and for the development of early pregnancy-dependent mammary tumors (1–4). The virus could be transmitted by the male as well as by the female. This gene, Mtv-2, has recently been mapped on chromosome 18, linked to the Tw (Twirler) locus. (R. Van Nie. Unpublished observations.) However, other investigators disagreed with a single gene model. Nandi and Helmich (5) reported that segregation of MMTV expression in crosses between GR and low mammary tumor strains was under control of two genes and Heston and Parks (6, 7) found that the development of mammary tumors and virus expression were transmitted as a multifactorial genetic trait.

Molecular biological studies showed that the number of MMTV proviral DNA copies in the GR strain exceeds that of other strains (8, 9). A subset of these proviruses was proven to be identical to Mtv-2 the induction gene for high amounts of virus (8). Assuming that the remaining proviral DNA copies could also give rise to virus expression, it became well possible that the total MMTV expression in the GR strain was controlled by several genes. In such a model the Mtv-2 locus should be responsible for high amounts of virus expression and early mammary tumors whereas other loci, probably also identical to proviral DNA copies, should induce lower amounts of virus, only detectable with sensitive techniques such as a radioimmunoassay (10).

The development of a GR/Mtv-2⁻ substrain, congenic to the GR but lacking the Mtv-2 gene for high virus expression and early mammary tumors (11), provided the possibility to study the genetic control over MMTV expression that is not controlled by the Mtv-2 gene. This report deals with the analysis and location of a gene for partial MMTV expression in the GR/Mtv-2⁻ strain.

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[1] *Abbreviations used in this paper:* BCI, BCII, first and second backcross populations; BSA, bovine serum albumin; MMTV, mouse mammary tumor virus; mRNA, messenger RNA, RIA radioimmunoassay; TNE, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl.

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Materials and Methods

Mice. BALB/c mice (BALB/c/HeA) and GR/Mtv-2⁻ mice were from the breeding colony of The Netherlands Cancer Institute, Amsterdam. The GR/Mtv-2⁻ strain (11) is in the eighth inbred generation.

Backcross Analysis. The first backcross population (BCI) consisted of female progeny mice from a [♀ (BALB/c × GR/Mtv-2⁻) × ♂BALB/c] cross. These mice were mated again to BALB/c males, generating a second backcross (BCII) population. The first litter BCII mice were grown up and then the mothers, the BCI population, were sacrificed at the next lactation period. BCII females were also sacrificed at the second lactation.

Preparation of Mammary Gland Extracts. Mammary glands were minced, suspended in 10 mM Tris-HCl, pH 9.2, 1 mM EDTA, 400 mM KCl, 1% (vol/vol) Triton X-100 and disrupted by douncing combined with freeze-thawing. The homogenate was incubated for 20 min at 37°C and centrifuged for 10 min at 27,000 *g*. The supernate was dialyzed against TNE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl) with 0.4% (vol/vol) Triton X-100. The dialyzed extracts were centrifuged for 1 h at 30,000 *g* and total protein concentration in the supernate was measured using bovine serum albumin (BSA) as a standard (12).

Labeling of Viral Proteins. MMTV gp52 and p27 were isolated as previously described (13, 14). The MMTV gag protein p10 was isolated by means of phosphocellulose chromatography. The envelope protein gp36 was kindly provided by Dr. F. Westenbrink, Rijswijk, The Netherlands (15).

MMTV proteins were labeled with ^{125}iodine according to Hunter and Greenwood (16). The final reaction mixture was composed of 15 μl protein in TNE buffer (0.5 mg/ml), 25 μl 0.5 M $NaHPO_4$ pH 7.4, 10 μl ^{125}iodine (Amersham Corp., Arlington Heights, Ill., carrier free, 100 mCi/ml in NaOH pH 9-11), and 10 μl chloramine T (1 mg/ml). Incubation was for 1 min at room temperature after which 5 μl $Na_2S_2O_5$ (10 mg/ml), and 50 μl 0.1 M NaJ were added to stop the reaction. Unbound iodine was removed from protein on a Sephadex G-50 (fine) column. Specific activities of labeled proteins ranged from 20,000-50,000 cpm/ng protein. The polyvalent rabbit anti-MMTV serum precipitated all four viral proteins at dilutions ranging from 1/5,000–1/20,000 (17).

Radioimmunoassay. Total protein concentration of tissue extracts was adjusted to 10 mg tissue protein per ml. Samples were serially diluted in 0.1 ml TNE buffer supplemented with 2 mg/ml BSA and 0.2% (vol/vol) Triton X-100. 10 μl of diluted anti-MMTV serum was added to each sample and incubated for 1 h at 37°C. Then, 40 μl of labeled protein (1 ng, 20,000–40,000 cpm) diluted in normal rabbit serum was added. After a 3-h incubation at 37°C, a titrated amount goat anti-rabbit gamma globulin serum was added. Immune complexes were allowed to form overnight at 4°C, collected by centrifugation (10 min, 3,000 *g*), washed three times in cold TNE buffer, and counted.

Electrophoretic mobility of Es-3 was examined according to published procedures (18). Briefly: hemolysates of erythrocytes were run on a 12% vertical starch gel in a Tris-EDTA-Borate buffer pH 3.6 and stained with 1 mg/ml 4 methylum umbelliferone propionate in a 0.1 M Na citrate buffer, pH 5.5. The solutions were applied on a filter paper overlays. After 15 min the gel could be scored for Es-3 under a long-wave UV lamp.

Results

Expression of the MMTV Genome in the GR/Mtv-2⁻ Strain. MMTV contains six structural proteins that are encoded on the viral genome, the envelope proteins gp52 and gp36, and the core proteins p27, p21, p14, and p10. Synthesis of envelope and core proteins proceeds independently from each other. The core, or gag, proteins are synthesized from a 35S messenger RNA (mRNA), initially as a precursor polypeptide of 73,000 dalton (16, 19). A 24S mRNA instructs synthesis of envelope, or env, proteins, also via a precursor polyprotein of 73,000 dalton (20). To measure the expression of individual representative genes of MMTV in GR/Mtv-2⁻ mice, two proteins from the env gene, gp52 and gp36, and two proteins from the gag gene, p27 and p10, were used as tracer antigens in the radioimmunoassay (RIA).

In Fig. 1 the results of competition assays for each of these viral proteins are shown. Comparison of the reactivity of tissue extracts with the reactivity of standard purified proteins of a known concentration allowed calculations of the amounts of viral proteins in tissue extracts. Purified disrupted MMTV also served as standard competing antigen. In extracts of mammary glands of lactating GR mice, high amounts of each of the viral proteins were detected, in the order of magnitude of 10 μg viral protein per mg total protein.

Extracts from lactating GR/Mtv-2⁻ mice did compete in the RIA for the MMTV gag proteins p27 and p10 at concentrations ranging from 200 to 2,000 ng viral protein per mg. The same extracts however did not react in the RIA for the env proteins gp52 and gp36. Up till a total protein concentration of 10 mg/ml, no inhibition was found. Apparently, the genome of MMTV was incompletely expressed in the mammary glands of the GR/Mtv-2⁻ strain. Levels of the gag proteins were relatively high but essentially no env proteins were detected. As a rule, env and gag proteins are found in concordant amounts, e.g. in the C3Hf strain (12). Results of a number of competition RIA's in individual GR/Mtv-2⁻ mice as well as in mice from other strains are summarized in Table I. It shows that essentially all GR/Mtv-2⁻ mice tested were positive for MMTV p27 expression, already at the first lactation.

To study the genetic transmission of the MMTV gag expression, an appropriate background strain had to be chosen. In a previous study we have shown that normal lactating mammary glands of several low mammary tumor strains, e.g. C57BL, 020

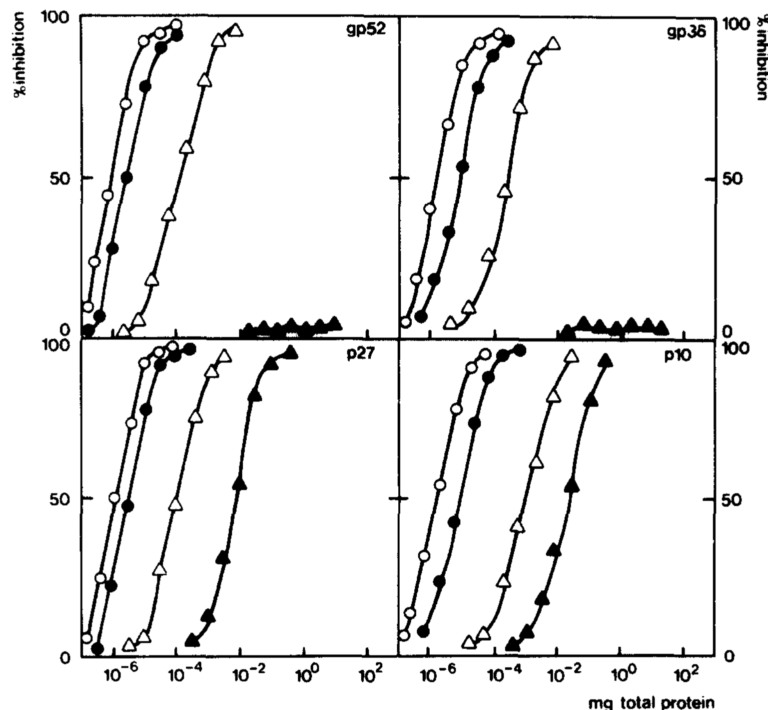

FIG. 1. Competition radioimmunoassay for individual MMTV structural proteins gp52, gp36, p27, and p10. (○) Standard purified protein; (●) disrupted MMTV; (△) extracts of mammary glands from GR mice; (▲) extracts of mammary glands from GR/Mtv-2⁻ mice.

TABLE I

Amounts of MMTV Viral Proteins in Extracts of Mammary Glands of Several Strains of Mice

Strain	Lactation	Number of mice positive/tested			
		$gp52^{env}$	$gp36^{env}$	$p27^{gag}$	$p10^{gag}$
GR/Mtv-2⁻	First	0/25	0/4	25/25 (210–1,800)	4/4 (20–800)
	Fourth			6/6	
BALB/c	First			0/6	
	Fourth			0/16	
(GR/Mtv-2⁻ × BALB/c)F_1	First	0/13		13/13 (350–1,600)	
	Fourth	0/12		12/12	
BALB/cfGR/Mtv-2⁻	Fourth	0/11		2/11 (2–10)	
GR	First	12/12 (10,000)		12/12 (10,000)	4/4

Range of amounts of viral proteins is given between parentheses in nanograms of viral protein per milligrams of tissue protein.

TABLE II

Backcross Analysis of the Genetic Transmission of MMTV p27 Expression in Mammary Glands of GR/Mtv-2⁻ Mice

Backcross I: (BALB/c × BALB/c GR/Mtv-2⁻)

	MMTV p27 expression	
	Positive	Negative
Es-3	42	40
a/a	0	36
a/B	42	4

TABLE III

Progeny Testing of MMTV p27 Expression in GR/Mtv-2⁻ Mice

Backcross II: (BALB/c × BALB/c GR/Mtv-2⁻) BALB/c

	Daughters from positive BCI mothers MMTV p27 expression	
	Positive	Negative
Es-3	36	43
a/a	2	43
a/B	34	0
	Daughters from negative BCI mothers MMTV p27 expression	
	Positive	Negative
Es-3	0	43
a/a	0	41
a/B	0	2

and BALB/c do not contain detectable levels of MMTV proteins gp52 and p27 (12). Data on BALB/c mice are also given in Table I. In a cross of the GR/Mtv-2$^-$ strain with the BALB/c, the MMTV p27 expression behaved as a fully dominant feature, since all F_1 hybrid daughters were positive, in levels comparable to those in the GR/Mtv-2$^-$ strain.

Extrachromosomal influences are known to interfere sometimes considerably in studies on genetic transmission of MMTV expression. Especially the BALB/c strain is extraordinary sensitive for replication of exogenously transmitted MMTV (21). Moreover, in the milk of GR/Mtv-2$^-$ mice MMTV antigens can be detected albeit at low levels ranging from 20 to 100 ng per mg. (A. Verstraeten. Personal communication.) It can be argued therefore that in crosses with GR/Mtv-2$^-$ mice as mothers, the milk transmitted MMTV rather than chromosomal inherited genes could give rise to virus expression in the progeny. This was tested by fosternursing newborn BALB/c mice to GR/Mtv-2$^-$ mothers and assaying the mammary glands for the presence of viral antigens. Except for two cases of very low amounts of p27 at late lactations, the fosternursed BALB/c mice did not show significant levels of virus expression. Therefore, the BALB/c strain was regarded as an appropriate strain for the mating experiments to study the genetic transmission of MMTV p27 in the GR/Mtv-2$^-$ strain.

Backcross Analysis of the MMTV p27 Expression in GR/Mtv-2$^-$ Mice. In the first backcross population MMTV p27 was measured in extracts of the mammary glands. Animals were scored as positive when the mammary gland contained more than 200 ng viral proteins per mg tissue protein and as negative when none or very little (0.5–1 ng) viral protein per milligram tissue protein was detected. Intermediate levels were not found in any of the backcross mice. In Table II it is shown that the segregation of animals positive or negative for p27 was very close to 50% (χ^2 = 0.04, P = 0.841), indicating that a single dominant gene governed the virus expression. The same backcross animals were assayed for a number of segregating chromosomal markers. A close linkage of the p27 expression was observed with the Es-3 locus, located on chromosome 11. The Es-3 genotype, a/a or a/B, segregated itself as one gene (χ^2 1.22, P 0.269) and only 4 recombinants between Es-3 and the gene for p27 expression were scored in 82 animals (χ^2 66.78, 5% recombination).

The one-gene model was further tested in a second backcross. Female BCI mice were mated to BALB/c males and offspring daughters were also tested at the second lactation for p27 reactivity in the mammary glands Table (III). Daughters from negative BCI mothers were again negative for viral antigens, whereas the progeny from positive BCI mothers again segregated very close to 50% (χ^2 0.62, P 0.431), as predicted by a single gene control. Also the linkage to the Es-3 locus persisted in the second backcross with a total of 2/79 (3%) recombinants. Levels of p27 expression in the backcross mice stayed within the same range as in the GR/Mtv-2$^-$ mice, indicating that virus expression was not restricted. Thus, also by progeny testing, the expression of MMTV gag proteins in the GR/Mtv-2$^-$ strain could be demonstrated to be governed by a single dominant gene, located on chromosome 11, close to the Es-3 locus.

Discussion

The gene for expression of MMTV gag proteins in the GR/Mtv-2$^-$ strain is the third MMTV induction gene that is identified in the mouse genome. Mtv-1, a gene

for moderate levels of MMTV proteins in the C3Hf and DBAf strains is located on chromosome 7 (22, 23). This gene is at least in the C3Hf strain also controlling the development of mammary tumors (22). Mtv-2, the gene for high amounts of MMTV and the early occurrence of mammary tumors in the GR strain has recently been mapped on chromosome 18. (Van Nie et al. Unpublished observations.) Molecular hybridization analysis, by reassociation kinetics as well as by hybridization to restriction enzyme fragments, has revealed that the Mtv-2 gene is identical to at least one proviral MMTV DNA copy (8, 24). Because of the absence of this MMTV proviral DNA in other strains, Mtv-2 has probably no allelic counterparts.

Out of the GR strain, a congenic strain has been created in our Institute. This strain, the GR/Mtv-2^- was developed by cross-intercross mating of a B10-GR hybrid to GR, and selecting for progeny mice which were negative for early mammary tumors and high levels of virus antigens in the milk (11). Virus expression was tested by immunodiffusion, a relatively insensitive technique. However, with more sensitive radioimmunoassays, viral antigens can still be detected in the GR/Mtv-2^- strain. Amounts of antigens are a 10- to 100-fold lower than in the GR and characteristically, only MMTV gag proteins are detected. The levels are sufficiently high to permit genetic analysis by crossing the strain to the virus negative BALB/c strain, and a clear 1:1 segregation, indicative of a single gene, is obtained. The gene is not linked with any of the other Mtv induction genes and is therefore called Mtv-3, located on chromosome 11. Since the GR/Mtv-2^- strain is congenic to the GR strain except for the Mtv-2 locus, Mtv-3 is presumed to be present also in the GR, and will give rise to viral antigens, albeit at lower amounts than those coded for by the Mtv-2 locus. Consequently, altogether two MMTV induction genes can be found in the GR when sensitive detection methods are employed, in accordance with studies by Heston et al. (6) and Bentvelzen et al. (10).

Mtv-3 is not associated with early mammary tumor development. The spontaneous mammary tumor incidence of the GR/Mtv-2^- strain in our colony is very low. Therefore, the results of Heston & Parks who observed a multifactorial pattern of inheritance of mammary tumors in the GR, cannot be ascribed to the Mtv-3 gene. Rather, extrachromosomal male transmission of MMTV can explain the high tumor incidence in segregating populations of crosses between GR and low mammary tumor incidence strains.

An unusual feature of the expression of the Mtv-3 gene is the absence of viral envelope glycoproteins, in the mammary gland extracts, whereas core proteins are present in relatively high amounts. The partial expression of the MMTV genome in the mammary gland might be explained by a gene deletion. Incomplete MMTV proviruses have been detected in the mouse genome with restriction enzyme analysis (25). Alternatively, the recent insight into the mechanism of RNA and protein synthesis may suggest that the deficiency in synthesis of envelope proteins is caused by a defective splicing mechanism. The 24S mRNA for envelope protein of retroviruses is derived from the 35S mRNA for core proteins by RNA splicing (26). Currently, the viral mRNA populations in mammary glands of GR/Mtv-2^- mice are fractionated and analyzed. The absence of gp52 also explains the lack of a humoral immune response to MMTV in the GR/Mtv-2^- strain (27). Production of similar levels of viral core proteins, accompanied by envelope proteins such as in the C3Hf strain usually elicits antibodies which are directed against the envelope proteins (28).

Thus far, three genes for MMTV expression have been located on different sites of the mouse genome. Only the Mtv-1 locus has been shown to have alleles in two, yet related, mouse strains, the C3Hf and DBAf. The different chromosomal location of Mtv-2 and Mtv-3 in the European GR strain supports the hypothesis that endogenous MMTV proviruses are the result of relatively recent, independent germ lines infections, rather than being evolved from ancient proviruses (29).

Summary

The GR/Mtv-2⁻ mouse strain is congenic to the GR strain but lacks the Mtv-2 gene for high amounts of mouse mammary tumor virus (MMTV) virion particles in the milk and early mammary tumors. With a sensitive competition radioimmunoassay for individual viral proteins of MMTV, substantial amounts of the gag proteins p27 and p10 could still be detected in extracts of the mammary glands of GR/Mtv-2⁻ mice, but essentially no viral envelope antigens. The genetic transmission of the MMTV gag expression in the GR/Mtv-2⁻ strain was investigated. In a cross with the virus-negative BALB/c strain, the MMTV p27 expression behaved as a dominant feature. Double backcross analysis proved that the p27 expression was governed by a single gene located on chromosome 11, close to the Es-3 locus. The gene was thereby not allelic to any of the previously described MMTV induction genes, Mtv-1 and Mtv-2, and is therefore called Mtv-3. It is concluded that the total MMTV expression in the GR strain is under control of two separate loci, Mtv-2 on chromosome 18, inducing high levels of complete virus particles and also early mammary tumors; and Mtv-3 on chromosome 11, coding for partial MMTV expression.

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