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The endogenous proviral mouse mammary tumor virus genes of the GR mouse are not identical and only one corresponds to the exogenous virus

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ABSTRACT

The endogenous proviral copies of mouse mammary tumor virus (MMTV) were selected from a gene library of GR mouse DNA. We obtained five different λ·MMTV recombinant clones. Four of them correspond to the 3' Eco RI fragments of the endogenous proviruses and one comprises an intact MMTV provirus with 2 to 3 kb of flanking mouse genomic DNA. Heteroduplex formation followed by S1 digestion under stringent conditions shows that there is nucleotide sequence heterology among the cloned endogenous proviral copies. Only one endogenous proviral copy, associated with the mtv-2 locus, was found to be totally homologous to the exogenous proviral DNA.

INTRODUCTION

Mouse mammary tumor virus (MMTV) is a murine retrovirus responsible for the induction of mammary adenocarcinomas (1). Exogenous MMTV is transmitted from the lactating mother to the off-spring via the milk. The viral RNA is reverse transcribed in infected cells and viral DNA is integrated into the host cell genome. Endogenous MMTV is genetically transmitted and is present in multiple gene copies in the germ line as well as in all somatic cells of inbred mouse strains. The GR strain of mice is characterized by a high incidence of mammary tumors (2) and a single genetic locus (mtv-2) has been assigned to the control of the complete expression of mouse mammary tumor virus particles (12). Although the GR mouse contains five copies of proviral DNA per haploid genome (3) only a single specific copy of the MMTV genes is associated with the mtv-2 locus (4). These results suggest the involvement of the MMTV copy present in the mtv-2 locus in viral expression, but it has not been unambiguously demonstrated that this proviral DNA copy gives rise to the

exogenous MMTV. Here we provide biochemical evidence for the identity of the exogenous MMTV with the MMTV proviral gene present in the mtv-2 locus. This conclusion has been made possible by the following experimental design. The endogenous proviral copies of the GR mouse have been cloned into λ recombinant molecules. The MMTV specific Eco RI fragments of the λ recombinants were denatured, renatured and heteroduplexes of the proviral MMTV gene copies were obtained. Using increasingly stringent conditions of S1 nuclease digestion we have shown that the endogenous proviral copies are very similar to each other but not identical. Heteroduplexes were also formed between cloned exogenous MMTV copies (5) and the endogenous MMTV genes of the GR mouse. Only the endogenous copy associated with the mtv-2 locus formed a heteroduplex resistant to the most stringent S1 nuclease digestion conditions.

MATERIALS AND METHODS

Molecular cloning of proviral MMTV DNA

DNA was isolated from the liver of a GR mouse (3) and partially digested with Eco RI. The average size of the DNA was 20 kb. The DNA was preparatively fractionated by agarose gel electrophoresis and 14 to 24 kb DNA was recovered. 3 μg of DNA were ligated to 2 μg of λ Charon 4A arms (6) and packaged in vitro into phage particles (7). 3 x 10^6 phage plaques were obtained. λ·MMTV recombinant phages were identified on nitrocellulose filter lifts by hybridisation to ^{32}P labeled MMTV cDNA and purified as described (5).

Heteroduplex formation and S1 nuclease analysis

λ·MMTV recombinant phage DNA was digested with Eco RI and extracted with phenol and chloroform. 0.5 μg of λ·MMTV DNA from the clones indicated in the Figure legends were mixed in pairs. The DNA was denatured at 70°C for 10 minutes in 50 μl of 70 % formamide, 0.3 M NaCl, 10 mM tris·HCl pH 8.5, and 1 mM EDTA. The DNA was allowed to reanneal in the same buffer at 37°C for 1 hour and was subsequently diluted with 500 μl of S1 digestion buffer. For the experiments shown in Figure 4, 5 and 6 this buffer consisted of 40 % formamide, 0.3 M sodium chloride, 0.03 M sodium acetate pH 4.5, 3 mM $ZnSO_4$, 5 μg denatured calf thymus DNA and

2 units (Sigma) of S1 nuclease. The digestion was carried out for 60 minutes at 45°C. In the experiment shown in Figure 3 the digestion conditions were altered as indicated in the Figure legend. After the S1 nuclease digestion 10 µg of denatured calf thymus DNA was added and the DNA was precipitated with ethanol. About 50 ng of λ·MMTV recombinant DNA was loaded onto each lane of a 0.8 % agarose gel and the MMTV specific DNA fragments were visualised after electrophoresis and Southern blotting (8) as described (3).

RESULTS

1. Molecular cloning of endogenous MMTV proviral DNA of the GR mouse

The combination of restriction enzyme digestion and Southern blotting analysis (8) has been used previously to map restriction sites of proviral MMTV genes from genomic DNA of several strains of mice (9, 10). Molecular amplification of proviral DNA copies makes possible a more comprehensive analysis of the differences and similarities between proviral genes and their flanking host DNA sequences (5). We have cloned the DNA of GR mouse cells into the λ Ch 4 A vector system (6). The recombinant phages were screened with MMTV cDNA and 36 λ·MMTV recombinants were obtained. The DNA of these recombinant λ phages was analysed by restriction digestion with Eco RI and compared to MMTV specific Eco RI fragments in the GR mouse DNA (Fig. 1 lane 1). Lanes 2-6 show the five different MMTV specific Eco RI fragments which were present in the recombinant phages. Four of them (clones 17, 18, 34, 55) correspond to 3' sides of proviral DNA. We have shown previously that all five copies of the proviral DNA in the GR mouse are cleaved once by Eco RI and the 5' fragments (left hand sides) and 3' fragments (right hand side) have been identified by hybridisation with site specific molecular probes (5). Four of the five 3' fragments present in mouse DNA have been cloned. The fifth type of MMTV containing clone obtained is shown in lane 6. This clone contains a left and a right hand Eco RI fragment and represents an intact proviral gene. Its right hand side corresponds to the fragment obtained in clone 55. Not all the clones shown in Fig. 1 were obtained with the same frequency. Of

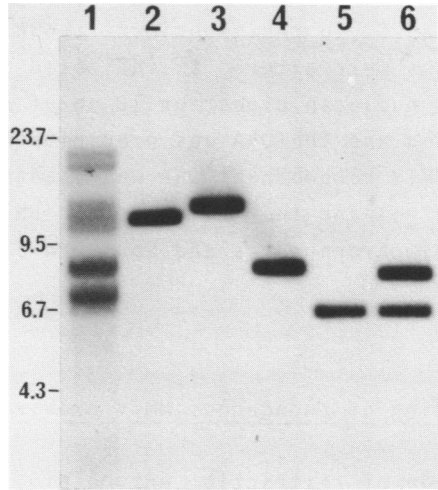

Figure 1. Molecular clones of endogenous proviral MMTV genes of the GR mouse. The five different λ·MMTV recombinant clones obtained from the library of GR mouse DNA were digested with Eco RI. 1 ng of DNA was electrophoresed on a 0.8 % agarose gel, transferred to a nitrocellulose filter and hybridized to ^{32}P MMTV cDNA. The cloned DNA is compared to GR mouse liver DNA digested with Eco RI. Lane 1: 10 μg GR mouse liver DNA, lane 2: clone 17, lane 3: clone 18, lane 4: clone 34, lane 5: clone 55 and lane 6: clone 40. Molecular weight markers are indicated in kb.

36 clones analyzed 3 corresponded to clone 17 (11 kb, lane 2), 25 to clone 18 (11.5 kb, lane 3), 2 to clone 34 (8.1 kb, lane 4, 3 to clone 55 (6.7 kb, lane 5) and 3 to clone 40 (7.8 and 6.7 kb lane 6). Since only fragments from partial Eco RI digests of GR DNA were introduced into the λ Ch 4 A arms additional flanking host DNA sequences were associated with clones 17, 18, 34 and 55. However, the 5' fragments of these proviruses were not obtained. In addition we did not obtain the 5' fragment of clone 40 by itself. The non-statistical yield of different cloned Eco RI fragments and the absence of the 5' specific Eco RI fragments of 4 of the 5 proviruses present indicate a non-random integration and propagation of the recombinant Eco RI fragments in λ phages.

Exogenous proviral copies were cloned from the DNA of a mammary tumor cell line as described previously (5). This cell

line is derived from the GR mouse (11) but has about 40 to 50 copies of the exogenous proviral DNA integrated into its genome. Five exogenous copies were obtained as λ recombinant molecules and all five have been shown to represent 3' fragments of the MMTV proviral gene with various lengths of host DNA between the end of the provirus and the nearest Eco RI site in the flanking DNA (5). Three of these exogenous MMTV clones were used in the experiments described in Figure 3, 4 and 6.

2. S1 nuclease digestion under stringent conditions detects differences among the heteroduplexes of proviral copies

Previous genetic analyses have shown that the proviralcopies of MMTV exhibit different biological properties (4, 12). These differences could be based on differential regulation of the proviral expression and/or on sequence differences of the proviral genes. The detection of sequence differences in proviral DNA could be informative. To circumvent the sequencing of the proviral genes we devised a method which allows us to detect sequence heterogeneities among proviral copies based on heteroduplex formation and S1 nuclease digestion. The principle of this method is outlined in Fig. 2. Two cloned MMTV containing Eco RI fragments are considered. These fragments can be distinguished from each other by the length of the host DNA flanking the MMTV provirus. The length of the host DNA sequence is defined by the distance of the nearest Eco RI site within the host DNA to the end of the provirus. Since the Eco RI site within the proviral DNA is located at the same position in each clone the overall size difference of the cloned MMTV specific Eco RI fragments has been used to deduce different genomic locations of proviral genes (3, 13). Two cloned fragments with different lengths of flanking host DNA are mixed and denatured in formamide at 70°C. Upon renaturation at 37°C three types of duplexes are formed, the two original homoduplexes and a heteroduplex bas-paired in the MMTV region of the molecular clones and single stranded in the adjacent host DNA. When these molecules are digested with S1 nuclease the reformed homoduplexes are expected to be resistant to digestion. The heteroduplex molecule is expected to be resistant in the MMTV specific part but not in the single stranded region containing the flanking host DNA. The length of the MMTV specif-

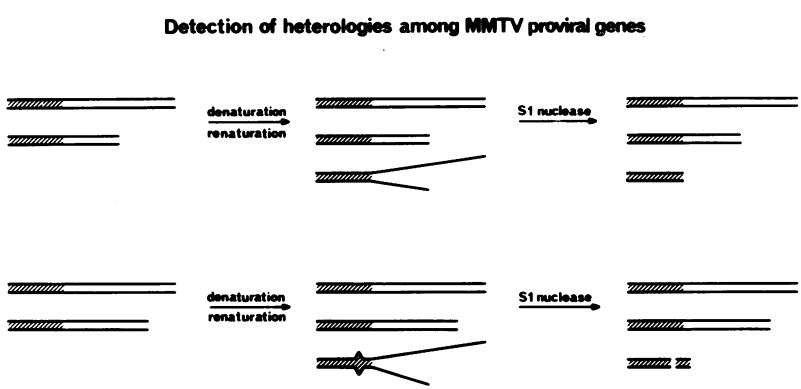

Figure 2. Experimental approach for the detection of DNA sequence heterologies among MMTV proviral genes. The upper line represents the heteroduplex formed between two DNA fragments sharing the proviral MMTV part (shaded area) and differing in the sequence of the flanking host DNA. Upon denaturation and renaturation S1 insensitive homoduplexes are reformed. A heteroduplex molecule differing in the host DNA sequences is reduced to the common MMTV region upon S1 nuclease digestion. The lower line illustrates the susceptibility of the heteroduplex region of MMTV specific DNA to S1 nuclease digestion in heteroduplex pairs with slight sequence heterology. Only the reformed homoduplexes persits whereas the MMTV region is digested into smaller fragments.

ic region from the internal Eco RI site to the 3' end of the provirus is 4 kb. This 4 kb fragment should be apparent after heteroduplex formation and S1 nuclease digestion. If the MMTV specific regions of different proviral copies are not identical then the MMTV portion of the heteroduplex might also be susceptible to S1 nuclease when the digestion is carried out under stringent conditions.

Fig. 3a shows the size of DNA fragments which were obtained when the 6.7 kb Eco RI fragment of endogenous proviral clone 55 (Fig. 1 lane 5) was mixed with a 5.3 kb Eco RI fragment of exogenous proviral clone 2 (Fig. 4, ref. 5) and subjected to denaturation, renaturation and S1 nuclease digestion. Lane 1 shows three major fragments of 6.7, 5.3 and 4.0 kb. These fragments were observed when the S1 nuclease digestion was carried out without formamide in 0.3 M NaCl. When the formamide concentration during S1 digestion was raised to 30 % (lane 2) or 40 %

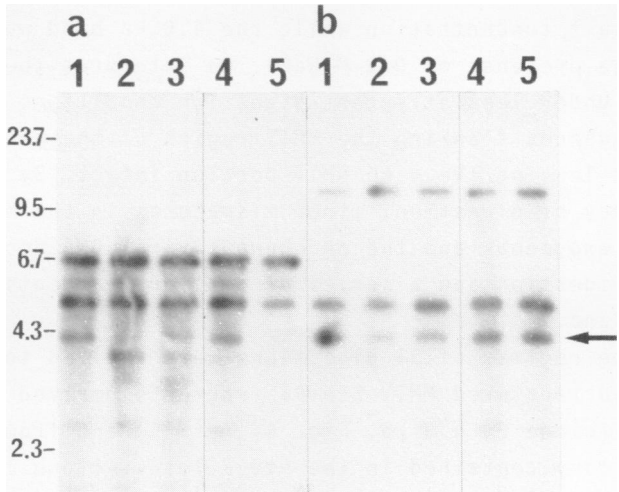

Figure 3. Analysis of homo- and heteroduplexes formed between pairs of MMTV DNA clones following different conditions of S1 nuclease digestion.

a) Eco RI digested DNA of λ·MMTV clone 55 (6.7 kb, Figure 1, lane 5) was mixed with Eco RI digested DNA of an exogenous λ·MMTV clone 2 (5.3 kb, Figure 4, ref. 5).

b) Eco RI digested DNA of λ·MMTV clone 17 (11.0 kb, Figure 1, lane 2) was mixed with the Eco RI digested DNA of the exogenous λ·MMTV described in a). After denaturation and renaturation of the DNA the DNA was digested with S1 nuclease in S1 nuclease digestion buffer containing: lanes 1, 0 % formamide; lanes 2, 30 % formamide; lanes 3, 40 % formamide and 0.3 M sodium chloride, 0.03 M sodium acetate pH 4.5, 3 mM $ZnSO_4$, 5 µg denatured calf thymus DNA and 2 units S1 nuclease. The digestions for lanes 4 contained 0.1 M sodium chloride and for lanes 5 0.02 M sodium chloride but no formamide. After S1 nuclease digestion the DNA was precipitated, electrophoresed on a 0.8 % agarose gel, transferred to a nitrocellulose filter and hybridized to $|^{32}P|$ MMTV cDNA. The MMTV specific sequences were visualized by autoradiography. Molecular weight markers are indicated in kb.

(lane 3) the reassociated homoduplexes of 6.7 and 5.3 kb were not affected. The band at 4.0 kb originating from the heteroduplex between the 5.3 and 6.7 kb Eco RI fragments is strongly reduced after S1 digestion at 30 % and 40 % formamide. Lanes 4 and 5 show the results obtained when S1 nuclease digestion of the homo- and heteroduplex molecules formed between the 6.7 and 5.3 kb fragments was carried out in 0.1 M NaCl and 0.02 M NaCl.

The reassociated homoduplexes of 5.3 and 6.7 kb were not affected by the salt concentration while the 4.0 kb band was only observed in the presence of 0.1 M NaCl. We interpret these results as follows. Under less stringent digestion conditions the single stranded sequences flanking the MMTV region of the heteroduplex are digested leaving the 4 kb MMTV portion intact. By increasing the stringency of digestion, minor mismatches in the MMTV portion between the exogenous and the endogenous viral DNA become sensitive to S1 digestion and a series of smaller fragments replaces the 4.0 kb band.

The same regimes of S1 digestions were applied to a pair of denatured and renatured MMTV Eco RI fragments derived from exogenous MMTV (clone 2, 5.3 kb, Fig. 4, ref. 5) and from the endogenous MMTV copy contained in the mtv-2 locus (clone 17, 11 kb, Fig. 1 lane 2). The 4.0 kb fragment formed after digestion of flanking host DNA was stable at 0, 30 and 40 % formamide (lanes 1, 2, 3, Fig. 3b) and also in 0.1 M and 0.02 M NaCl (lanes 4 and 5). This experiment provides evidence for the stability of the MMTV region to S1 nuclease under the various digestion conditions. The same result was obtained with heteroduplexes formed by pairs of exogenous proviral copies. The pairs of clones 4 and 6 and of clones 1 and 4 (see Fig. 4, ref. 5) yielded stable 4 kb heteroduplex bands when the S1 digestion was carried out in 40 % formamide (data not shown).

The difference in stability towards S1 digestion in the 4 kb fragment formed in Fig. 3a (between an endogenous copy probably not active in virus production in vivo and an exogenous MMTV) and the 4 kb fragment formed in Fig. 3b (between an exogenous MMTV and the endogenous copy of the mtv-2 locus) is therefore most likely based on slight sequence heterologies between the exogenous and endogenous MMTV which prevent perfect base pairing.

The appearance of the 4 kb MMTV fragment formed by heteroduplex formation and digestion of single stranded regions is dependent on the incubation time of heteroduplexes with S1 nudease. Fig. 4 A (lanes 6 to 10) shows the kinetics of formation of the 4 kb fragment formed by the heteroduplex of clone 17 (Fig.1, lane 2) and an exogenous proviral MMTV fragment homologous in

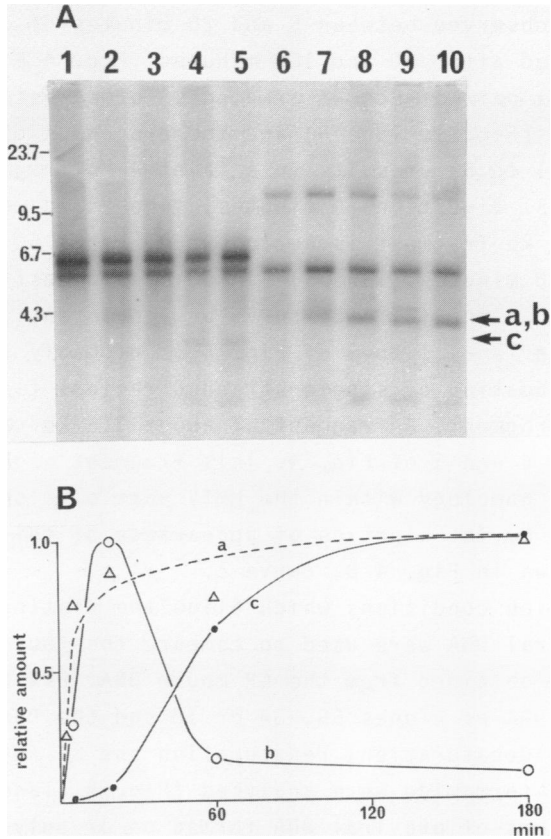

Figure 4. Kinetics of S1 nuclease digestion of cloned pairs of endogenous and exogenous MMTV Eco RI fragments after denaturation and renaturation.
A) Lanes 1 to 5: Eco RI digested DNA of clone 55 (Figure 1, lane 5) was mixed with DNA of exogenous clone 6 (Figure 4, clone 6, ref. 5). Lanes 6 to 10: Eco RI digested DNA of clone 17 (Fig. 1, lane 2) was mixed with DNA of exogenous clone 6. The DNA pairs were subjected to denaturation and renaturation and digested with S1 nuclease as described in Materials and Methods except for the digestion periods. DNA shown in lanes 1 and 6 was digested for 1 minute, lanes 2 and 7 for 5 minutes, lanes 3 and 8 for 20 minutes, lanes 4 and 9 for 60 minutes and lanes 5 and 10 for 180 minutes. The MMTV specific fragments were visualised as described in Fig. 3.
B) The lanes shown in A) were analysed by densitometry of the X-ray film. The relative hybridisation signals found in the 4 kb region of lanes 1 to 5 (o——o, curve b, also indicated by an arrow in A), the 4 kb region in lanes 6 to 10 (Δ---Δ, curve a) and the 3.6 kb region in lanes 1 to 5 (●——●), curve c) were measured and normalized versus the maximum hybridisation observed.

the common MMTV region. The maximum hybridisation signal in the 4 kb region is observed between 5 and 20 minutes of digestion and is maintained after 60 and 180 minutes (Fig. 4 B, curve a). The heteroduplex pair of Eco RI fragments formed between endogenous clone 55 (Fig. 1, lane 5) and the exogenous clone 6 (Fig. 4, lanes 1 to 5) results in a faint 4 kb fragment after short times of S1 digestion (5 minutes, lane 2 and 20 minutes, lane 3). This 4 kb fragment is not observed after 60 minutes (lane 4) and 180 minutes (lane 5) S1 nuclease digestion time. This is also shown in Fig. 4 B, curve b. The formation of the 4 kb fragment in lanes 1 to 3 of Fig. 4 is probably due to the more rapid degradation of single stranded regions flanking the MMTV specific fragment. A fragment of about 3.6 kb can be observed in lanes 4 and 5 of Fig. 4. This fragment might repreent a region of homology within the MMTV part of clone 55 and exogenous clone 6. The kinetics of appearance of this 3.6 kb fragment is shown in Fig. 4 B, curve c.

The digestion conditions which allow the distinction between MMTV proviral DNA were used to compare the four endogenous proviral clones obtained from the GR mouse DNA. DNA of clone 17 was mixed with DNA of clones 55, 34 or 18 and the DNA fragments resulting after denaturation, renaturation and S1 nuclease digestion in 40 % formamide were analysed (Fig. 5, lanes 3-5). The three combinations of proviral DNA formed no or only very little S1 resistant hybrid of 4 kb. DNA of clone 17 (lane 1) and of clone 55 (lane 2) was denatured, renatured and S1 nuclease digested. Only the reassociated homoduplexes of 11.0 and 6.7 kb appear as major bands. We conclude that each of the MMTV proviral regions of clones 18, 34 and 55 has slight sequence heterogeneities when compared to the DNA of clone 17.

The differences in the endogenous proviral copies among each other and the availability of a proviral sequence corresponding to the exogenous MMTV allowed us to correlate the exogenous sequence to a single endogenous sequence. Fig. 6 shows the MMTV specific fragments obtained after heteroduplex formation and S1 nuclease digestion, in the presence of 40 % formamide, of exogenous MMTV proviral DNA with the four cloned endogenous MMTV copies. Only clone 17 paired to three different exo-

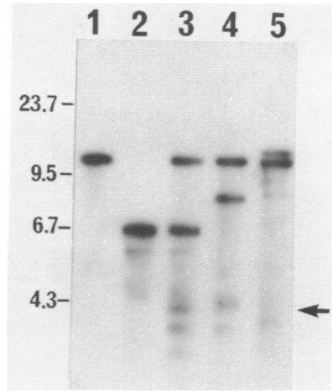

Figure 5. Digestion of cloned pairs of endogenous MMTV Eco RI fragments with S1 nuclease after denaturation and renaturation. Eco RI digested DNA of clone 17 (Figure 1, lane 2) was mixed with DNA of clone 55 (lane 3), clone 34 (lane 4), clone 18 (lane 5) and subjected to denaturation, renaturation and S1 nuclease digestion as described in Materials and Methods. The MMTV specific fragments were visualized as described in Figure 3. Lane 1 shows DNA of clone 17 and lane 2 DNA of clone 55 individually subjected to the same procedure. Molecular weight markers are indicated in kb.

genous MMTV copies (clones 2, 6 and 3 Fig. 4, ref. 5) could form a stable hybrid of 4 kb in the MMTV region (Fig. 5, lanes 4, 5 and 6). Clone 18 (Fig. 5, lane 3) does not form a stable 4 kb hybrid with the exogenous MMTV copy and clones 34 and 55 (Fig. 5, lanes 1 and 2) form only reduced amounts of 4 kb MMTV fragments in addition to a number of smaller bands. The residual 4 kb bands in Fig. 5, lanes 1 and 2 are possibly due to incomplete S1 digestion.

The same result was obtained with two other exogenous proviral copies (clones 3 and 6 in Fig. 4, in ref. 5). Both copies only formed a stable 4 kb heteroduplex with the endogenous clone 17 but not with the clones 18, 34 and 55 (data not shown).

Thus only the DNA of clone 17 is totally homologous to exogenous MMTV. This is consistent with previous results where it has been shown that the proviral copy contained in clone 17 is part of the mtv-2 locus, which is responsible for complete viral expression in the GR mouse (4).

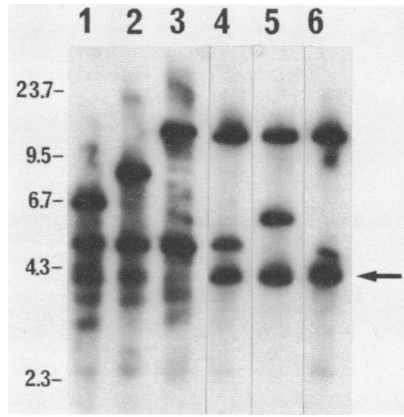

Figure 6. Digestion of cloned pairs of endogenous and exogenous MMTV Eco RI fragments with S1 nuclease after denaturation and renaturation. Eco RI digested DNA of (1) clone 55 (Figure 1, lane 5), (2) clone 34 (Figure 1, lane 4), (3) clone 18 (Figure 1, lane 2) and (4) clone 17 (Figure 1, lane 2) was mixed with DNA of the exogenous clone 2 (Figure 4, clone 2, ref. 5). The DNA was subjected to denaturation, renaturation and S1 nuclease digestion as described in Materials and Methods. The MMTV specific fragments were visualized as described in Figure 3. Lanes 5 and 6 show the fragments obtained by mixing the endogenous clone 17 (Figure 1, lane 2) with two different exogenous clones (clones 6 and 3 in Figure 4, ref. 5). Molecular weight markers are indicated in kb.

DISCUSSION

The diversity of MMTV with respect to immunological characteristics of viral proteins is well documented (14). The use of monoclonal antibodies specific for the major viral glycoprotein gp 52 has demonstrated type, group and class specificity of individual determinants (15). Based on the type and class specific antigenic determinants on the gp 52 and gp 36 of the MMTV produced by the C3Hf mice, it was possible to distinguish the C3Hf virus from the virus produced by C3H, GR and R III mice (16). These viral variants also differ in their oncogenic characteristics, i.e. their average latency period to induce tumors (17). The comparison of oligonucleotides generated by RNAse T1 from C3H and GR MMTV RNA has also indicated differences (18). A direct visualisation of differences in the proviral DNA has become possible through the Southern filter transfer technique. Digestion

of genomic DNA from various mouse strains with restriction enzymes and identification of internal MMTV fragments has revealed different units of endogenous proviral DNA (9, 10).

We have used molecular cloning of proviral DNA and a comparison of heteroduplex pairs to establish the non-identity of at least four of the five endogenous MMTV proviral genes of the GR mouse. S1 nuclease treatment of heteroduplex MMTV DNA containing molecules under stringent conditions cleaved the MMTV DNA when pairs of endogenous proviral copies were tested. S1 nuclease did not cleave when pairs of exogenous MMTV copies were annealed. We do not know the extent of diversity in the nucleotide sequence of endogenous copies which allows S1 nuclease cleavage in the presence of 40 % formamide. The stability of the same heteroduplex pairs of endogenous MMTV following S1 nuclease digestion in the absence of formamide suggests that the heterology is not very extensive. This method can be used to compare other closely related gene families for minor heterologies in nucleotide sequence.

Only one proviral copy characterised by Eco RI fragments of 11.0 and 7.0 kb has been found associated with the mtv-2 locus, the genetic locus which governs expression of MMTV virus in the GR mouse (4). This finding suggested that the provirus located in the mtv-2 locus was transcriptionally active and responsible for the virus production. However, the exact size of the genetic locus and nature of the DNA surrounding the MMTV provirus is not known. A more complex mechanism of transcriptional regulation involving control genes associated with the mtv-2 locus and affecting other proviruses remained a possibility. The comparison of heteroduplex stability during S1 nuclease digestion between pairs of endogenous and exogenous MMTV allows us to biochemically demonstrate the relatedness of the mtv-2 associated proviral copy with the exogenous MMTV. We conclude that the mtv-2 associated MMTV gene is transcribed in the GR mouse. Analysis of GR mouse DNA, DNA from a mammary tumor cell and MMTV infected rat cell line by Southern blotting has led Fanning et al. (19, 20) to a similar conclusion.

The immunological differences in the structural proteins of the C3Hf, C3H, GR and R III virus (16) might be related to their

different oncogenic potential. Analogously, the differences in primary DNA sequence observed among the endogenous copies of the GR mouse might be related to their different biological properties in vivo. Molecular cloning in conjunction with gene transfer into cultured cells will show if any of the endogenous proviruses in addition to the one contained in the mtv-2 locus can code for an oncogenic MMTV (21, 22).

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