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Genetics

Mapping the structural genes coding for the major urinary proteins in the mouse: Combined use of recombinant inbred strains and somatic cell hybrids

(DNA polymorphism/multigene family/hormonally responsive genes/Southern blot analysis)

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ABSTRACT **We have mapped the multiple (15–25) genes coding for the hormonally regulated major urinary proteins (MUPs) of the mouse by using a cloned cDNA probe. By Southern blot analysis of DNA from Chinese hamster–mouse somatic cell hybrids, all of the MUP genes were found to be on chromosome 4. Different inbred mouse strains showed DNA polymorphism in their MUP Southern hybridization pattern. Analysis of recombinant inbred strains derived from these parent strains has shown that all the polymorphisms are linked to the *MUP-a* locus on chromosome 4. The combination of these mapping techniques should be applicable to many cloned DNA sequences.**

The major urinary proteins (MUPs) are a family of small (≈20,000 daltons), closely related proteins that are produced in large amounts in the livers of mice and subsequently excreted in the urine. The MUPs are hormonally regulated. Under normal conditions, much greater amounts are found in the urine of male mice than females. This difference can be abolished by the administration of testosterone to the female mice. MUP mRNA levels are lower in untreated females, and they increase to male levels with androgen induction (1). Recently, other hormones have been implicated in MUP regulation (J. Knopf and W. A. Held, personal communication). Liquid hybridization studies with a purified cDNA probe have shown that there are 15–25 MUP genes in the mouse genome (1).

It is of interest to determine whether these genes are in tandem arrays such as the histone genes in sea urchin (2), clustered in an interspersed pattern on one or several chromosomes as the globin genes in man (3), or solitary genes at multiple loci in the manner of the actin genes in *Drosophila* (4). This information is pertinent because the MUP genes are currently being studied in terms of differential hormonal control and their developmental regulation. In each case, it is important to know how many and which genes are being expressed. The means by which the MUP genes relate to one another would be expected to depend on their chromosomal location and molecular organization. Also, knowledge of the organization of the MUP genes may shed light on the evolution of this multigene family.

This communication is not the first to report mapping studies of MUPs. Before the multigene nature of the MUP family was known, the two variant phenotypes, Mup-a^1 and Mup-a^2 (based on proteins produced by induced females), were mapped by Hudson *et al.* (5) to chromosome 4, five map units from the *brown* (*b*) locus. Because it was found that homozygous inbred strains of mice carrying either *Mup-*a^1 or *-*a^2 alleles produced all known MUP proteins when hormonally induced, albeit in varying proportions, Szoka and Paigen (6) proposed that *Mup-a* was a regulatory locus controlling the reponse to hormonal induction. Until now it remained unclear where the structural genes mapped. Because in this laboratory several cDNAs coding for MUP sequences have been cloned and identified by various criteria (7), we were able to map the structural genes at the DNA level. The mapping of the MUP family was done by using Southern blot hybridization. We took advantage of the unique combination of two mapping techniques available in the mouse—somatic cell hybrids and recombinant inbred (RI) mouse strains.

This communication reports that (*i*) the Southern blot pattern for the MUP genes is indicative of a multigene family; (*ii*) all MUP genes map to chromosome 4, as shown by analysis of Chinese hamster–mouse cell hybrid lines which selectively lose mouse chromosomes; (*iii*) inbred strains of mice show polymorphism for MUP at the DNA level which parallels completely the previously seen protein differences between strains; and (*iv*) availability of RI strains has allowed the mapping of all MUP DNA polymorphisms to the region of the *Mup-a* locus.

MATERIALS AND METHODS

DNA Preparation. Mouse liver DNA and Chinese hamster–mouse hybrid cell DNA were extracted essentially as described (8) except that, for the cell lines, DNA was extracted from whole cells rather than from nuclei.

Enzyme Restriction of DNA and Agarose Gel Electrophoresis. *Eco*RI and *Hin*dIII restriction endonucleases were purchased from New England BioLabs and Calbiochem. Reactions were carried out under conditions recommended by the manufacturers. Digested DNA samples were subjected to electrophoresis in 0.8% agarose gels less than 3 mm thick.

Southern Blot Analysis. DNA was denatured and transferred to nitrocellulose according to Southern (9). The baked filters were hybridized with a cloned MUP probe that had been nick-translated (10). The hybridization solution was 4× standard saline citrate (NaCl/Cit; 1× is 0.15 M NaCl/0.015 M sodium citrate)/5× Denhardt's solution (11)/0.1% $NaDodSO_4$/0.1% sodium pyrophosphate and containing 150 μg of denatured salmon sperm DNA per ml; 50 ml of hybridization solution was used for a 14 × 21 cm filter. Filters were hybridized overnight at 65°C after prehybridization for 3–4 hr in the same fluid. The filters were washed with 1 M NaCl/1× Denhardt's/0.1% $NaDodSO_4$/0.1% sodium pyrophosphate/45 mM Tris·HCl, pH 7.9, at 65°C for several changes and then with 2× NaCl/Cit/0.1% $NaDodSO_4$ at 65°C. Autoradiographs were developed

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Abbreviations: MUP, major urinary protein; RI, recombinant inbred; KAP, kidney androgen protein; NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate); kb, kilobase(s).

after exposure of Kodak XAR-5 film with an intensifying screen at −70°C.

Somatic Cell Hybrids. Cell hybrids between Chinese hamster and mouse spleen cells from BALB/c mice were generated and maintained as described (12). Under the conditions used, hybrid clones segregated different numbers and combinations of mouse chromosomes but retained all the hamster chromosomes. The mouse chromosome content of the hybrid clones was determined enzymatically and cytogenetically. Each hybrid clone was analyzed for the expression of 25 enzymes representing linkage group assigned to 16 of the 19 autosomes and the X chromosome as described (13, 14).

Trypsin/Giemsa banding was used to identify each of the mouse chromosomes as described (15). Individual clones were considered positive for a given chromosome if >15% of the metaphases examined contained the chromosome; the clone was scored negative for that chromosome if <5% of the metaphases contained the chromosome. If chromosomes were retained with a frequency between 5% and 15%, the data were not included in the segregation analysis of that chromosome. All analyses were carried out on parallel cultures of each hybrid clone so that enzyme, chromosome, and MUP gene data were correlated.

Inbred Strains and RI Strains. All mice were purchased from The Jackson Laboratory.

RESULTS

Complex Pattern of MUP Southern Blot. Southern blot analysis of mouse liver DNA hybridized with a cloned MUP probe revealed a complex pattern of more than 15 bands of varying intensities; this was the case whether *Eco*RI or *Hin*dIII was used. The number of bands resolved varied with the restriction enzyme used, the strain tested, and the duration of the electrophoresis (see Fig. 1, lanes 1 and 2, or Figs. 2 and 3, any lane). The overall pattern was consistent with these being a repetitive gene family (see *Discussion*).

In order to determine that the sensitivity of our blots was sufficient to detect a single copy gene sequence, we hybridized a separate filter with a cDNA probe from the kidney androgen protein (KAP) gene, shown by hybridization kinetics to be present in about a single copy in the mouse genome (16). Under conditions similar to those used for MUP, a single KAP band was evident with an intensity equal to that of one of the middle intensity bands of the MUP pattern (results not shown). Also, control experiments showed that all restriction endonuclease digestions were carried to completion. Because several MUP probes were available in our laboratory, we wanted to see if each of the probes hybridized to all the MUP bands and if the relative intensities remained constant. Four different cloned MUP cDNAs yielded the same banding pattern (results not shown). For the studies described here, the probes represent 50–75% of the length of the mRNAs.

Mapping MUP Genes at the Chromosomal Level with Somatic Cell Hybrids. Chinese hamster–mouse somatic cell hybrids that selectively lose mouse chromosomes provide a means of mapping mouse genes to a particular mouse chromosome (13, 14). Swan *et al.* (17) first applied the Southern technique with hamster–mouse hybrids to show that the variable and constant regions of mouse κ light chain genes both map to chromosome 6. The human insulin, prolactin, and growth hormone genes have been mapped in a similar manner (18, 19).

In order to utilize this strategy to map the MUP genes, it was necessary first to demonstrate that hybridization of the MUP cDNA probe to mouse DNA is readily distinguished from the hybridization pattern of MUP cDNA to homologous sequences in Chinese hamster DNA. This was the case (Fig. 1, lanes 1 and 2 vs. lane 11). The hamster DNA hybridizes weakly to the MUP probe, but the five or six bands observed were easily differentiated from the mouse bands by their lower intensity and, in several cases, distinct sizes. One of the smallest hamster bands was quite variable and was observed in several of the hybrid lines (Fig. 1, lanes 6, 8, and 10) but not in the hamster parent. This band has been found in the hamster parent in other gels yet always was absent in the mouse (lane 1).

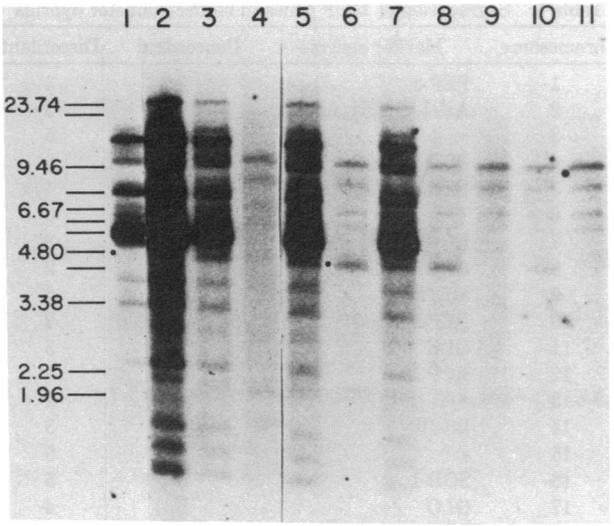

FIG. 1. Southern blot analysis of Chinese hamster–mouse hybrid line DNAs and parental mouse and Chinese hamster DNAs. Genomic liver DNA was digested with *Hin*dIII; then, 15 μg of DNA was loaded per lane and run at 30 mA and 45 V for 16 hr in a 0.8% agarose gel. After transfer to nitrocellulose, a nick-translated MUP cDNA probe (5×10^7 cpm; $\approx 1 \times 10^8$ cpm/μg), was added, along with nick-translated λ plasmid DNA (5×10^6 cpm). Hybridization was at 65° for 16 hr. The film was exposed for 5 days, except that lane 1 is a 40-hr exposure of lane 2. A mixture of *Eco*RI- and *Hin*dIII-digested λ DNA was run as a marker. Marker sizes are indicated in base pairs $\times 10^{-3}$. Lanes: 1, BALB/c mouse DNA, 40-hr exposure; 2, BALB/c mouse DNA; 3, hybrid 1; 4, hybrid 2; 5, hybrid 3; 6, hybrid 4; 7, hybrid 5; 8, hybrid 6; 9, hybrid 7; 10, hybrid 8; 11, E-36 (Chinese hamster). Hybrid clones 1, 3, and 5 retained chromosome 4. Hybrids 2, 4, 6, 7, 8, 9, and 10 had lost chromosome 4. The dots mark the positions of the hamster band discussed in the text.

The data for all 10 clones investigated are summarized in Table 1. The entire mouse MUP pattern either was present (Fig. 1, lanes 3, 5, and 7) or absent (Fig. 1, lanes 4, 6, 8, 9, and 10) for each clone tested, demonstrating that the MUP genes segregate together. The segregation of the MUP genes in the hybrid clones was compared with the segregation of the mouse chromosomes as determined enzymatically and cytogenetically on parallel cultures of each hybrid clone (Table 1). The MUP genes segregated concordantly with mouse chromosome 4. There were no exceptions, and all other chromosomes segregated discordantly with the MUP genes. The three hybrid clones that retained chromosome 4 had the MUP genes; the seven clones that lost chromosome 4 had only the hamster bands. These data assign the MUP genes to chromosome 4.

DNA Polymorphism in Inbred Strains. We were interested in determining whether polymorphisms exist in the MUP genes at the DNA level between different inbred mouse strains. Such DNA differences might relate to the known strain variability in MUP phenotypic expression (5, 6) and could be valuable for more definitive mapping of the MUP genes. The Southern blot analysis of DNA from six inbred mice strains is shown in Fig. 2A. Although the majority of the bands were common between lines and the patterns of intensities were strikingly similar,

Table 1. Segregation of MUP genes in mouse–hamster hybrids

Chromosome	Marker enzyme	Concordant	Discordant
1	PEP-3	7	3
2	AK-1/SODH/ACP-2	7	3
3	*	5	5
4	PGD/PGM-2	10	0
5	PEP-7/PGM-1	6	4
6	TPI	7	3
7	LDH-1/GPI/PEP-4	7	3
8	GR/APRT	8	2
9	ME/MPI	6	4
10	PEP-2/HK-1	8	2
11	GLK	7	3
12	ACP-1	7	3
13	*	8	2
14	ES-10	7	3
15	*	5	5
16	SOD-1	5	5
17	GLO	6	4
18	PEP-1	6	4
19	GOT	7	3
X	HPRT	3	7

The symbols for the marker enzymes, their chromosome assignments and the electrophoretic procedures used to separate the Chinese hamster and mouse enzymes have been described (15, 16). The presence or absence of mouse enzyme markers agreed with the presence or absence, respectively, of the particular mouse chromosome. Enzyme, chromosome, and Southern blot analyses were performed on triplicate cultures of the same passage for each hybrid clone.

* Chromosomes 3, 13, 15, and Y have no enzyme markers; their concordance/discordance was based on karyotypic analysis alone.

there were two distinct patterns. Variability was seen for five or six of the lower-intensity bands (Fig. 2*A*); these varied from ≈20 kilobases (kb) to slightly <2 kb. Of the limited number of inbred lines examined, each strain fell into one of two patterns. There were no new band patterns. No lines showed a recombinant genotype. Those mice that were classed as Mup-a^1 phenotype (BALB/cBy, C3H/HeJ, AKR/J, and DBA/2J) had one restriction pattern; those that were MUP a^2 (C57BL/6By, C57BL/6J, and C57L/J) had the other without exception.

Use of RI Strains for More Definitive Mapping. The inbred strains shown in Fig. 2*A* were chosen because they all were parent strains (progenitors) for RI strains. RI strains, useful for gene mapping, are available in the mouse and, to a lesser extent, the rat (20). The rationale and genetic basis for the construction of RI strains have been described (21–23). The final outcome is a group of separate strains that are homozygous at all loci but may differ from each other at any particular locus. Any two markers are more likely to be of the same parental type in one strain if they are closely linked. The advantage of RI strains is that they remain essentially "frozen" and the strain distribution pattern of any markers of interest can be compared for linkage to all previously mapped loci. Any characteristic (or any DNA sequence) can be mapped provided it is found to vary between the progenitors and to be linked to known markers. The Southern blot patterns of the multigene MUP family meet these criteria.

We used two different sets of RI strains, C×B from a cross of BALB/*c*By and C57*B*L/6 parents and the AK×L strains produced from the mating of an *AK*R mouse to a C57*L*/J one. The Southern blot patterns for the seven C×B strains plus the parents are shown in Fig. 2*B*. (*Hin*dIII digests); those for nine of the 18 AK×L strains (*Eco*RI digests) are shown in Fig. 3. Each RI strain gave a distinct band pattern identical to that found in one or the other parent; none had a pattern different from a parental one. In all, 25 strains were tested. None of the polymorphic bands segregated individually. The strain distribution pattern among RI strains for the Mup-a phenotype was published for the C×B strains (24); that for the AK×L strains was obtained from B. Taylor (personal communication). They are listed in Table 2 along with our results from Southern blots. The patterns were identical for all 25 strains. Thus, the variability in the MUP structural genes that we detected with our cloned DNA probe maps to the *Mup-a* locus, on chromosome 4. We have calculated the possibility of a false positive linkage to *Mup-a*; with 25 strains, that possibility is infinitely small (22).

One can estimate the degree of linkage of the MUP polymorphisms to the *Mup-a* locus by using (22) $P = (1 - [4r/(1 + 6r)])^n$ in which $1 - [4r/(1 + 6r)]$ is the probability of not getting recombination in one attempt, n is the number of attempts (25 here), r is the recombination frequency, and P is the confidence limit of the linkage. One map unit equals 1% recombination. In a case such as ours, in which no recombinants are observed, linkage of the DNA polymorphisms to the *Mup-a* lo-

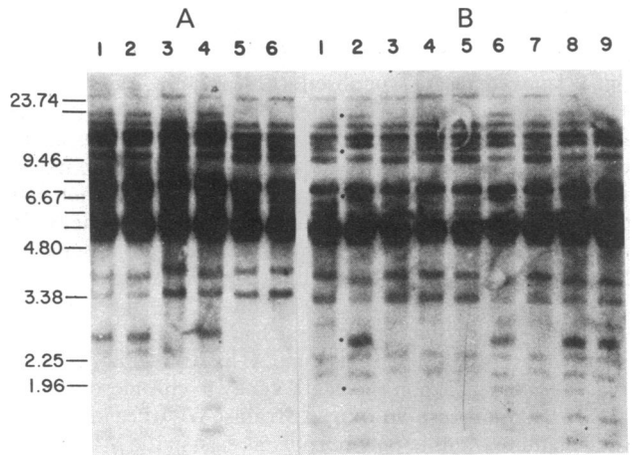

FIG. 2. Southern blot analysis of inbred mice strains and recombinant inbred strains (*Hin*dIII digests) probed with cloned MUP DNA. Conditions were as in Fig. 1, except gels were run for 20 hr. (*A*) Lanes: 1, C57BL/6J; 2, C57L/J; 3, BALB/cBy; 4, C57BL/6By; 5, AKR/J; 6, DBA/2J. (*B*) 1, BALB/cBy; 2, C×BD; 3, C×BE; 4, C×BG; 5, C×BH; 6, C×BI; 7, C×BJ; 8, C×BK; 9, C57BL/6By. Dots indicate bands that differ between a B and a C parental pattern. Markers as in Fig. 1.

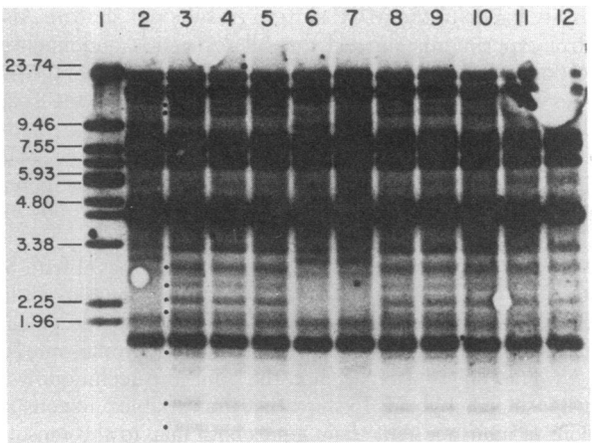

FIG. 3. Analysis of AK×L RI strains and parental DNA after *Eco*RI digestion. Conditions were as for Fig. 1, except that exposure was for 3 days. Lanes: 1, 2 ng of λ DNA digested with *Eco*RI and *Hin*dIII; 2, AKR/J; 3, AK×L 17; 4, AK×L 19; 5, AK×L 21α; 6, AK×L 24; 7, AK×L 25; 8, AK×L 28; 9, AK×L 29; 10, AK×L 37; 11, AK×L 38; 12, C57L/J. Dots indicate bands that differ between an A and an L parental pattern. Sizes are as in Fig. 1.

Table 2. Inheritance of chromosome 4 marker Mup-a and the MUP Southern blot strain distribution pattern

Progenitor or RI strain	MUP Southern blot pattern	Mup-a phenotype*
C57BL/6By, C×BD, C×BI, C×BK	B	B
BALB/cBy, C×BE, C×BG, C×BH C×BJ	C	C
AKR/J, AK×L 6, AK×L 8, AK×L 9 AK×L 12, AK×L 16, AK×L 24, AK×L 25	A	A
C57L/J, AK×L 5, AK×L 7, AK×L 13, AK×L 14, AK×L 17, AK×L 19 AK×L 21, AK×L 28, AK×L 29 AK×L 37, AK×L 38	L	L

* The strain distribution pattern of the C×B lines for the Mup-a phenotypes is published (24). The strain distribution pattern for AK×L for Mup-a is unpublished and was provided by B. Taylor (personal communication). B, C, A, and L are used as generic symbols for parental types inherited from C57BL/6By, BALB/cBy, AKR/J, and C57L/J, respectively. The seven C × B strains were analyzed with both *Eco*RI and *Hin*dIII. Both enzymes gave the same strain distribution pattern. The AK×Ls were done with *Eco*RI.

cus is within 0.7 map unit at the 50% confidence level. At the 95% confidence level, the tightness of linkage is 3 map units.

The closest markers to *Mup-a* that have been characterized for the C×B and AK×L strains are: *b*, 6 map units to one side of *Mup-a*; and *Lyb-2*, an alloantigen mapped with RI strains (25), 6 map units in the other direction. Both of these markers have strain distribution patterns for the 25 strains that are different from the MUP pattern reported here (ref. 24; B. Taylor, personal communication). Thus, this 12-map-unit distance represents the outermost limits of the variability in the MUP complex.

DISCUSSION

We have used somatic cell hybrids and RI mouse strains in conjunction with Southern blot hybridization to map the multiple structural genes coding for the MUPs in the mouse. Analysis of mouse–hamster hybrid clones has established that all of the MUP genes map to chromosome 4. Polymorphisms in the Southern blot patterns between inbred strains allowed finer mapping with RI strains. The polymorphic sequences all map to the region of the *Mup-a* locus.

It is not yet possible to state the exact arrangement of the MUP genes on the chromosome—to distinguish between a tandem or an interspersed arrangement. The evidence presented here rules out solitary locations on many chromosomes. The great complexity of the Southern blot pattern with multiple bands of varying intensities would argue against a simple tandem repeat of a single type of MUP gene. The results from the RI mapping presented here suggest a tight linkage at one location for at least some of the MUP genes. However, it is possible that all of the polymorphism detected by our Southern blots is hybridization to one divergent gene with the other MUP genes widely scattered on chromosome 4. *A priori*, one could argue that if polymorphisms are generated randomly, the chances of all changes occurring in one of the 15–25 genes are statistically very small unless, for example, a MUP pseudogene exists and has been under no selective pressure for a long evolutionary time.

Recent information received from A. J. Clark, P. M. Clissold, and J. O. Bishop (personal communication) aids us in the interpretation of this matter and in considering the complexity of the blot pattern. With genomic MUP clones identified in Bishop's laboratory, they found that (*i*) the average MUP gene (including introns) is small, 3–4 kb, and (*ii*) a single 3.8-kb *Eco*RI fragment containing much of the gene was present in several different genomic clones. We therefore believe it highly unlikely that the nine different polymorphic *Eco*RI fragments (Fig. 3), totaling ≈30 kb, are from one MUP gene. More likely they represent the variable sections of several MUP genes that all map in a cluster.

The 3.8-kb fragment corresponds exactly to the darkest band seen in the Southern patterns (Fig. 3). This band is much stronger than the single-copy KAP band we tested. It is likely that this band represents a conserved DNA sequence found in many of the MUP genes; the lightest bands, variable or not, could be single-copy sequences that represent whole genes or sections of genes that have diverged or sequences containing mostly flanking regions or introns. The fact that we see about as many polymorphic bands with the *Hin*dIII digest as with *Eco*RI could imply that there are twice as many polymorphic DNA stretches as any one digest shows. Alternatively, a single insertion or deletion could generate new *Eco*RI and *Hin*dIII fragments simultaneously.

It is likely that the MUP cDNA clones are detecting all the members of the MUP gene family in these experiments for the following reasons. First, the original determination that there are 15–25 MUP genes (1) was carried out by using a noncloned cDNA probe that hybridizes efficiently with all our cDNA clones (unpublished data). Also, in the experiments described here, conditions of hybridization were less stringent (temperature lower by 5°C and twice the salt concentration) than those under which gene number was determined. Thus, divergent MUP genes should be detected as were the homologous sequences in hamster and rat (Fig. 1) (1). Second, although it is known that the MUP gene is contained in a 3- to 4-kb DNA segment in several genomic clones, the cDNA MUP probe hybridizes to >100 kb of total genomic DNA. Finally, the products of *in vitro* translation of mRNA selected by each individual probe include all the known MUP proteins (unpublished data).

Because the amounts and relative proportions of MUPs excreted differ widely between strains and the strains appear to produce all the known MUPs after hormonal induction, we postulated (1) that the differences in MUP phenotypes might reflect differences in dosage of genes coding for different MUPs. One would predict from such a model that the bands on Southern blots would differ in total or relative intensity between strains. Within the limits of our resolution, this does not seem to be the case. No one strain has lighter bands than the others, nor does the pattern of intensity vary between strains. However, the polymorphic bands do differ between strains in a way that parallels the MUP phenotypic differences seen in the urine and reflected in the message populations (26). It is tempting to say that the DNA differences are responsible for the phenotypes seen. However, the relationship could be casual rather than causal, reflecting a serendipitous fixing of DNA polymorphisms which in no way affects the regulatory or structural differences responsible for the MUP-a^1 or -a^2 response.

The work reported here demonstrates the unique advantages of the mouse for genomic mapping of cloned sequences due to the availability of somatic cell hybrids and RI strains. Any DNA sequence can be mapped with interspecific hybrids provided that one can distinguish the hybridization patterns of the two species. Any polymorphic cloned sequence can be tested with RI strains and, with the increasing number of markers available, chances of successful mapping to a specific locus are good.

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