

Ancestral Polymorphism of *Mhc* Class II Genes in Mice: Implications for Balancing Selection and the Mammalian Molecular Clock

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ABSTRACT

To investigate the evolutionary dynamics at *Mhc* class II DR genes of mice (genus *Mus*), we sequenced the peptide binding regions (PBRs) of 41 DRB (=E β) genes and eight DRA (=E α) genes from 15 strains representing eight species. As expected trees of these PBR sequences imply extensive maintenance of ancestral DRB alleles across species. We use a coalescent simulation model to show that the number of interspecific coalescent events (c) observed on these trees was higher than the number expected for neutral genealogies and similar sample sizes and is more consistent with balancing selection than with neutrality. Patterns of ancestral polymorphism in mouse DRB alleles were also used to examine the tempo of synonymous substitution in the PBR of mouse class II genes. Both absolute and relative rate tests on DRA and DRB genes imply increased substitution rates at two- and fourfold degenerate sites of mice and rats relative to primates, and decreased rates for the DRB genes of primates relative to ungulate and carnivore relatives. Thus rates of synonymous substitution at *Mhc* DR genes in mammals appear to be subject to generation time effects in ways similar to those found at other mammalian genes.

IT has been known for a number of years that the α and β chains of several mammalian class II MHC genes show strikingly different patterns of molecular evolution. Molecular evolution in the β chains of the class II DR genes of humans and mice (=E locus) exhibit several important hallmarks expected for loci under balancing selection, in particular high ratios of non-synonymous to synonymous substitutions in the peptide-binding region (PBR) (HUGHES and NEI 1989; KLEIN *et al.* 1993; POTTS and WAKELAND 1993). In addition, both primate and rodent alleles are thought to be maintained for extremely long time periods by balancing selection, although there is controversy over exactly how long alleles persist at mammalian DRB loci (LUNDBERG and MCDEVITT 1992; HUGHES *et al.* 1994). Balancing selection is thought to maintain alleles and allele motifs across multiple speciation events (KLEIN 1987). Thus alleles of a given species are often more closely related to alleles of long-diverged species than they are to other alleles from the same species ("trans-species" evolution; GYLLENSTEN *et al.* 1990; KLEIN *et al.* 1993). By contrast, the α chains of DR and its orthologues are known to be monomorphic in populations, or nearly so, resulting in an absence of ancestrally maintained polymorphism and an excess of synonymous *vs.* nonsynonymous substitutions in the PBR (FIGUEROA *et al.* 1990; HUGHES and NEI 1990; HUGHES *et al.* 1994).

Many of these conclusions and the most precise mea-

surements of evolutionary dynamics for the DR locus rely on the most extensive database, that from primates (*e.g.*, KLEIN *et al.* 1993; SATTA *et al.* 1993, 1996; FIGUEROA *et al.* 1994; AYALA and ESCALANTE 1996). Furthermore, the wealth of fossil (MARTIN 1993) and molecular data on divergence times in primates makes calibration of absolute rates of evolution at *Mhc* genes relatively straightforward, despite the need for several methodological considerations particular to *Mhc* genes. For example, SATTA *et al.* (1993) concluded that the rate of synonymous substitution in primate DRB genes is surprisingly low despite the large sequence divergence found between alleles within and between primate species. From this and other data KLEIN (1987) and TAKAHATA *et al.* (1992) suggested that long persistence times, rather than intrinsically high mutation rates in the *Mhc* region, are likely responsible for the high diversity seen at DRB genes. However, other data from human populations imply a primary role for mutation in producing some of the current diversity (ERLICH and GYLLENSTEN 1991; ZANGENBERG *et al.* 1995).

To test the generality of some of these hypotheses in rodents and other mammals, we determined nucleotide sequences of the second exon of 41 DRB (=E β) and eight DRA (=E α) genes from a suite of rodent species in the genus *Mus*. Like primates, rodents, in particular mice, also have a well-studied temporal framework in which to calibrate substitution rates at *Mhc* genes (reviewed in SHE *et al.* 1990b; SILVER 1995). In addition to providing a substantial data base with which to study evolutionary trends in the second exon of the murine

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DR molecule, our comparative results from these data and from longer sequences sampled from multiple mammalian lineages suggest that the rates of synonymous substitution in mammals are variable.

MATERIALS AND METHODS

Mice: The wild-derived mouse strains from which we obtained E β and E α sequences have been described (WAKELAND *et al.* 1987; SHE *et al.* 1991). For the E β locus we obtained 41 sequences from DNA extracted from tail tips as follows: *M. domesticus* ($n = 19$ sequences), *M. musculus* ($n = 7$), *M. castaneus* ($n = 2$), *M. spicilegus* ($n = 4$), *M. spretus* ($n = 3$), *M. spretoides* ($n = 1$), *M. molossinus* ($n = 1$), *M. cervicolor* ($n = 1$), *M. cooki* ($n = 1$), *M. caroli* ($n = 1$), *M. platythrix* ($n = 1$). For the E α locus, we determined eight sequences total, two from *M. musculus* and one each from *spretus*, *spicilegus*, *cooki*, *cervicolor*, *spretoides* and *caroli*.

PCR procedures: For E β , two primers (forward primer sequence: 5' \rightarrow 3'; CATCTTCTTCCATCTCCCTCC; reverse primer GAAGGGCAAGCAAGACCT) were designed on the basis of sequences flanking the second exon of a *M. musculus* allele (BEGOVICH *et al.* 1990). The two primers used to amplify exon 2 of E α genes were designed from flanking intron sequences in MCNICHOLAS *et al.* (1982): CATCTTCTTCATCTCCCTCC (forward) and GAAGGGCAAGCAAGACCTC (reverse). PCR products obtained after 30–35 cycles of amplification were cloned into TA cloning vectors (Invitrogen) and sequenced on an Applied Biosystems 373A automated sequencer using ABI Dye-deoxy sequencing reagents and protocols provided by the manufacturer. In general two to three clones per amplification were determined. After multiple amplification and sequence determinations, sequence, number 4 from *M. spicilegus*, appeared to contain a stop codon in the second codon of the second exon, indicating either a recurrent *Taq* polymerase error or a nonfunctional DRB copy (*cf.* FIGUEROA *et al.* 1989), but was retained in the analyses because it did not display any other aberrant trends compared to other sequences. Sequences have been deposited in the Genbank and EMBL databases under accession numbers U89284–U89291 (DRA) and U88910–U88949 (DRB).

Simulation model and quantification of ancestral polymorphism: Phylogenetic trees suggesting maintenance of ancestral polymorphism by balancing selection and extended life-spans of allelic lineages are common for DRB exon 2 sequences, but there is as yet no method for quantifying the degree to which ancestral polymorphisms are being maintained or the degree to which the level of maintenance by selection exceeds that expected for neutral alleles. *Mhc* alleles are thought to persist in populations over long time periods under the combined forces of balancing selection and genetic drift (TAKAHATA 1990b; TAKAHATA and NEI 1990). If genetic drift eliminated ancestral alleles in different species, then we would expect the allelic lineages to show some degree of concordance with species boundaries and a higher incidence of monophyly of conspecific sequences over time (Figure 1). Even neutral alleles will show discordance with the species tree if divergence times have been short. However, if balancing selection was very strong relative to genetic drift, and there has been no significant loss of allelic lineages during the history of divergences between the species examined, we would expect alleles from within and between species to coalesce at random times in the distant past, before the divergence of species examined. In this case, the tree should show a high level of discordance with species boundaries.

One way to measure the level of discordance of the gene tree with species boundaries in such trees is to count the number of interspecific coalescent events and to compare this

number to that expected for trees under simple models of balancing selection. Phylogenetic trees of our sequences were estimated using the neighbor-joining and parsimony methods (SAITOU and NEI 1987; SWOFFORD 1991) in the computer programs MEGA (KUMAR *et al.* 1993) and PAUP (SWOFFORD 1991), respectively. Interspecific coalescent events are defined as instances in which alleles or lineages found in one species trace back to common ancestors of alleles found in a different species. We counted such events by assigning species names to tips of the tree and by using MacClade (MADDISON and MADDISON 1992) to determine the minimum number of times this "character" changed by parsimony on our trees. The number of interspecific coalescent events (c) observed on these trees was then compared to that expected for samples of allelic genealogies subject to balancing selection. This expected number is difficult to determine analytically (TAKAHATA *et al.* 1992), so we used simulations. We simulated two diploid, randomly mating ancestral populations ($N_e = 10^4$) having descended from a common ancestral population t/N_e generations ago, where t is the time in generations since divergence and N_e is the effective populations size in both the ancestral and descendant populations. We used a value of $N_e = 10^4$ so that we could compare our results for the behavior of c directly with those of SLATKIN and MADDISON (1989), who also used a value of $N_e = 10^4$. Although this value is likely smaller than the actual N_e for mouse species (see below), it is the ratio of t to N_e that is important. For various numbers of sampled alleles, we generated 10,000 coalescent trees for the neutral case, for two selection intensities, $s = 0.001$ and $s = 0.01$, and for a range of divergence times, from $t/N_e = 0.2$ to 8. Allelic genealogies were generated using the coalescent model outlined in TAKAHATA (1990a) and TAKAHATA and NEI (1990). For each set of trees, the mean number of interspecific coalescent events (c) was counted using the parsimony method (FITCH 1971; SLATKIN and MADDISON 1989; MADDISON and SLATKIN 1991) as implemented on MacClade (MADDISON and MADDISON 1991). The value of c for these trees thus served as an expected number under various sampling schemes and parameter values, and the value of c for the neutral cases served as a null model against which c values from pairs of species in our data can be tested. We did not record the accumulation of mutations on these trees because our value of c is concerned only with tree topology and not branch lengths. Indeed the test proposed here is similar to and inspired by the cladistic tests for gene flow and panmixis proposed by MADDISON and SLATKIN (1991). These tests also compare the observed number of interspecific coalescent events for neutral genes with the number found for various simulated scenarios or random trees.

Tests that use the allelic genealogy to test hypotheses on mechanisms such as gene flow or neutrality assume that the tree used to calculate the number of interspecific coalescent events is the true genealogy (see DISCUSSION). Since this assumption may be invalid in many cases, we have incorporated ambiguity in the reconstructed tree by generating 100 bootstrap sequence data sets (FELSENSTEIN 1985) and counting the number of interspecific coalescent events on trees generated from these. In this way uncertainty in the tree can simultaneously be incorporated into the analysis of interspecific coalescent events, as suggested for analysis of other characters by FELSENSTEIN (1988).

Rate tests and molecular clock analyses: Because *Mhc* alleles show persistence times much longer than the species that carry them (KLEIN *et al.* 1993), novel methods of analysis are required to measure absolute rates of point substitution (SATTA *et al.* 1993; UYENOYAMA 1995). SATTA *et al.* (1993) devised a method known as the "minimum-minimum" method to estimate rates of silent substitution in primate *Mhc* genes. They recommended calculating rates of synonymous

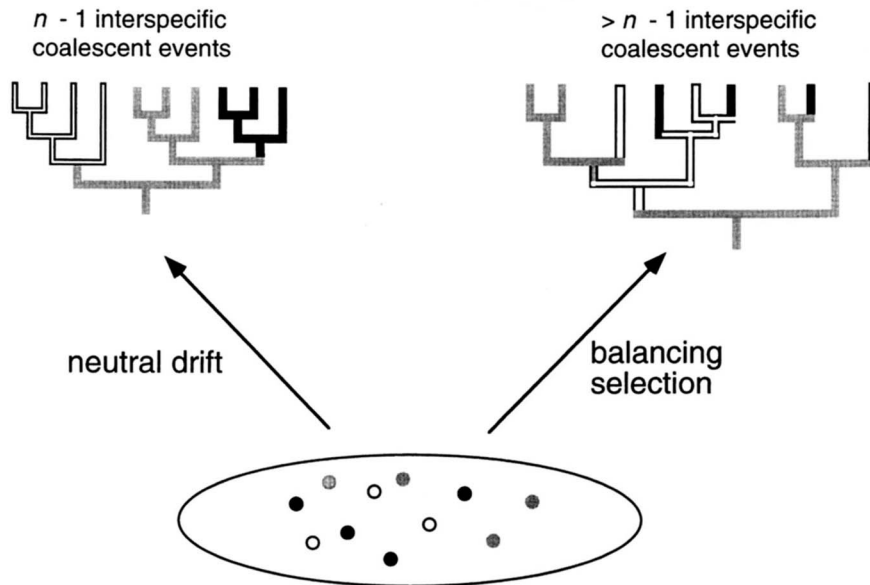


FIGURE 1.—Schematic diagram illustrating the effect of balancing selection on the number of inferred interspecific coalescent events when alleles are sampled from n number of species. At the bottom, colors of dots (alleles) in the ancestral allele pool do not indicate similarities or differences among alleles, but rather the different species in which they will eventually be found after speciation. On the left is depicted the expected pattern of interspecific allelic relationships under neutral drift after populations have diverged for $\geq 4N$ generations. On the right is depicted a pattern expected for alleles under balancing selection. See text for further explanation.

substitution by focusing on pairs of alleles between species that differ by the smallest number of estimated synonymous substitutions (D_m). Such pairs of alleles should have diverged close to the time of divergence of species, whereas more divergent alleles between species likely diverged long before the species in question did. If sampling of alleles within species has been dense enough taxonomically, this method yields a robust estimate of absolute evolutionary rates, calculated as half the slope of the line connecting D_m and the origin ($b_{mm} = D_m/2T$, where T is the divergence time), even if transspecies evolution is occurring (SATTA *et al.* 1993). We plotted D_m in exon 2 [calculated using the method of NEI and GOJOBORI (1986)] *vs.* divergence time for species pairs of mice and rats. The time scale for divergence between Mus species came from SHE *et al.* (1990b), JANKE *et al.* (1994) and SILVER (1995) and references therein. There is controversy over the divergence time of mice and rats. For this divergence time, we used two extremes, one based on paleontological data (10 mya; JAEGER *et al.* 1986), the other based on complete mtDNA sequences and other molecular data (35 mya; JANKE *et al.* 1994) and references therein. In addition we used alternate estimates of divergence times in primates as recommended (MARTIN 1993; SATTA *et al.* 1993).

We also collected sequences from exons 2–4 of 13 DRB genes and 11 DRA genes from Genbank. Both sets of sequences contained at least one representative of primates, ungulates, rodents, carnivores and a marsupial (Figure 2). To test the hypothesis of a molecular clock at silent sites in DRB and DRA genes of mammals, we extracted two- and fourfold degenerate sites from the sequences using the sequence display format in the program MEGA (KUMAR *et al.* 1993). We then compared the log likelihoods of sequence data made to conform to trees whose branch tips were constrained [DNAMLK option in PHYLIP; FELSENSTEIN (1994)] or not constrained (DNAML) to end contemporaneously (FELSENSTEIN 1988). If the likelihood of the sequence data when applied to a tree with the assumption of a clock was significantly lower than when applied to the same tree without a clock assumption, a molecular clock is rejected. In addition,

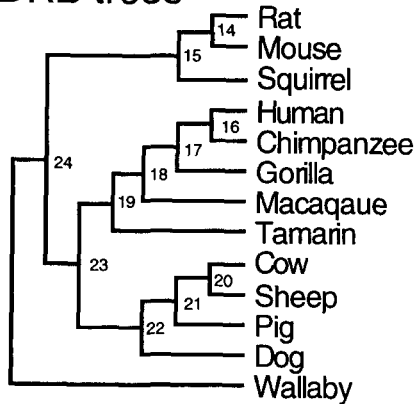
we employed the recently devised “two-cluster” test of TAKEZAKI *et al.* (1995) on sequence data consisting of two- and fourfold degenerate sites. This test measures the departure from equality (δ) of mean branch lengths found in sister groups (clusters) stemming from a common ancestor. The Kimura two-parameter method (KIMURA 1980) was used to calculate divergence at two- and fourfold degenerate sites for use in the two-cluster program. For both the maximum likelihood and two-cluster tests, fourfold degenerate sites alone were also analyzed separately.

The tree of placental mammals remains poorly resolved (ALLARD *et al.* 1996), but evidence suggests that rodents branched off before primates and ungulates (LI *et al.* 1990; Figure 2, tree 1). The placement of Lagomorpha (rabbits) is particularly uncertain, so throughout our clock analyses we assumed three other trees (Figure 2). Both a traditional close relationship of lagomorphs with rodents (Glires; tree 3), as well as an outgroup relationship of lagomorphs to nonrodent groups, (tree 4) are represented in this analysis, as in LI *et al.* (1990). In addition, we used a third tree corresponding to phylogenetic analysis of the DRB and DRA sequences themselves (Figure 2; trees 2 and 5). Even though these trees are not congruent with other lines of evidence (*e.g.*, squirrel and rodent sequences not monophyletic), they may incorporate some of the inconsistencies with the species tree expected for sequences under balancing selection (*e.g.*, within primates). However, when considering relationships *between* mammalian groups (*e.g.*, primates and ungulates) retention of ancestrally polymorphic *Mhc* alleles is unlikely to cause incongruence between gene and organismal trees.

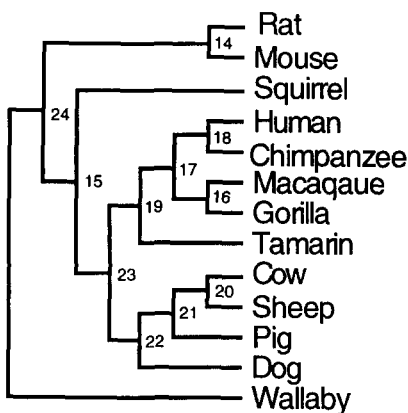
RESULTS

DRB sequences and trees: Our study brings the number of available DRB sequences in mice to ~ 50 (SAITO *et al.* 1983; BEGOVICH *et al.* 1990; FIGUEROA *et al.* 1990). In our compilation of sequences from eight *Mus* species, 97 of the 270 nucleotides and 55 of the 90 codons

DRB trees

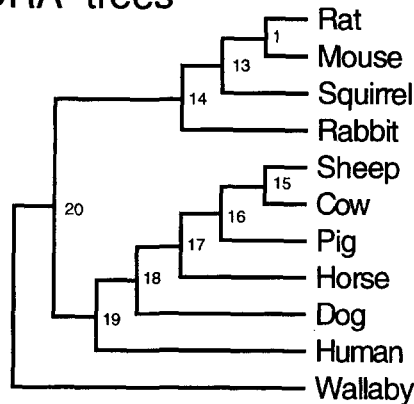


Tree 1

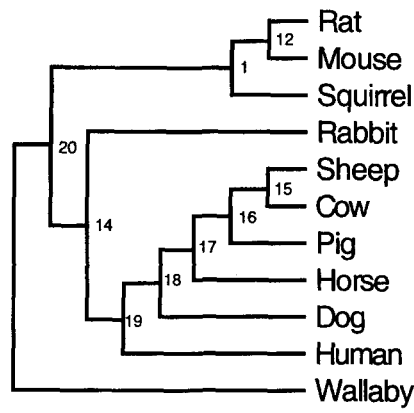


Tree 2

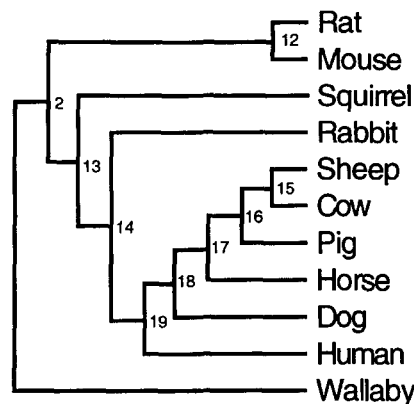
DRA trees



Tree 3



Tree 4



Tree 5

FIGURE 2.—Tree topologies of mammalian DRB and DRA genes assumed in the analysis of molecular clocks at two- and fourfold degenerate sites (Tables 3 and 4). The trees are plausible representations of current knowledge of mammalian relationships. DRB trees: tree 1 incorporates monophyly of rodent sequences, whereas tree 2 represents the best tree consistently obtained in neighbor-joining, maximum-likelihood and parsimony analyses. DRA trees: tree 3, Glires tree, indicating monophyly of rabbit and rodent sequences; tree 4, tree according to Li *et al.* (1990), indicating the rabbit sequence as outgroup to primate and ungulate sequences; tree 5, best tree obtained in multiple phylogenetic analyses of DRA coding sequences (exons 2–4). Branch lengths are not drawn to scale. Nodes numbers indicate points at which sister group branch lengths are compared to one another via two-cluster tests (see Table 4).

in the second exon are polymorphic. In three cases the same DRB amino acid sequence appeared in multiple species; one sequence in particular was found to occur in *M. domesticus*, *musculus*, *spretus*, and *castaneus*, which last shared a common ancestor a little over 1 mya (SHE *et al.* 1990a). Using a squirrel (Genbank accession number M97616) and human allele (DRB*0101) as outgroups, the phylogenetic relationships of the 50 alleles in trees of the 270-bp DRB exon 2 sequence exhibited little concordance with species boundaries, suggesting ubiquitous transspecies evolution (Figure 3A). Even a

rat DRB allele (ROBERTSON and MCMMASTER 1985) clusters within the mouse sequences when squirrel and human alleles are used as outgroups, implying that alleles have persisted since the divergence of rat and mouse (see next section).

Interspecific coalescent events and balancing selection: We can quantify the degree of transspecies evolution implied by these trees by first noting the species from which each allele was sampled and then using parsimony to count the number of interspecific coalescent events (c) one must invoke to explain the distribu-

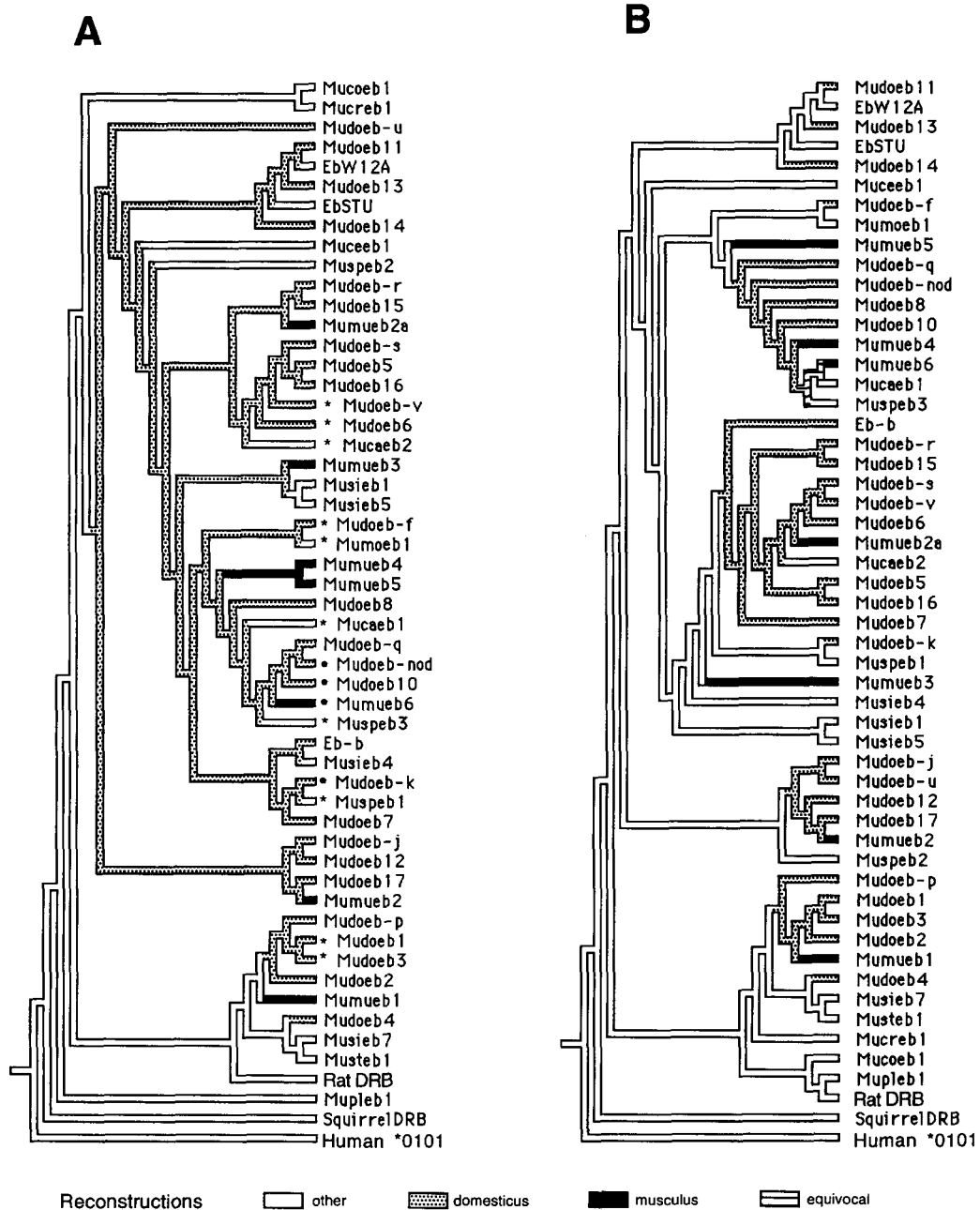


FIGURE 3.—(A and B) Neighbor-joining trees of 51 mouse DRB exon 2 sequences with sequences from a tassel-eared squirrel (*Sciurus aberti*), rat (*Rattus rattus*) and human as outgroups. Mus species names (first four letters of each Mus sequence) are indicated so as to conform with names given in SHE *et al.* (1990a,b) and are as follows: Mudo, *domesticus*; Mumu, *musculus*; Muce, *cervicolor*; Muca, *castaneus*; Mumo, *mollosinus*; Musi, *spicilegus*; Musp, *spretoides*; Mucr, *caroli*; Mupl, *platythrix*; Muco, *cooki*. Sequences beginning with Eb are *M. domesticus* alleles taken from Genbank (accession numbers available upon request). The shading in the branches indicates whether the ancestral sequences lead to alleles found in *M. domesticus* (stippled), *musculus* (black), or other Mus species (white). The trees were constructed in PHYLIP (Tamura-Nei distance, gamma rate parameter $\alpha = 0.5$). (A) Tree of alleles based on all 270 nucleotide sites. Asterisks indicate clusters of sequences that are identical; these sequences are not depicted as identical in the tree so as to help visualize reconstructions. The tree requires a total of 20 interspecific coalescent events between all reconstructed ancestral Mus species designations. (B) Tree based on 198 sites not contained in the 24 codons comprising the putative PBR (BROWN *et al.* 1993). This tree also requires 20 events to explain the distribution of alleles across Mus species.

tion of alleles across species in the tree. When there is complete concordance of alleles and species, this number will be $n - 1$ where n is the number of species (Figure 1). The trees in Figure 3 both indicate that one must invoke at least 20 interspecific coalescent events to explain the observed distribution of alleles. Removal

of 24 positions comprising the putative PBR (BROWN *et al.* 1993) before tree building does not influence this conclusion (Figure 3B). Because our simulation modeled allelic genealogy in two descendant species, we compared our observed value of c with those in the simulations by first removing all tips in the trees in

TABLE 1
Observed and expected number of interspecific coalescent events (c) for *Mhc* alleles sampled from pairs of mouse species

Species pair	n_1	n_2	Observed c	t/N_e	c , neutral	Best fit	
						s	c
<i>domesticus/musculus</i>	19	7	6	1	2.253 ± 0.752^a	0.001	6.103 ± 0.838^a
<i>domesticus/spicilegus</i>	19	4	3	2	1.387 ± 0.516^a	0.001	3.660 ± 0.538^a
<i>domesticus/spretus</i>	19	3	3	2	1.387 ± 0.516^a	0.001	3.660 ± 0.538^a
<i>domesticus/castaneus</i>	19	2	2	1	1.495 ± 0.500^a	0.01	1.962 ± 0.190^a
<i>musculus/spicilegus</i>	7	4	3	2	1.387 ± 0.516^a	0.001	3.660 ± 0.583^a
<i>musculus/spretus</i>	7	3	3	2	1.387 ± 0.516^b	0.001	3.660 ± 0.538^b
<i>musculus/castaneus</i>	7	2	2	1	1.495 ± 0.500^a	0.01	1.962 ± 0.190^a
<i>spicilegus/spretus</i>	4	3	3	2	1.387 ± 0.516^b	0.001	3.476 ± 0.808^b
<i>spicilegus/castaneus</i>	4	2	2	2	1.495 ± 0.500^a	0.01	1.962 ± 0.190^a
<i>spretus/castaneus</i>	3	2	2	2	1.495 ± 0.500^a	0.01	1.962 ± 0.190^a

Values for c are shown ± 1 SD. n_1 and n_2 , sample sizes for the left and right species in column 1, respectively; t/N_e , the ratio of the divergence time and effective population size; c , the number of interspecific coalescent events; s , selection coefficient. See text for further details.

^aThe simulations corresponding to $n_1 = 20$ and $n_2 =$ the smaller of the two sample sizes were used for comparison with observed results.

^bThe simulation $n_1 = 5$, $n_2 = 5$ was used for comparison with observed results.

Figure 3 except for those of the two species of interest. We then recounted c on these pruned trees. This protocol assumes that the best estimate of the allele tree for two species is the pruned tree originally built from data from all species, as opposed to the tree built using alleles for the two species (see also SLATKIN and MADDISON 1989). When pairs of species for which multiple alleles were sampled are considered separately, the value of c ranged from 6 for *musculus/domesticus* ($n = 19$ and 7, respectively) to 2 (e.g., trees involving *castaneus*, $n = 2$; Table 1). Thus, for example, even though only two alleles were sampled from *castaneus*, these alleles appear to coalesce before the common ancestor of *castaneus* and other species in the tree.

For the neutral cases in the simulations, the expected value of c is high at first, then asymptotes to 1 as t/N_e increases (Figure 4, A–D). This trend reflects the frequently made observation that if populations are recently diverged, the allelic lines from the two descendant species will not be monophyletic until an average of $t = 4N$ generations (NEIGEL and AVISE 1986; SLATKIN and MADDISON 1989; HUDSON 1990). Under balancing selection, the value of c is generally higher than for neutrality and asymptotes more slowly (Figure 4, A–D). For the population sizes we used ($N_e = 10^4$), the simulations suggest that neither the intensity of balancing selection nor the size of the larger sample of alleles (n_1) influences the value of c greatly. SLATKIN and MADDISON (1989) also found a weak dependence on the size of the larger sample in their simulations on gene flow. In general, the number of interspecific coalescent events can distinguish allelic genealogies under balancing selection from neutral genealogies unless t/N_e is < 1 or the sample sizes from each species are small ($n_1, n_2 < \sim 5$) is known.

An appropriate comparison of observed c values to simulated results requires an estimate of t/N_e for mice. Although these values are not known for many species, several estimates of both parameters in *Mus* have been made. Divergence time (t) can be derived from molecular data and varies from ~ 0.3 to 1.0 mya (see next section), whereas N_e is generally thought to be on the order of $1-5 \times 10^5$ (FERRIS *et al.* 1983; WILSON *et al.* 1985; NACHMAN *et al.* 1994; PRAGER *et al.* 1996; M. NACHMAN, personal communication). We take a conservative approach with respect to detecting balancing selection by using a value of 500,000 for N_e at the high end of the range (Table 1). Thus divergence times in units of t/N_e conservatively vary from around 1 for divergences of *musculus, domesticus* and *castaneus* from one another to 2 for divergences of *spretus* and *spicilegus* from other *Mus* in this study (Table 1). In all cases, the c value observed using the tree in Figure 3A is higher than that predicted from the value of c for the corresponding sampling scheme and time scale under neutrality in the simulations (Table 1), implying that the alleles are being maintained for periods longer than predicted by neutrality. Even when taking a conservative approach by comparing observed c values to results of simulations for larger sample sizes, most c values exceed those expected for neutrality and more closely resemble those for balancing selection (Table 1).

Our conclusions also need to be tempered by the fact that trees of exon 2 are suspect because they are relatively short sequences and have likely experienced recombination, gene conversion, and a high rate of convergence (GYLLENSTEN 1991; SHE *et al.* 1991; ZANGENBERG *et al.* 1995), all of which compromise the reliability of trees. Indeed, bootstrapping (FELSENSTEIN 1985) reveals only 12 branches that are significant at

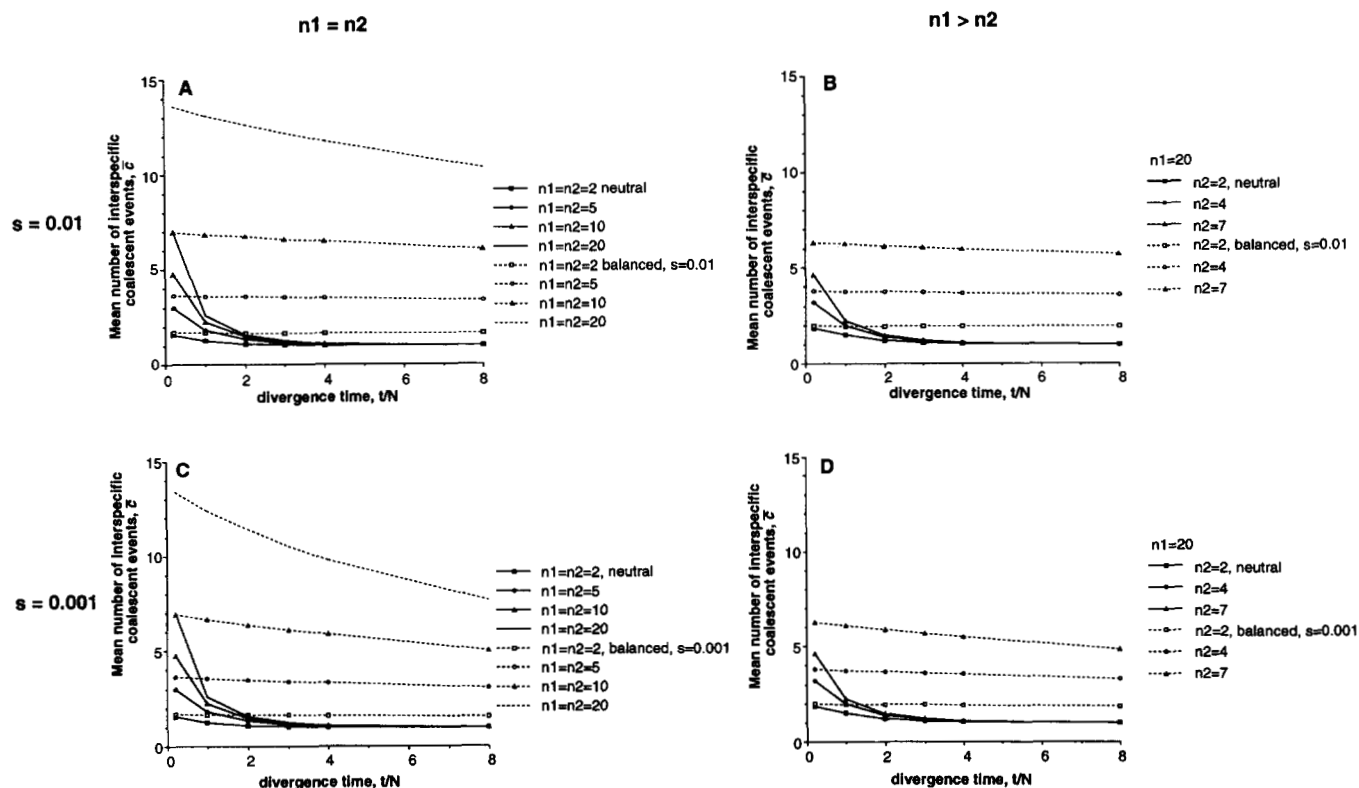


FIGURE 4.—Mean number of interspecific coalescent events (c) for alleles sampled from two species under neutrality and balancing selection. N_e was assumed to be 10,000 in all simulations. (A and B) Mean values of c for a selection intensity of symmetrical balancing selection of $s = 0.01$ for equal and unequal sample sizes, respectively. (C and D) Values of c for a selection intensity of symmetrical balancing selection of $s = 0.001$ for equal and unequal sample sizes, respectively. Each point represents the mean value of c over 10,000 trees simulated under the parameters indicated. See MATERIALS AND METHODS and RESULTS for further details.

the 90% level (Figure 3, A and B), and the rat sequence clusters within the mouse sequences only with 80% confidence. Furthermore, for the 19 *domesticus* sequences, trees of the α -helix and β -sheet significantly conflict with one another (Table 2), suggesting that the different domains are characterized by different evolutionary histories. [This pattern is, however, less pronounced in the seven *musculus* sequences, perhaps due to small sample size (Table 2)]. Although parsimony trees in which alleles are constrained to cluster by species with unspecified relationships between species are much longer (515 steps) than the most parsimonious trees for these data (337–338 steps, legend, Figure 3), these results suggest that the precise level of transspecies evolution in *E β* may be difficult to determine from these exon 2 sequences alone. Even if we assume that the tree of alleles is correct (Figure 3), parsimony reconstruction of amino acids suggests that convergent evolution has occurred within mice at a minimum of 28 of the 90 codons, with an additional three instances of convergence between mouse sequences and those of rats and humans. Phylogenetic analysis of alleles at the DQB (β) locus in these same strains suggested a similarly noisy pattern of evolution, presumably caused by intra-exonic recombination and motif shuffling (SHE *et al.* 1990a; McDEVITT 1995). Like available phylogenetic tests for gene flow and drift (SLATKIN and MADDISON

1989; MADDISON and SLATKIN 1991), conclusions about the effects of selection in these mouse DRB sequences depend critically on the accuracy of the phylogenetic tree.

Analysis of trees generated from 100 bootstrapped data sets showed that the number of interspecific coalescent events varied from 19 to 22 across data sets, (mean, 20.49) when only *Mus* species were considered. Thus, uncertainties in the tree seem to involve branches connecting alleles sampled from the same species (*e.g.*, within *domesticus*), rather than those connecting different species. The mean value of c across 1000 random trees of *Mus* sequences was similar (21.4), indicating that *Mus* alleles are distributed randomly across species and that, if the observed trees are correct, neutral drift, which would tend to decrease c , has played a negligible role in the history of these sequences.

Divergence in DRA: There were a total of six variable positions in the eight new mouse DRA exon 2 sequences; although these caused a total of four variable amino acids, they were found primarily in non-PBR regions of exon 2. Total Jukes-Cantor divergences between DRA exon 2 sequences between *Mus* species ranged from 0% (*e.g.*, *caroli vs. spretooides*) to 4.2% (*caroli vs. musculus*; a complete matrix of divergences is available upon request). The tree of the eight DRA sequences shows little resolution among *Mus* species (Fig-

TABLE 2

Winning sites tests of recombination in *Mus* DR genes, measured as conflicts between trees based on the α -helix and β -sheet of E β alleles

Species and region of exon 2	No. of sites favoring β vs. α tree	Total no. of steps over all sites favoring β vs. α tree
<i>M. domesticus</i> (<i>n</i> = 19 sequences)		
β sheet	10 vs. 1**	15 vs. 1**
α helix	0 vs. 12**	0 vs. 21**
<i>M. musculus</i> (<i>n</i> = 7 sequences)		
β sheet	4 vs. 3	4 vs. 3
α helix	1 vs. 4	1 vs. 4

The winning-sites test (FELSENSTEIN 1988; PRAGER and WILSON 1988) compares the number of sites that undergo fewer steps on (*i.e.*, favor) trees 1 and 2, in this case, the set of most parsimonious trees of the α helix and β sheets determined in PAUP. These tests were performed using the "compare two treefiles" option in MacClade (MADDISON and MADDISON 1992). ** $P < 0.01$.

ure 5). The sequences do not cluster according to species, but trees in which sequences were forced to cluster in a species-specific manner were not significantly worse than the best tree, and so maintenance of ancestral polymorphisms at the E α locus in mice cannot be unambiguously supported (legend, Figure 5).

Rates of synonymous substitution: To compare the rate of synonymous substitution in the second exon of rodent DRB alleles to that found in a recent study of primate DRB alleles (SATTA *et al.* 1993), we used the minimum-minimum method, in which the smallest number of synonymous substitutions, D_m , between alleles from pairs of species is plotted against species divergence time. The rate in exon 2 suggested for rodents and comparable estimates of b_{mm} in primates are shown in Figure 6, A and B. Using the mouse/rat divergence as a reference point for $b_{mm} = D_m/2T$, the estimates of synonymous substitution rates in rodent exon 2 varies from 1.2 to $4.3 \times 10^{-9} \pm 1.2$ substitutions/site/year depending on whether a divergence time of 35 or 10 mya is assumed. The corresponding rate for primates in the second exon is $0.9-1.2 \times 10^{-9} \pm 0.76$ substitutions/site/year, depending on the divergence time assumed for macaques (Figure 6). The synonymous rate estimated for the primate PBR is very close to that estimated for the entire gene (SATTA *et al.* 1993). These results suggest that the rodent synonymous rate may be as much as 4.7 ($=4.3/0.9$) times higher than the rate in primates for exon 2. If the lower bound of the rodent synonymous rate is correct, the rate differences between rodent and primate exon 2 sequences are statistically indistinguishable. Furthermore, if points involving comparisons among *Mus* alleles are considered, b_{mm} assumes a value of 0, since there are some alleles that are identical at synonymous sites which have a non-zero

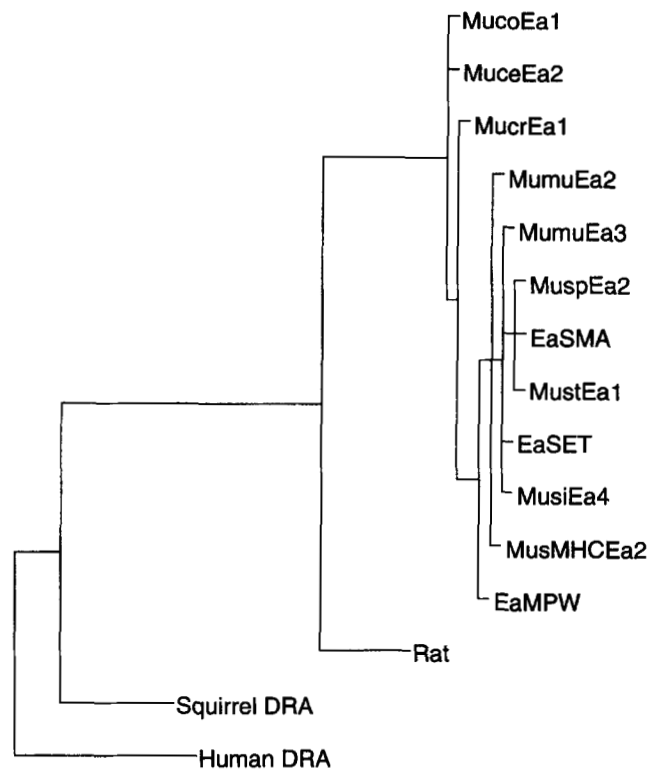


FIGURE 5.—Tree of DRB exon 2 alleles from the genus *Mus* and outgroups. Species designations are as in Figure 3; four alleles (EaMPW, MusMHCEa2, EaSET and EaSMA) and rat, tassel-eared squirrel and human (outgroup) sequences were obtained from Genbank. This tree was one of six equally parsimonious trees (79 steps) obtained in PAUP when branches of zero length were collapse. A similar tree was obtained using the neighbor-joining method [Tamura-Nei distance, gamma distribution of rates ($\alpha = 0.5$) used in the distance matrix]. Sequences that are depicted on the same vertical line are identical sequences.

divergence time. (In principle, b_{mm} always equals 0 for any pair of alleles found in different species that are identical at silent sites.) On the other hand, if the upper bound of the rodent rate is correct, the rodent synonymous rate significantly exceeds that in primates.

To pursue the issue of variable synonymous rates in mammals further, relative rate tests, which are not subject to uncertainties of absolute dating, were used. The results of maximum-likelihood tests of molecular clocks on various tree topologies for two- and fourfold degenerate sites of mammalian DRB genes are presented in Table 3. Regardless of the tree topology assumed, all analyses suggested that rates of silent substitution in mammalian DRB genes has not been constant. This result was confirmed and the particular lineages undergoing variable rates were identified using two-cluster tests, which were performed in parallel to each maximum likelihood test (Table 4). Regardless of the tree topology assumed, these tests rejected the hypothesis of similar rates among mammalian lineages and furthermore suggest faster and slower than average rates for the rodent and primate lineages, respectively (Table 4). In particular, for DRB the mouse/rat clade appears

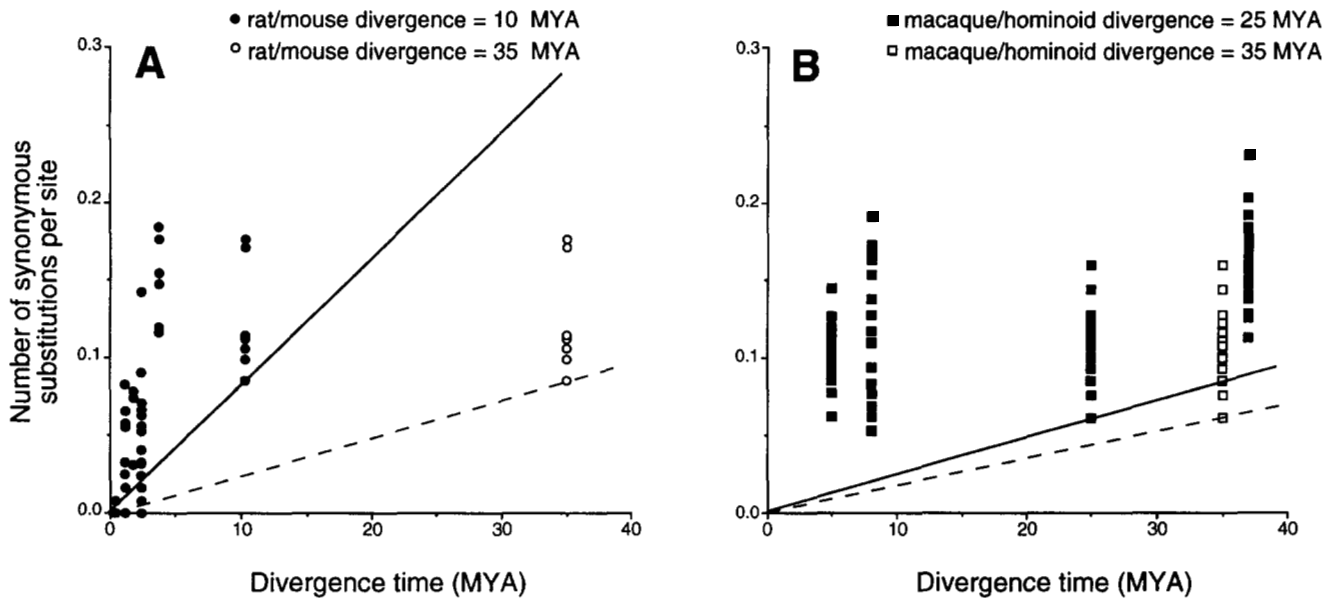


FIGURE 6.—Estimates of rates of synonymous substitution in the second exon of DRB genes in rodents (A) and primates (B) based on the minimum-minimum method of SATTA *et al.* (1993). In both graphs, black circles and squares indicate minimum divergence times of species in cases where divergence time is uncertain. Solid lines indicates maximum rates, and dashed line indicated minimum rates for each group.

to evolve faster than the squirrel sequence, and the primate clade appears to evolve slower than ungulate-carnivore relatives. In addition, variation at DRB fourfold degenerate sites alone suggests that the rat sequence has changed to a greater degree than the mouse DRB sequence (Table 4). Because there is a high level of base compositional bias at two- and fourfold degenerate sites in these sequences, the Kimura two-parameter estimates of sequence divergence at these sites are probably underestimates. However, the bias is of similar magnitude and direction for all the sequences (Table 3), suggesting that variation among sequences in base compositional bias cannot solely explain these results. Rejection of a clock by a maximum-likelihood analysis (Table 3), which takes base compositional bias into account in a way much better than do the Jukes-Cantor divergence

estimates we used (FELSENSTEIN 1994), lends confidence to the overall result.

Estimated numbers of synonymous substitutions between the 11 mammalian DRA sequences were very high, ranging from 9.5% (sheep *vs.* cow) to over 180% (182.7%, wallaby *vs.* mouse). The total Jukes-Cantor divergences ranged from 3.3% (sheep *vs.* cow) to 36.7% (wallaby *vs.* mouse). These numbers suggest that DRA in mammals is under strong stabilizing selection (HUGHES *et al.* 1994; Edwards *et al.*, unpublished data), but also raise the question why divergence in this gene is so high. Maximum likelihood analyses of two- and fourfold degenerate sites of the DRA sequences also rejected a molecular clock (Table 3), as did two-cluster tests (Table 4). These latter tests again revealed a statistically significant increase in rates in the lineage leading

TABLE 3

Maximum likelihood values for two- and fourfold degenerate sites of *Mhc* class II DRB and DRA genes on trees with and without the assumption of a molecular clock

Gene	Tree in Figure 2	Two- and fourfold degenerate sites		Fourfold degenerate sites	
		Without clock	With clock	Without clock	With clock
DRB	1	-842.07	-879.86 (7.62)*	-407.11	-424.96 (5.56)*
	2	-840.37	-864.77 (6.18)*	-408.30	-425.89 (4.82)*
DRA	3	-1062.29	-1072.01 (4.21)*	-536.91	-553.83 (5.12)*
	4	-1059.17	-1064.84 (3.33) ^{ns}	-533.26	-548.49 (4.90)*
	5	-1059.50	-1082.01 (6.51)*	-533.26	-546.58 (4.56)*

Values in parentheses are standard errors. * Rejection at $P < 0.05$ level; *ns*, not significant. The average frequency of A, C, G and T at the 142 two- and fourfold degenerate sites of DRB genes was 0.13, 0.39, 0.33, 0.15, respectively; at the 61 fourfold degenerate sites, 0.14, 0.23, 0.44, 0.18, respectively; at the 148 two- and fourfold degenerate sites of DRA, 0.12, 0.38, 0.25, 0.25, respectively; at the 68 fourfold degenerate sites, 0.16, 0.34, 0.25, 0.25, respectively.

TABLE 4

Two-cluster tests of a molecular clock at two- and fourfold degenerate sites of *Mhc* class II DRB and DRA sequences

Two- and fourfold degenerate sites							Fourfold degenerate sites						
Node	L	R	δ	SE	Z	CP(%)	Node	L	R	δ	SE	Z	CP(%)
DRB (<i>n</i> = 142 sites)							DRB (<i>n</i> = 61 sites)						
Tree 1							Tree 1						
14	Mouse	< Rat	0.033	0.027	1.216	77.38	14	Mouse	< Rat	0.119	0.058	2.067	96.06
15	Squirrel	< 14	0.088	0.039	2.265	97.62	15	Squirrel	> 14	0.000	0.058	0.007	0.00
20	Sheep	> Cow	0.059	0.032	1.811	92.98	20	Sheep	< 12	0.006	0.072	0.087	6.38
21	20	< Pig	0.017	0.039	0.430	33.28	21	20	> Pig	0.056	0.044	1.257	78.88
22	21	> Dog	0.016	0.042	0.376	28.86	22	21	< Dog	0.030	0.043	0.686	50.34
16	Chimp	< Human	0.017	0.018	0.939	64.76	16	Chimp	> Human	0.023	0.020	1.171	75.80
17	16	> Gorilla	0.006	0.012	0.511	39.00	17	16	> Gorilla	0.005	0.010	0.514	39.00
18	Macaque	< 17	0.003	0.014	0.199	15.06	18	Macaque	< 17	0.004	0.007	0.515	39.00
19	Tamarin	< 18	0.012	0.016	0.735	53.46	19	Tamarin	> 18	0.015	0.030	0.488	36.88
23	19	< 22	0.097	0.025	3.849	99.96	23	19	< 22	0.049	0.039	1.257	78.88
24	23	< 15	0.005	0.038	0.130	10.34	24	23	< 15	0.142	0.096	1.478	85.84
Tree 2							Tree 2						
14	Mouse	< Rat	0.033	0.027	1.216	77.38	14	Mouse	< Rat	0.119	0.058	2.067	96.06
18	Chimp	< Human	0.017	0.018	0.939	64.76	18	Chimp	< Human	0.023	0.020	1.171	75.80
16	Macaque	> Gorilla	0.001	0.009	0.139	10.34	16	Macaque	> Gorilla	0.003	0.003	0.984	67.30
17	16	< 18	0.004	0.015	0.274	21.28	17	16	< 18	0.006	0.011	0.535	40.38
19	Tamarin	< 17	0.012	0.016	0.734	53.46	19	Tamarin	< 17	0.014	0.030	0.488	36.88
20	Sheep	> Cow	0.059	0.032	1.811	92.98	20	Sheep	> Cow	0.006	0.072	0.087	6.38
21	20	< Pig	0.017	0.039	0.430	33.28	21	20	< Pig	0.055	0.044	1.257	78.88
22	21	> Dog	0.016	0.042	0.376	28.86	22	21	> Dog	0.030	0.043	0.686	50.34
23	22	> 19	0.097	0.025	3.85	99.96	23	22	> 19	0.049	0.039	1.257	78.88
15	Squirrel	< 23	0.043	0.026	1.644	89.90	15	Squirrel	< 23	0.055	0.060	0.924	64.24
24	15	< 14	0.027	0.049	0.552	41.76	24	15	< 14	0.130	0.099	1.313	80.98
DRA (<i>n</i> = 148 sites)							DRA (<i>n</i> = 68 sites)						
Tree 3							Tree 3						
12	Mouse	> Rat	0.035	0.051	0.692	50.98	12	Mouse	> Rat	0.085	0.091	0.943	65.28
13	12	> Squirrel	0.168	0.068	2.490	98.72	13	12	> Squirrel	0.291	0.135	2.149	96.76
14	13	> Rabbit	0.129	0.055	2.359	98.12	14	13	> Rabbit	0.190	0.102	1.872	93.86
15	Sheep	> Cow	0.002	0.019	0.125	9.56	15	Sheep	> Cow	0.005	0.038	0.131	10.34
16	Pig	< 15	0.039	0.028	1.374	82.94	16	Pig	< 15	0.046	0.052	0.898	62.66
17	Horse	> 16	0.006	0.036	0.175	13.50	17	Horse	> 16	0.007	0.066	0.111	8.76
18	Dog	> 17	0.002	0.030	0.071	5.58	18	Dog	> 17	0.005	0.052	0.104	7.96
19	Human	> 18	0.019	0.042	0.458	34.72	19	Human	> 18	0.040	0.052	0.773	55.88
20	19	< 14	0.107	0.078	1.366	82.62	20	19	< 14	0.193	0.210	0.921	64.24
Tree 4							Tree 4						
15	Sheep	> Cow	0.002	0.019	0.125	9.56	15	Sheep	> Cow	0.004	0.036	0.107	7.96
16	Pig	< 15	0.039	0.028	1.374	82.94	16	Pig	< 15	0.042	0.049	0.844	59.90
17	Horse	> 16	0.006	0.036	0.175	13.50	17	Horse	> 16	0.012	0.062	0.188	14.28
18	Dog	> 17	0.002	0.030	0.071	5.58	18	Dog	> 17	0.003	0.050	0.063	4.78
19	18	< Human	0.019	0.042	0.458	34.72	19	18	< Human	0.041	0.047	0.861	61.02
14	Rabbit	< 19	0.004	0.046	0.081	6.38	14	Rabbit	< 19	0.004	0.087	0.049	3.20
12	Mouse	> Rat	0.035	0.051	0.692	50.98	12	Mouse	> Rat	0.079	0.079	1.004	68.26
13	12	> Squirrel	0.168	0.068	2.490	98.72	13	12	> Squirrel	0.282	0.123	2.287	97.74
20	13	> 14	0.149	0.093	1.594	88.82	20	13	> 14	0.199	0.197	1.010	68.26
Tree 5							Tree 5						
15	Sheep	> Cow	0.002	0.019	0.125	9.56	15	Sheep	> Cow	0.005	0.038	0.131	10.34
16	Pig	< 15	0.039	0.028	1.374	82.94	16	Pig	< 15	0.046	0.052	0.898	62.66
17	Horse	> 16	0.006	0.036	0.175	13.50	17	Horse	> 16	0.007	0.066	0.111	8.76
18	Dog	> 17	0.002	0.030	0.071	5.58	18	Dog	> 17	0.005	0.052	0.104	7.96
19	18	< Human	0.019	0.042	0.458	34.72	19	18	< Human	0.040	0.052	0.773	55.88
14	19	> Rabbit	0.004	0.046	0.081	6.38	14	19	> Rabbit	0.001	0.089	0.014	0.80
13	Squirrel	< 14	0.033	0.057	0.583	43.80	13	Squirrel	< 14	0.142	0.108	1.314	80.98
12	Mouse	> Rat	0.035	0.051	0.692	50.98	12	Mouse	> Rat	0.085	0.091	0.943	65.28
20	12	> 13	0.222	0.128	1.736	91.64	20	12	> 13	0.452	0.361	1.250	78.50

The tests asks whether the difference (δ) in average branch length of lineages stemming to the left (L) and right (R) of nodes in a tree is significantly different from 0. The trees and node numbers indicated for each analysis are depicted in Figure 2. δ , SE of δ , and Z are statistics associated with the test of $\delta = 0$ (TAKEZAKI *et al.* 1995). CP is the confidence probability, or one minus the *P* value for the test, with larger percentages indicating increasing certainty of rejection of a molecular clock. Entries in bold indicate significant deviations from clock-like evolution above the indicated node.

to mice and rats relative to the squirrel sequence (Table 4). However, the increase detected in rodents depended somewhat on the tree topology assumed, and in the case of the unlikely tree 5 (Figure 2), no deviations from a clock were detected with two- and fourfold degenerate sites combined, or fourfold degenerate sites alone.

DISCUSSION

One of the most remarkable features of the evolution of vertebrate *Mhc* loci is the extended lifespan of alleles, which is thought to be a consequence of balancing selection acting on peptide binding region. While it has been claimed that some non-*Mhc* loci show evidence of balancing selection (*e.g.*, McDONALD and KREITMAN 1991), *Mhc* loci [and the self-incompatibility alleles in plants (RICHMAN and KOHN 1996)] are the only loci for which balancing selection has caused dramatic retention of ancestrally polymorphic alleles, so called "trans-species polymorphisms" (KLEIN 1987). Why loci other than *Mhc* loci do not exhibit this type of polymorphism is not clear. Available estimates of selection differentials between heterozygotes and homozygotes at *Mhc* loci vary somewhat depending on the method of estimation used (TAKAHATA *et al.* 1992; SATTA *et al.* 1994; HEDRICK and KIM 1997), and it is not immediately clear that the differences in evolutionary dynamics between *Mhc* loci and other genes under balancing selection are due to the intensity of selection. Although the apparent retention of ancestral polymorphism of *Mhc* alleles influences analysis of these genes in ways not apparent for other loci, it also offers opportunities for analysis of selection not possible for other loci. In this paper we have used patterns of ancestral polymorphism to characterize two aspects of *Mhc* evolution in mice, namely evidence of nonneutral evolution and rates of synonymous substitution.

Balancing selection: The new α and β chain sequences from *Mhc* class II DR molecules in mice have helped quantify the effects of balancing selection at these loci. It is well known that patterns of nucleotide substitution in the PBR of *Mhc* class II genes can be used to detect (HUGHES and NEI 1989) and estimate the strength of balancing selection (TAKAHATA *et al.* 1992). Although the mouse PBR sequences we have collected do exhibit one hallmark of balancing selection, namely an excess of nonsynonymous over synonymous substitutions (S. V. EDWARDS, K. CHESTNUT, E. K. WAKELAND, unpublished data), we have chosen here to investigate a different aspect of class II gene diversity, ancestral polymorphism, to detect balancing selection. Ancient allelic divergences indicating balancing selection at *Mhc* loci have been detected in several taxonomic groups (FIGUEROA *et al.* 1988; LAWLOR *et al.* 1988; GYLLENSTEN and ERLICH 1989; GRASER *et al.* 1996), but ancestral polymorphism even for neutral genes is expected if species divergence times have been short or if

population sizes are large. Our simulations have helped rule out the possibility of ancestral polymorphism for neutral genes by focusing on one index of ancestral polymorphism, the number of interspecific coalescent events, over a range of species divergence times. The number of interspecific coalescent events in our trees appears to be too high to be compatible with the number expected under neutrality.

We caution that our conclusions from our analysis of interspecific coalescent events (*c*) are contingent on the parameters being used for comparison with simulation results. It is likely that the estimate of t/N_e for these mice is not accurate, and deviations from the values given in our Table 1 could affect our conclusion. Furthermore, changes in population size over time could affect our results. Since we found little difference between mean *c* values for selection intensities of 0.01 and 0.001, this statistic is probably best used to reject neutrality for various sample sizes and times, rather than to determine precisely the intensity of balancing selection. SATTA *et al.* (1994) and TAKAHATA *et al.* (1992) have proposed other methods for measuring the intensity of selection at *Mhc* using other kinds of data. Furthermore, our test is specific to loci for which balancing selection causes ancestral polymorphisms to be retained for long periods of time. TAKAHATA and NEI (1990) and VEKEMANS and SLATKIN (1994) investigated the time to common ancestry (T_c) and average pairwise time of divergence (T_d) for alleles under balancing selection in a single population. The number of interspecific coalescent events is likely to be correlated with these statistics, although it is, of course, dependent on divergence times of species as well. Other tests for balancing selection for alleles that do not show maintenance of ancestral polymorphism are available (*e.g.*, HUDSON *et al.* 1987; FU and LI 1993; LI and FU 1994), but have not yet been applied to *Mhc* loci.

The high level of polymorphism in DRB in fact makes it difficult to evaluate the degree to which trans-species evolution is occurring. In addition to maintaining abundant diversity in the PBR, balancing selection likely causes significant convergence and homoplasy, as has been suggested for equine (GUSTAFSSON and ANDERSSON 1994) and primate (ERLICH and GYLLENSTEN 1991; O'HUIGIN 1995) DRB genes. This problem, in addition to the fact that exon 2 sequences are relatively short, compromises most studies in which phylogenetic analysis is based solely on exon 2 nucleotide sequences; in general, more stable markers, such as retroposons (MCCONNELL *et al.* 1988) and *Alu* sequences (MNUKOV'A FAJDELOV'A *et al.* 1994) are most useful for quantifying the extent of trans-species evolution at these loci. Importantly, several of the oldest suspected examples of trans-species evolution are inferred solely from exon 2 sequences, despite the fact that convergent similarities are suspected to occur between very ancient allelic pairs (TAKAHATA 1994). In addition, there has been little testing of the significance of these trees either by boot-

strapping or comparison with null models (KLEIN *et al.* 1993; FIGUEROA *et al.* 1994). What statistical testing has been done has attempted to document different questions, namely the different genealogical histories of various PBR subdomains (GYLLENSTEN *et al.* 1990; EDWARDS *et al.* 1995; Table 2) or other issues. It is possible that the noise apparent in DRB trees is due to functionally driven convergence due to selection, although it seems at least as likely that such convergence is a byproduct of the diverse mutational mechanisms apparent in DRB loci (WAKELAND *et al.* 1990; ERLICH and GYLLENSTEN 1991; SHE *et al.* 1991; MCDEVITT 1995). Although our bootstrap analysis suggested that the interpretation of the number of coalescent events was not drastically affected by uncertainties in the tree, this issue should be studied further. The noisiness of $E\beta$ evolution has important implications for population genetic as well as behavioral studies of *Mhc* loci, and will be important to consider in future evolutionary studies.

The α chain gene, DRA, is relatively conserved in our mouse sequences and appears to be under stronger stabilizing selection in mammals generally. We found no statistically robust evidence for maintenance of ancestral polymorphism in our mouse sequences or the larger mammalian data set. Nonetheless, the number of substitutions between various mammals in DRA is very high (see RESULTS). Polymorphism for DRA is known in some species (ALBRIGHT-FRASER *et al.* 1996). Further within- and between-species studies of DRA genes are needed.

Synonymous substitution rates: Our analyses of DRB and DRA sequences from mice and other mammals suggests that these genes have undergone increases in synonymous substitution rates in rodents. In addition, the synonymous rate in the DRB gene appears to have decreased in the lineage leading to primates. Indeed, visual inspection of previously published class II *Mhc* trees strongly suggest that rodent sequences have a higher rate of substitution than their primate homologues (*e.g.*, Figure 2 of FIGUEROA *et al.* 1990), although this hypothesis has yet to be tested quantitatively. To the extent that rates at two- and fourfold degenerate sites reflect underlying mutation rates (KIMURA 1983), these results suggest variable mutation rates at *Mhc* genes in mammals.

Variation in rates of evolution at synonymous sites are thought to be driven by variation in generation time (SARICH and WILSON 1973; WU and LI 1985) or variable DNA damage due to differences in metabolic rates (MARTIN and PALUMBI 1993); physiological and thermal constraints on proteins, which are suspected to influence rates of amino acid substitution in different vertebrates (ADACHI *et al.* 1993; MINDELL *et al.* 1996), would not apply to rate variation at silent sites. Both of the trends suggested by our analysis of DRB and DRA genes, the increase in rate in rodents and the decrease in primates, have been observed in numerous other studies of molecular clocks. Drawing on predictions of the

neutral theory, that generation time should influence the rate per year of neutral mutation, WU and LI (1985) first suggested that rates of evolution in rodent genes were accelerated relative to their primate homologues. Subsequent analyses (EASTEAL 1985; LI *et al.* 1990) suggested that the rate difference between rodents and primates was less than originally claimed; nevertheless, when the appropriate comparisons have been made, rate increases in rodents and decreases in primates have generally been observed (LI 1993; LI *et al.* 1996; but see EASTEAL 1988; KAWAMURA *et al.* 1991). In addition, we found evidence for rate differences between mice and rats, contrary to observations at other nuclear genes (O'HUIGIN and LI 1992).

Whereas most genes at which a deceleration has been detected appear to have begun their deceleration in the ancestor of hominoid primates or in humans, our tests of DRB evolution (Figure 6, Tables 3 and 4) imply that the slowdown began before the divergence of the higher primates and other Old World monkeys. The two-cluster tests did not detect a slower rate in human DRB relative to that of other primates in the data set, perhaps indicating a primate lineage-specific rate (*cf.* O'HUIGIN and LI 1992). If true, this suggests that the low synonymous rates estimated for DRB genes in primates (KLEIN *et al.* 1993; SATTA *et al.* 1993) may in fact represent slowdowns from a formerly faster mammalian rate. It may also be that the number of primate lineages compared was too low to detect substitution rate variation within primates.

Analysis of molecular clocks at *Mhc* loci is made more difficult because of the long persistence times of alleles, which decreases the correlation of species and gene divergence times (see also TAKAHATA and NEI 1990; UYENOYAMA 1995). Although our use of the D_m statistic is appropriate for *Mhc* loci, our rate estimates in the mouse sequences in principle could be much lower. There were instances in which identical alleles were found in species that diverged over 1 mya (see Introduction), and in one case alleles identical at synonymous sites occurred in *M. caroli* and *musculus*, which diverged 2.4 mya (Figure 6A), suggesting a low upper bound to synonymous substitution rates. However, there is a large sampling error in estimates of the number of nucleotide substitutions between closely related sequences and the rate estimate based on the divergence of rats and mice may be more reliable. Furthermore, recombination is thought to play a role in the evolution of class II *Mhc* sequences and appears to occur in some of the DRB sequences characterized here (Table 2). SATTA *et al.* (1993) removed from their analysis of primate DRB sequences alleles that they inferred to have participated in recombination events. Because of difficulties in distinguishing similarity in exon 2 due to recombination and due to convergence (TAKAHATA 1994), we did not attempt to identify alleles that might have recombined with other alleles in our analysis; thus recombination could obscure estimates of silent rates in this study. In

addition, the skewed base composition of third positions makes estimation of synonymous substitution numbers difficult, and current methods of estimation do not make the most efficient use of available data (MUSE 1996). However, evidence for variable rates remained after the relative rate analysis of DRB sites among multiple mammalian lineages. These tests were less subject to some of the assumptions about recombination, and presumably in these tests the differential effects of problems in distance estimation in primates and rodents were minimized.

Finally, it is possible that synonymous substitutions in DRA/B genes are not completely neutral, particularly because the gene as a whole is under positive selection. For example, at a codon, the synonymous substitution toward the most favorable codon may be preferred after an amino acid replacement. Deviation from strict neutrality of silent sites could mean that variation in substitution rates among lineages is due not only to differences in per year mutation rates but also to selection.

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