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Two Types of Molecular Evolution. Evidence from Studies of Interspecific Hybridization

(mammals/frogs/proteins/regulation/maternal-fetal immunology)

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ABSTRACT **To assess the significance of macromolecular sequence differences among species, we compared the serum albumins of 81 pairs of vertebrate species capable of producing viable hybrids. Micro-complement fixation experiments showed that the average difference between the albumins within such pairs was only 3 immunological distance units for placental mammals (31 pairs), but 36 units for frogs (50 pairs). Albumin immunological distance is strongly correlated with other measures of genetic distance, including those made with DNA annealing techniques. It therefore seems likely that mammalian species pairs capable of hybridization are far more similar at the macromolecular sequence level than is the case for most hybridizable frogs.**

We think the most likely explanation for the marked molecular restriction on hybridization among mammals is that the ratio of regulatory evolution to protein evolution is higher for mammals than for frogs. Mammals may have experienced unusually rapid regulatory evolution; indeed, this could be the factor responsible for their unusually rapid anatomical evolution.

There may be two major types of molecular evolution. One is the process of protein evolution, which goes on at about the same rate in all species. The other is a process whose rate is variable and which is responsible for evolutionary changes in anatomy and way of life. We propose that evolutionary change in regulatory systems accounts for evolution at and beyond the anatomical level.

This proposal emerges from attempts to explain the observation that protein evolution and anatomical evolution can proceed independently (1–3). This independence is illustrated by protein and anatomical studies on frogs and mammals. Frogs (Anura) are an ancient group that has undergone much protein evolution (1, 2, 4–7) but little anatomical evolution during its 150-million-year history. Although there are thousands of frog species living today, they are all rather alike in anatomy and way of life. By contrast, the placental mammals, which are only 75 million years old, have undergone extensive anatomical evolution. The diversity in anatomy and way of life represented by bats, whales, sloths, and people is unparalleled among frogs. Yet placental mammals have experienced less protein evolution than frogs have. While the rate of protein evolution is similar in the two groups, their rates of anatomical evolution differ greatly (1, 2). This remarkable contrast between protein evolution and anatomical evolution implies that protein evolution may not be at the basis of anatomical evolution.

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For the idea that evolutionary changes in regulatory systems may provide the basis for anatomical evolution, we are indebted to Wolpert (8), Britten and Davidson (9, 10), and above all, Ohno (11, 12). Accordingly, we suggest that the rapid anatomical evolution exhibited by placental mammals is attributable to rapid evolutionary changes in their developmental regulatory systems. Evidence in support of this idea is now presented.

Our evidence comes from studies on interspecific hybridization. For the past several years we have been comparing the blood proteins, albumin, transferrin, and hemoglobin, of a great variety of vertebrate species (1, 2, 7, 13–23) including numerous pairs of frog and of mammal species known to be capable of producing viable interspecific hybrids. This enabled us to estimate how similar the proteins of the parental species are in those cases where successful interspecific hybridization can occur. Hence, one can examine the problem of what relationship, if any, exists between hybridization potential and degree of protein sequence difference among species. At first thought, it might seem obvious that degree of protein similarity between the parental species should be a major factor affecting the probability of successful development of an interspecific zygote. The more similar the proteins of two species, the more likely it is, one might expect, that their genomes would be compatible enough to permit development of viable hybrids. However, our results do not fulfill this expectation. Mammals that can hybridize with each other differ only slightly at the protein level, whereas frogs that differ substantially in protein sequence hybridize readily. In order to explain this contrast, we review evidence suggesting that the principal molecular barriers to interspecific hybridization are regulatory differences between the parental genomes. Rapid regulatory evolution in mammals may account for both their rapid anatomical evolution and their rapid evolutionary loss of the potential for interspecific hybridization.

MATERIALS AND METHODS

Protein Purification and Antiserum Production. Serum or plasma samples were obtained from 31 pairs of mammalian species and 50 pairs of frog species known to produce viable interspecific hybrids. Albumin was purified from many of these species, usually by preparative polyacrylamide gel electrophoresis (2, 7, 13) and then injected into groups of three to six rabbits (of the New Zealand White or the Dutch Belted varieties). After a 3-month period of immunization by published methods (2, 14), antisera were collected and pooled in

inverse proportion to their micro-complement fixation titers. Transferrin was purified by Rivanol precipitation, ammonium sulfate fractionation of the supernatant, and preparative polyacrylamide gel electrophoresis (15). Hemoglobin was purified from hemolysates by polyacrylamide electrophoresis alone. The immunization procedure used for hemoglobin and transferrin was essentially the same as for albumin.

Measurement of Immunological Distance. Amino-acid sequence differences between proteins were measured immunologically. Each antiserum pool was tested for reactivity with the unpurified albumin present in serum from various species. Reactivity was measured by the quantitative micro-complement fixation method (24). The results are given in immunological distance units, which are defined elsewhere (14, 20, 24, 25). Immunological distance (y) is generally related to percent difference in amino-acid sequence (x) by the equation $y \cong 5x$ (24–28). For the particular case of albumin, there is direct empirical evidence that each unit of immunological distance is roughly equivalent to one amino acid substitution (23). Although micro-complement fixation measures only the approximate degree of sequence difference between homologous proteins, it is superior to conventional chemical methods in speed and economy (16, 24, 25).

Albumin Immunological Distance as a Measure of Genetic Distance. We worked mainly with serum albumin, not only because of our considerable experience with the study of species differences in this protein (1, 2, 7, 13–23), but also because albumin evolves faster than most other proteins. Whereas the average rate of protein evolution is 1 amino-acid substitution per 100 residues per 10^7 years (29–31), that of albumin appears to be twice as fast (13). Albumin is also nearly twice as large as the average protein, having about 580 amino acids in a single polypeptide chain (32). For these reasons, it is a useful protein for detecting sequence differences among closely related species.

Although we studied primarily albumin, protein evolution proceeds with sufficient regularity (22) to make us confident that species whose albumins differ greatly will also differ

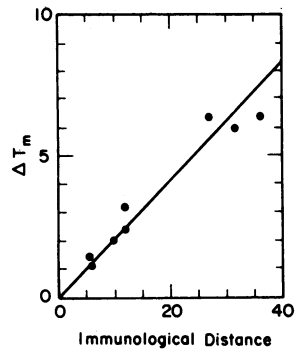

FIG. 1. Relationship between DNA evolution and albumin evolution in primates. The albumins of various species of catarrhine primates (i.e., humans, apes, and Old World monkeys) were compared by the micro-complement fixation method. Each immunological distance value plotted is the average of two reciprocal measurements. The nonrepeated fraction of the total genome DNA was also compared, using the same primate species and the methods described by Kohne *et al.* and Hoyer *et al.* (34, 35). The ΔT_m values plotted are taken from refs. 34 and 35; ΔT_m is thought to be related linearly to percentage difference in nucleotide sequence (34, 35).

TABLE 1. *Albumin differences within 31 pairs of placental mammal species that hybridize*

Pair	Immunological distance between albumins*
Primates	
Cercocebus albigena × *C. galeritus*	10
Cercocebus torquatus × *Macaca fascicularis*	5
Cercocebus torquatus × *Macaca nemestrina*	5
Cercocebus torquatus × *Mandrillus sphinx*	6
Cercopithecus aethiops × *C. mona*	4
Cercopithecus aethiops × *Macaca mulatta*	8
Cercopithecus cephus × *Erythrocebus patas*	5
Cynopithecus niger × *Macaca fascicularis*	4
Mandrillus sphinx × *Papio anubis*	4
Papio anubis × *P. cynocephalus*	0
Papio anubis × *P. hamadryas*	0
Papio anubis × *P. papio*	0
Papio cynocephalus × *P. hamadryas*	0
Papio cynocephalus × *P. papio*	0
Papio hamadryas × *P. papio*	0
Papio hamadryas × *P. ursinus*	0
Papio hamadryas × *Theropithecus gelada*	0
Papio papio × *P. ursinus*	0
Lemur fulvus × *L. macaco*	6
Carnivora	
Felis catus × *Felis libyca*	0
Canis familiaris × *C. latrans*	2
Canis familiaris × *C. lupus*	0
Ursus americanus × *U. arctos*	0
Ursus arctos × *Thalarctos maritimus*	3
Arctocephalus pusillus × *Zalophus californicus*	8
Perissodactyla	
Equus asinus × *E. caballus*	4
Equus burchelli × *E. caballus*	8
Artiodactyla	
Bison bison × *Bos taurus*	1
Cervus canadensis × *C. elaphus*	2
Odocoileus hemionus × *O. virginianus*	5
Odocoileus hemionus × *Axis axis*	8
Mean	3

* Values taken from ref. 19 and from Sarich (unpublished work). These results were obtained with antisera against the purified albumins of the following species: *Bison bison, Bos taurus, Canis familiaris, Cercocebus galeritus, Cercopithecus aethiops, Cervus canadensis, Equus caballus, Felis catus, Lemur fulvus, Macaca mulatta, Odocoileus hemionus, Papio anubis, Ursus americanus,* and *Zalophus californicus.*

substantially at other loci as well. Electrophoretic measures of genetic distance (33), for example, correlate strongly (r = 0.8) with immunological distances among the albumins of the same species. If two species differ electrophoretically at 50% of their loci, the immunological distance between their albumins is usually about 22 units (V. M. Sarich, L. R. Maxson, M. -C. King, K. Keeler, and A. C. Wilson, paper presented at the annual meeting of the Society for the Study of Evolution, Houston, Texas, December 1973).

Genetic distance can also be estimated from DNA hybridization experiments; the best method is to measure the melting (denaturation) temperature of heteroduplexes formed by an-

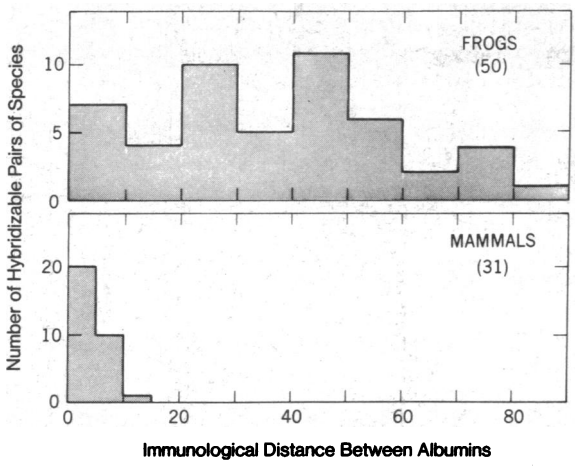

FIG. 2. Immunological distances between the serum albumins of species pairs capable of producing hybrids. The numbers of pairs are given in parentheses.

nealing non-repeated DNA sequences and to subtract it from the melting temperature of the homoduplexes. By comparing the albumins of the same species whose DNAs were so compared (34, 35), we find that there is a very strong correlation ($r = 0.9$) between melting temperature difference (ΔT_m) and albumin immunological distance. This is illustrated in Fig. 1. Hence we believe the immunological distance results given below are indicative of the overall degree of sequence resemblance among the genomes of the species compared.

RESULTS

Serum Albumin. Table 1 gives the results of the albumin comparisons for 31 pairs of placental mammal species. Gray (36) reports that every one of these pairs can produce viable, full-term interspecific offspring. The albumins of these pairs generally differ by about 3 units (range 0–10), which corresponds to a sequence difference of about 0.6%. It appears that if the albumin sequence difference found within a pair of mammalian species exceeds 2% (i.e., 10 units), the pair is very unlikely to produce a viable hybrid.

Sharply contrasting with the mammal results are the frog results given in Table 2. The 50 pairs of frogs listed are reported to produce interspecific offspring that successfully metamorphose from tadpole to adult (37–40). Yet the albumin differences within these pairs average 37 units (range 0–91), which is about 10 times greater than the average for hybridizable mammal pairs. Indeed, 42 of the 50 frog pairs§ showed albumin differences greater than those within any of the mammal pairs in Table 1. Fig. 2 summarizes the albumin results and illustrates the frog–mammal contrast.

Other Proteins. The large molecular differences within hybridizable frog pairs are not unique to albumin. Immunological comparison of the hemoglobins of several of the *Hyla* pairs in Table 2 showed immunological distances averaging half of the corresponding albumin immunological distances (7). This is consistent with the finding in other vertebrate groups, including mammals, that albumin generally evolves twice as fast as hemoglobin (13).

The small differences found within hybridizable mammal species pairs are also not unique to albumin. Fibrinopeptides, which are known to evolve extremely fast (31), do not differ much within hybridizable species pairs. We calculated that the average sequence difference between the fibrinopeptides of 13 such pairs is 1.8 amino-acid substitutions (a 5% sequence difference). In addition, we have obtained information on transferrins, which are known to evolve faster than albumin but slower than fibrinopeptides (V. M. Sarich, J. E. Cronin, E. M. Prager, and A. C. Wilson, unpublished work). The transferrins of 21 of the species pairs listed in Table 1 differ by an average of 8 units of immunological distance (a 1.6% sequence difference). Hence there is a parallel between the relative rates of evolution of the three polypeptides and the relative magnitudes of the sequence differences within pairs of hybridizable species. Accordingly, the 3 polypeptides give a consistent picture of the degree of amino-acid sequence difference within the various species pairs.

In summary, it appears that, as a general rule, protein sequence differences within mammalian pairs capable of hybridization are an order of magnitude smaller than the corresponding frog differences.

DISCUSSION

Regulatory hypothesis

We propose that the marked restriction on interspecific hybridization among mammals occurs because mammals, in contrast to frogs, have experienced rapid evolutionary change in the systems regulating expression of genes. If two species have very different mechanisms for regulating gene expression during embryonic development, it is unlikely that a healthy adult hybrid organism could develop. The hypothesis that the chief molecular barriers to development of hybrid organisms are regulatory ones is consistent with observations on somatic cell hybrids as well as the phenomenon of allelic repression in organismal hybrids.

Somatic Cell Hybrids Versus Organismal Hybrids. Somatic cells from extremely different animals can hybridize and grow well for many generations (41). Bird cells, for example, hybridize readily with those of mammals. Yet, at the protein level, the average degree of sequence difference within a pair of mammal species capable of organismal hybridization is at least 100 times smaller than that between birds and mammals. It is even possible for invertebrate cells to hybridize with those of mammals (42), despite sequence differences which are undoubtedly greater than those between birds and mammals.

Cell hybridization between distantly related species is much easier than organismal hybridization because cell hybrids are exempt from the requirement to develop into an organism. The process of embryonic development involves activation of most of the genes that were inactive in the sperm and egg (43, 44). For successful development of an interspecific zygote, the two regulatory systems (contributed by the egg and sperm genomes) controlling the expression of such genes must be compatible. As somatic cell hybrids are less subject to such a

§ The low number of frog values in the mammalian range (0–10 units) may result from the fact that frog populations differing from one another by 0–10 units of albumin immunological distance are rarely (except in the case of *Bufo* species) considered as separate species and hence did not fall within our purview. Thus the wide range of within-pair immunological distance values (0–91 units) found for frogs may be more significant than the average (36 units) in the comparison with mammals.

TABLE 2. *Albumin differences within 50 pairs of frog species that hybridize*

Pair	Immunological distance between albumins*
Bufo	
B. boreas × *B. alvarius*	25
B. boreas × *B. americanus*	30
B. boreas × *B. arenarum*	61
B. boreas × *B. calamita*	46
B. boreas × *B. coccifer*	49
B. boreas × *B. cognatus*	20
B. boreas × *B. compactilis*	24
B. boreas × *B. ibarrai*	43
B. boreas × *B. marmoreus*	46
B. boreas × *B. mazatlanensis*	42
B. boreas × *B. microscaphus*	23
B. boreas × *B. perplexus*	46
B. boreas × *B. punctatus*	4
B. boreas × *B. speciosus*	25
B. boreas × *B. spinulosus*	54
B. boreas × *B. terrestris*	29
B. boreas × *B. valliceps*	36
B. boreas × *B. viridis*	58
B. boreas × *B. woodhousei*	27
B. cognatus × *B. compactilis*	3
B. cognatus × *B. punctatus*	24
B. cognatus × *B. woodhousei*	19
B. marinus × *B. arenarum*	4
B. marinus × *B. paracnemis*	0
B. woodhousei × *B. americanus*	2
B. woodhousei × *B. hemiophrys*	3
B. woodhousei × *B. microscaphus*	5
B. viridis × *B. calamita*	45
Hyla and *Pseudacris*	
H. chrysoscelis × *H. cinerea*	42
H. chrysoscelis × *H. femoralis*	52
H. chrysoscelis × *H. gratiosa*	34
H. chrysoscelis × *H. squirella*	35
H. cinerea × *H. arborea*	57
H. cinerea × *H. avivoca*	45
H. cinerea × *H. squirella*	31
H. femoralis × *H. arenicolor*	63
H. femoralis × *H. cinerea*	66
H. femoralis × *H. gratiosa*	51
H. femoralis × *H. squirella*	54
H. gratiosa × *H. squirella*	42
H. regilla × *P. triseriata*	70
H. crucifer × *P. triseriata*	70
H. crucifer × *P. nigrita*	91
P. brachyphona × *P. nigrita*	38
P. brachyphona × *P. ornata*	76
Rana	
R. pipens × *R. capito*	10
R. pipiens × *R. palustris*	17
R. pipiens × *R. areolata*	21
R. temporaria × *R. japonica*	29
Xenopus	
X. laevis × *X. mulleri*	18
Mean	36

* Values taken from refs. 2 and 7 and from Maxson (unpublished work). These results were obtained with antisera against the purified albumins of the following species: *Bufo boreas*, *B. cognatus*, *B. marinus*, *B. viridis*, *B. woodhousei*, *Hyla cinerea*, *H. crucifer*, *H gratiosa*, *H. femoralis*, *H. regilla*, *H. squirella*, *Pseudacris brachyphona*, *Rana pipiens*, *R. temporaria*, and *Xenopus laevis*. The antisera to *B. cognatus*, *B. marinus*, and *B. woodhousei* were supplied by Dr. S. Guttman.

requirement, it is understandable that cell hybridization, but not organismal hybridization, can take place between extremely distantly related species.

Breakdown of Gene Regulation in Organismal Hybrids. Additional evidence that interspecific differences in developmental regulatory systems may be the major barrier to organismal hybridization comes from studies on gene expression in organismal hybrids. In the fish hybrids, *Lepomis* × *Micropterus*, for example, the glucose-6-phosphate dehydrogenase present is encoded exclusively by the paternal allele; expression of the maternal allele is completely repressed (45). Such allelic repression is indicative of a breakdown in gene regulation. A converse example is provided by alcohol dehydrogenase in quail × chicken hybrids; here, the maternal allele is expressed while expression of the paternal allele is delayed or suppressed totally (46). Similar observations have been reported for other cases of hybridization between distantly related species (see ref. 45 for a review). Indeed, at some loci in such hybrids, neither the maternal nor the paternal allele is expressed (45).

Allelic repression is most often encountered in extreme hybrids. Three of the eight loci tested in the *Lepomis* × *Micropterus* hybrids exhibited this phenomenon (45). Allelic repression is reported to be less common in hybrids between taxonomically very similar species (45). Thus, the extent of allelic repression may be correlated with taxonomic distance between the parental species. This leads one to expect that hybrids between very distantly related species could not develop because the breakdown in gene regulation would be so extensive.

We are impressed with the regulatory hypothesis because it explains why mammals have experienced both rapid anatomical evolution and rapid evolutionary loss of the potential for interspecific hybridization. However, it is important to be aware that an alternative hypothesis may explain the restriction on hybridization among placental mammals.

Immunological hypothesis

This hypothesis appeals to immunological interaction between the mammalian mother and fetus. Such interaction has been the subject of several reviews (e.g., 47, 48). According to this hypothesis, if the proteins of the placental mother and fetus differed as much in sequence as does the average frog species pair in Table 2, the mother would make antibodies against the hybrid fetus, thereby causing abortion. Obviously this phenomenon cannot occur in most frogs, as both fertilization and embryonic development take place outside the mother in the great majority of species, including all those in Table 2. This hypothesis has the corollary that if lethal immunological interaction between mother and fetus were circumvented, hybrids between mammalian species pairs as distinct in protein sequence as the frog pairs of Table 2 should be obtainable. The consequence is staggering, as this would mean that mammalian species pairs with albumin immunological distances of up to 50 units would often be similar enough at the molecular level to form viable hybrids once the postulated immuno-

logical barrier were eliminated. Such pairs of mammals include: (*1*) Any pair of anthropoid primates (17–19), e.g., man and monkey; (*2*) Any pair of arctoid carnivores (14), e.g., dog and seal; (*3*) Any pair of pecoran artiodactyls (V. M. Sarich, A. Bennett, and A. C. Wilson, unpublished albumin studies), e.g., sheep and giraffe.

Elimination of the hypothetical immunological barrier can in principle be achieved, for example, by use of immunosuppressants or by growing the fetus *in vitro*. In fact, immunosuppressive experiments have already been attempted with two interspecific crosses in which the hybrid fetus normally dies during pregnancy. These are the goat × sheep cross (49) and the ferret × mink cross (50). No significant improvement in hybrid survival resulted from such treatment. Given continued rapid progress in both our understanding of the immune response and the development of *in vitro* fetal growth techniques (51), it should soon be possible to conduct more definitive tests of the immunological hypothesis. Until this is done, the available evidence (49, 50) leads us to think that the immunological hypothesis is probably incorrect.

Conclusions

We therefore propose that (*a*) the chief molecular barriers to interspecific hybridization are the regulatory system differences between the maternal and paternal genomes, which must function in concert if an interspecific zygote is to develop, and (*b*) anatomical evolution is due chiefly to regulatory system changes, macromolecular sequence changes usually being rather inconsequential.

Further evidence consistent with the regulatory hypothesis will appear in the next issue of these PROCEEDINGS (53). That evidence, derived from studies on chromosomal evolution in frogs and mammals, will focus attention on the phenomenon of gene rearrangement as a possible means of achieving new systems of regulation.

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