Pattern of Nucleotide Substitutions in Growth Hormone-Prolactin Gene Family: A Paradigm for Evolution by Gene Duplication

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ABSTRACT

The growth hormone-prolactin gene family in mammals is an interesting example of evolution by gene duplication. Divergence among members of duplicated gene families and among species was examined by using reported gene sequences of growth hormone, prolactin and their receptors. Sequence divergence among species was found to show a general tendency in which a generation-time effect is pronounced for synonymous substitutions but not so for nonsynonymous substitutions. Divergence among duplicated genes is characterized by the relatively high rate of nonsynonymous substitutions, *i.e.*, the rate is close to that of synonymous ones. In view of the stage- and tissue-specific expression of duplicated genes, some of the amino acid substitutions among duplicated genes is likely to be caused by positive Darwinian selection.

THE growth hormone gene family in mammals is interesting from the standpoint of evolutionary biology, as its mammalian genomic locus has recently expanded and contains several genes expressed in different tissues (CHEN *et al.* 1989; KARIN, CASTRILLO and THEILL 1990). While most mammals have only one growth hormone gene and one prolactin gene, the human genome contains a cluster of recently duplicated genes of growth hormone and its close relative, chorionic somatomammotropin, and the sequences of these genes have now been determined (CHEN *et al.* 1989). Prolactin also belongs to the growth hormone superfamily, and the bovine genome has been shown to contain several genes that encode prolactin-like proteins (YAMAKAWA *et al.* 1990; TANAKA *et al.* 1991).

The role of gene duplication in evolution is important particularly in mammals (OHNO 1970; OHTA 1980). GOODMAN (1976) found that amino acid substitutions are accelerated following the gene duplication separating myoglobin and hemoglobin and that separating α and β hemoglobins and proposed that the acceleration was caused by selection. LI (1985) examined more loci, including growth hormone genes, and found that amino acid substitution is often accelerated following duplication. He argued that it is very difficult to determine whether positive selection is responsible, or whether relaxation of selection constraint is the real cause of the acceleration. There are now more data to clarify the pattern of nucleotide substitutions in detail. I have been studying various models of evolution by gene duplication and have suggested that positive Darwinian selection is needed for acquiring new genes, whereas, once gene families

are established, positive selection is likely to be unimportant (OHTA 1988a,b). In this report, the theory is examined by calculating the number of amino acid altering nucleotide substitutions and that of synonymous substitutions among various members of this family. Special attention was paid to the acceleration of amino acid substitution in relation to gene duplication. Some sequences of growth hormone and prolactin receptors were also studied to supplement the analysis.

DATA ANALYSIS

Nucleotide sequences were obtained from the genetic databases maintained at the National Institute of Genetics, which include GenBank, DDBJ (DNA Database of Japan), and EMBL. Four sequences of the human growth hormone family and one sequence of the growth hormone gene of each of six mammalian species (goat, pig, mink, rat, mouse and bovine) were used. As for the prolactin family, five sequences of bovine and one sequence each of four species (mouse, rat, human and pig) were used. They are listed in Table 1. In addition, receptor gene sequences were compared. One sequence of growth hormone receptor gene of each of three species (human, pig and mouse) and one sequence of prolactin receptor gene of each of three species (human, rabbit and rat) were used. They are also listed in Table 1.

For the acquisition and analysis of the data, the ODEN Package created by Y. INA and installed in DDBJ was used. The numbers of nonsynonymous and synonymous substitutions were estimated by the method of NEI and GOJOBORI (1986) that is in the ODEN Package. This method divides the nucleotide

TABLE 1

Sequences used in the study

Growth hormone ^a	Prolactin ^b	Receptor ^c
HUMGHCSA ^d (human)	BOVMRNAPRO (bovine)	MUSGHRBPA (mouse)
GH1, GH2 (growth hormone)	BOVPRL (bovine)	PIGPGHRC (pig)
CS1, CS2 (chorionic somatomammotropin)	BOVPRCI (bovine)	HUMGHR (human)
GOTGH (goat)	BOVPROL (bovine)	RATPRLRO (rat)
MVIMKGGH (mink)	BOVPROLA (bovine)	RABPRLR (rabbit)
PIGGH (pig)	PIGPPLAC (pig)	HUMPRLR (human)
RATGH1 (rat)	MUSPRLHR1 (mouse)	
BOVGHC (bovine)	RATLACM (rat)	
	HUMLACT (human)	

^a Data from CHEN *et al.* (1989), VNENCAK-JONES and PHILLIPS (1990), YATO *et al.* (1988), SHOJI *et al.* (1990), VIZE and WELLS (1987), BARTA *et al.* (1981), TAKEUCHI *et al.* (1990).

^b Data from TANAKA *et al.* (1991), MILLER, THIRION and MARTIAL (1980), NILSON *et al.* (1980), SCHULER and HURLEY (1987), YAMAKAWA *et al.* (1990), SCHULZ-AELLEN, SCHMID and MOVVA (1989), LINZER and TALAMANTES (1985), COOKE *et al.* (1980, 1981).

^c Data from LEUNG *et al.* (1987), SMITH, KUNYOSHI and TALAMANTES (1989), CIOFFI, WANG and KOPCHICK (1990), SHIROTA *et al.* (1990), BOUTIN *et al.* (1989), EDERY *et al.* (1989).

^d Another gene notation by CHEN *et al.* (1989): GH-N = GH1, GH-V = GH2, CS-A = CS1 and CS-B = CS2.

substitutions into synonymous and nonsynonymous categories, and then the multiple hit is estimated under the assumption of random mutability among the four kinds of bases. For the growth hormone and prolactin genes, this assumption may not be quite satisfied, but the bias is likely to be not serious for the present purpose. The standard error of the estimate was calculated by the method of KIMURA and OHTA (1972) that is in the ODEN Package.

SEQUENCE DIVERGENCE AMONG SPECIES

The results of the analysis are presented in Table 2. As for the synonymous substitutions, the growth hormone gene family appears to evolve according to the standard rate [see KIMURA (1983, p. 95) and LI and GRAUR (1991, pp. 69–70)]. Let us look at the pattern of nucleotide substitutions among the species. As has been done by KIMURA (1983, pp. 76–78), and GILLESPIE (1991, pp. 113–119), the star phylogeny, *i.e.*, radiation of mammalian species, is examined below. Figure 1 represents star phylogenies of the synonymous and nonsynonymous substitutions for growth hormone, prolactin, growth hormone receptor and prolactin receptor. As can be seen from the figure, the branch lengths differ considerably in each tree. The synonymous trees suggest that the primate line is shortest, and the rodent line is longest as has previously been recognized (LI, TANIMURA and SHARP 1987). This is the so-called generation time effect which will be discussed later. For the nonsynonymous trees, this tendency is not so clear.

By using the star phylogenies, the variations of the substitution rate can be examined. A convenient measure is the dispersion index, *i.e.*, the ratio of the variance to the mean of the number of substitutions. GILLESPIE (1991, pp. 115–118) suggested that the lineage effect or generation time effect should be removed in estimating the dispersion index. For this,

he calculated the weighting factors so that their average is unity, and each factor represents the lineage effect from available data. His weighting factors based on synonymous substitutions were used here because the effect of natural selection is minimal. They are: 1.611 for rodents, 0.762 for artiodactyls and 0.627 for primates.

Table 3 shows the estimates of the dispersion index; R_u is the unweighted index, and R_s is the value weighted by the synonymous substitution. R_s values for the prolactin receptor are not given because the star phylogeny includes the rabbit line, and the weight factor is not available. The slight difference of R_u and R_s for the growth hormone and prolactin from the values in Table 3.5 of GILLESPIE (1991) is caused by using the rat line instead of the mouse line, and by the different method used for estimation of the numbers of synonymous and nonsynonymous substitutions. In general, the previous conclusion (GILLESPIE 1991) was confirmed, *i.e.*, the dispersion index is larger than the value expected under the Poisson process for both the synonymous and nonsynonymous changes, but the index often becomes small by removing the lineage effect for the synonymous substitutions.

SEQUENCE DIVERGENCE AMONG DUPLICATED GENES

Let us now turn our attention to the sequence divergence among duplicated genes on the chromosome. Results in Table 2 immediately tell us that the ratio of nonsynonymous to synonymous substitution numbers is higher for the duplicated genes than for the species comparisons. In order to clarify this point, the relationship between synonymous and nonsynonymous substitution numbers was plotted. Figure 2 presents the relationship for the case of the human growth hormone gene family. The abscissa (x) is the number of synonymous substitutions, and the ordinate

TABLE 2

Estimated numbers of synonymous and nonsynonymous nucleotide substitutions per site with standard errors

Genes compared	Nonsynonymous site	Synonymous site	Genes compared	Nonsynonymous site	Synonymous site
Growth hormone, among-species ^a			Prolactin, among-species		
Goat-pig	0.049 ± 0.011	0.302 ± 0.057	Mouse-rat	0.078 ± 0.014	0.222 ± 0.046
Goat-mink	0.054 ± 0.011	0.438 ± 0.074	Mouse-human	0.259 ± 0.027	0.756 ± 0.119
Goat-rat	0.068 ± 0.013	0.646 ± 0.104	Mouse-pig	0.238 ± 0.026	0.932 ± 0.151
Goat-mouse	0.068 ± 0.013	0.574 ± 0.093	Mouse-bovine	0.315 ± 0.031	0.841 ± 0.133
Goat-human	0.204 ± 0.024	0.687 ± 0.110	Rat-human	0.232 ± 0.025	0.933 ± 0.151
Goat-bovine	0.002 ± 0.002	0.055 ± 0.021	Rat-pig	0.206 ± 0.024	1.002 ± 0.166
Pig-mink	0.007 ± 0.004	0.333 ± 0.061	Rat-bovine	0.259 ± 0.027	0.816 ± 0.130
Pig-rat	0.028 ± 0.008	0.591 ± 0.096	Human-pig	0.096 ± 0.015	0.462 ± 0.076
Pig-mouse	0.026 ± 0.008	0.573 ± 0.093	Human-bovine	0.157 ± 0.020	0.567 ± 0.090
Pig-human	0.174 ± 0.022	0.569 ± 0.093	Pig-bovine	0.108 ± 0.016	0.481 ± 0.079
Pig-bovine	0.046 ± 0.011	0.315 ± 0.058	Prolactin, among-duplicated genes ^b		
Mink-rat	0.030 ± 0.008	0.522 ± 0.086	BOVMRNAPRO-BOVPRCI	0.303 ± 0.030	0.268 ± 0.054
Mink-mouse	0.028 ± 0.008	0.475 ± 0.080	BOVMRNAPRO-BOVPROLA	0.320 ± 0.031	0.232 ± 0.050
Mink-human	0.183 ± 0.023	0.533 ± 0.087	BOVMRNAPRO-BOVPROL	0.577 ± 0.050	0.656 ± 0.108
Mink-bovine	0.051 ± 0.011	0.439 ± 0.074	BOVMRNAPRO-BOVPRL	0.406 ± 0.038	0.564 ± 0.093
Rat-mouse	0.007 ± 0.004	0.160 ± 0.038	BOVPRCI-BOVPROLA	0.136 ± 0.018	0.098 ± 0.030
Rat-human	0.169 ± 0.024	0.781 ± 0.123	BOVPRCI-BOVPROL	0.629 ± 0.054	0.703 ± 0.116
Rat-bovine	0.066 ± 0.013	0.649 ± 0.104	BOVPRCI-BOVPRL	0.443 ± 0.040	0.628 ± 0.102
Mouse-human	0.199 ± 0.024	0.736 ± 0.119	BOVPROLA-BOVPROL	0.686 ± 0.058	0.646 ± 0.108
Mouse-bovine	0.066 ± 0.013	0.577 ± 0.094	BOVPROLA-BOVPRL	0.438 ± 0.040	0.616 ± 0.102
Human-bovine	0.201 ± 0.024	0.652 ± 0.104	BOVPROL-BOVPRL	0.355 ± 0.034	0.632 ± 0.103
Growth hormone, among-duplicated genes			Growth hormone receptor		
GH1-GH2	0.032 ± 0.009	0.164 ± 0.039	Mouse-pig	0.146 ± 0.011	0.604 ± 0.054
GH1-CS1	0.070 ± 0.013	0.111 ± 0.031	Mouse-human	0.170 ± 0.012	0.490 ± 0.046
GH1-CS2	0.070 ± 0.013	0.120 ± 0.033	Pig-human	0.080 ± 0.008	0.268 ± 0.029
GH2-CS1	0.101 ± 0.016	0.128 ± 0.034	Prolactin receptor		
GH2-CS2	0.101 ± 0.016	0.157 ± 0.038	Rat-human	0.172 ± 0.011	0.504 ± 0.045
CS1-CS2	0.000	0.024 ± 0.014	Rat-rabbit	0.174 ± 0.013	0.776 ± 0.074
			Human-rabbit	0.127 ± 0.010	0.488 ± 0.047

^a Human growth hormone gene is GH1.^b Bovine prolactin gene is BOVPRL.

FIGURE 1.—Star phylogenies of growth hormone, prolactin and their receptors. The number beside each branch is the estimated total number of substitutions. In the figure: r, rat; b, bovine; h, human; m, mouse; p, pig and rb, rabbit.

TABLE 3
Estimates of the index of dispersion

Gene	Nonsynonymous		Synonymous	
	R_e	R_s	R_e	R_s
Growth hormone	29.6	51.3	2.1	10.9
Prolactin	24.1	3.3	7.2	0.9
Growth hormone receptor	48.7	13.9	53.0	11.2
Prolactin receptor	12.9		35.3	

The subscripts e and s refer to the equal and the silent weights (GILLESPIE 1991).

FIGURE 2.—Relationship between the estimated number of synonymous substitutions (abscissa, x) and that of nonsynonymous substitutions (ordinate, y) for growth hormone genes. (●) Among-species comparisons; (□) among-duplicated gene comparisons. Regression lines are shown.

(y) is that of nonsynonymous substitutions. In the figure, the values for sequence comparisons among species are also shown. The squares are for the among-duplicated gene comparisons, and the solid circles, for the among-species comparisons. The regression lines and correlation coefficients for the two groups are given in the upper part of the figure. The regression coefficient is much higher for the duplicated genes than for the among-species comparisons, and the difference is statistically significant. It is interesting to see that, in among-species comparisons, all six high values of y involve the human growth hormone sequence. Note that the regression coefficient is the estimate for the proportion of neutral amino acid substitutions under the neutral theory (KIMURA 1983). This is because synonymous substitutions are considered to be neutral. Thus, the neutral proportion among amino acid substitutions is much higher in comparisons of duplicated genes than in among-species comparisons. In other words, selective constraint is relaxed in duplicated genes (LI 1985).

An alternative explanation for the acceleration of amino acid substitution in duplicated genes is positive Darwinian selection (OHTA 1991). Differentiation of

FIGURE 3.—Relationship between the estimated number of synonymous substitutions (abscissa, x) and that of nonsynonymous substitutions (ordinate, y) for prolactin genes. (●) Among-species comparisons; (□) among-duplicated gene comparisons. Regression lines are shown.

the chorionic somatomammotropin gene from the growth hormone gene in the human lineage would have accompanied positive selection, which will be discussed later.

Prolactin genes showed a very similar picture in the study. Figure 3 gives the relationship between the synonymous and nonsynonymous substitutions for the duplicated genes of bovine, together with the relationship for the among-species comparisons. The regression coefficient for duplicated genes is again larger than that for the species comparisons, and the difference is significant.

DISCUSSION

The gene families of human growth hormone and bovine prolactin are good examples of recent expansion. Both gene families show acceleration of the amino acid substitutions relative to the synonymous substitutions. The duplicated genes are differentially used in time and space (CHEN *et al.* 1989; SCHULER and HURLEY 1987; KARIN, CASTRILLO and THEILL 1990; YAMAKAWA *et al.* 1990) and would have diverged to acquire slightly different functions and specificities. For example, among the members of the human growth hormone gene family, only one, GH1, is expressed in the anterior pituitary, and CSH1 and CSH2 are expressed only in placental trophoblasts (CHEN *et al.* 1989). Thus I suggest that the positive Darwinian selection is a likely cause for the acceleration of amino acid substitutions. However, it is difficult to determine the relative importance of positive selection and relaxation from constraint to acceleration.

The star phylogeny and the dispersion index presented in the previous section follow the general pat-

tern of molecular evolution, *i.e.*, the generation time effect is found in the synonymous changes, but a more erratic pattern is observed in the nonsynonymous trees as in the other loci (GILLESPIE 1991). The growth hormone phylogeny in the human line is most rapid in both synonymous and nonsynonymous trees. This might be caused by gene duplication in the human lineage. Evolution by gene duplication sometimes accompanies gene conversion involving short DNA segments, which provides favorable mutant combinations (*e.g.* see IRWIN and WILSON 1990; FITCH *et al.* 1991; BASTEN and OHTA 1992). When the conversion occurs between nonallelic members, gene divergence may increase, and the subsequent phylogenetic tree might look like an accelerated substitution pattern. Large multigene families with diverse members often show such a pattern, *i.e.*, hypervariability at the reactive center of serin proteases and their inhibitors, and of immunoglobulins are characterized by the high rate of not only amino acid-altering but also synonymous nucleotide substitutions (OHTA and BASTEN 1992; OHTA 1993).

The star phylogenies of prolactin, and receptors showed the normal pattern. If the synonymous substitutions faithfully reflect the mutation rate according to the neutral theory (KIMURA 1983), the amino acid substitutions are likely to be influenced by very mild selection and become less dependent on generation time, *i.e.*, the nearly neutral theory would apply to the substitution process (OHTA 1976, 1992). It is here notable that R_e values are quite large, even if the index may decrease in size through weighting.

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