cetaceans would help distinguish between causes of low mtDNA diversity that operate maternally (such as cultural hitchhiking or selection on the mtDNA genome) or nonmaternally (such as population bottlenecks).

The apparently greater role of cultural inheritance among cetaceans compared with nonhuman terrestrial mammals is likely ultimately linked to environmental differences. Compared with most terrestrial environments, the ocean can support large body sizes, has low travel costs and no barriers, contains dispersed and patchy food, and transmits sound very efficiently. The behavior and social structure of cetaceans seem to have evolved distinctive features in this setting. These features include vocal learning, large home ranges, lack of territoriality, and bisexual group philopatry (*1*). Cultural transmission may be another such feature favored by the environment of the matrilineal whales.

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39. The number of daughters of a female was Poisson distributed with mean Ks/P, where P is the current female population size, s is the selective advantage of the female (1.1 for females with the trait and 1.0 for those without), and K is the carrying capacity (200,000 females). All descendants of females with a particular mtDNA haplotype carried that haplotype, except a proportion μbg {where μ is the mutation rate, set to 7.5×10^{-9}/year [A. R. Hoeltzel, J. M. Hancock, G. A. Dover, *Mol. Biol. Evol.* **8**, 475 (1991)], b is the number of base pairs being considered (300), and g is the generation length (15 years)} carrying novel haplotypes, different from the maternal haplotype at one base pair. The four populations, initially mitochondrially homogeneous, ran for 330,000 generations to stabilize haplotype and nucleotide diversities and then, in each situation (Fig. 1, A to D), for an additional 300 generations to indicate the dynamics of the nucleotide diversity. Runs in which the cultural trait drifted to extinction were discarded.

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Zebrafish *hox* Clusters and Vertebrate Genome Evolution

Angel Amores, Allan Force, Yi-Lin Yan, Lucille Joly, Chris Amemiya, Andreas Fritz, Robert K. Ho, James Langeland, Victoria Prince, Yan-Ling Wang, Monte Westerfield, Marc Ekker,* John H. Postlethwait*†

HOX genes specify cell fate in the anterior-posterior axis of animal embryos. Invertebrate chordates have one *HOX* cluster, but mammals have four, suggesting that cluster duplication facilitated the evolution of vertebrate body plans. This report shows that zebrafish have seven *hox* clusters. Phylogenetic analysis and genetic mapping suggest a chromosome doubling event, probably by whole genome duplication, after the divergence of ray-finned and lobe-finned fishes but before the teleost radiation. Thus, teleosts, the most species-rich group of vertebrates, appear to have more copies of these developmental regulatory genes than do mammals, despite less complexity in the anterior-posterior axis.

HOX cluster genes encode DNA binding proteins that specify fate along the anterior-posterior axis of bilaterian animals (*1*). Remarkably, the order of *HOX* genes along the chromosome reflects the order they act along the body (*2*). Invertebrate chordates have one *HOX* cluster and little axial diversity, but tetrapods have four clusters and substantial axial complexity (*3*). Tetrapod clusters arose by duplications of an ancestral cluster containing 13 genes (*4*). Although it is widely assumed that vertebrates have four *HOX* clusters, initial studies of teleost fish, the most diverse group of vertebrates, revealed unexpected *HOX* genes (*5–8*). To understand this problem, we isolated *hox* clusters from the zebrafish *Danio rerio*.

To complement previous surveys of zebrafish *hox* gene fragments (*7, 8*), we identified genomic DNAs in P1 artificial chromosomes (PACs), using degenerate primers to amplify homeoboxes (*9*). We then identified overlap-

ping PACs in chromosome walks, inventoried their *hox* gene content using redundant primers, sequenced gene coding regions, and analyzed gene phylogenies (*10*). These experiments identified seven *hox* clusters containing 40 of the 41 previously identified zebrafish *hox* genes, seven new *hox* genes, one *hox* pseudogene, and *evx1* (Fig. 1). Although we tried to find all genes in each cluster, it is possible that additional genes or pseudogenes exist that do not amplify with our primers.

Phylogenetic analysis of sequence data (*11*) assigned zebrafish genes to one of 13 paralogy groups. Groups 4 and 9 appear in each mammalian cluster and in four zebrafish clusters, so we joined the nucleotide sequences of these groups, removed nonalignable sequence, and constructed a phylogenetic tree. The results showed (Fig. 2A) that each of these four clusters is orthologous to one of the four mammalian clusters. Hence, the duplication events that produced the four mammalian clusters occurred before the divergence of ray-finned and lobe-finned lineages about 420 million years ago (*12*).

Further analysis revealed the origin of the other three zebrafish *hox* clusters. The group 6 tree showed that zebrafish has two orthologs of mammalian *HOXB6*, called *hoxb6a* and *hoxb6b* (Fig. 2B). The group 5 nucleotide tree confirmed duplicate *hoxb* clusters (Fig. 2C). Likewise, zebrafish has two orthologs of mammalian *HOXC6*, called *hoxc6a* and *hoxc6b* (Fig. 2B). To investigate *HOXA* clusters, we joined and aligned the homeodomains of groups 9, 11, and 13, which allows comparison with the pufferfish *Fugu* (for which only the amino acid sequence of the homeobox is available). This tree (Fig. 2D) shows that zebrafish has two clusters orthologous to the mammalian *HOXA* cluster. These data suggest that all *hox* clusters duplicated in the lineage that led to zebrafish after it diverged from the lineage that led to tetrapods, with subsequent loss of one *hoxd* cluster. The divergent *Fugu Hoxd* cluster (*5*) branches with high bootstrap value (965) with the *HOXA* clusters of other vertebrates (Fig. 2D). We conclude that *Fugu* has two orthologs of the tetrapod *HOXA* cluster and no described *Hoxd* cluster.

Comparative analysis of cluster content illuminates the history of *HOX* cluster duplication. The (AB)(CD) model (*13*) suggests two sequential duplications, giving a proto-*AB* clus-

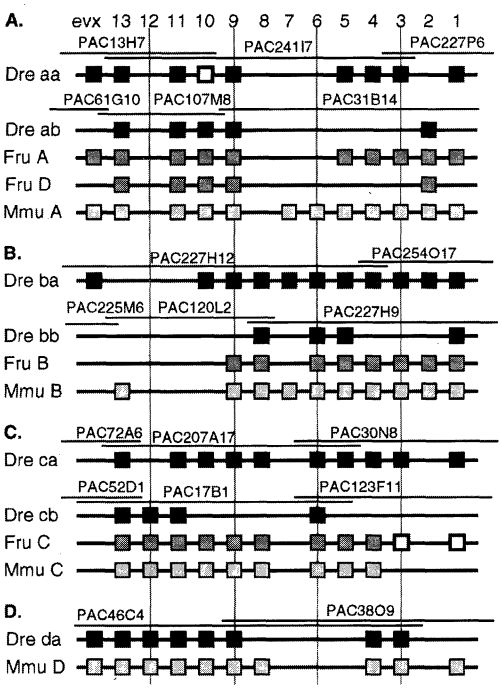

Fig. 1. Organization of vertebrate *HOX* clusters. Each horizontal thick line represents a cluster, designated by species abbreviation followed by cluster name. Species designations are as follows: zebrafish (Dre), black squares; *Fugu* (Fru), gray squares; mouse (Mmu), pale gray squares. Parts (**A**) to (**D**) display *HOX* clusters from different species. Clusters are organized from the 5′ end (paralogy group 13) to the 3′ end (paralogy group 1), with the *even-skipped* homologs of the *evx* family at the 5′ end of the clusters. Clones from the PAC library (*19*) are shown above each zebrafish cluster. The known content of each PAC is represented by black or gray squares (genes) and open squares (pseudogenes). The orphan *hox* genes *hoxx4*, *hoxx9*, and *hoxy6* (*7*, *8*) are synonymous with *hoxa4a*, *hoxa9a*, and *hoxc6b*. Chromosome walks show that genes formerly thought to represent the *hoxa* cluster (*7*) are split into the *hoxab* and *hoxbb* clusters.

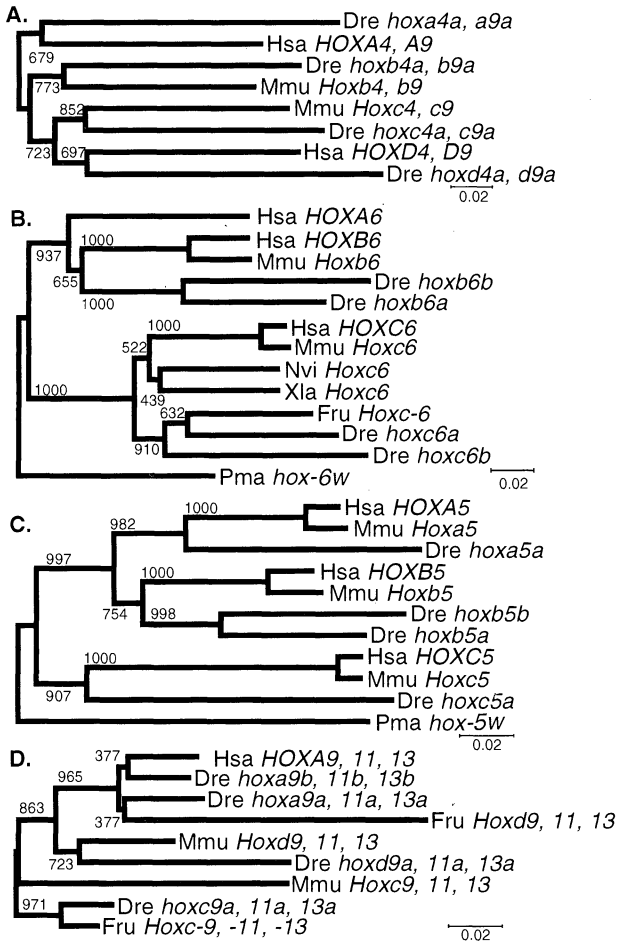

Fig. 2. Phylogenetic analysis. (**A**) The tree constructed by joining homeodomain sequences of group 4 and 9 genes shows that zebrafish (Dre) has orthologs of each human (Hsa) and mouse (Mmu) *HOX* cluster. (**B**) The group 6 tree shows that zebrafish has two copies of mammalian *HOXB* and *HOXC* clusters. Furthermore, *Fugu* (Fru) *Hoxc6* is closely related to just one of the zebrafish genes, suggesting that duplication occurred before the divergence of *Fugu* and zebrafish lineages. This tree is rooted on the lamprey (*Petromyzon marinus*, Pma) *hox-6w* sequence. (Xla, *Xenopus laevis;* Nvi, *Notophthalmus viridescens*.) (**C**) The group 5 tree confirms cluster orthologies and duplications. (**D**) The tree constructed by joining homeodomains of groups 9, 11, and 13 shows that zebrafish has two orthologs of the mammalian *HOXA* cluster, and the *Fugu Hoxd* cluster branches with *HOXA* clusters of other vertebrates. Numbers at nodes indicate bootstrap values for 1000 runs.

A. Amores, A. Force, Y.-L. Yan, Y.-L. Wang, M. Westerfield, J. H. Postlethwait, Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA. L. Joly and M. Ekker, Loeb Institute of Medical Research, Ottawa Civic Hospital, Ottawa, Ontario K1Y 4E9, Canada. C. Amemiya, Center for Human Genetics, Boston University School of Medicine, Boston, MA 02118, USA. A. Fritz, Department of Biology, Emory University, Atlanta, GA 30322, USA. R. K. Ho, Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA. J. Langeland, Biology Department, Kalamazoo College, Kalamazoo, MI 49006, USA. V. Prince, Organismal Biology and Anatomy, University of Chicago, Chicago, IL 60637, USA.

*The experiments reported here were conducted in the laboratories of these authors.

†To whom correspondence should be addressed.

ter and a proto-*CD* cluster after the first event. The alternative (D(A(BC))) model (*14*) suggests three duplications, the first producing the *D* and proto-*ABC* clusters, the second giving the *A* and proto-*BC* clusters, and the third providing the *B* and *C* clusters. Cladistic analysis of cluster content favors the (AB)(CD) model (Fig. 3). For example, loss of group 12 is a shared derived characteristic of teleost and tetrapod *HOXA* and *HOXB* clusters, and loss of groups 2 and 7 unites *HOXC* and *HOXD* clusters. This model minimizes the number of convergent gene losses and is also independently supported by sequence analysis (Fig. 2C).

Superimposed on shared gene loss is lineage-specific loss. For example, fish have lost genes present in mammals (*hoxa6*, *hoxa7*, *hoxd1*, and *hoxd8*). Reciprocally, mammals have lost paralogs present in teleosts (*hoxb10a* and *eve1*). We conclude that the degeneration of *HOX* clusters continued in both lineages after the divergence of ray-finned fish and the lobe-finned ancestors of tetrapods. Furthermore, *hox* cluster degeneration may be ongoing, at least in fish, because *hoxc1a* and *hoxc3a* are active in zebrafish but their orthologs are pseudogenes in *Fugu* (*5*) and are absent from mammals; likewise, *hoxa10a* is a pseudogene in zebrafish but has normal structure in *Fugu* and mouse.

When did the latest *HOX* cluster duplication occur in the zebrafish lineage? The pattern of shared gene loss suggests that the last common ancestor of zebrafish and *Fugu* already had duplicated *HOX* clusters (Fig. 3E). Gene phylogenies support this conclusion, because *Fugu Hoxc-6*, the only informative full-length sequence available (*5*), is more closely related to zebrafish *hoxc6a* than it is to *hoxc6b* (Fig. 2B). In addition, the presence of two *HOXA* clusters in *Fugu*, one related to the zebrafish *hoxaa* and the other to the *hoxab* cluster (Fig. 1), supports a shared duplication. The presence in killifish (*7*) of five group 9 and four group 1 genes as in zebrafish, rather than four group 9 and three group 1 genes as in mammals, is consistent with the hypothesis that the killifish lineage also

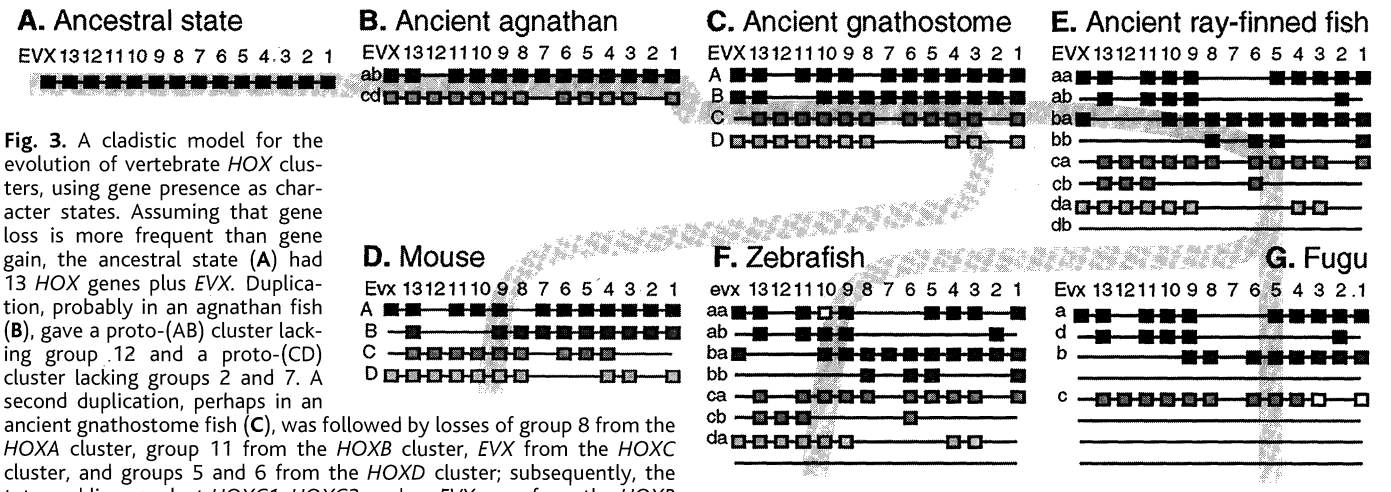

Fig. 3. A cladistic model for the evolution of vertebrate *HOX* clusters, using gene presence as character states. Assuming that gene loss is more frequent than gene gain, the ancestral state (**A**) had 13 *HOX* genes plus *EVX*. Duplication, probably in an agnathan fish (**B**), gave a proto-(AB) cluster lacking group 12 and a proto-(CD) cluster lacking groups 2 and 7. A second duplication, perhaps in an ancient gnathostome fish (**C**), was followed by losses of group 8 from the *HOXA* cluster, group 11 from the *HOXB* cluster, *EVX* from the *HOXC* cluster, and groups 5 and 6 from the *HOXD* cluster; subsequently, the tetrapod lineages lost *HOXC1*, *HOXC3*, and an *EVX* gene from the *HOXB* cluster (**D**). Finally, an apparent duplication event produced eight clusters in a ray-finned fish (**E**), followed by further shared and unique losses in zebrafish (**F**) and *Fugu* (**G**) lineages.

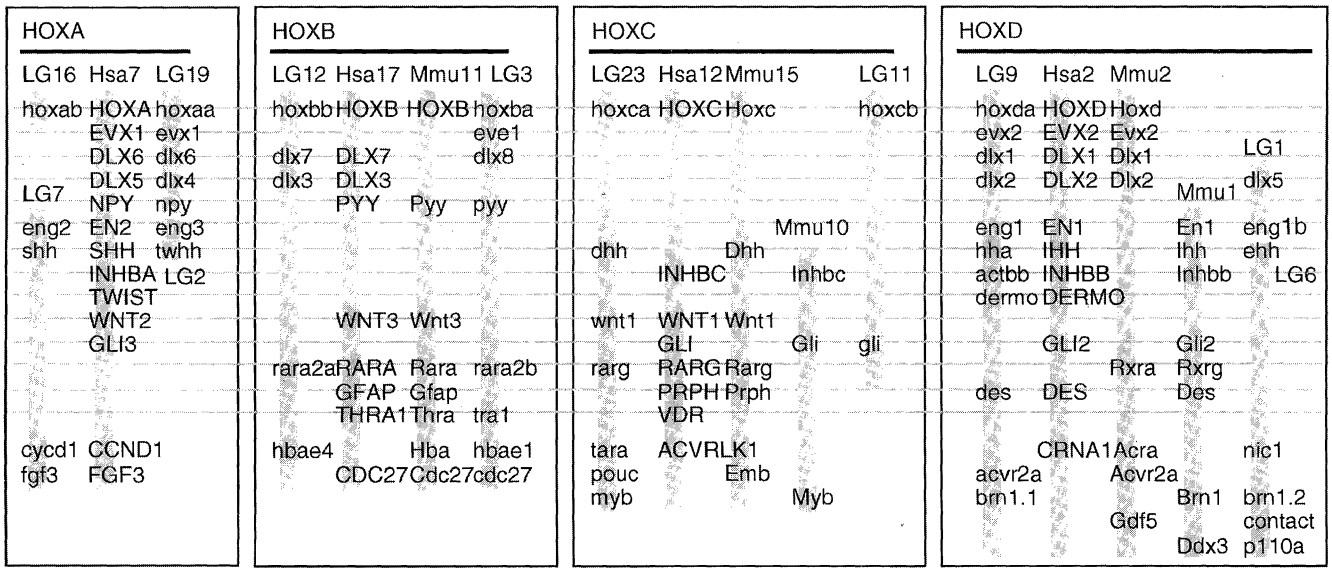

Fig. 4. *HOX* cluster duplication involved large chromosome segments. The diagram shows syntenic relationships among *HOX* containing chromosomes of human (Hsa), mouse (Mmu), and zebrafish linkage groups (LG). Vertical gray lines indicate a group of genes on the same chromosome (syntenic loci), with order ignored to facilitate the comparison of orthologs and paralogs. Horizontal gray lines connect presumed orthologs within chromosome groups as well as paralogs between chromosome groups.

experienced an "extra" duplication event. This suggests that a fish-specific *HOX* cluster duplication occurred before the divergence of *Fugu* and zebrafish lineages more than 150 million years ago (*15*), but after the divergence of ray-finned and lobe-finned lineages. Goldfish, salmonids, and some other teleosts have experienced additional, more recent polyploidization events (*16*). Genomic analysis of basally branching ray-finned fish, such as sturgeons, *Amia*, or *Polypterus*, is necessary to clarify the timing of the *HOX* duplication event.

To determine whether "extra" fish *hox* clusters result from tandem duplication or chromosome duplication in fish, or cluster loss in tetrapods, we mapped zebrafish *hox* clusters; cloned, sequenced, and mapped four new genes whose orthologs are syntenic with *HOX* clusters in mammals (*dhh*, *evx1*, *eng1b*, and *gli*); and mapped four previously unmapped zebrafish genes [*dlx5*, *dlx6*, *dlx8*, and *pl10a*; see (*11*)] whose orthologs are linked to *HOX* clusters in mammals. These experiments showed that zebrafish has two copies of each *HOX* chromosome segment in mammals (Fig. 4). For example, the human and mouse *HOXB* chromosomes have six and four genes, respectively, whose apparent orthologs map on one of the two zebrafish chromosomes containing *hoxba* or *hoxbb* (Fig. 4). Each of these two chromosomes also has one copy of other duplicate genes, including *dlx7/dlx8*, *rara2a/rara2b*, and *hbae4/hbae1* (*11, 17*). We conclude that zebrafish has two copies of this mammalian chromosome segment. Because similar results were obtained for the other clusters (Fig. 4), we infer that *hox* cluster duplication in ray-finned fish occurred by whole chromosome duplication. Although we found a single *hoxd* cluster in zebrafish, mapping experiments identified the predicted duplicate chromosome segments (Fig. 4), suggesting secondary loss of one *hoxd* duplicate.

These results suggest two rounds of *HOX* chromosome duplication (probably whole genome duplication) before the divergence of ray-finned and lobe-finned fishes, and one more in ray-finned fish before the teleost radiation. Because gene duplicates often have a subset of the functions of the ancestral gene (*18*), mutations in duplicate genes may reveal essential functions that otherwise might remain hidden. For example, if a gene is essential for distinct early and late functions, a lethal mutation knocking out the early function might obscure the late function in a mutant mammal, but both functions would be evident if the two functions assort to different zebrafish gene duplicates. The conclusion that the genetic complexity of *hox* clusters in teleost fish has exceeded that of mammals for more than 100 million years calls into question the concept of a tight linkage of *HOX* cluster number and morphological complexity along the body axis. However, because teleosts are the most species-rich group of vertebrates and exhibit tremendous morphological diversity, it is tempting to speculate that the duplication event detected here may have provided gene copies that helped spur the teleost radiation.

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9. DNA pools (plate pools, then row and column pools) from an arrayed zebrafish PAC library (*19*) were screened with redundant primers (Hox-F: 5′-CGNAAGAARMGNGTNCCNTAYAC-3′; Hox-R: 5′-CATNCKNCKRTTYTGRAACCANAT-3′; Hox-Fi: 5′-CTNGARYTNGARAARGARTTYCA), where N is A, C, T, or G, R is A or G, M is A or C, Y is C or T, and K is T or G. Ends of positive PACs were sequenced and specific primers used to find overlapping clones. Positive PACs were amplified with redundant primers; products were cloned and sequenced, and gene specific primers were used to obtain sequence directly from PAC DNA.
10. Unambiguously alignable sequences were obtained using CLUSTAL X (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and trees were generated by the neighbor-joining method [N. Saitou and M. Nei, *Mol. Biol. Evol.* **4**, 406 (1987)]. A lamprey (*Petromyzon marinus*) cDNA library screened with redundant *hox* gene primers provided an outgroup. For accession numbers, see (*11*).
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Regulation of the Proinflammatory Effects of Fas Ligand (CD95L)

Jian-Jun Chen, Yongnian Sun, Gary J. Nabel*

Fas ligand (CD95L) inhibits T cell function in immune-privileged organs such as the eye and testis, yet in most tissues CD95L expression induces potent inflammatory responses. With a stably transfected colon carcinoma cell line, CT26-CD95L, the molecular basis for these divergent responses was defined. When injected subcutaneously, rejection of CT26-CD95L was caused by neutrophils activated by CD95L. CT26-CD95L survived in the intraocular space because of the presence of transforming growth factor–β (TGF-β), which inhibited neutrophil activation. Providing TGF-β to subcutaneous sites protected against tumor rejection. Thus, these cytokines together generate a microenvironment that promotes immunologic tolerance, which may aid in the amelioration of allograft rejection.

The CD95 protein (also called Fas or APO-1) is a cell surface receptor that activates the death signaling pathway in cells. Its physiological ligand, CD95L, can transduce this signal upon cell contact (*1*). The CD95-CD95L system has been implicated in the clonal deletion of autoreactive lymphocytes in peripheral lymphoid tissues and in the elimination of autoreactive lymphocyte populations (*2*), thus contributing to homeostasis