Reports

Evolution of the Hemoglobin S and C Genes in World Populations

Abstract. *A polymorphic* Hpa *I endonuclease recognition site on the 3′ side of the β-globin gene was used to analyze the evolution of the β-globin gene mutants S and C. Study of the worldwide distribution of the normal and variant* Hpa *I sites showed that the mutation which resulted in the variant 13.0-kilobase fragment arose in a localized region in West Africa. It predated the hemoglobin S and C mutations, both of which arose separately from a chromosome with the variant 13.0-kilobase* Hpa *I site. In contrast, the sickle genes in other parts of Africa and in Asia are associated with the normal 7.6-kilobase* Hpa *I fragment, indicating that the sickle mutations in these other areas arose separately from that in West Africa.*

Restriction endonuclease analyses have expanded our knowledge of the molecular structure and arrangement of the normal and mutant human globin genes (*1–4*), and have also revealed that variations in DNA sequences near the globin genes occur frequently (*2–5*). These variations appear to be common in the noncoding region of the DNA, and they provide a new class of markers for genetic analysis (*5*). Previously we described a polymorphism in an *Hpa* I endonuclease recognition site adjacent to the β-globin structural gene (*5*), and demonstrated its usefulness in the prenatal diagnosis of sickle cell anemia (*6*). We have now studied the relation of this polymorphic *Hpa* I site to the hemoglobin S and hemoglobin C genes in various world populations, and have derived from these data new information on the evolution of these mutations.

When normal human DNA is digested with the restriction endonuclease *Hpa* I, the β-globin gene is contained in a DNA fragment 7.6 kilobases (kb) long (Fig. 1). The polymorphic *Hpa* I recognition site we detected was on the 3′ side of the β-globin gene, approximately 5000 nucleotides away from that gene (*5*). We found two variants: one where the *Hpa* I site was located closer to the β-globin gene, yielding an *Hpa* I fragment 7.0 kb long; and another where the *Hpa* I site was farther away from the β-globin gene, resulting in a fragment 13.0 kb long (*7*). The genotypes of the β-globin fragments in a diploid cell could thus be classified as 7.6/7.6, 7.6/7.0, 7.6/13.0, 13.0/13.0, and the like.

We initially found the *Hpa* I polymorphism in DNA obtained from American black subjects (Table 1). Whereas normally the β-globin gene is contained in the 7.6-kb fragment and occasionally in the 7.0-kb fragment, it is rarely associated with the 13.0-kb fragment. In contrast, the majority of the β-globin genes from individuals with the sickle mutation are associated with the 13.0-kb fragment, and less frequently with the 7.6-kb and the 7.0-kb fragment. From the data of Table 1, we calculated by the gene-counting method (*8*) that in the American black the frequency of association of the A gene with the 7.6-kb fragment (A-7.6) is 0.879, A-7.0 is 0.087, and A-13 is 0.034 whereas the frequency of S-7.6 is 0.306, S-7.0 is 0.015, and S-13.0 is 0.679. The normal and variant *Hpa* I fragments are inherited in a Mendelian manner. The *Hpa* I sites appear to be tightly linked to the β-globin gene, since we have so far not observed crossover in the families studied.

Since the majority of American blacks came from West Africa, it seemed plausible that the mutation which resulted in the 13.0-kb variant originated there. We therefore studied the relationship between the *Hpa* I site and hemoglobin C, a β-globin gene mutation that occurs frequently in West Africa in a geographically more confined area than hemoglobin S (*9–11*). Studies of American black subjects with hemoglobin CC, AC, and SC mutations showed that the 13.0-kb *Hpa* I fragment is also frequently associated with the C gene (*5*, *12*) (Table 1).

The β^S and β^C mutations affect the codon GAG (G, guanine; A, adenine) which encodes for glutamic acid at the sixth amino acid of the β-globin chain (*13*). The β^S mutation is encoded by GUG (U, uracil) (the codon for valine), and the β^C mutation by AAG (lysine). The β^S and β^C genes most likely arose independently from the β^A gene in that a mutation of A to C or A to S would each involve only one nucleotide change, while a S ↔ C mutation would necessitate the improbable event of two nucleotide changes per triplet. Since both the S and C genes are linked to the 13.0-kb *Hpa* I variant, we concluded that the mutation which produced this *Hpa* I variant

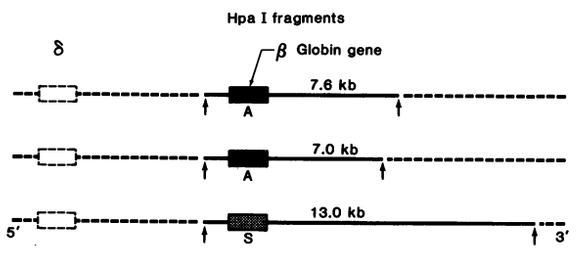

Fig. 1. The three types of DNA fragments containing the β-globin gene. The variations in size are due to changes in the *Hpa* I site on the 3′ side of the β-globin gene. In the American black population, the 7.6- and 7.0-kb fragments are usually associated with the A genotype, and the 13.0-kb fragment is associated with the S genotype.

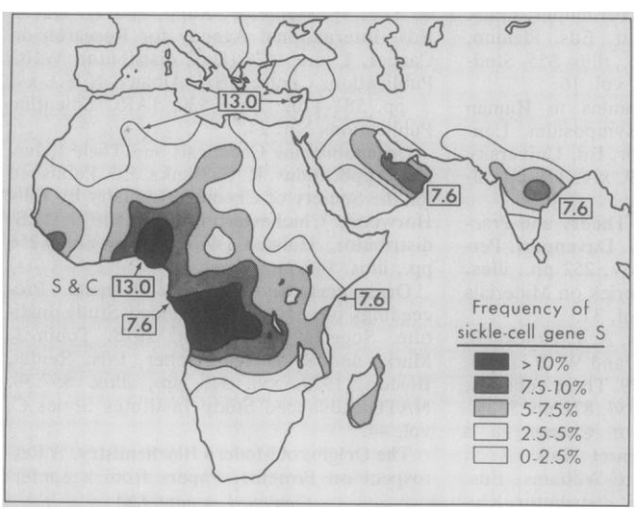

Fig. 2. Geographic distribution of the *Hpa* I β-globin gene containing fragments and their relationship to the hemoglobin S and C genes. The figure of the frequency of sickle gene is adapted from Cavalli-Sforza and Bodmer (*11*).

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arose before the mutations of hemoglobins S and C, both of which then arose separately from a chromosome containing the 13.0-kb *Hpa* I site. Further study is needed to clarify which of the two β-globin mutations arose first.

The sickle mutation is found in a large geographic area which includes Africa, the Mediterranean region, and Asia (*9–11*). To determine whether the *Hpa* I genotypes in these areas are similar to those in West Africa, we extended our studies to Sicily, Cyprus, Gabon, Kenya, Saudi Arabia, and India (*14*) (Table 1). The 13.0-kb fragment is present in both Mediterranean islands. In Sicily, where more samples were studied, the association between the 13.0-kb fragment and the sickle gene is high. In contrast, the sickle gene was associated with the 7.6-kb and not the 13.0-kb fragment in the four other areas we studied—Gabon in West Africa, Kenya in East Africa, Saudi Arabia in the Middle East, and the Valmiki, Khonda Reddi, and Koya Dora tribes of India in Asia (*15*).

There has been much debate as to whether the sickle gene in world populations arose from a single or from multiple mutational events (*16, 17*). We could use the *Hpa* I site to analyze this question. There are two possible explanations for the association of the 7.6-kb and 13.0-kb *Hpa* I fragments with the sickle gene: (i) the sickle gene arose as a single mutational event and the variant *Hpa* I sites resulted from a crossover of the DNA in the region between the β-globin gene and the *Hpa* I recognition site; or (ii) the sickle mutation occurred more than once; once on a chromosome with the 13.0-kb recognition site, as in West Africa, and at least once on a chromosome with the 7.6-kb recognition site in other geographic areas (*18*). The studies in India are particularly informative in this regard. The Valmiki, Khonda Reddi, and Koya Dora tribes in southern India are geographically isolated. Previous studies of blood and serum types in the Khonda Reddi and Koya Dora, and in other southern Indians with the sickle gene, showed little or no similarities to blood and serum types in Africans (*19*). This lack of similarity in these genetic markers, together with the fact that the *Hpa* I sites, tightly linked to the β^S gene, are also different, favors the conclusion that the Indian and West African sickle mutations arose as separate events. The fact that separate mutational events produced the same abnormal hemoglobin is not surprising in light of our present knowledge of spontaneous mutations in the unstable hemoglobin syndromes, where multiple mutational events producing the same amino acid substitution have been well described (*20*). The similarity in the *Hpa* I sites does not distinguish between the possibility that the sickle genes in Kenya and Saudi Arabia arose separately from the Indian, or that they were derived from the same origin, as has been postulated (*16*). It may be useful to search for other polymorphic restriction endonuclease sites in these populations to answer this question.

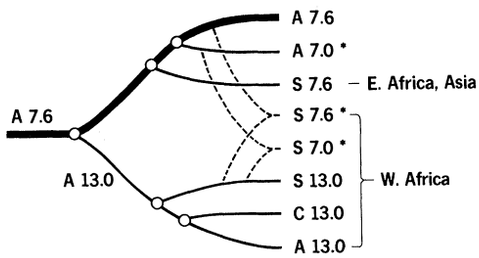

Fig. 3. Evolution of the *Hpa* I site and the β-globin genes. The letters A, S, and C represent the β-globin genotypes and the numbers indicate the lengths of the β-globin gene containing *Hpa* I fragments in kilobases. No time scale is implied in this diagram. The divergence of the S gene before the C gene indicated in this diagram is arbitrary. Asterisks denote where the precise mode of evolution is not certain; —, normal; —, variants; and - - -, recombinants.

These data on the geographic distribution of the 13.0- and 7.6-kb fragments associated with the S and C gene (Fig. 2) allowed us to draw a preliminary evolutionary tree of the *Hpa* I site and its relation to the A, S, and C hemoglobin genes (Fig. 3). In most sectors of the world population, the β-globin gene is normally contained in a 7.6-kb *Hpa* I fragment. In West Africa, a mutation affecting the *Hpa* I recognition site on the 3′ side of the β^A-globin gene produced a 13.0-kb fragment (A-13.0). The S and C mutations arose separately, each from a chromosome that contained the variant 13.0-kb *Hpa* I site (S-13.0 and C-13.0). A higher proportion of 13.0-kb fragments is associated with the S and C genes than with the A gene because the S and C genes protected against malaria, and hence increased the frequency of S-13.0 and C-13.0. The incidence of A-13.0 remained low because this association did not offer any selective advantage. The increase in incidence of the 13.0-kb *Hpa* I fragment, due to its close linkage to the S and C gene, is an example of "genetic hitch-hiking" (*21*).

The fact that the S and C genes are both associated with the 13.0-kb *Hpa* I fragment localizes the origin of the mutation that gave rise to the 13.0-kb fragment to a small geographic area in the

Table 1. The *Hpa* I β-globin gene fragment in several populations with hemoglobins S and C.

Hemoglobin type	No.	*Hpa* I β-globin pattern					Association of hemoglobin S or C genes with the 13.0-kb fragment
		7.6/7.6	7.6/7.0	7.0/13.0	7.6/13.0	13.0/13.0	
United States							Present
AA	43	30	9	0	4	0	
AS	86	29	3	4	49	1	
SS	58	4	0	2	22	30	
CC	5	0	0	0	0	5	
AC	4	0	0	0	3	1	
SC	15	0	0	0	6	9	
Sicily							Present
AA	3	2	0	0	1	0	
AS	7	0	0	0	7	0	
Sβ-thal	6	0	0	0	6	0	
SS	2	0	0	0	0	2	
Cyprus							Present
AA	6	5	1	0	0	0	
AS	2	1	0	0	1	0	
Gabon							Absent
AS	9	9	0	0	0	0	
Kenya							Absent
AA	7	5	1	0	0	1	
SS	8	8	0	0	0	0	
Saudi Arabia							Absent (*15*)
AA	2	2	0	0	0	0	
SS	4	4	0	0	0	0	
India							Absent
AA	17	16	1	0	0	0	
AS	34	29	1	0	0	0	
SS	3	3	0	0	0	0	

Upper Volta or Ghana, where the C gene is highly concentrated (*11*). In this area, the frequency of the 13.0-kb fragment is expected to be high and studies in Nigeria show that, indeed, the 13.0-kb fragment is very frequently associated with the S gene (*22*). In Gabon, just south of Nigeria and Ghana, the sickle gene is already associated with the 7.6-kb fragment. The Algerian sickle gene has also been associated with the 13.0-kb *Hpa* I fragment (*15*). While additional studies in other countries of North Africa are necessary, it seems likely that the sickle gene with the 13.0-kb *Hpa* I site spread from the Ghana-Nigeria area through North Africa to the Mediterranean islands.

The sickle genes in other parts of Africa, Saudi Arabia, and India, all predominantly associated with the 7.6-kb *Hpa* I site, most likely arose as one or more mutational events separately from the West African sickle gene. As the 7.6-kb fragment is the normal pattern in worldwide populations, the selective advantage of the sickle gene for malaria is not adequate to raise appreciably the proportion of S-7.6 relative to A-7.6, and S-7.6 remains low compared to A-7.6.

The evolution of the other *Hpa* I genotypes is less certain. For example, the 7.0-kb variant could have arisen from a chromosome that contained either the 7.6-kb or the 13.0-kb *Hpa* I site. Other combinations, such as the association of the 7.6-kb site or 7.0-kb site with the S gene, could be the result of separate mutations or of recombination. The classification of these genotypes will necessitate further studies of their distribution in areas of the world where there is a frequency of these *Hpa* I variants.

Polymorphism of restriction endonuclease sites has already provided a new approach for prenatal diagnosis by analysis of the linkage of normal and variant restriction sites to structural genes (*6*). Thus, the *Hpa* I polymorphism can be utilized for the intrauterine detection of sickle cell anemia. Previously, the common β-globin chain abnormalities, β-thalassemia and sickle cell anemia, were diagnosed in utero primarily with fetal blood sampling and analysis of the type or quantity of β-globin chain synthesized in the fetal blood (*23*). The acquisition of fetal blood is a complex procedure, requiring specially trained physicians and elaborate and sophisticated equipment. It also carries a 5 to 10 percent risk to the fetus. In those families where the polymorphic *Hpa* I site is associated with the sickle gene, it is now possible to use the well-established procedure of amniocentesis for prenatal diagnosis (*6*). Amniotic fluid cells are fetal in origin, and DNA can be isolated from these cells for restriction endonuclease analysis. The technique is very sensitive. At the 16th week of gestation, when amniocentesis is usually performed, enough DNA can be isolated directly from the cells without the necessity of cell culture in most cases.

Thus, restriction endonuclease site polymorphism constitutes a new class of genetic marker for studying human diseases. The *Hpa* I polymorphism has provided new information regarding the evolution of the hemoglobin S and C genes and has furnished strong evidence for the multicentric origin of the sickle gene. Detailed studies of the interrelation between the structural mutants and the restriction endonuclease sites in localities where the variants occur frequently will undoubtedly provide additional information on the evolution and migration of these genes in Africa, Europe, and Asia.

It has been suggested that the frequency of the 13.0-kb *Hpa* I fragment varies in different parts of the United States [(*5*, *12*) and this report]. This needs to be confirmed by studying a larger number of subjects. If the difference is real, the *Hpa* I polymorphism may also serve as a marker for tracing the origin and migration of the black population in North America.

The polymorphism that we discovered has provided a useful means for linkage analysis of the sickle gene. In about 70 percent of the pregnancies at risk in the American black population, it is now possible to diagnose sickle cell anemia prenatally with the relatively simple and well-established method of amniocentesis. In localities such as Nigeria and Ghana in West Africa, where the 13.0-kb *Hpa* I fragment originated, an even higher proportion of the sickle gene is expected to be linked to the 13.0-kb fragment, making prenatal diagnosis by amniocentesis possible in most pregnancies at risk. Several other polymorphic restriction endonuclease sites around the β-globin gene region have been reported: *Hind* III sites in the intervening sequence of the $^{G}\gamma$ and $^{A}\gamma$ gene, a *Pst* I site in the intervening sequence of the δ gene, and a *Bam* HI site in the sequence 3′ to the β-globin gene (*1–3*, *24*). The *Bam* HI site can be used for linkage analysis and diagnosis of certain patients with β-thalassemia (*24*), while the *Hind* III sites for sickle-cell anemia and β-thalassemia (*25*). By using a combination of restriction endonuclease sites, it is possible that the globin disorders can be diagnosed in a large number of cases.

An increasing number of human genes are being isolated with recombinant DNA technology and more and more genomic DNA's are being isolated from libraries of cloned human DNA. It will be possible to use polymorphism of restriction endonuclease sites to study the evolution of many genes, to characterize and diagnose other genetic disorders, and to map human variations.

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Survival of *Escherichia coli* Host-Vector Systems in the Mammalian Intestine

Abstract. *Survival in the mouse and human intestine of* Escherichia coli *host-vector systems used and proposed for recombinant DNA technology was assessed. There was no detectable survival of severely disabled* E. coli *K12 strain χ1776 in mice or in human subjects 24 hours after ingestion. The same strain bearing the plasmid pBR322, however, was recovered from human subjects for 4 days in amounts of six organisms for every million ingested. Nondisabled* E. coli *K12 strain χ1666, with or without pBR322, survived in 10^4-fold greater numbers and for 2 days longer, with better recovery of the plasmid-containing derivative. Although the plasmid-bearing strains were recovered for longer periods, no intestinal colonization was noted. Despite the presence of pBR322 for a maximum of 6 days in the human intestine, there was no evidence that it was transferred from either bacterial host to endogenous aerobic fecal bacteria.*

Certain *Escherichia coli* host-vector systems, designated EK2(HV2), have been approved for use in recombinant DNA research. Mutations in both host and vector have been introduced so as to improve biologic containment (*1*-*5*). One particular host cell, χ1776, developed by Curtiss and colleagues (*1*) was derived to be unable to survive outside the defined laboratory environment. It is sensitive to bile acid, resistant to nalidixic acid, and multiply auxotrophic (Dap^-, Thy^-, Met^-, Thr^-, Bio^-). Plasmid vectors, such as pBR322 (*2*), have been constructed that are incapable of directing transfer of themselves to other bacterial hosts and are also poorly transferred by other plasmids.

We have reported the recovery of χ1776 from mice for 2 days after inoculation by different parenteral routes (*6*). The mean lethal dose (LD_{50}) was 10^3- to 10^4-fold greater than that of naturally occurring *E. coli*, but not markedly different from that of a laboratory *E. coli* K12 strain. In the present studies, we compared in mice and in human subjects the survival in the intestinal tract of χ1776 and its plasmid pBR322-bearing derivative, χ2236, with that of a conventional laboratory EK1 strain, *E. coli* K12, χ1666 (Ara^-, Nal^R) (*7*) and its pBR322-bearing derivative, D20-5.

When doses of 5×10^9 χ1776 cells were given by gastric intubation to ten germ-free mice, no intestinal colonization was found. The organism was detected only in fecal samples analyzed within the first 24 hours after inoculation. Similarly no intestinal colonization was found with χ2236 given in food, by gastric intubation, or by rectal administration. In contrast, χ1666 and D20-5 colonized in germ-free mice at 10^9 cells per gram of feces. Inoculations of 5.2×10^9 cells of χ1666 did not lead to colonization of the intestinal tract of conventional mice; cells were recovered only up to 3 days after gastric intubation.

Studies in human subjects were performed on groups of four male volunteers housed in the Clinical Study Unit of Tufts-New England Medical Center Hospital. They were permitted to leave the room only once each day for 1 to 2 hours. No physical contact with other individuals was permitted. An attachment on the toilet ensured that all fecal samples would be collected in an autoclavable plastic bag. These samples were immediately placed in the refrigerator in the room until being taken for analysis.

In the first experiment, three of four individuals drank 1.9×10^9 viable cells of χ1776 in milk on two separate occasions, 6 hours apart. After each dose, skin, nose, and throat swabs were cultured in supplemented eosin-methylene blue broth (EMB) with nalidixic acid (Nal) or on agar plates (EMB-Nal) (*8*) to identify the presence of the ingested organism. Each fecal sample was processed within 1 hour of passage. It was weighed, mixed thoroughly in the plastic bags, and sampled on EMB-Nal agar to determine the number of endogenous and ingested *E. coli* and enterococci per gram of feces.

Up to 1 hour after ingestion, χ1776 was detected in the nose and throat;

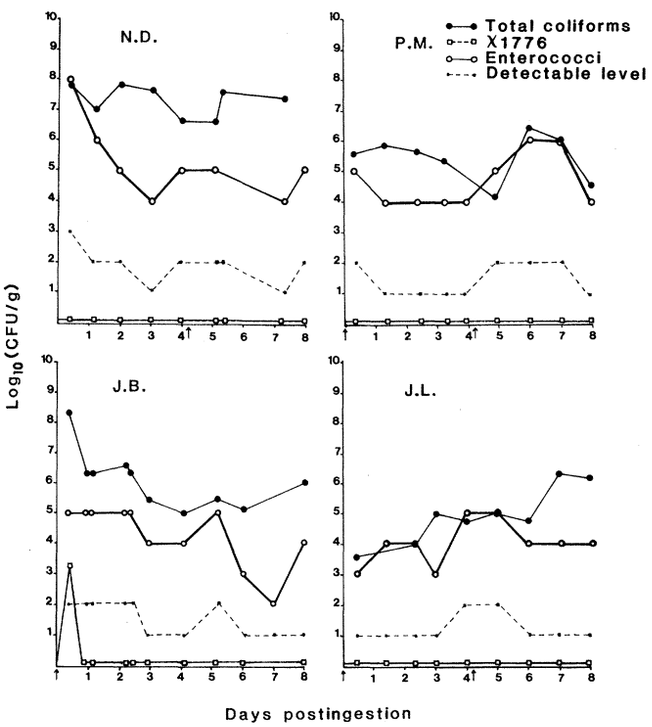

Fig. 1. Survival of χ1776 after the ingestion of 3.8×10^9 organisms on day 0 and 1.5×10^{10} organisms on day 4 (arrow). The minimal detectable level for each sample is designated by the dashed line. The ordinate is number of colony-forming units (*CFU*) of bacteria per gram (wet weight) of feces.

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