cluster and the bound biotin residue, or about 12 Å (*8*). Because the avidin molecule itself is small and lacks identifiable structural features, we did not attempt to relate the position of the gold cluster to any specific feature of the protein structure.

Further applications of gold cluster labeling are likely to involve larger molecules in which the positions of labeled sites must be determined relative to features of the biological structure itself; effective resolution will be limited by radiation damage to the biological structure. With the use of negatively stained specimens and reduced electron doses, resolution in the range of 15 to 20 Å should be obtainable. The combination of specific labeling with heavy-metal cluster compounds and detection by STEM offers a powerful new method for studying the locations and interactions of subunits and functional sites in biological macromolecules.

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Hemoglobin Parchman: Double Crossover Within a Single Human Gene

Abstract. *Structural analysis of a new variant hemoglobin revealed tryptic peptides with the amino acid composition of normal δ-globin, except for two internal peptides, which had the compositions of normal β-globin. The most likely explanation for these findings is that a double, nonhomologous crossover between the δ- and β-globin genes had occurred.*

The closely linked human δ- and β-globin genes are located on the short arm of chromosome 11 (*1*) and are expressed in the ratio 1:40 (*2*). Nonhomologous crossing-over between these genes results in a family of fusion genes that produce the Lepore and Miyada type hemoglobins. The Lepore hemoglobins are characterized by globin chains with a δ-amino terminus, a β-carboxyl terminus, and reduced synthesis, resembling that seen in normal δ-globin synthesis (*3*). The reciprocal products of the Lepore hemoglobin genes, the Miyada type hemoglobins, have β-amino and δ-carboxyl termini and also exhibit reduced synthesis (*2*). We found, in a black patient, an abnormal hemoglobin that appeared to have arisen as a result of a double nonhomologous crossing-over between the δ- and β-globin genes. The hybrid globin chain had a δ-amino terminus, an internal β-chain fragment, and a δ-carboxyl terminus. This hemoglobin, which we call hemoglobin Parchman, is the only fusion hemoglobin gene which, on the basis of protein structure, unequivocally contains the intervening sequence 1 (IVS-1) of the β-globin gene, and thus may offer additional insight into the role of untranslated DNA in the differential expression of the δ- and β-globin genes.

The patient was not anemic, had no evidence of hemolytic disease, and had very mild microcytosis (mean corpuscular volume, 79 fl). There was no evidence of hemoglobin instability. There was an imbalance in globin chain synthesis with a deficit of α chain relative to β chain ($\alpha/\beta = 0.75$), consistent with the presence of α-thalassemia. Gene mapping studies indicated that the patient had heterozygous α-thalassemia 2, since digestion of cellular DNA with the restriction endonuclease Eco RI yielded two DNA fragments, 19 and 23 kilobase pairs (kbp) long, containing α-globin genes after hybridization with a complementary DNA probe for α-globin (*4*). Hemoglobin studies showed that hemoglobin A_2 was lower than normal (1.2 percent) and hemoglobin F was elevated (4.5 percent). The reason for the increase in hemoglobin F is not known. An additional hemoglobin fraction migrated in the same position as sickle cell hemoglobin (hemoglobin S) on alkaline electrophoresis and eluted near

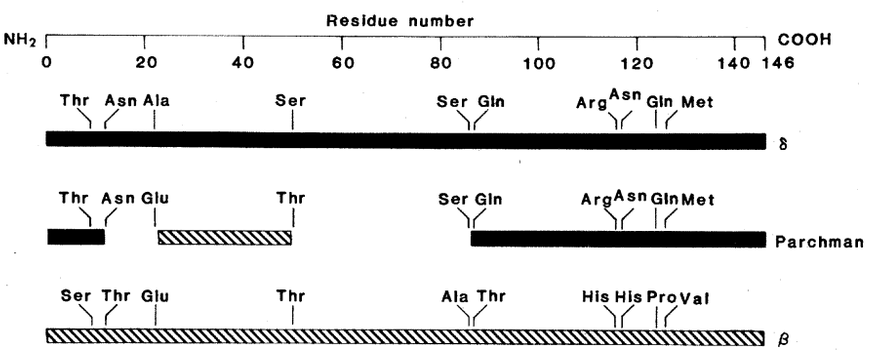

Fig. 1. The position of the pertinent amino acids of hemoglobin Parchman. The δ chain differs from the β chain by ten amino acids. The positions of these amino acids are shown for the δ chain, which is the non-α chain of hemoglobin Parchman, and for the β chain. Hemoglobin Parchman was purified by DEAE-Sephadex column chromatography (*10*), and its constituent chains were separated by chromatography on columns of *O*-(carboxymethyl)cellulose (*11*). The purified non-α chain was digested with trypsin, and the resulting peptides were separated by chromatography on Beckman PA-35 resin and by high-performance liquid chromatography (HPLC) (*12*). Peptides were repurified by column chromatography on Bio-Rad AG50W × 2 resin and by HPLC. Purified peptides were hydrolyzed in 5.7*M* HCl for 22 and 72 hours and analyzed on a Dionex amino acid analyzer with DC-4A resin and *O*-pthalaldehyde fluorescence detection. The amino acids shown are Thr, threonine; Asn, asparagine; Ala, alanine; Ser, serine; Arg, arginine; Gln, glutamine; Met, methionine; Glu, glutamic acid; His, histidine; Pro, proline; and Val, valine. Automatic sequence analysis of T-3 revealed the sequence of Val-Asn-Val-Asp-Glu (Asp, aspartic acid), demonstrating glutamic acid at position 22.

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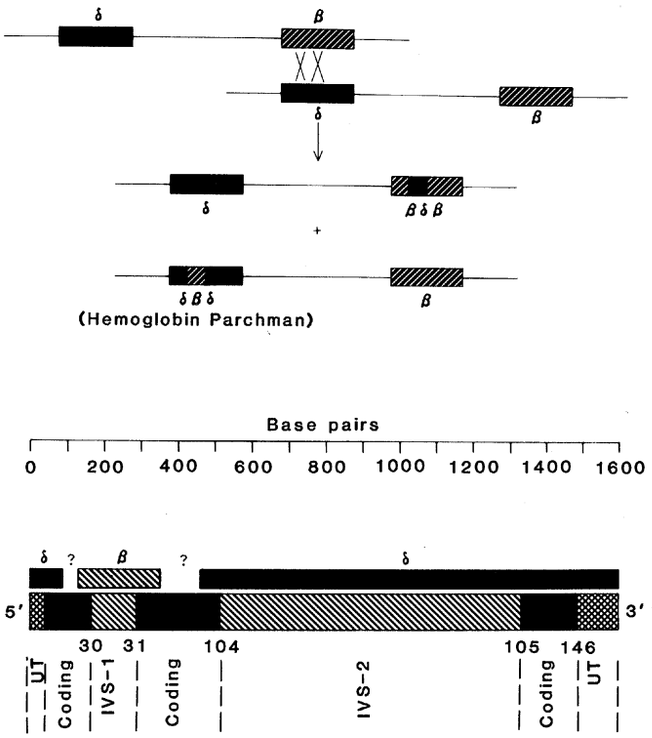

Fig. 2. Origin of the hemoglobin Parchman non–α-globin gene by double nonhomologous crossing-over between δ- and β-globin genes. The reciprocal chromosome is also shown. An expanded diagram of the gene for the non-α chain of hemoglobin Parchman depicting the intervening sequences IVS-1 and IVS-2 and untranslated flanking sequences (*UT*) is shown in the lower panel.

the position of hemoglobin S after DEAE-Sephadex column chromatography. This hemoglobin constituted 1.6 percent of the hemolyzate. Hybridization with canine hemoglobin revealed that the abnormality of this fraction was in the non–α-globin chain.

The human δ-globin chain differs from the β-globin chain by ten amino acids. These ten amino acids are distributed in seven tryptic peptides (T-2, T-3, T-5, T-10, T-12b, T-12c, and T-13). The amino acid compositions of the purified tryptic peptides were those expected from a normal δ-globin chain except for the two internal peptides T-3 and T-5, which had the compositions expected for a normal β-chain (Fig. 1). The simplest explanation of these results is that a double nonhomologous crossover had taken place (Fig. 2), the first occurring between the codons for amino acid residues 12 and 22 and the second between the codons for residues 50 and 86. An alternative, but less likely, explanation is that hemoglobin Parchman is a δ-globin variant with two separate amino acid substitutions.

An analysis of known restriction endonuclease sites in and surrounding the δ- and β-globin genes indicates that a double crossover, such as we propose, would not delete known restriction sites or add new ones that would allow the hemoglobin Parchman gene to be detected by gene mapping (*5*, *6*). For example, when the proband's DNA was cleaved with the restriction endonuclease Xba I—which cuts outside of the δβ gene complex—and analyzed with a plasmid probe containing DNA complementary to the β gene (*4*), only the usual 10.8-kbp fragment was seen. This result, as expected, indicated no major genetic additions or deletions in this complex.

The δβδ gene of hemoglobin Parchman appears to share most of the properties of the normal δ-globin gene. When the proband's peripheral blood was incubated with [^{3}H]leucine, the purified hemoglobin Parchman constituted 0.9 percent of the total hemoglobin radioactivity. Chain separation of purified hemoglobin Parchman revealed that all of the radioactivity was associated with the α chain. Thus, the δβδ chain of hemoglobin Parchman, like the δ chain of hemoglobin A_2, the δβ chain of the Lepore hemoglobins, and the βδ chain of the Miyada type hemoglobins, is not synthesized in reticulocytes (*7*, *8*). The output of the δβδ gene is similar to that of the normal δ-globin gene and is somewhat less than that observed for the Lepore and Miyada type hemoglobins (*2*, *3*). Since the affected chromosome does not contain a normal δ-globin gene, the result is a half-normal level of hemoglobin A_2. The β-globin gene of the affected chromosome is intact and therefore there is no deficit in β-chain synthesis. In contrast, the Lepore hemoglobin chromosome lacks a normal δ gene, which accounts for the low hemoglobin A_2 levels, and the normal β gene is also absent, producing the phenotype of β-thalassemia. In the Miyada type hemoglobins, both a normal δ- and normal β-globin gene are present on the affected chromosome, but there is evidence suggesting that the β-globin gene when *cis* to the βδ-globin gene is capable of only limited output (*8*). The mild microcytosis and low ratio of α to β biosynthesis in our patient are due to heterozygous α-thalassemia 2, a condition prevalent in blacks but incidental to the presence of the δβδ gene in this case.

On the basis of the sites of this double crossover, which were deduced from the protein structure, the hybrid globin gene contains the IVS-1 of the β-globin gene, which falls between the codons for amino acid residues 30 and 31 (*6*). The 5′ untranslated sequences flanking the coding portions of the gene and containing putative promoter regions and the origin of gene transcription, as well as the 3′ untranslated DNA containing the polyadenylate addition site, are those of the δ-globin gene.

Although the nucleotide sequence of the δ-globin gene has been completed (*6*), its examination does not provide an explanation for the low level of expression of this gene relative to that of the β-globin gene from which it arose. Hybrid genes such as this δβδ gene and the Lepore δβ genes may provide additional insights into the role of intervening sequences and untranslated DNA in the differential expression of the δ- and β-globin genes. The Lepore and Miyada type hemoglobins are synthesized in reduced amounts. Hemoglobin Parchman is the only fusion hemoglobin that, on the basis of its protein structure, unequivocally has a β-IVS-1 (*9*). Yet its total synthesis approximates that expected from a single δ-globin allele and in the heterozygote is considerably lower than that found in Lepore or Miyada type hemoglobins. The presence of δ-IVS-1 is, therefore, alone insufficient to explain the reduced synthesis of the δ-globin gene. Since the Lepore and Miyada type hemoglobins as well as hemoglobin Parchman are all synthesized in reduced amounts, it would appear likely that more than one region of untranslated DNA is responsible for the low output of the δ-globin gene.

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Angiogenesis Induced by "Normal" Human Breast Tissue: A Probable Marker for Precancer

Abstract. *Normal human breast lobules, freshly isolated by precision microdissection of tissue stained with methylene blue chloride, were assayed for their ability to induce neovascularization (angiogenesis) in rabbit irises. Histologically, normal lobules from cancerous breasts induced angiogenesis twice as often as lobules from noncancerous breasts, suggesting that preneoplastic transformation is diffuse.*

The ability to evoke blood vessel formation (angiogenesis) is a common property of malignant tissue, including that of the breast (*1*). Without the benefit of neovascularization, tumors can grow only to spheroids 1 to 2 mm in diameter, the limit of nutriment diffusion (*2*). Most cancers and epithelial hyperplasias of the breast are believed to originate in the hormone-sensitive lobules located on the branching duct system that drains secretions to the nipple (Fig. 1A) (*3*, *4*). Although normal tissues are rarely angiogenic when transplanted to a suitable host (*5*), we now report that normal-appearing lobules from cancerous breasts are significantly more angiogenic than lobules from noncancerous breasts (*6*).

Multiple random samples were obtained 3 cm or more from the invasive ductal carcinoma in mastectomy specimens from 26 patients and from benign breast tissue in biopsy material from 34 patients. About 12 hours or less after surgery the excised tissues were cut into 2-mm-thick slices and submerged in methylene blue chloride (1 mg per 100 ml) at 4°C for 90 minutes (*7*). The blue-stained lobules (Fig. 1B) were then separated from the surrounding collagenous stroma under a dissecting microscope.

The lobules were transplanted onto the irises of New Zealand White female rabbits (2 kg) under anesthesia (*8*). The anterior chamber of selected eyes had been drained, causing the cornea to collapse. This pressed the transplant against the iris, where it adhered. Seventy-two percent of the lobules were ~ 1 mm in diameter and were transplanted in toto; of the bigger lobules, 1-mm fragments were transplanted. Since the excised breast tissues were originally handled without strict aseptic technique, they were rinsed in four batches of sterile saline before being cut into smaller pieces. Otherwise, aseptic technique was used throughout. The irises were observed daily for 5 days with a magnifying lens for the appearance of delicate capillaries growing toward the transplant (Fig. 1, C and D). On day 5 the rabbits were killed and the preparations were fixed and processed for light microscopic examination (Fig. 1E).

As shown in Table 1, 44 of the 155 normal-appearing lobules from cancerous breasts were angiogenic by day 5 (28 percent), compared to 46 of 307 lobules from noncancerous breasts (15 percent) ($P < .001$). Angiogenesis was observed sooner in lobules from cancer patients. By day 3, 21 percent of the lobules from cancerous breasts had elicited new vessel formation, compared to only 7 percent of the lobules from noncancerous breasts ($P < .0001$).

Lobules in older women tend to be smaller than those in younger women. Therefore, the data were analyzed to evaluate the effect of age and lobule size on angiogenesis. The analysis showed no significant effect (Table 2).

Usually the mastectomy specimens arrived at the laboratory in aseptic condition and the tissue used in the experiments was removed with sterile techniques. Therefore, the reported differences in angiogenicity between the two types of specimens cannot be attributed to bacterial contamination. Furthermore, the transplants did not display an acute inflammatory response.

To determine whether differential delays between excision and transplantation played a role in the observed differ-

Table 1. Angiogenicity of histologically normal lobules from cancerous and noncancerous breasts. Yates' correction for continuity was applied in making the statistical comparisons.

Breast type	Number of lobules eliciting angiogenesis	
	Three days after transplantation	Five days after transplantation
Cancerous	32 of 155 (21 percent)	44 of 155 (28 percent)
Noncancerous	21 of 307 (7 percent)*	46 of 307 (15 percent)†

*Significantly different from corresponding value for cancerous breast lobules [χ^2 (1) = 17.992, $P < .0001$]. †χ^2 (1) = 10.958, $P < .001$.

Table 2. Effects of patient age and lobule size on the angiogenicity of histologically normal lobules from cancerous and noncancerous breasts. Values in parentheses are percentages. Differences are not statistically significant.

Lobule source	Number of lobules eliciting angiogenesis			
	Age of patient		Lobule size	
	< 50 years	> 50 years	≤ 1 mm	> 1 mm
Cancerous breasts	26 of 95 (27)	18 of 60 (30)	30 of 112 (26)	14 of 43 (32)
Noncancerous breasts	43 of 279 (15)	3 of 28 (10)	33 of 224 (14)	13 of 83 (15)

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