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Genetics

Gene deletions in α thalassemia prove that the 5′ ζ locus is functional

(ζ globin genes/embryonic hemoglobin/hemoglobin Bart's hydrops fetalis)

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ABSTRACT **The deletions in the ζ-α globin gene cluster in two infants with the hemoglobin Bart's hydrops fetalis syndrome (homozygous α thalassemia 1) have been mapped by restriction endonuclease analysis using a ζ-specific probe. DNA from a Thai infant lacked the ψα1 gene and both α genes, but the ζ genes were present. A Greek infant's DNA had also lost the 3′ ζ1 gene. Because ζ globin was synthesized in the infant's cord blood, this indicates that the 5′ ζ2 gene recently identified by Lauer *et al.* [Lauer, J., Shen, C. J. & Maniatis, T. (1980) *Cell*, in press] must be functional.**

During normal human development the embryonic hemoglobins Gower 1 ($\zeta_2\epsilon_2$), Gower 2 ($\alpha_2\epsilon_2$), and Portland ($\zeta_2\gamma_2$) are found in significant amounts only before about 10 weeks of gestation (1, 2). Analysis of the hemoglobin of embryos of various gestational ages shows a coordinated change-over from ζ and ε to α and γ chain synthesis at about the time when the liver replaces the yolk sac as the main site of fetal erythropoiesis (3). At birth only trace amounts (about 0.1%) of hemoglobin Portland can be detected in normal infants (4).

Recently Lauer *et al.* (5) have shown by analysis of cloned human DNA that there are two ζ genes and an α-like pseudogene (ψα1) linked together within a 21-kilobase (kb) region 5′ to the two normal α genes situated on chromosome 16 (6). However, nothing is known about the functional properties of these ζ genes or about the regulation of the ζ-α gene complex.

Globin gene deletions have recently been shown to be the cause of several of the thalassemias, diseases characterized by defective hemoglobin synthesis (for review see ref. 7). Two of the most common forms of α thalassemia are of this type: α thalassemia 2, which results from a deletion of one of the α globin genes (−α/), and α thalassemia 1, which results from a loss of both of them (−−/) (8–11). In the homozygous state for α thalassemia 1, the hemoglobin Bart's hydrops fetalis syndrome, all four α genes are deleted (−−/−−), precluding synthesis of any normal fetal ($\alpha_2\gamma_2$) or adult ($\alpha_2\beta_2$) hemoglobin. Infants with this condition are stillborn or die within a few hours of birth and their hemoglobin consists mainly of hemoglobin Bart's (γ_4) with about 10–20% hemoglobin Portland (5, 12). This is the only condition known in which embryonic ζ chain synthesis persists beyond the yolk sac stage of development.

In order to learn more about the function of the two ζ globin genes and to determine the extent and likely cause of the deletions that produce α thalassemia 1, we have analyzed the DNAs of Greek and Thai infants with the hemoglobin Bart's hydrops fetalis syndrome. In the Thai infant both α genes and the ψα1 gene were deleted, leaving the two ζ genes intact, whereas in the Greek infant the 3′ ζ1 gene had also been removed. Because this infant produced hemoglobin Portland ($\zeta_2\gamma_2$), this is conclusive proof that the 5′ ζ2 gene identified by Lauer *et al.* (5) is a functional gene.

MATERIALS AND METHODS

Hemoglobin Analysis. Hematological studies were carried out by using standard techniques. Hemoglobin electrophoresis was performed in starch gels buffered by Tris/EDTA/borate, pH 8.6 (13).

Restriction Endonuclease Analysis. Spleen and buffy coat DNA was prepared by phenol/chloroform extraction (14). DNA (15 μg) was digested with 15–20 units of the restriction endonucleases *Bam*HI, *Bgl* II, *Eco*RI, *Hin*dIII, *Hpa* I, or *Kpn* I in the appropriate buffer (as specified by the supplier) at 37°C for approximately 24 hr. The digested DNA was then electrophoresed in 0.8% agarose gels and transferred to nitrocellulose filters by a modification of the method of Southern (15). The DNA on the filters was hybridized with a ^{32}P-labeled probe for 2–3 days and then washed under stringent conditions (11) and autoradiographed for 1–7 days.

Preparation of Probes. *α and β probes.* α and β probes were prepared by nick-translation of α plasmid JW101 and β plasmid JW102 (16) by a modification of the method of Maniatis *et al.* (17).

ζ probe. ζ probe was prepared by nick translation of a 5′ end fragment isolated from the plasmid pBRζ (5). Plasmid DNA (12 μg) was digested with 20 units of *Hin*cII at 37°C for 24 hr, and then with 20 units of *Pvu* II at 37°C for 24 hr. This mixture was then extracted with phenol and precipitated with ethanol prior to end-labeling with [α-^{32}P]dGTP. The restriction endonuclease-treated plasmid DNA and 1 μl buffer (0.1 M Tris·HCl, pH 7.4/0.1 M $MgCl_2$/0.5 M NaCl/0.01 M dithiothreitol) were added to the vacuum-dried [α-^{32}P]dGTP (25 μCi of [α-^{32}P]dGTP, 400 Ci/mmol, purchased from the Radiochemical Centre, Amersham, England; 1 Ci = 3.7×10^{10} becquerels) together with 1 unit of *Escherichia coli* DNA polymerase Klenow (Boehringer Mannheim) in a final reaction volume of 10 μl, and the mixture was incubated for 15 min at room temperature. The reaction mixture was then loaded onto a 1-mm 6% acrylamide gel (made up in 90 mM Tris/90 mM boric acid/2.5 mM EDTA, pH 8.3) and run in gel buffer at 20 V/cm for about 3 hr at room temperature. The gel was autoradiographed and the region of gel corresponding to the fastest band on the autoradiograph was cut out and eluted at room temperature in 600 μl of 0.3 M sodium acetate for 16 hr. A small sample of the eluate was run on a 0.8% agarose gel with *Hin*dIII digested λ markers to size the eluted fragment. The fragment was calculated to be 0.365 kb, which is in good agreement with the 0.350 kb expected from the restriction map of the pBRζ (J. Lauer, personal communication). The remainder of the eluted DNA was precipitated with ethanol. The nick-translation re-

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Abbreviations: kb, kilobase(s); HPFH, hereditary persistence of fetal hemoglobin.

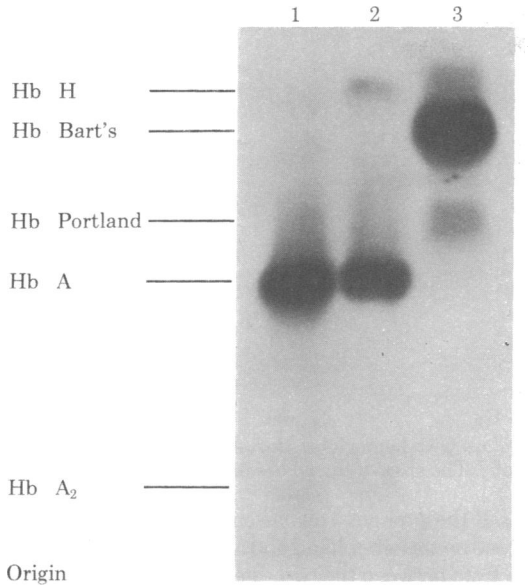

FIG. 1. Starch gel electrophoresis of hemolysates from: lane 1, a normal adult; lane 2, an individual with hemoglobin H disease; lane 3, the cord blood of the Greek infant with hemoglobin Bart's hydrops fetalis.

action was carried out as for α and β probes except that no DNase was added.

RESULTS

Patients and Hemoglobin Analysis. The studies described here were carried out on two infants with the hemoglobin Bart's hydrops fetalis syndrome. One was of Greek origin, and the hemoglobin constitution and hematological and globin chain synthesis data from the parents (to be presented elsewhere) indicated that they both had α thalassemia 1. The hemoglobin composition of the infant's cord blood was typical of homozygous α thalassemia 1 ($--/--$); starch gel electrophoresis (Fig. 1) showed mainly hemoglobin Bart's, a small amount of hemoglobin H (β_4), and about 10% hemoglobin Portland (verified by the identification of specific ζ-chain peptides). Moreover, no α chains were synthesized in cord blood cells incubated with [^{3}H]leucine. The other infant, from Thailand, had the clinical and hematological findings and hemoglobin constitution typical of the hemoglobin Bart's hydrops syndrome as it occurs in Southeast Asia (18).

Restriction Endonuclease Analysis. DNA from the Greek infant and both parents was digested with *Bam*HI, an enzyme that produces a 14-kb fragment containing both α genes from normal DNA (19). When the parents' DNA was analyzed by blot hybridization (15), using a ^{32}P-labeled α-specific probe, a single band of 14.0 kb was demonstrated, which is consistent with a heterozygous α thalassemia 1 genotype ($--/\alpha\alpha$) (11). Similar hybridization analysis of the infant's DNA produced no α-specific bands at all, confirming the absence of normal α genes. In particular, there was no evidence of the small α gene fragment that has been described in a few Mediterranean patients with α thalassemia (11, 20).

Table 1. Sizes of ζ-specific fragments obtained after restriction endonuclease digestion of DNA from the Greek and Thai infants

Enzyme	Fragment size, kb		
	Normal	Greek infant	Thai infant
*Bam*HI	5.9	5.9	5.9
	10.8		≈20
Bgl II	11.3	13.9	
	12.6		
*Eco*RI	5.0	5.0	5.0
	≈23		17.2
*Hin*dIII	13.5	13.5	13.5
	16.5		≈20
Hpa I	11.7	11.7	11.7
			13.0
Kpn I	11.0	11.0	
	23.5		

A map of the physical organization of the ζ and α gene cluster has been constructed by Lauer *et al.* (5) (Fig. 2). Blot hybridization of restriction enzyme digests of normal DNA with a ^{32}P-labeled 350-base-pair DNA fragment ("ζ probe") isolated from a cloned genomic fragment containing the 3′ ζ1 gene [pBRζ (5)] gave the pattern of ζ-specific fragments listed in Table 1. These results are in good agreement with the fragment sizes predicted from the map shown in Fig. 2.

Digestion of the Greek infant's DNA with five of the enzymes listed in Table 1—*Eco*RI, *Hin*dIII, *Bam*HI, *Kpn* I, and *Hpa* I—gave only a single ζ-specific band in each case. These fragments were exactly the same size as those containing the 5′ ζ2 gene obtained from normal DNA with these enzymes (Fig. 3), showing that this gene is present in the hydrops DNA and that the restriction sites for these enzymes immediately to the right of the ζ2 gene have been maintained. Because no other ζ-specific bands were seen, the 3′ ζ1 gene that is present in a second fragment in digests of normal DNA must be deleted. Digestion with *Bgl* II gave a single fragment of approximately 13.9 kb, slightly larger than either of the two fragments obtained from normal DNA (12.6 and 11.3 kb) and indicating that the *Bgl* II site to the right of the 5′ ζ2 gene has been removed. The deletion thus starts between the *Hpa* I and the *Bgl* II sites situated between the ζ genes and extends beyond the two α genes. It must therefore span at least 14.5 kb. Furthermore, the first *Bgl* II site to the right of the ζ–α gene cluster is 7.4 kb from the *Bgl* II site between the α genes (21). If this site defines the 3′ end of the 13.9-kb fragment obtained from the hydrops DNA, the deletion extends for 17.4 kb (i.e., 12.6 + 11.3 + 7.4 − 13.9).

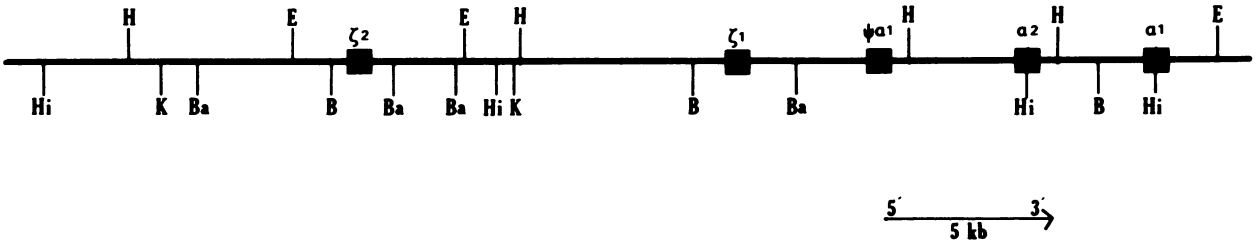

FIG. 2. The restriction endonuclease map of the ζ–α globin gene cluster and surrounding DNA (5). B, *Bgl* II; Ba, *Bam*HI; E, *Eco*RI; H, *Hpa* I; Hi, *Hin*dIII; K, *Kpn* I.

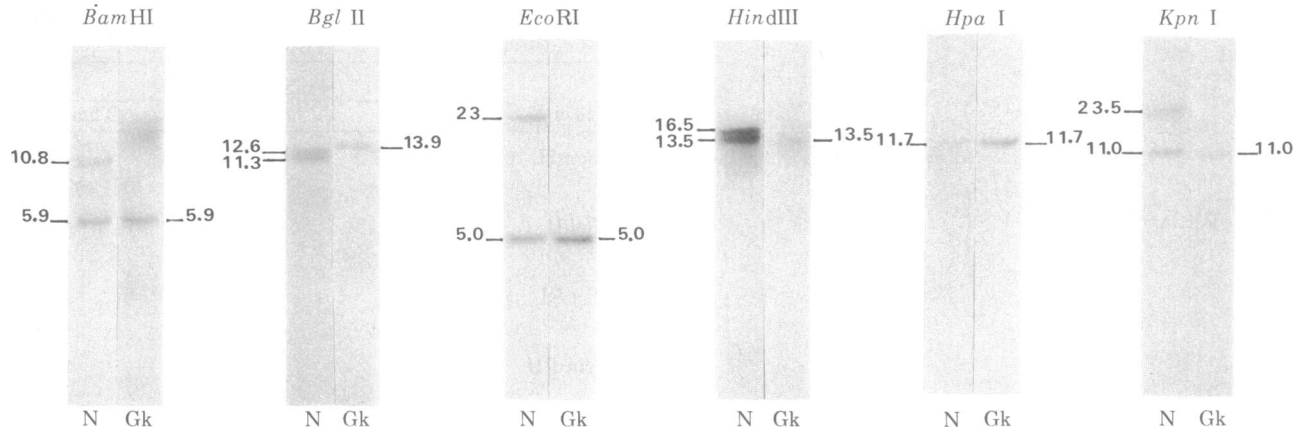

FIG. 3. Autoradiograph of DNA from a normal individual (N) and the Greek infant with hemoglobin Bart's hydrops fetalis (Gk) digested with the six restriction endonucleases shown and hybridized with the ζ-specific probe. The sizes of the DNAs in the bands are shown in kb.

DNA from the Thai infant gave a different pattern of restriction endonuclease fragments. Digestion with *Bam*HI, *Eco*RI, *Hpa* I, and *Hin*dIII gave two ζ-specific bands (Fig. 4, Table 1). In each case the smaller fragment was the same size as the 5′ fragment containing the ζ2 gene obtained from normal DNA. Because the ζ genes are not cut by these enzymes (5), the presence of these two fragments indicates that the 5′ ζ2 gene and at least part of the 3′ ζ1 gene are maintained. None of these enzymes produced a second ζ-specific fragment of the same size as that containing the ζ1 gene obtained from normal DNA with these enzymes, so the *Bam*HI, *Eco*RI, *Hpa* I, and *Hin*dIII sites 3′ to the ζ1 gene must all have been removed. Thus the breakpoint of the deletion must lie between the ζ1 gene and the *Bam*HI site to the right of this gene (Fig. 2).

In normal DNA the ζ1 and ζ2 genes are each found in 11.7-kb *Hpa* I fragments. However, restriction endonuclease digestion of the Thai DNA gave one fragment of 11.7 kb and a new fragment of 13.0 kb, presumably containing the ζ1 gene. In normal DNA the first *Hpa* I site to the right of the α1 gene is 18.8 kb from the site between the ψα1 and α2 genes (which defines the 3′ end of the ζ1 fragment) (5, 11). The minimum size of the deletion in the Thai DNA is thus 17.5 kb (i.e., 11.7 + 18.8 − 13.0).

Both the Greek and Thai deletions may have arisen by an unequal crossing-over mechanism similar to that which has produced the hemoglobin Lepore and α thalassemia 2 genes (11, 22). The distance between the ζ2 gene and the ψα1 gene is about 17 kb (Fig. 2), the estimated minimum sizes of the deletions. If the ψα1 gene on one chromosome had aligned with the ζ2 gene on the other hand and a crossover occurred between the *Hpa* I site between the two ζ genes on one chromosome and the *Bgl* II site to the 3′ side of the α genes on the other, a contracted chromosome would be formed with only the ζ2 gene remaining, as in the Greek infant (Fig. 5). In the Thai infant, the crossover would have to have occurred between the *Hin*dIII site to the right of the α genes and the *Bam*HI site to the right of the ζ1 gene on the other chromosome (Fig. 5). However, the evidence presented here does not preclude variations of this crossover model—for example, alignment of ζ2 with α1—nor can a more complicated double-crossover event be excluded.

DISCUSSION

The results presented here provide clear evidence that the ζ2 gene identified by Lauer *et al.* (5) is functional. Furthermore, we have shown that the α thalassemia 1 haplotypes in these Greek and Thai cases of the hemoglobin Bart's hydrops syndrome are characterized by different gene deletions, although it seems possible that they may both have arisen by the same mechanism of unequal crossing-over between misaligned ζ and ψα1 genes as discussed above (Fig. 5).

All the infants with the hemoglobin Bart's hydrops syndrome that have been adequately studied to date have had significant amounts of hemoglobin Portland in their cord blood (4, 12, 18). In the present study it is clear that in the Greek infant the 3′ ζ1

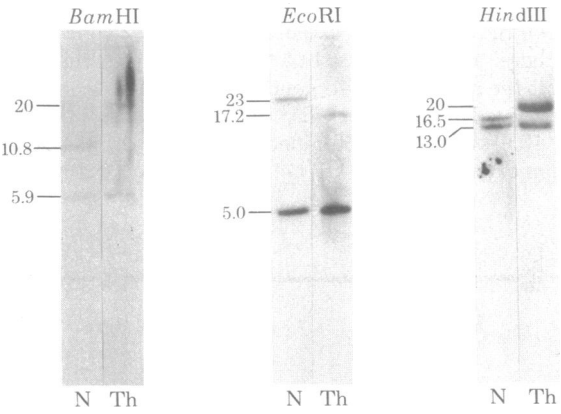

FIG. 4. Autoradiograph of DNA from a normal individual (N) and the Thai infant with hemoglobin Bart's hydrops fetalis (Th) digested with the three restriction endonucleases shown and hybridized with the ζ-specific probe.

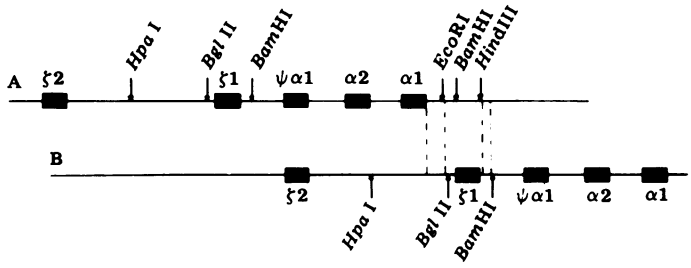

FIG. 5. A hypothetical model for the formation of the α thalassemia 1 deletions described in the text, showing only the relevant restriction endonuclease sites. The ψα1 gene on chromosome A has misaligned with the ζ2 gene on chromosome B. In the Greek infant the crossover must occur between the α1 gene on chromosome A and the *Bgl* II site shown on chromosome B, whereas in the Thai infant the crossover could occur between the ζ1 gene and the *Bam*HI site to the right of this gene on chromosome B. The actual site is further limited, by the result of the *Hin*dIII digest, to between the *Hin*dIII site on chromosome A and the *Bam*HI site on chromosome B.

globin gene has been deleted along with the $\psi\alpha 1$ and both α genes. Because hemoglobin Portland was found in the cord blood of this infant, the 5′ $\zeta 2$ gene must be functional.

Lauer *et al.* (5) have determined the nucleotide sequence of part of the $\zeta 1$ gene. The amino acid sequence predicted from this agrees exactly with the peptide compositions of residues 72 to 95 of ζ chains isolated from normal embryonic hemoglobin (unpublished data). It seems likely, therefore, that this gene codes for a protein that is similar if not identical to normal embryonic ζ globin. Furthermore, all the available evidence (2, 4, 23) indicates that the structures of the ζ chains of hemoglobin Portland obtained from infants with the hemoglobin Bart's hydrops syndrome and the ζ chains of hemoglobin Gower 1 from normal embryos are the same. Thus either the two ζ genes are very similar or the 3′ gene is normally inactive. The more likely possibility is that both are functional. Obviously more information about the $\zeta 1$ and $\zeta 2$ gene sequences and the structures of the ζ chains produced in normal and α thalassemic embryos is needed to clarify this point.

It is interesting that the ζ genes, which are normally inactive after about 10 weeks of gestation, remain functional in the hemoglobin Bart's hydrops syndrome. It seems unlikely that this is a nonspecific result of severe intrauterine anemia because the other embryonic hemoglobin gene, the ϵ gene, does not remain active; a careful search has failed to demonstrate either hemoglobin Gower 1 or Gower 2 in this condition (4, 12, 18).

There is an interesting parallel between this phenomenon and the continued activity in adult life of the γ chain genes in some forms of hereditary persistence of fetal hemoglobin (HPFH) and $\delta\beta$ thalassemia, which are also caused by substantial deletions of the genome 3′ to the active gene (see ref. 7 for review). Clearly it will be important to characterize other examples of the hemoglobin Bart's hydrops syndrome because, again by analogy with HPFH, it seems likely that different deletions in the α gene cluster may give rise to a variety of related phenotypes depending on the precise location and extent of the deletions. Furthermore, if this interpretation of the mechanism for persistent ζ chain production is correct, one would anticipate that heterozygous carriers for α thalassemia 1, or individuals heterozygous for both α thalassemia 1 and 2—i.e., those with hemoglobin H disease—should have detectable levels of hemoglobin Portland, although in much smaller amounts than those observed in the hemoglobin Bart's hydrops syndrome, because in this case the ζ chain production would be derived from only a single chromosome. It would also follow that any cases of α thalassemia 1 or hemoglobin H disease that are not associated with deletion defects would not produce hemoglobin Portland.

Despite the fact that α thalassemia 1 is known to occur relatively frequently in Mediterranean races, we know of only one previous report of the hemoglobin Bart's hydrops syndrome in this population (24). Hence it seems possible that the case we have studied is in some way atypical, perhaps because it involves a comparatively rare α thalassemia 1 haplotype. If there were a more common form in this population due to a defect involving both ζ genes, no hemoglobin Portland could be synthesized in homozygotes, a situation not compatible with fetal survival.

In any case it is clear that the deletion forms of α thalassemia 1 are heterogeneous and this offers an excellent opportunity for analyzing the regulation of the ζ and α globin genes in a manner exactly analagous to the way in which the various deletions in the γ–δ–β globin gene cluster can be related to the HPFH and $\delta\beta$ thalassemia phenotypes.

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