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RESTRICTION ENDONUCLEASE ANALYSIS OF HUMAN GLOBIN GENES IN CELLULAR DNA

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Summary: Using samples of human cellular DNA digested with restriction endonucleases Eco RI, Hind III, Hinc II, Bam HI, Alu I, or Hae III, we were able to localize globin gene fragments separated by agarose gel electrophoresis. The fragments were transferred to nitro-cellulose filters and identified by hybridization to [^{32}P]cDNA for total adult globin mRNA. The α-globin gene fragments were specifically identified by their presence in normal controls and absence in DNA from homozygous α-thalassemia, a genetic disorder due to deletion of α-globin genes. In addition, the patterns with Hind III indicate a 4.1 kb distance between the centers of the normal duplicated α-globin gene loci.

The inherited group of human disorders called the thalassemia syndromes are characterized by absent or decreased synthesis of one or more of the globin chains of hemoglobin [1]. Recent studies using hybridization of radioactive globin cDNA to sheared cellular DNA in solution have indicated that some patients with α-thalassemia, hereditary persistence of fetal hemoglobin and β-thalassemia have deletions of DNA sequences for specific structural globin genes which account for lack of production of the corresponding proteins [2-6]. In contrast, in β^{o}-thalassemia, where there is absent synthesis of the β-globin chain, the structural genes for β-chain are present [6,7].

The development of methods for localizing specific DNA sequences by restriction endonuclease digestion and subsequent gel electrophoresis [8] provides a new approach to the study of human genetic diseases. These techniques have been used for detection, sizing, mapping and cloning of mouse and rabbit globin DNA sequences [9-11]. Using similar methods, we have com-

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pared restriction endonuclease digests of normal human DNA to DNA from an infant with hydrops fetalis due to homozygous α-thalassemia. Our studies using several restriction endonucleases identify the fragments containing globin genes and demonstrate specific deletion of α-gene fragments in homozygous α-thalassemia.

MATERIALS AND METHODS

Globin mRNA was prepared using peripheral blood containing 60 per cent reticulocytes from a 23 year old man with pyruvate kinase deficiency. Globin synthesis studies [12] of his reticulocytes showed equal α- and β-chain production. The globin mRNA was prepared by a sequence of acid precipitation, phenol-chloroform extraction, double passage through oligo-dT cellulose and sucrose density gradient centrifugation, using modifications of methods previously described [13]. The resulting poly[A] containing 9-11S RNA was used as a template for oligo$(dT)_{12-18}$-primed synthesis of radioactive cDNA by reverse transcriptase from avian myeloblastosis virus [14], with [^{32}P]GTP(2-3000 Ci/mmole; Amersham Corp., Illinois) as the source of radioactivity.

High molecular weight DNA was isolated [15] from the spleen of a fetus with hydrops fetalis due to homozygous α-thalassemia and from spleens removed from children with hemolytic anemia. The DNA was cleaved with an excess of a specific restriction endonuclease in each reaction at 37°C for 24 hours, using buffer conditions described by the enzyme suppliers (New England Biolabs, Inc. and Bethesda Research Labs, Inc.). Solutions containing 10-20 μg of DNA fragments in 20 μl aliquots were separated by electrophoresis in a 1% neutral, horizontal agarose gel employing a tris-acetate buffer system [16]. Electrophoresis was performed at 20 milliamps constant current for 16-18 hours at room temperature. The DNA samples were placed so that normal and α-thalassemia samples treated with the same enzyme were in adjacent wells. Restriction endonuclease fragments from λ and ϕX174 phage DNA were used as molecular weight markers. Following electrophoresis the DNA fragments were denatured with alkali, neutralized and transferred to a cellulose nitrate filter as described by Southern [8]. The DNA on the filters was annealed for 24-48 hours at 65°C with 1 to 5ng/ml of high specific activity globin cDNA, and the filters were subsequently washed [8]. Radioactive bands were located by radioautography with Kodak XRP-1 film and Dupont Cronex Hi-Speed intensifying screens.

RESULTS AND DISCUSSION

Since analysis of hybridization in solution has demonstrated deletion of α-globin DNA sequences in homozygous α-thalassemia [2,3], any globin specific fragments found in restriction endonuclease digests of normal DNA but absent in α-thalassemia DNA may be identified as containing α-globin genes. Figure 1 shows the patterns of globin specific fragments obtained after digestion with various endonucleases. Distinct differences between α-thalassemia and controls were found with Eco RI, Hind III, Hinc II, Bam HI and Alu I, but not with Hae III. A single 20.5 kb α-globin DNA fragment

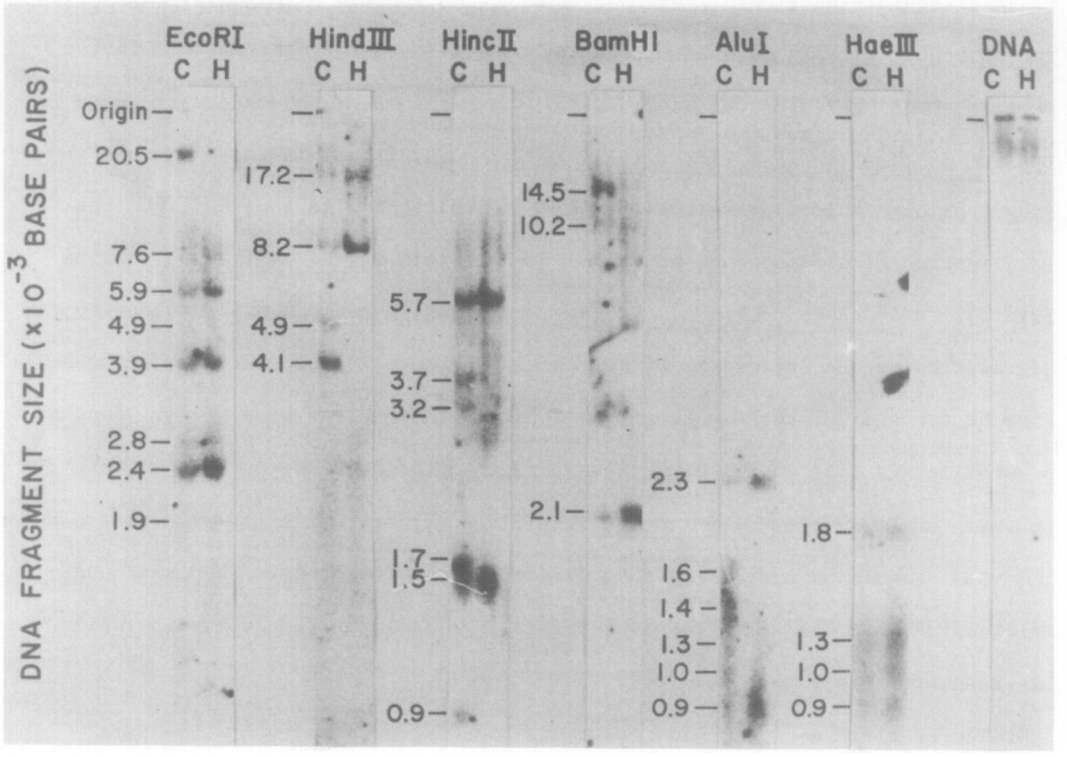

Figure 1. Autoradiographic detection of globin genes in restriction endonuclease digests of human DNA. DNA from a control (C) and a fetus with homozygous α-thalassemia (H) was cleaved with various restriction endonucleases (Eco RI, Hind III, Bam HI, Alu I and Hae III). The resultant DNA fragments were separated by electrophoresis on a 1% agarose gel [16] and transferred to cellulose nitrate filters as described by Southern [8]. Radioactive cDNA against globin mRNA from an adult was used for detection of DNA fragments containing globin gene sequences as described in Materials and Methods. The DNA specimens on the electrophoretic strip on the right are from undigested samples.

is present in the Eco RI digest of normal DNA but absent in the α-thalassemia pattern. This finding differs from that described in a recent report which noted two α-chain fragments, 8.7 and 7.8 kb, in Eco RI digests of normal DNA [17]. In that report the authors state that a large amount of DNA hybridizing to their radioactive probe was found close to the origin when electrophoresis was performed on neutral gels and that the use of an alkaline gel system resolved this problem. We observed no such difficulties in employing a neutral gel system. In the previous study [17] a large amount of low molecular weight

material containing globin genes was observed in the Eco RI digests, suggesting the presence of contaminant nucleases or alkali-induced fragmentation; in the present study only discrete bands containing globin gene sequences were seen in the Eco RI digests (Fig. 1, Eco RI lanes). In addition, in the present study the high molecular weight of DNA before digestion is apparent from the localization of globin genes adjacent to the origin in the undigested samples (Fig. 1, "DNA" lanes on right). These variations in methods which relate to degradation of DNA during the experimental procedure may account for the presence of two smaller α-chain fragments found in this recent study [17]. Another possibility is that an RNA linker is present in the 20.5 kb Eco RI fragment. If DNA with an RNA linker were treated with alkali prior to agarose gel fractionation, the RNA would be hydrolyzed to generate two DNA fragments. The existence of alkali-labile RNA sites in mitochondrial DNA isolated from mammalian cells has been previously reported [18].

The specific α-globin gene fragments found after digestion with other enzymes (Fig. 1), identified by differences in the patterns of normal and α-thalassemia DNA, are the following: Hind III (4.9, 4.1 kb), Hinc II (3.7, 3.2, 1.7 and 0.9 kb), Bam HI (14.5 kb) and Alu I (1.6, 1.4, 1.3 kb). There is a clear difference in intensity of the two Hind III α-globin DNA bands in normal DNA, with the 4.1 much darker than the 4.9 kb band, indicating hybridization to more α-globin cDNA molecules. A variety of genetic data [19] and results of cDNA-driven hybridization experiments [20] indicate that most humans studied have duplication of α-globin loci. The most likely explanation for our finding of unequal intensity of α-globin fragments is that there are twice as many separate α-globin sequences in the 4.1 kb band than in the 4.9 band. Since Hind III cuts at the center of double-stranded α-globin cDNA [21,22], the two α-globin loci are therefore approximately 4.1 kb apart, measured from their centers. The enzyme should produce at least three α-globin DNA bands, while we have only identified two. The third may have been small enough to migrate off the gel (less than about 500 bp), or it may overlap other globin

DNA bands.

The identification of β-globin DNA fragments is complicated by cross-hybridization between radioactive β-globin cDNA and DNA sequences for δ- and γ-globin. The cDNA in this study was prepared using total globin mRNA from an adult. This mRNA contains α- and β-chain molecules in approximately equal amounts, with little or no γ- or δ-chain mRNA. However, there is extensive amino acid sequence homology between β- and δ-globin chains, and cDNA-driven hybridization reactions indicate that β-cDNA will hybridize to β- and δ-genes [2,5]. There is less amino acid sequence homology between β- and γ-chains, and hybridization studies show only slight cross-hybridization [13]. Because of cross-hybridization of β- and δ-globin DNA, each of the 5.9, 3.9 and 2.4 kb bands present in the Eco RI digests of both normal and α-thalassemia DNA presumably contain either β- or δ-globin genes, or both. The putative β- and δ-gene fragments detected here are similar in size to those recently reported using a partially purified β-globin cDNA probe (6.6, 4.5, 3.0 kb)[17]. The less intense bands (7.6, 4.9, 2.8, 1.9 kb) in the Eco RI DNA digests [Fig.I] may represent cross-hybridization of radioactive β-cDNA to γ-globin genes, or hybridization of ribosomal cDNA contaminants [23-24] to multiple copy rDNA genes. The major bands in the other digests which represent β- and δ-globin genes are the following: Hind III (17.2, 8.2 kb), Hinc II (5.7, 1.5 kb), Bam HI (10.2, 2.1 kb) and Alu I (2.3, 1.0, 0.9 kb). Since Hae III fragments of 1.8, 1.3, 1.0 and 0.9 kb were present in both DNA preparations, they must each contain at least β- or δ-chain DNA sequences.

These studies demonstrate deletion of α-globin gene sequences in several different restriction enzyme digests of splenic DNA from homozygous α-thalassemia. Since new fragments hybridizing to globin cDNA did not appear in these digests, the deletion of material corresponding to α-chain mRNA is either complete, or the remaining sequences are too short to be detected by these methods. The identification of α-, β- and δ- gene fragments in these different enzyme digests will facilitate partial purification of the fragments in preparation for mo-

lecular cloning and further analysis for duplication of loci, ordering of genes, intervening sequences in globin genes, and mutations in the many genetic forms of thalassemia. Precise identification of all globin gene fragments and mapping of the loci should be possible using cloned double-stranded cDNA for α-, β- and γ-globin [22], and these studies are in progress. These methods may allow prenatal diagnosis of homozygous α-thalassemia and other thalassemic disorders by restriction endonuclease mapping of DNA from amniotic fluid fibroblasts obtained by amniocentesis.

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