Vol. 120, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1984 Pages 116-123

S_1-NUCLEASE MAPPING OF THE GENOMIC LEPORE-BOSTON DNA DEMONSTRATES THAT THE ENTIRE LARGE INTERVENING SEQUENCE OF THE FUSION GENE IS OF β-TYPE

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Received January 16, 1984

Several reports have suggested but not proven that the large intervening sequence of Lepore δ-β fusion gene was of β-type (3-5). A method able to detect rearrangements as small as 4 nucleotide pairs directly into genomic DNA (6) has been applied to the total DNA of a heterozygous Lepore-Boston patient in order to identify accurately the origin of the large intervening sequence of the δ-β fusion-gene. Hybrid duplexes were formed between genomic Lepore DNA and single-stranded DNA used as probes, then submitted to S_1-nuclease treatment. Our data demonstrate that the entire large intervening sequence of the Lepore fusion gene is of β-type. Moreover, no large modification was detected in any δ-and β parts of the δ-β fusion gene.

In hemoglobin Lepore-Boston, the non-α-chains have a normal length but an abnormal amino acid sequence corresponding to the sequence of the δ-chain from residue 1 to 87 and that of the β-chain from residue 116 to 146 (1). There are no means to discriminate δ- from β- globin chains between the positions 87 and 116. Analysis of Lepore DNA by gene mapping has confirmed that the non-α-chain of hemoglobin Lepore is indeed the product of a δ-β fusion gene (2) with a deletion of approximatively 7 kb of the entire δ-β intergenic region. Our group (3) and two others (4, 5) have recently demonstrated that the non-homologous exchange to explain the δ-β Lepore recombination is localized between the δ-codon 87 and the 5' end of the large intervening sequence (IVS2) occurring in the resultant fusion gene. From these data, it has been concluded that IVS2 of the δ-β fusion gene must be mainly derived from the β-IVS2 but we could not exclude the possibility of various nucleotide changes intervening into this IVS2 as well as the other part of the Lepore δ-β fusion gene.

In order to identify such small changes which cannot be revealed when they do not arise within a restriction site, we have developped a new method suitable to detect rearrangements as small as 4 nucleotide pairs directly from a genomic DNA (6). In this technique, molecular probes are obtained by subcloning human globin regions into replicative forms (RF) of filamentous phages (fd 103 or M13). The viral (+) strand being only secreted into the supernatant of the cultures, two complementary full-length single-stranded probes result, each of importance

0006-291X/84 $1.50

as a probe in further studies (7-11 for review see 12, 13). The recombinant single-stranded DNA are used to obtain duplexes by hybridization with genomic DNA. Hybrids were then submitted to the action of S_1-nuclease which recognises and digests regions remaining as single-stranded DNA. In this report we have applied this method to improve directly the structure of genomic DNA of a heterozygous Lepore-Boston patient with that of the δ- and β-globin gene regions cloned from normal human subjects. No mismatch was detected between the δ- and β- gene probes and the overlapping regions of the Lepore δ-β fusion gene. Thus our data confirm that the entire IVS2 is of β-type as well as no large modification occurs into any parts of the δ-β fusion gene compared to the homologous normal δ- and β- globin gene sequences.

MATERIALS AND METHODS

1. Sources and preparations of human genomic DNA

High molecular weight DNA was obtained either from the placenta of a Caucasian subject (Normal DNA) or from the spleen of a heterozygous Lepore-Boston patient who was a 10-year-old female of Italian origin. She was diagnosed as bearing a β^+ thalassemic mutation on one chromosome and a Lepore-Boston on the other one by her family studies (14). DNA extractions were prepared by standard methods as described previously (15).

2. Double-stranded probes

- A 2.2 kb Pst I fragment containing the δ- globin gene cloned in pBR 322 was obtained from Maniatis (16).
- A 5.2 kb Eco RI fragment containing the 5' flanking region of the β-globin gene and the β-globin gene downstream to codon 122 was initially cloned in λWES phage from DNA of the normal French female, then subsequently subcloned in pBR 322 (3). Location of cloned fragments and abbreviations of the different clones are summarized in fig. 1.

Every of the cloned fragments was isolated from their cloning vectors by appropriate endonuclease digestion and purified either by sucrose gradient centrifugation or by electroelutions from preparative agarose gels using standard methods.

3. Restriction analysis

High molecular weight DNA were digested to completion with several restriction endonucleases (2-3u/μg of DNA) according to the suppliers instructions (BRL, BOEHRINGER) then electrophoresed on agarose gels (1-2 %, agarose from Miles); Southern blots were essentially carried out as described previously (17) using ^{32}P-probes labeled *in vitro* by nick-translation (1.5-2.0 x 10^8 dpm/μg). The filters were autoradiographed at - 70° C using Kodak-XO mat AR films and Agfa-Gevaert cassettes with intensifying screens (Agfa-Gevaert S). Lambda DNA and pBR 322 DNA digested with appropriate restriction enzymes and hybridized separately were used as size markers.

4. Single-stranded clones and heteroduplex formation

Each of the double-stranded fragments, cloned in pBR 322, was subcloned into filamentous coliphages fd 103 (18) using the appropriate endonuclease sites. The procedure for subcloning and selection of recombinant phages were as described (8, 18). Phage growth, isolation of recombinant single-stranded DNA, heteroduplex formation and S_1-nuclease mapping of the genomic DNA were extensively described in Chebloune et al. (6). Following steps (agarose electrophoresis, Southern transfers, hybridizations with ^{32}P probes labeled by nick-translation, washes and autoradiographies) were carried out as above mentioned in "Restriction analysis".

RESULTS

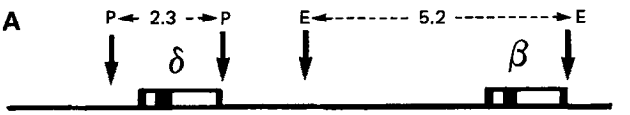
1. Restriction enzyme analysis of Normal (N) and heterozygous Lepore-Boston (L) DNA.

Patterns obtained with either normal or heterozygous Lepore-Boston DNA digested with Pst I, Xba I and Bgl II were compared when the cloned double-stranded D δ 2.3 (see fig. 1) was used as probe. The Lepore patient was found heterozygous for a 2.6 kb Pst I fragment, a 3.8 kb Xba I fragment and a 5.2 kb Bgl II fragment (fig. 2A). These sizes correspond to those of fragments carrying the entive δ-β fusion gene and limited from the 5' to 3' ends respectively by Pst I, Xba I or Bgl II sites (fig. 2B). These values agree with the presence of a large intervening sequence (IVS2) in the Lepore-Boston fusion gene as described previously (2-5).

2. S_1-nuclease mapping of the Lepore genomic DNA

a. The δ-globin gene region

Total genomic DNA either from the normal subject or from the heterozygous Lepore-Boston patient was hybridized with the recombinant single-stranded DNA F δ 2.3 containing the normal δ-globin gene (fig. 1). After S_1-nuclease assay, electrophoresis and transfer, the Southern blot was revealed by the double-stranded 2.3 kb fragment isolated from its pBR 322 cloning vector and nick-translated. Only one band of 2.3 kb was detected with the normal DNA whereas two bands of 2.3 kb and 0.75 kb were observed with the heterozygous Lepore-Boston DNA (fig. 3 A I). The 2.3 kb band observed in both normal and Lepore-Boston DNA corresponds to perfect duplexes between the single-stranded DNA



B

Fragments cloned	Abbreviations used for double-stranded probes in pBR322	Abbreviations used for single-stranded probes in fd 103
2.3 kb Pst I	D δ 2.3	F δ 2.3 (+) / F δ 2.3 (−)
5.2 kb Eco RI	D β 5.2	F β 5.2 (+) / F β 5.2 (−)

Figure 1 : Location of fragments cloned (A) and abbreviations of clones used either as double-stranded probes or single-stranded probes (B). The two possible orientations of a double-stranded fragment cloned in filamentous phage fd 103 allow to obtain each strand of the fragment inserted [coding (+) or anti-coding (-)] as two complementary single-stranded probes recombined each other with the same secreted viral strand. P = Pst I sites ; E = Eco RI sites ; the fragment sizes are given in kilobase pairs (kb).

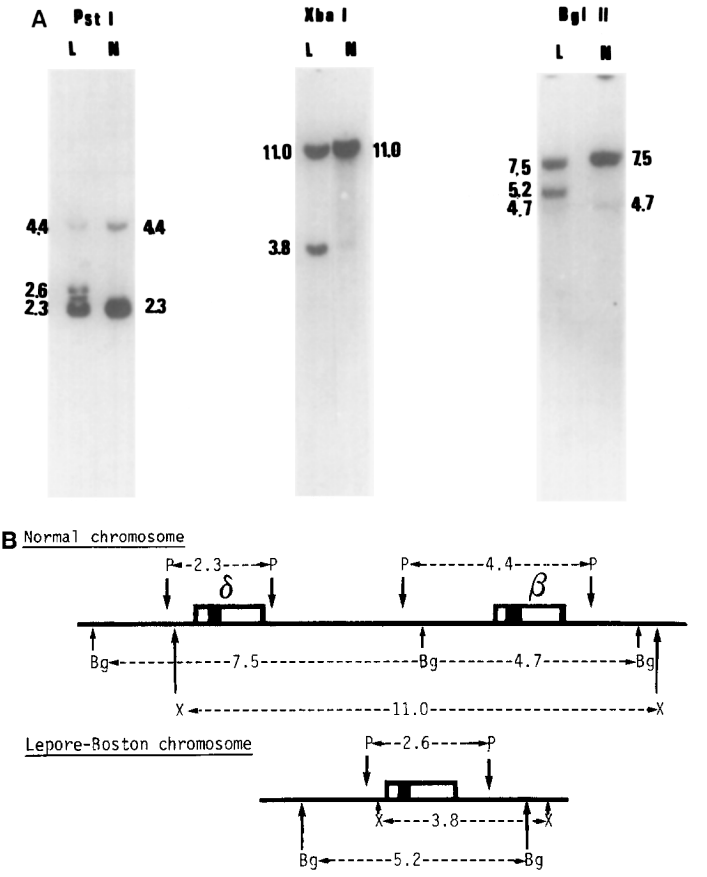


Figure 2 : Restriction enzyme analysis of genomic normal (N) and heterozygous Lepore-Boston (L) DNA. 20 µg DNA were digested with Pst I (P), Xba I (X) and Bgl II (Bg), electrophoresed in either 1 % or 1.5 % agarose gel and Southern transfered. Filters were hybridized with ^{32}P labeled D δ 2.3 probe (see fig. 1). Locations of the 2.6 kb Pst I fragment, 3.8 kb Xba I fragment and 5.2 kb Bgl II fragment, present in Lepore DNA but absent in normal DNA are shown in (B). The protein-encoding regions (or exons) [filled boxes], and the intervening sequences (IVS1 and IVS2) or introns [open boxes] are indicated.

used as probe and the complementary strand of the genomic DNA. Thus no mismatch was recognized by S_1-nuclease in the normal chromosome of the Lepore patient. Alternatively, the 0.75 kb band represents S_1-nuclease resistant duplexes obtained between the single-stranded probe and the Lepore chromosome. From this last result, one can conclude that the fragment of 0.75 kb originated from normal δ-globin gene was extended downstream the Pst I site located in the 5' flanking region of the δ-β fusion gene to the 3' part of the exon II (fig. 3 B and fig. 4).

b. The β-globin gene region

A similar strategy was used to study the remaining β-globin gene sequences

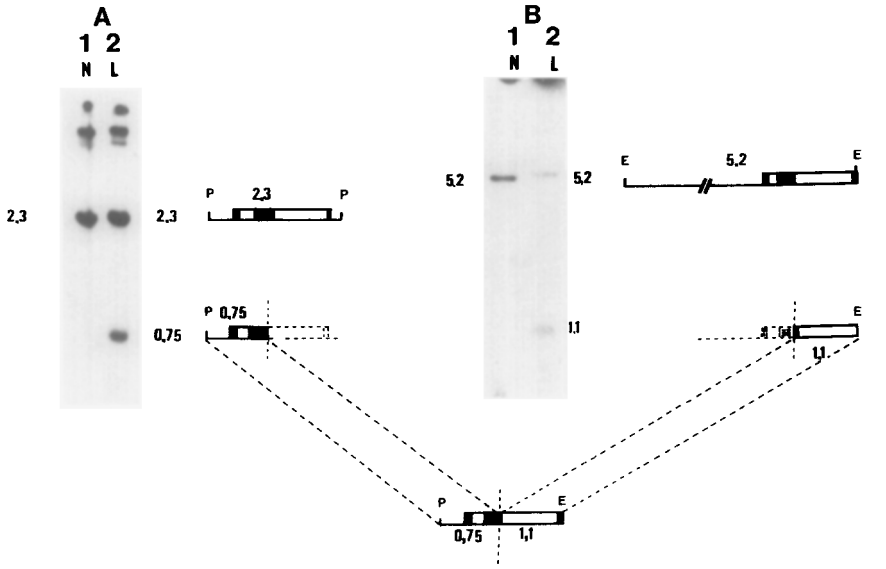


Figure 3 : S_1-nuclease mapping of the δ- and β-globin gene regions in genomic Normal (N) and Lepore-Boston (L) DNA.
20 µg of denatured genomic DNA from the normal subject (N) and the heterozygous Lepore-Boston patient (L), were hybrized with 200 ng of single-stranded F δ 2.3 (A) or F β 5.2 (B) probes. Duplexes were digested with 500 units of S_1 nuclease, electrophoresed in 1.5 % agarose gel and Southern transfered. The filters were hybridized either with 32P nick-translated D δ 2.3 fragment (A) or with 32P nick translated D β 5.2 fragment (B) used as probe to detect hybrids formed. Bands of high molecular weight in (A) corresponds to faint contaminants of double-stranded DNA fd 103 recombinants carried by single-stranded F δ 2.3 DNA preparation. The 0.75 kb (A) and 1.1 kb (B) bands correspond to the overlapping regions hybridized in heteroduplexes formed between genomic Lepore DNA and respectively the F δ 2.3 and F β 5.2 probes. The fragment sizes are given in kilobase pairs (kb). E = Eco RI ; P = Pst I.

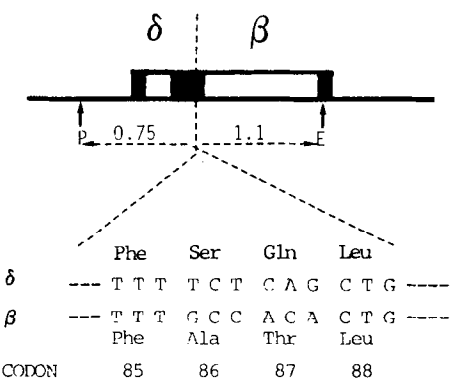


Figure 4 : Comparison of nucleotide sequences between the δ- and the β-globin-genes in the region of the codons 85-88. The 5 nucleotide changes observed must produce the mismatch recognized by S_1-nuclease leading to a cutting site in heteroduplexes formed between Lepore DNA and single-stranded probes used (see fig. 3). This cutting site represents the 5' limit of the overlapping sequences between the δ- β fusion gene and the F δ 2.3 probe as well as the 3' limit of the overlapping sequences between the δ- β gene and the F β 5.2 probe.

into the Lepore δ-β fusion gene. Using the Fβ5.2 probe, only one band of 5.2 kb was observed for the hybrids formed with normal DNA (fig. 3 A lane 1) whereas a supplementary band of 1.1 kb were detected with Lepore-Boston DNA (fig. 3 A II lane 2). If we apply the same arguments as those developped for the δ-globin gene region, the 1.1 kb band must correspond to the overlapping sequences between the F β 5.2 probe and the Lepore δ-β fusion gene. Similarly, this result shows that the fragment of 1.1 kb originates from normal β-globin gene and extends upstream the Eco RI site located at the 5' end of the third exon to the 3' part of the exon II of the δ-β fusion gene (fig. 3 B and fig. 4).

DISCUSSION

Using our S_1-nuclease mapping method which could detect rearrangements as small as 4 nucleotide pairs (6), we have not observed structural modifications of the Lepore δ-β fusion gene either in its 5' part of δ-type or in its 3' part of β-type. Obviously we cannot exclude changes smaller than 4 nucleotides in the δ-β fusion gene compared to the homologous regions of normal δ- and β-globin genes, particularly into IVS1 or IVS2. Nucleotide sequences already published (19-21) show that no difference has been observed between codon 87 and 104 comparatively in the δ- and β-globin genes whereas five nucleotide changes can be observed in the region of codons 86 and 87 (fig. 4). Thus, in the Lepore DNA, the two supplementary bands of 0.75 kb and 1.1 kb respectively observed with the F δ 2.3 and F β 5.2 probes must result from the S_1-nuclease cuts in this area. Consequently to these fragment sizes, the entire IVS2 of the Lepore δ-β fusion gene is of β- origin.

The several known resultant Lepore δ-β fusion genes as well as the "anti-Lepore" genes are expressed at a low level, although somewhat higher than that of the δ-globin gene itself (22-28). The reasons for the low level of synthesis of these fusion genes are not known. These low levels of expression might result either from the relative instability of abnormal globin mRNA or from the defective transcription of the fusion gene promoters. Although the upstream sequences to the genes (the so-called CAAT and TATA boxes) have been shown to play a role for efficient transcription of most structural genes (see 2-9 for review), there is evidence for regulatory sequences in addition to the CAAT and TATA boxes. For example, deletion of the human γ- and δ-globin genes results in the disease γ-δ-β thalassemia in which none of these genes is expressed even though the β-globin gene and almost 3 kb of DNA upstream from it appear to be intact (30). On the other hand, the anti-Lepore hemoglobins are present at much lower levels than hemoglobin A and at approximately the same levels as the Lepore hemoglobin despite the presence of 5' β-flanking sequences presumably containing regulatory sequences identical to those of the normal β-globin gene.

With such a view that transcriptional regulation of a globin fusion gene as well as a normal globin gene must be modulated by more than one region of untranslated DNA, the δ-origine of IVS1 has been correlated with a reduced synthesis in several fusion genes resulting from a δ-β cross-over event (4) : Lepore-Boston gene (codon 87 of δ-gene, Ava II site at the 5' end of the βIVS2 (3-5)), Lepore-Baltimore gene (codon 50 of δ-gene - codon 86 of β-gene) and anti-Lepore Miyada gene (codon 12 of β-gene - codon 22 of δ-gene (22-27)). Thus, it would be interesting to determine using our S_1-mapping technique if the ISV1 in the Lepore-Hollandia gene (codon 22 of δ-gene - codon 50 of β-gene) and the anti-Lepore P Nilotic (codon 22 of β-gene - codon 50 of δ-gene) are of δ- or β- orogins. However, a δ- β- δ fusion gene has been recently described producing the hemoglobin Parchman (31). In spite of the presence of a β- IVS1 in this δ- β- δ gene, the level of the product approximates that expected from a single δ-globin gene allele. Therefore, the occurrence of a δ- IVS1 in most of the other fusion genes above-mentioned could be alone insufficient to explain the reduced synthesis from them.

Recently two reports have suggested modifications of the current models of transcription of globin genes. Several intitiation sites upstream the canonical cap site of ε-globin gene have been identified until 4.5 kb upstream from the translation initiation codon ATG (32) as well as in the 5' flanking region of the human β-globin gene (33). In this last case, these RNAs are transcribed *in vivo* by RNA polymerase III but extend into the mRNA-globin coding region that is transcribed by polymerase II. If such polymerase III transcripts are synthesized from the 5' flanking regions of other genes and play a role to produce normal levels of mRNA as suggested by the authors the defective production of fusion genes might be associated to structural rearrangements of such transcribed 5' region upstream the concerned genes. The S_1-nuclease mapping technique that we have applied to the Lepore δ- β fusion gene might be available to test directly from genomic DNA, using appropriate single-stranded DNA as probe, the integrity of such regions upstream the genes as well as their downstream non-coding parts.

ACKNOWLEDGEMENTS

We thank C. Faure for her technical assistance. This work was supported by research grants from CNRS and INSERM.

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