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Research Article

Globin messenger RNA (mRNA) isolated from three patients homozygous for hemoglobin Lepore is shown to have a marked reduction of the amount of beta-like globin mRNA (Lepore-globin mRNA sequences) compared with alpha-globin mRNA by molecular hybridization. The relative amounts of alpha- and Lepore mRNA are similar to the amounts of alpha- and Lepore globin synthesized in intact cells and by isolated mRNA in a cell-free system. It is also demonstrated that Lepore-globin mRNA can completely hybridize to full-length or nearly full-length beta-globin specific complementary DNA and protect it from nuclease digestion, indicating close homology between the delta-mRNA sequences present in Lepore mRNA and the beta-complementary-DNA probe. We have also quantitated the numbers of beta-like globin gene sequences in genomic Lepore DNA by molecular hybridization and demonstrated a reduction in their number consistent with the Lepore gene being a delta beta-gene fusion product.

Defects in DNA and Globin Messenger RNA in Homozygotes for Hemoglobin Lepore

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ABSTRACT Globin messenger RNA (mRNA) isolated from three patients homozygous for hemoglobin Lepore is shown to have a marked reduction of the amount of β-like globin mRNA (Lepore-globin mRNA sequences) compared with α-globin mRNA by molecular hybridization. The relative amounts of α- and Lepore mRNA are similar to the amounts of α- and Lepore globin synthesized in intact cells and by isolated mRNA in a cell-free system. It is also demonstrated that Lepore-globin mRNA can completely hybridize to full-length or nearly full-length β-globin specific complementary DNA and protect it from nuclease digestion, indicating close homology between the δ-mRNA sequences present in Lepore mRNA and the β-complementary-DNA probe. We have also quantitated the numbers of β-like globin gene sequences in genomic Lepore DNA by molecular hybridization and demonstrated a reduction in their number consistent with the Lepore gene being a δβ-gene fusion product.

INTRODUCTION

In recent years, the use of highly radioactive complementary DNA (cDNA)[1] specific for α-,β-, and γ-human globin sequences has permitted the quantitation of messenger RNA (mRNA) and DNA sequences in normal human cells and those of patients with different forms of thalassemia (1–12). We report here the analysis of Lepore-globin mRNA and genomic DNA in three patients homozygous for hemoglobin (Hb) Lepore. This disorder, in the homozygous state, is characterized by a complete absence of HbA ($\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$). The only β-like globin in these patients is a δβ-fusion product (Lepore globin) containing δ-sequences at the *N*-terminal end and β-sequences at the *C*-terminal end, derived by an unequal crossover between the δ- and β-globin chain genes (13). Patients homozygous for Hb Lepore show a phenotype similar to that of patients with homozygous β-thalassemia, with severe anemia associated with deficient or absent β-globin synthesis, and with a marked relative excess of α-globin. There are several types of Hb Lepore, depending upon the site of the crossover (14). Two of the patients described in this paper are of the Boston type, where the change from the δ- to the β-like sequence has occurred between position 87 and 116 of the non-α-chain (15).

There is a marked decrease of Lepore-mRNA content compared with α- and γ-mRNA by hybridization with full-length or nearly full-length β-cDNA probes. Such decreased Lepore-mRNA content is comparable to the decrease in Lepore-globin synthesis obtained in the same patients in intact cells and when isolated mRNA from these patients is translated in a wheat germ cell-free system. These results provide evidence that the structural sequences of Lepore mRNA are present in reduced amounts in Lepore homozygotes, and that the decreased synthesis of the Lepore globin is a result of a decreased amount of Lepore mRNA and not of defective translation of a normal number of Lepore-

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[1] *Abbreviations used in this paper*: cDNA, complementary DNA(s); Co, mean concentration of RNA; Hb, hemoglobin; mRNA, messenger RNA(s); t, time in seconds.

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globin mRNA molecules. In addition, Lepore mRNA hybridizes completely to β-cDNA and protects it from nuclease digestion, indicating that the δ-sequences present in Lepore mRNA are closely related to those of β-mRNA.

Finally, the number of β-like genes in genomic DNA from these homozygous Lepore patients is approximately one-half that found in normal subjects, supporting the genetic and biochemical data that the Lepore gene is a fusion product of the δ- and β-genes, and that the untranscribed parts of the δ- and β-genes are deleted in these disorders.

METHODS

Patients. The three patients are all of Southern Italian extraction and are homozygous for Hb Lepore. P.P. is 5 yr old, has been splenectomized, has 11% Hb Lepore, and requires frequent transfusions. L. P. is 13 yr old, is not splenectomized, has 19% Hb Lepore, and requires infrequent transfusions. The last transfusion was 3 mo before obtaining samples for RNA and DNA analysis. Parents of L.P. and P.P. have 8.0% and 7.0% Hb Lepore, respectively. The Lepore globin in these patients, analyzed by peptide analysis, are of the Boston type (14). A third patient, M.M., has a chronic hemolytic anemia with hypochromic microcytic cells, and has been splenectomized; 20% of his Hb is Lepore, the remainder is fetal Hb, and no HbA or HbA_2 are detectable. The Lepore globin from this patient has not been analyzed to date.

Preparation of Lepore RNA and cellular DNA. Poly-A-containing RNA was prepared from washed, whole blood cells as previously described (16, 17). The RNA was translated in a wheat germ cell-free system, and its product was analyzed by cellulose acetate electrophoresis as described (18, 19). Cellular DNA was extracted from Epstein-Barr-virus-transformed lymphocytic cell lines from normal subjects and from one of the homozygous Lepore patients (L.P.) as previously described (20).

Full-length cDNA preparation. Rabbit and human cDNA were prepared with conditions designed to obtain a population of homogeneous, intact, full-sized cDNA molecules (21). The cDNA reaction was stopped by adding 10 mM EDTA; the solution was then adjusted to 0.3 M NaOH, incubated at 68°C for 1 min, and then layered on a 5–20% alkaline sucrose gradient (0.1 M NaOH, 0.9 M NaCl) and centrifuged at 4°C for 19 h at 49,000 rpm in an SW 50.1 rotor. The fractions corresponding or greater in size to 7S DNA were pooled, neutralized, and precipitated with ethanol. The cDNA was collected by centrifugation at 10,000 rpm for 1 h at −15°C in an HB4 rotor; the pellet was then resuspended in 3 mM NaCl, 1 mM EDTA, and 50 mM Tris-HCl (pH 8). The cDNA were characterized by size using polyacrylamide gel electrophoresis in 98% formamide as described (22). Rabbit mRNA was iodinated according to methods previously described (23). 97.4% of the ^{125}I-labeled mRNA (sp act: 1.6×10^5 cpm/μg) was found to be sensitive to combined digestion with RNAase A and T_1. The characterization of the purity of the human α-, β-, and γ-cDNA was as described previously (8, 18). Globin cDNA was hybridized with mRNA under conditions previously described (24). The melting temperature of the mRNA:cDNA hybrids was obtained as previously described (25) with micrococcal nuclease to assay thermal stability of the hybrids. The hybridization of globin cDNA to cellular DNA was performed as previously described (20).

RESULTS

Globin synthesis in homozygous Lepore. The ratio of Lepore to α-globin synthesis is 0.06 in P.P., and 0.12 in L.P. (Fig. 1, Table I). There is more γ-globin synthesis in L.P. than P.P. as well.

Characterization of full-size cDNA. Rabbit cDNA and α- and β-human cDNA were shown by polyacrylamide gel electrophoresis under denaturing conditions to be similar in size and to contain ≅650–700 nucleotides. The rabbit cDNA was shown to protect 96% of ^{125}I-radiolabeled mRNA from nuclease digestion at a cDNA:mRNA ratio (wt/wt) of about 1:1. Thus, the rabbit and human cDNA are representative of all the sequences in their respective globin mRNA, homogeneous in size, and of a length similar to that of the

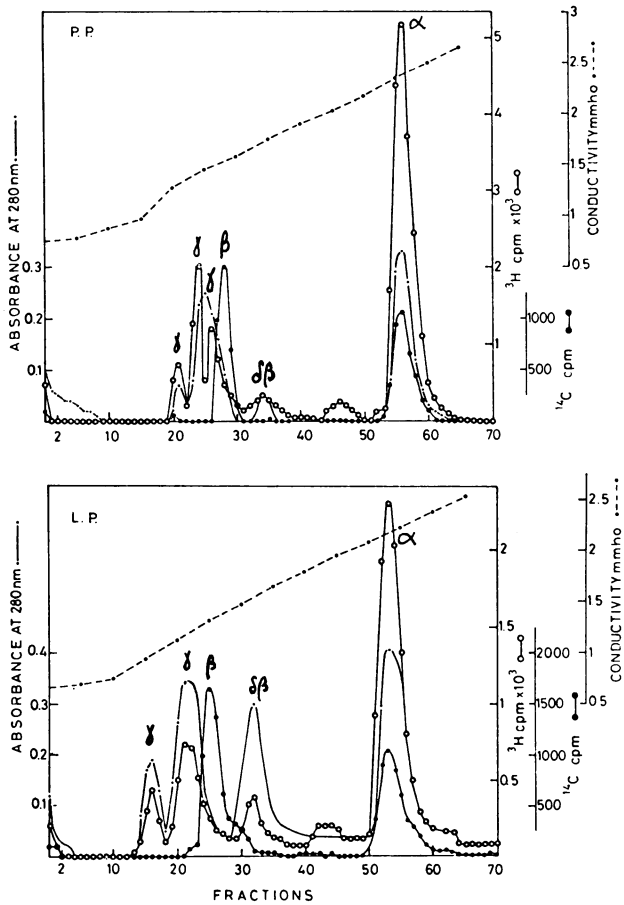

FIGURE 1 Carboxymethylcellulose column chromatography of globin from two patients homozygous for Hb Lepore. The reticulocytes of the two patients were labeled with [^{3}H]-leucine. ^{14}C-labeled globin from a patient with autoimmune hemolytic anemia, was added as carrier. The β-globin peak is between fractions 26–31 (P.P.) and 23–30 (L.P.). Preceding the β-peak are two or three peaks identified by fingerprinting as having γ-chain structure and no significant nonglobin protein. Following the β-peak is the Lepore globin, identified by fingerprinting as Boston in type (15).

TABLE I
Relative Amounts of Globin mRNA

	Lepore:α		γ:α	
	Globin chain*	mRNA‡	Globin chain*	mRNA‡
L.P.	0.12	0.10	0.42	0.70–1.0
P.P.§	0.06	0.025	0.38	0.25
M.M.§	—	0.15	—	0.57

The RNA content value was calculated from the Cot derived from the counts shown in Fig. 2. The Cot, defined as the middle point between the lower and higher plateau of the curve, were: (L.P.) α: 10^{-2}; β: 7×10^{-2}; γ: 1.2×10^{-2}; (P.P.) α: 1.4×10^{-2}; β: 6×10^{-1}; γ: 6×10^{-2}; (M.M.) α: 4×10^{-2}; β: 2.6×10^{-1}; γ: 7×10^{-2}.

* By carboxymethylcellulose column.

‡ By hybridization.

§ Splenectomized.

mRNA. Although there is no evidence in this or previous studies that the cDNA is indeed complete and contains the 5′-ended sequences of mRNA, the designation of "full-length cDNA" will be used.

Characterization of Lepore RNA. Translation of one of the Lepore RNA (M.M.) in a wheat germ cell-free system shows only α-, Lepore-, and γ-globin chains with a ratio of α-:Lepore synthesis of ≅6:1 (data not shown). When Lepore RNA is hybridized to purified α- and β-cDNA, the Lepore mRNA hybridizes completely to β-cDNA and can completely protect all of the sequences present in the β-probe from micrococcal nuclease digestion (Fig. 2). This indicates a close structural similarity of those δ-nucleotide sequences present in Lepore mRNA with those in β-cDNA. The hybridization kinetics in Fig. 2 show that in all three patients there is a marked decrease of β-like mRNA sequences as compared with α-mRNA. To further characterize the β-cDNA:Lepore-mRNA hybrid, its melting temperature was compared with that of a β-cDNA: normal RNA hybrid. No significant differences were detected (Fig. 3, Table II).

On the other hand, hybridization of the Lepore mRNA to γ-cDNA shows different results. The Cot (Co, mean concentration of RNA times t, time in seconds) with γ-cDNA in the two patients with more severe clinical features (M.M., P.P.), is less than with α-cDNA; the γ-mRNA:α-mRNA ratios are 0.25 and 0.57 (Fig. 2). By contrast, in the mildest patient (L.P.), the Cot with α- and γ-cDNA are close to unity and consistent with the increased γ-globin and lesser anemia in this patient. We have previously shown that there is no hybridization of γ-cDNA with β-mRNA under the conditions used (8).

Characterization of Lepore DNA. When cellular DNA from one of the Lepore patients (L.P.) was hybridized to β-cDNA, a significant decrease in the hybridization plateau to 38% was obtained compared with that of 55% with DNA from lymphocyte cell lines of normal subjects (Table III). Because only 80% of the β-cDNA is hybridized, even in a vast excess of cell DNA, 80% was taken as the value equivalent to 100% hybridization in calculating the relative number of globin genes. With this correction factor, the number of copies of β-like genes in Lepore DNA is ≅50% that of normal DNA. The percent is higher than that in DNA from patients homozygous for hereditary persistence of fetal Hb and δβ-thalassemia known to have extensive deletion of β-like genes (11) (Table III). The decrease in β-like sequences in homozygous Lepore DNA is consistent with the hypothesis that this syndrome is associated with the presence of a single δβ-fusion gene per haploid chromosome set and with deletion of parts of the δ- and β-globin structural gene sequences in this condition.

DISCUSSION

It has been previously shown, with separated α-, β-, and γ-human globin cDNA, that it is possible to dis-

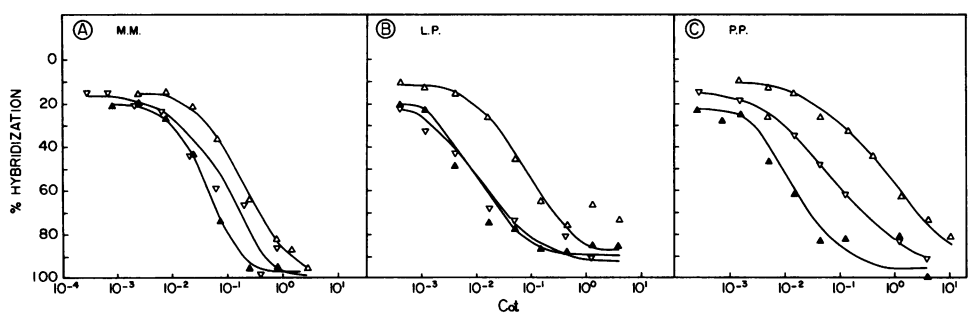

FIGURE 2 Hybridization of purified α-, β-, and γ-cDNA probes with total poly-A-containing RNA from three patients homozygous for Hb Lepore. Between 100 pg and 2 μg of RNA was hybridized to 178 pg of human globin cDNA (sp act: 14×10^3 cpm/ng). The time of incubation for all the hybridizations was 4 h. (▲), α-cDNA; (△), β-cDNA; (▽), γ-cDNA.

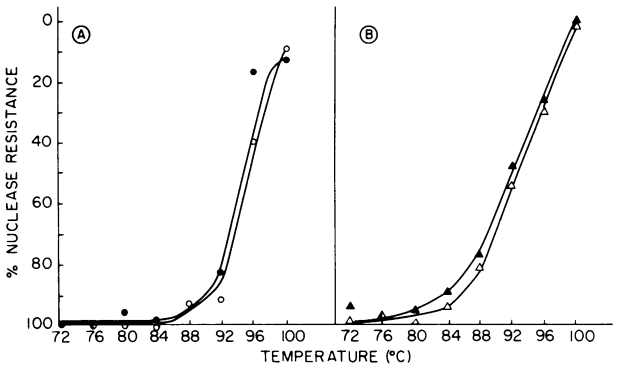

FIGURE 3 (A) Melting temperatures of the α-cDNA:α-mRNA and (B) β-cDNA:β-mRNA hybrids. (○), α-cDNA:nonthalassemic-RNA hybrid; (●), α-cDNA:Lepore-(M.M.) RNA hybrid; (△), β-cDNA:nonthalassemic-RNA hybrid; (▲), β-cDNA:Lepore-RNA hybrid. The conditions used to determine the thermal denaturation of the cDNA:RNA hybrids were previously described (25), except that the assay was performed with micrococcal nuclease instead of S_1 nuclease.

tinguish different defects in mRNA in patients with β^+-, β^0-, and $\delta\beta$-thalassemia (1, 2, 11, 25–28). In β^+-thalassemia, there is decreased β-mRNA similar to that reported here for Lepore mRNA in Lepore homozygotes. In β^0-thalassemia, there is either absent or abnormal β-mRNA in β^0-patients from Ferrara, Italy, abnormal β-mRNA in β^0-patients from Catania, Sicily and China (11, 25–27); or absent β-mRNA in Italian (28), Algerian (29), and Israeli (30) patients. In at least some cases of $\delta\beta$-thalassemia, there are absent δ- and β-mRNA (28). This paper demonstrates that reduced Lepore-globin synthesis is associated with a reduced amount of structural Lepore-globin mRNA sequences in the poly-A-containing cell RNA, and cannot be explained by defective translation of normal amounts of Lepore mRNA. Similar results were recently obtained by others (31) using total cellular RNA from a patient of Italian ancestry homozygous for Hb Lepore. The decrease in structural Lepore-mRNA sequences is reflected in a similar relative reduction in Lepore-mRNA translational activity in a wheat germ cell-free system. The parallel decrease in Lepore-globin mRNA translational activity and content in reticulocytes is similar to the close correlation between the reduced translational activity of β-mRNA and the amount of β-mRNA sequences in β^+-thalassemia. It remains unclear whether the decreased Lepore-globin mRNA content is a result of decreased transcription, abnormal processing, or accelerated degradation of Lepore mRNA.

These studies also indicate a considerable degree of nucleotide homology between human δ- and β-sequences. The complete protection of β-cDNA by Lepore mRNA suggests that δ-like sequences present in Lepore mRNA are homologous to those of β-mRNA. It is still possible that the δ-sequences at the 3′-end

TABLE II
Melting Temperature of cDNA:mRNA Hybrids

RNA	cDNA	Tm
		°C
Normal	α	94.8
Lepore (M.M.)	α	94.2
Normal	β	92.6
Lepore (M.M.)	β	91.8

Tm is the temperature at which 50% of the cDNA:RNA hybrids are protected from micrococcal nuclease digestion. Over 90% of the cDNA:RNA hybrids were protected from nuclease digestion at temperatures below 80°C.

of δ-mRNA not represented in the Lepore mRNA may not be as closely homologous with β-cDNA. The results also provide insights into the molecular defect in thalassemia at the gene level. We and others have previously shown that in β^+- and β^0-thalassemia, structural β-globin genes are present in normal amounts (5, 10). It is postulated by us and others (32, 33) that regulatory DNA sequences in the linked γ-δ-β-gene sequences are responsible for the expression of these genes. A role is postulated for the DNA sequences between the γ-, δ-, and β-genes in limiting γ-globin gene expression in the β-thalassemias. We and others (6, 7, 11, 12) have previously shown that extensive deletion of β-globin genes, and probably δ-genes as well, in $\delta\beta$-thalassemia and hereditary persistence of fetal Hb is associated with greater γ-globin gene expression, in contrast to β^0- and β^+-thalassemia in which no deletion of δ- and β-structural genes is detectable (5, 10). Similarly, in the Lepore homozygote the presence of a fusion-gene product including the *N*-terminal end of δ-globin suggests that DNA sequences between γ- and δ-genes are intact and may account for the

TABLE III
Relative Amounts of Globin Genes

Source of DNA	Percentage of hybridization	
	α-cDNA	β-cDNA
	%	%
Normal	55–60	52–55
Lepore	58–54	40–37
Hereditary persistence of fetal Hb	55–56	19–21
$\delta\beta$-thalassemia	56–53	28–27

150 μg of cellular DNA, prepared as described previously (20), was hybridized with 45 pg of full-length α- or β-cDNA (sp act: 14×10^3 cpm/ng) and analyzed by hydroxylapatite chromatography. A similar "plateau" level of hybridization was documented at two different times of incubation in these experiments to insure maximal hybridization. Cot values of 5×10^3 were attained. The Lepore patient studied was L.P.

inadequacy of γ-globin compensation in homozygous Lepore disease. The inadequate compensation by γ-globin gene expression in the Lepore homozygote suggests that deletion in the region between the δ- and β-genes (missing in Lepore) does not increase γ-globin gene expression. It is of interest that in the two brothers with homozygous Lepore disease, there are differences in γ-globin mRNA content, γ-globin synthesis, and clinical severity (Table I).

The decreased number of β-like globin genes in Lepore DNA compared with normal DNA provides biochemical evidence for deletion of the untranscribed and untranslated δ- and β-genes, and the presence of a δβ-gene fusion product in Lepore DNA. Recent analysis of Lepore DNA by restriction enzyme digestion of cellular DNA from the cell line of patient L.P. (34, 35) indicates that two specific DNA restriction fragments containing β-like globin genes are absent. Further studies indicate that the deletion is of the 3'-end of the δ-structural gene and the 5'-end of the β-structural gene. Additional analysis suggests that at least one and perhaps two intragenic intervening DNA sequences not represented in β-globin mRNA are detectable in the δ- and Lepore-globin structural genes (35). Similar intervening sequences have been reported in mouse (36) and rabbit β-globin genes (37); in the mouse it has been shown that these intervening sequences are transcribed into nuclear globin RNA precursors (38), although they are not present in mature mouse globin mRNA. It is possible that differences in either the number, nucleotide sequence, or length of the intervening sequences in δ- and Lepore genes as compared with β-genes may lead to decreased relative stability during the processing of the nuclear precursors of the δ- and Lepore-globin mRNA. Alternatively, differences in these intervening sequences could either be important in the transcriptional regulation of these genes, or be of no functional significance.

It has been shown previously that both δ- (39, 40) and Lepore-globin biosynthesis in a patient heterozygous for Lepore (41) are markedly reduced in intact reticulocytes as compared with bone marrow cells. Lepore globin synthesis in the reticulocytes of our patients is higher than in this report (41). It is unlikely that this is a result of the presence of nucleated erythrocytes, because P.P. (splenectomized) shows less Lepore-globin synthesis than L.P. (non-splenectomized). Because of the common 5'-δ-nucleotide sequence of the δ- and Lepore-globin mRNA, it was initially postulated that this region of the mRNA might be responsible for the decreased biosynthesis of these chains. Alternatively, regulatory sequences adjacent to this region, but not translated, could reduce δ- or Lepore-globin synthesis. However, studies of globin synthesis in reticulocytes of a patient heterozygous for Hb Miyada, an anti-Lepore (βδ) chain, by Roberts et al. (42) also showed markedly decreased synthesis of the anti-Lepore product despite a presumed normal β-sequence at the 5'-end of the βδ-gene and adjacent to it. Thus, it is difficult to understand how the 5'-end of the δ-gene could influence the decreased biosynthesis of the Lepore chain. However, if regulatory sequences are present at both the 5'- and 3'-ends of these fusion genes, the level of synthesis of Lepore and anti-Lepore would be expected to be intermediate between that of normal β- and normal δ-synthesis.

Differences in the intervening sequences in the δ- and Lepore genes as compared with β-genes suggest another possible explanation for the decreased synthesis of δ- and Lepore mRNA. In the unequal crossovers resulting in the formation of the Lepore and Miyada genes, part or all of the intervening region may be derived from the δ-gene and part or none from the β-gene. This may lead to unstable mRNA precursors, decreased globin mRNA, and decreased synthesis of these globins. This hypothesis would explain our finding of decreased Lepore mRNA and is consistent with the findings of decreased biosynthesis of δ- and Miyada globins as well. On the other hand, some or all of the Lepore syndromes could be a result of changes in regulatory sequences outside of the globin structural genes. The β^+-thalassemias, in which β-globin mRNA sequences are also decreased in amount (1, 2), may also be associated with midgene defects or deletions in the β-gene or, alternatively, may be a result of altered regulatory gene sequences outside of the β-structural gene.

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