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THE $^{M}\gamma$ CHAIN OF HUMAN FETAL HEMOGLOBIN; ITS IDENTIFICATION AND OCCURRENCE

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SUMMARY

High-performance liquid chromatographic procedures have been used in the detection and identification of a new γ chain of human fetal hemoglobin (Hb). This $^{M}\gamma$ chain is characterized by a Leu→Met replacement at position γ141; no other structural variations have been observed. The $^{M}\gamma$ chain has been detected in red cell lysates of subjects with a heterozygosity for one of many types of so-called hereditary persistence of fetal hemoglobin conditions, which are characterized by an increased level of Hb F in adult life, in sickle cell anemia, and in a few cord blood samples. At present it is not possible to definitely identify the genetic cause of this newly discovered heterogeneity; an infidelity in translation or the existence of an unrecognized γ globin gene should be considered.

INTRODUCTION

High-performance liquid chromatographic (HPLC) procedures have become increasingly popular for the detection, quantitation, and identification of normal hemoglobins (Hbs) and their variants (for review see refs. 1 and 2). During the past few years, several minor Hbs or Hb chains have been noted in some of these chromatograms; only a few have been adequately identified[3]. Here, we report the detection and identification of a previously unrecognized γ chain of human fetal Hb (Hb F). This $^{M}\gamma$ chain resembles the $^{A}\gamma$ chain but has a Leu→Met substitution at position γ141. It appears in numerous chromatograms of red cell lysates from adults with elevated levels of Hb F, and perhaps also in cord blood red cell lysates.

MATERIALS AND METHODS

Blood samples

These were collected in vacutainers with EDTA as anticoagulant and transported in ice to the laboratory in Augusta, GA, U.S.A. Various subjects were studied; these are discussed in more detail in the Results section. Informed consent was obtained.

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Hematological and Hb analyses

Hematological data were collected with an automated cell counter. Lysates of red cells, washed three times with a 0.9 g/dl sodium chloride solution, were studied for the possible presence of Hb abnormalities using methods described before[4]. Hb A_2 was quantitated by micro column chromatography[5], and Hb F by an alkali denaturation procedure[6]. Blood, washed red cells, and hemolysates were stored at 4°C for less than 14 days, or in liquid nitrogen for an indefinite period.

Chromatographic procedures

Quantitation of Hb components was by cation-exchange HPLC[7]. Larger quantities of Hb F were isolated by DEAE-cellulose chromatography[8]. The separation of the polypeptide chains of 100–300 μg of Hb on a Vydac C_4 column followed the method of Shelton *et al.*[9]. Developer A consisted of acetonitrile–water (60:40), with a final trifluoroacetic acid (TFA) concentration of 0.1%. Developer B was acetonitrile–water (20:80) (0.1% TFA). A gradient of 50% A to 60% A was applied for 70 min at a flow-rate of 1 ml/min at room temperature. Separation of the globin chains on a large scale made use of a 300 × 21.5 mm Bio-Sil TSK-ODS-120T (C_{18}) column; as much as 10–15 mg Hb was applied. The developers were: developer A, acetonitrile–water (52:48) (0.1% TFA) and developer B, acetonitrile–water (35:65) (0.1% TFA), and the gradient was 55% A to 75% A in 150 min at a flow-rate of 6 ml/min and at room temperature. Protein zones from various identical chromatograms were combined, concentrated by freeze-drying under reduced pressure and analyzed.

Structural analyses

Isolated chains were digested with TPCK-trypsin for 6 h at pH 8.9 and at 37°C. The resulting soluble peptides were separated by reversed-phase HPLC as described before[10]. The amino acid composition of each tryptic peptide was determined with a fully automated Beckman Spinco 121M amino acid analyzer. The sequences of a few peptides were determined by the ultra micro sequencing procedure of Chang *et al.*[11]. Isolated Hb F from some subjects was also treated with cyanogen bromide. Lyophilized globin was dissolved in 70% formic acid and treated with a 10-fold excess of cyanogen bromide for 24 h at room temperature[12]. The resulting cyanogen bromide peptides (CB) were isolated on the 300 × 21.5 mm Bio-Sil TSK-ODS-120T column using developer A (100% water with 0.1% TFA) and developer B [acetonitrile–water (75:25), containing 0.1% TFA], and a gradient of 100% A to 50% A in 60 min at a flow-rate of 8 ml/min and at room temperature. Isolated cyanogen bromide peptides were identified by amino acid analysis.

RESULTS

Identification of the $^{M}\gamma$ chain

This unusual chain was first noted in red cell lysates and isolated Hb F fractions from members of a Black family with the Greek type of $^{A}\gamma\beta^{+}$-HPFH (hereditary persistence of fetal hemoglobin)[13]. These heterozygotes had about 15% Hb F (normal persons : < 1%) with γ chains being mainly of the $^{A}\gamma$ type [this $^{A}\gamma$ (75Ile; 136Ala) differs from $^{G}\gamma$ (75Ile; 136Gly) only at position 136; the two γ chains are the products

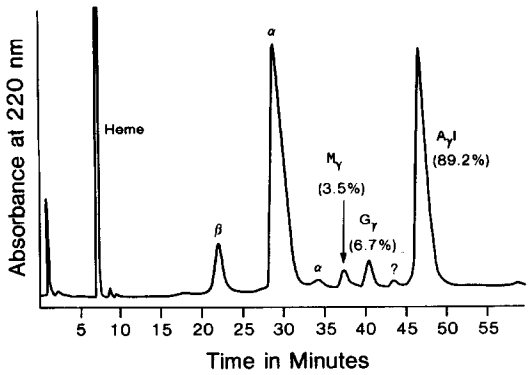

Fig. 1. Separation of globin chains from isolated Hb of subject RDH with an $^{A}\gamma\beta^{+}$-HPFH heterozygosity by reversed-phase Vydac C_4 HPLC. See text for details.

of duplicated γ globin genes, located on chromosome No. 11; for further information see refs. 14–16]. Fig. 1 illustrates the separation of the globin chains on a Vydac C_4 column; 100 μg Hb F, isolated from a red cell lysate of one of the heterozygotes, was applied. Four minor zones eluted between the α and the $^{A}\gamma$ chains; the second, labelled $^{M}\gamma$, and the third, labelled $^{G}\gamma$, are the peaks of interest. Separation of these two zones is probably greatly facilitated by the small prcentage of $^{G}\gamma$; if this is much higher, the chance of overlap increases. Preparative HPLC yielded the same two fractions, although the separation of the α and the $^{M}\gamma$ zones was less complete (Fig. 2). Other differences between the chromatograms of Figs. 1 and 2 included the position of the heme component and the possible absence of the fourth minor protein zone in front of the $^{A}\gamma^{I}$ zone. The $^{M}\gamma$, $^{G}\gamma$, and $^{A}\gamma^{I}$ zones of nearly 20 chromatograms from the same subject (RDH) were combined, freeze-dried, and digested with trypsin. The resulting tryptic peptides were separated by reversed-phase HPLC; Fig. 3 illustrates the three chromatograms. The $^{M}\gamma$ digest contained numerous γ chain peptides and also several

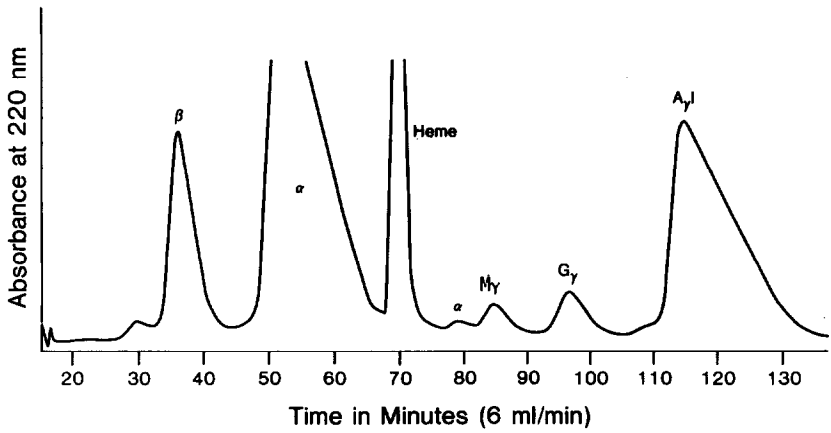

Fig. 2. Separation of the globin chains by reversed-phase HPLC using the large Bio-Sil TSK-ODS column. The same isolated Hb F was analyzed as was used in the chromatogram of Fig. 1. See text for details.

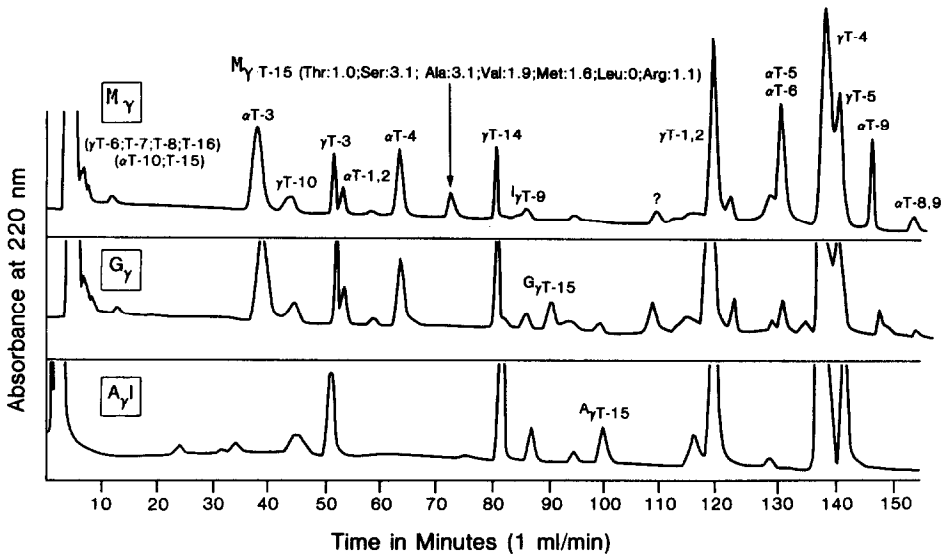

Fig. 3. Separation by reversed-phase HPLC[10] of tryptic peptides from digests of the $^{M}\gamma$, $^{G}\gamma$, and $^{A}\gamma^{I}$ zones isolated from preparative chromatograms as shown in Fig. 2. Identification of the various peptides is given only for those in the $^{M}\gamma$ digest to avoid confusion. Exceptions are the $^{G}\gamma$T-15 and $^{A}\gamma$T-15 peptides, which are specific for the $^{G}\gamma$ and $^{A}\gamma$ chains.

α chain peptides; the presence of peptides αT-1,2, αT-3, αT-4, αT-5, αT-6, αT-8,9, αT-9, αT-10, and αT-15, indicate considerable contamination of the $^{M}\gamma$ chain with the normal α chains. Even the $^{G}\gamma$ digest contained several α chain tryptic peptides (αT-3, αT-4, αT-5, αT-6, αT-9) suggesting a continuous elution of small amounts of α chain from the Bio-Sil TSK-ODS column behind the major α chain zone (Fig. 2). Nevertheless, all soluble tryptic γ chain peptides were identified in the digests of all three γ chains, namely T-1,2; T-3; T-4; T-5; T-6; T-7; T-8; T-9; T-10; T-14; T-15, and T-16 (the core peptides T-11, T-12, and T-13 were not recovered). Peptide T-9 of all three digests had the expected composition with one isoleucyl residue (at position γ75). The differences between the three chains were located within the γT-15 peptide; this fragment in the $^{A}\gamma^{I}$ digest contained Thr 1, Ser 3, Gly 0, Ala 3, Val 2, Met 1, Leu 1, and Arg 1, that in the $^{G}\gamma$ digest had the same composition except for one Gly and two Ala residues, but that of the $^{M}\gamma$ digest lacked the leucine residue and had an extra methionine residue (the actual composition is listed in Fig. 3). This Leu→Met substitution adequately explains the positions of the unusual chain (called $^{M}\gamma$) in the chromatograms shown in Figs. 1 and 2, and that of the abnormal T-15 peptide in the top chromatogram of Fig. 3.

The structural abnormality was further confirmed by sequence analysis of the isolated $^{M}\gamma$ T-15 peptide; a sequence of

Met-Val-Thr-Ala-Val-Ala-Ser-Ala-Met(?)-Ser-(Ser,Arg)

was obtained, but unfortunately the identification of the second methionine residue was less definite. Therefore, we treated isolated Hb F from subject RDH and Hb F

from a control cord blood sample with cyanogen bromide and separated the CB peptides on a Bio-Sil TSK-ODS-120T column following the procedure outlined in the Materials and Methods section. The following C-terminal CB peptides were expected:

	133 134 135 136..... 137 138 139 140 141 142 143 144 145 146
Control	Met-Val-Thr-Gly(or Ala)-Val-Ala-Ser-Ala-Leu-Ser-Ser-Arg-Tyr-His
	←——$^{AG}\gamma$CB-3——→
	133 134 135 136.....137 138 139 140 141.....142 143 144 145 146
RDH-F	Met-Val-Thr-Ala.....-Val-Ala-Ser-Ala-Leu(Met)-Ser-Ser-Arg-Tyr-His
	←——$^{A}\gamma$CB-3——→
	←——$^{M}\gamma$CB-3^{A}——→ ←——$^{M}\gamma$CB-3^{B}——→

Fig. 4 illustrates the chromatograms. The $^{A}\gamma$ and/or $^{G}\gamma$CB-3 peptides were eluted as several zones in both chromatograms at about 31–33 min, while two minor zones were observed in the RDH-F chromatogram which eluted at about 22 min and at about 28 min, respectively. The amino acid composition data, listed in Table I, readily identified the mixture of the $^{A}\gamma$ and $^{G}\gamma$CB-3 peptides in the control, the $^{A}\gamma$CB-3 peptide in RDH-F (note that the γ chain composition of this Hb F is mainly $^{A}\gamma$; Fig. 1), the $^{M}\gamma$CB-3 peptide [impure; the low recoveries of Gly (0.4 res.), Leu (0.4 res.),

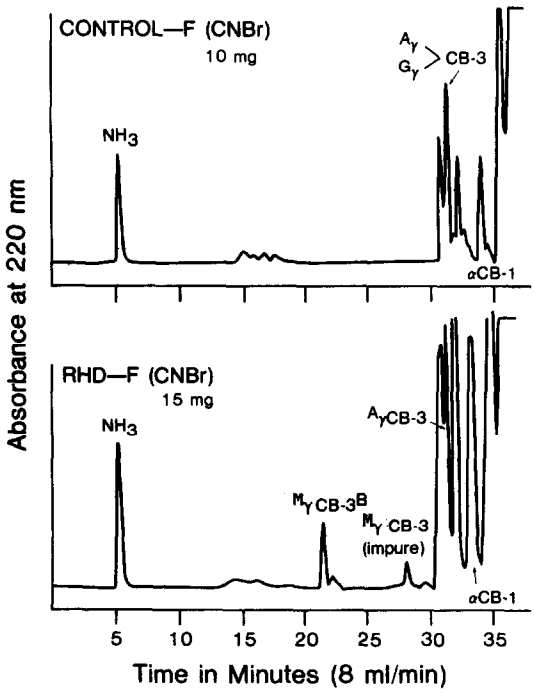

Fig. 4. Separation of cyanogen bromide peptides of isolated Hb F samples by reversed-phase HPLC as decribed in the text.

TABLE I

AMINO ACID COMPOSITION OF THE γCB-3 PEPTIDES*

Amino acid	*Control*	*Patient RDH*		
Threonine	0.97	0.96	1.06	
Serine	2.88	2.85	2.94	1.88
Glycine	0.60	0.12	0.40	
Alanine	2.66	2.83	2.70	
Valine	1.97	1.94	2.18	
Methionine	0	0	0.64	
Leucine	1.11	1.02	0.42	
Tyrosine	1.17	1.20	0.94	0.98
Histidine	1.03	1.09	0.94	1.15
Arginine	0.87	1.11	1.18	0.96
Peptide	$^{AG}\gamma$CB-3	$^{A}\gamma$CB-3	$^{M}\gamma$CB-3**	$^{M}\gamma$CB-3B
Residues	γ134-146			γ142-146

* In mole/peptide.
** Impure and very small.

and Met (0.6 res.) may suggest a mixture of the $^{M}\gamma$CB-3, $^{G}\gamma$CB-3 and $^{A}\gamma$CB-3 peptides], and the $^{M}\gamma$CB-3^{B} peptide. The Ser_2, Tyr_1, His_1, Arg_1 composition of this fragment indicates that it originates from positions γ142-γ146, inclusive, and supports the Leu→Met replacement at position γ141.

The occurrence of the $^{M}\gamma$ chain

As many as 13 members of the H Family with an $^{A}\gamma\beta^{+}$-HPFH heterozygosity were tested for the presence of the $^{M}\gamma$ chain; it was observed in all chromatograms. Its quantity varied, and averaged 9.0% of the total quantity of γ chain with an S.D. of 3.3%, which amounts to 1–1.5% of the total Hb level. The variation in $^{M}\gamma$ level is to some extent technical in nature and depends on storage conditions; data on freshly collected samples appear most reliable. Structural characteristics of the $^{M}\gamma$ chain were confirmed for two additional members of the Family H. The $^{M}\gamma$ chain was also seen in chromatograms of red cell lysates from a Chinese subject with a different type of $^{A}\gamma\beta^{+}$-HPFH[17], and from a Caucasian subject with a somewhat comparable condition[18].

Two types of $(\delta\beta)^{\circ}$-$^{G}\gamma^{A}\gamma$-HPFH have been observed in Blacks which are characterized by large deletions including the δ and β globin genes (ref. 19, and references quoted). As a result, adult heterozygotes produce some 30% Hb F and adult homozygotes have 100% Hb F. One Black family with type I HPFH (characterized by 45–50% $^{G}\gamma$ in the Hb F) was studied; all five heterozygotes had the $^{M}\gamma$ chain in amounts varying between 5 and nearly 22%. These quantitative data should be viewed with some caution as partial overlap with $^{G}\gamma$ and, occasionally, with residual α must be considered. All heterozygous members of five families with the $(\delta\beta)^{\circ}$-$^{G}\gamma^{A}\gamma$-HPFH (type II) had variable quantities of $^{M}\gamma$; in some chromatograms its presence was barely noticeable, while in others it amounted to over 20% of total γ chain. Fig. 5 illustrates such a difference, while the third chromatogram shown in this figure

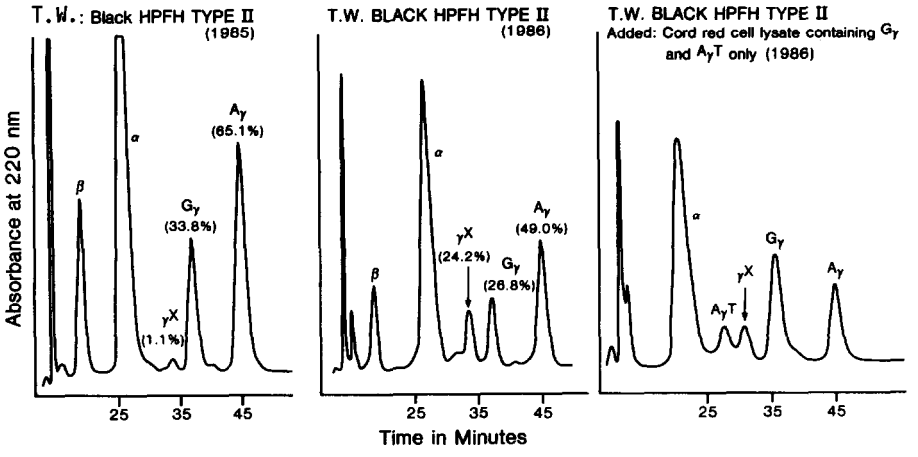

Fig. 5. Separation of γ^X (= $^M\gamma$), $^G\gamma$ and $^A\gamma$ chains present in Hb F isolated from a subject with a deletional type of HPFH by reversed-phase HPLC on Vydac C_4 columns. The two collections were about one year apart; addition of an $^A\gamma^T$ chain ($^A\gamma$, 75Thr, 136Ala, 141Leu) further defines the chromatographic mobility of the $^M\gamma$ chain.

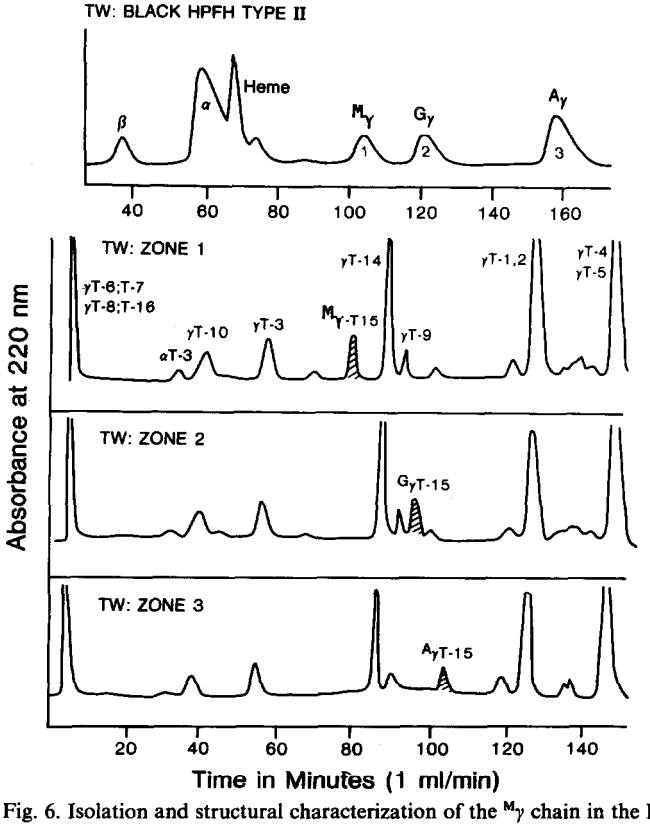

Fig. 6. Isolation and structural characterization of the $^M\gamma$ chain in the Hb F of T.W. Methodology is the same as in the experiments described in Figs. 2 and 3. The sample is from T.W. collected in 1986 (see Fig. 5, second chromatogram).

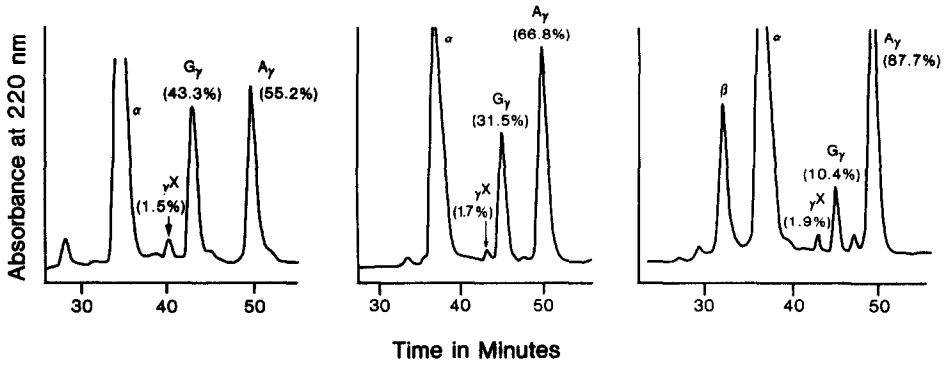

Fig. 7. Demonstration of the γ^X (= $^M\gamma$) chain in the Hb F of patients with sickle cell anemia by reversed-phase HPLC on a Vydac C_4 column. Left: adult SS with the most common SS haplotype (19/19); middle: adult with a combination of haplotypes 19 and Mor (ref. 22) characterized by high $^A\gamma$ production; right: mother of this patient with the AS condition (Mor β^S haplotype) and slightly elevated Hb F (2.7%).

indicates that the $^M\gamma$ chain and the frequently occurring $^A\gamma^T$ chain [*i.e.* an $^A\gamma$ chain with an Ile→Thr substitution at $\gamma 75^{20,21}$] can be separated in this chromatographic system. Further characterization of the $^M\gamma$ chain that was seen in the 1986 blood sample of subject T.W. followed the procedures used for its original identification. These data, shown in Fig. 6, clearly identify the types of γ chain observed in each of the three zones.

The Hb F seen in variable quantities in sickle cell anemia (SS) patients may also contain the $^M\gamma$ chain; its quantity is low and difficult to determine because of partial overlap with the $^G\gamma$ chain. Fig. 7 provides a few examples which include that of the mother of SS patient M.N., who has a slightly elevated Hb F (2.7%) with mainly $^A\gamma$ chains (the subjects have been discussed in detail in ref. 22).

The $^M\gamma$ chain is rarely observed in chromatograms of cord blood red cell lysates, perhaps because it is absent or it elutes with the $^G\gamma$ chain. Fig. 8 illustrates two examples: Cord No. 59005 might contain some 4% $^M\gamma$ chain, while a larger quantity could be present in Cord No. 250. Chromatography on the large Bio-Sil TSK-ODS

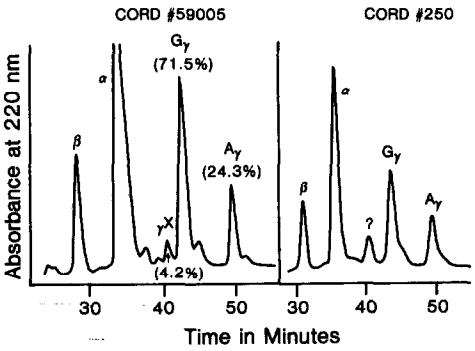

Fig. 8. Demonstration of the γ^X (= $^M\gamma$) chain in cord blood red cell lysates by reversed-phase HPLC on a Vydac C_4 column.

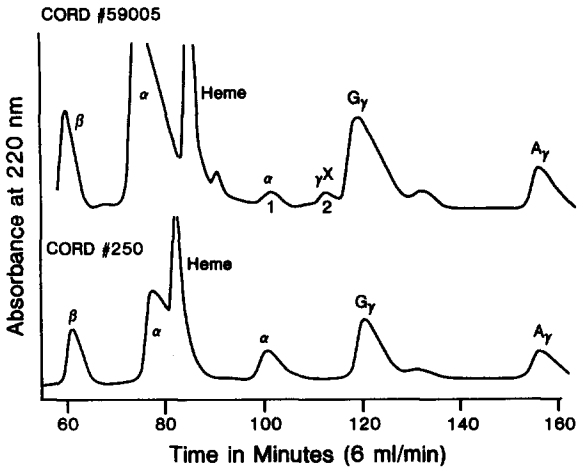

Fig. 9. The separation of the γ^X (= $^M\gamma$), the α, and the $^G\gamma$ chains in cord blood red cell lysates by reversed-phase HPLC on the large Bio-Sil TSK-ODS column.

column confirmed the suspicion for the presence of $^M\gamma$ in sample No. 59005 but not in sample No. 250 (Fig. 9), while structural analyses (Fig. 10) provided definitive proof.

DISCUSSION

Analyses such as the ones described above clearly demonstrate the usefulness of HPLC procedures. These techniques provide data in a relatively short period of time, the availability of larger-sized columns allows isolation of significant amounts of a specific protein, while the use of volatile developers allows a rapid isolation of

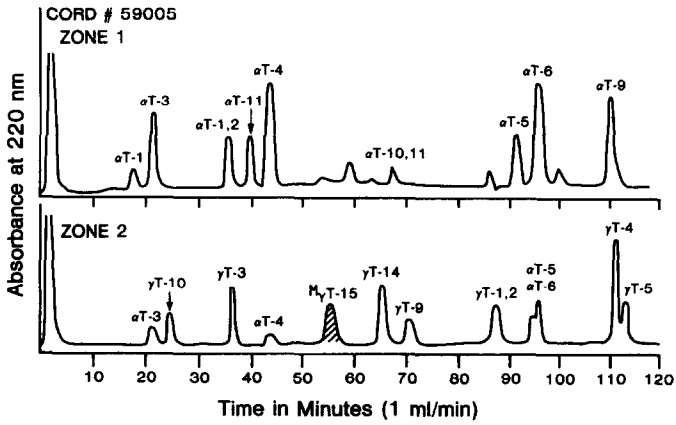

Fig. 10. Separation of tryptic peptides of digests from the protein in zones 1 and 2 (Fig. 9) by reversed-phase HPLC[10]. The fragment eluting at 55–57 min in the digest of zone 2 was identified as the abnormal T-15 without a leucine residue and with an extra methionine residue.

the protein from its solvent. Indeed, the discovery and characterization of the unusual $^{M}\gamma$ chain was only possible because of the availability of these methods.

The Leu→Met substitution at position γ141 in the $^{M}\gamma$ chain has been demonstrated through amino acid analysis of isolated tryptic and cyanogen bromide peptides, and through sequence analysis. Thus far, no additional substitutions have been observed, although the core peptides (residues 83–120) were not analyzed. The $^{M}\gamma$ chain has been demonstrated in red cell lysates of numerous individuals with reversed-phase HPLC procedures, and has been structurally characterized in five samples (three $^{A}\gamma\beta^{+}$-HPFH heterozygotes, one $^{G}\gamma^{A}\gamma(\delta\beta)^{\circ}$-HPFH heterozygote, one cord blood). Data such as shown in Figs. 8 and 9 caution against the identification of the $^{M}\gamma$ chain by reversed-phase HPLC alone as misinterpretation of the chromatographic pattern can easily be made. Fortunately, the $^{A}\gamma^{T}$ and $^{M}\gamma$ chain will separate in this system (Fig. 5), while the position of the $^{M}\gamma$ chain in the chromatogram obtained with the Bio-Sil TSK-ODS-120T column system is characteristic and reproducible, eliminating confusion with minor α chain contaminants (Fig. 9).

Data obtained thus far indicate a great variability in the quantities of the $^{M}\gamma$ chain. This may in part be due to technical difficulties in separating the chain from related globin chains, or to modifications during storage. It appears best to analyze the samples soon after collection and to store red cell lysates in liquid nitrogen. Table II lists quantitative data for members of three families, many of whom were studied on two occasions. Factors contributing to the considerable variability may be: con-

TABLE II

QUANTITIES OF THE $^{M}\gamma$ AND $^{A}\gamma$ CHAINS IN ADULT HETEROZYGOTES*

Subject	*Conditions*	F_{AD} (%)	*1st Collection*		*2nd Collection*	
			$^{M}\gamma$	$^{A}\gamma^{I}$	$^{M}\gamma$	$^{A}\gamma^{I}$
V.R.	Black $^{A}\gamma$-HPFH	10.1	7.0	87.5	38.2	58.7
P.R.		13.4	9.0	78.0	10.2	82.7
B.N.		11.6	8.0	84.5	30.2	64.8
B.H.		12.0	9.5	82.0	14.6	79.2
R.D.H.		11.8	6.5	87.0	3.1	90.3
E.H.		16.4	–	–	2.2	87.8
F.H.		18.8	4.0	84.5	2.3	93.8
J.H.		14.3	5.0	85.5	3.8	93.8
N.H.		17.8	4.0	78.0	4.9	90.6
T.W.	Black $^{G}\gamma^{A}\gamma$-HPFH (II)	20.7	14.2	57.3	24.2	49.0
E.W. Sr.		19.5	17.5	59.8	0	75.0
E.W. Jr.		23.1	11.9	49.8	0	66.6
G.W.		22.4	4.6	60.3	0	66.9
V.W.		21.6	13.4	52.6	11.2	56.1
Q.E.B.	Black $^{G}\gamma^{A}\gamma$-HPFH (I)	18.2	7.3	47.7	2.0	54.1
G.B.		19.4	21.6	34.0	5.1	46.6
R.B.		21.5	8.5	44.4	2.8	48.1
K.B.		–	17.5	36.5	–	–
A.B.		–	5.0	43.4	–	–

* In % of total γ; data by reversed-phase HPLC on Vydac C_4 columns.

tamination with α and partial overlap with $^{G}\gamma$; the possibility of different rates of synthesis should also be considered. It is of considerable interest to note that an increase in $^{M}\gamma$ is accompanied by a decrease in $^{A}\gamma$ but not in $^{G}\gamma$.

There are several possibilities to consider when an explanation is to be sought for the existence of the $^{M}\gamma$ chain. This chain could be a simple mutant of $^{A}\gamma$ like so many other $^{A}\gamma$ variants; however, its widespread occurrence and its presence in all members of families who are affected with a genetic anomaly such as one of the different HPFH conditions, makes this not probable. Ambiguity in translation of the $^{A}\gamma$ gene is a distinct possibility which cannot, at present, be excluded, but is supported by the observation of a direct correlation between the quantities of the $^{M}\gamma$ and $^{A}\gamma$ chains (Table II). The third possibility assumes a specific genetic factor which causes the observed effect; this $^{M}\gamma$ gene, if it exists, is probably located outside the β globin gene cluster of chromosome No. 11. Additional studies are presently being conducted to find answers to some of these interesting questions.

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REFERENCES

1 T. H. J. Huisman (Editor), *The Hemoglobinopathies*, Churchill Livingstone, Edingburgh, 1986.
2 T. H. J. Huisman, in Z. Deyl (Editor), *Separation of Biopolymers and Supramolecular Structures*, Elsevier, Amsterdam, 1986, in press.
3 W. A. Schroeder, J. B. Shelton, V. Huynh and J. R. Shelton, *Hemoglobin*, 10 (1986) 239.
4 T. H. J. Huisman and J. H. P. Jonxis, *The Hemoglobinopathies Techniques of Identification*, Marcel Dekker, New York, 1977.
5 C. T. H. Schleider, S. M. Mayson and T. H. J. Huisman, *Hemoglobin*, 1 (1977) 503.
6 K. Betke, H. R. Marti and I. Schlicht, *Nature (London)*, 187 (1959) 1877.
7 T. H. J. Huisman, J. B. Henson and J. B. Wilson, *J. Lab. Clin. Med.*, 102 (1983) 163.
8 W. A. Schroeder and T. H. J. Huisman, *The Chromatography of Hemoglobin*, Marcel Dekker, New York, 1980.
9 J. B. Shelton, J. R. Shelton and W. A. Schroeder, *J. Liq. Chromatogr.*, 7 (1984) 1969.
10 J. B. Wilson, H. Lam, P. Pravatmuang and T. H. J. Huisman, *J. Chromatogr.*, 179 (1979) 271.
11 J. Y. Chang, D. Brauer and B. Wittmann-Liebold, *FEBS Lett.*, 93 (1978) 205.
12 R. L. Lundblad and C. M. Noyes, in *Chemical Reagents for Protein Modification*, Vol. 1, CRC Press, Boca Raton, FL, 1984, pp. 49–50.
13 H. J. Huang, T. Stoming, H. F. Harris, F. Kutlar and T. H. J. Huisman, *Amer. J. Hematol.*, in press.
14 W. A. Schroeder, T. H. J. Huisman, J. R. Shelton, J. B. Shelton, E. F. Kleihauer, A. M. Dozy and B. Robberson, *Proc. Natl. Acad. Sci. U.S.A.*, 60 (1968) 537.
15 J. L. Slightom, A. E. Blechl and O. Smithies, *Cell*, 21 (1980) 627.
16 S. H. Shen, J. L. Slightom and O. Smithies, *Cell*, 26 (1981) 191.
17 R. Gelinas, M. Bender, C. Lotshaw, P. Waber, H. Kazazian and G. Stamatoyannopoulos, *Blood*, 67 (1986) 1777.
18 T. H. J. Huisman, W. A. Schroeder, H. R. Adams, J. R. Shelton, J. B. Shelton and G. Apell, *Blood*, 36 (1970) 1.
19 A. Kutlar, M. B. Gardiner, M. G. Headlee, A. L. Reese, M. P. Cleek, S. Nagle, P. K. Sukumaran and T. H. J. Huisman, *Biochem. Genet.*, 22 (1984) 21.
20 V. Grifoni, H. Kamuzora, H. Lehmann and D. Charlesworth, *Acta Haematol.*, 53 (1975) 347.
21 T. H. J. Huisman, F. Kutlar, T. Nakatsuji, A. Bruce-Tagoe, Y. Kilinc, M. N. Cauchi and C. Romero Garcia, *Hum. Genet.*, 71 (1985) 127.
22 Y. Hattori, F. Kutlar, A. Kutlar, V. C. McKie and T. H. J. Huisman, *Hemoglobin*, in press.