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Genetics

Human $^{T}\gamma$ globin chain is a variant of $^{A}\gamma$ chain ($^{A}\gamma^{Sardinia}$)

(silent globin mutations/Nonidet P-40 isoelectric focusing/Nonidet P-40 electrophoresis)

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ABSTRACT **Isoelectric focusing, cellulose acetate electrophoresis, and carboxymethylcellulose chromatography in the presence of Nonidet P-40 allow the separation of pure γ globin chains into two fractions. Amino acid analysis of their cyanogen bromide fragment 3 (γCB3) identifies these fractions as the separated $^{G}\gamma$ (Gly-136) and $^{A}\gamma$ (Ala-136) globin chains. Fingerprint and amino acid analyses of the γTp9 tryptic peptide from the purified $^{A}\gamma$ and $^{G}\gamma$ fractions from two different patients demonstrate that the commonly occurring $\gamma^{Sardinia}$ variant (γ 75 isoleucine → threonine), also known as $^{T}\gamma$ chain, has alanine in position 136. From this analysis we suggest that the $^{T}\gamma$ gene is an allele of the $^{A}\gamma$ locus ($^{A}\gamma^{Sardinia}$) rather than a third γ locus.**

The γ globin chains of human fetal hemoglobin are a heterogeneous mixture of two major species differing in position 136, which is occupied either by a glycyl residue ($^{G}\gamma$ chain) or by an alanyl residue ($^{A}\gamma$ chain); all normal individuals of different geographic areas or races so far examined have these two chains, a fact suggesting, with other genetic and molecular hybridization evidence, that these chains are the product of at least two nonallelic structural genes (1–5).

The $^{G}\gamma$ and $^{A}\gamma$ chains, differing only by a neutral amino acid residue, have identical charge and size, thus preventing their separation by standard chromatographic and electrophoretic techniques. So far their quantitation could be obtained only by analysis of a specific cyanogen bromide fragment including the variant residue for its glycine and alanine contents. Limits inherent to this method prevent biosynthetic studies using radioactive amino acids and do not allow its application to extensive surveys.

In the course of studies on the separation by isoelectric focusing (IEF) of human globin chains, we observed that chromatographically pure γ globin chains were split into two bands upon addition of the detergent Nonidet P-40 (NP-40) (6); on the basis of comparison of the relative intensities of the bands and the $^{G}\gamma/^{A}\gamma$ ratios in different samples, we suggested that these bands might represent separated $^{G}\gamma$ and $^{A}\gamma$ chains, and that this technique could be used for biosynthetic studies (7). By using a chromatographic technique we have now been able to prepare amounts of material corresponding to each band, adequate for demonstrating unambiguously that these two bands are in fact separated $^{G}\gamma$ and $^{A}\gamma$ chains.

Moreover, the recent discovery in a large proportion of normal and thalassemic subjects (8) of a variant γ chain having threonine instead of isoleucine (8, 9) at position 75 ($^{T}\gamma$ chain or $\gamma^{Sardinia}$) suggested that in at least some individuals gene duplication may have occurred. Demonstration that this is the case must first await the clarification of the nature of $^{T}\gamma$ chain (as to the amino acid in position 136); we now show that $^{T}\gamma$ chain is a variant of $^{A}\gamma$ chain ($^{A}\gamma^{Sardinia}$).

METHODS

CM-Cellulose Chromatography of Globin Chains. Purified α, β, and γ globin chains were obtained by CM-cellulose chromatography (10) of acid/acetone-precipitated total globin chains (10). For radioactive experiments, the globin chains were labeled by incubating 1 ml of peripheral blood with 100 μCi (1 Ci = 3.7×10^{10} becquerels) of lyophilized L-[4,5-^{3}H]leucine (110 Ci/mmol) and 2 mg of glucose at 37°C for 1 hr. For analytical experiments, a 5–32.5 mM sodium phosphate gradient (pH 6.8) of 300 ml was used; when indicated, 3% (vol/vol) NP-40 was included. For preparative fractionation of $^{G}\gamma$ and $^{A}\gamma$ chains, 20–50 mg of purified γ globin chains was loaded onto a 6 × 2.5 cm column and eluted with an 800-ml 5–19 mM sodium phosphate gradient containing 3% NP-40. Thirty-microliter aliquots from every second fraction were analyzed by IEF as described below, and fractions to be further studied were again precipitated by acid/acetone.

IEF. Samples, dissolved in 8 M urea/3% NP-40/10% (vol/vol) 2-mercaptoethanol, were focused in 6% acrylamide slab gels containing 8 M urea, 2% pH 6–8 and 0.2% pH 3.5–10 Ampholine (LKB), and 3% NP-40 (6, 7, 11), and were stained with Coomassie brilliant blue G-250 (7, 11). The pI values were determined at room temperature (22°C) and after correction for 8 M urea (12). Quantitation of the bands was obtained by scanning the gel with a Joyce–Loebl densitometer.

Cellulose Acetate Electrophoresis. Electrophoresis on cellulose acetate (Cellogel, Chemetron, Milano, Italy) was performed by using a modification of L. F. Bernini's unpublished technique (personal communication); globins, dissolved in electrophoresis buffer (6 M urea/0.1 M Tris·HCl, pH 7.2/10 mM EDTA/3% NP-40/1% 2-mercaptoethanol), were run for 16 hr, 100 V, 22°C. At the end of the run, proteins were stained with amido-schwartz.

Chemical Analysis of γ Globin Chains. The γ136 Gly/Ala ratio was determined after cleavage of γ chains with a 100-fold molar excess of cyanogen bromide in 70% (wt/vol) formic acid at 22°C for 24 hr. Cyanogen bromide was eliminated by freeze-drying and the residue was fingerprinted on paper at pH 6.4 in order to separate the γCB3 fragment as described by Bernini *et al.* (13) and by Kamuzora *et al.* (14). The $^{T}\gamma$ chain determination was obtained by tryptic digestion and fingerprinting on paper (15, 16) of γ globin chains. After staining of the peptides with cadmium/ninhydrin reagent (17) the peptides Tp9a (Ile-75) and Tp9b (Thr-75) were eluted in methanol

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Abbreviations: IEF, isoelectric focusing; NP-40, Nonidet P-40; γCB3, cyanogen bromide fragment 3 of the γ chain; γTp9a, tryptic peptide 9 of the γ chain Ile-75; γTp9b, tryptic peptide 9 of the γ chain Thr-75.

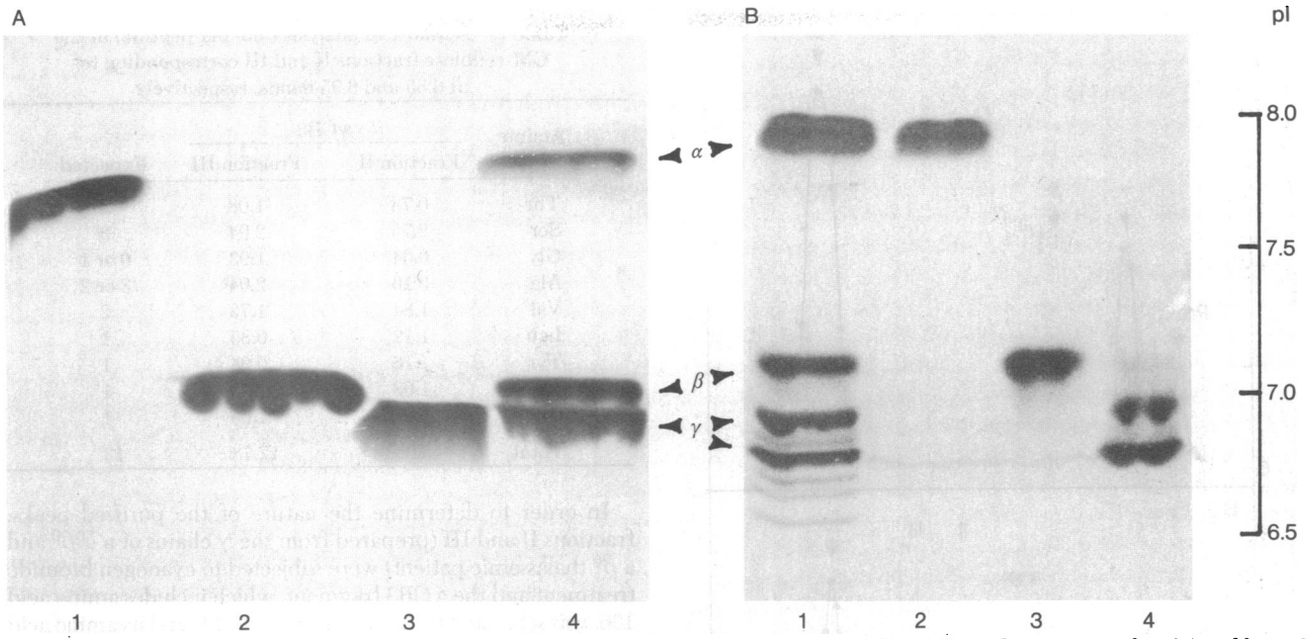

FIG. 1. Separation of human globin chains by IEF in the absence (*A*) and in the presence (*B*) of NP-40. Lanes 1, 2, and 3 of *A* and lanes 2, 3, and 4 of *B* show chromatographically pure α, β, and γ globin chains, respectively. Lanes 4 of *A* and 1 of *B* show unfractionated globins.

and the eluates were read spectrophotometrically at 500 nm. For amino acid analysis of Tp9a and Tp9b, these peptides were stained with a solution containing 2 mg of Fluram (Hoffmann–La Roche) in 100 ml of 0.1% pyridine in acetone, identified under UV light, eluted with 5% (wt/vol) acetic acid, vacuum dried, and repurified by ascending chromatography on paper in a solvent composed of 98% formic acid, secondary butanol, and water (30:140:30 vol/vol) (8). Amino acid analysis of these peptides and of the γCB3 fragment was performed on an LKB model 4101 amino acid analyzer after elution of the peptides directly from the paper with 6 M HCl and their hydrolysis under reduced pressure at 110°C for 24 hr.

RESULTS

The separation of human globin chains by IEF in the presence or absence of NP-40 is shown in Fig. 1; whereas α and β chains focus as rather homogeneous bands both in the presence and absence of NP-40, the γ chain marker is split into two bands of similar intensities, of pI 6.95 and 6.85, upon addition of NP-40. The same phenomenon can be observed also by gel electrophoresis (Fig. 2); again, the γ chain separates into two components of approximately similar intensities. The latter observation suggests that the detergent might be the agent effective in splitting the γ chains, rather than any peculiar interaction with Ampholines, and prompted a study of globin chain separation by CM-cellulose chromatography in the presence of NP-40.

Fig. 3 shows the elution profile of radioactive α and γ chains synthesized *in vitro* by reticulocytes from a β^0 thalassemic patient; with the traditional chromatography (Fig. 3*A*) (10) the γ peak represents approximately 35% of the α chain, while the pre-γ peak (*N*-acetylated γ chains) is about 11%; in contrast (Fig. 3*B*), when 3% NP-40 is added to the elution gradient, three radioactive peaks appear in the γ region; they represent 8%, 16%, and 19% of the α chain, respectively. These values are consistent with the hypothesis that peak I represents the pre-γ chains, whereas peaks II and III correspond to the split γ chains.

To test this possibility the protein fractions from each of these peaks were precipitated with acid/acetone and analyzed by IEF and cellulose acetate electrophoresis; Fig. 3*B inset* shows that upon focusing fraction II gives rise to a single band of pI 6.85, whereas fraction III bands at pI 6.95; fraction I, on the other hand, focuses as a single band at pI 6.75, the value of acetylated γ chains purified by conventional CM-cellulose methods (not shown) and a minor band at pI 6.85 (contaminating peak II protein). The same result (not shown) was obtained by autoradiography. By cellulose acetate electrophoresis, fraction II corresponds to the slow γ band and fraction III to the fast one. Thus, this modification of the chromatographic technique appears to be able to separate the apparently uniform γ chain peak into two species, which correspond exactly to those that are identified by IEF of pure unfractionated γ globin chains. The pI 6.85 protein elutes at 9.5 mM sodium phosphate,

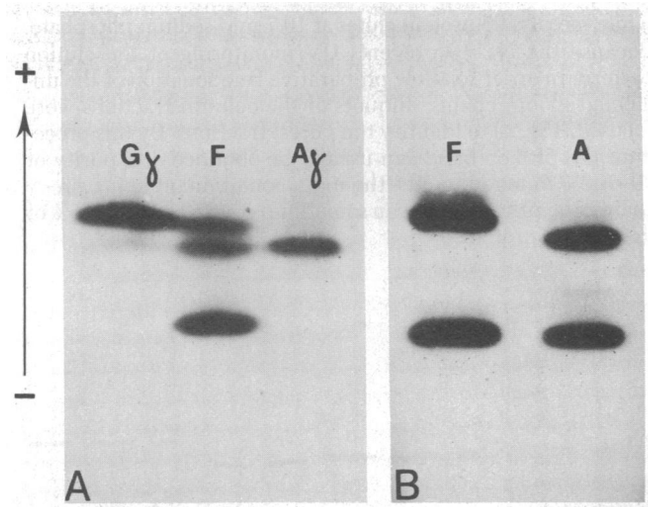

FIG. 2. Cellulose acetate electrophoresis of human globin chains in the presence (*A*) and in the absence (*B*) of NP-40. The γ chains, which in the absence of the detergent migrate as a single fast-moving band (*B*, upper band of lane F), in the presence of NP-40 split into two bands (*A*, upper bands of lane F). Purified CM-cellulose fractions II and III (see Fig. 3) are run in lanes $^{A}\gamma$ and $^{G}\gamma$. Lane A of *B* shows adult globin chains α (lower band) and β (upper band).

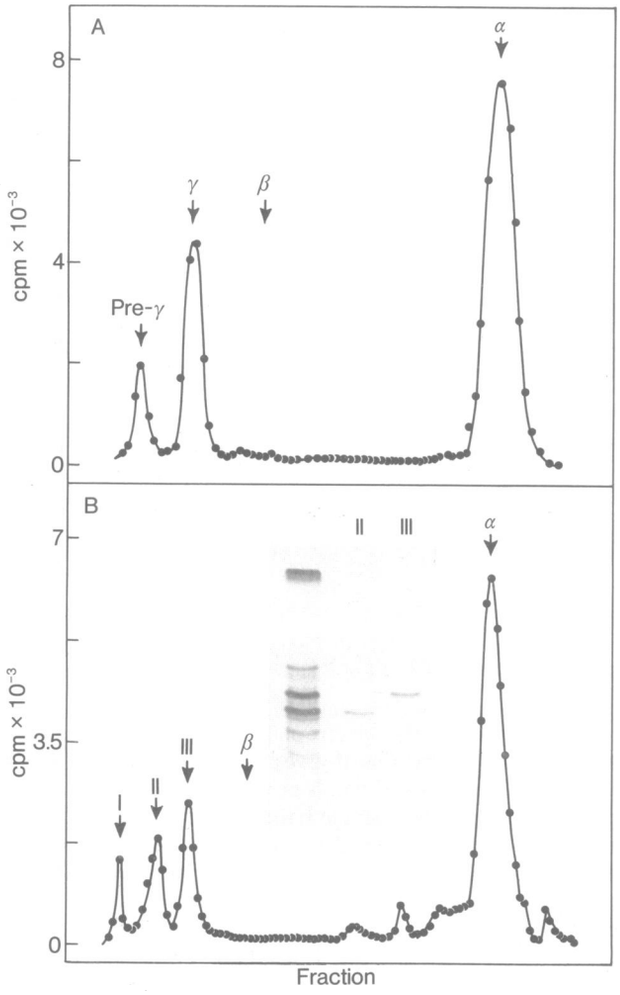

FIG. 3. CM-cellulose chromatography of fetal globin chains in the absence (*A*) and in the presence (*B*) of NP-40. Upon addition of the detergent, γ chains elute in three fractions (I, II, and III) that focus at pI 6.75 (not shown), 6.85, and 6.95, respectively (see *Inset*). The first lane of the *Inset* shows unfractionated fetal globin focused in parallel.

while the pI 6.95 protein elutes at 10.7 mM sodium phosphate. Because the NP-40 prevents UV monitoring of the elution pattern, in order to allow preparative fractionation of the unlabeled globin chains, aliquots of the column fractions were focused (Fig. 4) to identify the purest fractions. By this procedure the pI 6.85 band can usually be obtained at a purity of 90–100% in mg amounts, the main contaminant being pre-γ chains; the pI 6.95 band can sometimes contain some 5–15% of the pI 6.85 protein.

Table 1. Amino acid analysis (mol per peptide) of the CM-cellulose fractions II and III corresponding to pI 6.85 and 6.95 bands, respectively

Amino acid	γCB3		
	Fraction II	Fraction III	Expected
Thr	0.78	1.08	1
Ser	2.75	2.94	3
Gly	0.04	1.03	0 or 1
Ala	3.16	2.04	3 or 2
Val	1.84	1.73	2
Leu	1.12	0.85	1
Tyr	1.16	0.94	1
Hys	1.08	1.12	1
Arg	1.15	1.05	1
Total	13.08	12.78	13

In order to determine the nature of the purified peaks, fractions II and III (prepared from the γ chains of a $\delta^0\beta^0$ and a β^0 thalassemic patient) were subjected to cyanogen bromide treatment and the γCB3 fragment, which includes amino acid 136, was separated by fingerprinting (13, 14) and its amino acid composition was analyzed. Table 1 shows that the pI 6.85 band contains essentially no glycine, while alanine is present in the proportion of 3 nmol/nmol of peptide; on the other hand, band pI 6.95 contains 1 nmol of glycine and 2 nmol of alanine per nmol of peptide. These results indicate that the pI 6.85 band is the $^{A}\gamma$ chain, whereas the pI 6.95 band is the $^{G}\gamma$ chain. The same results were obtained with purified fractions II and III from three additional patients (Table 2).

The same experiment was repeated with purified fractions from a subject whose γ chains contained about 30% of the variant $^{T}\gamma$ chain; the $^{G}\gamma/^{A}\gamma$ ratio (as determined by amino acid analysis and IEF) was approximately 1. Again, the purified fractions II and III demonstrated almost integral values for alanine and glycine, respectively (Table 2), indicating that the isoleucine-threonine substitution does not shift the $^{T}\gamma$ chain from one band to the other. By fingerprint analysis of the two fractions (Fig. 5) it is possible to determine the presence of isoleucine or threonine at position 75; whereas the $^{G}\gamma$ band shows almost exclusively the typical isoleucine-containing upper T9 peptide, the $^{A}\gamma$ band demonstrates essentially only the threonine-containing lower T9 peptide. Because proteins recovered after NP-40 chromatography give a somewhat blurred fingerprint pattern, the relevant Tp9a and Tp9b peptides were further characterized by amino acid analysis, which confirmed their identities unambiguously, by showing the expected amounts of threonine and isoleucine (see Table 3).

These results demonstrate that the $^{T}\gamma$ chain has alanine at

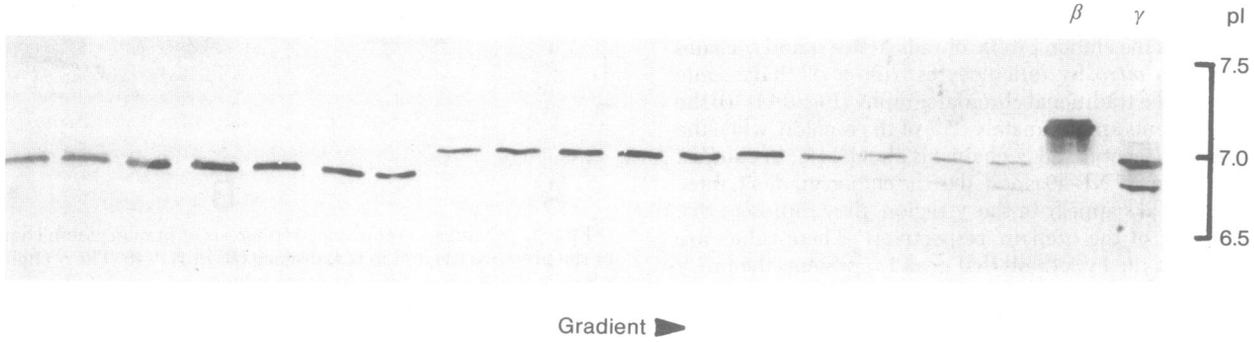

FIG. 4. IEF of gradient fractions eluted from a preparative CM-cellulose column in the presence of NP-40. Lanes β and γ indicate β and γ globin markers run in parallel.

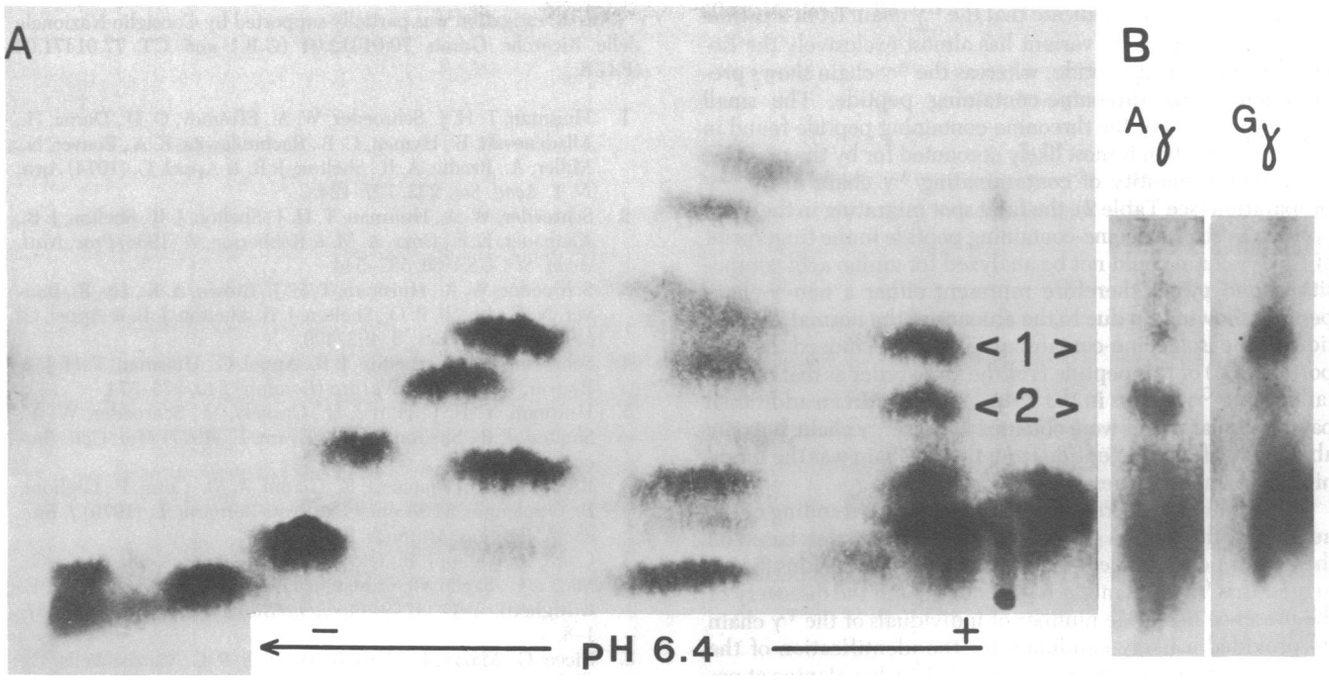

FIG. 5. Tryptic fingerprint on paper (15, 16) of whole non-aminoethylated Hb F from a β^0 thalassemic patient (*A*) and of CM-cellulose-purified fraction II and III (*B*). Peptides 1 and 2 are γTp9(Ile-75) and γTp9(Thr-75), respectively. (*B*) Relevant neutral fingerprint areas (18, 19) of purified CM-cellulose fraction II (lane $^{A}\gamma$) and fraction III (lane $^{G}\gamma$).

position 136. This conclusion is further strengthened by analysis of other thalassemic patients with high levels of $^{T}\gamma$ chains (Table 2 and results not shown).

DISCUSSION

The existence of two types of γ chains ($^{G}\gamma$ and $^{A}\gamma$) has been known for almost 10 years, but the lack of any technique capable of separating them has greatly limited the study of the regulation of their expression during development and in different pathologic conditions. After the initial observation that NP-40 splits purified γ globin chains into two components (pI 6.85 and 6.95 proteins) in IEF experiments (6, 7), we have now shown that the same result can also be obtained by NP-40 acetate electrophoresis and NP-40 CM-cellulose chromatography. The latter technique has enabled us to prepare purified chains corresponding to the $^{A}\gamma$ and $^{G}\gamma$ chains, confirming our previous indirect conclusion based on genetic analysis (7).

The molecular basis for the "Nonidet effect" is unknown, but we have suggested (7) that NP-40, an uncharged ligand, alters the pI of γ globin chains by binding to them and either masking some charged groups on the polypeptide chains or altering the intrinsic pK of some ionizable groups in the neighborhood of the binding sites. Because alanine is slightly more hydrophobic than glycine, the binding of NP-40 to the 133–141 $^{A}\gamma$ residue stretch (Met-Val-Thr-Ala-Val-Ala-Ser-Ala-Leu) might be favored. Because purified γ chains of individuals having the $^{T}\gamma$ chains still show only two bands in IEF, it appears that the same effect does not operate to separate the isoleucine-containing molecule from threonine-containing molecule. Therefore it is possible to separate in a highly purified state the $^{G}\gamma$ from the $^{A}\gamma$ chains of patients having high levels of the $^{T}\gamma$ variant and to assign this variant to either $^{G}\gamma$ or $^{A}\gamma$ chain by analyzing the tryptic map.

Table 2. $^{T}\gamma$, $^{G}\gamma$, and $^{A}\gamma$ determination in different patients

Thalassemia patient	Chain	$^{T}\gamma$, %	mol per γCB3		IEF
			Gly	Ala	$^{G}\gamma/(^{G}\gamma + ^{A}\gamma)$
L. (β^0)	γ total	0	0.7	2.29	0.6
	$^{A}\gamma$	0	0.04	3.16	0.02
	$^{G}\gamma$	0	1.03	2.04	0.99
T. ($\delta^0\beta^0$)	γ total	0	0.5	2.42	0.46
	$^{A}\gamma$	0	0.07	2.82	0.05
	$^{G}\gamma$	0	ND	ND	0.9
C. (β^0)	γ total	45	0.61	2.41	0.5
	$^{A}\gamma$	90	0.15	2.91	0.1
		95	0.05	3.1	0.05
	$^{G}\gamma$	10	0.86	2.16	0.85
		15	1.12	2.04	0.95
M. (β^0)	γ total	25	0.65	2.37	0.6
	$^{A}\gamma$	55	ND	ND	0.07
	$^{G}\gamma$	ND	ND	ND	ND

For discussion of determination of errors in $^{G}\gamma/^{A}\gamma$ and $^{T}\gamma$ ratios see refs. 1, 2, and 8. A band at pI 6.82 may sometimes contaminate the $^{A}\gamma$ chain in IEF experiments; the main contaminant of purified $^{A}\gamma$ chains is the pI 6.75 band (mainly acetylated γ chains); we have assumed that its $^{G}\gamma/(^{G}\gamma + ^{A}\gamma)$ ratio is the same as that of total γ chains. ND, not determined.

Table 3. Amino acid analysis (mol per peptide) of the γTp9 peptides from the $^{G}\gamma$ and $^{A}\gamma$ chains (numbers 1 and 2 in Fig. 5*B*)

Amino acid	γTp9(Ile-75) from $^{G}\gamma$ chain	γTp9(Thr-75) from $^{A}\gamma$ chain	Expected
Asp	1.08	1.12	1
Thr	0.89	1.72	1 or 2
Ser	1.09	1.04	1
Gly	1.12	1.18	1
Ala	1.00	1.09	1
Val	0.88	0.84	1
Ile	0.84	0	1 or 0
Leu	1.88	1.79	2
Lys	1.02	0.96	1
Total	9.80	9.74	10

Fig. 5 and Table 3 indicate that the $^{G}\gamma$ chain from a patient with high levels of $^{T}\gamma$ variant has almost exclusively the isoleucine-containing peptide, whereas the $^{A}\gamma$ chain shows predominantly the threonine-containing peptide. The small amount (5–10%) of the threonine-containing peptide found in the $^{G}\gamma$ preparation is most likely accounted for by the presence of an equal quantity of contaminating $^{A}\gamma$ chain in the $^{G}\gamma$ preparation (see Table 2); the faint spot migrating in the same position as the isoleucine-containing peptide in the fingerprint of the $^{A}\gamma$ chain could not be analyzed for amino acid composition, and might therefore represent either a non-γ-chain peptide showing up due to the absence of the normal T9 peptide, or the isoleucine-containing T9 peptide; indeed the proportion (5%) of this peptide is of the same order as that of contaminating $^{G}\gamma$ chains in the $^{A}\gamma$ preparation. In an additional patient similar results were obtained with the $^{G}\gamma$ chain, but only about 50% of the T9 peptide from the $^{A}\gamma$ chain was the threonine-containing species.

These data may be relevant to further understanding of the number of the human globin genes. Genetic models based on the rate of postnatal decay of $^{G}\gamma$ variant globin chains (5, 20) suggest that $^{G}\gamma$ gene might be duplicated and the discovery of the presence in a large number of individuals of the $^{T}\gamma$ chain (8) provided a likely candidate for the identification of the duplicated gene. Our data, showing that $^{T}\gamma$ has alanine at position 136 and that $^{T}\gamma$ (as inferred from fingerprint analysis of purified $^{A}\gamma$ chains) is present in amounts that probably account for the total of the $^{A}\gamma$ chain of at least one patient, do not support this hypothesis but rather suggest that the $^{T}\gamma$ gene is a common allele of the $^{A}\gamma$ gene in the Italian population; according to this interpretation patient C. should be a $^{T}\gamma$ homozygote, whereas patient M. should be a $^{T}\gamma/^{A}\gamma$ heterozygote. This interpretation is consistent with liquid hybridization data (unpublished results) obtained with $cDNA_{\gamma}$ (21) demonstrating two γ globin genes per haploid genome both in the patient described here with high levels of $^{T}\gamma$ chain and in patients with no $^{T}\gamma$ chains. However, final conclusions that $^{A}\gamma$ gene is not duplicated will require extensive liquid hybridization and gene mapping experiments in a number of different subjects.

The observation that IEF and cellulose acetate electrophoresis in the presence of NP-40 split purified γ globin chains into two bands that can be demonstrated by direct chemical analysis to correspond to the separated $^{G}\gamma$ and $^{A}\gamma$ chains provides a tool for biosynthetic studies of these chains and for extensive surveys of their ratios during development and in different pathological conditions. Moreover, the demonstration that $^{T}\gamma$ is a variant $^{A}\gamma$ chain ($^{A}\gamma^{Sardinia}$) provides a very commonly occurring mutant for genetic studies of the γ gene cluster.

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1. Huisman, T. H. J., Schroeder, W. A., Efremov, G. D., Duma, H., Mladenovski, B., Hyman, C. B., Rachmilewitz, E. A., Bouver, N., Miller, A., Brodie, A. R., Shelton, J. R. & Appel, G. (1974) *Ann. N. Y. Acad. Sci.* **232**, 107–124.
2. Schroeder, W. A., Huisman, T. H. J., Shelton, J. R., Shelton, J. B., Kleihauer, E. F., Dozy, A. M. & Robberson, B. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 537–544.
3. Schroeder, W. A., Huisman, T. H. J., Brown, A. K., Uy, R., Bouver, N. G., Lerch, P. O., Shelton, J. R., Shelton, J. B. & Appel, G. (1971) *Pediatr. Res.* **5**, 493–499.
4. Schroeder, W. A., Shelton, J. R., Appel, G., Huisman, T. H. J. & Bouver, N. G. (1972) *Nature (London)* **240**, 273–274.
5. Huisman, T. H. J., Harris, H., Gravely, M., Schroeder, W. A., Shelton, J. R., Shelton, J. B. & Evans, L. (1977) *Mol. Cell. Biochem.* **17**, 45–55.
6. Righetti, P. G., Gianazza, E., Gianni, A. M., Comi, P., Giglioni, B., Ottolenghi, S., Secchi, C. & Rossi-Bernardi, L. (1979) *J. Biochem. Biophys. Methods* **1**, 45–57.
7. Comi, P., Giglioni, B., Ottolenghi, S., Gianni, A. M., Ricco, G., Mazza, U., Saglio, G., Camaschella, C., Pich, P. G., Gianazza, E. & Righetti, P. G. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1–8.
8. Ricco, G., Mazza, U., Turi, R. M., Pich, P. G., Camaschella, C., Saglio, G. & Bernini, L. F. (1976) *Hum. Genet.* **32**, 305–313.
9. Griffoni, V., Kamuzora, H., Lehmann, H. & Charlesworth, D. (1975) *Acta Haematol.* **53**, 347–355.
10. Clegg, J. B., Naughton, M. A. & Weatherall, J. D. (1966) *J. Mol. Biol.* **19**, 91–108.
11. Righetti, P. G., & Righetti, A. B. B. (1975) in *Isoelectric Focusing*, eds. Arbuthnott, J. P. & Beeley, J. A. (Butterworth, London), pp. 114–131.
12. Ui, N. (1973) *Ann. N. Y. Acad. Sci.* **209**, 198–209.
13. Bernini, L. F., Fiorelli, G., Matuonto, V., Bianchi, G. & Chiesa G. (1971) *Atti riunioni gruppo studio dell'eritrocita* (Cilag, Milano, Italy), p. 25.
14. Kamuzora, H., Ringelmann, B., Konotey-Ahulu, F. I. D., Lehmann, H. & Lorkin, P. A. (1975) *Acta Haematol.* **53**, 315–320.
15. Sick, K., Beale, D., Irvine, D., Lehmann, H., Goodall, P. T. & McDougall, S. (1967) *Biochim. Biophys. Acta* **140**, 231–242.
16. Beale, D. (1967) *Biochem. J.* **103**, 129–140.
17. Dreyer, W. J. & Bynum, E. (1967) *Methods Enzymol.* **11**, 32–39.
18. Ingram, V. M. (1958) *Biochim. Biophys. Acta* **28**, 539–545.
19. Baglioni, C. (1961) *Biochim. Biophys. Acta* **48**, 392–396.
20. Schroeder, W. A., Bannister, W. H., Grech, J. L., Brown, A. K. Wrightstone, R. N. & Huisman, T. H. J. (1973) *Nature (London) New Biol.* **244**, 89–90.
21. Old, J., Clegg, J. B., Weatherall, D. J., Ottolenghi, S., Comi, P., Giglioni, B., Mitchell, J., Tolstoshev, P. & Williamson, R. (1976) *Cell* **8**, 13–18.