Organization of the α-Globin Genes in the Chinese α-Thalassemia Syndromes

STEPHEN H. EMBURY, ROGER V. LEBO, ANDRÉE M. DOZY, and YUET WAI KAN,
Howard Hughes Medical Institute Laboratory and Hematology Service, San Francisco General Hospital, Department of Medicine and Laboratory Medicine, University of California, San Francisco, San Francisco, California 94110

ABSTRACT The α-thalassemia syndromes are a group of inherited anemias, the clinical severity of which has been shown to increase with the number of α-globin structural genes deleted. Employing restriction endonuclease gene mapping, we defined the organization of the α-globin genes in cellular DNA from Chinese subjects with various α-thalassemia syndromes. The four α-globin genes of normals are at two loci located on a 23.0-kilobase pair (kb) Eco RI fragment. In deletion type hemoglobin-H disease the 5′ α-globin locus is deleted and the single 3′ α-globin locus is found on a 19.0-kb Eco RI fragment. In α-thalassemia-2 there are two α-globin genes on a 23.0-kb Eco RI fragment and one on a 19.0-kb fragment. In α-thalassemia-1 and the nondeletion type of hemoglobin-H disease the two α-globin genes are at two loci on one chromosome and none reside on the other chromosome.

INTRODUCTION

The α-thalassemia syndromes are a group of inherited anemias usually caused by deletion of the α-globin structural genes (1, 2). In the Asian population, the clinical severity of the α-thalassemia syndromes increases with the number of the α-globin genes deleted. Nonthalassemic individuals have four copies of α-globin genes per diploid genome, whereas α-thalassemia trait (α-thalassemia-1) subjects have two, deletion type of hemoglobin-H disease subjects have one, and homozygous α-thalassemia (hydrops fetalis) subjects have none (3). It is known indirectly from family studies that silent carrier (α-thalassemia-2) subjects have three α-globin genes (4). An exception to this pattern is the nondeletion type of hemoglobin-H disease, in which two α-globin genes are present, but which is clinically as severe as the deletion type of hemoglobin-H disease where only one α-globin gene persists (5).

We used the restriction endonuclease mapping technique of Southern (6) to define these syndromes further. In this method, cellular DNA is first digested with one or more restriction endonucleases that cleave DNA at a specific nucleotide sequence. The DNA fragments are separated according to size by agarose gel electrophoresis and transferred from the gel onto nitrocellulose filters. The filters are hybridized with a radioactive DNA sequence that is complementary to the α-globin gene. The α-globin gene-containing DNA fragments can then be detected by autoradiography. With this method we define the α-globin genes in the α-thalassemia syndromes.

METHODS

DNA was prepared from leukocytes, placentas, cultured fibroblasts, and cultured Epstein-Barr virus-transformed B lymphocytes (7) of normal α-thalassemia-2, α-thalassemia-1, deletion type of hemoglobin-H disease, nondeletion type of hemoglobin-H disease (5), and hydrops fetalis subjects, as described (8). Aliquots of 7–10 μg DNA were digested at 37°C for 4–7 h with the restriction enzymes Eco RI, Hpa I, Hin dIII, or their combination, according to conditions specified for each enzyme by the manufacturer (New England Biolabs, Beverly, Mass.).

The digested DNA samples were electrophoresed and transferred onto nitrocellulose filters (Millipore Corp., Bedford, Mass. or Schleicher & Schuell, Inc., Keene, N. H.) by a method modified from Southern (6, 8). The filters were hybridized and autoradiographed and the complementary DNA (cDNA)[1] prepared as described (8).

3′-specific, 5′-specific, and total α-globin gene probes were prepared from hybrid plasmid JW 101 that contains a synthetic copy of the human α-globin gene in pMB 9 (9). The plasmid was cultured and isolated (10) according to NIH guidelines. For total α-probe a 1.6-kilobase pair (kb) DNA fragment con-

Dr. Kan is an Investigator of the Howard Hughes Medical Institute.
Received for publication 9 March 1979 and in revised form 9 April 1979.

[1] *Abbreviations used in this paper*: cDNA, complementary DNA; kb, kilobase pairs.

taining all the α-globin cDNA sequences was excised by digestion with Mbo II according to the conditions specified by the manufacturer (New England Biolabs) and isolated by agarose gel electrophoresis (11). To prepare 3′- and 5′-specific globin gene probes, we digested the JW 101 plasmid with Hin dIII and isolated by agarose gel electrophoresis (11) the 0.5-kb fragment that contained the 3′ α-globin gene sequences and the 5.2-kb fragment which contained the 5′ α-globin gene sequences.[2] These plasmid fragments were labeled with ^{32}P by nick translation to a 1–4 × 10^8-sp act cpm/μg DNA. The 200-μl reaction mixture contained 0.5 μg plasmid DNA and 12.5 U DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg/ml gelatin, 5 μM [α-^{32}P]dCTP and [α-^{32}P]dTTP (New England Nuclear, Boston, Mass. or Amersham Corp., Arlington Heights, Ill.) and 15 μM unlabeled dATP and dGTP, and was incubated at 22°C for 1–4 h. The reaction mixture was made 20 mM with EDTA and the radioactive DNA separated from unincorporated nucleotides by chromatography on Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N. J.).

RESULTS

When normal DNA was digested with Eco RI, (Fig. 1A) a DNA fragment of ≅23.0 kb fragment containing the α-globin genes was generated. The same size fragment was also obtained from digestion of the DNA of α-thalassemia-1 and the nondeletion type of hemoglobin-H disease. This DNA fragment contained both α-globin loci (12). Thus, nonthalassemic subjects had such a fragment on both chromosomes, whereas Chinese subjects with α-thalassemia-1 and the nondeletion type of hemoglobin-H disease had these two loci on a single chromosome. In the deletion type of hemoglobin-H disease, the single remaining α-globin gene was found on a fragment about 19.0 kb in length. In α-thalassemia-2 both the 23.0- and 19.0-kb fragments were present, a result compatible with the presence of two α-loci on one chromosome and one on the other. Both these fragments contained 5′ and 3′ sequences (Table I). We did not detect any DNA that reacted with the α-probe in homozygous α-thalassemia.

When normal DNA was digested with the enzyme Hpa I, two α-specific fragments, 14.5 and 4.1 kb in length, were generated (Fig. 1B). Each contained both 5′ and 3′ sequences (Table I), and therefore the complete α-globin gene. Both fragments were seen in DNA from the α-thalassemia-2, α-thalassemia-1 and the nondeletion type of hemoglobin-H disease. In contrast, only the 14.5-kb fragment was found in the deletion type of hemoglobin-H disease. We observed some variation in the size of the larger fragments, a result most likely due to a polymorphism and apparently unrelated to thalassemia.[3]

Digestion of the normal DNA with Hin dIII yielded

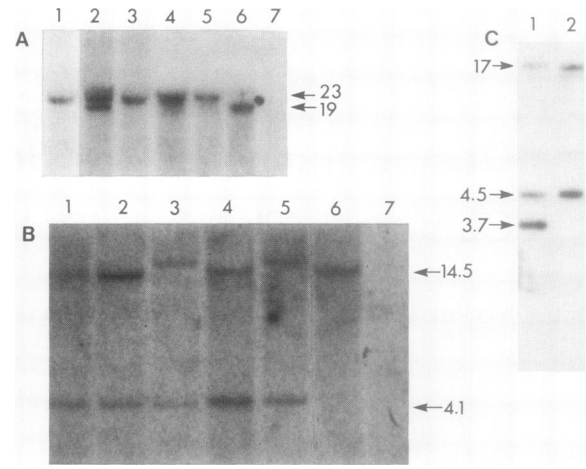

FIGURE 1 Autoradiographs show restriction fragments containing α-globin genes. Sizes of the fragments in kb are indicated by arrows in the margins. (A) Eco RI digested DNA samples after hybridization with globin specific cDNA. This photograph is of only the α-specific fragments and does not show the β-, δ-, and γ-specific fragments. The DNAs are from the following subjects: (*1*) nonthalassemic; (2) α-thalassemia-2; (*3, 4*) α-thalassemia-1; (*5*) nondeletion type of hemoglobin-H disease; (6) deletion type of hemoglobin-H disease; and (7) hydrops fetalis. (B) Hpa I digested DNA samples following hybridization with total α-specific probe. The DNAs are from subjects numbered as in A. (C) Hin dIII digested DNA samples after hybridization with total α-specific probe. The DNAs are from the following subjects: (*1*) nonthalassemic; and (2) deletion type of hemoglobin-H disease.

three α-globin gene-containing fragments, 17.0, 4.5, and 3.7 kb in length (Fig. 1C). Because Hin dIII is known to cut within the α-globin structural gene sequences (between the codons corresponding to amino acids 90 and 91) (13), the finding of three fragments confirmed the duplication of the α-loci. Table I shows that the 17.0 kb fragment hybridized only with the 5′ probe, the 4.5-kb fragment hybridized only with the 3′ probe, and the 3.7-kb fragment hybridized with both probes. This result is compatible with the interpretation that the 3.7-kb fragment contains the 3′ end of the 5′ α-locus, the DNA in the intergenic region, and the 5′ end of the 3′ α-locus. This fragment could be called the "bridging fragment" and defines the distance between the two α-loci. In α-thalassemia-2, α-thalassemia-1, and the nondeletion type of hemoglobin-H disease the same three fragments were seen. In the deletion type of hemoglobin-H disease a DNA fragment of about the same size as the bridging fragment was missing and only a 17.0-kb fragment containing the 5′ α-sequences and a 4.5-kb fragment containing the 3′ α-sequences was seen.

To clarify the nature of the deletion, a series of double digests was performed to locate these restriction sites relative to one another. These filters were hybridized with 5′ specific, 3′ specific, or total α-probes to establish the

[2] Forget, B. Personal communication.

[3] Embury, S. H., A. M. Dozy, and Y. W. Kan. Unpublished data.

TABLE I

Sizes and Orientation of α-Specific DNA Fragments Generated by Various Restriction Endonucleases

Enzymes*	Fragment size	Normal DNA‡	Deletion type Hb-H DNA‡
	kb		
Eco RI	23.0	5′ + 3′	—
	19.0	—	5′ + 3′
Hpa I	4.1	5′ + 3′	—
	14.5	5′ + 3′	5′ + 3′
Hin dIII	17.0	5′	5′
	3.7	5′ + 3′	—
	4.5	3′	3′
Hin dIII + Eco RI	17.0	5′	5′
	3.7	5′ + 3′	—
	1.8	3′	3′
Eco RI + Hpa I	4.1	5′ + 3′	—
	4.8	5′ + 3′	5′ + 3′
Hin dIII + Hpa I	3.4	5′	—
	0.7	3′	—
	3.0	5′	5′
	4.5	3′	3′

* The enzyme or combination of enzymes employed to generate the α-specific DNA fragments whose size is listed.
‡ The orientation of the DNA fragments as determined by hybridization with the 3′- and 5′-specific α-probe.

5′-3′ orientation of each fragment, and the results are shown in Table I and in Fig. 2. The Hin dIII + Eco RI digest showed the size and orientation of the 17.0- and 3.7-kb Hin dIII fragments to be unchanged, but the 3′ 4.5-kb Hin dIII fragment was cleaved by Eco RI to a 1.8-kb fragment. The Hpa I + Eco RI digest revealed the 4.1-kb Hpa I fragment to be unchanged, but the 14.5-kb Hpa I fragment was cleaved by Eco RI to a 4.8-kb fragment that contained both 5′ and 3′ sequences. The Hin dIII + Hpa I digest showed the 3′ 4.5-kb Hin dIII fragment to be unchanged, the 5′ 17.0-kb Hin dIII fragment to be cleaved by Hpa I to a 3.4-kb fragment and the 3.7-kb Hin dIII bridging fragment to be cleaved by Hpa I to a 0.7-kb fragment that hybridized only with the 3′ probe and a 3.0-kb fragment that hybridized only with the 5′ probe. In deletion type hemoglobin-H disease the 3.4- and 0.7-kb fragments were missing. Because these two deleted fragments were derived from the 4.1-kb or 5′ Hpa I fragment, it was the 5′ α-globin gene that had been deleted. The absence of the 3.7-kb fragment on the single Hin dIII digest indicates that one of the two Hpa I sites located on either side of the 5′ α-globin gene was included in the deletion.

DISCUSSION

In this paper we have extended our preliminary observations on the organization of the α-globin structural

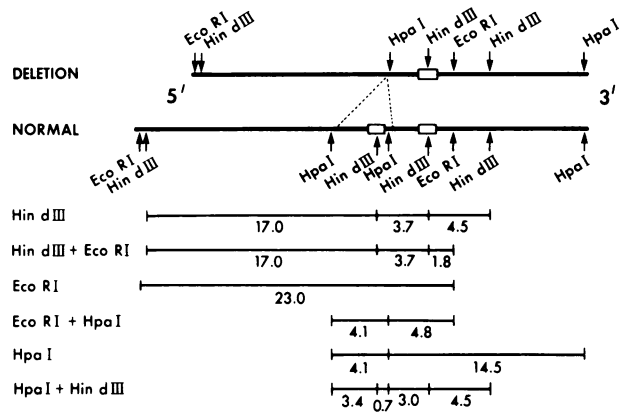

FIGURE 2 Restriction endonuclease maps of the α-globin genes of the normal (two α-locus) and the deletion-type (single α-locus) chromosome. The lengths in kb of α-specific DNA fragments generated by digestion with these enzymes and their combination are shown below the map of the normal chromosome.

genes in normal and the Chinese α-thalassemia syndromes (14). Our results were in agreement with those of Orkin (12) on the arrangement of the two normal α-globin loci and that the distance from the Hin dIII site of one locus to that of the other was 3.7 Kb. In addition, by using specific 5′ and 3′ sequences as probes, we unambiguously establish the 5′ and 3′ orientation of the α-loci.

These results also establish the nature of the deletion that result in the single α-globin locus in deletion type hemoglobin-H disease. Gene mapping showed that a piece of DNA that contains the 5′ α-globin locus is deleted. The deletion results in the loss of the 4.1-kb Hpa I and the 3.7-kb Hin dIII fragments seen in normals.

These findings provide a method for detection of the α-thalassemia-2 gene. Thus, the previously hematologically undetectable silent carrier state can be diagnosed by the findings of two α-globin fragments on Eco RI digests and could be represented by the genotype (-α/αα). Also, the two molecular arrangements giving rise to the α-thalassemia-1 phenotype could also be distinguished. If both α-globin genes on the same chromosome are deleted (--/αα), Eco RI and Hpa I will show the normal pattern. On the other hand, if the α-thalassemia-1 phenotype is a result of the deletion of the two 5′ α-globin loci, (homozygous α-thalassemia-2 or -α/-α), Eco RI digest will show only the 19.0-kb band and Hpa I only the 14.5-kb band. The finding of the --/αα genotype in Chinese α-thalassemia-1 subjects explains the occurrence of homozygous α-thalassemia in this group. This disease is not found in the black population because the α-thalassemia-1 phenotype is a result of the deletion of the two α-globin loci on opposite chromosomes (-α/-α) (15).

These results also confirm the findings previously

shown by liquid hybridization that both α-globin genes loci are intact in the nondeletion type of hemoglobin-H disease (5). Our results from mapping with these and other enzymes have not detected any difference between this genotype and the normal. However, possible differences may be found by more extensive restriction mapping, or by cloning these genes for further structural and functional analysis.

The findings described have already been shown to be of practical clinical value. The sensitivity of this technique allows it to be used as a more rapid means of prenatal diagnosis (16). The detection of the hemoglobin-H genotype in a patient with sickle cell trait permitted the correct diagnosis that had previously not been made because of the paucity of β-globin tetramers (17).

We have studied with single enzyme digests several Chinese patients with deletion type hemoglobin-H disease and obtained results similar to those of this individual. However, even within the same racial group, different mutational events may have occurred. Orkin et al. (18) obtained a different restriction pattern from our study in a Chinese subject with hemoglobin-H disease. Also, in the Mediterranean population with hemoglobin-H disease, heterogeneity in α-globin gene numbers and restriction patterns have also been detected (18, 19). It thus seems likely that, as in β-thalassemia, the mechanism that leads to abnormal gene function in α-thalassemia is heterogeneous.

ACKNOWLEDGMENTS

We thank Dr. Bernard Forget for his generous gift of the plasmid JW 101, and Judy C. Chang for her helpful discussion.

This work was supported in part by grants AM 16666 and HL 20985 from the National Institutes of Health, and the National Foundation–March of Dimes.

REFERENCES

1. Taylor, J. M., A. M. Dozy, Y. W. Kan, H. E. Varmus, L. E. Lie-Injo, J. Ganesan, and D. Todd. 1974. Genetic lesion in homozygous α thalassaemia (hydrops fetalis). *Nature (Lond.).* **251**: 392–393.
2. Ottolenghi, S., W. G. Lanyon, J. Paul, R. Williamson, D. J. Weatherall, J. B. Clegg, J. Pritchard, S. Pootrakul, and W. H. Boon. 1974. The severe form of α thalassemia is caused by a haemoglobin gene deletion. *Nature (Lond.).* **251**: 389–392.
3. Kan, Y. W., A. M. Dozy, H. E. Varmus, J. M. Taylor, J. P. Holland, L. E. Lie-Injo, J. Ganesan, and D. Todd. 1975. Deletion of α-globin genes in haemoglobin-H disease demonstrates multiple α-globin structural loci. *Nature (Lond.).* **255**: 255–256.
4. Wasi, P., S. Na-Nakorn, and A. Suingdumrong. 1964. Hemoglobin H disease in Thailand: a genetical study. *Nature (Lond.).* **204**: 907–908.
5. Kan, Y. W., A. M. Dozy, R. Trecartin, and D. Todd. 1977. Identification of a nondeletion defect in α-thalassemia. *N. Engl. J. Med.* **297**: 1081–1084.
6. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503–517.
7. Pattengale, P. K., R. W. Smith, and P. Gerber. 1974. B-cell characteristics of human peripheral and cord blood lymphocytes transformed by Epstein-Barr virus. *J. Natl. Cancer Inst.* **52**: 1081–1086.
8. Kan, Y. W., and A. M. Dozy. 1978. Polymorphism of DNA sequence adjacent to human β-globin structural gene: relationship to sickle mutation. *Proc. Natl. Acad. Sci. U. S. A.* **75**: 5631–5635.
9. Wilson, J. T., L. B. Wilson, J. K. de Riel, L. Villa-Komaroff, A. Efstratiadis, B. G. Forget, and S. M. Weissman. 1978. Insertion of synthetic copies of human globin genes into bacterial plasmids. *Nucleic Acids Res.* **5**: 563–581.
10. Bolivar, F., R. L. Rodriguez, M. C. Betlach, and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles I. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene.* **2**: 75–93.
11. Tabak, H. F., and R. A. Flavell. 1978. A method for the recovery of DNA from agarose gels. *Nucleic Acids Res.* **5**: 2321–2332.
12. Orkin, S. H. 1978. The duplicated human alpha globin genes lie close together in cellular DNA. *Proc. Natl. Acad. Sci. U. S. A.* **75**: 5950–5954.
13. Orkin, S. H. 1978. Selective restriction endonuclease cleavage of human globin genes. *J. Biol. Chem.* **253**: 12–15.
14. Embury, S. H., A. M. Dozy, and Y. W. Kan. 1978. Heterogeneity between Hb-H deletion and non-deletion syndromes further defined by restriction endonuclease gene mapping. *Blood.* **52**(Suppl. 1): 111. (Abstr.)
15. Dozy, A. M., Y. W. Kan, H. M. Koenig, and S. H. Embury. 1979. Alpha globin gene organization in blacks precludes the severe form of α thalassemia. *Clin. Res.* **27**: 507A. (Abstr.)
16. Kan, Y. W., and A. M. Dozy. 1978. Antenatal diagnosis of sicklecell anaemia by DNA analysis of amniotic-fluid cells. *Lancet.* **II**: 910–912.
17. Matthay, K. K., A. M. Dozy, W. C. Mentzer, and Y. W. Kan. 1978. Hemoglobin H disease with sickle cell trait. *Blood.* **52**(Suppl. 1): 115. (Abstr.)
18. Orkin, S. H., J. Old, H. Lazarus, C. Altay, A. Gurgy, D. J. Weatherall, and D. G. Nathan. 1979. The molecular basis of alpha thalassemia: frequent occurrence of dysfunctional alpha loci detected by restriction endonuclease mapping. *Cell.* In press.
19. Kan, Y. W., A. M. Dozy, G. Stamatoyannopoulos, M. G. Hadjiminas, Z. Zachariades, M. Furbetta, and A. Cao. 1978. Heterogeneity in the molecular pathology of α thalassemia. *Clin. Res.* **26**: 500A. (Abstr.)