Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 9431–9435, December 1990
Medical Sciences

Inactivation of human α-globin gene expression by a *de novo* deletion located upstream of the α-globin gene cluster

(α-thalassemia/upstream regulatory region/genetic disease/anemia)

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Communicated by Y. W. Kan, August 27, 1990

ABSTRACT **Synthesis of normal human hemoglobin A, $\alpha_2\beta_2$, is based upon balanced expression of genes in the α-globin gene cluster on chromosome 16 and the β-globin gene cluster on chromosome 11. Full levels of erythroid-specific activation of the β-globin cluster depend on sequences located at a considerable distance 5′ to the β-globin gene, referred to as the locus-activating or dominant control region. The existence of an analogous element(s) upstream of the α-globin cluster has been suggested from observations on naturally occurring deletions and experimental studies. We have identified an individual with α-thalassemia in whom structurally normal α-globin genes have been inactivated in cis by a discrete *de novo* 35-kilobase deletion located ≈30 kilobases 5′ from the α-globin gene cluster. We conclude that this deletion inactivates expression of the α-globin genes by removing one or more of the previously identified upstream regulatory sequences that are critical to expression of the α-globin genes.**

Transcription of globin genes is a tightly controlled process resulting in high-level, erythroid-specific expression. Synthesis of functional human hemoglobin tetramers, $\alpha_2\beta_2$, depends upon the balanced expression of the two α-globin genes, α2 and α1, and the β-globin gene, which are located on chromosomes 16 and 11, respectively (1). Appropriate expression of the β-globin genes is determined by promoter sequences and associated sequence motifs in close proximity to the structural genes (2–7) acting in concert with a distinct set of transcriptional control elements flanking the β-globin gene cluster. This latter set of erythroid-specific but developmentally stable elements (8, 9) includes four specific DNase I hypersensitive sites located between 6.1 and 18.0 kilobases (kb) upstream from the 5′ border of the β-globin gene cluster. These sequences, referred to as the locus activating region (LAR; ref. 10 and 11) or dominant control region (DCR; ref. 9), appear to be critical for the transcriptional activation of the β-globin genes. Evidence for their function is derived from the ability of a subset of these elements to activate the expression of linked genes (globin or heterologous) in erythroid tissue of transgenic mice (9, 12–14). The importance of these regions *in vivo* is confirmed by the observation in a subset of β-thalassemic individuals that deletion or regions encompassing these LAR elements can inactivate β-globin genes in cis (15–17). In a similar fashion, erythroid-specific DNase I hypersensitive sites have been located 30–50 kb upstream from the α-globin gene cluster, and functional assays have localized sequences in this region that activate expression of the α-globin genes (18). The importance of this region *in vivo* is suggested by studies of two thalassemic individuals with extensive deletions that overlap this region and down-regulate α-globin gene expression; in the first case, $(\alpha\alpha)^{RA}$, a 62-kb deletion extends from within the cluster (coordinate +10 with respect to the ζ2 mRNA cap site) to a position 52 kb 5′ to the cluster (coordinate −52) (19), and in the second case, $(\alpha\alpha)^{TI}$, the deletion extends from coordinate −30 and continues in a 5′ direction to the telomere of chromosome 16p (20). This report characterizes a discrete *de novo* deletion between coordinates −28 to −67 (outer limits) relative to the α-globin cluster. This deletion, which silences otherwise normal α-globin genes in cis, removes the previously described elements (18) that are critical to the *in vivo* expression of the α-globin genes.

Table 1. Hematologic status and α-globin genotypes

	Father (AJ)	Mother (EJ)	Daughter No. 1* (IJ)	Daughter No. 2 (AsJ)
Hematologic data				
Hb, g/dl	17.6	14.0	9.9	15.5
RBCs, × 10^{-6}/ml	5.5	4.8	5.0	5.4
MCH, pg	32.0	29.0	19.6	28.9
Hb H, %	0	0	11.1	0
α-Globin genotype	αα/αα	$-\alpha^{3.7}/\alpha\alpha$	$-\alpha^{3.7}/(\alpha\alpha)^{IJ}$	αα/αα

RBC, erythrocytes; MCH, mean corpuscular hemoglobin.
*Proposita.

MATERIALS AND METHODS

Control Patient Samples. Control studies were carried out on the genomic DNA or reticulocyte RNA of previously studied individuals with established genotypes: αα/αα and $-\alpha^{3.7}/\alpha\alpha$ (patients 3 and 1, respectively, described in ref. 21) and $--/-\alpha^{3.7}$ [a Cambodian male with hemoglobin H (Hb H) disease; S.A.L., unpublished data].

DNA Analysis and Mapping. Isolation of total genomic DNA from peripheral blood leukocytes, restriction digestion, gel analysis, Southern transfer and hybridization (with a ψ-ζ gene fragment probe, pHpζ-Hind III.Pst I) were all carried out as described (22). Haplotype analysis (see Fig. 3) was carried out by Southern blotting (for details, see ref. 19) with a series of probes that detect a defined set of polymorphic markers in and surrounding the α-globin gene cluster located within 16p13.3. These markers include the following: α-globin 5′ hypervariable region (HVR) (23), L1.1 (M. A. Vickers, J. P. Bennett, and D.R.H., unpublished results; polymorphism at coordinate −14 of the α-globin cluster), inter-ζ HVR (24), α-globin 3′ HVR (25), and EKMDA2-I (26) using the subclone EKA0.9 (27).

Gene Cloning and Sequencing. Total genomic DNA from the proposita (IJ) was digested with *Bam*HI and size frac-

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Abbreviations: HVR, hypervariable region; LAR, locus-activating region.

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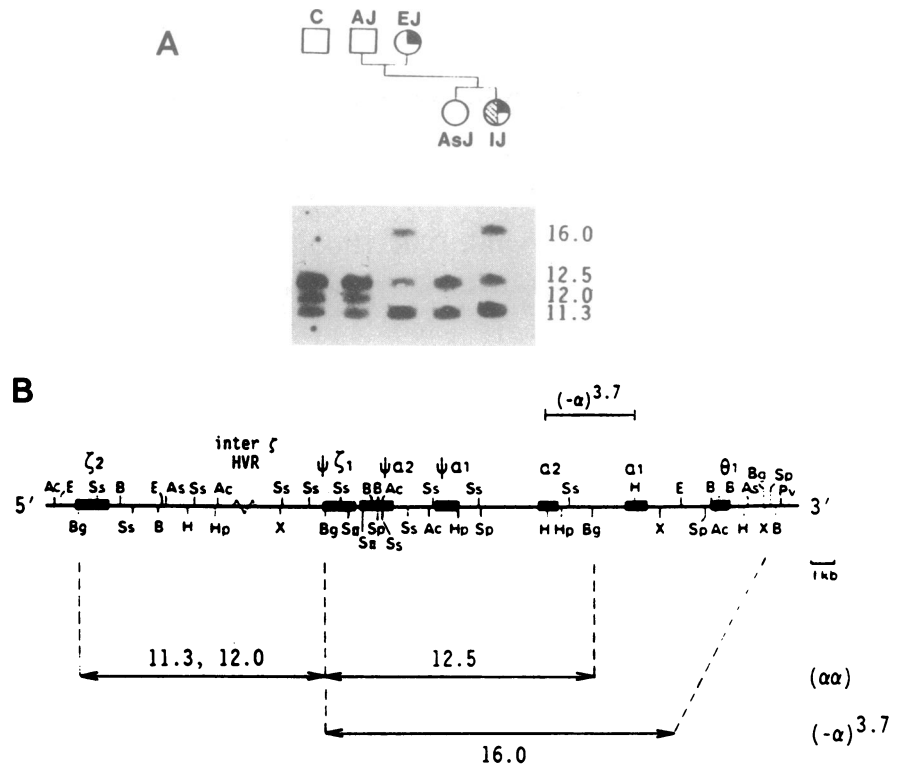

FIG. 1. Southern blot analysis of the α-globin gene cluster. (*A*) Southern analysis of α-globin gene organization in members of the reported kindred. Total genomic DNA from the normal control (C), the father (AJ), mother (EJ), the proposita (IJ), and her sister (AsJ) were digested with *Bgl* II and analyzed by Southern blotting with a ψ-ζ gene fragment probe. The 16.0-kb band contains the ψ-ζ gene and a single α-globin gene on the $-\alpha^{3.7}$ deletion chromosome, noted by the filled upper right quadrant of the circle, whereas the 12.5-kb fragment corresponds to a DNA region containing the ψ-, ζ-, and α2-globin genes on the normal chromosome. The 12.0-kb and 11.3-kb bands contain the ζ-globin gene and differ in length due to an inter-ζ HVR polymorphism (24). The $(\alpha\alpha)^{IJ}$ determinant in the proposita is indicated by the crosshatched left half of the circle. (*B*) Map of the normal (αα) and $-\alpha^{3.7}$ deletion containing α-globin gene clusters. The predicted *Bgl* II (Bg) hybridizing fragments and the position of the $-\alpha^{3.7}$ deletion are indicated. Genes and pseudogenes are indicated as filled rectangles. The inter-ζ HVR is shown as a zigzag. Ac, *Acc* I; As, *Asp* 718; Pv, *Pvu* II; SII, *Sst* II; Sp, *Sph* I; Ss, *Sst* I. The remaining enzymes are as indicated in Fig. 4.

tionated on a sucrose density gradient. Fractions containing the 14-kb and 10-kb fragments were cloned in the EMBL3 vector, and α-globin recombinant phage plaques were identified by hybridization to the α-globin gene probe (1.5-kb *Pst* I fragment of pBRα1.5). The α1- and α2-globin genes on the 14-kb (αα) *Bam*HI fragment were separately subcloned on

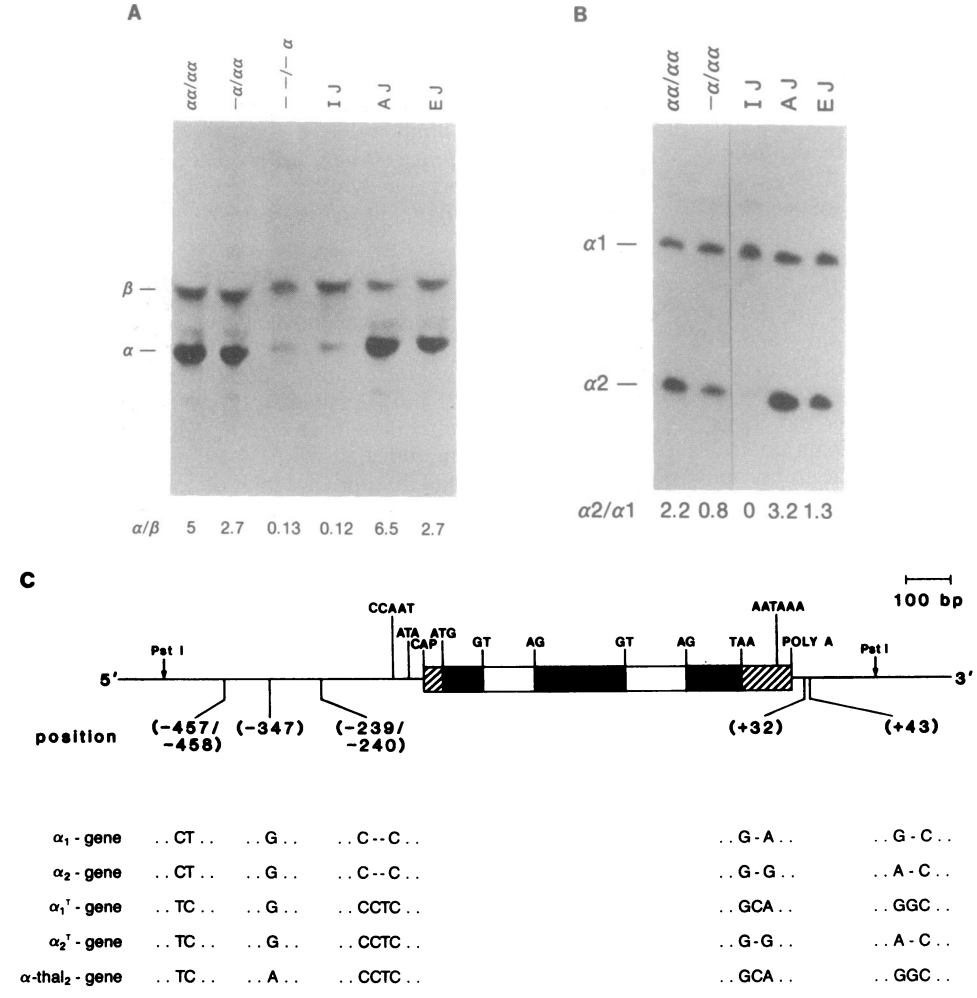

FIG. 2. Analysis of reticulocyte mRNA. (*A*) *In vitro* translations. Equal quantities of total reticulocyte RNA from each of the family members and controls (αα/αα, $-\alpha^{3.7}/\alpha\alpha$, $--/-\alpha^{3.7}$; see *Materials and Methods*) were translated *in vitro* and analyzed by Triton X-100/acid/urea electrophoresis. The identity of each sample, the positions of α- and β-globin, and the ratios of α-globin to β-globin syntheses are noted. (*B*) Detection and relative quantitation of α1- and α2-globin mRNAs. Equal quantities of total reticulocyte RNA were analyzed, and the end-labeled digestion products, specifically representing α1- or α2-globin mRNAs, were resolved and autoradiographed. The origin of each sample and the positions of the α1- and α2-globin mRNA-specific fragments are indicated, and the ratio of the α2-globin to α1-globin mRNA specific bands are shown at the bottom of each respective lane. (*C*) Sequence analysis of the three α-globin genes of the proposita. The sequences of the three genes from the proposita $[(\alpha\alpha)^{IJ}$ genes, labeled $\alpha1^T$ and $\alpha2^T$, and the $-\alpha^{3.7}$ gene, labeled α-thal$_2$] were identical to those of reported normal α1- and α2-globin genes (37, 38) with the exceptions as noted. The positions of the consensus sequences known to be necessary for normal expression are indicated above the gene diagram. bp, Base pairs.

1.5-kb *Pst* I fragments and sequenced by using a combination of universal and α-globin-specific primers (28). Compressions were resolved by using deaza-dGTP or dITP.

RNA Analysis. Total reticulocyte RNA was extracted from acid-precipitated polysomes (29). *In vitro* translations were carried out in the presence of [^{35}S]methionine (1400 Ci/mmol; 1 Ci = 37 GBq; Amersham), analyzed on a Triton X-100/acid/urea slab gel, and quantitated by soft-laser densitometry as described (30, 31). As the absolute α-globin to β-globin synthetic ratio in control samples can demonstrate inter-assay variation, all comparisons were made intra-assay, and each study was repeated at least three times. Analysis of relative α2- and α1-globin mRNA levels was carried out by a reverse transcription mapping assay exactly as described (30).

Expression Studies. α-Globin gene expression was assayed by transient expression in mouse erythroleukemia cells, and quantitative analysis of total cellular RNA isolated 48 hr posttransfection was carried out by a reverse transcription/polymerase chain reaction assay (32). The normal α-globin gene was marked by site-specific mutation (33) of two bases—CG to AA at the 11th and 12th bases 3′ from the translation termination codon. In control experiments it was demonstrated that these substitutions had no adverse effect on expression.

RESULTS

The subject of the present report is an adult German woman (IJ) with Hb H disease (Table 1). This severe form of α-thalassemia usually results from the loss of three of the four α-globin genes (−−/−α) by coinheritance of a mild α-thalassemia defect from one parent (denoted as −α) combined with a severe α-thalassemia defect from the other parent (denoted as −−) (34, 35). Southern blot analysis of genomic DNA from the patient's mother (EJ) demonstrates that she is heterozygous for a common deletion of 3.7 kb, which removes one of the two α2-globin genes (genotype: $-\alpha^{3.7}/\alpha\alpha$) (Fig. 1). This is compatible with her normal hematologic values (Table 1). By Southern blot analysis, the α-globin gene clusters of the father (AJ) and sister (AsJ) appear to be structurally normal (genotype: αα/αα) as are their hematologic parameters. In contrast, the genotype of the proposita (IJ), −α/αα, is inconsistent with her Hb H phenotype, suggesting the presence of only a single functional α-globin gene. As nonpaternity is exceedingly unlikely on the basis of minisatellite DNA analysis (data not shown) using a probe to the HVR 3′ to the α-globin gene cluster (36), the most parsimonious explanation is a spontaneous loss of function of both α-globin genes on the paternally derived αα chromosome.

De novo loss of α-globin gene expression was confirmed by RNA analysis. *In vitro* translations of reticulocyte RNA from the father and the mother resulted in α-globin to β-globin synthesis ratios similar to those seen in the αα/αα and $-\alpha^{3.7}/\alpha\alpha$ controls (Fig. 2*A*). In contrast, this synthetic ratio in the proposita was severely depressed to a level usually associated with that in individuals expressing a single α-globin gene [(−−/−α) as shown in Fig. 2*A*] (30).

The α-globin gene expression in this family was further characterized by determining the relative mRNA expression of the two α-globin loci, α1 and α2 (Fig. 2*B*). In normal individuals, peripheral reticulocytes contain about 2.6-fold more α2- than α1-globin mRNA (range, 2.6 ± 0.6), whereas in individuals heterozygous for the $-\alpha^{3.7}$ deletion this ratio is 1 (range, 1.0 ± 0.3) (21, 39). The ratios of α2-globin to α1-globin mRNA of 3.2 and 1.3 in the father and mother, respectively, are consistent with their genotypes (21). In contrast, there is a total absence of α2-globin mRNA in the reticulocytes of the proposita. Although not directly assessed by this approach, the very low α-globin to β-globin chain synthesis ratio (Fig. 2*A*) suggests functional loss of the α1 gene as well. These results therefore suggest that both α-globin genes on the paternal chromosome have been inactivated by a cis-acting mutation and that the genotype of the proposita can be designated $-\alpha/(\alpha\alpha)^{IJ}$.

To define the presumed cis-acting thalassemic defect(s), each of the α-globin genes on the $(\alpha\alpha)^{IJ}$ chromosome as well as the single α-globin gene on the $-\alpha^{3.7}$ chromosome was cloned and sequenced. The sequences differed from the normal corresponding genes (37, 38) at a limited number of sites not known to be of functional significance (Fig. 2*C*). Several of these substitutions have been previously reported in unrelated individuals (19) and most likely represent sequence polymorphisms.

To confirm that at least one of the α-globin genes on the $(\alpha\alpha)^{IJ}$ chromosome could function normally when removed from the context of the cluster, the α2-globin gene, which is not expressed at all *in vivo* (Fig. 2*B*), was studied. The 1.5-kb *Pst* I genomic fragment containing this gene (Fig. 2*C*) was electroporated into mouse erythroleukemia cells along with an equal molar quantity of the normal α2-globin gene containing a hybridization marker (see *Materials and Methods*). The relative levels of mRNA from the normal α2 and $\alpha2^{IJ}$ genes as determined by a quantitative reverse transcriptase/

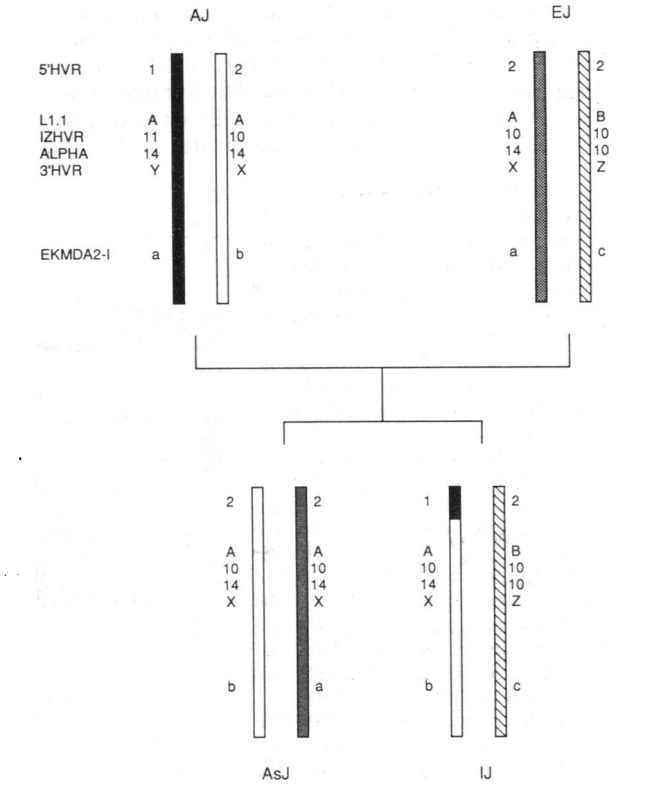

FIG. 3. Crossover 5′ to the α-globin gene cluster in the paternal α-globin gene cluster based on extended haplotype analysis. DNA from each family member was analyzed by using a series of polymorphic marker systems that have been localized within 16p13.3 (see *Materials and Methods*). The chromosome that each sister inherited from each parent is indicated by the distinct shading patterns. For the ALPHA (α-globin *Bam*HI fragment) and IZHVR (inter-ζ HVR *Bgl* II fragment) polymorphism, the numbers represent the respective restriction fragment length polymorphism size (in kb) for each chromosome. For other loci, arbitrary assignments to a figure or letter is given to the polymorphic fragment. The position of the crossover in the paternal chromosomes as deduced from the data in this figure may have occurred at any point between 5′ HVR and IZHVR, and the assignment 5′ to the noninformative L1.1 marker is based upon subsequent mapping of the deletion.

polymerase chain reaction assay (32) yielded a ratio of $\alpha 2^{IJ}$ to $\alpha 2$ (normal) of 1.14.

The above studies demonstrate that a *de novo* mutation inactivated the α-globin genes in an otherwise intact $\alpha\alpha$ gene cluster. The possibility of a deletion in the region adjacent to the cluster was next investigated. The analysis of the region flanking the α-globin gene cluster was facilitated by a long-range map of the encompassing chromosome 16p subtelomeric segment (40, 41). Segregation analysis of the two sets of paternal chromosomes was carried out using hybridization probes that recognize hypervariable markers flanking the cluster (refs. 25–28; data summarized in Fig. 3). This analysis demonstrates that one of the two daughters inherited a paternal $\alpha\alpha$ chromosome that had undergone a crossover 5′ to the α-globin genes in a region flanked by two informative polymorphic markers, 5′ HVR and inter-ζ HVR, located at coordinates −74 and +9, respectively (Fig. 3). It is most probable that this rearranged paternal $\alpha\alpha$ chromosome was inherited by the proposita IJ, because more detailed mapping of $(\alpha\alpha)^{IJ}$ in this region reveals a further abnormality: a discrete deletion extending from position −28/−33 kb to −64/−67 kb.

The breakpoint was initially detected by using the probe L2 and the restriction enzyme *Asp* 718 (40). It was then mapped in detail with probes RA1.0 and L4 (40), which flank the deletion (Fig. 4 and Table 2). Aside from this deletion, the map of the cluster and its 5′ and 3′ flanking regions are entirely normal (Figs. 2*C*, 3, and 4 and data not shown). By accurately measuring the size of the smallest breakpoint fragment on the $(\alpha\alpha)^{IJ}$ chromosome that hybridizes in common to both RA1.0 and L4 probes (5.2-kb *Bam*HI fragment, see Table 2) and knowing the coordinates of the *Bam*HI restriction sites in the native cluster (−68.5 and −28; ref. 40), it can be concluded that the deletion is ≈35 kb. This deletion most likely represents an unequal crossover during meiosis in the paternal germ cells, although a mitotic event resulting in germline mosaicism cannot be ruled out.

DISCUSSION

The studies in this report indicate that the α-globin genes can be inactivated by a deletion of regulatory elements that lie far upstream from the α-globin gene cluster. In contrast to previously described deletions of this region [$(\alpha\alpha)^{RA}$ (19) and $(\alpha\alpha)^{TI}$ (20)], the presently described deletion, $(\alpha\alpha)^{IJ}$, is relatively small, the entire α-globin cluster remains intact, and much of the 5′ flanking region is preserved. The effects of the $(\alpha\alpha)^{IJ}$ deletion are consistent with the fact that segments within the region localized by this deletion have the capacity to enhance α-globin gene expression in stable transformant assays and in transgenic mice (18). Nevertheless, it is still possible that other accessory sequences that normally synergize the activity of this positive regulatory region, but do not exert an effect on their own, are present in the $(\alpha\alpha)^{IJ}$ chromosome.

The $(\alpha\alpha)^{IJ}$ mutation bears strong parallels to the 30-kb deletion located 9.5 kb 5′ from the β-globin cluster, which silences β-globin gene expression and contributed to the definition of the β-globin gene cluster LAR (17). It cannot be concluded from the present data that the $(\alpha\alpha)^{IJ}$ deletion inactivates the entire cluster since the expression of the embryonic ζ-globin gene was not studied. However, the loss of expression from the $\alpha 2$-globin locus and the probable loss of $\alpha 1$-gene expression as well (see above) suggest that this is

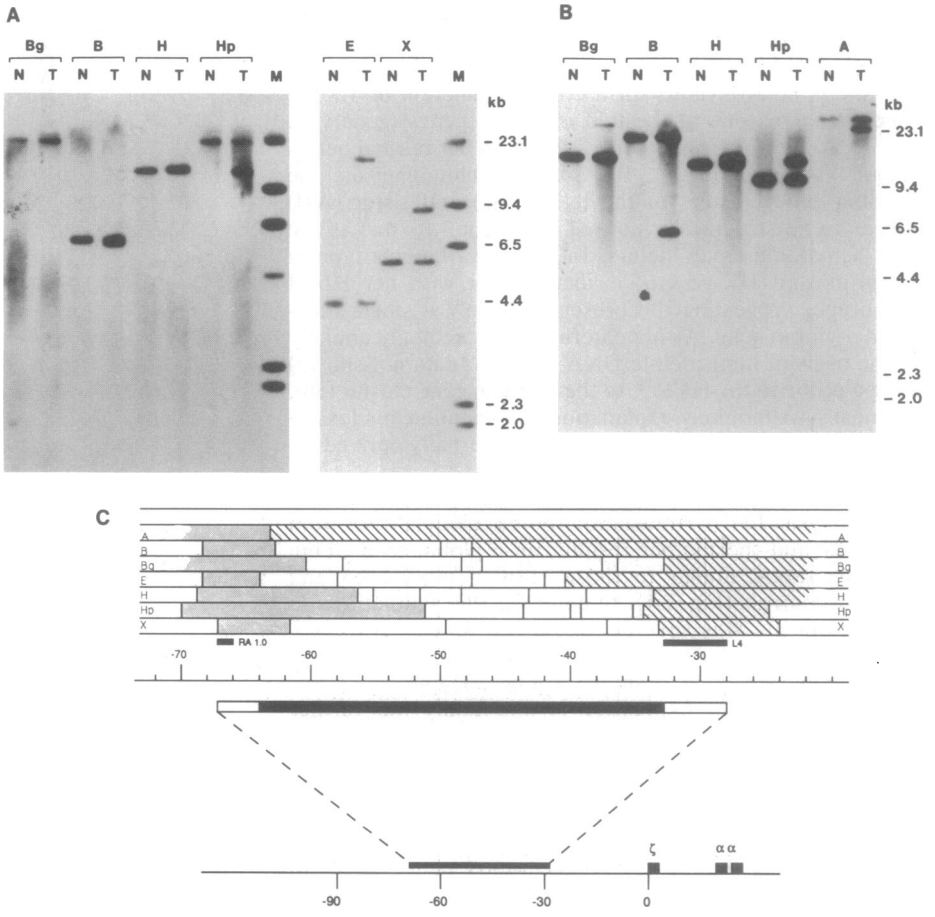

FIG. 4. Extent and position of the deletion 5′ to the $(\alpha\alpha)^{IJ}$ cluster. (*A* and *B*) Southern blot analyses of the deletion endpoints. The extent of the deletion upstream from the α-globin gene cluster on the $(\alpha\alpha)^{IJ}$ chromosome was mapped with probes RA1.0 and L4, which detect the 5′ and 3′ ends of the $(\alpha\alpha)^{IJ}$ breakpoint, respectively. DNA isolated from the proposita IJ was analyzed in parallel with that of a known normal ($\alpha\alpha/\alpha\alpha$). Each lane contains either the normal (N; $\alpha\alpha/\alpha\alpha$) or thalassemic [T; $-\alpha^{3.7}/(\alpha\alpha)^{IJ}$] DNA digested with the indicated enzymes. Restriction enzymes are A, *Asp* 718; B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa* I; and X, *Xba* I. The filters in *A* were hybridized with the RA1.0 probe and the filters in *B* were hybridized with the L4 probe. The locations of these probes are indicated in *C*. The molecular weight markers (M) are λ phage DNA digested with *Hin*dIII. The data from these analyses are summarized in Table 2. (*C*) Position and extent of the deletion in the $(\alpha\alpha)^{IJ}$ chromosome. At the bottom, the α-globin complex is diagramed showing the functional ζ- and α-globin genes; the position of the $(\alpha\alpha)^{IJ}$ deletion is indicated by the black bar. The coordinates are in kb, with 0 representing the $\zeta 2$-globin mRNA cap site. At the top is a detailed restriction map of the region between −20 and −70 showing the normal fragments spanning the 5′ (stippled) and 3′ (hatched) breakpoints. Lack of a vertical line indicates that the fragments extend beyond the region shown (for specific fragment sizes, see Table 2). The extent of the $(\alpha\alpha)^{IJ}$ deletion is shown by the thick black bar, with the region of uncertainty around each breakpoint shown in white bar. The positions of probes RA1.0 and L4 used to define this deletion are indicated. The enzymes are as defined in *A* and *B*.

Table 2. Fragment sizes from the α-globin cluster mapping studies

Enzyme	Fragment size, kb			
	αα/αα	$-\alpha^{3.7}/(\alpha\alpha)^{IJ}$		
	$(\alpha\alpha)$	$-\alpha^{3.7}$*	$(\alpha\alpha)^{IJ}$	Probe
Bgl II	13.0	13.0	≥23	L4
*Bam*HI	19.0	19.0	5.2	L4
*Hin*dIII	12.5	12.5	13	L4
Hpa I	9.4	9.4	≈13	L4
Asp 718	>40	>40	≈20	L4
*Eco*RI	≈23	≈23	≈17	L4
Xba I	9.0	9.0	9.0	L4
Bgl II	21	21	≥23	RA1.0
*Bam*HI	5.5	5.5	5.2	RA1.0
*Hin*dIII	12.6	12.6	≈13	RA1.0
Hpa I	≈19	≈19	≈13	RA1.0
*Eco*RI	4.0	4.0	≈17	RA1.0
Xba I	5.5	5.5	9.0	RA1.0

Breakpoints common to the probes L4 and RA1.0 are indicated by underlining.

*The presence of the $-\alpha^{3.7}$ deletion in this case has no effect on the sizes of the bands observed in the region of the complex detected by the L4 and RA1.0 probes.

likely to be the case. Thus at present three deletions have been described that lie entirely upstream of the α-globin cluster and inactivate the structurally normal α genes that lie in cis [$(\alpha\alpha)^{RA}$ (19), $(\alpha\alpha)^{TI}$ (20), and $(\alpha\alpha)^{IJ}$ (present report)]. The parallel positioning and long-range effects of these mutations are reminiscent of similar deletions from the upstream region of the β-globin cluster that remove the β-globin LAR (15–17). The $(\alpha\alpha)^{IJ}$ deletion is a discrete deletion that removes all sequences between −28 and −67, including the previously described positive regulatory region, localized to the segment spanning coordinates −28 to −65 (18). Thus this deletion lends support to the hypothesis (18–20) that this region contains sequences that serve at least some of the critical functions attributed to the β-globin LAR. Finally, it seems likely that other currently unexplained forms of α-thalassemia with intact structural genes may be due to related deletions that remove this positive regulatory region.

We thank Dr. C. R. Müller for establishment of permanent cell lines, Drs. E. Kohne and E. Kleihauer for hematological data, the patients for invaluable cooperation, Drs. N. E. Cooke and R. Nussbaum for critical readings of the text, and Susan Kelchner for expert secretarial assistance. This investigation was supported in part by the Deutsche Forschungsgemeinschaft.

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