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Repetitive DNA sequences near three human β-type globin genes

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ABSTRACT

Five repetitive DNA sequences, of average length 259 bp, have been identified in the intergenic regions which flank three human β-type globin genes. A pair of inverted repeat sequences, separated by 919 bp, was found 1.0 kb to the 5' side of the epsilon-globin gene. Each contains a homologous Alu I site. Another repetitive sequence, with the same orientation as the inverted repeat sequence closest to the epsilon-globin gene, lies about 2.2 kb to the 5' side of the delta-globin gene. A pair of inverted repeat sequences, with the same relative orientations as the other pair and separated by about 800 bp, was found about 1.5 kb to the 3' side of the β-globin gene.

INTRODUCTION

Different β-type globin chains are synthesized in humans at sequential stages of development (1). The embryonic epsilon chains made in the yolk sac are replaced during foetal development by γ chains produced in the liver. Subsequently synthesis of foetal chains stops at birth whilst synthesis of the adult chains (β and delta) is established in the bone marrow.

These changes are due to the differential expression of the series of evolutionarily related genes which code for the β-type globin chains. Recombinant DNA techniques have allowed the cloning of human DNA fragments which contain the linked β- and delta-globin genes (2), one (3) or both (4) γ-globin genes, and the epsilon-globin gene (5). Each also contains several kilobases (kb) of DNA surrounding the coding regions. The relation of intergenic sequences to the control of expression of the β-type globin genes and to overall organisation of the human genome is of great interest.

Single copy, repetitious and inverted repeat (IR) sequences

are interspersed throughout most of the human genome (6-8) in a pattern similar to that found in other eukaryotes (9-11). Cloned gene fragments enable the arrangement of such sequences to be mapped relative to each other and to known coding regions.

In this paper we identify and characterise by electron microscopy, Southern blot hybridisation and restriction endonuclease mapping five repetitive DNA sequences, including two IR sequences, in the intergenic regions near the β-, delta- and epsilon-globin genes.

MATERIALS AND METHODS

Restriction endonucleases. These were obtained as follows - Bam HI, Hind III, Hpa II, Pst I and Sal I from Bethesda Research Laboratories; Alu I, Bgl II, Hae III and Xba I from New England Biolabs; and Eco RI from Miles Laboratories Ltd. Reactions were carried out using the incubation mixtures and conditions recommended by the supplier.

Recloning of human genomic DNA fragments. HβG2, kindly provided by Dr T Maniatis, is a 14.45 kb human genomic DNA fragment, which contains the β- and delta-globin genes, in λ Charon 4A (2). The genomic region consists of five Eco RI fragments each of which was recloned into the Eco RI site of pAT153. The cloning vector pAT153, which was derived from pBR322 by removal of two contiguous Hae II fragments (totalling 705 bp) that contain the mobility region (12), was kindly provided by Prof D Sherratt.

Eco RI-digested HβG2 was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) to prevent self-ligation (13). Ligation was then carried out for 18h at 4°C with T4 ligase (Bethesda Research Laboratories), 10μg/ml Eco RI-digested pAT153 and a 10:1 molar excess of each HβG2 Eco RI fragment over the plasmid.

Competent E coli HB101 cells (14) were prepared by a modification of Norgard's method (15). A log phase culture of HB101 cells was harvested by centrifugation, gently resuspended at half the original volume in transformation buffer (100 mM $CaCl_2$, 250 mM KCl, 5 mM $MgCl_2$, 5 mM Tris HCl, pH 7.6) and left on ice for 20 minutes. After recentrifugation, the cells were resuspended in 1/10th volume of buffer with 15% glycerol and

stored at - 70°C for up to 3 months. These cells gave a transformation efficiency of greater than 10^6 colonies/µg of pAT153 DNA. 200 µl of HB101 cells and 50 ng of ligated plasmid in 200 µl of transformation buffer were left on ice for 25 minutes, then subjected to a 'heat shock' of 5 minutes at 37°C. After addition of 1 ml L-broth and incubation for 1 h at 37°C, the cells were plated on L-agar containing 100 µg/ml ampicillin and 10 µg/ml tetracycline and incubated overnight at 37°C.

56 of the 1600 colonies picked were positive by Grunstein-Hogness hybridisation (16) to Eco RI-digested HβG2 labelled with ^{32}P by nick-translation (17). Five different recombinant plasmids, identified by Eco RI digestion of alkaline-extracted DNA (18), were designated pJP1-5 as described in the Results. Their orientations in pAT153 were determined from the size of fragments produced by Pst I or Bam HI-digestion.

An 8.0 kb human genomic Hind III fragment containing the epsilon-globin gene - the epsilon fragment - cloned in λ788 (5) was kindly provided by Dr N J Proudfoot and Dr F E Baralle. It was recloned in the Hind III site of pAT153 by a method similar to that described above. The plasmid was treated with alkaline phosphatase, then ligated at 11°C for 18 h at 25 µg/ml with an equimolar amount of the Hind III-treated epsilon fragment. 360 of the 400 colonies picked hybridised to epsilon fragment labelled with ^{32}P by nick-translation. One recombinant clone, designated pMG1, was selected for further study. The 5' to 3' orientation of the epsilon fragment, determined by restriction endonuclease mapping, was the same as the clockwise direction in which the map of the parental plasmid pBR322 is represented (19).

Recloning experiments were carried out according to GMAG guidelines under Category II containment conditions.

Electron microscopy. For self-annealing of restriction endonuclease treated DNA, the epsilon fragment in λ788 was digested either with Hind III or Xba I and the epsilon fragment in pAT153 was treated with Bgl II or Eco RI. In the case of Eco RI, the DNA was partially digested so that on average it was cut only once per molecule. The DNA was denatured, reannealed and prepared for electron microscopy as previously described (20),

with pBR322 as an internal standard (19).

For restriction endonuclease digestion of self-annealed DNA, 0.15 µg of pMG1 linearised with Hind III was denatured at 100°C for 1 minute without the addition of formamide (20). After reannealing at 37°C for 20 minutes, the incubation mixture was adjusted to that appropriate for the restriction endonuclease to be used. 1 unit of Alu I or Bgl II was added for 30 minutes at 37°C, and the DNA was spread with formamide as previously described (20).

Heteroduplex mapping (21) was carried out with pJP1, pJP3 and pJP5 digested with Bam HI, pJP2 and pJP4 with Sal I, and pMG1 with Bam HI or Sal I as appropriate.

A Wang 2262 digitizer interfaced with a Wang 2200S computer programmed by Dr Bryan Young, Beatson Institute was used for length measurements and data analysis.

Restriction mapping and Southern blot hybridization. For restriction mapping, pJP1 and pJP5 were digested with one or more of the following enzymes - Eco RI, Hae III, Hpa I and Xba I - and the products analyzed by electrophoresis on 1.5% ME agarose gels (Miles Research Laboratories Ltd).

For Southern blot hybridization, the recloned genomic fragments were first purified from pAT153 by digestion with Eco RI or Hind III as appropriate, gel electrophoresis on 1% LGT agarose gels (Miles Research Laboratories Ltd) and extraction with $NaClO_4$ (22). After electrophoresis on 3% ME agarose gels, fragments produced by restriction endonuclease treatment were transferred to 0.1 µm pore size nitrocellulose filter paper (Sartorius) by Southern's method (23). Cross-hybridizations to genomic fragments, ^{32}P labelled by nick-translation (17), were carried out for 18h at 42°C in hybridization buffer (1xDenhardt's reagent (24), 100 µg/ml salmon sperm DNA (Sigma) with either 3xSSC/50% formamide for high stringency (HS) and low stringency (LS) conditions or 5xSSC/30% formamide for extra low stringency (XLS) conditions). Filters were washed for 1h either in 2xSSC/0.1% SDS at 24°C (XLS) or 50°C (LS), or in 0.03xSSC/0.1% SDS at 50°C (HS) before autoradiography. Filters were reused after removal of bound probe by 0.1xSSC/70% formamide at 70°C for 2-4h.

RESULTS

Size and position of inverted repeat sequences near the epsilon-globin genes. A loop of single stranded DNA and a duplex DNA stem (Fig 1) were observed by electron microscopy in self-annealed molecules of the 8.0 kb human epsilon-globin genomic fragment (which will be referred to as the 'epsilon fragment') cloned in either λ788 or pAT153. This foldback structure revealed the presence of a pair of inverted repeat (IR) sequences which was able to hybridise intramolecularly, flanking a heterologous DNA region. The size of the stem was 227 ± 39 base pairs (bp) and the loop was 919 ± 134 bases (n=35), giving a total of 1.37 kb for the whole foldback structure.

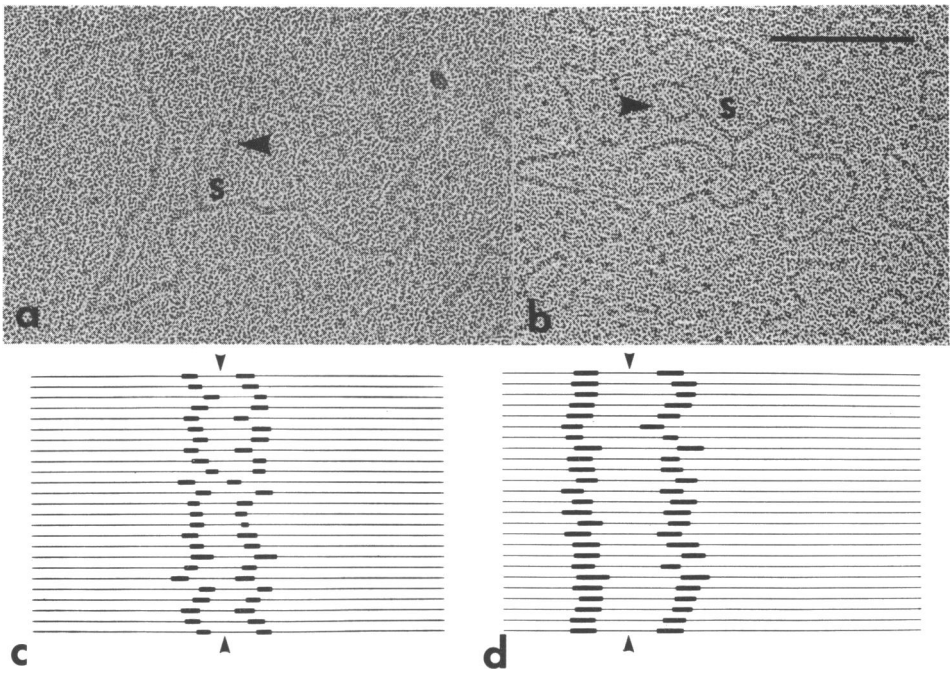

Figure 1. Self-annealed (a) Hind III-digested and (b) Xba I-digested cloned epsilon fragment DNA, showing stem (s) and loop (arrow) of foldback DNA structure. Bar represents 0.25 μm. The position of the intramolecularly hybridising regions in (c) 8.0 kb Hind III fragments and (d) 4.6 kb Xba I fragments are plotted (bars) as fraction of total length of each self-annealed molecule (not corrected for length difference between single and double stranded DNA).

The position of the IR sequences within the epsilon fragment was determined relative to two previously mapped restriction endonuclease sites (5). After treatment with Hind III or Xba I, the DNA was self-annealed and the position of the foldback structure was plotted. The centre of the structure was 46.8 ± 1.9% (n=25) along the 8.0 kb Hind III epsilon fragment (Fig 1a,c) which was equivalent to 3.7 kb from a Hind III site. This corresponded to a position close to the central Eco RI site (Fig 3) or to one about 600 bp to the 3' side of this site. (The terms 3' or 5' give relative map positions on the criterion of the direction of transcription of the globin genes). Xba I cut asymmetrically relative to the Hind III sites and in this case (Fig 1b,d) the centre of the foldback structure was 30.1 ± 1.7% (n=25), or 1.4 kb along the 4.6 kb restriction fragment. This correlated with the position closest to the Eco RI site.

This assignment was confirmed by partial Eco RI digestion of the epsilon fragment cloned in pAT153, followed by self-annealing. When a molecule which was cut only at the Eco RI site between the pair of IR sequences was denatured and reannealed, the palindromic sequences could undergo intramolecular hybridization. The small loop was observed to be cut (Fig 2a) to produce two arms which were found to be 41.7 ± 3.1% and 58.3 ±

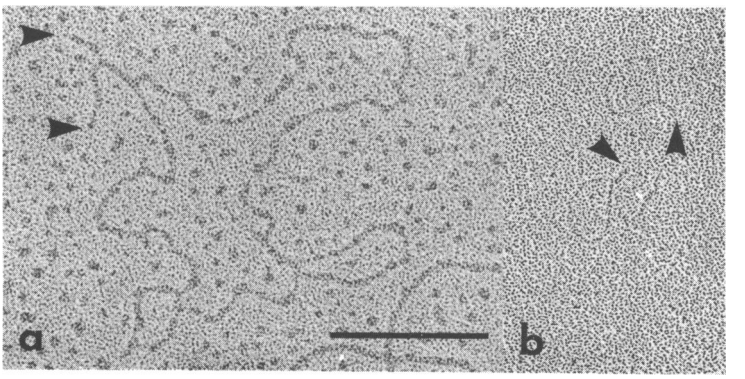

Figure 2. (a) Epsilon fragment DNA, cloned in pAT153, partially digested with Eco RI and self-annealed. Foldback loop is cut (arrows). (b) Self-annealed epsilon fragment DNA treated with Alu I, showing cut duplex stem (arrows) attached to loop. Bar represents 0.25 μm.

3.1% of the total length. These represent mean lengths of 383 and 536 bp. These results could not determine the orientation of the slight asymmetry of the IR sequences with respect to the Eco RI site.

Bgl II sites in the foldback structure. The data described above showed that the 1.37 kb foldback structure is located about the central Eco RI site in the epsilon fragment. Proudfoot and Baralle (5) have mapped two Bgl II sites in this region, one on each side of the Eco RI site, and this suggested that one site might be present in each IR sequence. However these sequences are 919 bp apart whereas the Bgl II sites are only 600 bp apart. If restriction sites are present in a pair of IR sequences which contain no mismatched bases, then each pair of sites in equivalent locations can hybridize intramolecularly to form a single restriction site in the stem of the foldback structure (20). When self-annealed epsilon fragment DNA was digested with Bgl II, only full-sized stem and loop structures were observed although other duplex DNA in the preparation was cut to the extent expected with Bgl II. Conversely, when epsilon fragment was treated with Bgl II and then self-annealed, no foldback structures were seen. This indicates that this enzyme cuts at least once in the region between the IR sequences.

These data localize the IR sequences with respect to previously mapped sites (5). We find that the repetitive sequence closest to the epsilon-globin gene is separated from the 5' end of the gene (F E Baralle, personal communication) by about 1.0 kb of DNA (Fig 3).

Alu I restriction sites mapped by electron microscopy. Analogous experiments to those described for Bgl II have been carried out with Alu I. When self-annealed epsilon fragment DNA was treated with this restriction endonuclease the stem of the foldback structure was cut (Fig 2b) with about one third to one half (75-120 bp) remaining attached to the loop. A homologous pair of Alu I sites have thus been mapped, one in each IR sequence. The cut duplex stem attached to the rest of the denatured epsilon fragment was not identified in the preparation.

Detailed mapping with other restriction endonucleases has revealed some differences between the IR sequences (unpublished

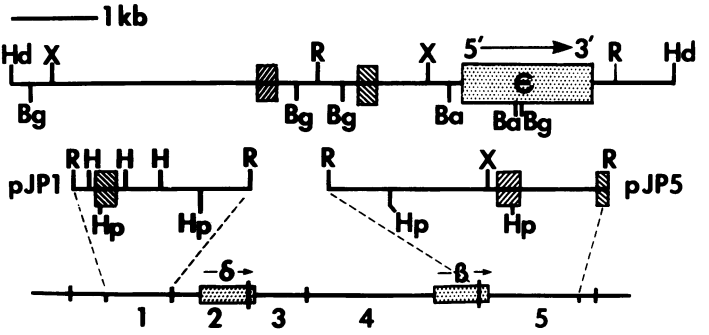

Figure 3. Map of epsilon fragment and the human genomic Eco RI fragments from pJP1 and pJP5, all to scale indicated, showing position of epsilon-globin gene (stippled) and its direction of transcription (Dr F E Baralle, personal communication). The position and orientation of the repetitive sequences are shown by cross-hatching. The genomic delta-β globin gene region (2) is displayed beneath, not to the same scale. Position of Bam HI (Ba), Bgl II (Bg), Eco RI (R), Hind III (Hd) and Xba I (X) sites in the epsilon fragment from (5), and Hae III (H), Hpa II (Hp) and Xba I (X) sites in pJP1 and pJP5 from our data.

data) which are currently being investigated.

Homology with sequences which flank the delta- and β-globin genes. HβG2 was produced by partial digestion of human genomic DNA with Hae III and Alu I, followed by the addition of synthetic Eco RI linkers, and cloning in λ Charon 4A(2). The genomic region contains four internal Eco RI sites so that on treatment with this enzyme it yields five fragments, C-G (2), which we have recloned in the Eco RI site of pAT153. pJP1 contains the 2.05 kb fragment F which was originally cut by the Hae III/Alu I digest from the 3.1 kb genomic Eco RI fragment and it lies to the 5' side of the delta-globin gene. Similarly, pJP5 contains the 3.2 kb fragment D from the 3.6 kb genomic fragment which lies to the 3' side of the β-globin gene. pJP2, pJP3 and pJP4 contain respectively the 2.25 kb E fragment, the 1.75 kb G fragment and the 5.2 kb C fragment, which lie between the F and D fragments (Fig 3).

A study of sequence homologies between the epsilon fragment in pMG1 and the pJP1-5 genomic fragments was carried out by

Southern blot hybridization and heteroduplex mapping. Data which show that the IR sequences in the epsilon fragment hybridized to sequences in pJP1 and pJP5 are described in detail below. pJP3 showed no hybridization to any other fragment, whereas pMG1, pJP2 and pJP4 appeared to cross-hybridize in the 5' coding regions (data not shown).

Heteroduplex mapping. Heteroduplexes between the epsilon fragment cloned in the Hind III site of pAT153 and the HβG2 fragments in the Eco RI site of pAT153 can be readily interpreted as these two sites are separated by only 30 bp in this vector (19).

The orientation of the genomic Eco RI fragment in pJP1 and the epsilon fragment in pMG1 were found to be the same with respect to the pAT153 segments (data not shown). Heteroduplexes between Bam HI-treated pJP1 (which is cut only in the pAT153 segment, 0.38 kb from the Eco RI site) and Bam HI-treated pMG1 (which in addition is cut in the epsilon fragment) showed a long DNA duplex, due to hybridization between the vector region, and a short duplex of 252 ± 41 bp (n=20) in the genomic region (Fig 4a). Length measurements of the single stranded DNA showed that the region of homology was between 4.08 ± 0.20 kb from the 5' junction of the epsilon fragment with the vector and 1.07 ± 0.15 kb from the Bam HI site at the 5' side of the gene. This agreed with the mapped position of the repetitive sequence next to the epsilon-globin gene, and was supported by the observation that a foldback structure was not present in the epsilon fragment when interstrand hybridization had occurred. The homologous region in pJP1 was 250 ± 38 bp from the 5' junction with the vector and 1.91 ± 0.14 kb from the Bam HI site (ie 1.53 kb from the 3' plasmid junction).

The orientation of the genomic Eco RI fragment in pJP5 and the epsilon fragment in pMG1 were found to be the same with respect to the pAT153 segments (data not shown). Heteroduplexes between Bam HI-treated pJP5 (which is cut only in the pAT153 segment) and Bam HI-treated epsilon fragment also showed a short duplex of 297 ± 37 bp (n=20) in the genomic DNA segment (Fig 4b). The epsilon fragment was found to hybridize between 2.97 ± 0.21 kb from the 5' junction with the vector and 2.00 ± 0.23 kb from

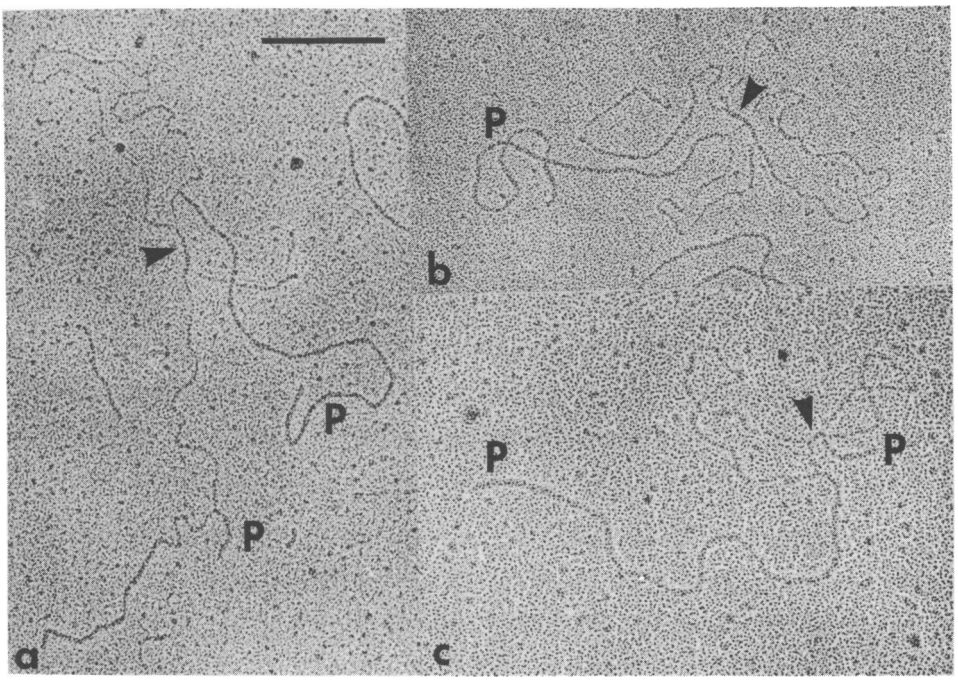

Figure 4. Heteroduplexes of (a) pJP1 and (b) pJP5, each with the epsilon fragment cloned in pAT153, and (c) pJP1 with pJP5. Arrows show hybridization between genomic regions. Long and short duplexes (P) are due to hybridization between the pAT153 segments. Bar represents 0.25 μm.

the Bam HI site at the 5' side of the epsilon-globin gene, agreeing with the previously mapped position of the IR sequence furthest from the gene. Again, interstrand hybrids precluded the presence of foldback structures in the epsilon fragment strand. The hybridizing region in pJP5 was 1.85 ± 0.13 kb from the 5' junction with the vector and 1.30 ± 0.22 kb from the Bam HI site (ie 0.92 kb from the 3' plasmid junction).

It was concluded that sequences about 2.2 kb to the 5' side of the delta-globin gene and about 1.5 kb to the 3' side of the β-globin gene are homologous to the IR sequences. The repetitive sequences in pJP1 and pJP5 are also inverted relative to each other and were not expected to hybridize in heteroduplexes

between these plasmids. This was the case, but a short genomic hybridising region of 132 ± 29 bp (n=20) was observed (Fig 4c). In pJP1 this was 205 ± 42 bp from the 5' plasmid junction and 1.72 ± 0.24 kb from the 3' junction, and we believe that this is part of the region which also hybridised to the epsilon fragment IR sequence. The hybridising region in pJP5 was at the 3' junction with the plasmid which, together with the shorter length of the duplex region, suggests that the Hae III/Alu I digest originally cut a longer homologous region during the cloning procedure. (Heteroduplexes between this region and the epsilon fragment were not observed, but this could have been because the shorter sequence allowed strand displacement by the full-length complementary epsilon fragment IR.) A pair of IR sequences, separated by about 800 bp, is thus also present to the 3' side of the β-globin gene with the same relative orientation as the IR sequences to the 5' side of the epsilon-globin gene.

Southern blot hybridisation. A 440 bp Hae III fragment, 160 bp from the 5' end of the genomic fragment in pJP1 (Fig 3), hybridised to ^{32}P-labelled epsilon fragment and pJP5 genomic probes under high stringency (data not shown) and low stringency conditions (Fig 5(a)-(c)). This location is in good agreement with that revealed by heteroduplex mapping. The Hae III fragment contains a Hpa II site, 300 bp from the 5' end of the genomic Eco RI fragment in the region previously mapped as a repetitive sequence. The 300 bp Hpa II/Eco RI fragment and the neighbouring 1.2 kb Hpa II fragment hybridised to both probes under low stringency conditions, but under high stringency conditions the pJP5 probe no longer hybridised to the 300 bp fragment, although the epsilon probe still did so (Fig 5(d)-(h)). Hybrid stability may be decreased if the part of the repetitive sequence in the 300 bp Hpa II/Eco RI fragment is short and the limits of accuracy of mapping this site do not exclude this possibility. However it also appears that the 5' end (relative to the globin gene) of the pJP1 repetitive sequence shows more mismatch with the pJP5 repetitive sequences than with the epsilon fragment IR sequences whereas the larger 3' ends appear relatively homologous in all these sequences.

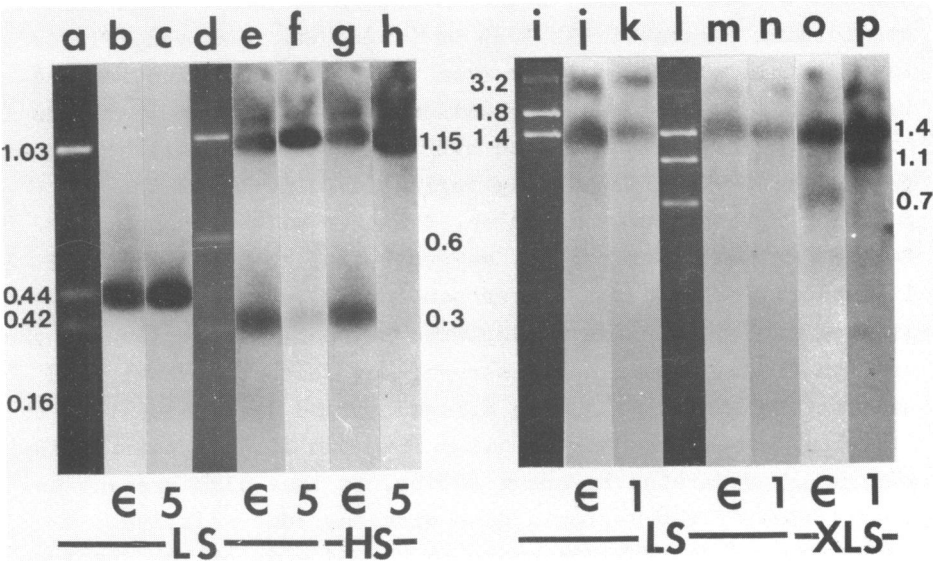

Figure 5. The Eco RI genomic fragments of pJP1 digested with Hae III or Hpa II((a)-(h)) and pJP5 digested with Xba I or Hpa II ((i)-(p)). Ethidium bromide staining ((a), (d), (i) and (l)) or Southern blot hybridization with purified epsilon fragment (ϵ), pJP1 (1) or pJP5 (5) genomic fragments, under conditions or high (HS), low (LS), or extra low (XLS) stringency as indicated on figure. Size of fragments in kb was determined using Hinf I-digested pAT153 fragments as standards.

In pJP5, a 300 bp region which is delimited by an Xba I and a Hpa II site (Fig 3) hybridized to both probes at high stringency (data not shown) and low stringency conditions (Fig 5(i)-(n)). This position is in good agreement with that revealed by heteroduplex mapping except that the Hpa II site was found to map in the complete pJP5 repetitive sequence. It is not known whether this site is homologous to the one in the pJP1 repetitive sequence. Under extra low stringency conditions two more hybridising regions were detected. The 700 bp 5' Hpa II/Eco RI fragment hybridized only to epsilon probe, probably due to partial homology between the 3' structural gene regions. The 1.1 kb 3' Hpa II/Eco RI fragment hybridized to pJP1, but not to the epsilon probe (Fig 5(o)-(p)). This fragment contains the parts of two repetitive sequences (Fig 3)

which are more or less equivalent to the 5' region of the pJP1 repetitive sequence. If these sequences are short it could affect hybrid stability, but it does appear that in this case the level of mismatch to the epsilon IR sequences is greater than to the pJP1 repetitive sequence. It is not clear why the reciprocal pJP1/pJP5 hybridisations occur at different levels of stringency, but one possibility is that the Hpa I sites are not homologous.

As expected, ^{32}P-labelled pJP1 and pJP5 genomic fragment probes were found to hybridise only to those restriction fragments of the epsilon fragment which contain the IR sequences (data not shown).

DISCUSSION

Five repetitive DNA sequences, which include two pairs of IR sequences, of average length 259 bp have been mapped in the intergenic regions near the human β-, delta-, and epsilon-globin genes. Although evidence has been found for some sequence differences between members of the family, the overall conservation of homology suggests that these sequences may be important for the regulation or function of the globin genes or in higher orders of organisation of the human genome. Three other lines of research may be pertinent to further investigations into these questions. First, deletions 1.8 kb to the 3' side of the β-globin gene and 4 kb to the 5' side of the delta-globin gene allow the expression to different extents of the γ-globin genes (25-27). These regions, each of which contains a repetitive sequence, have been proposed as the boundaries of a chromosomal domain in a model for the regulation of globin gene expression (28). Second, the same two repetitive sequences - one in pJP1 and the complete one in pJP5 - map close to and have the same relative orientations as two DNA sequences which are complementary to *in vitro* transcripts by RNA polymerase III (29). Another sequence with the same properties was identified 1.5 kb to the 5' side of the $^{G}\gamma$-globin gene. We have not investigated the presence of repetitive sequences in this region. Third, a family of highly reiterated sequences, with an average size of 300 bp and mostly containing an Alu I site (30) have recently

been implicated in a wider role in the organisation of the human genome (31). Similarities between the sequence of members of the Alu family and the region near the $^{G}\gamma$-globin gene which is transcribed by RNA polymerase III were also found (31). The repetitive sequences we have found are on average slightly shorter than the Alu family and the presence of an Alu I site in both epsilon fragment IR sequence is not definitive on its own.

Repetitive sequences have also been identified in the intergenic regions of β-type globin genes of chicken (32) and rabbit (33). They have a similar distribution, including the presence of IR sequences, and have a size of a few hundred base pairs. Comparative studies will show whether such sequences are a general feature of globin gene and genomic organization, and their evolutionary relationships.

While this paper was in preparation, Fritsch et al (34) described the characterisation of a series of clones containing all five human β-type globin genes. They have identified seven repetitive sequences, four of which agree in their general position with those we have mapped. They also found two sequences flanking the γ-globin genes and one further to the 5' side of the delta-globin gene in a region we were unable to examine. We have found an additional partial sequence to the 3' side of the β-globin gene, and have also characterised the size and orientation of the repetitive sequences we have examined.

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