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Cloning specific segments of the mammalian genome: Bacteriophage λ containing mouse globin and surrounding gene sequences

(RPC-5 chromatography/preparative gel electrophoresis/restriction endonuclease mapping)

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ABSTRACT **We have developed a general approach to the cloning of specific segments of the mammalian genome that involves a two-step purification of *Eco*RI fragments of mammalian DNA and their *in vitro* insertion into a suitably constructed EK2 derivative of bacteriophage λ. The combination of fragment purification, exclusion of parental-type recombinants, and simple phage screening techniques permits the isolation of virtually any gene segment for which there is an identifying hybridization probe. We illustrate the approach by describing the cloning of an approximately 7000-base-long segment of mouse DNA containing globin and surrounding gene sequences.**

The prospect of using recombinant DNA technology (1–4) to clone a given segment of the mammalian genome offers a means of understanding gene structure and function as well as of achieving certain practical benefits. The difficulty in accomplishing this, however, arises from the great complexity of the mammalian genome. A given mammalian gene may represent as little as one ten-millionth of the organism's total DNA. In order to overcome this difficulty, we have used a two-step DNA fractionation scheme and a versatile EK2 phage λ cloning vector which, together with efficient phage screening techniques, bring virtually any segment of the mammalian genome within cloning range. This strategy is illustrated by the isolation of a 7000-base-long *Eco*RI fragment of the mouse genome containing a globin gene and surrounding nucleotide sequence.

MATERIALS AND METHODS

Isolation and purification of mouse DNA

Chromatography. High-molecular-weight DNA was isolated from MOPC-149 plasmacytomas by the method of Polsky *et al.* (5) which involves extraction of frozen tissue with high salt, sodium dodecyl sulfate, and phenol followed by ribonuclease A digestion, proteinase K digestion, and phenol extraction. The DNA was cleaved with *Eco*RI (5, 6), extracted with phenol, and precipitated with ethanol at −20°. Fifty milligrams of *Eco*RI-digested DNA in 1.25 M sodium acetate (NaOAc)/50 mM Tris·HCl (pH 7.5)/1 mM EDTA were applied to a 200-ml RPC-5 column, prepared according to method C of Pearson *et al.* (7). A 2-liter gradient of 1.45–1.60 M NaOAc in 50 mM Tris·HCl (pH 7.5)/1 mM EDTA was used to elute the DNA (8). In order to detect fragments that contained globin sequences, one-tenth volumes of individual fractions were pooled, sonicated, and concentrated by ethanol precipitation. Hybridizations were carried out for 96 hr in 10 mM Tris·HCl (pH 7.5)/1 mM EDTA/1 M NaCl at 68° in the presence of 1400 cpm of globin [^{32}P]cDNA (specific activity 3×10^6 cpm/μg). The fractions were assayed for globin-specific hybridization by treatment with S1 nuclease (9).

Electrophoresis. Appropriate column fractions were pooled, concentrated by precipitation with ethanol, dissolved in 40 mM Tris-acetate (pH 7.8)/1 mM EDTA, and applied to an automated, horizontal 1% preparative agarose gel system (5). Approximately 10-ml fractions were collected hourly for 160 hr. One-tenth volume of each fraction was pooled in groups of four fractions and hybridized in the presence of 600 cpm of globin [^{32}P]cDNA (specific activity 4.5×10^7 cpm/μg) as described above. Globin sequence-containing fractions were pooled, concentrated by ethanol precipitation, and dissolved in 10 mM Tris·HCl (pH 7.4)/0.1 mM EDTA.

Preparation, screening, and hybridization properties of recombinant phage

The certified EK2 vector, bacteriophage λgt*WES*·λB (10, 11) was propagated in an *Escherichia coli* derivative of strain ED 8656 (12), referred to here as LE 392. Appropriate experiments were carried out in accordance with the NIH Guidelines in a P3 facility. DNA was prepared according to Tiemeier *et al.* (8). After *Eco*RI digestion of λgt*WES*·λB DNA, the outer "arms" were separated from the internal λB *Eco*RI fragment by chromatography on RPC-5 (8). This isolation reduces the background of parental type phage in recombinant pools to approximately 1%.

Recombinants of λgt*WES* arms and purified mouse *Eco*RI fragments were annealed, ligated, and transfected as described (8). Phage were screened for globin-specific inserts by two procedures. In the first, individual recombinants were transferred to Millipore filter grids according to Kramer *et al.* (13). In the second procedure, sets of 800–1000 individual plagues were transferred directly from nutrient agar plates to replica Millipore filters according to Benton and Davis (14). The Millipore filters from either procedure were preincubated for 4 hr in Denhardt's solution (15) plus 3× SSC (SSC is 0.15 NaCl/0.015 Na citrate) and then in Denhardt's solution plus 6× SSC, and 0.5% sodium dodecyl sulfate in the presence of 2 to 5×10^5 cpm of globin [^{32}P]cDNA per ml (specific activity 2.5×10^7 cpm/μg). After 16 hr at 68°, the filters were washed extensively at 52° in 0.1× SSC/0.05% sodium dodecyl sulfate, dried, and autoradiographed. When pCR1·βM9 (pCR1–mouse β globin) (16), pCR1·αM10 (pCR1–mouse α globin) (16), or pβG1 (pMB9–rabbit β globin) (17) was used as probe, the DNA was labeled to a specific activity of 2×10^7 cpm/μg using *E. coli* DNA polymerase (18) and denatured at 100°C for 5 min.

Hybrid phage DNA was prepared from 2-ml lysates as de-

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Abbreviations: kb, kilobase; SSC, 0.15 M NaCl/0.015 M sodium citrate.

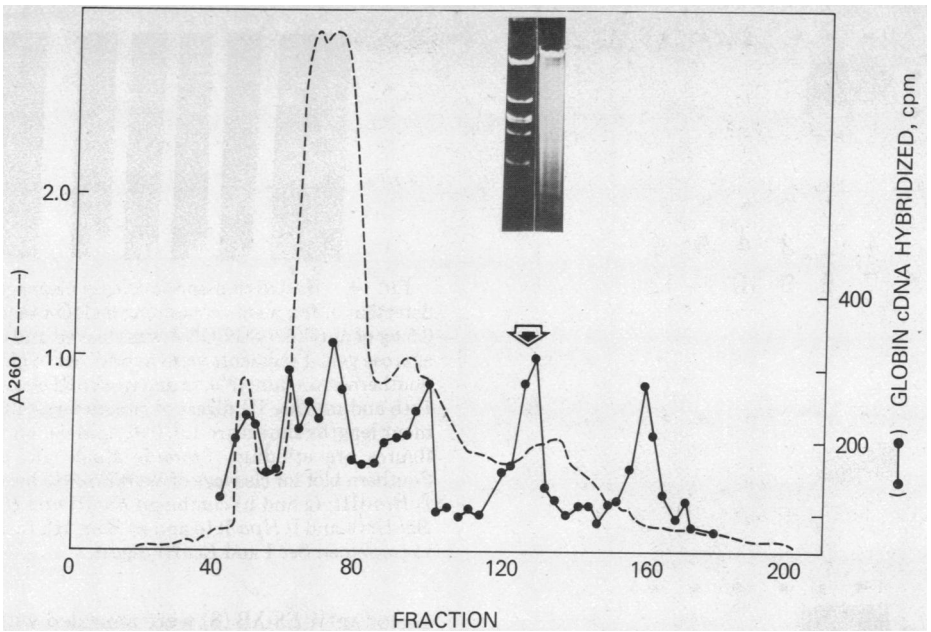

FIG. 1. RPC-5 chromatography of *Eco*RI-digested mouse genomic DNA. - - -, A_{260}; ●—●, S1-resistant hybridization of globin [^{32}P]cDNA. *Inset* above fractions 124–130 illustrates an ethidium bromide-stained 1% analytical agarose gel of an *Eco*RI digest of (*Left*) 0.5 μg of wild-type λ*cI*857 DNA, where migration is from top to bottom and the kilobase pair sizes of individual fragments are 21.3, 7.36, 5.79, 5.40, 4.69, and 3.3 kilobases, and (*Right*) 2 μg of fraction 130.

scribed (8). Approximately 0.1–0.2 μg of DNA was sheared by sonication in the presence of 50 μg of salmon sperm DNA per ml and concentrated by ethanol precipitation. The DNA was hybridized to 1200 cpm of globin [^{32}P]cDNA for 4 hr at 68° in 30 μl under conditions described above, and S1-resistant radioactivity was determined. An equal amount of DNA of pCR1·β globin, pCR1·α globin, λ*cI*857, and a non-globin-containing λgt*WES*·mouse hybrid (MC20 B4) served as controls.

Restriction Endonuclease Mapping. Restriction endonuclease mapping of λgt*WES*·βG2 DNA was performed with 0.5 μg of DNA in 50 μl under standard conditions for *Eco*RI (6), *Hin*dIII (19), *Sst* I (10), *Bgl* II (20), *Hpa* I, (20), and *Bam* HI (21). The fragments were analyzed on 1% agarose gels (22), visualized by ethidium bromide straining, and transferred to Millipore filters by the procedure of Southern (23). The filters were preincubated and hybridized as above in the presence of 2×10^5 cpm of globin [^{32}P]cDNA per ml, washed, and autoradiographed.

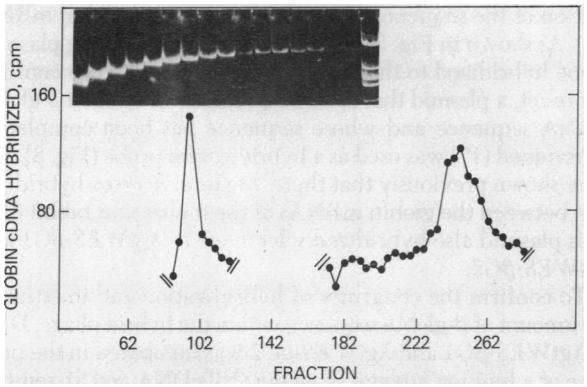

FIG. 2. Detection of globin-containing fragments obtained from a preparative discontinuous electro-elution gel. Approximately 2 mg of DNA from fractions 124–130 of the RPC-5 chromatography (Fig. 1) were electrophoresed through a preparative 1% agarose discontinuous electro-elution gel. ●—●, S1-resistant hybridization of pooled fractions to globin [^{32}P]cDNA. *Inset* contains an ethidium bromide stain of an analytical 1% agarose gel containing 50 μl of individual fractions, which correspond to the fractions on the abscissa. Size markers are provided by an *Eco*RI digest of λ*cI*857 as in Fig. 1.

RESULTS

Purification of *Eco*RI fragments containing mouse globin sequences

Cleavage of total mouse genome DNA with *Eco*RI would be expected to generate approximately 10^6 fragments. Such complexity would require the screening of several million recombinants made from total mouse DNA in order to find a globin (or other unique) segment. Even with an exceptionally efficient hybrid phage screening technique (14), this would be a formidable task. We, therefore, combined two sequential purification steps, RPC-5 chromatography (8, 24, 25) and preparative agarose gel electrophoresis using an especially designed apparatus (5), to purify the mouse globin genes. The advantage of these procedures, in addition to the high capacity and resolving power of each, is that they are complementary. The discrimination of the former is only slightly dependent upon size, whereas the latter is almost entirely dependent on this property.

The fractionation of 50 mg of *Eco*RI-digested mouse DNA by RPC-5 chromatography is shown in Fig. 1. Pooled fractions were hybridized to a mixture of α and β globin cDNA under conditions of cDNA excess in hopes of detecting embryonic globin gene sequences as well as those expressed in the adult mouse. Six individual hybridizing fractions were detected. The DNA in fractions 124–130 (Fig. 1) was enriched approximately 20-fold over total genomic DNA and was chosen for further purification. As shown by electrophoretic analysis (*inset*, Fig. 1), this fraction contains *Eco*RI fragments that vary considerably in size [as do all fractions eluted from RPC-5 (8)], making gel electrophoresis a logical second dimension. Approximately 2 mg of DNA obtained from fractions 124 to 130 of the RPC-5 column (Fig. 1) were applied to a 1% agarose gel in an automated preparative electrophoresis device we have recently constructed (5). Fractions were collected in 1-hr intervals by

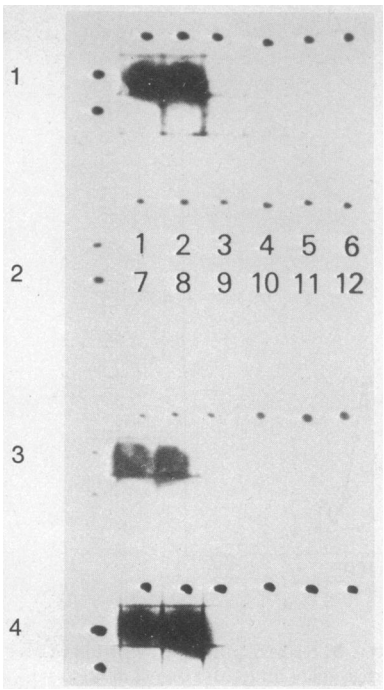

FIG. 3. *In situ* hybridization of λgt*WES*·βG1 and λgt*WES*·βG2 to α and β globin-containing chimeric plasmids. Replica filters were prepared from phage suspensions according to Kramer *et al.* (13) and hybridized to the [^{32}P]DNA probes as follows: filter 1, mouse α and β globin cDNA; filter 2, pCR1·mouse α globin; filter 3, pCR1·mouse β globin; filter 4, pMB9·rabbit β globin. The individual grids, numbered in filter 2, contained: (*1*) λgt*WES*·βG2, (*2*) λgt*WES*·βG1, (*3–8*) λgt*WES*·mouse DNA hybrids that were negative in the original screening, (*9*) λgt*WES*·mouse rDNA, (*10–12*) λgt*WES*·mouse DNA hybrids that contain reiterated sequences.

completely draining and then refilling the collection chamber by means of an electronic timer. The resulting DNA was analyzed by hybridization to globin [^{32}P]cDNA (Fig. 2). Two different size classes of DNA contained sequences that hybridized to globin cDNA. The first peak, in fractions 96–100, migrated in a 1% analytical agarose gel as a 7.0-kilobase (kb) fragment (Fig. 2, *inset*). The second peak, from fractions 238 to 242, migrated as a fragment greater than 14 kb in length. The smaller 7.0-kb fragment is the one described in these studies.

Isolation of a recombinant containing a globin sequence

The electrophoresis fraction most enriched for globin sequences (Fig. 2, fractions 96–100) can be estimated to have been 500-fold purified. This estimate is made from the relative proportion of the DNA in that pool after adjusting for the recovery of DNA and globin hybridizing sequences. Purified arms from the EK2 vector λgt*WES*·λB (8) were annealed with the DNA from this pool, ligated, and used for transfection. Since the recovery of nonhybrid phage is very low in this procedure, one expects the frequency of phage carrying the globin sequence to be one in 2000 with this enriched DNA.

Table 1. Hybridization of globin [^{32}P]cDNA to hybrid phage DNA

Hybrid DNA	% hybridization*
λgt*WES*·MβG1.0	45.3
λgt*WES*·MβG2.0	42.5
λgt*WES*·MC20 B4	4.2
λ*cI*857	5.5
pCR1·α $\text{globin}_{\text{mouse}}$	43.1
pCR1·β $\text{globin}_{\text{mouse}}$	51.6

Hybridization of 0.1–0.2 μg of phage or plasmid DNA was performed in the presence of 1200 cpm of globin [^{32}P]cDNA.
* S1 nuclease-resistant.

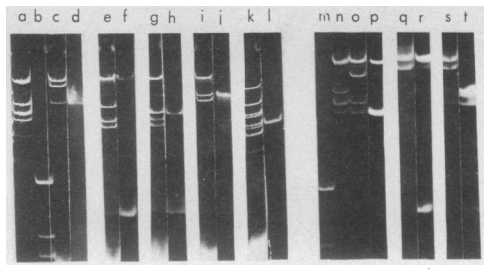

FIG. 4. Restriction endonuclease cleavage of λgt*WES*·βG2 and detection of fragments containing β globin sequences. Approximately 0.5 μg of λgt*WES*·βG2 DNA was cleaved and electrophoresed on 1% agarose gels. Fragments were hybridized to globin [^{32}P]cDNA by the Southern procedure (23). (a and n) *Eco*RI cleavage of λ*cI*857 as in Fig. 1; (b and m) *Hae* III digest of simian virus 40 DNA, where the fragment lengths shown are 1.5, 0.95, and 0.6 kb. The following pairs of figures are ethidium bromide stains and autoradiographs of a Southern blot for cleavage of λgt*WES*·βG2 by: (c and d) *Eco*RI; (e and f) *Hin*dIII; (g and h) combined *Eco*RI and *Hin*dIII digest; (i and j) *Bgl* II; (k and l) *Hpa* I; (o and p) *Bam* HI; (q and r) *Sst* I; and (s and t) combined *Sst* I and *Eco*RI digest.

Approximately 4300 plaques from this transfection were individually picked and screened by the procedure of Kramer *et al.* (13) using globin [^{32}P]cDNA. Three positive clones were detected. Transfectants were also pooled from plates with 800–1000 plaques, replated at about 1000–2000 plaques per plate, and screened according to Benton and Davis (14) by transferring DNA from the plaques to Millipore filters, where they were probed with globin [^{32}P]cDNA. About half of the pools contained several positive clones.

Identification of a β Globin-Like Sequence. The cDNA probe used to detect positive clones contained approximately equal amounts of mouse α and β globin sequences (Fig. 3). In order to determine which sequence was contained within the cloned fragment, two independently isolated positive clones, λgt*WES*·βG1 and λgt*WES*·βG2, were transferred to a replica set of Millipore filters along with negative controls. The filters were hybridized with ^{32}P-labeled plasmids that contained a portion of the sequence of either mouse α or β globin mRNA (16). As shown in Fig. 3, only the β globin-containing plasmid probe hybridized to the two recombinant phage. To confirm this result, a plasmid that contains 576 bases of a rabbit β globin mRNA sequence and whose sequence has been completely determined (17) was used as a hybridization probe (Fig. 3). We have shown previously that there is extensive cross-hybridization between the globin mRNAs of the mouse and rabbit (26). This plasmid also hybridized selectively to λgt*WES*·βG1 and λgt*WES*·βG2.

To confirm the congruity of hybridization and to estimate the amount of β-globin sequence within the hybrid phage, DNA of λgt*WES*·βG1 and λgt*WES*·βG2 was incubated in the presence of a limiting amount of globin [^{32}P]cDNA and S1-resistant hybridization was determined (Table 1). Both phage DNA protected approximately 45% of the mixed α and β globin cDNA probe, comparable to the extent of hybridization of the pCR1·β mouse globin plasmid, which Rougeon and Mach (16) have estimated contains at least 540 base pairs of the β globin mRNA sequence.

Arrangement of Globin Hybridizing Sequence within the Fragment. The region of the 7-kb cloned fragment that hy-

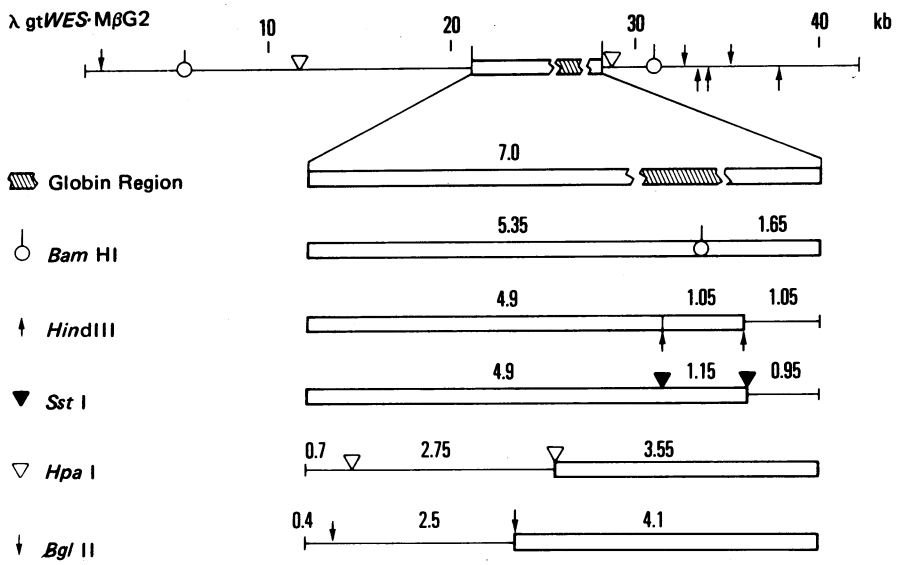

FIG. 5. Location of restriction endonuclease sites and globin sequences in λgt*WES*·MβG2. The top line represents the DNA genome of the hybrid. The 7000-base-long insert is illustrated as an open rectangle set off by vertical lines. The symbols in the left and right arms represent restriction enzyme sites. There are seven additional *Hpa* I sites in the arms; only those directly adjacent to the insert are presented here. The insert is presented on an expanded scale in the second through the sixth lines. In this case, the numbers refer to the size of fragments in kilobase pairs generated by digestion with the restriction enzyme shown on the left. Fragments that hybridize to β globin nucleic acid probes are represented as open rectangles (see Fig. 4). Fragments that do not hybridize are simply represented as horizontal lines. The hatched area within the insert displayed on the first and second lines represents an estimate of the region containing β globin sequences based on this hybridization data and R-loop mapping in the electron microscope (S. M. Tilghman, D. C. Tiemeier, B. M. Peterlin, and P. Leder, unpublished).

bridizes to globin was localized by digesting λgt*WES*·βG2 with several restriction enzymes and transferring the fragments to a nitrocellulose filter for hybridization (23) (Fig. 4). The *Hin*dIII fragments were ordered from an analysis of a *Hin*dIII-*Eco*RI double digest. The *Sst* I fragments were ordered from an *Eco*RI–partial *Sst* I digest (Fig. 4, lanes s and t). The 6.1-kb and 2.1-kb partial products, indicated by lines in lane t, allow the ordering of the *Sst* I cleavage sites as in Fig. 5. The positions of the other sites were determined by a comparison of the size of new insert fragments to known adjacent enzyme sites in λgt*WES* left and right arms (Figs. 4 and 5).

The hybridization data localize the globin sequences to the region between 3.5 kb and 6.0 kb from the left end of the insert. The enzymes *Bam* HI, *Hin*dIII and *Sst* I cut within the region of hybridization and yield two globin sequence-containing fragments, whereas *Hpa* I and *Bgl* II do not cut within the region of hybridization (compare Figs. 4 and 5). The positions of these sites are indicated diagrammatically in Fig. 5. The separately isolated hybrid λgt*WES*·βG1 contains this fragment in the same orientation within the phage DNA, but a second unrelated 7.0-kb fragment is also included in this recombinant phage (data not shown).

DISCUSSION

The Cloning Procedure. The extent to which a mammalian fragment must be enriched prior to cloning depends upon the number of recombinants that can be reasonably screened. The EK2 vector that we have used, λgt*WES*·λB, has two major advantages in this respect. First, the frequency of formation of parental recombinants is very low, which ensures that over 99% of the phage generated carry an inserted foreign fragment. Second, with the development of the rapid replica plating technique of Benton and Davis (14), it is possible to screen 2 to 5×10^4 recombinant phage in a single day.

While the range of the screening technique can be extended with additional effort, it appears that any unique fragment purified approximately 100-fold from mammalian DNA can be *easily* identified. Using sequential RPC-5 chromatography and preparative agarose gel electrophoresis, this degree of purification is often exceeded by an order of magnitude. An occasional fragment falls into range after only a single dimension of purification (8).

The only limitation of which we are aware relates to the size of fragments that may be inserted into λgt*WES*·λB. Theoretically, the small fraction of mammalian fragments below 1.0 kb cannot be inserted into viable phage particles (3, 11). In practice, however, we find that small fragments can be incorporated as multiple inserts. On the other hand, the theoretical upper limit on insert size, 14–15 kb, is determined by the capacity of the phage head. Again, in practice, we have cloned larger fragments, for example, the 15-kb globin-containing sequence shown in Fig. 2 (D. C. Tiemeier, S. M. Tilghman, F. Polsky, J. G. Seidman, M. H. Edgell, and P. Leder, unpublished result). While the efficiency of transfection is reduced by using uniformly large fragments, only recombinant phage appear as plaques. This places a strong selection on rare events which may permit the cloning of genomic segments beyond the theoretical limit.

The Cloned Mouse Fragment. That this fragment contains an authentic β globin sequence is supported by the hybridization of the pCR1–mouse β globin and pMB9–rabbit β globin to the hybrid and by its S1-resistant hybridization to half of a mixed α, β globin cDNA probe. Furthermore, globin mRNA appears fully annealed to the complementary cloned sequence when viewed in the electron microscope.* The hybridization and restriction endonuclease mapping data presented in this report are insufficient to determine whether adult or embryonic

* S. M. Tilghman, D. C. Tiemeier, B. M. Peterlin, and P. Leder, unpublished.

β chain is encoded within the cloned fragment. The cloned globin structural sequence pCR1-mouse β (adult) (16) contains a *Bam* HI cleavage site (our unpublished observations) that corresponds to amino acids 98–100 and a known *Bam* HI site in the rabbit β chain (25). The identical amino acid sequence is known to occur in all four mouse β chains (27–32). Therefore, regardless of the β globin sequence contained in λgt*WES*·MβG2, *Bam* HI should split its globin-hybridizing sequence, and it does (Figs. 4 and 5).

On the other hand, the globin structural sequence contained in pCR1-mouse β globin contains neither a *Hin*dIII site nor an *Sst* I site (our unpublished observations). Yet the 7.0-kb genomic fragment contains both very near the *Bam* HI site. Each splits the globin-hybridizing sequence so that they would appear to be within the globin coding region. It is possible that λgt*WES*·MβG2 contains a β globin sequence other than the one contained within pCR1-mouse β globin. However, this observation is also consistent with an unusual R-loop structure we observe by electron microscopy which can be interpreted as an interruption in the structural gene.* The identity and structural organization of this β-like globin sequence will require a detailed sequence analysis.

The mouse β globin genes, together with the adult and embryonic α genes, are of particular interest in that their expression must be coordinately regulated in the yolk sac and liver during development and in erythrocyte differentiation during adult life. The availability of a cloned segment of the genome which potentially carries adjacent regulatory sequences should permit a detailed examination of the factors that determine the coordinated expression of these interesting genes.

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