A 66-Base Pair Insert Bridges the Deletion Responsible for a Mouse Model of β-Thalassemia*

(Received for publication, November 26, 1985)

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The breakpoints of the deletion responsible for the Hbb(th-1) mouse model of β-thalassemia have been isolated. A 3709 (±2)-base pair (bp) region, including the entire β major globin gene and 2 kilobases of 5' flanking region, is deleted. A novel 66 (±2)-bp sequence, ending in a stretch of 25 dA:dT base pairs, was found to bridge the deletion. A region of the normal murine genome, containing the first 43 bp of the deletion-associated insert (DAI), but lacking the 25-bp dA:dT sequence, was isolated. All normal mice tested contain this DAI-like element and several inbred strains contain an additional DAI-like element. The sequence spanning the Hbb(th-1) deletion may be a reverse transcript of this region.

Many deviations from normal heredity are the result of recombinatorial events (Lewin, 1985). These may be manifest as an unusual combination of traits within an individual, the production of novel protein products (e.g. immunoglobulins, Tonegawa, 1983), or the appearance of mutations (e.g. hemoglobin Lepore (Baird et al., 1981). The analysis of the primary nucleotide sequence at the sites of recombination has provided clues relating to the mechanisms by which such rearrangements might take place (Baltimore, 1985; Hochtl and Zachau, 1983).

Deletions in the globin loci can produce easily identified, non-lethal mutations (Collins and Weissman, 1984). Indeed, many of the mammalian deletions for which primary sequence data are available involve the human globin loci. Some of these are believed to confer a selective advantage upon the heterozygote (Weatherall and Clegg, 1972), and many of the best characterized deletions have persisted in populations for long periods of time. Over time, the regions surrounding these events may have undergone further mutation and/or recombination, making it difficult to define the original event.

A mouse model of β-thalassemia has been described and the genetic locus associated with it has been designated Hbb(th-1) (Skow et al., 1983). In the homozygous state these animals have a trierythro, hypochromic anemia with a profound reticulocytosis. Their peripheral smears are remarkably similar to those seen in human β-thalassemia (Skow et al., 1983). Chromatographic separation of denatured globin chains reveals that the β major globin chain, which normally represents 80% of the total adult β-globin in this Hbb(d) variety of mouse (Russell and McFarland, 1974), is absent.

Despite their anemia, mice homozygous for the Hbb(th-1) allele survive to adulthood and are able to reproduce (Popp et al., 1984). The underlying defect responsible for the Hbb(th-1) phenotype, which arose spontaneously, is the deletion of the β major globin gene (Skow et al., 1983). Both the β minor globin gene (3' to β major) and the β-H3 pseudogene (5' to β major) are intact. On the basis of the restriction and hybridization patterns of genomic DNA, the deletion was estimated to be 3.3–3.6 kb, covering the entire β major globin coding region (Skow et al., 1983).

The chromosomal region corresponding to the Hbb(th-1) locus has now been isolated. The sequence of the DNA spanning the breakpoints of the deletion has been determined. Analysis of this portion of the genome of the mouse model of β-thalassemia provides several clues relating to the mechanism by which this, and possibly other, deletions arose.

MATERIALS AND METHODS

Restriction Fragment Probes—Probes for areas related to the murine β major globin gene were prepared as restriction enzyme digests of the 7.4-kb EcoRI fragment described by Konkel et al. (1978). Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories, and digestions were performed according to the conditions specified by the supplier. The fragments were separated by agarose gel electrophoresis, electroeluted, extracted with phenol and chloroform, and then ethanol-precipitated. Radiolabeled probe was generated by nick translation (Rigby et al., 1977) using [α-32P]dATP as substrate.

Southern Blotting—DNA was extracted from the livers of the various mouse lines by the method of Gross-Bellard et al. (1973). Restriction enzyme digests of genomic DNA were transferred to nitrocellulose (Schleicher & Schuell, BA 85) after agarose gel electrophoresis in the manner described by Southern (1975). Restriction digests of recombinant phage DNA were transferred simultaneously to two sheets of nitrocellulose by sandwiching the gel between the two sheets after soaking both the gel and the nitrocellulose in 20 × SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate). Blots were baked under vacuum at 80 °C for 2 h and stored dessicated until use. They were then prehybridized for 4–18 h in 6 × SSC, 5 × Denhardt (1966) solution, 0.5% sodium dodecyl sulfate (SDS). Labeled probe was denatured by boiling for 10 min and was added to the prehybridization mixture, and the incubation continued for an additional 12–18 h. Blots were washed four times for 20 min at room temperature in 2 × SSC, 0.5% SDS, twice for 20 min in 0.2 × SSC, 0.5% SDS at the hybridization temperature, air-dried, and exposed to x-ray film (XAR, Kodak) for periods ranging from 5 min (for phage DNA) to 5 days (for murine genomic DNA).

Genomic Libraries—A genomic library of liver DNA extracted from a β-thalassemic mouse was constructed from an EcoRI partial digestion of the DNA cloned into a Charon 30 vector (Rimm et al., 1980).

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1 The abbreviations used are: kb, kilobase(s); DAI, deletion-associated insert; bp, base pairs; SDS, sodium dodecyl sulfate.
and plated on the BNN45 Escherichia coli sub strain. A library of a Sau3AI partial digest of DBA/2J liver DNA was constructed in an EMBL3B vector (Frischauf et al., 1983) and grown on BNN45. Plaques were transferred to nitrocellulose and hybridized to probe by the method of Benton and Davis (1977). Positive plaques were picked, grown in the same sub strain of E. coli, and characterized by restriction enzyme digestion and hybridization pattern.

**Sequence Determination**—Restriction fragments of isolated λ clones were sub cloned into pUC8. These fragments were further sub cloned into M13mp18 and M13mp19. Primary nucleotide sequences were then determined by the technique of Sanger et al. (1977) using a synthetic 15-base primer (Bethesda Research Laboratories).

**Synthesis of Oligomeric DNA Probes**—The 18-base sequence, TAAGTGAATAATTTTGT, was synthesized by a modification of the solid-phase phosphotriester technique using a Vega Polynucleotide Synthesizer (Miyoshi et al., 1980a, 1980b). The 20-bp sequence, AATGTGTATTAACAAAAGA, was synthesized by the phosphite-triester method (Beaucage and Caruthers, 1980). The oligonucleotides were deprotected by acidification, purified by reverse phase high pressure liquid chromatography using a modification of the method of Dembek et al. (1981) and Kempe et al. (1982). The fragments were terminally labeled using T4 polynucleotide kinase (P-L Biochemicals) (Maniatis et al., 1978) using [γ-32P]ATP (3000 Ci/mm, 110TBq/mm) as the phosphate donor. The sequences of the oligonucleotides were verified by chemical sequence analysis (Maxam and Gilbert, 1980).

**Hybridization with Oligomeric Probes**—Southern blots were prepared and prehybridized as described above for hybridization with restriction fragments. The probe was diluted 100-fold in hybridization solution and incubated at 65 °C for 10 min. The filters were then hybridized at 47 °C (18-mer), or 52 °C (20-mer) for 18-24 h. The filters were then washed three times in 6 X SSC at 0 °C for 15 min each, twice at the hybridization temperature with 3 X SSC, 0.5% SDS, and twice at room temperature with 6 X SSC. The filters were dried in air, covered with plastic wrap, and exposed to x-ray film for periods of 5 min (phage) to 4 days.

**Computer Analysis**—Some of the computer resources used to carry out these studies were provided by the BIONET National Computer Resource for Molecular Biology, whose funding is provided by the Biomedical Research Technology Program, Division of Research Resources, National Institutes of Health, Grant 1U41RR-01685-02. Sequence data were input to an APPLE II computer using the GelPad System (K&H BioSoft, Frederick, MD). The sequence data were then transmitted to the BIONET system for analysis. The GEL program was used for sequence assembly and IFIND for comparison to the databases.

**RESULTS**

A 3.7-kb Deletion Is Responsible for the Hbb(th-1) Allele—Restriction analysis of genomic DNA had previously shown...
that a simple 3.3-3.6-kb deletion spanning the β major globin gene accounts for the absence of this protein in mice homozygous for the Hbb(th-1) allele (Skow et al., 1983). Analysis of the novel band pattern generated by DNA extracted from β-thalassemic mice on genomic Southern blots probed with the non-deleted, β major specific, 5′ and 3′ flanking regions generated the restriction map shown in Fig. 1a. The deletion encompasses the β major globin coding region: 1.16 kb of 5′ flanking region and 0.3–0.6 kb of 3′ flanking region.

A fragment extending from an XbaI site (2 kb 3′ to the cap site) to a BglII site (1 kb further downstream) was used to screen a genomic phage library of the β-thalassemic mouse. This region is beyond the deletion in the Hbb(th-1) genome and produces a unique set of bands on genomic Southern blots of β-thalassemic DNA that are different from those generated by normal DBA/2J DNA (data not shown).

The clones identified by this probe had the restriction pattern and hybridization pattern expected at the breakpoint of the deletion in the Hbb(th-1) genome. The insert contained an 8.8-kb EcoRI fragment and a 1.85-kb BglII fragment, both of which hybridized to the XbaI-BglII fragment 3′ to the β major globin gene (Fig. 1b). The pattern of the cloned region was consistent with that previously determined by genomic Southern blots. A diffuse hybridization pattern, characteristic of a repeated sequence, was obtained (data not shown).

Since this restriction fragment contains a 25-bp dA:dT stretch that could be expected to hybridize with repeat sequences (Schmid and Jelinek, 1982), the oligonucleotide, TA-GTGTAAATTTTTGTT, corresponding to the -1727 to -1731. Thus the deletion is 3709 ± 2 base pairs long. (The ambiguity is caused by the 4 dA:dT base pairs at the 3′ end of the normal sequence, any one of which could be the breakpoint). The BALB/c and DBA/2J sequences are identical 3′ to the breakpoint for at least 250 bp. This confirms the conservation of β-globin sequences between these two strains of mice.2

Sixty-six (±2) base pairs are inserted at the deletion site. This deletion-associated insert (DAI) is a T-rich (82% A+T) and ends in a 25-bp dA:dT sequence but does not contain an identifiable poly(A) addition signal (AAATTAAA). No sequences similar to it have been described in the vicinity of any murine β-globin gene (Konkel et al., 1978; Citron et al., 1984). Comparison of this sequence with the GenBank® database (release 31.0) using the algorithm of Wilbur and Lipman (1983) failed to identify it.

The Normal Murine Genome Contains a Small Number of DAI-like Sequences—Insertions often accompany deletions but they are frequently so small that their origins cannot be identified. The Hbb(th-1) DAI sequence was large enough to allow its use as a probe. The 124-base pair MnlI fragment consisting of the 66-base pair insert, 21 base pairs of 5′ flanking DNA, and 35 base pairs of 3′ flanking DNA (described above), was used as a probe for genomic Southern blots. A diffuse hybridization pattern, characteristic of a repeat sequence, was obtained (data not shown).

Since this restriction fragment contains a 25-bp dA:dT stretch that could be expected to hybridize with repeat sequences (Schmid and Jelinek, 1982), the oligonucleotide, TA-GTGTAAATTTTTGTT, corresponding to the (−) strand of the left-most 18 base pairs of the insert (orientation defined by the direction of β-globin transcription) was synthesized, purified by high pressure liquid chromatography, end-labeled with polynucleotide kinase, and used to probe restriction enzyme digests of DNA extracted from normal, DBA/2J mice and β-thalassemic mice (Fig. 4). Hybridization of this probe produced a relatively complex pattern with some apparent differences between normal and thalassemic samples. A second probe was then synthesized, AATGTGTATTA-GCCAAAAGA, corresponding to the (−) strand of the 20 base pairs adjacent to the dA:dT region of the insert. This oligonucleotide was purified by high pressure liquid chromatography, labeled, and similarly used as a probe for restriction digests of genomic DNA (Fig. 4). Using this sequence, which does not overlap the 18-base probe, a less complex pattern is generated. BamHI generates only two hybridizing bands in both the β-thalassemic and the DBA/2J genomes. A 15-kb band is present in both genomes. The hybridizing band at 8.3 kb in the β-thalassemic DNA corresponds to the location of

Fig. 3. Sequence at the Hbb(th-1) deletion breakpoint. The sequence of the HindIII-XbaI fragment isolated from the Hbb(th-1) genome (th-1), portions of the normal DBA/2J β major globin gene (β-major), were determined using the clones described. Only a small portion of the alignment with the normal sequences is shown. The 4 A:T base pair differences at position 1725 in the normal 3′ flanking sequence make the assignment of the 3′ breakpoint ambiguous. The numbering of the normal sequence begins with +1 at the cap site. The undetermined sequence is the inserted region. The sequence in italics (GGTTTC) is the hexanucleotide common to several deletions (see “Discussion”).

2D. Kuebbing, unpublished observations.
DNA extracted from a β-thalassemic (T), normal DBA/2J (D), and heterozygote between a β-thalassemic and C57Bl/6J mouse (H) were digested with BamHI and multiple Southern blots prepared in parallel (strip blots). These were then hybridized with the indicated probe each related to the DAI. The 190-bp and 3.7-kb probe were isolated from plaque A42.

**FIG. 4.** Pattern of hybridization of genomic Southern blots with various probes related to the DAI and DAI-like elements. DNA extracted with from plaque A42. from plaque A42, was selected for further analysis (Fig. 5). DNA was isolated from this phage, digested with a variety of restriction enzymes (both singly and in combination), and transferred to nitrocellulose as described under “Materials and Methods.” The resulting Southern blots were then independently hybridized to the two oligomers probes described above. With every combination of restriction enzymes tried, the same restriction fragments hybridized to both probes (data not shown). Thus, the two probe sequences appeared to be close to one another.

The 410-base pair region (extending from an RsaI site to a BglII site) which hybridizes to both oligomeric probes, derived from plaque A42, was sequenced by subcloning several subfragments into an M13 vector (Fig. 6). This fragment contains a sequence that is identical to the first 43 base pairs of the DAI sequence found at the site of the Hbb(th-1) deletion. However, it does not contain a dA:dT stretch nor does it contain a canonical poly(A) addition signal.

**FIG. 5.** A region of the normal murine genome that contains a DAI-like sequence. The 2.5-kb PscI fragment which contains the hybridizing region (box) is expanded below. The SalI sites are not indicated but are present at the ends of the insert and are derived from the phage vector. The arrow indicates the region which hybridizes to both synthetic oligomers described in the text. Restriction enzymes: A, AccI; G, BglIII; H, HindIII; P, PvuII; R, RsaI.

The deleted β major globin gene in the Hbb(th-1) genome. A 2.7-kb BamHI band is present in normal DBA/2J DNA but absent in the β-thalassemic genome.

The 20-base probe hybridized to a subset of the bands that hybridize to the 18-base probe. As expected, the longer probe is more specific. It is surprising, however, that all fragments that hybridized to the 20-base probe also hybridize to the 18-base probe. This suggests that the association of the 18- and 20-base sequences is not limited to the DAI. These two independent probes are close to each other elsewhere in the genome.

A Normal Genomic Sequence Homologous to the DAI Lacks a String of dA:dT—The normal genomic sequence that corresponds to the DAI may represent the recombinatorial partner which, along with the β major globin gene, generated the Hbb(th-1) allele. On the basis of Southern blots (Fig. 4), no more than two such areas exist in the normal genome. By isolating such a region, we might determine the relationship between the inserted fragment and its precursor.

The previously described EMBL-3B library of the normal DBA/2J genome was screened with the 18- and 20-base oligomeric probes. A plaque which hybridized to both oligomers, designated A42, was selected for further analysis (Fig. 5). DNA was isolated from this phage, digested with a variety of restriction enzymes (both singly and in combination), and transferred to nitrocellulose as described under “Materials and Methods.” The resulting Southern blots were then independently hybridized to the two oligomers probes described above. With every combination of restriction enzymes tried, the same restriction fragments hybridized to both probes (data not shown). Thus, the two probe sequences appeared to be close to one another.

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**FIG. 6.** Sequence of a normal DAI-like element. The sequence of the 410-base pair BglII fragment, subcloned from plaque A42, was determined as described under “Materials and Methods.” The box encloses a sequence that is identical to the S' end of the DAI. The bases in italics are those complementary to the synthetic oligomers described.
β-globin gene, or its absence could reflect a polymorphism at that site. To identify the genealogy of these elements, Southern blots of a variety of mouse strains were probed with the 190-bp Sau3A-AluI fragment (Fig. 7). The 15-kb BamHI fragment was present in all strains tested. The 2.7-kb fragment was present in some strains (DBA/2J, DBA/1J, NZB/J, AKR/N, STS/J, and C58/J) and absent from others (C57BL/6J, C57/LJ, and BALB/cJ). Closely related strains share the same pattern. Neither C57BL/6J nor C57/W has a deletion (Spritz and Orkin, 1982). The 2.7-kb band; both DBA/1J and DBA/2J have it. This pattern of divergence is consistent with at least one ancestral rearrangement in this region, prior to the divergence of BALB/cJ and AKR/J.

**DISCUSSION**

A previously described mouse model of β-thalassemia (Skow et al., 1983) is the result of a deletion of the β major globin gene. The molecular details of the deletion have not been investigated. The deleted DNA (3709 ± 2 bp) is replaced by 66 ± 2 bp of DNA that apparently originated in another part of the murine genome.

**Several Deletions Share a Common Hexanucleotide**—The hexanucleotide at the 5′ breakpoint, GGTTTTC, includes the first five bases deleted in the Hbb(th-1) mutation, is found close to the breakpoint of several other deletions (Table I). The cleavage between the two guanines and the six nucleotides that follow are identical to the deletion in an albumin pseudogene (Spritz and Orkin, 1982). The normal nonamer sequence is replaced (in part) by the hexamer GGTTTTC. Despite (or perhaps before) this change, this pseudogene appears to have undergone a V-J joining. This same hexanucleotide appears in various elements of the murine immunoglobulin rearrangement switch region, S(1mu) (Gerondakis et al., 1984), and is also found 2 bp 3′ to a site at which the spleen focus-forming virus genome differs from the Moloney murine leukemia virus genome by a deletion of 810 base pairs (Clark and Mak, 1983). Recently, the 9q+ (reciprocal of the Ph(1) chromosome) breakpoint has been sequenced. This breakpoint (which is also associated with a deletion) occurs at the same (GGTTTTC) hexanucleotide.

The appearance of this hexanucleotide, GGTTTTC, near several deletion breakpoints suggests that it might play some role in a recombinatorial process which has led to these heritable mutations. Based upon the limited data available, this sequence may be a recognition site for an enzyme that is active in germ cells, possibly related to meiotic recombination. The appearance of this sequence in immunoglobulin rearrangement regions and areas involved in T cell receptor rearrangement implies that either the somatic and germ line recombinatorial mechanisms share certain features (including some degree of sequence specificity) and/or that germ line rearrangements can be the manifestations of the action of the somatic recombinatorial machinery acting on an unusual substrate.

**Structure of the Insertion**—The association of short inserts with heritable deletions is a common (Table I) although not invariant finding. Short, novel sequences are also associated with somatic recombinatorial events, e.g. the “N sequences” of immunoglobulin rearrangement (Alt and Baltimore, 1982) and the translocation of cellular oncogenes (Gerondakis et al., 1984). Some of these sequences seem to be duplications of neighboring regions of the genome (e.g. the albuminemic rat (Esumi et al., 1983) and the Indian β-thalassemia mice (Spritz and Orkin, 1982) genes). Others do not have a clear origin but are generally too short and contain too little information to specify their origins.

The 43-base pair Hbb(th-1) DAI is large enough to be a meaningful probe for the detection of matching sequences in the normal murine genome. However, initial attempts to identify a genomic source for the Hbb(th-1) insertion sequence, using restriction enzyme fragments, suggested that it

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**Fig. 7. Polymorphism of the DAI-like element in several mouse strains.** DNA from various inbred mouse strains was digested with BamHI, and the resulting Southern blots hybridized with the 190-bp Sau3A-AluI fragment, containing the DAI-like element. isolated from plaque A42 (see Fig. 5).
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**TABLE I**

| Gene    | Genus | Deletion                        | Insertion       |
|---------|-------|---------------------------------|-----------------|
| β-Globin| Mus   | GAGTTGCC...                     | (See Fig. 3)    |
| Albumin | Rattus| GAGTTCCCGG                      | CGAGCCTA        |
| β-Globin| Homo  | CATGATGTAAGAAGTTTTCAATT         | AAGTAGA         |
| δ-β-Globin| Homo | ATAGGAGGTTTTACC...              | AAATAA          |
| δ-β-Globin| Homo | GAGTTTC... 15 bp... CTGG       |                 |

Genealogy of the DAI Sequence—All mice tested have a copy of the DAI sequence that migrates as a 15-kb BamHI fragment (Fig. 7). A second copy, seen as a 2.7-kb BamHI fragment, is present in some strains but absent in others. When probes isolated from the phage clone of the 15-kb copy are used to probe genomic Southern blots, the hybridization to this 2.7-kb band remains strong with increasing probe size, whereas the signal that corresponds to the Hbb(th-1) breakpoint grows weaker as the DAI sequence becomes a smaller fraction of the total probe (Fig. 4). Thus, these two normal copies of the DAI appear to share a longer region of sequence homology than the 43 base pairs inserted at the Hbb(th-1) breakpoint.

The kinase pattern suggested by the Southern blot is consistent with the genealogy of these inbred mouse strains (Staats, 1980). Both DBA strains have the additional band and only one C57 strain lack it. Since the C57 and C58 strains are related only through their paternal genes, the 2.7-kb BamHI fragment must have come from the female in this 1921 mating.

The genealogy of the strains can be used to locate the point of divergence with respect to this additional copy of the DAI sequence in the evolutionary history of these inbred mouse strains. The progenitor of the AKR and DBA strains (which contain the 2.7-kb fragment) diverged from the progenitor of the BALB/c and C57 strains (which have a single copy of the DAI) in the mid-19th century. Further analyses using wild mice and related rodent species will be necessary to decide whether this divergence constituted an insertion or a deletion of the second DAI-like region.

**How Did the β-Thalassemic Mouse Arise?**—Models attempting to surmise the events that generated the Hbb(th-1) allele must account for the deletion, the insertion, and the da:dT stretch (a putative reverse transcript). Many heritable deletions can be understood as mismatched meiotic recombinatorial events (e.g., β-thalassemia (Higgs and Weatherall, 1983)) or intramolecular gene conversions (Michelson and Orkin, 1983). There is no evidence that the process that generated the mouse model of β-thalassemia involved a homologous recombination. The ends of the deletion are not homologous with one another, and the only detectable region of homology between the DAI and the β major globin region is between the da:dT sequence of the DAI and the region between 1728 and 1731 consisting of 4 da:dT base pairs at the 3' insertion site for the DAI.

The da:dT stretch suggests that the DAI arose from an RNA precursor. There are three models (not mutually exclusive) that account for all of the data: Damage repair, immunoglobulin type rearrangement, and transposition. The data are insufficient to distinguish between these models. The characterization of other deletions and the further analysis of this element should help to clarify the mechanisms involved in the process that generate these events.

**Acknowledgments**—We thank Dr. K. Huppi for the Charon 30 phage library; Drs. K. Huppi, L. D'Hoostelaere, and M. Potter for supplying the DNAs from various mouse strains used in Fig. 7; Dr. Martin Egitis for his thoughtful comments on the manuscript; and Dr. Patricia Berg for many helpful discussions.

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