Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 6735–6739, October 1987
Biochemistry

Molecular cloning of a human glycophorin B cDNA: Nucleotide sequence and genomic relationship to glycophorin A

(oligonucleotide mapping/K562 erythroleukemia cells/gene structure)

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Communicated by Harvey A. Itano, July 6, 1987 (received for review June 2, 1987)

ABSTRACT **Here we describe the isolation and nucleotide sequence of a human glycophorin B cDNA. The cDNA was identified by differential hybridization of synthetic oligonucleotide probes to a human erythroleukemic cell line (K562) cDNA library constructed in phage vector λgt10. The nucleotide sequence of the glycophorin B cDNA was compared with that of a previously cloned glycophorin A cDNA. The nucleotide sequences encoding the NH_2-terminal leader peptide and first 26 amino acids of the two proteins are nearly identical. This homologous region is followed by areas specific to either glycophorin A or B and a number of small regions of homology, which in turn are followed by a very homologous region encoding the presumed membrane-spanning portion of the proteins. We used RNA blot hybridization with both cDNA and synthetic oligonucleotide probes to prove our previous hypothesis that glycophorin B is encoded by a single 0.5- to 0.6-kb mRNA and to show that glycophorins A and B are negatively and coordinately regulated by a tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate. We established the intron/exon structure of the glycophorin A and B genes by oligonucleotide mapping; the results suggest a complex evolution of the glycophorin genes.**

The major sialoglycoproteins of the human erythrocyte membrane belong to a family of at least three related glycoproteins termed glycophorins A (MN), B (Ss), and C (D-glycoprotein) (1, 2). The complete amino acid sequences of the glycophorin A (3, 4) and B (5) apoproteins have been determined by peptide sequencing. These studies have shown that glycophorins A and B share some amino acid homology, particularly in the NH_2-terminal region.

To date, two human glycophorin cDNAs have been isolated and sequenced: glycophorin C cDNA by Colin *et al.* (6) and glycophorin A cDNA by us (7). We recently showed by genomic DNA hybridization using oligonucleotide probes that glycophorins A and B are encoded by separate and distinct genes (8). However, they appear to share a substantial degree of nucleotide sequence homology both in the NH_2-terminal region of the apoprotein and in the leader peptide. The nucleotide and deduced amino acid sequences of glycophorin C (6) show very little if any homology to either glycophorin A or B, but the proteins do show similarity in carbohydrate structure (1, 2).

We previously showed that the expression of glycophorins A (8, 9) and B (8) is negatively and coordinately regulated in human erythroleukemic K562 cells by the tumor-promoter phorbol 12-myristate 13-acetate (PMA) at the level of mRNA abundance, although the identification of mRNA for glycophorin B was tentative.

In an effort to further delineate the relationships and regulated expression of the glycophorins at the nucleic acid level, we describe here the cloning and nucleotide sequence of a glycophorin B cDNA.* We used the cDNA to examine the structure and regulation of the glycophorin mRNAs by PMA. Furthermore, by oligonucleotide mapping we deduced the intron–exon structure of the glycophorin A and B genes.

EXPERIMENTAL PROCEDURES

Materials and Hybridization Probes. All radioactively labeled compounds were purchased from New England Nuclear. Restriction enzymes were from Bethesda Research Laboratories. Synthetic oligonucleotides were constructed on an Applied Biosystems (Foster City, CA) automated 380A DNA synthesizer and purified by reverse-phase high-performance liquid chromatography. For hybridization probes, 22- or 27-mer oligonucleotides were end-labeled by standard protocols (19) with [γ-^{32}P]ATP (>7000 Ci/mmol; 1 Ci = 37 GBq) and purified by either of the two following methods. For cDNA library screening, probes were purified by NACS ion-exchange columns supplied by Bethesda Research Laboratories. For gene mapping, oligonucleotide probes were purified by electrophoresis in 15% polyacrylamide/8.3 M urea gels. The specific activities of all oligonucleotide probes were $\geq 2 \times 10^9$ cpm/μg. cDNA probes were made by random-oligonucleotide-primed extension (10) using a kit supplied by Pharmacia and had specific activities of $0.5–2 \times 10^9$ cpm/μg.

Cell Culture and Isolation of Nucleic Acids. Human erythroleukemic K562 cells (11) and human promyelocytic HL-60 cells (12) were cultured in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal bovine serum. Treatment of cells with PMA was performed as described (9, 13). Total cellular RNA was prepared by the guanidinium thiocyanate method of Chirgwin *et al.* (14). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (15). Genomic DNA was prepared from K562 cells as described by Friedrich and Lehman (16).

Construction and Screening of the cDNA Library. Double-stranded cDNA was prepared, by the method of Gubler and Hoffman (17), from a total K562 cell poly(A)$^+$ RNA template primed with oligo(dT). Internal *Eco*RI sites were protected by *Eco*RI methylase (Bethesda Research Laboratories) and the cDNA was blunt-ended by phage T4 DNA polymerase without the use of nuclease S1. *Eco*RI linkers (Pharmacia) were ligated to the cDNA, using conditions supplied by the manufacturer and digested with *Eco*RI. The resulting linker·cDNA fraction was purified and size-selected by sucrose gradient centrifugation. The cDNA was then ligated to *Eco*RI-digested and dephosphorylated λgt10, packaged, and transfected into *Escherichia coli* C600 Hfl (Stratagene, San

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Abbreviation: PMA, phorbol 12-myristate 13-acetate.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02982).

Diego, CA). The complexity of the library was estimated to be $\geq 8 \times 10^5$. All selected clones were found to be recombinants with intact *Eco*RI sites. Screening for desired clones was performed by differential hybridization using two 27-mer oligonucleotide probes, as described for colony hybridization by Woods (18).

Hybridization of Oligonucleotide Probes to Purified DNA and RNA. For hybridization to λ phage, DNA was prepared by a rapid polyethylene glycol precipitation method (19), restricted with *Eco*RI, resolved by 1.3% agarose gel electrophoresis, and transferred to nitrocellulose as described by Southern (20). Hybridization was then conducted exactly as described above for cDNA library screening. For RNA blots, poly(A)$^+$ RNA was resolved by denaturing agarose/formaldehyde gel electrophoresis (21), transferred to nitrocellulose filters, and hybridized to 27-mer oligonucleotide probes as described (7). *In situ* hybridization of oligonucleotides to restricted genomic DNA immobilized in dried agarose gels has also been described (8).

Nucleotide Sequencing. Sequencing of the cDNA was performed by the phage M13/dideoxyribonucleotide chain-termination method of Sanger *et al.* (22), using a kit supplied by Pharmacia. Following use of universal primers, sequencing was continued by use of sequentially constructed 16-mer oligonucleotides (23). The nucleotide sequence was determined for both strands of the cDNA.

RESULTS AND DISCUSSION

Construction and Screening of the cDNA Library. A human erythroleukemic K562 cell cDNA library was constructed from a total poly(A)$^+$ RNA template primed with oligo(dT) as described by the Gubler and Hoffman modification (17) of the original RNase H/DNA polymerase I method of Okayama and Berg (24). The cDNA was treated with *Eco*RI methylase, ligated to *Eco*RI linkers, restricted, and cloned into a λgt10 vector. Approximately 4×10^5 recombinant clones were replica-plated and hybridized in duplicate to an exact-sequence oligonucleotide probe (GC), which was determined by us previously to hybridize to both the glycophorin A and B mRNAs (8). In all, five clones were found to hybridize reproducibly to this probe. After plaque purification, λ DNA was prepared from each of the clones, restricted with *Eco*RI, and tested by Southern blot hybridization for the ability to hybridize differentially to the above GC probe and another oligonucleotide probe, GE. The latter probe was purposely constructed to achieve two important criteria. First, probe GE was specific to a region of the nucleotide (and amino acid) sequence of glycophorin A absent in glycophorin B. Second, this probe was oriented 3′ (or towards the COOH terminus) with respect to the first probe, GC (see also Fig. 4). Therefore, any glycophorin A-specific cDNA molecule primed from the 3′ end of the mRNA (i.e., using oligo(dT) as a primer as we did) would have to hybridize to both of the oligonucleotide probes, whereas any glycophorin B cDNA would be expected to hybridize to the first (GC) probe but not to the second (GE) probe. One cDNA clone out of the five clones satisfied these criteria and was selected as a glycophorin B cDNA and designated λHGpB-1. The size of this cDNA was estimated to be approximately 480 base pairs, which [minus a full poly(A)$^+$ tail] is reasonably close to our estimate of the size of the glycophorin B mRNA by RNA blot hybridization (500–600 base pairs); this suggests that we have isolated a full- or near full-length cDNA.

Nucleotide Sequence of the Glycophorin B cDNA. The nucleotide sequence of clone λHGpB-1 was determined by M13 cloning/dideoxyribonucleotide termination (20) as described above. The nucleotide and deduced amino acid sequences for glycophorins A and B are compared in Fig. 1. It can be seen that there is almost a perfect nucleotide and amino acid identity in the leader peptide and the NH_2-terminal 26 amino acid residues encoded by the glycophorin A and B cDNAs. The only difference in the leader-peptide region is at amino acid residue −7, where a single nucleotide change predicts alanine vs. glutamic acid in glycophorins A and B, respectively. The amino acids at positions 1 and 5 differ for our published sequence of the glycophorin A cDNA pHGpA-3. This is due to polymorphisms of glycophorin A giving rise to blood-group antigens M and N. Clone pHGpA-3 encodes the M-group antigen (7). During the course of this present work we have partially sequenced an additional glycophorin A cDNA, which encodes the N-group activity and which is identical to glycophorin B in residues 1 and 5 (4, 25) (see Fig. 1*C*). This indicates that K562 cells carry the MN phenotype.

In our previously isolated glycophorin A cDNA, we proposed (7) that the ATG encoding the methionine residue at position −19 was the translation initiation codon. We have now found a stop codon 30 nucleotides 5′ to the single methionine at position −19 in the second glycophorin A cDNA, thus providing evidence that this methionine represents the initiation codon. Because the same residues were found in the glycophorin B cDNA, we propose that we have isolated the complete structural gene for both glycophorin A and glycophorin B.

In addition, we have made several corrections to the 3′-end nucleotide sequence of the glycophorin A cDNA encoding amino acid residues 123–126 and have extended the nucleotide sequence to include the COOH-terminal 5 amino acid residues by sequencing the glycophorin A cDNA described above.

As predicted by amino acid sequencing of glycophorin A and B peptides, a segment (amino acid residues 27–55) of glycophorin A is absent from glycophorin B. Because of this deletion, glycophorin B lacks the Asn-Asp-Thr sequence, which is the N-glycosylation site for glycophorin A at residues 26–28. This result is consistent with the fact that glycophorin B contains only oligosaccharides linked O-glycosidically to serine or threonine (25). The next region, encoding residues 56–100 of glycophorin A and 27–71 of glycophorin B, showed some homology. Residues 60–72 of glycophorin A and residues 31–43 of glycophorin B are quite different, whereas residues 80–100 of glycophorin A and 51–71 of glycophorin B are strikingly similar in both the amino acid and nucleotide sequence. It is important that the above-mentioned area of nonhomology contains such a large degree of nucleotide heterogeneity that it is unlikely to have arisen by random mutation of a duplicated gene precursor (see below). Two discrepancies between the deduced sequence and established amino acid sequence of glycophorin B apoprotein were noted. First, amino acid residues 50 and 65 in the deduced sequence are cysteine and threonine respectively, whereas both were found to be serine by amino acid sequence analysis, although assignment of serine to position 50 was tentative (5). Another difference is that our deduced sequence has an additional residue (alanine) at the COOH terminus. This brings the total number of amino acid residues in glycophorin B to 72.

Position 29 in the amino acid sequence of glycophorin B is responsible for another polymorphism, giving rise to Ss group activity, S having a methionine and s having a threonine (4). The latter was indicated in the glycophorin B cDNA examined in this study.

Blot Hybridization Analysis of Glycophorin mRNAs. Previously, we found that glycophorin A was encoded by three electrophoretically distinct mRNAs of 2.8, 1.7, and 1.0 kilobases (kb), whereas we proposed that glycophorin B was encoded by a single 0.6-kb mRNA (7). Having cloned and sequenced a glycophorin B cDNA, we now were able to test this hypothesis. The results of several experiments in which

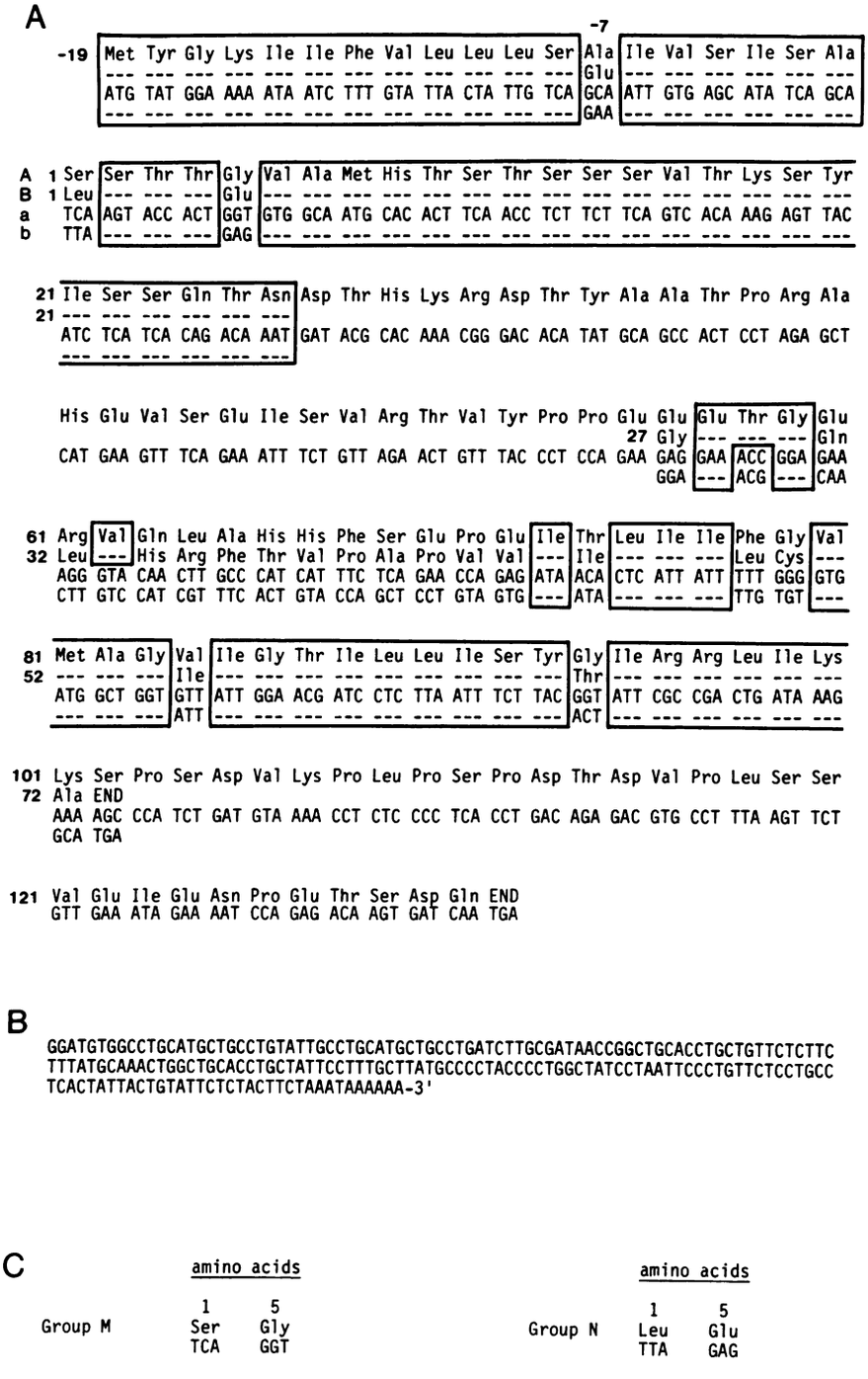

FIG. 1. (*A*) Comparison of the nucleotide sequences of the coding regions of the glycophorin A and glycophorin B cDNAs and their deduced amino acid sequences. Each set of four lines shows (from top to bottom) the amino acid sequences of glycophorins A and B followed by the cDNA sequences from which they were deduced, as indicated specifically by the letters A, B, a, and b to the left of position 1. Regions of identical sequences are shown by dashes and are boxed in. (*B*) The 3′ noncoding region of the glycophorin B cDNA, beginning with the nucleotide following the stop codon TGA. (*C*) Glycophorin A cDNA sequences at codons for amino acids 1 and 5, defining the MN blood-group antigens.

various probes were successively hybridized to RNA from control K562 (−), PMA-treated K562 (+), and promyelocytic HL-60 cells are shown in Fig. 2. When a synthetic oligonucleotide specific only to the glycophorin B cDNA (oligo GB-1) was used as a probe, it was found to hybridize exclusively and strongly to a 0.5- to 0.6-kb mRNA. After removal of the probe, the filter was rehybridized using a glycophorin A cDNA probe (pHGpA-3). This probe hybridized to four mRNA species; the smallest was presumed to be the glycophorin B mRNA, since it comigrated with the mRNA to which the glycophorin B-specific oligonucleotide hybridized. The cross-hybridization of the pHGpA-3 probe to the glycophorin B mRNA is due to the homology present in the 5′ region of the cDNAs as discussed above. When the glycophorin B cDNA λHGpB-1 was used as a probe, strong hybridization to the glycophorin B mRNA was noted. However, in this case cross-hybridization of the glycophorin B cDNA to the glycophorin A mRNAs occurred but was much less pronounced, perhaps because of steric conformations required for cross-hybridization.

For all of the probes, there was a marked decrease in hybridization to RNA isolated from PMA-treated K562 cells.

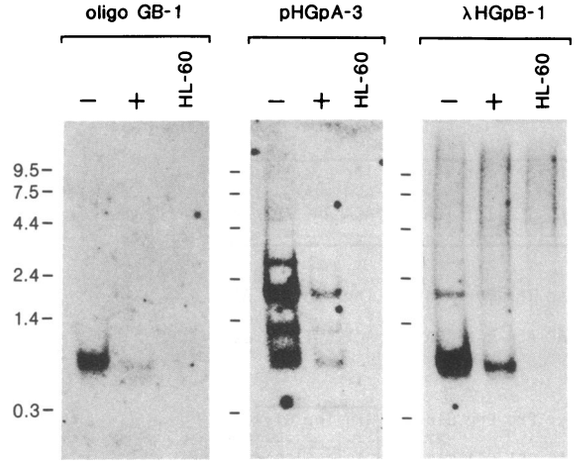

FIG. 2. Blot hybridization analysis of glycophorin B mRNA. Samples (8 μg) of poly(A)$^+$ RNA from control (−) and PMA-treated (+) K562 cultures and from HL-60 cells were resolved by denaturing 1.3% agarose/formaldehyde gel electrophoresis. Following transfer to nitrocellulose, the blot was sequentially hybridized to several glycophorin probes. The first probe was a 27-mer synthetic oligonucleotide, GB-1, complementary only to the glycophorin B mRNA encoding amino acid residues 32–40. Hybridization and low-stringency washing were as described (7). The final wash was in 6× SSC/0.1% NaDodSO$_4$ at 58°C for 30 min. (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.) After removal of the first probe (H_2O, 100°C for 15 min), the blot was hybridized to a glycophorin A cDNA probe (pHGpA-3, whole plasmid) prepared by random-oligonucleotide-primed extension. Hybridization and washing were as described by Thomas (26) except that dextran sulfate was omitted. The high-stringency wash was conducted in 0.1× SSC/0.1% NaDodSO$_4$ at 50°C for 45 min. After removal of this probe, the filter was rehybridized to a glycophorin B cDNA probe (λHGpB-1 cDNA insert) exactly as described for pHGpA-3. Positions and sizes (kb) of RNA markers (purchased from Bethesda Research Laboratories) are shown at left.

Further, none of the probes hybridized to RNA prepared from human nonerythroid, promyelocytic HL-60 cells. The

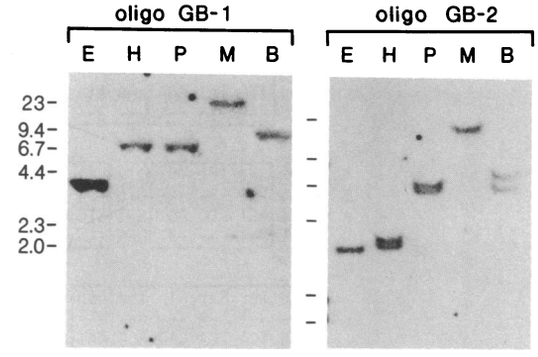

FIG. 3. Oligonucleotide gene-mapping experiments. Genomic DNA was prepared from K652 cells, digested by various restriction enzymes (E, *Eco*RI; H, *Hin*dIII; P, *Pvu* II; M, *Msp* I; B, *Bgl* II), resolved in 0.8% agarose gels, and hybridized *in situ* to seven perfect-sequence synthetic oligonucleotide probes derived from the nucleotide sequence of the glycophorin A and B cDNAs (see legend to Fig. 4). Results are shown for oligonucleotide probes GB-1 and GB-2. Markers at left show positions and sizes (kb) of *Hin*dIII-digested λ DNA.

results of the RNA experiments clearly show that the single 0.5- to 0.6-kb RNA encodes glycophorin B and that levels of glycophorin A and B mRNA are negatively and coordinately regulated by PMA.

Genomic Relationship Between Glycophorins A and B. By hybridization of synthetic oligonucleotide probes to restricted human genomic DNA, it was possible to establish the intron/exon relationships of the glycophorin A and B genes. We had used this type of oligonucleotide mapping to establish that glycophorins A and B are encoded by separate and distinct genes (8). In the present investigation it was possible to construct oligonucleotide probes from regions of the nucleotide sequences common to both genes as well as those specific to glycophorin A and in particular to glycophorin B. In brief, seven different 22- and 27-mer oligonucleotide probes were hybridized directly to dried agarose gels con-

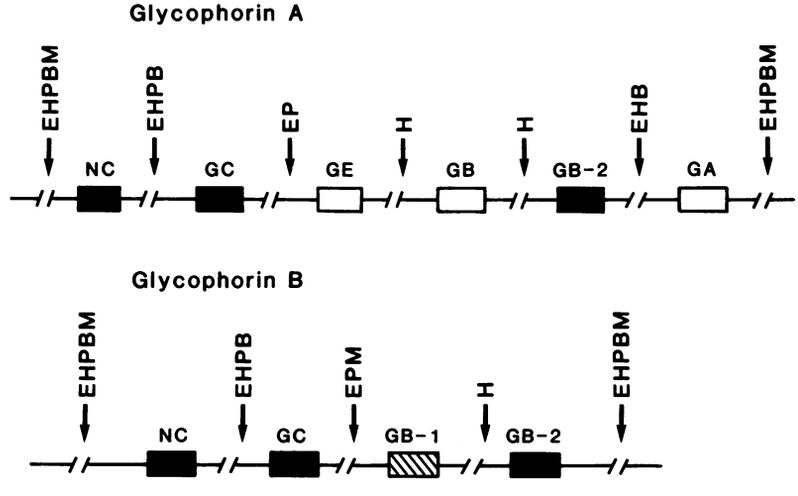

FIG. 4. Deduced intron/exon map of the human glycophorin A and B genes. Black boxes represent exons with homology to both glycophorin A and B. Open and hatched boxes are exons specific to either glycophorin A or B, respectively. Designations above each exon refer to the oligonucleotides used in the mapping experiments. GA, GB, and GE are 27-mers encoding amino acids 112–120, 63–71, and 28–36, respectively, which are exclusive to glycophorin A. Oligonucleotides GC, GB-2, and NC are common to glycophorins A and B: GC is a 27-mer encoding amino acids 18–26 of both glycophorin A and glycophorin B; GB-2 is a 27-mer encoding residues 85–93 of glycophorin A and 56–64 of glycophorin B; NC is a 22-mer encoding residues −20 to −14, which reside in the leader peptide of glycophorins A and B. GB-1, a 27-mer, is exclusive to glycophorin B and encodes amino acid residues 32–40. Arrows indicate cleavage sites for the five restriction enzymes (abbreviated as in Fig. 3); the actual distances of the restriction sites from the intron/exon boundaries cannot be determined from these experiments, as indicated by the discontinuities in the lines representing the introns.

taining restricted and denatured human genomic DNA (8). None of the five restriction enzymes used in this study cut within the coding region of the glycophorin cDNAs, and therefore any cleavage sites must reside within introns.

When restricted genomic DNA is probed with an oligonucleotide specific to either glycophorin A or glycophorin B, it would be expected to hybridize to single fragments only. An example of this situation is shown in Fig. 3 (*Left*), for oligonucleotide probe GB-1, which is specific to glycophorin B. In contrast, when DNA is probed with an oligonucleotide constructed from regions in the cDNA where there is exact nucleotide identity between glycophorin A and glycophorin B cDNAs, the hybridization pattern would be expected to show one or two hybridizing DNA fragments. An example of this situation is shown in Fig. 3 *Right*, for oligonucleotide probe GB-2, which hybridizes to two DNA fragments in three of the five restriction digests.

Most important, when the hybridization pattern changes for any set of oligonucleotide probes, it is clear that the region of DNA to which the probes correspond must belong to different exon structures. This situation is illustrated in Fig. 3: with the enzyme *Hin*dIII the size of the hybridizing DNA fragment differs for the probes GB-1 and GB-2, making it clear that there must be at least one intron separating these sites.

The use of oligonucleotides instead of restriction fragments of the cDNA has the primary advantage that probes can be constructed from essentially any desired portion of the cDNA without the need to have, by chance, suitable restriction sites. Note, however, that the actual distances of the restriction sites from the intron/exon junctions could not be deduced. Also, the number of deduced exons must be considered a minimal value. Further examination of the glycophorin gene family will require the isolation and analysis of genomic cDNA clones.

The results from all of the oligonucleotide gene-mapping experiments are summarized in Fig. 4. The restriction enzymes used and specificities of the probes are described in the figure legend. For simplicity, each exon is identified by the name of the oligonucleotide that is specific to it. The results show that the coding regions of the glycophorin A and B genes are interrupted by at least five and three introns, respectively. The data further suggest that some of the corresponding exons can be considered to specify protein domains. For example, from 5′ to 3′, the exon NC corresponds to the leader peptide in the protein. The next exon, GC, which is identical for both glycophorin A and B, encodes the extracellular portions of the proteins, which are highly glycosylated. The next two exons, GE and GB, are specific only for glycophorin A and are absent from the glycophorin B coding sequence. The situation is reversed for exon GB-1, which is present in the glycophorin B coding sequence but absent from the glycophorin A sequence. These regions of the genes are followed by a homologous exon, GB-2, which indicates the transmembrane domain. Last is exon GA, encoding the cytoplasmic region of glycophorin A which is much shorter or absent in glycophorin B.

Comparison of the structures of the glycophorin A and B genes shows that they share some regions of exon homology but that each gene has at least one exon that is specific to itself. It can also be seen that the differences in the homology of the exon structures are orientationally mixed; for example, exons GE and GB, which are A-specific, are situated between exons GC and GB-2, which are homologous in the glycophorin A and B genes. The reverse situation is also indicated, as the homologous exon GB-2 is situated between two A-specific exons, GB and GA. A similar mixed exon/intron organization is also observed for the glycophorin B gene; i.e., exon GB-1 is between the two glycophorin A/B homologous exons GC and GB-2.

The complex organization of the glycophorin A and B genes argues against a simple two-step evolution by duplication of one of the genes followed by its divergence and suggests a more complex type of gene evolution.

A more complex evolution is also suggested by the finding that the exons specific to each gene show very little if any nucleotide homology. One possibility is that these genes originated by the duplication of a precursor gene that contained all or most of the current exons. This might have been followed by the heterogeneous loss of particular exons from one or both genes. Further studies are necessary to elucidate the evolution of the glycophorin gene family.

We thank Dr. Juha Vittala for useful discussions and Dr. Richard Maki for critical reading of the manuscript. We thank Candy Farmer for secretarial assistance. This work was supported by Grant R01 CA33000, awarded by the National Cancer Institute.

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