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Physical linkage of the genes for platelet membrane glycoproteins IIb and IIIa

(subchromosomal localization/pulsed-field electrophoresis/fibrinogen receptor/integrins)

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ABSTRACT The fibrinogen receptor on human platelets is a prototypic member of the integrin family and is composed of subunit glycoproteins IIb (gpIIb) and IIIa (gpIIIa) in a 1:1 stoichiometric ratio. We have isolated cDNA clones for gpIIb and gpIIIa and localized both genes to chromosome 17. In the current study, several approaches were used to localize and map the genes for gpIIb and gpIIIa. A preliminary evaluation of subchromosomal localization was performed by using a panel of mouse-human somatic cell hybrids that contain different amounts of the long arm of human chromosome 17. Southern hybridization to the DNA of these hybrids shows that both genes map near the thymidine kinase gene. *In situ* hybridization to intact human chromosomes localized both genes to the 17q21-22 region. To better define the physical distance between the two genes, we examined the genomic hybridization pattern of each cDNA probe to high molecular weight restriction fragments separated by pulsed-field gel electrophoresis. Serial hybridizations of the same filter have allowed construction of long-range *Mlu* I and *Sfi* I restriction maps spanning more than 500 kilobases. Finally, nonoverlapping portions of the cDNAs for both gpIIb and gpIIIa were used to probe *Sfi* I digests of genomic DNA separated by field-inversion gels. This confirmed that the genes are physically linked within the same 260-kilobase *Sfi* I fragment and suggests that the gene for gpIIb is located on the 3' side of the gene for gpIIIa. These results suggest that coordinate expression of gpIIb and gpIIIa may depend on physical proximity.

Regulated expression of heteropolymeric proteins requires a mechanism for coordinating the synthesis of individual subunits. Subunit genes that have evolved from duplication of a common ancestor may contain conserved *cis*-acting control elements that respond to alterations in a single diffusible factor (1–4). Coordinate expression of nonhomologous subunits must be achieved by a different mechanism.

The platelet fibrinogen receptor is a heterodimer of glycoproteins IIb (gpIIb) and IIIa (gpIIIa) (5, 6) that correspond to the α and β subunits in the integrin superfamily of membrane-adhesion receptors (7). *In vitro* translation of RNA from human erythroleukemia (HEL) cells (8) and isolation of individual cDNA clones for gpIIb and gpIIIa (9, 10) have shown that gpIIb and gpIIIa are transcribed from separate mRNAs. Analysis of these cDNA clones illustrates a general rule for integrins—although there is a high degree of sequence conservation between different α subunits and different β subunits, there is little or no similarity between the α and β groups. Nonetheless, the 1:1 stoichiometry of gpIIb and gpIIIa in normal platelets (5, 6) and the similar reduction in concentration of both subunits in the hereditary bleeding

disorder Glanzmann thrombasthenia (11–15) imply that there are cellular mechanisms for coordinate expression of gpIIb and gpIIIa. Northern analysis of RNA gel blots reveals similar levels of gpIIb and gpIIIa mRNA in HEL cells (16), suggesting possible coordinate expression at the level of transcription.

Few of the genes for integrin subunits have been mapped, and none have been found to be linked (18, 31). During previous studies of gpIIb and gpIIIa gene structure, we discovered that both genes were located on human chromosome 17 (16, 19). By using a combination of physical and somatic cell genetic techniques, we now report the subchromosomal localization and orientation of the gpIIb and gpIIIa genes. Both are contained on a 260-kilobase segment that maps to the 17q21-22 region. This close physical linkage is unlikely to be coincidental and suggests that regulated expression may be based on physical proximity.

MATERIALS AND METHODS

cDNA Probes. A partial-length gpIIb cDNA clone (16) was used to obtain a 3.3-kb full-length clone for gpIIb. This was sequenced and is identical to that described by Poncz *et al.* (9). The gpIIIa cDNA is 3.3 kb long and contains the entire coding region except the 5' most 30 base pairs (19). Both gpIIb and gpIIIa cDNA clones were obtained from HEL-cell cDNA libraries and were subcloned into pBS (Stratagene Cloning Systems) and pIBI31 (International Biotechnologies), respectively. Purified cDNA inserts were prepared by standard techniques (20). Probes used include the 1.3-kb gpIIb fragment on the 5' side of the internal *Acc* I site, the 2.0-kb gpIIb fragment on the 3' side of the internal *Acc* I site, the 2.2-kb gpIIIa fragment on the 5' side of the internal *Eco*RI site, and the 1.5-kb gpIIIa fragment on the 3' side of the internal *Eco*RI site.

Human-Mouse Hybrid DNA. The parent hybrid line WL contains the long arm of human chromosome 17 fused to a mouse chromosome. This line and the daughter lines 12B, 12A, 12D, and 12G have been characterized (21).

Southern Blotting and Hybridization. Genomic DNA was digested with restriction endonucleases, separated by electrophoresis through 0.8% agarose gels, and transferred to a nylon membrane by standard methods (16). Hybridizations were performed in a solution of 2 \times SSC (300 mM NaCl/30 mM sodium citrate, pH 7.0), 1% NaDodSO₄, salmon sperm DNA at 100 μ g/ml, and 10% (wt/vol) dextran sulfate with ³²P-labeled probe at 2 \times 10⁶ cpm/ml at 65°C. Filters were washed in 2 \times SSC/1% NaDodSO₄ at 65°C for 60 min with

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Abbreviations: gpIIb, glycoprotein IIb; gpIIIa, glycoprotein IIIa; HEL, human erythroleukemia.

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two changes. Filters to be rehybridized were "stripped" with $0.1 \times \text{SSC}/1\% \text{NaDodSO}_4/50\%$ (vol/vol) formamide at 65°C for 30 min and exposed to film for 4 days to ensure complete removal of the first probe.

In Situ Hybridization. Plasmids containing 2.2 kb of gpIIIa cDNA and 3.3 kb of gpIIb cDNA were radiolabeled by nick-translation (22) with $[^3\text{H}]\text{dCTP}$ plus $[^3\text{H}]\text{dTTP}$ to specific activities of $\approx 3.7 \times 10^7$ and $\approx 3.0 \times 10^7$ dpm/ μg of DNA, respectively. *In situ* hybridization to human metaphase chromosomes was performed as described (22, 23).

Pulsed-Field Gel Electrophoresis. DNA from HEL cells was prepared in agarose plugs at a concentration of 10^7 cells per ml (24). A $50\text{-}\mu\text{l}$ plug was incubated with 20 units of the indicated restriction enzyme in a total volume of $100\ \mu\text{l}$ and the reaction was terminated after 8 hr by the addition of 1 ml of $50\ \text{mM Tris}\cdot\text{HCl}$, pH 8.0/ $50\ \text{mM EDTA}$. Electrophoresis was performed at 13°C at $330\ \text{V}$ ($10\ \text{V}/\text{cm}$) in $0.5 \times \text{TBE}$ buffer ($45\ \text{mM Tris base}/45\ \text{mM boric acid}/1.25\ \text{mM EDTA}$, pH 8.3) by using a commercial apparatus (LKB Pulsaphor). Total electrophoresis time was 40 hr with a switching interval of 100 sec.

Field-Inversion Gel Electrophoresis. DNA from HEL cells was prepared as above and digested with *Sfi* I. Electrophoresis was as described (25) with the following modifications. Field strength was $240\ \text{V}$ ($7\ \text{V}/\text{cm}$) in $0.5 \times \text{TBE}$ buffer containing ethidium bromide at $0.5\ \mu\text{g}/\text{ml}$, with recirculation of buffer through a heat exchanger to maintain the gel temperature at 13°C . The electric field was periodically inverted by computer-controlled relays according to a program that increased the forward interval exponentially from 10 to 60 sec during a 4-hr cycle, such that 40% through the cycle, half of the increase in forward switching time had been achieved. The reverse interval was maintained at one-third of the forward interval, and the field was interrupted between each inversion by a period equal to 2% of the forward interval. The total electrophoresis time was 16 hr.

RESULTS

Somatic Cell Hybrid Analysis. A preliminary evaluation of subchromosomal localization was performed by using a panel of mouse-human somatic cell hybrids that contain different amounts of the long arm of human chromosome 17 (21). WL, the parent hybrid line, contains all of human chromosome 17q; lines 12B and 12A carry the human thymidine kinase, galactokinase, and procollagen $\alpha 1(\text{I})$ genes; 12D carries the thymidine kinase and galactokinase genes; and 12G carries only the thymidine kinase gene (Fig. 1A). All three genes have been mapped centromere-proximal to 17q22 (21).

DNA from each cell line was digested with *Eco*RI, fractionated on a 0.7% agarose gel, and transferred to a nylon filter. Hybridization with a gpIIIa cDNA probe revealed 11.0-kb, 7.8-kb, and 3.8-kb fragments in all the cell lines (Fig. 1B, lanes 1-5). Comparison with the pattern generated by total mouse and human genomic DNA (Fig. 1B, lanes 6 and 7) showed that the 11.0-kb and 7.8-kb fragments were of mouse origin and the 3.8-kb fragment was of human origin. The same blot, washed and then hybridized with a gpIIb cDNA probe, detected a prominent 4.5-kb fragment in all cell lines as well as a less intense 6.4-kb fragment; comparison with the control lanes showed the 6.4-kb fragment originated from the mouse and the 4.5-kb fragment was from the human gene (Fig. 1C). These results demonstrate that the gpIIIa and gpIIb genes are located in the same region of chromosome 17q21-22, centromere-proximal to the galactokinase and procollagen $\alpha 1(\text{I})$ genes.

In Situ Hybridization of Human Chromosomes. To exclude the possibility that cosegregation of the gpIIIa and gpIIb genes in the hybrid cell lines might be due to retention of a small, unrecognized fragment of human DNA, we performed

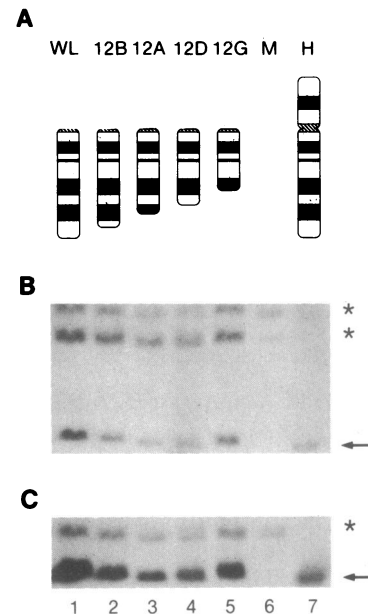


FIG. 1. Analysis of mouse-human hybrid chromosomes. (A) Karyograms depicting the amount of human chromosome 17 in each cell line and controls. Total mouse (M) and human (H) genomic DNA are indicated. (B) Southern blot analysis with the 5' 2.2-kb gpIIIa cDNA probe. (C) Southern blot analysis with a full-length 3.3-kb gpIIb cDNA probe. The filter used in B was stripped and then rehybridized. An * indicates mouse bands, and an \leftarrow indicates human bands. Lanes: 1, WL; 2, 12B; 3, 12A; 4, 12D; 5, 12G; 6, M; 7, H.

direct *in situ* hybridization to intact human chromosomes. By using a gpIIIa cDNA probe, 203 metaphase cells were counted and scored. Seventeen percent had silver grains on the long arm of chromosome 17 (Fig. 2A). Although the subchromosomal distribution was more diffuse than with the gpIIb probe, the majority of grains were found over 17q21.3-q22. The results of a similar experiment performed with a cDNA probe for gpIIb are shown in Fig. 2B. Of 114 metaphase cells counted and scored, 29% had grains on chromosome 17q with the highest density over 17q21.3. These results confirm the somatic cell hybrid mapping data and identify locations for the gpIIIa and gpIIb genes that are virtually indistinguishable.

Pulsed-Field Gel Electrophoresis. The limits of resolution by cytogenetic mapping are one to two orders of magnitude greater than the distances usually considered as significant in terms of physical linkage. To directly address this issue, we examined the genomic hybridization pattern of each cDNA probe to high molecular weight restriction fragments separated by orthogonal-field gel electrophoresis (Fig. 3). Careful alignment of autoradiograms produced from serial hybridizations of the same filter revealed that the gpIIIa (Fig. 3A) and gpIIb (Fig. 3B) probes detected several identical fragments produced by *Mlu* I (470 kb and 530 kb), *Nar* I (280 kb), and *Sfi* I (260 kb). The *Sfi* I digestion contained the smallest overlapping fragment, placing a maximum distance of 260 kb between coding sequences for the gpIIIa and gpIIb genes.

Field-Inversion Gel Electrophoresis and Construction of a Long-Range Restriction Map. Apparent identity of the fragments detected by each probe was confirmed by performing the experiment several times under different fractionation conditions and with DNA from different sources (foreskin fibroblasts, HeLa cells, and HEL cells). Both probes hybridized to multiple fragments in the 100- to 1000-kb range, detecting apparently identical high molecular weight fragments produced by the enzymes *Bss*HII, *Mlu* I, and *Sfi* I (data not shown). Field-inversion gel electrophoresis allowed the best resolution of the HEL cell DNA fragments resulting

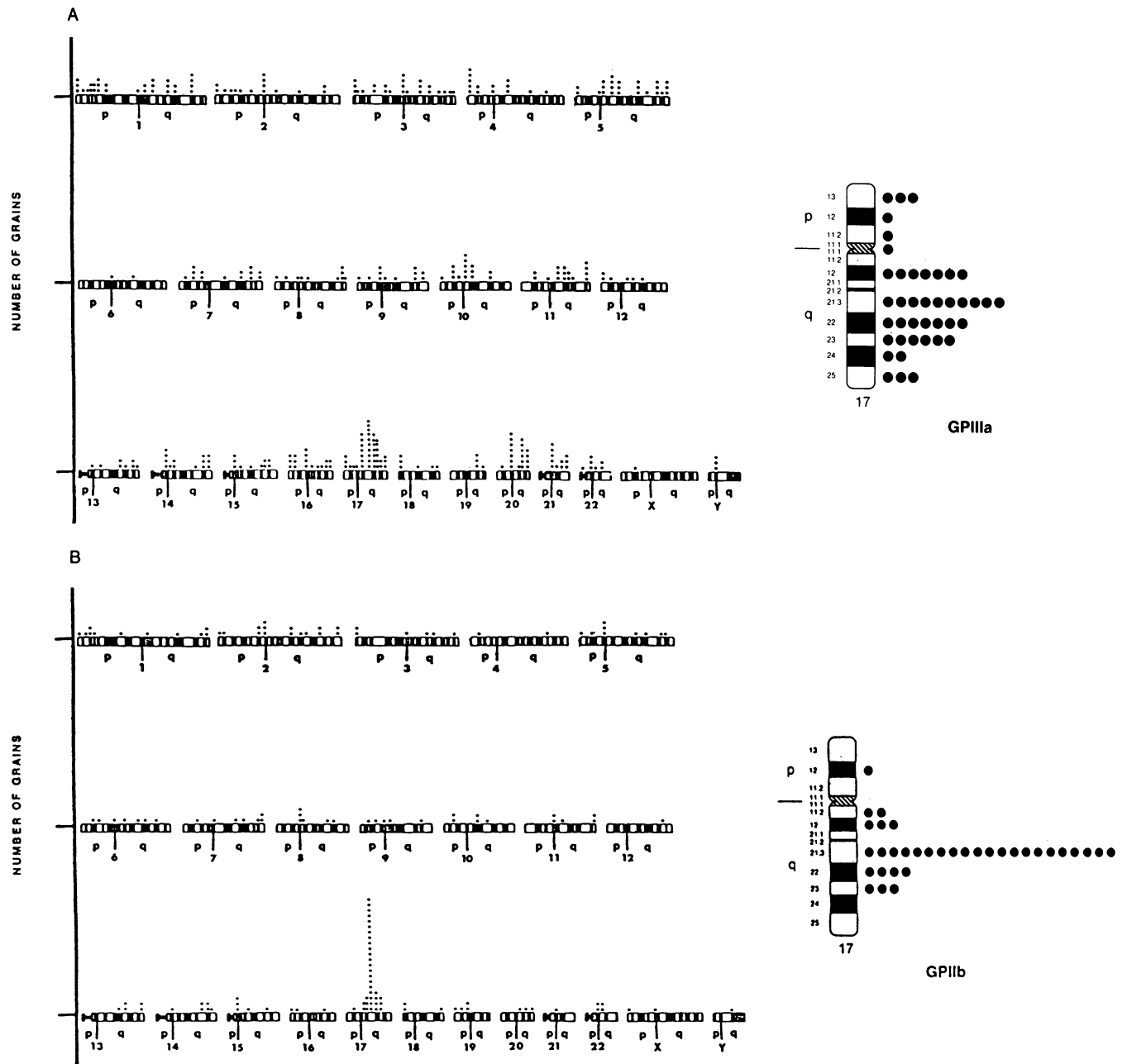


FIG. 2. Grain distributions resulting from *in situ* hybridization of chromosomes from a normal human male. Each dot represents a grain observed over a specific region. Note excess of grains over band 17q21.3. (A) Hybridization with gpIIIa-containing plasmid probe. (B) Hybridization with gpIIb-containing plasmid probe.

from *Sfi* I digestion. By using nonoverlapping portions of both gpIIIa and gpIIb cDNAs as probes, Southern analysis revealed a common 260-kb fragment (Fig. 4A, lanes 1–4). However, only the 5' gpIIIa probe hybridized to an additional 125-kb fragment (Fig. 4A, lane 1). These results confirm the overlapping *Sfi* I fragment seen in the previous experiment, suggest that the gene for gpIIb is oriented on the 3' side of the gene for gpIIIa, and permit construction of a long-range *Sfi* I map (Fig. 4C).

The largest fragments seen in Fig. 3 are likely to be partial cleavage products, perhaps due to a variable degree of secondary DNA modification (27), because additional experiments with portions of each cDNA as hybridization probes detect the same fragments as are detected by the full-length cDNAs (data not shown). Had complete digestion occurred, the shorter cDNA probes would have only detected subsets of these fragments. Comparison of the *Mlu* I pattern produced by each probe (Fig. 4B) allowed construction of a

long-range restriction map based on these partial digests (Fig. 4C). Probes for both cDNAs identified overlapping 470-kb and 530-kb *Mlu* I fragments. The difference in length of these fragments, 60 kb, corresponds to the difference in length between the two smaller fragments identified by gpIIIa (180 kb and 240 kb). The small fragments detected only by the gpIIb probe (290 kb, 320 kb, and 390 kb) must extend in the opposite direction.

DISCUSSION

Pulsed-field gel electrophoresis has been used to establish linkage relationships for several mammalian genes (28–30). Because such conclusions are based upon coincidental detection of seemingly identical restriction fragments with unrelated probes, the possibility that each probe produces similar hybridization patterns by chance must always be considered. Factors that suggest physical linkage of two

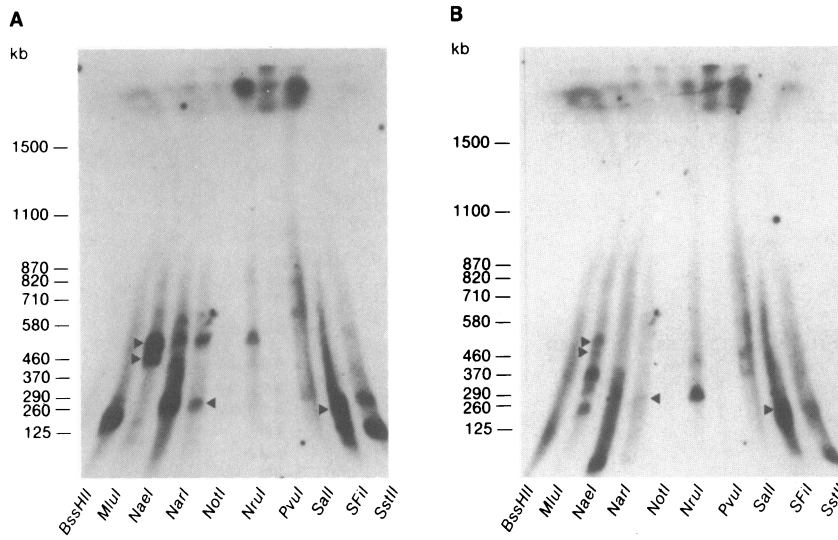


FIG. 3. Pulsed-field gel electrophoretic analysis of gpIIIa and gpIIb genes. DNA was digested with the restriction endonucleases indicated. (A) Hybridization with two pooled gpIIIa cDNA probes: a 2.2-kb probe on the 5' side of the internal *EcoRI* site plus a 1.5-kb probe on the 3' side of the internal *EcoRI* site (19). (B) Hybridization with the 3.3-kb gpIIb cDNA. Prior to the experiment, the filter used in A was stripped. Arrowheads indicate overlapping fragments. Size markers were estimated with chromosomes from the yeast strain HY-1 (26). Ethidium bromide stains of the agarose gels revealed inadequate DNA in the *Not I* and *Pvu I* digests (data not shown).

unrelated probes are the previous localization of the two probes to a small subchromosomal region, the presence of overlapping bands produced by more than one enzyme or enzyme combination, and similar banding patterns seen with partial enzyme digests. In cases where the data allow construction of a long-range restriction map, there should be no inconsistencies between maps generated from each probe. All of these criteria are met by the present study. In particular, our somatic cell mapping and *in situ* hybridization data narrow the total target size for gpIIIa and gpIIb genes to less than 1% of the haploid genome or approximately 30,000 kb, in which it is exceedingly unlikely that two probes would share several comigrating restriction fragments by chance. Although final confirmation of the long range *Sfi I* and *Mlu I* restriction map will depend on isolation of genomic DNA, the data presented here conclusively demonstrate physical linkage of gpIIIa and gpIIb coding sequences.

The analysis of the *Sfi I* digests with nonoverlapping portions of each cDNA suggests that the gene for gpIIb is on the 3' side of the gene for gpIIIa. An alternative interpretation of this data is that the additional 125-kb fragment identified by the 5' gpIIIa probe (Fig. 4A, lane 1) represents a homologous region of the genome not adjacent to the 260-kb *Sfi I* fragment. Since under these experimental conditions, the

gpIIIa probe has not hybridized to other homologous integrin β subunits (19), such an alternative explanation would require a highly conserved sequence in the 17q21-22 region.

Sosnoski *et al.* (31) colocalized the genes for gpIIb and gpIIIa to 17q21-23 region by using somatic cell hybrids and *in situ* hybridization. Our data confirm their results and narrow the distance between the two genes to a maximum of 260 kb.

Other genes that have been previously mapped to this region of chromosome 17 include the *HOX2* homeobox gene cluster, the procollagen $\alpha 1(I)$ gene, the galactokinase gene, and the *ERBA* oncogene (32-35). A region of conserved synteny for all of these genes exists on mouse chromosome 11 (36), baboon chromosome 16 (37), and African green monkey chromosome 19 (37); and it would not be surprising if subunit genes for the fibrinogen receptor in these other species are also physically linked.

In the hereditary bleeding disorder Glanzmann thrombasthenia, both gpIIb and gpIIIa are absent or markedly reduced and platelets from affected patients fail to bind fibrinogen and aggregate (11-15). The Glanzmann phenotype is likely to be caused by a heterogeneous group of genetic defects (38). For example, a defect in the expression of one gene may result in the inability of the other subunit to be expressed in the platelet membrane. Close physical linkage between the genes

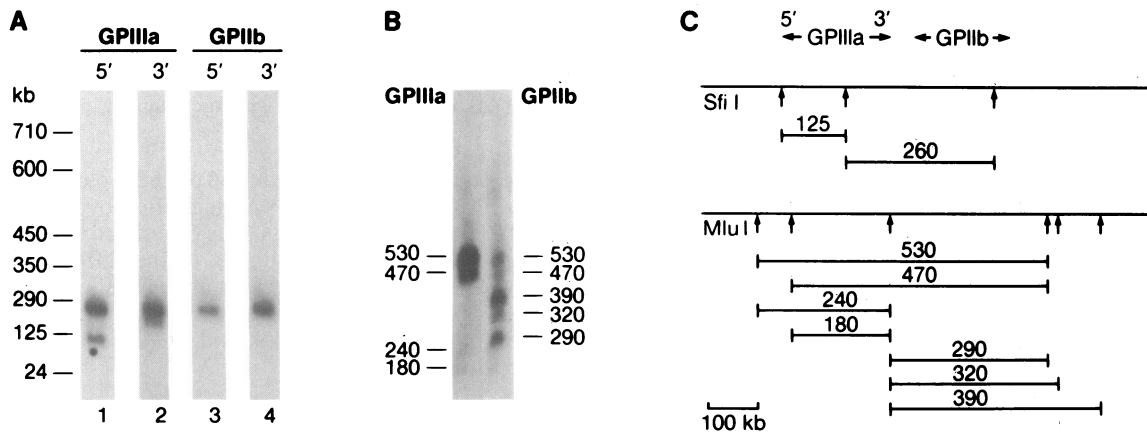


FIG. 4. Southern hybridization analysis of large fragment DNA and long-range restriction map. (A) Field-inversion gel analysis of gpIIIa and gpIIb genes. cDNA probes were as follows. Lanes: 1, a 2.2-kb gpIIIa cDNA on the 5' side of the internal *EcoRI* site; 2, a 1.5-kb gpIIIa cDNA on the 3' side of the internal *EcoRI* site; 3, a 1.3-kb gpIIb cDNA on the 5' side of the internal *Acc I* site; 4, a 2.0-kb gpIIb cDNA on the 3' side of the internal *Acc I* site. The same filter was hybridized with all four probes. Size markers are as described in Fig. 3. (B) The same *Mlu I* digests as in Fig. 3 A and B. (C) Long-range restriction map based on the *Sfi I* and *Mlu I* digests. The horizontal arrows indicate that the 5' and 3' extents of gpIIIa and gpIIb are not certain. The *Sfi I* map demonstrates orientation of gpIIIa with respect to gpIIb since only the 5' gpIIIa probe identified the 125-kb band. The *Mlu I* map is constructed from the partial *Mlu I* digests seen in B and, like the *Sfi I* map, is aligned below the indicated extents of gpIIIa and gpIIb.

for gpIIb and gpIIIa could be coincidental; but considering the coordinate expression of gpIIb and gpIIIa in both normal and Glanzmann platelets, it seems other explanations must be entertained. Any regulatory mechanism for expression that depends on physical proximity could serve as a selective pressure to establish and maintain this linkage. A *cis*-acting signal that mediates coordinate expression is an obvious candidate for such a selective pressure, perhaps in the form of a tissue-specific enhancer (4) or derepression of a large chromatin domain (39). However, although 260 kb is the maximum distance between the genes for gpIIIa and gpIIb, we do not know precisely how close they are and they may be too far apart to be controlled by a single *cis* element.

gpIIb and gpIIIa correspond to α and β subunits in the integrin superfamily (17) that includes the vitronectin and fibronectin receptors, the chicken integrin complex, the group of leukocyte-adhesion proteins lymphocyte function-associated antigen 1 (LFA-1), Mac-1, and p150,95, the very late antigens (VLA) group of proteins on lymphocytes, and the position-specific antigens found in *Drosophila* imaginal disks (for reviews, see refs. 7 and 38). Most integrin subfamilies combine different α subunits with a common β subunit (7), implying that cellular mechanisms exist for independent regulation of each subunit. Few of the integrin genes have been mapped (17, 18), and only gpIIIa and gpIIb have been found to be linked. It will be interesting to see whether the linkage of gpIIIa and gpIIb genes has been conserved in other vertebrates and whether other integrin subunit genes are physically linked.

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