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Leukocyte Adhesion Deficiency

ABERRANT SPLICING OF A CONSERVED INTEGRIN SEQUENCE CAUSES A MODERATE DEFICIENCY PHENOTYPE*

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Leukocyte adhesion deficiency (LAD) is a heritable deficiency of the LFA-1, Mac-1, p150,95 family of leukocyte $\alpha\beta$ heterodimers (the leukocyte integrins). We have studied the defect in patients who synthesize an aberrantly small form of the β subunit common to all three proteins. S_1 nuclease protection showed the presence of a 90-nucleotide mismatch in RNA from patients and relatives, correlating with inheritance of the disease. Use of the *Taq* polymerase chain reaction to amplify this region of RNA after first strand cDNA synthesis and sequencing showed an in-frame deletion of 90 nucleotides in the extracellular domain. Thus, this highly conserved region, 63% and 53% identical in amino acid sequence to two other β subunits of the integrin family, is required for association of the β subunit with α subunits. The 90-nucleotide region corresponds to a single exon present in both the normal and patient genome. The patient DNA has a single G to C substitution in the 5′ splice site. This results in the direct joining of nonconsecutive exons in an unusual type of abnormal RNA splicing. A small amount of normally spliced message, detected by S_1 nuclease protection and *Taq* polymerase chain reaction, encodes a normal sized β subunit which is surface-expressed and accounts for the low levels of leukocyte integrin expression observed in these patients, and hence the moderate phenotype.

Leukocyte adhesion deficiency (LAD)[1] is a heritable, often fatal disease which is characterized by severe, recurrent bacterial and fungal infections of the skin, mucous membranes, and intestines (reviewed in Anderson and Springer, 1987; Todd and Freyer, 1988; Fischer *et al.*, 1988; Anderson *et al.*, 1988; Kishimoto and Springer, 1988). Leukocytes fail to mobilize to sites of infection; necrotic and indolent lesions are largely devoid of leukocytes, despite the observation that these patients have chronic leukocytosis. Lymphocytes, monocytes, and granulocytes from these patients show a wide spectrum of defects in adhesion-related immune function, such as antigen presentation, cell-mediated cytolysis, and cell migration. LAD is due to deficient expression of the LFA-1, Mac-1, and the p150,95 family of leukocyte adhesion glycoproteins.

LFA-1, Mac-1, and p150,95 are members of the integrin superfamily of adhesion proteins, which includes the fibronectin receptor and the platelet IIb-IIIa glycoprotein (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Kishimoto *et al.*, 1987a). All the members of this superfamily are $\alpha_1\beta_1$ heterodimers. There are three integrin subfamilies, each defined by a common β subunit, that share multiple distinct α subunits. Thus, the leukocyte integrin β subunit (95,000 daltons; also called CD18) is shared by the α subunits of LFA-1, Mac-1, and p150,95 (180,000, 170,000 and 150,000 daltons, respectively; also called CD11a, -b, and -c, respectively) (Sanchez-Madrid *et al.*, 1983). The leukocyte integrin β subunit (Kishimoto *et al.*, 1987b; Law *et al.*, 1987) shares 45% and 37% overall amino acid identity with the β subunits of the chicken fibronectin receptor (Tamkun *et al.*, 1986) and platelet *gp* IIb-IIIa (Fitzgerald *et al.*, 1987), respectively. The sequence is conserved along the entire length, with the highest conservation in the transmembrane domain and a stretch of 241 amino acids in the extracellular domain.

Recently, we have shown that heterogeneous mutations in the β subunit common to the leukocyte integrins cause LAD (Kishimoto *et al.*, 1987c). Five phenotypes of β subunit mRNA expression and protein precursor synthesis were observed. Heterogeneity has also been observed in the extent of the deficiency at the cell surface. LAD patients have been categorized as severely deficient (<1% normal levels of expression) or moderately deficient (3–10% of normal) (Anderson *et al.*, 1985). The severity of the clinical complications is reflected in the extent of the deficiency. Severely deficient patients usually due early in childhood from overwhelming microbial infections, unless they receive bone marrow transplants (reviewed in Fisher *et al.*, 1988). Moderately deficient patients suffer from severe, recurrent infections, but can survive to adulthood with medical care. The molecular basis for this heterogeneity is unclear. In patients with an apparent defect in mRNA transcription, two severely deficient patients had no detectable β subunit mRNA expression or protein precursor synthesis, while one moderately deficient patient had low levels of mRNA expression and precursor synthesis (Kishimoto *et al.*, 1987c). Thus, quantitative differences in RNA synthesis may cause the severe and moderate phenotypes. However, the majority of both moderate and severe deficiency LAD patients analyzed have normal levels of β subunit precursor synthesis (Kishimoto *et al.*, 1987c; Dana *et al.*, 1987; Dimanche *et al.*, 1987).

In this paper, we have examined the genetic basis for the defect in four related patients which results in the synthesis of an aberrantly small β subunit precursor (Kishimoto *et al.*,

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[1] The abbreviations used are: LAD, leukocyte adhesion deficiency; PHA, phytohemagglutinin A; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pairs; bp, base pairs; nt, nucleotides; PCR, polymerase chain reaction.

1987c) and have examined why it results in the moderate phenotype. We show that an unusual RNA splicing defect results in an in-frame deletion of 30 amino acids from the extracellular region that is highly conserved among other integrin β subunits. A small amount of normally spliced message accounts for the low levels of LFA-1 surface expression, and hence the moderate deficiency phenotype.

EXPERIMENTAL PROCEDURES

Patients and Kindred

Four related LAD patients, with a moderate deficiency form of the disease, and their kindred were studied (Anderson *et al.*, 1985). One of these patients, patient 6, is now 40 years old, and the only LAD patient known to father children. Two of his children have the LAD disease (patients 7 and 8), while the other two have been shown to be heterozygous carriers of the defect (Anderson *et al.*, 1985; Kishimoto *et al.*, 1987c). The parents are consanguinous and related to patient 4, who is from the same isolated rural area. See Kishimoto *et al.*, 1987c, for the family tree. The clinical histories of these patients have been described (Anderson *et al.*, 1985). Epstein-Barr virus-transformed B cell lines and phytohemagglutinin A (PHA)-stimulated T lymphoblasts were established from healthy controls, LAD patients, and kindred and maintained as described previously (Anderson *et al.*, 1985; Kishimoto *et al.*, 1987c).

Cell Labeling

Cell Surface Iodination—PHA blasts in log phase of growth were washed three times in phosphate-buffered saline and resuspended at 4×10^6/ml. 2.5 mCi of ^{125}I was added to 1 ml of cells, and the entire mixture was then added to IODO-GEN (Pierce)-coated glass vials for 10 min with intermittent agitation. The cells were washed twice in Hanks' balanced salt solution and once in Hanks' balanced salt solution containing 10% FCS. The cell pellets were lysed in 0.5% Triton X-100, 0.5% Nonidet P-40, 50 mM Tris (pH 8.0), 0.15 M NaCl, and 1 mM freshly added phenylmethylsulfonyl fluoride (lysis buffer) for 20 min at 4 °C. Nuclei were pelleted at 12,000 × *g* for 15 min at 4 °C. All cell samples were labeled to similar levels, as judged by trichloroacetic acid-precipitable counts.

Biosynthetic Labeling—2×10^7 PHA blasts were pulse-labeled at 5 × 10^6 cells/ml for 2 h at 37 °C with 250 μCi of [^{35}S]methionine and 250 μCi of [^{35}S]cysteine as described previously (Kishimoto *et al.*, 1987c). Cell samples were labeled to similar levels, as judged by trichloroacetic acid-precipitable counts.

Immunoprecipitation

Cell lysates were incubated with 5 μl of normal rabbit serum for 2 h at 4 °C and then extensively precleared with a protein A-Sepharose slurry (1:1 in 50 mM Tris (pH 8.0), 0.15 M NaCl, and 0.025% azide (TSA)).

LFA-1 was immunoprecipitated from equal amounts (3.6×10^6 trichloroacetic acid-precipitable counts) of lysate from ^{125}I-surface-labeled control and LAD patient cells with 15 μl of anti-β subunit specific (TS1/18) monoclonal antibody (mAb) coupled to Sepharose. In some cases, as indicated, a 20-fold excess of lysate (7.2×10^7 counts) was utilized. Lysates were diluted with lysis buffer so that the precipitation volume was the same in all cases. Control precipitations were performed with 15 μl of protein A-Sepharose slurry. Lysates were incubated for 3 h at 4 °C with gentle rotation. The immunoprecipitates were washed three times with lysis buffer, two times with TSA, and then heated to 100 °C for 10 min in 40 μl of 0.5% SDS.

The β subunit precursor was immunoprecipitated from biosynthetically labeled cells with 1 μl of rabbit anti-β subunit serum and 15 μl of protein A-Sepharose as described previously (Kishimoto *et al.*, 1987c), except that lysates were not denatured in SDS.

N-Glycanase

To one-half of an immunoprecipitate (20 μl in 0.5% SDS), 1 μl of 2 M β-mercaptoethanol was added and then heated to 100 °C for 10 min. The sample was diluted to 50 μl in a buffer containing a final concentration of 5 units/ml of *N*-glycanase (Genzyme), 50 mM Tris (pH 8.8), 1 mM 1,10-phenanthroline, and 3% Triton X-100, and then incubated at 37 °C for 16 h. The reaction was precipitated in 250 μl of cold acetone at −20 °C for 16 h, using 20 μg of tRNA as carrier.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Immunoprecipitates were subjected to SDS-8% PAGE under reducing conditions, as described previously (Kishimoto *et al.*, 1987c). The gel was divided into two, and the portion containing ^{125}I-labeled samples was exposed to preflashed XAR film (Kodak) with intensifying screens, and the portion containing ^{35}S-labeled samples was subjected to fluorography.

RNA

Total RNA was isolated from Epstein-Barr virus-transformed cell lines by standard methods using guanidinium isothiocyanate (Chirgwin *et al.*, 1979) as previously described (Kishimoto *et al.*, 1987, a and c). Poly(A)$^+$-RNA was selected on oligo(dT)-cellulose columns, as described (Aviv and Leder, 1972).

S1 Nuclease Protection

The S1 nuclease protection assay was performed basically as described (Davis *et al.*, 1986). Restriction enzyme fragments of the β subunit cDNA were subcloned into the M13 vector in the antisense orientation. The identity and orientation of each fragment was confirmed by DNA sequencing (not shown). Antisense single strand probes were uniformly labeled with [^{32}P]dATP by Klenow enzyme extension of the universal M13 primer. The M13 vector was cleaved in the universal polylinker by restriction enzyme digest to release the cDNA insert. The probe was heat-denatured and gel-purified on a standard DNA sequencing gel (5% acrylamide, 0.25% bisacrylamide, 8% urea in TBE). The probe was visualized by short autoradiography, then excised, and eluted in 500 mM ammonium acetate, 10 mM Mg^{2+} acetate, 1 mM EDTA, and 0.1% SDS for 2 h at 37 °C.

50,000 cpm of the probe was added to 30 μg of total RNA or tRNA control. The mixture was precipitated in ethanol and resuspended in 20 μl of 80% formamide hybridization solution containing 20 mM Tris (pH 7.4), 0.4 M NaCl, and 1 mM EDTA. Hybridization was carried out for 16 h at 55 °C. Portions of the DNA probe which did not hybridize to RNA were cleaved with 200 units of S1 nuclease (Boehringer Mannheim) in 500 μl of 0.3 M NaCl, 1.67 mM $ZnSO_4$, and 30 mM Na^+ acetate (pH 4.5) for 1 h at 37 °C. The digests were extracted with phenol:chloroform and precipitated in ethanol. Samples were loaded on a standard DNA sequencing gel. An *Eco*RI and *Hin*fI digest of pBR322 was end-labeled with ^{32}P and used as markers. In some experiments, ^{35}S sequencing reactions were also used as markers. Gels were dried and exposed to preflashed XAR film with an intensifying screen.

Densitometry

The autoradiogram from Fig. 2C was scanned with a dedicated laser densitometer (Ultroscan XL, LKB). The lanes with samples from the patient were normalized against samples from the heterozygous relatives, with the assumption that the heterozygous relatives have half-normal levels of normally spliced message.

Oligonucleotides

Three oligonucleotides were synthesized against the deletion region: oligonucleotide 650, a sense strand 30-mer corresponding to positions 1102–1132 (5′-GAGCTGTCTGAGGACTCCAGCAATGT GGTC-3′); oligonucleotide 716, an antisense 17-mer, corresponding to positions 1154–1138 (5′-TTGTAAGCATTCTTAAT-3′); oligonucleotide 717, a sense strand 17-mer, corresponding to positions 1067–1084 (5′-AACTCACCGAGATCATC-3′).

Four oligonucleotides were directed against sequences outside of the deletion region. Oligonucleotide 747, a sense strand 24-mer corresponding to positions 958-981 (AGCAACGAATTCGACTACCC ATCG) and containing a natural *Eco*RI site (underlined); oligonucleotide 742, an antisense 24-mer corresponding to positions 1238–1215 (5′-CCATTGCTGCAGAAGGAGTCGTAG-3′) and containing a natural *Pst*I site (underlined); oligonucleotide 748, a sense strand 30-mer corresponding to positions 1026–1055 (5′-CATCTTCGCGGT-GACCAGTAGGATGGTGAA-3′); and oligonucleotide 740, an antisense 18-mer corresponding to positions 1213–1196 (5′-TGACTTT-CAGGGTGTCGG-3′).

Taq Polymerase Chain Reaction

The *taq* polymerase chain reaction protocol used to amplify genomic DNA sequences (Saiki *et al.*, 1988) was adapted to amplify RNA sequences. A standard first strand cDNA reaction was per-

formed with 2.5 μg of poly(A)$^+$ RNA from LAD patient 4. The reaction was primed with 500 ng of the antisense oligonucleotide 742, which contains a natural *Pst*I restriction site. First strand cDNA synthesis was performed in 100 μl of 50 mM Tris (pH 8.8), 50 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTPs, and 140 units of reverse transcriptase (Life Sciences) for 45 min at 42 °C. The *Taq* polymerase chain reaction was primed by the addition of 2.5 units of *Taq* polymerase (New England Biolabs), 1 μl of 2% gelatin, 500 ng of the sense strand oligonucleotide 747 corresponding to nucleotides 958–981, which contains a natural *Eco*RI site. One hundred μl of mineral oil was layered on top to prevent evaporation. The sample was amplified by 30 rounds of heat denaturation at 95 °C for 1 min, by oligonucleotide annealing at 50 °C for 2 min, and primer extension at 70 °C for 1.5 min. The amplified product was incubated a final 5 min at 70 °C, extracted with phenol:chloroform, and precipitated in ethanol.

In some experiments, the *Taq* polymerase chain reaction was primed with the sense strand oligonucleotide 717 corresponding to the putative deletion region, in order to select for normally spliced messages. In some cases, the *Taq* polymerase chain reaction product was reamplified as above, with omission of the first strand cDNA reaction.

Southern Analysis

One-tenth of the *Taq* polymerase chain reaction product was loaded onto 1.2% agarose gels with *Hae*III-digested φX174 DNA as markers. The DNA was transferred to nylon membrane in 0.4 M NaOH as previously described (Reed and Mann, 1985). Oligonucleotides were end-labeled with [γ-^{32}P]ATP using polynucleotide kinase. Filters were prehybridized in 6 × SSC, 0.05% pyrophosphate, and 20 μg/ml tRNA for 2 h at 37 °C and then hybridized with labeled oligonucleotide for 16 h at 36 °C. Filters were washed twice at 22 °C for 15 min in 6 × SSC and 0.05% pyrophosphate, and twice in the same solution at 68 °C or 48 °C for 30-mer and 17-mer oligonucleotides, respectively.

DNA Sequencing

Taq polymerase chain reaction reactions were cleaved with *Eco*RI and *Pst*I and cloned into the M13 vector. Inserts from the λgt10 genomic library were subcloned into the *Eco*RI site of M13. *Taq* polymerase chain reaction and genomic inserts were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the universal M13 primer and a specific oligonucleotide 717, respectively.

Partial Genomic Library Construction

High molecular weight DNA from patient 4 was isolated by standard methods (Davis *et al.*, 1986) and digested to completion with an excess of *Eco*RI. The digested DNA (120 μg) was loaded into a preparative well of a 1% agarose gel, with *Bst*NI-digested pBR322 as markers. Four broad strips of DNA, corresponding roughly to 1.1–1.3 kb, 1.3–1.5 kb, 1.5–1.7 kb, and 1.7–1.9 kb, were excised and electroeluted. Aliquots of the eluted DNA were run on a 1% agarose gel and transferred to nylon membranes. Filters were hybridized with a 30-mer oligonucleotide derived from the putative deletion region. A band of approximately 1.7 kb was identified in the 1.5–1.7- and 1.7–1.9-kb fractions. The two fractions were pooled and cloned into the *Eco*RI site of λgt10 (Promega Biotech). The library was packed *in vitro* (Stratagene), and approximately 500,000 recombinants were plated with C600 hfl host cells.

Screening of Genomic Libraries

Duplicate filters were prehybridized and then hybridized with the nick-translated 1.7-kb *Eco*RI fragment of the normal β subunit genomic clone[2] for 16 h at 68 °C in 6 × SSC, 0.5% nonfat dry milk, 1% SDS, 10 mM EDTA, and 100 μg/ml denatured salmon sperm DNA. The filters were washed twice for 15 min at 22 °C in 2 × SSC, 0.5% SDS, twice for 20 min at 68 °C in 0.5 × SSC, and then exposed to XAR film with an intensifying screen. Fourteen positives were picked and plaque-purified. Positives were rescreened with the oligonucleotide 650 against the putative deletion region, as described for Southern analysis.

[2] X. Hollander and T. A. Springer, unpublished observations.

RESULTS

Definition of the Defect at the Protein Level—Patients 4, 6, 7, and 8 are members of an extended family with several consanguinous marriages from an isolated rural region (Kishimoto *et al.*, 1987c). We have previously shown that these patients synthesize an aberrantly small β subunit precursor which is degraded. Removal of the *N*-linked oligosaccharides shows that the defect is in the protein backbone rather than in glycosylation (Kishimoto *et al.*, 1987c). To examine the small amount of LFA-1 that is expressed on the cell surface of lymphoblasts from these moderately deficient patients, LFA-1 was immunoprecipitated from ^{125}I-surface-labeled T cell blasts from patient 6 and a healthy control. As expected, the patient expressed barely detectable levels of LFA-1 (Fig. 1*A*, *lane 2*). However, when 20 times more LAD patient cell lysate was used, the β subunit from the LAD patient was detectable and shown to be of normal size (Fig. 1*A*, compare *lane 3* with *lanes 1* and *4*). *N*-Glycanase digestion of the *N*-linked carbohydrates showed that the protein backbone was also of normal size (Fig. 1*A*, compare *lane 7* with *lanes 5* and *8*). In contrast, [^{35}S]methionine labeling of the patient β subunit precursor showed that the predominant species is about 5000 daltons smaller than normal, both for the intact precursor (Fig. 1*B*, compare *lanes 2* and *1*) and after deglycosylation (Fig. 1*B*, compare *lanes 4* and *3*), as previously described (Kishimoto *et al.*, 1987c). These results suggest that most of the β subunit is synthesized as an aberrantly small precursor that is degraded; however, the small amount of LFA-1 which reaches the cell surface contains a grossly normal β subunit.

Definition of the Defect at the mRNA Level—A deletion in the protein backbone should be reflected in the mRNA message. To test this hypothesis, total RNA from patient 4 and

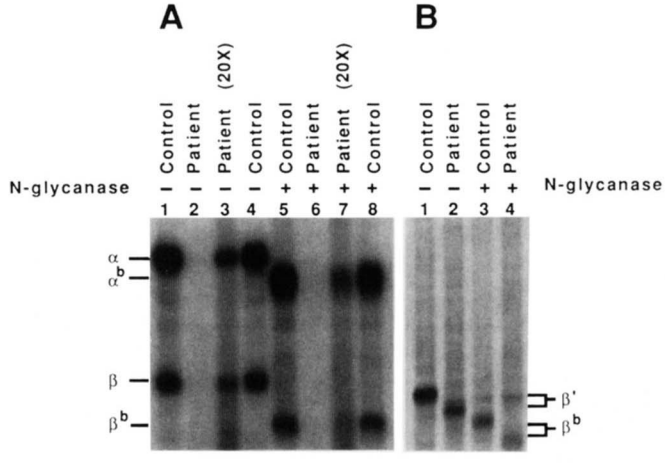

FIG. 1. **Analysis of the mature and precursor forms of the β subunit polypeptide.** *A*, the small amount of cell surface LFA-1 from LAD patient 6 contains a grossly normal β subunit. PHA blasts from a healthy control (*lanes 1*, *4*, *5*, and *8*) and LAD patients (*lanes 2*, *3*, *6*, and *8*) were surface-labeled with ^{125}I. LFA-1 was immunoprecipitated with LFA-1 mAb coupled to Sepharose. In some cases, a 20-fold excess of lysate was used (*lanes 3* and *7*). One-half of each sample was digested with *N*-glycanase (*lanes 5–8*). *B*, the predominant β subunit precursor from LAD patients is aberrantly small. PHA blasts from a healthy control (*lanes 1* and *3*) and LAD patient 6 (*lanes 2* and *4*) were pulse-labeled for 2 h with [^{35}S]methionine and [^{35}S]cysteine. The β subunit precursor was immunoprecipitated with rabbit anti-β subunit serum and protein A-Sepharose. One-half of each sample was digested with *N*-glycanase (*lanes 3* and *4*). Samples from A and B were subjected to SDS-8% PAGE on a single gel. The gel was divided and subjected to autoradiography with intensifying screen (*A*) or fluorography (*B*). β and β′, mature and precursor β subunit, β^b, β polypeptide backbone.

three other unrelated patients, 1, 2, and 14, were analyzed by S1 nuclease protection. Four fragments of the β subunit cDNA, which span 94.5% of the coding region, were subcloned into M13 to allow synthesis of uniformly labeled single strand antisense probes (Fig. 2*A*). Hybridization with total RNA followed by S1 nuclease digestion revealed only one gross mismatch. Probe 3 (nucleotides 965–1728; numbered as in Kishimoto *et al.*, 1987b) protected a 763-nt fragment in normal RNA (Fig. 2*B*, *lane 2*), but protected aberrant fragments of approximately 597 nt and 76 nt with RNA from patient 4 (Fig. 2*B*, *lane 4*), suggesting a deletion of approximately 90 nt. No mismatches were detected with the other three probes in RNA samples from any of the LAD patients (Fig. 2*B*, *lanes 10–13* and data not shown).

To further map the mismatch and to follow its inheritance, probe 5 (nucleotides 965–1232) was hybridized to RNA from patient 6 and members of his immediately family. S1 nuclease protection of RNA from the only normal sibling (normal surface expression of LFA-1) showed the expected 267-nt protected fragment (Fig. 2*C*, *lanes 4* and *10*). Analysis of the patient gave bands of 101 nt and 76 nt (Fig. 2*C*, *lanes 8* and *11*). Analysis of the three heterozygous siblings (half-normal expression of LFA-1) and the heterozygous parents showed the normal and two aberrant fragments (Fig. 2*B*, *lanes 3*, *5*, *6*, *7*, and *9*). These results confirmed the 90-nt deletion and allowed us to map the deletion to nucleotides 1066 to 1156 in the normal cDNA. In addition to the aberrant fragments, a small amount of the normal 267-nt protected fragment was reproducibly observed ($N = 4$) in RNA from these patients (Fig. 2*C*, *lanes 8* and *11*). Scanning the autoradiogram by densitometry shows 3% of control levels, using the ratio of aberrant to normal fragments found for heterozygous relatives to normalize for the amount of loaded RNA. A small amount of undigested probe, probably due to incomplete resuspension of the precipitated probe, was detectable in some lanes. However, the amount of undigested probe present was variable and did not correlate with the small amount of normal protected fragment observed in patient 6 (Fig. 2*C*, compare *lanes 8* and *11*). Furthermore, the normal protected fragment observed was not due to spillover from adjacent lanes (Fig. 2*C*, *lane 11*). These results suggested that a small amount of normal message may account for the normal-size β subunit polypeptide associated with the low level of surface LFA-1 expression.

We adapted the polymerase chain reaction with the thermostable *Taq* polymerase to further study the β subunit mRNA message from these patients. The antisense oligonucleotide 742 (Fig. 3*A*), which spans the *Pst*I site at position 1232, was used to prime a standard first strand cDNA reaction using reverse transcriptase. The RNA:DNA heteroduplex was denatured at 95 °C, and the polymerase chain reaction was driven by the addition of *Taq* polymerase and the sense strand oligonucleotide 747 spanning the *Eco*RI site at position 965 (*PCR*-1, Fig. 3*A*). After 30 rounds of amplification, a single band of 190 bp was detected by ethidium bromide staining (Fig. 3*B*) and Southern analysis with oligonucleotide 748 (Fig. 3*C*). The size of the *Taq* polymerase chain reaction product was 90 bp smaller than expected for the wild type message. Sequence analysis of eight M13 clones showed the presence of an in-frame 90-nt deletion, as suspected, without insertion of any extraneous sequence (Fig. 3*D*). The presence of a normal β subunit product of 280 bp could not be detected by Southern analysis of the *Taq* polymerase chain reaction product or screening of 26 independent M13 plaques using oligonucleotide probe 650 directed against the deletion region sequence (data not shown). However, this might have been due to selection of the more abundant deletion message during first strand cDNA synthesis or the subsequent 30 rounds of *Taq* polymerase chain reaction amplification; slightly greater

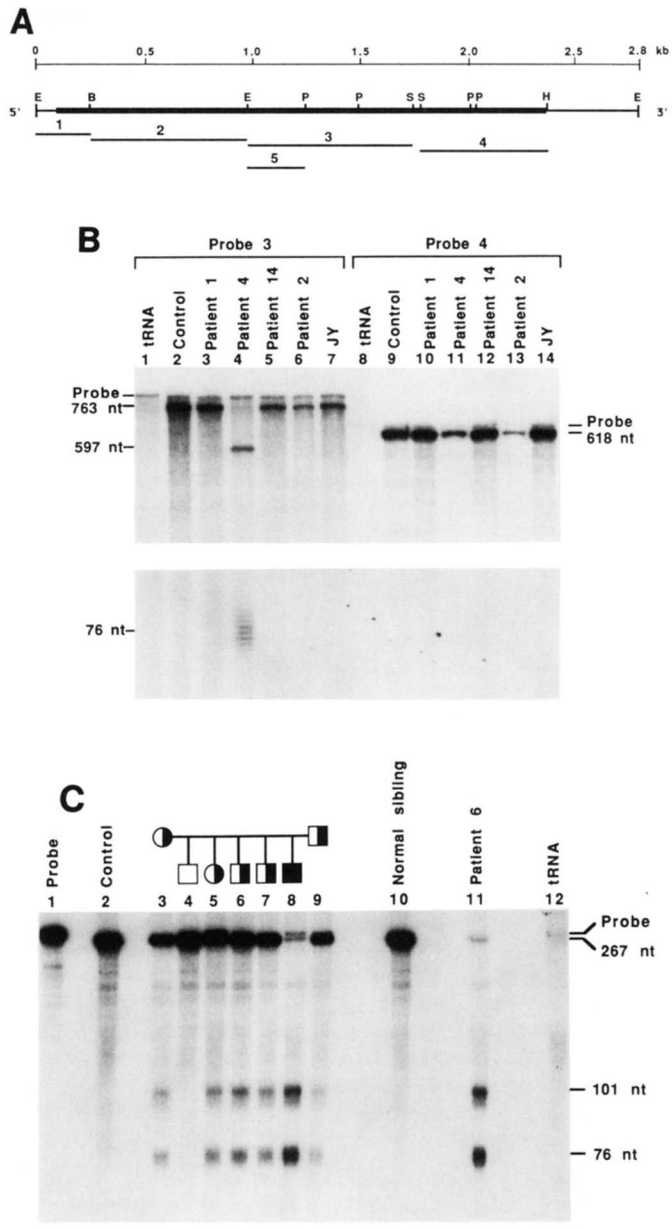

FIG. 2. **S1 nuclease protection.** *A*, restriction enzyme fragments of the β subunit cDNA were subcloned into M13 to generate uniformly ^{32}P-labeled antisense probes. *Probes 1*, *2*, *3*, and *4* span the entire coding region (*thick line*) except for 42 bp between adjacent *Sma*I (*S*) sites. *Probe 5* is a subfragment of *probe 3*. The identity and orientation of each fragment was confirmed by DNA sequencing (not shown). *E* = *Eco*RI, *B* = *Bam*HI, *P* = *Pst*I, and *H* = *Hgi*AI. *B*, total RNA from a healthy control (*lanes 2* and *9*), four unrelated LAD patients (*lanes 3–6* and *10–13*), and the JY cell line (*lanes 7* and *14*), and control tRNA (*lanes 1* and *8*) were hybridized to uniformly ^{32}P-labeled probes and subjected to S1 nuclease digestion. Protected fragments were size-fractionated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography with preflashed XAR film. The lower portion of the autoradiogram, showing a protected fragment of about 76 nt from patient 4 with *probe 3* (*lane 4*), was exposed longer. No other bands were detectable in the intervening portion of the autoradiogram. The size of protected fragments was estimated from standards generated by *Eco*RI- and *Hin*fI-digested pBR322. *C*, total RNA from a healthy control, patient 6 (*lanes 8* and *11*), one normal sibling (*lanes 4* and *10*), and heterozygous siblings (*lanes 5–7*) and parents (*lanes 3* and *9*) were analyzed by S1 nuclease protection with *probe 5*, as described above.

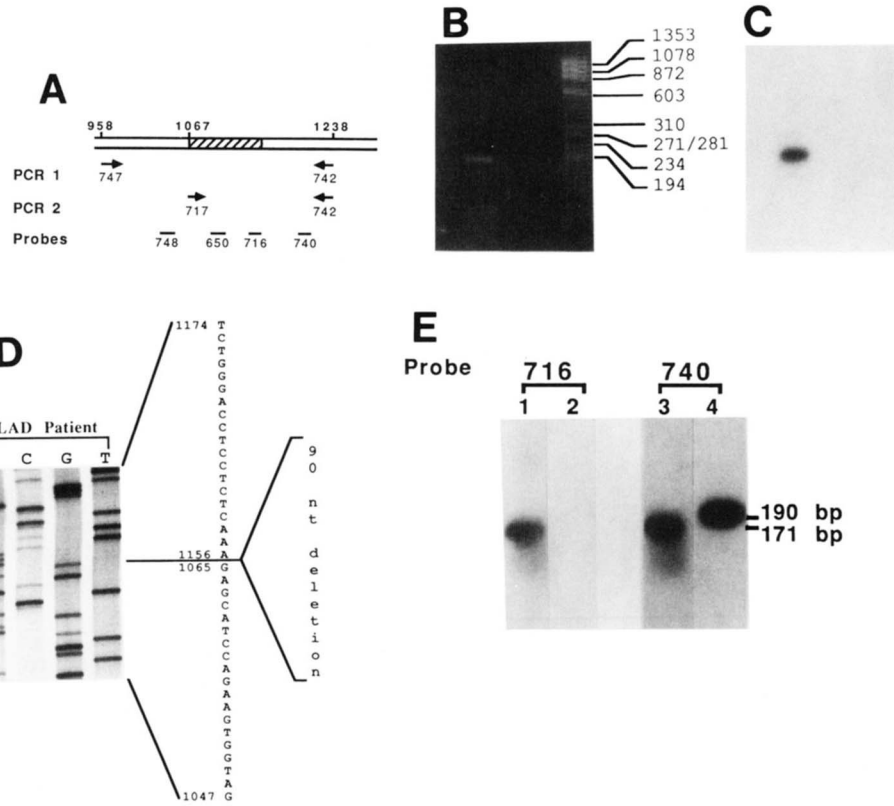

FIG. 3. ***Taq* polymerase chain reaction (*PCR*) products.** *A*, first strand cDNA, primed with antisense oligonucleotide 742, was generated from 2.5 μg of poly(A)$^+$ RNA from patient 4. In the first polymerase chain reaction reaction (*PCR-1*), the sequence between oligonucleotides 742 and 747, containing the putative deletion region (*hatched*), was amplified by 30 cycles of *Taq* polymerase chain reaction. A 280-bp product is expected for wild type message. In the second polymerase chain reaction reaction (*PCR-2*), the sequence between oligonucleotides 717 and 742 was amplified by 60 cycles of *Taq* polymerase chain reaction, after first strand cDNA synthesis primed with oligonucleotide 742. *B*, agarose gel electrophoresis of the PCR-1 product with ethidium bromide staining. *Hae*III-digested bacteriophage φX174 was run as size standards. *C*, Southern blotting of the PCR-1 product with ^{32}P-end-labeled oligonucleotide 748. *D*, the sequence of the PCR-1 product. *E*, comparison of the PCR-1 and PCR-2 products. The PCR-1 product (*lanes 2* and *4*) and the PCR-2 product (*lanes 1* and *3*) were analyzed by Southern blotting with oligonucleotides 716 (*lanes 1* and *2*) and 740 (*lanes 3* and *4*), corresponding to deletion region and nondeletion region, respectively.

efficiency in synthesis of the shorter 190-bp fragment could account for its over-representation.

In an effort to select any potential normal message in RNA from patient 4, a second *Taq* polymerase chain reaction was primed with oligonucleotide 717, which corresponds to 17 bp within the deletion region, and oligonucleotide 742 (*PCR-2*, Fig. 3*A*). Analytical agarose gel electrophoresis with ethidium bromide staining showed no detectable band. The *Taq* polymerase chain reaction product was subjected to a further 30 cycles of the polymerase chain reaction, using the same oligonucleotides. This time, a 171-bp product was detectable by Southern analysis, using oligonucleotide probes 716 (Fig. 3*E*, *lane 1*) and 740 (Fig. 3*E*, *lane 3*), which correspond to sequences in the deletion and nondeletion regions, respectively. The size of the band correlates with the expected size from wild type message. In contrast, the 190-bp PCR-1 product hybridized with oligonucleotide 740 (Fig. 3*E*, *lane 4*), but not with oligonucleotide 716 (Fig. 3*E*, *lane 2*). These results confirm that a small portion of normal β subunit message is present in RNA from these patients.

The 30 amino acids encoded by the deletion region map to the middle of the extracellular domain, outside of the cysteine-rich homologous repeats (Fig. 4). Comparison of this region to other integrin β subunits shows 63%, 53%, and 50% amino acid identity (Fig. 4). This region is part of the highly conserved 241 amino acid segment of the extracellular domain

Definition of the Defect at the Genomic Level—The presence of a small amount of normal size β subunit polypeptide on the cell surface and the small amount of apparently normal β subunit message suggested aberrant mRNA splicing. To test this hypothesis, we examined the intron/exon organization of the normal β subunit gene. A 40-kb cosmid clone containing a portion of the normal β subunit gene was recently isolated.[2] A 1.7-kb *Eco*RI fragment from the cosmid clone hybridized with a 30-mer oligonucleotide directed against the deletion region (data not shown). Sequence analysis showed that the 90-bp sequence corresponding to the deletion region is encoded on a single exon (data not shown). The intron/exon splice sequences closely follow the consensus splice donor and acceptor sites (Fig. 5*A*).

We predicted that the 90-bp exon should be present in the patient's genome and that a mutation in one of the splice sites should be found. Southern analysis of *Eco*RI-digested genomic DNA from patient 4, probed with oligonucleotide probe 650 (Fig. 3*A*) against the deletion region, showed a specific band of 1.7 kb (data not shown). A partial genomic library from the patient's DNA was constructed. High molecular weight DNA was digested to completion with *Eco*RI and loaded onto a preparative agarose gel. DNA from a broad band, corresponding to 1.5–1.9 kb, was cut out, electroeluted, and cloned into λgt10 vector. 500,000 recombinations were screened with the 1.7-kb *Eco*RI fragment from the normal genomic clone and the 30-mer oligonucleotide directed against the deletion region. Fourteen plaques, which hybridized to both probes, were picked. Sequence analysis showed that the 90-bp exon (not shown) and the 3′ splice site (Fig. 5*A*) were intact and identical with control sequences. However, the 5′ splice site contained a single G to C substitution in the third position of the intron sequence (Fig. 5*B*). This result indicates that a mutation in the conserved mRNA splice site leads to improperly spliced message.

DISCUSSION

We have shown that a RNA splicing defect results in the synthesis of an aberrantly small β subunit precursor and subsequently leukocyte adhesion deficiency. Four related patients with this defect (Kishimoto *et al.*, 1987c) have a moderate deficiency form of the disease (Anderson *et al.*, 1985). We have previously shown that the defect affects the protein backbone rather than glycosylation. No gross deletion was detected by Northern analysis (Kishimoto *et al.*, 1987c). However, we have shown here, by S1 nuclease protection, that the

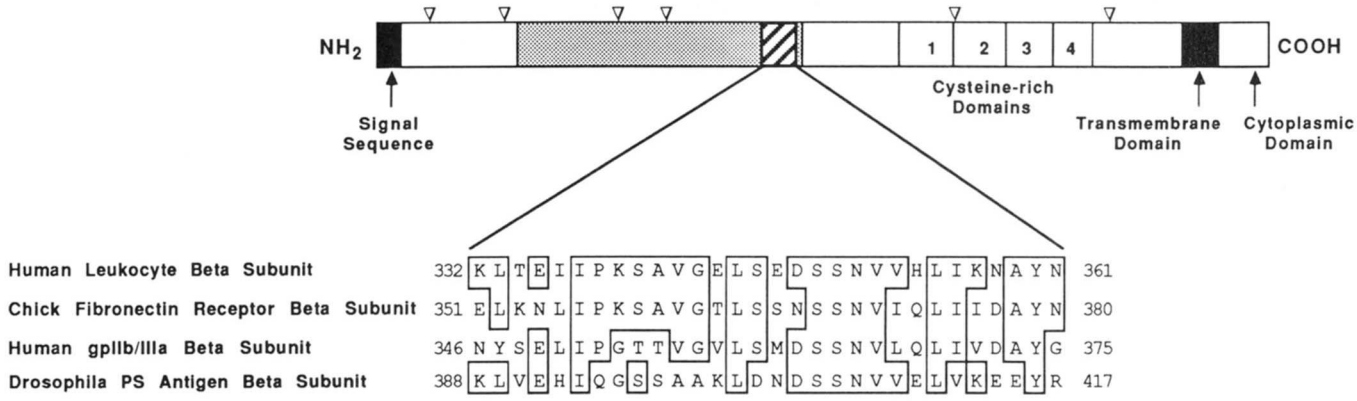

FIG. 4. **Schematic of the β subunit polypeptide showing the region corresponding to the 30-amino-acid deletion (*hatched box*) and the 241-amino-acid highly conserved segment of the extracellular domain (*shaded*).** The position of potential *N*-glycosylation sites are indicated with *triangles*. The 30-amino-acid sequence is compared to the corresponding sequences from the β subunits of the chick fibronectin receptor (Tamkun *et al.*, 1986), human Gp IIb-IIIa (Fitzgerald *et al.*, 1987), and *Drosophila* position-specific antigen (MacKrell *et al.*, 1988).

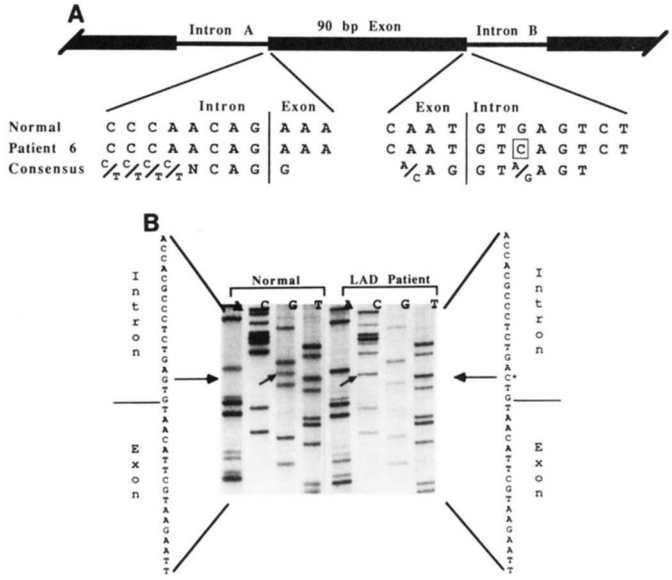

FIG. 5. **Splice site sequences of the 90-bp exon.** *A*, a 90-bp exon, corresponding precisely to the deletion region, was cloned from genomic DNA from a control and patient 4. The sequence of both exons was identical with the nucleotides 1066 to 1156 of the β subunit cDNA (not shown). The 3′ splice site of intron A and the 5′ splice site of intron B were sequenced and compared to consensus splice sequences. The splice sites of control and patient 4 were identical except that a single G to C substitution (*box*) was detected in the 5′ splice site. No other substitutions were detected in the first 60 bp of the 5′ end of intron B or 93 bp of the 3′ end of intron A (not shown). *B*, sequencing reactions showing the 5′ splice site of intron B from a control and patient 4. The G to C substitution in the third position of the intron is shown (*arrows*).

β subunit mRNA from these patients contains a deletion of 90 nt. A 30-amino-acid deletion would account for a 3270-dalton decrease in polypeptide size, which is close to the 5000-dalton difference estimated by SDS-PAGE (Kishimoto *et al.*, 1987c). The 90-nt region is encoded on a separate exon in the genomic DNA from both control and patient, demonstrating that there is not a gross deletion in the patients' genomic DNA. Furthermore, a single G to C substitution was detected in the 5′ splice sequence from patient DNA.

Survival of LAD patients is greatly influenced by the extent of deficiency of the leukocyte integrins. Severely deficient patients (<1% of normal expression) often die in the first 2 years of childhood, while moderately deficient patients (3–10% of normal expression) are less prone to life-threatening infections and can survive to adulthood. In the case of deficiency of β subunit mRNA and protein precursor, the extent of deficiency correlates with moderate and severe phenotype (Kishimoto *et al.*, 1987c). However, among 13 patients synthesizing normal quantities of mRNA and protein precursor, it has been unclear why some are of the moderate and some of the severe deficiency phenotype (Kishimoto *et al.*, 1987c; Dimanche *et al.*, 1987, Dana *et al.*, 1987).[3] In the case reported here, a S1 nuclease protection assay showed a small amount of protected message of normal size from these patients. *Taq* polymerase chain reaction is a powerful technique which has been utilized to amplify specific genomic DNA sequences (Kogan *et al.*, 1987; Saiki *et al.*, 1988). We adapted the *Taq* polymerase chain reaction to amplify sequences from mRNA after classical first strand cDNA synthesis. When oligonucleotides corresponding to sequences outside of the deletion region were used to prime the *Taq* polymerase chain reaction, only the 90-bp deletion product was detected. However, an apparently normal β subunit product could be specifically selected when the *Taq* polymerase chain reaction was primed with an oligonucleotide corresponding to sequences within the deletion region. These results correlate with our finding that the LFA-1 expressed in low levels on the cell surface contains a normal size β subunit polypeptide. Taken together, these results suggest that an aberrant splicing event causes a 90-nt deletion in about 97% of the β subunit message; however, about 3% of normally spliced message apparently accounts for low levels of LFA-1 expression, hence the moderate phenotype.

Our results show that a G to C substitution at position 3 of the 5′ splice site can disrupt but not totally abolish normal splicing. Aberrant splicing defects have been reported in a number of diseases, notably the β-thalassemias (Orkin and Kazazian, 1984; Padgett *et al.*, 1986). Mutations in the first two positions (GT) of 5′ splice site totally abolish normal splicing and cause a severe form of β-thalassemia (Baird *et al.*, 1981; Kazazian *et al.*, 1984; Orkin *et al.*, 1982; Treisman *et al.*, 1982, 1983). The first two positions are conserved in virtually all splice sites (Padgett *et al.*, 1986). In contrast, mutations in the four following nucleotides of the 5′ splice site, which are not universally conserved, result in at least a

[3] A. Wardlaw, T. K. Kishimoto, and T. A. Springer, unpublished observations.

small portion of normally spliced message (Cheng *et al.*, 1984; Kazazian *et al.*, 1984; Orkin *et al.*, 1982; Treisman *et al.*, 1983; Atweh *et al.*, 1987), as observed here.

Mutations in the 5′ splice site commonly activate one or more cryptic splice sequences within the upstream exon or intron (Orkin and Kazazian, 1984; Padgett *et al.*, 1986). In the case reported here, however, there is no apparent use of a cryptic splice site. It appears unlikely that normal splicing of intron A would create a cryptic splice site which is then joined to the 3′ splice site of intron B, since the sequence generated by splicing of intron A (AG:AAACTC) is a poor candidate for a 5′ splice site (Padgett *et al.*, 1986). Thus, it appears that the 3′ splice site of intron B and the 5′ splice site of intron A are directly spliced together, resulting in joining of nonconsecutive exons. Mutations in the first and second position of the 5′ splice site in a dihydrofolate reductase gene (Mitchell *et al.*, 1986) and in phenylketonuria (Marvit *et al.*, 1987), respectively, result in deletion of the preceding exon, similar to the results reported here. The only other examples of this type of aberrant splicing of which we are aware were detected only as minor products in one case of β-thalassemia (Treisman *et al.*, 1982) and in a case of an aberrantly rearranged immunoglobulin gene (Seidman and Leder, 1980). These splicing events are curious because intact splice sites in the intron preceding that with the mutation are present, but are not used normally. As previously proposed (Marvit *et al.*, 1987), this may be because the spliceosome complex does not bind to the mutant 5′ splice site, and interaction between it and the spliceosome bound to the preceding 3′ acceptor site is required for splicing of the preceding intron to occur normally. In other words, exons as well as introns may be looped out during splicing.

LFA-1, Mac-1, and p150,95 are structurally and functionally related to the other integrin adhesion proteins. The homology between the β subunits which define the three integrin subfamilies is particularly striking. The leukocyte integrin β subunit (Kishimoto *et al.*, 1987; Law *et al.*, 1987) shares 45% and 37% amino acid identity with the β subunits of the fibronectin receptor (Tamkin *et al.*, 1986) and the platelet IIb-IIIa glycoprotein (Fitzgerald *et al.*, 1987). The most highly conserved regions are the transmembrane domain and a stretch of 241 amino acids in the extracellular domain. Many features are thought to be common to all integrins, such as binding of ligands, heterodimeric structure, and interaction with the cytoskeleton (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Kishimoto *et al.*, 1987a). However, the structure:function relationships of the conserved domains are unclear. Normal biosynthesis of the leukocyte integrins requires αβ precursor association before further processing of *N*-linked oligosaccharides and transport to the cell surface (Ho and Springer, 1983; Springer *et al.*, 1984). In all the LAD patients which synthesize a β subunit precursor, association with the α subunit is not detectable (Springer *et al.*, 1984; Kishimoto *et al.*, 1987c; Dimanche *et al.*, 1987). Thus, the heterogeneous mutations observed in the common β subunit precursor probably affect αβ association. The 30-amino-acid deletion is located within the highly conserved 241-amino-acid region of the extracellular domain. Since the 30-amino-acid segment is required for αβ association, it may be present at a contact site between the α and β subunits. This segment is highly conserved in evolution; the β subunit of a *Drosophila* integrin protein shares 35% amino acid identity overall with the human leukocyte integrin β subunit, but 50% identity in the 30-amino-acid sequence (MacKrell *et al.*, 1988). Both the α and β subunits of integrin proteins appear to contribute to ligand binding, since the α subunit regulates ligand specificity and the β subunit can be cross-linked to ligand peptides (D'Souza *et al.*, 1988). Analysis of other naturally occurring mutations in patients, as well as *in vitro* mutagenesis, should help define the role of this highly conserved region in αβ interaction and function.

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REFERENCES

Anderson, D. C., Schmalsteig, F C., Finegold, M. J., Hughes, B. J. Rothlein, R., Miller, L. J., Kohl, S., Tosi, M. F., Jacobs, R. L., Waldrop, T. C., Goldman, A. S., Shearer, W. T., and Springer, T. A. (1985) *J. Infect. Dis.* **152,** 668–689

Anderson, D. C., and Springer, T. A. (1987) *Annu. Rev. Med.* **38,** 175–194

Anderson, D. C., Smith, C. W., and Springer, T. A. (1988) in *Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., and Frederickson, D. S., eds), in press

Atweh, G. F., Wong, C., Reed, R., Antonarakis, S. E., Zhu, D., Ghosh, P. K., Maniatis, T., Forget, B. G., and Kazazian, H. H. (1987) *Blood* **70,** 147–151

Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 1408–1412

Baird, M., Driscoll, C., Schreiner, H., Sciarrata, G. V., Sansone, G., Niazil, G., Ramirez, F., and Bank, A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4218–4221

Cheng, T., Orkin, S. H., Antonarakis, S. E., Potter, M. J., Sexton, J. P., Markham, A. F., Giardina, P. J. V., Li, A., and Kazazian, H. H. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 2821–2825

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299

Dana, N., Clayton, L. K., Tennen, D. G., Pierce, M. W., Lachmann, P. J., Law, S. A., and Arnaout, M. A. (1987) *J. Clin. Invest.* **79,** 1010–1015

Davis, L. G., Dibner, M. D., and Battey, J. F. (1986) *Basic Methods in Molecular Biology.* Elsevier/North-Holland, New York

Dimanche, M. T., Le Deist, F., Fischer, A., Arnaout, M. A., Griscelli, C., and Lisowska-Grospierre, B. (1987) *Eur. J. Immunol.* **17,** 417–419

D'Souza, S. E., Ginsberg, M. H., Lam, S. C.-T., and Plow, E. F. (1988) *J. Biol. Chem.* **263,** 3943–3951

Fisher, A., Lisowska-Grospierre, B., Anderson, D. C., and Springer, T. A. (1988) *Immunodef. Rev.* **1,** 39–54

Fitzgerald, L. A., Steiner, B., Rall, S. C., Jr., Lo, S., and Phillips, D. R. (1987) *J. Biol. Chem.* **262,** 3936–3939

Ho, M.-K., and Springer, T. A. (1983) *J. Biol. Chem.* **258,** 2766–2769

Hynes, R. O. (1987) *Cell* **48,** 549–554

Kazazian, H. H., Orkin, S. H., Antonarakis, S. E., Sexton, J. P., Boehm, C. D., Goft, S. C., and Waber, P. G. (1984) *EMBO J.* **3,** 593–596

Kishimoto, T. K., and Springer, T. A. (1988) in *Immunodeficiency and Skin* (Fritsch, P., ed), in press

Kishimoto, T. K., Miller, L. J., and Springer,T. A. (1987a) in *Leukocyte Typing III* (McMichael, A., ed) pp. 896–898, Springer Verlag-New York, New York

Kishimoto, T. K., Hollander, N., Roberts, T. M., Anderson, D. C., and Springer, T. A. (1987c) *Cell* **50,** 193–202

Kogan, S. C., Doherty, M., and Gitschier, J. (1987) *N. Engl. J. Med.* **317,** 985–990

Law, S. K. A., Gagnon, J., Hildreth, J. E. K., Wells, C. E., Willis, A. C., and Wong, A. J. (1987) *EMBO J.* **6,** 915–919

MacKrell, A. J., Blumberg, B., Haynes, S. R., and Fessler, J. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 2633–2637

Marvit, J., DiLella, A. G., Brayton, K., Ledley, F. D., Robson, K. J. H., and Woo, S. L. C. (1987) *Nucleic Acids Res.* **15,** 5613–5628

Mitchell, P. J., Urlaub, G., and Chasin, L. (1986) *Mol. Cell. Biol.* **6,** 1926–1935

Orkin, S. H., and Kazazian, H. H., Jr. (1984) *Annu. Rev. Genet.* **18,** 131–171

Orkin, S. H., Kazazian, H. H., Antonarakis, S. E., Goff, S. C., Boehm, C. D., Sexton, J. P., Waber, P. G., and Giardina, P. J. V. (1982) *Nature* **296,** 627–631

Padgett, R. A., Grabowski, P. J., Kornarska, M. M., Seiler, S., and Sharp, P. A. (1986) *Annu. Rev. Biochem.* **55,** 1119–1150

Ruoslahti,E., and Pierschbacher, M. D. (1987) *Science* **238,** 491–497

Saiki, R., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science* **239,** 487–491

Sanchez-Madrid, F., Nagy, J., Robbins, E., Simon, P., and Springer, T. A. (1983) *J. Exp. Med.* **158,** 1785–1803

Seidman, J. G., and Leder, P. (1980) *Nature* **286,** 779–783

Springer, T. A., Thompson, W. S., Miller, L. J., Schmalstieg, F. C., and Anderson, D. C. (1984) *J. Exp. Med.* **160,** 1901–1918

Tamkun, J. W., DeSimone, D. W., Fonda, D., Patel, R. S., Buck, C. Horwitz, A. F., and Hynes, R. O. (1986) *Cell* **46,** 271–282

Todd, III, R. F., and Freyer, D. R. (1987) *Hematology/Oncology Clin. North Am.* **2,** 13–31

Treisman, R., Proudfood, N. J., Shander, M., and Maniatis, T. (1982) *Cell* **29,** 903–911

Treisman, R., Orkin, S. H., and Maniatis, T. (1983) *Nature* **302,** 591–596