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The Gene for Human Erythrocyte Protein 4.2 Maps to Chromosome 15q15

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Summary

Protein 4.2 (P4.2), one of the major components of the red-blood-cell membrane, is located on the interior surface, where it binds with high affinity to the cytoplasmic domain of band 3. Individuals whose red blood cells are deficient in P4.2 have osmotically fragile, abnormally shaped cells and moderate hemolytic anemia. cDNA clones from both the 5′ and the 3′ coding regions of the P4.2 gene were used to map its chromosomal location by fluorescence in situ hybridization. The probes, individually or in combination, gave specific hybridization signals on chromosome 15. The hybridization locus was identified by combining fluorescence images of the probe signals with fluorescence banding patterns generated by Alu-PCR (R-like) probe and by DAPI staining (G-like). Our results demonstrate that the locus of the P4.2 gene is located within 15q15.

Introduction

The normal red blood cell (RBC) membrane is stabilized by an underlying cytoskeletal network of proteins which determines both the membrane strength and deformability. The major membrane components are spectrin, ankyrin, protein 4.1, protein 4.2 (P4.2), actin, and protein 4.9. P4.2, which represents ∼5% of the protein mass of the human RBC membrane, is of interest because the RBCs of P4.2-deficient individuals exhibit abnormal morphology and increased fragmentation, indicating defective membrane-cytoskeletal interactions (Rybicki et al. 1988). We (Sung et al. 1990) and others (Korsgren et al. 1990) have recently determined the complete cDNA sequence for human P4.2, and in this cDNA we have identified a mutation associated with P4.2 deficiency (Bouhassira et al., in press).

We now report the use of clones from both the 5′ and the 3′ coding regions of the P4.2 gene to map its chromosomal location. A recently developed hybridization technique that permits the simultaneous identification of the hybridization signals and their band location (Baldini and Ward 1991) was used in the present study. The results presented demonstrate that the P4.2 gene maps to 15q15.

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Material and Methods

Metaphase Chromosome Preparations

Metaphase-chromosome spreads from a healthy individual were obtained from phytohemagglutinin-stimulated peripheral blood cells incubated for 72 h. Ethidium bromide (2.5×10^{-5} M; Sigma, St. Louis) and colcemid (0.1 μg/ml) were added for 45 min and 15 min, respectively, before harvesting. Hypotonic treatment of cells, fixation in methanol-acetic acid, and preparation of chromosome spreads followed standard cytogenetic procedures. Chromosomes were dehydrated through 70%, 90%, and 100% ethanol and were stored at 4°C until used (i.e., as long as 1 mo).

Probe Preparation

Two probes were prepared which encoded the entire cDNA sequence. Clone 12 encodes 0.8 kb of

cDNA sequence which includes the 5′ end, and clone 7 encodes 1.8 kb of cDNA which includes the 3′ end (Sung et al. 1990). Cloned DNA was amplified using the PCR essentially as described by Saiki et al. (1988), with oligonucleotide primers as described by Bouhassira et al. (in press).

Biotinylation of Probes

Probes were labeled with biotin-11-dUTP (Sigma, St. Louis) by nick-translation (Brigatti et al. 1983). Unincorporated nucleotides were separated from probe by using Sephadex G-50 spin columns (Sambrook et al. 1989). To ensure a probe size range of 200–400 nucleotides, an aliquot of each DNA was heat-denatured and run on a 1.2% agarose gel (nondenaturing) with appropriate size markers. Both the 5′ and the 3′ probes were used in a concentration of 7.5–10 ng/μl.

In Situ Hybridization

Chromosomal preparations were hybridized in situ essentially according to a method described by Lichter et al. (1990). The hybridization was performed at 37°C in 2 × SSC; 50% (v/v) formamide, 10% (w/v) dextran sulfate, 8–10 ng probe/μl, and 0.8 μg/μl sonicated salmon sperm DNA. In some experiments the hybridization cocktail also contained sonicated human genomic DNA (0.3 μg/μl). Post-hybridization washing was at 42°C in 2 × SSC, 50% formamide (× 3) followed by three washes in 0.1 × SSC at 60°C. In situ hybridization banding (ISHB) used for chromosome identification and locus assignment was generated by adding digoxigenin-11 (Boehringer Mannheim, Indianapolis)-labeled Alu-PCR products to the hybridization mixture (3–6 ng/μl). This produces an R-like banding (Alu-PCR banding) pattern which is suitable for gene mapping studies (Baldini and Ward 1991). The Alu-PCR products were prepared from human DNA by using a single Alu primer (primer 517 in Nelson et al. 1990) for sequence amplification. The PCR products were nick-translated, and the probe size was adjusted to 200–400 bp. Separately denatured hybridization mixtures of P4.2 and the PCR-Alu products were mixed together just prior to being layered onto the slides, and hybridization continued for 16–18 h at 37°C.

Biotin-labeled DNA was detected by using fluorescein isothiocyanate (FITC)-conjugated avidin DCS (5 μg/ml) (Vector Laboratories, Burlingame, CA); digoxigenin-labeled DNA was detected by using a rhodamine-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis). Chromosome identification was also confirmed by simultaneous DAPI (diamidinophenylindole) staining (0.2 μg/ml for 5 min), which produces a G-like banding pattern.

Digital Microscopy

Epifluorescence images were obtained using a widefield microscope (Zeiss Axioskop-20, 63 × 1.25 NA Plan Neofluar oil immersion objective, 50 W HBO mercury source) coupled to a thermoelectrically cooled CCD camera (Photometrics CH220). Camera control and image acquisition were performed using an Apple Macintosh IIx computer. Image processing was done on a Macintosh IIci computer equipped with an accelerated 24-bit color system (SuperMac Spectrum-24 PDQ). The 8-bit grey-scale images were recorded sequentially using DAPI, FITC, and rhodamine filter sets, custom manufactured by C. Zeiss, Germany, to minimize image offsets. Exposure times were 30 s for FITC, 1 s for DAPI, and 5 s for rhodamine. Systematic offsets were improved by a factor of 5, relative to standard Zeiss 4879 series filter (0.2 ± 0.04 μm vs. 1 μm in object space). Position noise due to mechanical shocks was removed by pixel shifting in the computer. Alignment of FITC and rhodamine pairs made use of the weak bleed-through of FITC fluorochrome into the rhodamine channel. Thus, contrast-enhanced rhodamine images of the chromosome spreads invariably contained many small background FITC objects suitable for image alignment. Alignment of the DAPI images was done by overlaying them on the corresponding rhodamine banding images by pixel shifting until the DAPI (G-like) and rhodamine (R-like) banding patterns interdigitated symmetrically. In practice, shifts rarely exceeded 4 pixels (± 0.2 μm). Merging of DAPI, FITC, and rhodamine images employed software (Gene Join Maxpix) by Tim Rand and Gwyn Ballard in the laboratory of David C. Ward. This software assigns user-designated pseudocolor and grey-scale values to each of the source images, then generates on output ("merged") image based on the most intense source image at each pixel location. Thus, objects with higher scaled brightness (e.g., probe signals) override the banding or counterstaining signals in the corresponding image location. Object displayed in the output image retain the pseudocolor assigned to them and therefore appear distinct and easily identifiable. The merging software permits any color to be assigned to any fluorochrome. Colors were chosen to give the best photographic contrast. Montages were prepared using the PixelPaint

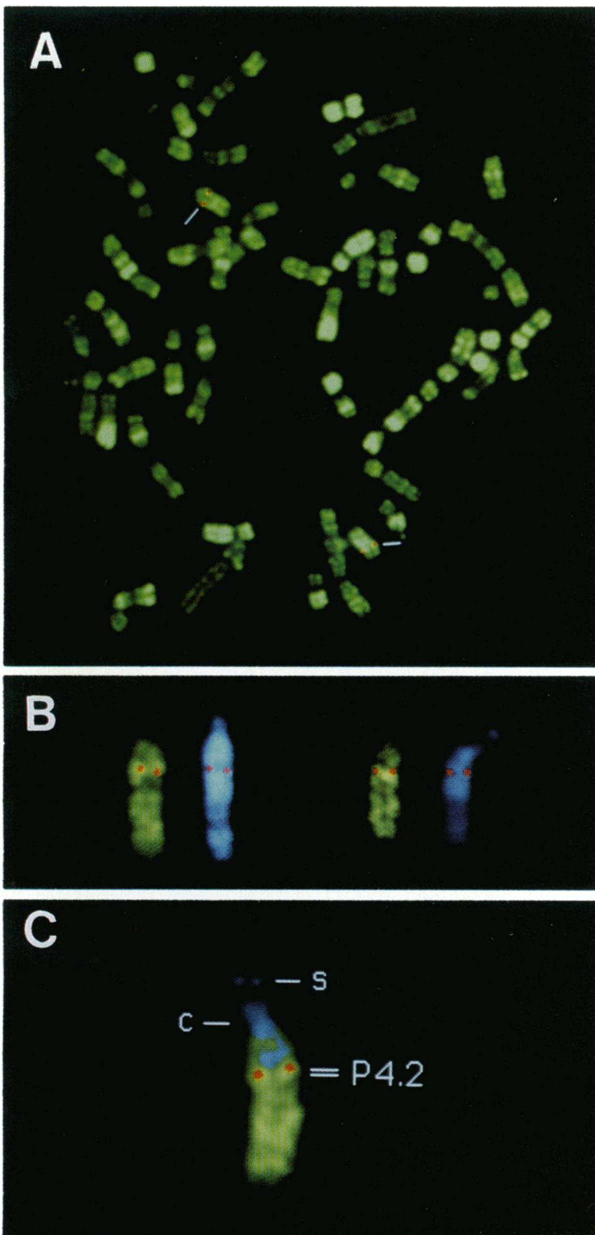

Figure 1 *A,* Double-label fluorescence image of metaphase chromosome spread. Green/yellow signal denotes R-like banding with Alu-PCR (see text). Red signals denote hybridization loci of P4.2 probe. FITC and rhodamine images were recorded sequentially and then were aligned, merged, and pseudocolored by computer. FITC fluorescence (P4.2 signals) is pseudocolored red, and rhodamine (Alu-PCR banding) is pseudocolored green/yellow for best photographic contrast. *B,* Alu-PCR R-like banding (green/yellow) and DAPI G-like banding (blue) applied to two nonhomologous chromosome 15s in which P4.2 locus (red) is visualized by in situ hybridization using 3′ and 5′ probes combined. DAPI staining serves primarily to identify chromosome 15. The P4.2 locus maps to the positive R band at 15q15 and to the negative DAPI band at this location. *C,* Complementary use of Alu-PCR banding and DAPI staining: image of panel *A* merged with DAPI counterstain image. DAPI (blue) assists chromosome identification by staining all chromatin, including regions that are essentially devoid of Alu sequences. Merging parameters are adjusted so that DAPI overrides low-intensity regions of the Alu-PCR banding (green-yellow). s = satellite DNA; and c = centromere region.

Professional software package (SuperMac, Sunnyvale, CA). Final images in 24-bit color were photographed directly from the display monitor.

Results

In chromosome spreads with low nonspecific FITC background, the combined 3′ and 5′ probe (2.6 kb total) hybridization signal was transiently visible, under the microscope, to the naked eye. Bleaching reduced the signal below visual threshold in a few tens of seconds. The 3′ probe signal alone could sometimes be seen by eye; however, the signal for the 5′ probe alone (0.8 kb) was too weak to be visible. It was, however, far above the noise level of the cooled CCD camera, and whether it could be successfully imaged depended on its contrast against the granular background of nonspecific fluorescence, which varied considerably between chromosome spreads.

For the combined probes, hybridization signals (symmetrical doublets on each homologue) were distinguishable in 83% (25/30) of chromosome spreads examined. With the 3′ probe (1.8 kb) alone, 70% were distinguishable symmetrical doublets; with the 5′ probe (0.8 kb), less than 10% were. The combined probe gave clear signals on both homologues in ∼70% of spreads examined. Of these, 85% were near-symmetrical doublets aligned approximately perpendicular to the chromosome axis (fig. 1*A*). The remaining 15% showed varying degrees of asymmetry and were rejected.

Identification of the chromosome carrying the P4.2 signal was achieved first by DAPI staining, which produces characteristic bright blue fluorescent bands in the satellite region of chromosome 15 and a low-contrast but distinct G-like banding pattern below the centromere (fig. 1*B*). The Alu-PCR banding (green/yellow in fig. 1*B*) observed is also characteristic of chromosome 15. The hybridization locus of P4.2 was visualized against the DAPI banding by merging corresponding FITC and DAPI images (fig. 1*B*). The P4.2 probe (red in fig. 1*B*) lies on the negative DAPI G band q15. This assignment was confirmed by merging the probe image with the R-like banding pattern (green/

yellow in fig. 1*A–C*) generated by Alu-PCR. In this case, the probe signal is seen to overlay the positive R-band at q15 (fig. 1*C*).

Discussion

In the present study, chromosomal in-situ hybridization was used to localize the P4.2 gene. The hybridization locus was visualized against both the G-like and R-like banding patterns, produced by DAPI staining and Alu-PCR banding, respectively. With the chromosome preparations used, the Alu-PCR products produced an R-type banding at the 400-band level of resolution. However, it does not stain regions of constitutive heterochromatin, which contain very few Alu sequence repeats (Korenberg and Rykowski 1988; Lichter et al. 1990; Baldini and Ward 1991). Thus, acrocentric chromosomes, including chromosome 15, appear "decapitated" when Alu-PCR banded, while metacentric and submetacentric chromosomes appear cut in two. This limits the utility of Alu-PCR in identifying acrocentric chromosomes, without complementary methods. The ability of DAPI to brightly stain centromere regions and satellite DNA and to generate a weak G-type banding pattern makes it a useful complement to Alu-PCR for gene mapping.

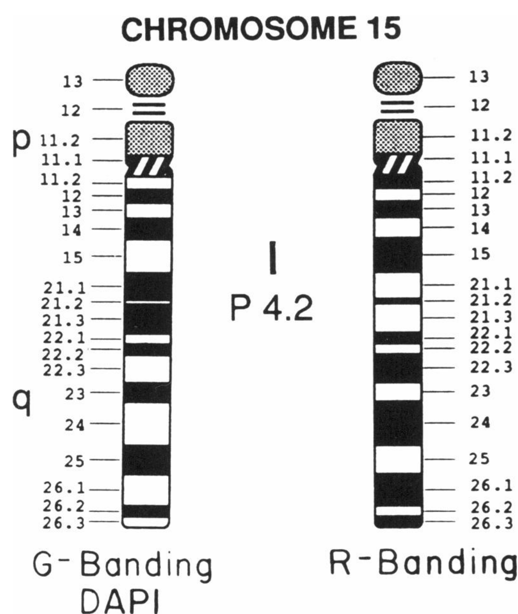

Figure 2 Ideogram of chromosome 15: G-like banding generated by DAPI staining (*left*) and R-like banding pattern obtained using Alu-PCR (*right*). The bold line in the middle shows the location of the P4.2 locus on chromosome 15.

On the basis of both DAPI and Alu-PCR banding methods, the hybridization loci of both the 3′ and 5′ probes for the P4.2 gene appear to be at 15q15, regardless of whether the probes were used alone or in combination. This assignment was relatively easy when the two probes were used in combination (2.6 kb) but was considerably more difficult when they were used singly. It appears that, with state-of-the-art detectors such as cooled CCD cameras, the ability to map genes by in-situ hybridization using small probes is generally limited not by detector noise or even by photon statistics but by the nonspecific, highly variable background fluorescence encountered. The background frequently contains fluorescent regions that are either as bright as or brighter than the probe signals. Reliable identification of probe signal depends on correlations such as visual recognition of doublets or the appearance of corresponding intensities on both homologous chromosomes. In either case, it may be necessary to change the microscope focus slightly to successfully visualize all signals present. In some cases, gene mapping by this method will not be possible until improved methodologies for background suppression are available.

Further evidence for the assignment of P4.2 to chromosome 15 comes from mouse mapping data when interspecific back-crossing with human erythrocyte P4.2 is used. The results demonstrated the assignment of the human P4.2 locus to mouse chromosome 2 (N. G. Copland and N. A. Jenkins, personal communication). The mouse chromosome 2 region where human P4.2 is mapped contains nine other genes located on human chromosome 15, indicating synteny between these regions of human and mouse chromosomes (Nadear 1990; O'Brien 1990).

Assignment of the P4.2 gene to 15q15 further illustrates the diversity of chromosomal locations for erythrocyte membrane proteins. For example, protein 4.1 maps to lp34-36.2 (Conboy et al. 1986), anion exchange protein (AE1, band 3) maps to 17q21-qter (Showe et al. 1987; Lux et al. 1989), glycophorin C maps to 2q14-q21 (Mattei et al. 1986), and ankyrin maps in the region of 8p11.2 (Lux et al. 1990).

Physical assignment of genes to metaphase chromosomes can contribute to genetic studies. For example, it is of interest to note that t(15;17)(q22;q11-12) is a very specific chromosomal rearrangement associated with acute promyelocytic leukemia (APL) (Rowley et al. 1977). Recently a cluster of 18-kb repeated sequences were located on chromosome 17, in the region of the breakpoint associated with APL. These se-

quences lie within a region ∿2 Mb (Moore et al. 1989). Clustering of repeated sequences appears to be important in a study of cancer genes. Whether any copies of repeated sequences are located adjacent to chromosome 15q15 sequence is not known yet. Further work may elucidate whether position effect of t(15;17) and/or tandem repeats have influence on the expression of P4.2 in patients with APL.

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