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Full length mouse glycophorin gene constructed using recombinant polymerase chain reaction

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SUMMARY Recently, an incomplete cDNA clone for a major mouse glycophorin gene, pGP315, and a genomic clone, pGX7 (which contains the first exon and nucleotide sequences around the transcription start sites) was isolated and sequenced by Matsui et al. (1). Since there were no available restriction sites for the construction of a full length mouse glycophorin A gene, the recombinant PCR technique was adapted to splice together the above two partial sequence clone inserts to obtain a full length recombinant DNA fragment (1053 bp) containing the proper sequence of mouse glycophorin A cDNA. The PCR reconstructed DNA fragments were verified by: gel electrophoresis to contain the expected sizes, hybridization to probes made from the DNA components before recombination, and confirmed by the restoration of a previously destroyed restriction enzyme site. The corrected gene sequence for pGP315 is also reported.

Malaria parasites invade erythrocytes by interacting with specific receptors on the erythrocyte membrane. Glycophorin, which is the major sialoglycoprotein found on the surface of mature human red blood cells, has been indicated as a putative receptor for human malaria parasites. However, the receptor system for rodent malaria parasites remains largely unexplored (2). Appropriately, the mouse provides an important model system for malaria research. Well characterized "glycophorin" clones in a model mammalian system would provide a powerful tool with which to approach these issues.

It became the intent of this laboratory to isolate and/or construct a full length mouse glycophorin A cDNA clone containing 5' untranslated regions. Screening for this full length clone had been somewhat unsuccessful using available libraries and traditional technology. Attempts to ligate overlapping regions of mouse glycophorin A-like sequences found in a genomic clone, pGX7, and in a cDNA clone, pGP315, at a common PvuII restriction enzyme site, were infeasible (due to G-C talling sequence modification of DNA inserts during construction of the cDNA library from which pGP315 was isolated, (personal communications). Using clones previously isolated and generously provided by the laboratory of Dr. Obinata, we were able to construct useful full length recombinant cDNA sequences by adapting novel recombinant polymerase chain reaction (PCR) methodologies (3-7). These sequences will be used for further studies investigating the role of glycophorin in malaria.

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MATERIALS AND METHODS

Clones and strains. The pGX7 clone was isolated from a mouse genomic library constructed in pUC 18 (M. Obinata, personal communication). pGX7 contains a 6.5 kb insert for which the partial sequence has been analyzed (1). The promoter region and the first exon for the mouse glycophorin A gene were included in this cloned insert. The pGP315 clone was isolated from an anemic mouse spleen cDNA library constructed in pBR322(1). The DNA sequence of this 1.5 kb insert (1) indicated that the clone contains an incomplete gene, lacking 15 bp of the 5' coding region and the preceding 5' upstream non-coding region. Host strains were grown in Luria Broth and plasmid DNA preparations were conducted according to Maniatis et al. (8). ØX174 DNA marker was purchased from New England Biolabs.

PCR. Primers designed for this experiment are as follows: P_1 - (20-mer) 5' CTG CTG TGA CAA CAT CAG GT 3'; P_3 - (21-mer) 5' GAA TTC CAC TTT CCT GGA AGA 3'; P_4 - (25-mer) 5' GAA TGA CCT GAT GTT GTC ACA GCA G 3'; P_7 - (19-mer) 5' GGC AAG GCC TTC CTA AGG C 3'; P_8 - (23-mer) 5' CAT TGA TTG AAG TCT TGT TTT CC 3'. All primers were synthesized by the DNA Factory (San Diego, CA). All PCR were carried out in an automated DNA thermal cycler (Perkin Elmer, Cetus Corporation, Norwalk, CT.) using a PCR Kit from Perkin Elmer. The composition of the reaction buffer, dNTP concentrations and Taq enzyme were as recommended by the manufacturer. 100 ng of each template DNA was used. PCR were performed with a profile of: 1 minute of denaturing at 94°C, 2 minutes of annealing at 50°C, and 3 minutes of extension at 72°C for 35 cycles, followed by 10 minutes of terminal extension and cooling to room temperature.

Gel analysis and sequencing. Separation, electroelution and purification of DNAs were conducted during electrophoresis on either a 1% agarose gel (BRL) at 7.1 V/cm or on an 4% Nusieve (FMC) gel at 8.9 V/cm in 1X TAE buffer unless other wise specified. DNA bands were visualized with 0.5 ug/ml Ethidium bromide. For Southern Blot analysis, conducted as in Gu et al. (9), the gels were blotted onto a nylon membrane (Sartorius) and hybridized to the specified alpha-^{32}P dATP nick translated probe (BRL kit) according to the manufacturer's instructions. Direct sequencing of double stranded DNAs were performed with Sequenase Version 2.0 (USB) according the manufacturer.

RESULTS AND DISCUSSION

Construction of the full length mouse glycophorin A gene. The strategy or reconstructing the full length mouse glycophorin A gene is schematically diagramed in Fig. 1. Five PCR primers (P_3, P_7, P_1, P_4 and P_8, see details in Fig. 2 and Materials and Methods) were generated and the herein described recombinant PCR protocol was followed. The primers P_3 and P_7 correspond to insert sequences found only in the genomic clone, pGX7. The primer P_1 is a forward primer for the sequences found to overlap in both the genomic clone, pGX7 and in the cDNA clone, pGP315

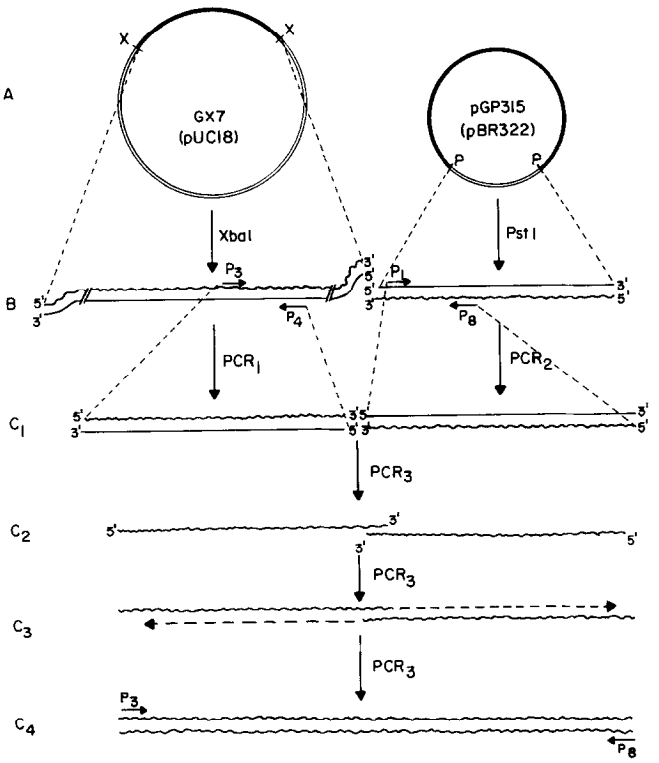

Figure 1. Strategy for constructing a full length mouse glycophorin gene. (Panel A) Two clone inserts from pGX7 and pGP315 were cut out at the insertion sites with XbaI and PstI, respectively for use in this PCR protocol as described in Materials and Methods. (Panel B) A 544 bp segment between PCR primers, P_3 and P_4, (Fig. 2) was amplified from the pGX7 insert during PCR_1. A 524 bp segment between PCR primers, P_1 and P_8 (Fig. 2) was amplified from the pGP315 insert during PCR_2. (Panel C_{1-4}) A third PCR was performed using the mixed two purified segments as templates and P_3 and P_8 as a pair of primers. C_1 - the purified products of PCR_1 and PCR_2 are combined and denatured; C_2 - the overlapping sequences between the two amplified fragments (Fig. 2) permitted hybrid annealing to occur in the complementary regions of the denatured DNA strands ; C_3 -the extension process for creating a complete double stranded chimeric molecule is denoted by the dotted arrows; C_4 - the completed amplification of the chimeric glycophorin DNA molecule between P_3 and P_8 is the final result. X - XbaI; P - PstI. P_1, P_3, P_4 and P_8 are primers (see details in Fig. 2, and Materials and Methods).

(Fig. 2,#). The P_4 primer is the reverse primer for most of that identical region (Fig. 2). Primer P_8 corresponds to insert sequences found only in the cDNA clone, pGP315.

Two clone inserts, one from the genomic sequence, pGX7, and one from the cDNA sequence, pGP315, were cut out with XbaI and PstI, respectively and the reactions PCR_1 and PRC_2 (Fig. 1) were conducted. During the third PCR illustrated in Fig. 1, two kinds of annealing could have occurred between the available complementary overlapping PCR amplified regions corresponding to nt530 to nt554 (see Fig. 2). However, since DNA synthesis and extension occurs in the 5'-3' direction, only the 3' end annealed DNA fragments (depicted as wavy lines in Fig. 1), were able to create a double stranded chimeric DNA molecule that would permit self-extension and ultimately continue to be amplified in this PCR_3 protocol (Fig. 1, C_2-C_4) using P_3 and P_8 as a pair of primers.

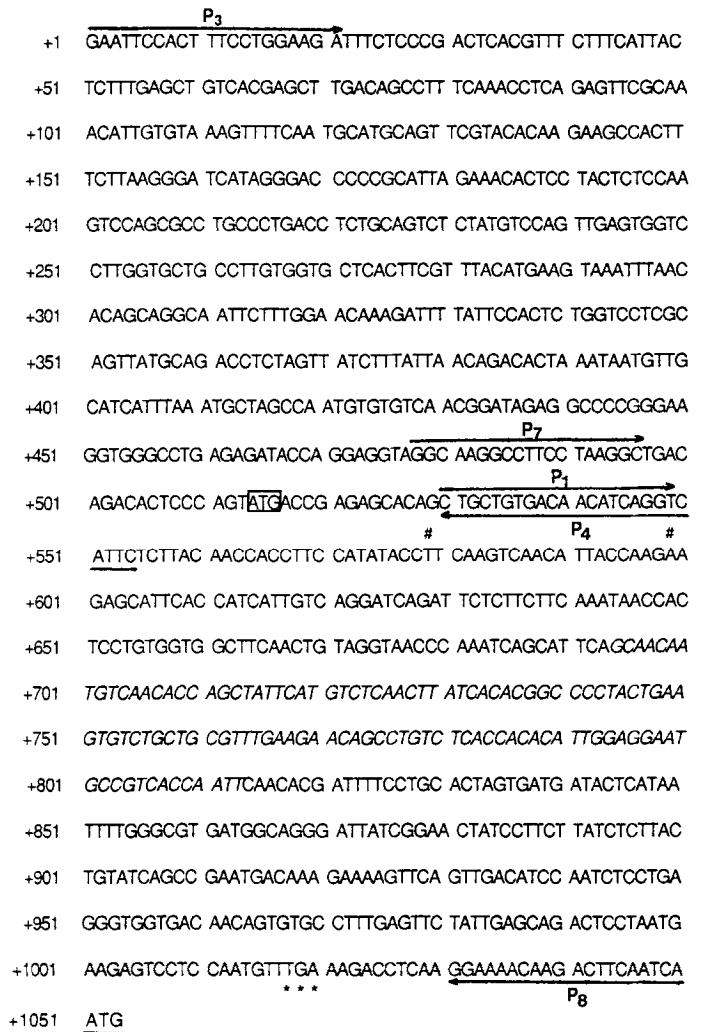

Figure 2. Sequence of the recombinant full length mouse glycophorin gene. Primer sequence locations along the nucleic acid sequence of the recombinant glycophorin gene are indicated. The coding region starts at the boxed ATG and stops at the TGA underscored with ***. The segment between the G (nt 529) and the T (nt 549) underscored with # is identical to the corresponding regions of both the pGX7 genomic sequence and the pGP315 cDNA sequence. Location of the primers designed for this experiment are indicated as arrows. *Italic* sequences (nt 694 - nt 753 and nt 754 - nt 813) represent the herein corrected transposed sequences reported in reference 1.

Characterization of the recombinant full length gene. The resultant recombinant P_3/P_8 mouse glycophorin A gene (Fig. 2, nt1 - nt1053) was expected to contain 1053 bp given the sequence information obtained from Matsui et al. (1). When agarose gel electrophoresis of the PCR products was analyzed, the resultant DNA fragment sizes revealed were as expected (Fig. 3A, lane 4). A 554 bp product (Fig. 3A, lane 3) resulting from PCR_1 (Fig. 1, C_1) was the expected product from a P_3/P_4 sequence amplification (see Fig. 2, nt1 - nt554) given the pGX7 partial sequence (1). A 524 bp fragment (Fig. 3A, lane 2) was also demonstrated, as expected, as the product of PCR_2 (Fig. 1, C_1), the amplification sequence of

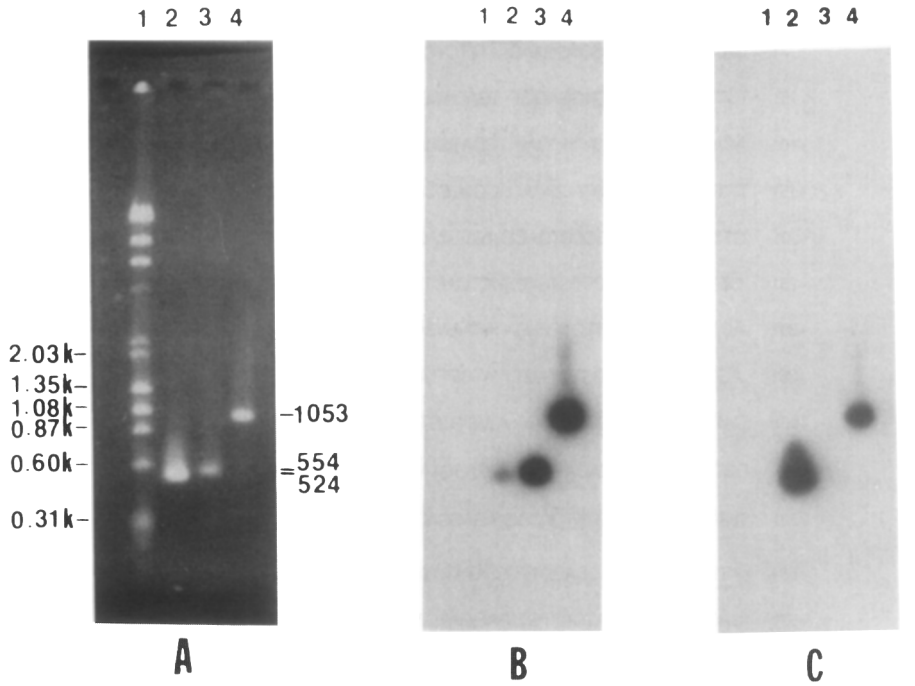

Figure 3. Agarose gel analysis of the $PCR_{1,2\ \&\ 3}$ products. The sizes (bp) of PCR products before and after recombination were analyzed as described in Materials and Methods. The sizes of DNA standards are shown in the left margin and the interpolated sizes of the PCR products in the right margin. (A) Lane 1 - HindIII digested lambda DNA combined with HaeIII digested ØX174 RF DNA; lane 2 - 1 µl (1/50) of the PCR_2 amplified segment (524 bp) between P_1 and P_8 using the pGP315 insert as template; lane 3 - 3 µl (1/17) of the PCR_1 amplified segment (554 bp) between P_3 and P_4 using the pGX7 insert as template; lane 4 - 2 µl (1/25) of the PCR_3 amplified recombinant DNA fragment (1053 bp) between P_3 and P_8 using the combined products of PCR_1 and PCR_2 as templates. The gel shown in (A) was submitted to Southern blot analysis and hybridized to specified alpha-^{32}P labelled probes. The probe used in (B) was the 554 bp PCR_1 amplified product (1.6 X 10^7 cpm/10 ml). The film was exposed for 7 hrs. with an intensifying screen at -70° C. The probe used in (C) was the 524 bp PCR_2 amplified product (1.6 X 10^7 cpm/10 ml). The exposure time was 3 hrs. with an intensifying screen at -70° C.

P_1/P_8 (see Fig. 2, nt530 - nt1053). To confirm that the recombinant PCR product contained the desired sequences, Southern blot analysis of this agarose gel was conducted (Figs. 3B and 3C). Both the P_3/P_4 (PCR_1) and the P_1/P_8 (PCR_2) products were nick translated with alpha-^{32}P dATP and used as probes independently in Fig. 3B and 3C, respectively. As demonstrated for the recombinant 1053 bp fragment, inclusion of both the 554 bp (P_3/P_4) genomic sequence (Fig. 3B, lane 4) and of the 524 bp (P_1/P_8) cDNA sequence (Fig. 3C, lane 4) was quite evident. Each probe identifies itself as a positive control (Figs. 3B, lane 3 and 3C, lane 2). Some recognition of the overlapping primer sequences in Fig. 3B, lane 2 is also indicated. Furthermore, direct sequencing of the pGP315 clone insert determined by the dideoxy method, without subcloning the insert (data not shown), revealed that the sequence order of two segments reported in reference 1 (Fig. 1, nt 181 - nt 240 and nt 241 - nt 300) was transposed. This transposition is corrected in this paper (Fig. 2, *italic* sequences nt 694 - nt 753 and nt 754 - nt 813).

A similar recombinant PCR procedure was conducted using a different forward primer in the PCR_3 reaction. P_7 is a 19-mer primer found between nt478 - nt496, upstream of the initiation codon (Fig. 2) in the genomic sequence only. Using P_7 and P_4 as a pair of primers and pGX7 as the template, the resultant PCR product was 77 bp (PCR_4, P_7/P_4) as shown in Fig. 4, lane 3. A successful recombinant PCR sequence between P_7 and P_8 (PCR_5) should yield a recombinant DNA fragment of 576 bp. As visualized in Figure 4, lane 5, when P_7/P_4 and P_1/P_8 fragments are used as templates with P_7 and P_8 as primers, the resultant product is the expected size. The sizes (bp) of PCR products before and after recombination were analyzed by electrophoresis on a 4% Nusieve gel (Fig. 4, lanes 3, 4 and 5). The lower band in Fig. 4, lane 5 is most likely a dimer of the primers used in the PCR_5 reaction.

The reported PvuII restriction site common to both the genomic and cDNA sequences had been modified in pGP315 during the original clone construction. This PvuII site was functionally restored in the recombinant glycophorin DNA molecule as demonstrated by the

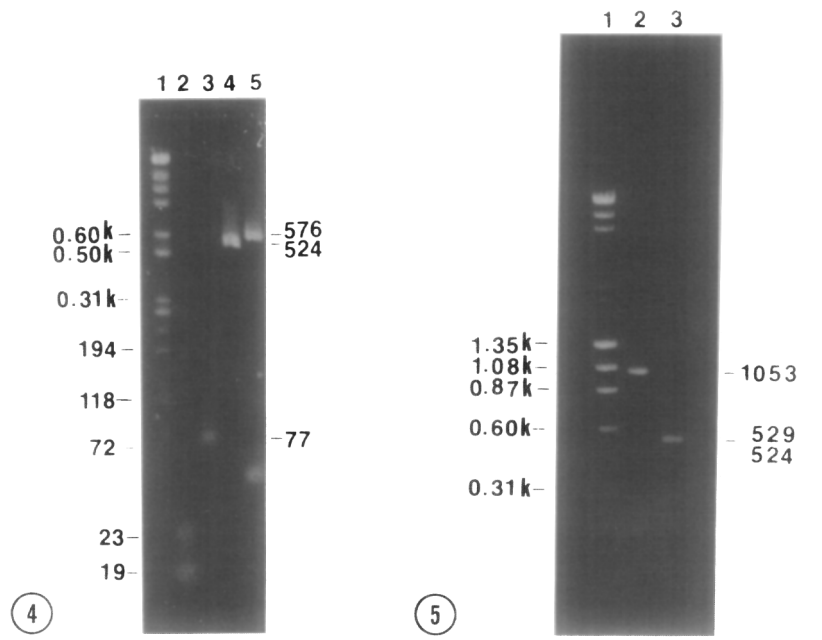

Figure 4. Nusieve gel analysis of a smaller PCR recombinant glycophorin DNA molecule. The size (bp) of PCR products before and after recombination was analyzed on a 4% Nusieve gel as described in Materials and Methods. The size of DNA standards are shown in the left margin and the interpolated sizes of the PCR products in the right margin. Lane 1 - HindIII digested lambda DNA marker combined with HaeIII digested ØX174 RF DNA, plus a 0.5 kb control DNA fragment (Perkin-Elmer); lane 2 - 23-mer and 19-mer DNA size markers; lane 3 - 5 μl (1/10) of the PCR_4 amplified product (77 bp); lane 4 - 3 μl (1/17) of the PCR_2 amplified product (524 bp); lane 5 - 5 μl (1/10) of the PCR_5 amplified recombinant DNA product (576 bp) using the PCR_4 and PCR_2 products combined as templates.

Figure 5. Restoration of the Glycophorin DNA PvuII site. The samples were analyzed on a 1.5% agarose gel as described in Materials and Methods. Lane1 - DNA standards are as described in Fig. 3; lane 2 - the recombinant glycophorin DNA (purified PCR_3 product- 1053 bp) undigested; lane 3 - results of a PvuII digestion (2.5 units/μg DNA) of 70 ng of the recombinant DNA product yields two DNA fragments (524 and 529 bp).

successful PvuII digestion of the 1053 bp fragment. Prior to recombinant PCR, pGP315 had no demonstrable PvuII site. Using the 1053 bp recombinant PCR glycophorin DNA (Fig. 5, lane 2), a PvuII digestion now yields two DNA fragments of 524 bp and 529 bp which are indistinguishable on this agarose gel (Fig 5, lane 3). The presented evidence demonstrating restoration of the PvuII site in the recombinant PCR_3 product is additional confirmation of the success of our modified recombinant PCR procedure and an indicator that this technique is a practical alternative method for splicing DNA fragments having no common restriction site.

In conclusion, up to 10.8 kb DNA fragments have been amplified for single template PCR (10), however, the maximum length of recombinant product which the recombinant PCR technique can amplify is still yet to be defined. From our results, the recombinant mouse glycophorin gene (1053 bp, Fig. 2) has been the longest recombinant construct so far reported in the literature. These genes will be useful in further studies investigating the interaction between glycophorin and murine malaria merozoites.

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