The committed bone marrow megakaryocyte (MK) progenitor undergoes a series of highly regulated stages of development resulting in a large multi-nucleated platelet-producing cell. We studied the development of the mRNA for two α granule proteins, fibronectin (FN) and fibrinogen γ chain (γ-FIB), and a cytoskeletal protein, actin, in MKs from marrow of Sprague-Dawley rats. By the method of in situ RNA:RNA hybridization, we showed that mRNAs for the α granule proteins were expressed most abundantly in a population of 15-μm diameter promegakaryocytes and in cells as small as 10-μm whose identity as immature MKs was inferred by positive staining for platelet- and MK-specific markers. γ-FIB and FN mRNAs were present in reduced abundance in a small proportion of intermediate MKs; however, little or no expression was seen in mature platelet-marrow.

Our results suggest that γ-FIB is expressed in a tissue-specific manner in that the predominant form of the mRNA is synthesized in both marrow and liver, whereas the alternatively spliced γ chain mRNA is expressed only in liver. In the present study, we determine if marrow expression of FN and γ-FIB mRNA is correlated with developmental stages of MKs, comparing FN and γ-FIB with the cytoskeletal protein actin in rat MKs by the technique of in situ hybridization. Our data indicate that FN and γ-FIB mRNAs are transcribed early, while that of actin is transcribed continuously throughout MK development.

MATERIALS AND METHODS

Construction of templates and riboprobe synthesis. All recombinant DNA (rDNA) methodology was performed as described. The probes were made using the pGEM family of RNA transcription vectors from Promega (Madison, WI). The plasmid pY A/B was generated by cloning the fragment encompassing nucleotides 1101 to 2053 of rat γB-FIB cDNA, a kind gift from Dr. Gerald Crabtree, Stanford University, into the pBluescript plasmid. To generate the sense RNA after truncation at the HindIII site, an appropriate restriction endonuclease (see Table 1) and RNA transcript and truncation with EcoRI produced the sense transcript. The rat FN clone pFN-1 was used as a negative control probe. To generate the sense RNA after truncation at the HindIII site, an appropriate restriction endonuclease (see Table 1) and RNA transcript and truncation with EcoRI produced the sense transcript.

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were performed using sterile plasticware. BM was removed in a large plug from the femur and allowed to clot. The clot was sliced into 2- to 3-mm sections and immediately fixed in formalin (10% TES-coated formaldehyde in phosphate-buffered saline [PBS]). The clot was embedded in paraffin, sectioned (5 μm), and mounted onto TES-coated (3-aminopropyltriethoxysilane) slides. Alternatively, the marrow was removed by flushing the cavity with 1 mL of CATCH buffer (25 mmol/L HEPES-buffered [pH 7.0], Ca<sup>2+</sup>-free Hanks-balanced salt solution with 0.38% citrate, 150 mmol/L sodium phosphate, pH 6.0, after cytocentrifugation, and then stained for acetylcholinesterase as described. After resuspending, the cells were fixed in formalin (15°C using Kodak D-19 developer and Kodak rapid fix, counter stained in Meyer’s hematoxylin and eosin, and examined under ultraviolet and darkfield microscopy using a Nikon Optiphot. Slides were photographed using Kodak Ektachrome 100 color slide film (Kodak) or Ektar 125 color print film. The relative abundance of mRNAs was quantitated in the same fashion from photographic slides. Background levels of sense probes were quantitated in the same manner.

**Table 1. Vectors Constructed for In Situ Hybridizations**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Gene</th>
<th>Species</th>
<th>Size Bases</th>
<th>Vector</th>
<th>Enzyme for Truncation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyA+B+</td>
<td>γ-FIB</td>
<td>Rat</td>
<td>1,060</td>
<td>pGEM3</td>
<td>HindIII</td>
</tr>
<tr>
<td>pFN+2</td>
<td>FN</td>
<td>Rat</td>
<td>525</td>
<td>pGEM2</td>
<td>HindIII</td>
</tr>
<tr>
<td>pA+</td>
<td>Actin</td>
<td>Drosophila</td>
<td>1,800</td>
<td>pGEM2</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

Acetylcholinesterase activity staining. Acetylcholinesterase activity has been shown to be a useful marker for rat MKs, particularly immature MKs that cannot be detected with conventional staining methods. Rat marrow was collected in CATCH buffer as described above, fixed in formalin or 10% formaldehyde in phosphate-buffered saline (PBS). The clot was embedded in paraffin, sectioned (5 μm), and mounted onto TES-coated (3-aminopropyltriethoxysilane) slides. Alternatively, the marrow was removed by flushing the cavity with 1 mL of CATCH buffer (25 mmol/L HEPES-buffered [pH 7.0], Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free Hanks-balanced salt solution with 0.38% citrate, 1 mmol/L adenosine, and 2 mmol/L theophylline) to prevent activation and degranulation of MKs. After resuspending, the cells were fixed in 1.5 mL of cytosin collection fluid (Shandon, Pittsburgh, PA). The fixed marrow was applied to TES-coated slides by cytocentrifugation (Shandon cytocentrifuge) at 400 to 450 rpm for 3 minutes at room temperature to form a single layer of cells. Prepared slides were stored in 95% ethanol at 4°C.

**Preparation of antiplatelet antiserum.** Rat blood was collected from the abdominal aorta into 1/9 vol 3.2% sodium citrate. Platelet-rich plasma was prepared by centrifugation of blood at 250g at 10°C for 15 minutes. Platelets were pelleted from platelet-rich plasma at 1,000g, gently resuspended with a sterile plastic pipette in 1 vol HEPES-buffered modified Tyrode’s solution, pH 6.5, without added Ca<sup>2+</sup>, Mg<sup>2+</sup>+, or bovine serum albumin (BSA), then washed three times and resuspended in a final volume of 0.5 mL Tyrode’s buffer. Resuspended platelets from individual rats were emulsified in an equal volume of Freund’s complete adjuvant (Life Technologies, Gaithersburg, MD) and 1 mL was injected subcutaneously in four to six sites along the dorsal side of a 2- to 3-month-old New Zealand white rabbit (Jackson Labs, Bar Harbor, ME). Rabbits were boosted monthly for 3 months then bimonthly for up to 1 year. Rabbit antisera against rat platelets was adsorbed with rat red blood cells (RBCs) and cryoprecipitate, and the adsorbed antisera tested monospecific for rat platelets by immunofluorescence microscopy.

**Immunofluorescence microscopy.** Goat antiplatelet factor 4 was obtained from Atlantic Antibodies (Scarborough, ME), rabbit anti-FIB from Dako Corporation (Carpinteria, CA), and rabbit anti-FN from Behring Diagnostics (La Jolla, CA). Secondary antibodies and immunofluorescence microscopy were as described. Cells were permeabilized with 0.5% Triton X-100 (Sigma, St Louis, MO) for analysis of α-granule proteins, FIB, FN, and platelet factor 4. Cells were not permeabilized for analysis of platelet-specific membrane markers.

**In situ hybridization.** In situ hybridization was as described. All cytosin cell preparations were postfixed in formalin after rehydration from 95% ethanol for retention of mRNAs. The cytosin marrows were not treated with proteinase K; however, clot sections were treated with 1 μg/mL proteinase K. The concentration of probe used was normalized to the smallest probe, which was applied at 0.3 μg/mL/μ probe complexity. Final concentrations of 50% formamide, 0.3 mol/L NaCl, 20 mol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, 1× Denhardt’s (0.02% each BSA, Ficoll, and polyvinylpyrrolidone), yeast tRNA 500 μg/mL, and 10% dextran sulfate were used. After probe application, the hybridization mix was covered with a silanized coverslip and the slides were incubated submerged in mineral oil at 45°C for 14 to 18 hours. After hybridization, excess probe was digested with RNase A and RNase T1, followed by stringency washes at 57°C for cross-species probes, and at 60°C for species-specific probes. The slides were dipped in NBT-2 emulsion (Kodak, Rochester, NY) and exposed in a light tight box for 5 weeks at 4°C. The slides were developed at 15°C using Kodak D-19 developer and Kodak rapid fix, counter stained in Meyer’s hematoxylin and eosin, and examined under fluorescence and darkfield microscopy using a Nikon Fluophot (Nikon, Tokyo, Japan) with an oil darkfield condensor or light and darkfield microscopy using a Nikon Optiphot. Slides were photographed using Kodak Ektachrome 100 color slide film (Kodak) or Ektar 125 color print film. The relative abundance of mRNAs localized to MKs was determined by counting the silver grains from laser copier enlargements of 2 × 2 in photographic slides. Background levels of sense probes were quantitated in the same manner.

**Table 2. Criteria for Staging MKs**

<table>
<thead>
<tr>
<th>MK Stage</th>
<th>Diameter (μm)</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Acetylcholinesterase</th>
<th>α Granule</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>10-14</td>
<td>2 N</td>
<td>Thin rim</td>
<td>+</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>Promegakaryocyte</td>
<td>14-20</td>
<td>4-8 N</td>
<td>Smaller in size</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Intermediate</td>
<td>&gt; 20</td>
<td>Bi- or tri-lobed</td>
<td>than nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mature</td>
<td>&gt; 20</td>
<td>8-64 N</td>
<td>Equal in size</td>
<td>Abundant, platelet-producing</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig 1. Acetylcholinesterase staining of MKs. Marrow cytospin preparations were fixed in glutaraldehyde, then stained for acetylcholinesterase activity. The large MK in (e) (arrow) is characteristic of a fully differentiat ed, platelet-producing MK.

Fig 4. Comparison of the relative abundance of FN, γ-FIB, and actin mRNA in MKs. Marrow clot sections (5 μm) were hybridized with the following probes: FN (pFN+2) (A, E, and F); negative control (B and D); γ-FIB (pγA/B+) (C and G); actin (pA+) (H). The hybridization signal is represented by the dark silver grains. Cells were counterstained with hematoxylin and eosin after photographic development. The amount of probe applied was normalized to account for probe complexity. All panels were photographed under lightfield illumination. In (E), the arrowheads point to immature MKs smaller than 14 μm with little observable cytoplasmic area, as defined in Table 2. In (A) and (B), the small, thick arrowheads point to promegakaryocytes of 15 to 20 μm with an increased rim of cytoplasm. In (B through D, F, and G), the thin arrowheads point to intermediate-staged MKs greater than 20 μm. In (A, B, and D, and F through G), the large, thick arrowheads point to mature MKs with abundant cytoplasmic area.
RESULTS

The MK is morphologically recognizable as such once it has undergone endomitosis. The criteria used in this study to define and identify MKs of increasing stage of maturation are detailed in Table 2. Staining for acetylcholinesterase activity and indirect immunofluorescence microscopy with platelet-specific markers were applied to further identify MKs. The intermediate and mature MKs stained positively (orange-brown) for acetylcholinesterase activity and in addition, cells of 10 to 20 μm were identified as immature MKs on this basis (Fig 1). Platelet α granule proteins (FIB, FN, and platelet factor 4) were stained in immature and promegakaryocytes of 12 to 25 μm (Fig 2a, b, and d) as well as in larger more mature forms (not shown). As expected, fluorescence with rabbit antirat platelet antiserum occurred most clearly in the intermediate (Fig 2f) and mature MKs (Fig 2g) and in platelets (Fig 2e). Smaller nucleated cells did not react with the rabbit antirat antiserum or with monoclonal antibody (MoAb) (7E3) against glycoprotein (GP) IIb-IIIa (a kind gift of Dr Barry Coller, State University of New York, Stonybrook) (not shown). While immature or promegakaryocytes may react positively with some heterologous antplatelet antisera, that used in this study was consistently negative for such cells.

Probes were constructed in RNA transcription plasmids (Table 1) to examine the developmental expression of the mRNAs for FN, γ-FIB, and cytoplasmic actin in rat marrow fixed immediately ex vivo. Mature and intermediate MKs were easily identified by morphology (Table 2) in these cytospin preparations. Cells of 15-μm diameter often demonstrated bi-lobed nuclei using light microscopy (Fig 3c) allowing for their identification as promegakaryocytes. Immature MKs of 10- to 14-μm diameter were not identifiable as such by morphology alone, and their MK lineage was inferred by correlating positive results of in situ hybridization as reflected by an accumulation of silver grains with positive staining for MK-specific markers.

Expression of γ-FIB (Fig 3a through d) and FN (Fig 3f and g) mRNA was detected in a select population of marrow cells of 10 to 35 μm in diameter. Expression of γ-FIB mRNA was most abundant in 15-μm diameter promegakaryocytes with an average density of 26 silver grains per cell (Table 3). Although the density of silver grains in these promegakaryocytes was similar to that in intermediate MKs (Fig 3a), the much smaller cytoplasmic surface area of the promegakaryocytes suggests a greater relative abundance of γ-FIB expression (Table 3). Similarly, FN mRNA expression was detected in the immature to intermediate MKs (Fig 3f and g). All sense strand probes
Fig 3. In situ hybridization of marrow cells. Marrow was fixed in cytospin collection fluid containing ethanol, applied to slides by cytocentrifugation, and postfixed in formalin for retention of mRNAs. The following probes were used: γ-FIB (p−A/B−) (a through d); negative control (pFN-1) (e); FN (pFN+2) (f and g); actin (pA+) (h through j). The hybridization signal is represented by the bright silver grains localized over cells. All panels except (c) and (h) were photographed under darkfield illumination; (c) under lightfield; and (h) under fluorescence. Silver gains under lightfield examination are dark (c); silver grains are not visualized under fluorescence examination (h). The bar in (a) represents 20 μm for all panels except (c) where the bar represents 5 μm.
showed background levels of silver grain density, eg, as indicated with the pFN-1 probe (Fig 3c, remaining sense strand probes not illustrated). Hybridization with the actin probe (pA+) was positive for actin mRNA in MKs of all morphologically identifiable stages of development (Fig 3h and i). Actin mRNA expression increased with progressive maturation of MKs (Fig 3j). The results of in situ hybridization of the marrow cytosin preparations are summarized in Table 4. The data suggests that FN and γ-FIB mRNA expression is inversely correlated with actin expression as the MK matures.

This developmental expression was more apparent when the in situ hybridized marrow clot sections were examined under high magnification using light microscopy (Fig 4, see page 562), which allowed identification of both cell morphology as described in Table 2 and of positive mRNA signal. The relative abundance of mRNAs for FN, γ-FIB, and actin was evaluated in MKs at different stages of maturation. The concentration of probe was normalized to probe length. Immature MKs and promegakaryocytes could be identified by their positive signal for FN (Fig 4 A and E). Expression of FN (Fig 4F) and γ-FIB (Fig 4C and G) was detected in intermediate MKs, but there was little or no expression of these mRNAs in mature MKs (Fig 4 F and G). A clot section was hybridized with the actin probe applied at full saturation to measure the absolute amount of actin mRNA expression in MKs of all morphologically identifiable developmental stages. The results confirmed that the most mature MKs demonstrated the highest levels of actin expression (Fig 5). The relative abundance of the three mRNAs indicates that FN is expressed in 2.5-fold to 3-fold higher levels than γ-FIB, but that both are in lower relative abundance than actin mRNA (Fig 4F, C, G, and H and Table 4).

The hybridization conditions were optimized for detection of mRNA expression in MKs by controlling the degree of proteinase K treatment and the time of exposure to photographic emulsion. A significant amount of FN and γ-FIB expression was observed in or between other marrow cells, but this was significantly less than that in MKs (Table 5). The relative abundance of FN and γ-FIB in MKs was twofold and fivefold greater, respectively, than in non-MK cells of equal (25 μm) diameter. The γ-FIB and FN expression in non-MK areas most likely represents residual mRNA expression in platelets or platelet aggregates, as further supported by a similar pattern of GP IIb mRNA expression in rat marrow clot sections (not shown).

**DISCUSSION**

Platelets contain residual mRNAs and vestigial protein-synthesizing machinery, presumably remnants of the MK cytoplasm. However, it appears that some platelet proteins are synthesized by the MK, while others are adsorbed or endocytosed from plasma into the MK or platelet. It is also unclear at which stage of MK development these proteins are synthesized or acquired. Studies to address the origin of platelet proteins suggest that von Willebrand factor, actin, platelet factor 4, FIB, factor V, and thrombospondin are biosynthesized by the MK, while others are primarily endocytosed from plasma into the MK or platelet such as IgG, albumin, and FIB.

There is some debate as to whether the MK synthesizes FIB, takes it up from plasma, or both. Several groups have demonstrated metabolic labeling and immunopurification of FIB in cultured MKs, in newly formed platelets, and in platelets that lack the FIB membrane receptor (Glanzmann's thrombasthenia) where FIB is synthesized but not stored. In addition, FIB mRNAs have been identified in purified rat MKs. However, Nachman et al found actin but not FIB biosynthesis in MKs. Recent data by Cramer et al suggested that FIB expression in MKs is uncoordinated and occurs at a later stage of development than the expression of other α granule proteins. Their study showed that in cultured MKs, FIB expression is dependent on an exogenous source of FIB. Several different studies suggest that endocytosis is the primary mechanism for appearance of FIB in platelet and MK α granules.

Studies to address these questions are hampered by the difficulties associated with isolation, purification, and culturing of MKs. MKs comprise only about 0.05% of nucleated marrow cells and are difficult to isolate because of their heterogeneous size and fragile membrane. Even though significant advances have been made in the isolation and in vitro culturing of MKs, the use of purified or cultured MKs for studying developmental expression of platelet proteins has three potential drawbacks. First, in vitro culture conditions of MKs may not completely duplicate in vivo requirements for developmental expression. Indeed, serum components have been shown to inhibit MK development in culture. Second, direct use of purified MKs may prevent identification of stage-specific proteins because of the lack of representation of all developmental stages in the purified...
Fig 5. Abundance of actin mRNA in MKs of morphologically identifiable developmental stages. A clot section was hybridized with the actin probe (pA+) applied at full saturation to measure the absolute amount of actin mRNA expression. (A) Lightfield of (B) darkfield illumination. The bar in (A) represents 60 μm in both panels. Arrowheads point to representative intermediate MKs and arrows point to representative mature MKs.

Finally, low abundance mRNAs may be lost by nuclease degradation during purification of MKs and isolation of RNA (personal observation). Therefore, to eliminate the drawbacks of MK purification or the complexity of culture conditions required for MK maturation in vitro, we chose in situ hybridization for direct analysis of MK mRNAs.

We report here that α granule mRNAs encoding FN and γ-FIB are expressed in cells identified as immature MKs and promegakaryocytes using indirect immunofluorescence with platelet-specific markers and staining for acetylcholinesterase activity. The data suggest that immature MKs synthesize α granule mRNAs for FN and γ-FIB early in development, with progressively decreasing amounts in more mature MKs (Table 4). In contrast, the synthesis of actin mRNA continues throughout all stages of MK development and, in particular, at significant levels in the large, fully differentiated MKs. These results indicate that MK mRNAs are synthesized in a developmentally regulated manner. In addition, the abundant expression of actin in fully differentiated MKs suggests that mature MKs are metabolically active for such developmentally specialized proteins.

The appearance of α granules in MKs occurs primarily after DNA synthesis ceases but is initiated in cells still synthesizing DNA. The α granules arise from the Golgi apparatus and the appearance of some α granule proteins is localized to immature granules in the Golgi zone of the MK. We propose that the transcription of α granule protein mRNAs occurs early in MK maturation to allow for translation, packaging, and storage of the protein. Once α granules form, transcription ceases; alternatively, changes in mRNA stability occur such that mRNAs encoding α granule proteins degrade. Actin is a ubiquitous protein involved in cell shape and intracellular movement of organelles and comprises about 20% of total platelet protein. When platelets are activated, shape changes occur which appear to be regulated by contractile proteins including actin, and MKs also undergo shape changes in

Table 5. Comparison of the Relative Abundance (mean ± SE) of FN and γ-FIB in Intermediate MKs (25 μm) Relative to Other Nucleated Marrow Cells

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Silver grains Localized to MK</th>
<th>Silver Grains in or Between Non-MK</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN</td>
<td>12</td>
<td>27.2 ± 3.9</td>
<td>5.4 ± 1.0</td>
</tr>
<tr>
<td>γ-FIB</td>
<td>14</td>
<td>10.6 ± 2.5</td>
<td>4.9 ± 1.0</td>
</tr>
</tbody>
</table>

Non-MK silver grain density was determined from cellular areas that correspond to the surface area of each MK analyzed for FN and γ-FIB.
response to platelet agonists. Because the level of actin expression remains high in the large, fully differentiated MKs, our data suggest that MKs remain biologically active to synthesize contractile proteins that are required for platelet production and activation.

The proposed developmental expression of α granule mRNAs is consistent with the early expression of other α granule proteins in MKs cultured in vitro as determined by Cramer et al. Our report is the first to demonstrate the expression of FN and γ-FIB mRNAs in immature MKs by in situ hybridization. Our findings do not support the mechanism of uptake of FIB by platelet and MK α granules. However, the physiologic relevance of uptake compared with biosynthesis of FIB is unknown, and further, expression of the γ chain alone does not directly prove the synthesis of whole FIB by MKs. We have recently demonstrated that the predominant form of γ-FIB is expressed in rat brain and lung, which is consistent with the ubiquitous expression of the γ chain promoter described by Baumhueter et al. The γ chain is conserved across species but differs significantly from the Aα and Bβ chains. Baumhueter et al have recently demonstrated expression of only rat fibrinogen γ chain mRNA in lung and Aα and Bβ chains in kidney. The manner and physiologic relevance of γ chain expression alone, or its expression in much greater abundance than that of Aα and/or Bβ chains may help to resolve the issue of whether FIB (or γ-FIB only) is synthesized and/or endocytosed by MKs.

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Developmental expression of mRNAs encoding platelet proteins in rat megakaryocytes

MA Courtney, MH Stoler, VJ Marder and PJ Haidaris