Molecular Cloning and Developmental Expression of Mouse p130, a Member of the Retinoblastoma Gene Family*

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With sequence homology to the SV40 T antigen-binding domain of the retinoblastoma protein (Rb), p107 and p130 constitute two additional members of the Rb family. To explore the potential function of p130 in mouse development, we cloned the full-length mouse cDNA for p130 and characterized p130 mRNA expression in mice. The deduced mouse p130 protein sequence shares a higher degree of similarity with mouse p107 than with mouse Rb. In adult mice, p130 mRNA is found in all tissues examined. Levels of p130 mRNA vary among different adult tissues, with the highest level in testis. Within testis, p130 mRNA is found predominantly in Leydig cells. Additionally, p130 expression in testis correlates with sexual maturation, suggesting p130 is important for the development of testis and, in particular, Leydig cells. In situ hybridization shows that in post coitus day 12.5 and 14.5 mouse embryos, distribution of p130 mRNA is quite uniform with the exception of a few tissues. Little differences in mRNA levels of either p130 or p107 were found between normal and Rb-deficient embryos, suggesting that p130 and p107 are expressed independently of Rb. Our data are consistent with the hypothesis that p130 and p107 do not compensate for the loss of Rb and support the view that p130 is related to, yet distinct from, the Rb gene.

The retinoblastoma protein (Rb) family is comprised of Rb, p107, and p130 (1). The gene for human P130 has been cloned through its association with E1A (2), cyclins, and CDK2 (3), and sequence homology to RB and P107 (4). Structurally, P130 contains the SV40 T antigen-binding (T-binding) domain characteristic of the RB protein family, which is composed of subdomains A and B, separated by a spacer region. The protein sequences of P130 and RB are conserved in subdomains A and B and exhibit little similarity outside this region. The P107 protein sequence, on the other hand, shares homology with the P130 sequence over its entire span. The primary structures of the three human proteins suggest that P130 is more closely related to P107 than to RB (2–4).

Like Rb and p107, overexpression of p130 suppresses cell growth in vitro (5, 6). A major target of the Rb family members in inhibiting cell proliferation is the transcriptional factor E2F (7), which regulates expression of many genes important for S phase progression such as DNA polymerase α, dihydrofuran reductase, and thymidine kinase (8, 9). The Rb family proteins bind to E2F and repress E2F’s function as a transcriptional activator. The T-binding domain of the Rb family proteins plays a primary role in their function. Through this domain, Rb, p107, and p130 interact with E2F and other cellular factors. Viral oncoproteins such as SV40 T antigen, adenovirus E1A, and papilloma virus E7 inhibit the function of p130, p107, and Rb by association with the same domain of those proteins, disrupting complexes formed between cellular factors and the Rb family proteins (1).

Despite some shared functional and structural features, p130 possesses certain functions distinct from Rb. The retinoblastoma gene (RB) is frequently mutated in tumors (1). Mutations in the P130 gene, on the other hand, have yet to be documented in human primary tumors. p130 also differs from Rb in its respective binding efficiencies toward distinct E2F family members (10–12). In the glioblastoma cell line T98G, overexpression of p130, but not Rb, inhibits cell growth (6). These observations implicate functional differences between p130 and Rb.

The ubiquitous expression of Rb suggests Rb may function in all cell types (13–15). However, only a limited spectrum of human tumors contains RB mutations. In the Rb-deficient mouse embryos, abnormalities are observed in specific cell types such as developing neurons, erythroblasts, and lens epithelial cells (16–19). The limited spectrum of human tumors with RB mutations and the limited phenotype of RB nullizygous embryos raise the question whether there is functional redundancy among p107, p130, and Rb, such that p130 and p107 could compensate for the loss of Rb in certain cell types. This compensation hypothesis was proposed in an in vitro study (20). In the Rb-deficient skeletal myoblast cell line CC42, p107 levels were elevated compared with C2C12 cells, a myogenic cell line with normal RB. It was then suggested that the up-regulation of p107 partially fulfilled the requirement of Rb for skeletal muscle cell differentiation in vitro. Because skeletal muscle tissue in the Rb-deficient mouse embryos was grossly normal, it is of interest to determine if p107 or p130 acts to compensate for the loss of Rb in skeletal muscle in vivo.

In this study, to examine the functions of p130 in vivo, we cloned the mouse cDNA for p130 and characterized its expression during mouse development. In addition, we also addressed the question whether p107 and p130 acted to compensate for the loss of Rb in the Rb-deficient mouse embryos, conferring grossly normal phenotypes to certain tissues.

EXPERIMENTAL PROCEDURES

Animals—Mouse mating was set up to obtain embryos of the desired stages. Rb nullizygous mouse embryos were generated as described previously (18). The mouse strain used was an outbred C57BL/6
Library Screening and DNA Sequencing—A λZAPII cDNA library from mouse embryos (Stratagene) was screened using human P130 cDNA as probes as described by Sambrook et al. (21). DNA sequencing was performed following the dideoxynucleotide chain termination method of Sanger et al. (22). Both strands of p130 cDNA were sequenced. Protein sequence similarities were analyzed by the Lipman-Pearson method, using DNA Star software, version 1.4.

RNA Extraction, RNase Protection Assay, and Northern Blot Analysis—Total RNA from adult mouse tissues was isolated using a guanidine thiocyanate/acid phenol extraction method (23). Poly(A)-selected RNA from adult tissues was obtained from total RNA by oligo-(dT) affinity chromatography. Antisense RNA probes were made using T7 or T3 RNA polymerase in the in vitro transcription (Promega). 32P-Labeled RNA transcripts of the expected length were gel purified. RNA probes were hybridized to total RNA or mRNA overnight and then digested with RNase A (5 μg/ml) and T1 (100 units/ml) following the manufacturer’s recommendations (Ambion, Austin, TX). Protected fragments were analyzed by electrophoresis in a 5% denaturing polyacrylamide gel followed by autoradiography.

For Northern blot analysis, mRNA were separated by electrophoresis in agarose gels containing formaldehyde. RNA transfer and hybridization were performed as described by Sambrook et al. (21).

In Situ Hybridization—Embryos at various stages and adult mouse testes were placed in ice-cold, freshly prepared 4% paraformaldehyde in phosphate-buffered saline overnight. Paraffin sections were prepared following successive dehydration and embedding in paraffin. In situ hybridization was performed according to Cox et al. (24). Hybridization was carried out at 42°C overnight, followed by washing in 0.1× SSC at 42°C. Autoradiography or color reaction was performed subsequently.

In the study of p130 expression in mouse embryos, the p130 sense and antisense probes were 50-mer oligonucleotides corresponding to

![Fig. 1. Nucleotide sequence of mouse p130 cDNA and the deduced protein sequence.](http://www.jbc.org/Downloaded from)
nucleotides 3146–3195 of p130 cDNA. They were labeled at the 3' end with [α-32P]dATP and TdT to a specific activity of approximately 10^9 cpm/μg and purified through an NENsorb column (DuPont NEN). In the study involving mouse testes, the p130 sense and antisense RNA probes were labeled with digoxigenin-11-UTP. Hybridization signals were visualized following the manufacturer’s suggestions (Boehringer Mannheim), using an alkaline phosphatase-conjugated secondary antibody.

RESULTS

Cloning of Mouse p130 cDNA—To clone mouse p130, we screened a cDNA library from mouse embryos with human P130 DNA probes. Positive phage plaques were isolated, and the cDNA inserts were sequenced. The complete sequence of the largest cDNA insert is shown in Fig. 1. It contains a continuous open reading frame of 3408 nucleotides and encodes a protein of 1135 amino acids with a deduced molecular mass of 127.5 kDa. Comparison of this protein sequence with human P130 indicates that we have isolated the full-length cDNA for mouse p130. The predicted mouse and human P130 protein sequences are 91% identical in their entire span.

Like their human counterparts, mouse p130 shares a higher degree of similarity with mouse p107 than with mouse Rb.
The A and B subdomains of mouse p130 are conserved with those of mouse Rb and p107. Little similarity exists in the N termini, the C termini, and the spacer regions of mouse p130 and Rb. On the other hand, the N termini of mouse p130 and p107 are well conserved, suggesting that this region is important for the function of p130 and p107.

p130 Is Differentially Expressed in Adult Mouse Tissues—We used RNase protection assays to measure p130 mRNA levels in different adult mouse tissues. RB was included in the RNase protection assays as a comparison with p130. GAPDH, a housekeeping gene, was also included as an internal standard. Antisense RNA probes corresponding to nucleotides 2865–3221 of mouse p130 cDNA and nucleotides 2982–3150 of mouse Rb cDNA (14) were used in this study. p130 mRNA was present in all tissues examined, albeit at different levels (Fig. 3). Tissues with relatively high levels of p130 mRNA are lung, thymus, kidney, uterus, and spleen. The highest level of p130 mRNA was found in adult testis. Quantitation by a PhosphorImager revealed that the p130 mRNA level in adult testis is about four times higher than the level in lung, the second highest tissue, whereas the Rb mRNA level in testis is similar to the levels found in several other tissues. Testis remains the tissue with the highest level of p130 mRNA when RNA quantitation was based on 28 S ribosomal RNA (data not shown). This observation suggests that even though p130 mRNA is ubiquitously expressed, its level is subject to regulation by tissue-specific factors.

Cell Type and Developmental Stage-specific Expression of p130 in Testis—The high level of p130 mRNA in adult testis suggests the importance of p130 for this organ. We used Northern blots to characterize p130 expression in testis development (Fig. 4). Two distinct bands corresponding to approximately 4.7

\[ \text{Fig. 6. Expression of p130 mRNA in mouse embryos from gestation day 9.5 to 16.5. mRNA was extracted from whole embryos, and its p130 contents were examined by RNase protection assay. Probes against p130 and GAPDH were added together to the hybridization mixtures.} \]

\[ \text{Fig. 7. In situ hybridization of p130 on RB heterozygous (RB } +/- \text{) and RB nullizygous (} -/- \text{) mouse embryos. A, E12.5, RB } +/- \text{; B, E12.5, RB } -/- \text{; C, E14.5, RB } +/- \text{; D, E14.5, RB } -/- \text{; E, E14.5, RB } +/- \text{. Antisense p130 probes were used in A, B, C, and D. In E, in situ hybridization was performed with sense p130 probes as a negative control. Abbreviations: co, colon; h, heart; hb, hindbrain; ie, inner ear; k, kidney; lu, lung; lv, liver; sc, spinal cord; tg, trigeminal ganglion. RB } +/- \text{ embryos are grossly normal and phenotypically indistinguishable from wild type embryos.} \]
and 2.1 kb, respectively, were observed in testes from 25-day and 83-day-old mice. The 4.7- and 2.1-kb bands could represent differentially spliced forms of p130 mRNA. The levels of p130 mRNA were very low in testes from 5-day, 8-day, and 15-day-old mice. In contrast, the levels of both the 4.7- and 2.1-kb species were greatly elevated in testes from 25-day and 83-day-old mice. Thus, p130 is developmentally regulated in testis. p130 mRNA expression in testis correlates with sexual maturation.

To identify the cell types in testis that express mRNA for p130, we performed in situ hybridization on adult testis. Intense cytoplasmic staining was found in the interstitial Leydig cells that were located between seminiferous tubules (Fig. 5). Thus the Leydig cell population is the predominant source of p130 mRNA in testis.

Expression of p130 mRNA in Developing Mouse Embryos—We examined the expression of p130 in midgestation mouse embryos by RNase protection assay and in situ hybridization. Similar levels of p130 mRNA were detected in embryos from gestation day 9.5 to 16.5 (Fig. 6). To identify the specific tissues that express p130 mRNA, we performed in situ hybridization on E12.5 and E14.5 mouse embryos (Fig. 7, A and C). p130 mRNA was expressed ubiquitously in embryos of both stages. In E12.5 embryos, slightly higher levels of p130 mRNA were found in the ventricular zone of the brain, trigeminal ganglion, heart, and dorsal spinal cord. In E14.5 embryos, p130 mRNA distribution was relatively uniform.

p130 and p107 mRNA Are Not Up-regulated in Rb-deficient Mice—The RB nullizygous mouse embryos die on or before gestation day 15.5. Developmental abnormalities in the erythroblast lineage, the nervous system, and lens epithelial cells were evident in RB nullizygous embryos of gestation day 12.5–15.5. Other tissues, however, were phenotypically normal (16–19). To examine if p130 and p107 could compensate for the loss of Rb in Rb-deficient embryos by up-regulating their expression and thus reduce the severity of the phenotype, we compared p130 and p107 mRNA levels in RB nullizygous embryos of gestation day 12.5–14.5 with the levels in age-matched wild type and RB heterozygous embryos. RNase protection assays showed that p130 and p107 mRNA levels were not increased in the head, trunk, limb, or liver of the RB nullizygous embryos compared with their counterparts in wild type embryos (Fig. 8). In situ hybridization revealed no apparent differences in p130 mRNA levels between the phenotypically normal RB heterozygous embryos and RB nullizygotes in tissues that are either affected by RB mutations, such as the nervous system, or tissues that are not affected by RB mutations, such as lung and heart (Fig. 7, A and B; C and D). p107 mRNA levels were also very similar between RB nullizygous mouse embryos and normal mouse embryos of gestation day 12.5–14.5 when examined by in situ hybridization (data not shown). These data indicate that p130 and p107 do not compensate for the loss of Rb by up-regulating their mRNA levels.

**DISCUSSION**

In this study, we cloned the cDNA for mouse p130 and characterized p130 mRNA expression in mouse development. Similar levels of p130 and p107 mRNA were observed between RB nullizygous embryos and normal embryos in tissues that are not affected by RB mutations, suggesting that the limited phenotypic defects seen in RB nullizygous mouse embryos could not be due to compensation by p107 or p130. Our results indicate that p130 and p107 mRNA levels are regulated independently of Rb in embryogenesis.

Sequence comparison of the three mouse proteins Rb, p107, and p130 reveals that mouse p130 is more closely related to p107 than to Rb. Conservation between p130 and Rb is limited to the A and B subdomains, while p130 and p107 are conserved in their entire length. Similar conclusions on their three human counterparts were also drawn (2–4). The sequence information suggests p130 and p107 may serve unique functions and do not overlap entirely with Rb functions. This view is reinforced by several observations. First, p130 and p107 both associate with E2F-2, and E2F-3 (11, 25). Second, the RB family members restrain E2F-2, whereas RB associates with E2F-1, E2F-2, and E2F-3 (11, 25). Second, the RB gene is mutated in many tumors. In contrast, no mutations in the RB gene or the p130 gene have been reported in human primary tumors (1–7). Third, we did not observe an apparent compensation of p130 and p107 for loss of Rb during mouse development. Fourth, mice deficient in p130 or p107 were viable, in contrast to the embryonic lethality caused by RB gene mutations. Schneider et al. (20) reported that the RB-deficient mouse skeletal myoblast cell line CC42 cells expressed elevated levels of p107 compared with C2C12 cells, a myogenic cell line with wild type Rb (20). The authors proposed that the increased levels of p107 acted to assume certain functions of RB to allow myogenesis to occur. In our study, we did not detect elevated mRNA levels of p107 and p130 in the skeletal muscles of RB nullizygous mouse embryos. The discrepancy in observations made by us and Schneider et al. (20) could reflect differences between in vivo and in vitro conditions.

The expression of p130 in testis is of interest. Testis was the one adult tissue that expressed the highest levels of p130 mRNA. A significant portion of p130 mRNA in testis was from Leydig cells. Additionally, expression of p130 was greatly increased in sexually mature mice relative to prepubescent mice. Kim et al. (26) observed the highest level of mouse p107 mRNA in adult testis. We compared levels of p130, p107, and Rb

![Fig. 8](http://www.jbc.org/Downloaded from http://www.jbc.org by guest on July 25, 2018)
mRNA in adult mouse testes with 9-day-old mouse testes. Levels of p107 and Rb mRNA, unlike p130 mRNA, were only modestly higher in adult mouse testes (data not shown). Our data suggest p130 may play important functions in tests development and, in particular, Leydig cells.

A 4.7-kb band and a 2.1-kb band were seen in the p130 Northern blots using testsis mRNA. The two transcripts, which were also present in other adult tissues (data not shown), were likely the results of alternative splicing, given the precedence of alternative spliced forms of Rb and p107 mRNA (14,26). The functional significance of the shorter transcripts of the three mouse RB family genes is currently unknown. Observations made in this study support the notion that the functions of Rb and p130 are distinct. The exact roles of p130 in vivo are not yet clear and cannot be simply extrapolated from those of Rb. The molecular cloning of mouse p130 cDNA and characterization of p130 mRNA expression pattern should facilitate future studies on the function of p130.

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Note Added in Proof—During the review process of the manuscript, a study describing the murine p130 cDNA was published (Pertile, P., La Thangue, N. B. (1994) Curr. Opin. Cell Biol. 6, 443–450).

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