Retinoblastoma Gene Promoter Directs Transgene Expression Exclusively to the Nervous System*

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In human, germ line mutations in the tumor suppressor retinoblastoma (Rb) predispose individuals to retinoblastoma, whereas somatic inactivation of Rb contributes to the progression of a large spectrum of cancers. In mice, Rb is highly expressed in restricted cell lineages including the neurogenic, myogenic, and hematopoietic systems, and disruption of the gene leads to specific embryonic defects in these tissues. The symmetry between Rb expression and the defects in mutant mice suggest that transcriptional control of Rb during embryogenesis is pivotal for normal development. We have initiated studies to dissect the mechanisms of transcriptional regulation of Rb during development by promoter lacZ transgenic analysis. DNA sequences up to 6 kilobase pairs upstream of the mouse Rb promoter, isolated from two different genomic libraries, directed transgene expression exclusively to the developing nervous system, excluding skeletal muscles and liver. Expression of the transgene in the central and peripheral nervous systems, including the retina, recapitulated the expression of endogenous Rb during embryogenesis. A promoter region spanning ~250 base pairs upstream of the transcriptional starting site was sufficient to confer expression in the central and peripheral nervous systems. To determine whether this expression pattern was conserved, we isolated the human Rb genomic region and generated transgenic mice expressing lacZ under control of a 1.6-kilobase pair human Rb promoter. The human Rb promoter lacZ mice also expressed the transgene primarily in the nervous system in several independent lines. Thus, transgene expression directed by both the human and mouse Rb promoters is restricted to a subset of tissues in which Rb is normally expressed during embryogenesis. Our findings demonstrate that regulatory elements directing Rb expression to the nervous system are delineated within a well-defined core promoter and are regionally separated from elements, yet to be identified, that are required for expression of Rb in the developing hematopoietic and skeletal muscle systems.

The tumor suppressor Rb1 was originally identified as a gene that predisposes individuals to retinoblastoma in infants and osteosarcoma and other malignancies in later stages of life (1). Rb was subsequently found to be frequently inactivated in human cancers either by mutations in the gene itself or by alterations in upstream factors that control the activity of the protein (2). During most of the G1 phase of the cell cycle, hypo-phosphorylated Rb inhibits progression into S phase by forming specific complexes with certain transcription factors. One of the main partners of Rb, E2F1, regulates expression of genes required for the transition into S phase and DNA synthesis (3). Mitogenic signals are propagated through the G1 cyclins and cyclin-dependent kinases, which phosphorylate and inactivate Rb, thereby allowing progression into S phase (4). Differentiation signals inhibit the G1 cyclins and associated cyclin-dependent kinases, thereby maintaining Rb in the under-phosphorylated, active state. Active Rb is required for permanent withdrawal from the cell cycle and suppression of cell death during the onset of differentiation (5–7). There is also evidence that under-phosphorylated Rb can interact with differentiation factors such as MyoD and myogenin during myogenesis (8) and C/EBPβ during adipogenesis (9) and promote transcriptional activation of responsive genes (10).

Another major mechanism of regulation of Rb is at the transcriptional level. Although initial analysis indicated that Rb is ubiquitously expressed in adult mouse tissues (11), subsequent studies revealed that Rb is up-regulated during differentiation of various cell types in vitro (12, 13). By using in situ hybridization analysis, we have shown (14) that levels of Rb transcripts are temporally and spatially regulated during embryogenesis. High expression of Rb is restricted to a subset of tissues during development including the nervous system, hematopoiesis, skeletal muscles, and lens. The pattern of Rb expression correlates very well with the phenotype of Rb mutant mice. Rb knockout mice die at embryonic day 13–14 with specific defects in cell cycle exit, cell survival, and terminal differentiation during neurogenesis, hematopoiesis, and lens development (15–17). A defect in terminal myogenesis was also observed in Rb mutant embryos partially rescued to birth by a Rb minigene composed of the mouse Rb promoter and Rb cDNA (7, 18). Interestingly, whereas the mouse Rb minigene only suppresses the Rb−/− neurogenic defects, a human Rb minigene could completely rescue the Rb−/− phenotype (16, 20).

The correlation between Rb expression and the phenotypes

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1 The abbreviations used are: Rb, retinoblastoma; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; lacZ, β-galactosidase; E, embryonic day; kb, kilobase pairs; bp, base pairs; 3′-UTR, 3′-untranslated region; 5′-CR, 5′ conserved region; CP, core promoter; HS, hypersensitive site.
of Rb mutant embryos suggests the existence of a mechanism that ensures that Rb is expressed in the appropriate tissues when cells receive signals to exit the cell cycle and terminally differentiate. Analysis of transcriptional regulation of Rb during embryogenesis may unravel this developmental program. Here, we initiated studies to dissect the transcriptional regulation of Rb during embryogenesis. We show that two independent DNA segments containing the mouse Rb promoter can direct expression of a linked reporter gene, lacZ, to the nervous system, excluding the myogenic and hematopoietic systems. A region of ~250 bp upstream of the transcription starting site is sufficient to confer lacZ expression during neurogenesis in the central and peripheral nervous systems including the retina. Transgene expression under control of the human Rb promoter was also detected primarily in the nervous system in mouse transgenic lines. These findings delineate the promoter region required for expression of Rb during neurogenesis and indicate that different mechanisms, yet to be defined, govern Rb expression during hematopoiesis and skeletal myogenesis.

EXPERIMENTAL PROCEDURES

Isolation of Mouse and Human Rb Promoter Genomic Regions—A genomic phage library, generated in AFixII from mouse 129/ev DNA (purchased from Stratagene), was screened by plaque lift hybridization, using nylon membranes (DuPont) as recommended by the manufacturer. A BglII-SfiI fragment from the mouse Rb promoter was used as a probe. A recombinant phage, \( \lambda-129-1 \), was plaque-purified, and the phage DNA was extracted using Qiagen kits. The physical map of \( \lambda-129-1 \) was determined by multiple digestions with restriction enzymes. A 6-kb SfiI fragment was subcloned into pBluescript, yielding a polymerase chain reaction product of 649 bp.

A human placenta genomic library (purchased from CLONTECH) was screened with a 455-bp non-NC region from the human genomic Rb promoter as a probe (21, 22), and several recombinant phages were isolated and purified. A 4.5-kb BamHI fragment containing the human Rb promoter region was identified by Southern blot of BamHI-digested recombinant phage and was subcloned into pBluescript (pHR.B-B). This BamHI fragment included 2-kb human Rb promoter and approximately 2.5 kb of the first exon and part of the first intron. Physical mapping and DNA sequencing verified that this DNA fragment contained the human Rb promoter.

Generation and in Vitro Verification of Transgenic Constructs—To create lacZ transgenic constructs, we generated a plasmid pEZ25/26, a derivative of pBluescript, in which the Saci-KpnI linker of pBS was replaced by a linker with following sites: SpeI, XhoI, NotI, NcoI, BamHI, SpeI. The NotI site, located at the N terminus of lacZ in this construct, pEZ25/26, is in frame with the NotI and EagI sites in the mouse and human Rb promoters, respectively. A lacZ-SV40 poly(A) cassette was introduced between the NcoI and BamHI sites yielding pEZ25/26.lacZ. To create mRbP(129).lacZ, a 4.5-kb Rb promoter region, previously isolated from mouse L-cell genomic library (23), was subcloned from the recombinant phage into pEZ25/26.lacZ. To create pmRbP-(129).lacZ, the NotI fragment from pmRbP(Not-Not) was subcloned into a NotI-linearized pEZ25/26.lacZ. The SfiI deletion was made by cutting with SfiI/1 (50 °C) followed by XhoI (at 37 °C), blunt ending with Klenow, and re-ligation. To create hrRbP.lacZ, an XhoI-EagI fragment was purified from phRb.B-B and subcloned into XhoI plus NotI-digested pEZ25/26.lacZ. Transgene plasmid DNA was purified on Qiagen mini-columns. COS7 cells cultured in Dulbecco’s modified Eagle’s medium plus 10% serum in 60-mm dishes were transfected with 8 μg of plasmid DNA using the calcium-phosphate method, as described (24). Twenty-four hours after transfection, the medium was aspirated, and the cells were fixed for 5 min in 4% paraformaldehyde (PFA) and treated with X-gal solution as described (25). Intensive X-gal staining confined primarily to the cytoplasm was observed microscopically within 1–2 h.

Generation and Analysis of Transgenic Mice—To release the insert DNA fragments, pmRbP(129).lacZ, pmRbP-(129).lacZ, and pmRbP.(129).lacZ were digested with SpeI and phRbP.lacZ with BamHI. The DNA inserts were extracted from agarose gels with a Qiagen kit and treated on Elutip-D columns (Schleicher & Schuell) from Xymotech. Microinjection of DNA and generation of chimeric mice were done in the transgenic mouse facilities at the Hospital for Sick Children and the Ontario Cancer Institute. Transgenic mice were initially identified by Southern blot hybridization and later genotyped by polymerase chain reaction. lacZ was amplified with a forward primer EZ74 (5’ GTT CCG TCA TAG CGA TAA CG) and reverse primer EZ64 (5’ TCA ATC CGG TAG GTT TTC CG), yielding a polymerase chain reaction product of 649 bp.

Analysis of Embryos and X-Gal Staining—For timed pregnancy and embryo staging, the mornings of vaginal plug observations were considered as E0.5. Pregnant females were sacrificed by cervical dislocation, the embryos were retrieved and fixed in 4% PFA for 30 min to 2 h depending on the developmental stage as described (25). The embryos were washed several times in phosphate-buffered saline and treated overnight with X-gal solution as in Ref. 25. Stained embryos were photographed under a stereomicroscope (Leica). To identify internal organs, the embryos were dissected sagittally and examined microscopically. Some embryos were dehydrated, embedded, and sectioned at 20

FIG. 1. A mouse Rb promoter from L-cells directs transgene expression exclusively to the nervous system. a, a schematic structure of the mRbP minigene containing 4.5 kb of mouse Rb promoter isolated from mouse L-cells, the Rb cDNA, and a human growth hormone sequence and polyadenylation site at the 3' end. b, the mRbP(L).lacZ transgene contains the same Rb promoter region fused to the bacterial β-galactosidase gene (lacZ) with an SV40 polyadenylation site at the 3' end. c–e, mRbP(L).lacZ transgenic mice express lacZ exclusively in the nervous system as visualized by staining with X-gal (blue). c, expression of mRbP(L).lacZ in the brain and spinal cord in a E12.5 embryo. d, expression of mRbP(L).lacZ in vibrissae in an E13.5 embryo. e, sagittal sections of a E12.5 mRbP(L).lacZ transgenic embryo reveal X-gal staining in the spinal cord (top) and dorsal root ganglia (DRG, bottom) but not in skeletal muscles such as the tongue (T) muscles. Abbreviations: SC, spinal cord; Vi, vibrissae; 4V, fourth ventricle; T, tongue; DRG, dorsal root ganglia.
Sections were deparaffinized with xylene, rehydrated with ethanol, counter-stained with eosin, dehydrated again, and mounted with Permount (Fisher).

In Situ Hybridization—In situ hybridization analysis for Rb was performed as described (14). The Rb plasmid DNA was linearized with Bgl II, and 35S-labeled antisense riboprobe was prepared with T7 polymerase and used at 2 \times 10^{5} \text{ cpm}/ml.

DNase I-hypersensitive Analysis—Brains and carcasses were recovered from staged embryos. Nuclear preparation, chromatin digestion with DNase I, and DNA extraction were performed as described (26). DNA samples were further digested with Sac I or Bgl II to detect the CP or 5'-CR, respectively, and analyzed by Southern blot hybridization using dextran sulfate and GeneScreen Plus nylon membranes as recommended by the supplier (Dupont) as described (23).

RESULTS

Mouse Rb Promoter Region from L-cell Genomic Library Directs Transgene Expression Exclusively to the Nervous System—We previously described the isolation and in vitro characterization of a mouse Rb promoter region isolated from a mouse L-cell genomic library (23). To explore the expression pattern of the Rb promoter during embryogenesis, the promoter (L) was placed upstream of the Escherichia coli \( \beta \)-galactosidase (lacZ) reporter gene, and five mRbP(L).lacZ transgenic founders were created (Fig. 1; Table I). F1 transgenic males were then used to set up timed pregnancies with wild type females to obtain staged embryos. X-gal staining of E13.5 mRbP(L).lacZ embryo revealed expression in the nervous system but not in developing muscles or liver. 4V, fourth ventricle.

Table I: Differential expression of endogenous Rb versus mouse and human Rb promoter transgenes

| Line                  | CNS | PNS | Retina | Vibrasea | Liver | Muscles | Others |
|-----------------------|-----|-----|--------|----------|-------|---------|--------|
| mRbP(L).lacZ 19       | +   | +   | +      | -        | -     | -       | -      |
| 37                    | +   | +   | +      | -        | -     | -       | -      |
| 5                     | +   | +   | +      | -        | -     | -       | -      |
| 27                    | +   | +   | +      | -        | -     | -       | -      |
| 29                    | +   | +   | +      | -        | -     | -       | -      |
| mRbP(129).lacZ 10      | +   | +   | +      | -        | -     | -       | -      |
| 2                     | +   | +   | +      | -        | -     | -       | -      |
| mRbPΔSfi.lacZ 3        | +   | +   | +      | -        | -     | -       | -      |
| 7                     | +   | +   | +      | -        | -     | -       | -      |
| 16                    | +   | +   | +      | -        | -     | -       | -      |
| hRbP.lacZ 5            | Low | Low | Low    | -        | Low   | Some    | Heart, kidney, skin |
| 15                    | Low | Low | Low    | -        | Low   | Some    | ID, PS, Int.     |
| 22                    | +   | +   | +      | -        | -     | Low     | Low hear, ID    |
| 7                     | +   | +   | +      | -        | -     | Some    | Kidney, ID      |
| 21                    | +   | +   | +      | -        | -     | -       | Kidney, ID      |

Endogenous Rb

Expression of endogenous Rb was determined by in situ hybridization (Fig. 3 and see Ref. 14). Expression of mouse and human Rb transgenes in the indicated lines was determined by X-gal staining (see Figs. 1–3, 5, and 6). CNS, central nervous system; PNS, peripheral nervous system; ID, intervertebral disk; PS, palate shelf; Int., intestine.

FIG. 2. A mouse Rb promoter isolated from 129/sv genomic library directs transgene expression exclusively to the nervous system. a, schematic presentation of a recombinant phage, \( \lambda \)-129-1, encompassing the Rb first exon, promoter region, and first intron. The \( \lambda \)-129-1 clone was isolated from a mouse 129/sv tissue library using the indicated BglII-SfiI genomic fragment from the mouse Rb promoter (L) as a probe. b, schematic presentation of the mRbP(129).lacZ transgene. c, whole mount X-gal staining of E13.5 mRbP(L).lacZ embryo. d, whole mount X-gal staining of E13.5 mRbP(129).lacZ embryo. Note the apparent identical X-gal staining in the mRbP(L).lacZ and mRbP(129).lacZ lines. Positive staining was observed in the ganglion layer of the retina (R), cranial nerves such as the trigeminal ganglia (Tg), dorsal root ganglia (DRG), the vibrissae (Vi), and throughout the brain and spinal cord (SC). No expression was observed in the liver (L) or skeletal muscles where endogenous Rb is normally expressed. e, a mid-sagittal dissection of E13.5 mRbP(129).lacZ embryo revealing expression in the nervous system but not in developing muscles or liver. 4V, fourth ventricle.
and 5 transgenic embryos revealed exclusive X-gal staining in the central (brain, spinal cord, Fig. 1e, top) and peripheral (dorsal root ganglia, Fig. 1e, bottom) nervous systems. X-gal staining was also detected in the follicles of vibrissae (Fig. 1d) where endogenous Rb is normally expressed (14). In contrast to endogenous Rb, no X-gal staining was detected in the liver or any muscle mass throughout embryogenesis (Fig. 1, c and e, top; Table I).

The expression pattern of the mRbP(L).lacZ transgenes is consistent with the phenotypes of the partially rescued mRbRb: Rb−/− fetuses (7, 18, 27). In these fetuses, the neurogenic, but not hematopoietic, skeletal muscle and lens defects in Rb−/− embryos are rescued by an Rb minigene containing the same mouse Rb promoter (L) fused to the mouse Rb cDNA (Fig. 1a). The mRbP:Rb−/− embryos survive to birth and exhibit aberrant myogenesis and other developmental defects, whereas the neurogenic phenotype is specifically suppressed (7, 18, 27).

Mouse Rb Promoter Region from 129/sv Genomic Library Also Directs Transgene Expression Exclusively to the Nervous System—The specific expression of the mouse Rb promoter (L) in the nervous system could reflect the absence of essential regulatory elements for expression in other tissues. Alternatively, this promoter DNA from a mouse L-cell genomic library could hammer mutations, deletions, or rearrangements that might have disrupted important regulatory elements required for faithful expression of the transgene. To address these issues, we re-isolated the mouse Rb promoter region from a 129/sv-mouse tissue genomic library (Stratagene) (Fig. 2a). A 6-kb Rb promoter fragment from the recombinant phage was subcloned into a lacZ cassette, yielding mRbP(129).lacZ (Fig. 2b), and three transgenic founders mRbP(129).lacZ 1, 2, and 10 were created (Table I). Whole mount X-gal staining revealed that the three independent transgenic lines expressed lacZ exclusively in the nervous system, excluding the liver and skeletal muscles, in a manner that was indistinguishable from the mRbP(L).lacZ lines (Fig. 2, c versus d and e; Table I).

The initial X-gal analysis was performed at E13.5 when endogenous Rb expression is maximal (14). To determine the temporal expression pattern of the mRbP(129).lacZ transgene during embryogenesis, embryos were collected from E9.5 to E17.5 and subjected to whole mount X-gal staining followed by cross-section analysis. The patterns of X-gal staining were then compared with endogenous Rb expression at similar developmental stages as detected by in situ hybridization analysis (Fig. 3). This comparison revealed that independent mRbP(129).lacZ lines expressed lacZ in the nervous system in a pattern that recapitulated the expression of endogenous Rb during neurogenesis. Specifically, both endogenous Rb and the mRbP.lacZ transgenes were expressed in the developing neural tube and brain folds, the spinal cord, dorsal root ganglia, other cranial nerves, and the ganglion layer of the retina (Fig. 3 and Table I). Even in younger or older embryos, there was no evidence of X-gal staining in the liver and skeletal muscles where Rb is normally expressed, suggesting that regulatory regions required for expression of Rb in these tissues are located outside the first 6 kb of the mouse Rb promoter.

Identification of Homologous DNA Sequences and DNase I-hypersensitive Sites around the Rb Promoter—We next sought to identify regulatory DNA sequences that might be involved in controlling Rb expression during neurogenesis. The mouse Rb core promoter (CP) is located about 200 bp upstream of the translation initiation codon and overlaps the major transcription starting site (Fig. 4a) (23). The human and mouse Rb core promoter sequences are virtually identical (21, 23). They both lack characteristic TATA and CAAT elements and contain consensus-binding sites for Sp1, ETS, ATF, and E2F (23, 28, 29).

Analysis of natural mutations in low penetrant retinoblastoma patients (30) and mutagenesis in vitro (23) revealed that the Sp1, ETS, and ATF consensus sites are positively required for transcription initiation and for faithful expression of the transgene. To address these issues, we re-isolated the mouse Rb promoter region from a 129/sv-mouse tissue genomic library (Stratagene) (Fig. 2a). A 6-kb Rb promoter fragment from the recombinant phage was subcloned into a lacZ cassette, yielding mRbP(129).lacZ (Fig. 2b), and three transgenic founders mRbP(129).lacZ 1, 2, and 10 were created (Table I). Whole mount X-gal staining revealed that the three independent transgenic lines expressed lacZ exclusively in the nervous system, excluding the liver and skeletal muscles, in a manner that was indistinguishable from the mRbP(L).lacZ lines (Fig. 2, c versus d and e; Table I).

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was found in the CP in brain and carcass tissues from E13.5 (Fig. 4, b–d) and E17.5 (not shown) embryos. This region consisted of a major HS band corresponding to the CP as well as two smaller HS fragments, indicating the presence of destabilized nucleosomal regions within and immediately upstream of the CP (Fig. 4d). Two additional bands of ~1.5–2.5 kb were identified, suggesting the existence of HS sites within the first intron of Rb. In contrast, analysis of the 5'-CR and upstream DNA sequences revealed no obvious HS regions in chromatin extracted from E13.5–E17.5 brain tissues (data not shown). A very faint signal in the predicted location of the 5'-CR was detected in nuclei isolated from carcasses of E13.5 embryos (data not shown). Thus, the main HS region in the Rb promoter appears to surround the CP; the 5'-CR may be important for certain cell lineages in carcasses or other embryonic or adult tissues.

Sequences Required for Expression of Rb in the Nervous System Are Independent of the 5'-CR and Confined to the First 250 bp Upstream of the Transcriptional Starting Site—The presence of multiple HS sites around the Rb core promoter but not at the 5'-CR, suggested that the core promoter alone may direct transgene expression to the nervous system, independently of the 5'-CR. To examine this possibility directly, we generated a deletion construct, mRb\_DSfi.lacZ, in which the 5'-CR and most upstream DNA sequences were removed (Fig. 5a). Five mRb\_DSfi.lacZ transgenic founders were generated, three of which expressed the transgene (numbers 3, 7, and 16). The mRb\_DSfi.lacZ 16 transgene was expressed in the nervous system as well as other tissues such as isolated groups of muscles and the heart (Table I). In contrast, both mRb\_DSfi.lacZ 3 and 7 lines expressed lacZ only in the nervous system in a pattern that appeared identical to mRbP\_129.lacZ and mRb-FIG. 4. Conserved regions and DNase I-hypersensitive sites in the Rb promoter. a, human and mouse Rb promoters share two regions of high homology. The core promoter (CP) contains consensus-binding sites for Sp1, ETS, ATF, and E2F. Mutation in these elements established their importance in vitro (23, 29). Mutation in the Sp1 and ATF sites were identified in low penetrance retinoblastoma patients (30). The 5'-conserved region (5'-CR) contains consensus sites for bHLH and MYB factors. There are also bHLH, GATA, and NF\_kB sites in the two promoters in non-conserved regions adjacent to the 5'-CR. As indicated, sequence analysis of the 5'-CR identified an A to G difference in the 129\_sv sequence compared with the published sequence of mouse L cells (23), which increases the homology between the human and mouse 5'-CR. b–d, DNase I-hypersensitive analysis of the mouse Rb core promoter in carcass and brain tissues from E13.5 embryos. Chromatin was partially digested with increasing amounts of DNase I, and the DNA was purified, digested with SacI, and Southern-blotted onto nylon membranes. The blots were hybridized with a 32P-labeled SacI-BglII probe shown in d. Identical results were obtained in other experiments in which a PCR fragment of 364 bp upstream of the BglII site was used as a probe (not shown). A major DNase I-hypersensitive region was found at about 1.2 kb downstream of the SacI site corresponding to the core promoter of Rb. Additional HS sites were detected upstream and downstream (within the first intron) of the core promoter.
P(L).lacZ transgenic mice (Fig. 5, b–i). At various developmental stages, the mRbΔSfi.lacZ 3 and 7 transgenes were detected in the central and peripheral nervous systems, follicles of vibrissae, and retina but not in liver, skeletal muscles, or other tissues (Fig. 5, b–i). Thus, the Rb 5′-CR is dispensable for expression in the nervous system, and a segment of 450 bp encompassing the Rb core promoter contains all the regulatory elements required to direct Rb expression to the central and peripheral nervous systems during embryogenesis.

We note that the relative levels of transgene expression within the nervous system varied among and within several lines. For example, mRbΔSfi.lacZ 7 showed weak expression in the follicles of vibrissae, whereas mRbΔSfi.lacZ 3 was highly expressed in this tissue (Fig. 5, c and i); within the mRb(129).lacZ 10 line, some embryos exhibited intense staining in the vibrissae, whereas other embryos, in the same litter, showed little expression (Table I and data not shown).

The Human Rb Promoter Region Directs Transgene Expression Primarily to the Nervous System—The exclusive expression pattern of the mRbP.lacZ transgenes in the nervous system (Figs. 1–3 and 5) is consistent with our observation that the Rb 5′-CR directs expression in the developing nervous system to the central and peripheral nervous systems, follicles of vibrissae, and retina like the mRbP.lacZ lines (Fig. 6, c–m, and Table I). Three of these transgenic lines (numbers 7, 21, and 22) exhibited X-gal staining primarily in the nervous system like the mRbP.lacZ lines (Fig. 6, c–i). The 21 and 7 lines also exhibited some expression in the kidney, and number 22 was expressed at low level in the heart. The hRbP.lacZ 15 line exhibited high level of expression in the retina and low levels in the central and peripheral nervous systems (Table I). The hRbP.lacZ line 5 was expressed in the spinal cord, the cartilage of palate shelf, intervertebral disks, intestines, and isolated groups of skeletal muscles (Fig. 6, j–m, and data not shown). The expression pattern of hRbP.lacZ 5 may represent activation of cryptic elements in the human Rb promoter that was used to rescue the Rb−/− lethal defect (32). However, the hRbP.lacZ minigene utilizes an SV40 polyadenylation signal (Fig. 6b), whereas the human Rb minigene contains a β-globin cassette at the 3′ end (32).

Five transgenic hRbP.lacZ lines were established, and the expression patterns of lacZ were determined during embryogenesis (Fig. 6, c–m, and Table I). Three of these transgenic lines (numbers 7, 21, and 22) exhibited X-gal staining primarily in the nervous system like the mRbP.lacZ lines (Fig. 6, c–i). The 21 and 7 lines also exhibited some expression in the kidney, and number 22 was expressed at low level in the heart. The hRbP.lacZ 15 line exhibited high level of expression in the retina and low levels in the central and peripheral nervous systems (Table I). The hRbP.lacZ minigene was transferred to a lacZ cassette yielding hRbP.lacZ (Fig. 5). This construct is similar to the human Rb minigene that was used to rescue the Rb−/− lethal defect (32). However, the hRbP.lacZ minigene utilizes an SV40 polyadenylation signal (Fig. 6b), whereas the human Rb minigene contains a β-globin cassette at the 3′ end (32).
man promoter in mouse tissues. Importantly, four of five hRb-P.lacZ lines directed transgene expression primarily to the nervous system but not the liver and skeletal muscles where endogenous Rb is highly expressed. Thus, both the human and mouse Rb core promoters evolved to direct Rb expression to the nervous system. Other, yet to be defined, regulatory elements, located outside the first 6 kb upstream of the Rb core promoter, may be required for expression of Rb during hematopoiesis and skeletal myogenesis.

**DISCUSSION**

Although Rb is expressed in all adult tissues (11), the gene is highly expressed only in a subset of cell lineages during development (Fig. 3, e, g, and i) (14). The correlation between Rb expression and the spectrum of developmental defects in Rb null mice suggests the existence of a mechanism that coordinates the expression of Rb during embryogenesis with the onset of terminal differentiation in certain tissues. We initiated a transgenic analysis, described herein, with the goal of defining regulatory elements that control Rb expression during embryogenesis and elucidating this developmental program. We found that mouse Rb promoter DNA sequences up to 6 kb in length directed transgene expression exclusively to the central and peripheral nervous systems, including the developing retina. Our results suggest that regulatory regions required for expression of Rb in the liver and skeletal muscles where Rb is normally expressed may be physically separated from regulatory elements that control Rb expression in the nervous system. This regulatory arrangement, also found in other genes (33), is consistent with the notion that an ancestor Rb might have initially evolved to exert neurogenic functions and was later recruited by other tissues that acquired appropriate regulatory elements elsewhere in the Rb locus. The regulatory DNA sequences required for transcription of Rb in the liver, lens, and skeletal muscles may be located in other genomic regions surrounding the Rb gene. Alternatively, the 3'-UTR of Rb may control the stability of the Rb message in the liver, lens, and skeletal muscles.

Arguably, the Rb promoter contains all the necessary regulatory elements, but the lacZ cassette suppresses the Rb promoter specifically in the liver, lens, and skeletal muscles but not in the nervous system. We consider this possibility unlikely because mouse Rb promoter minigenes, containing the Rb cDNA rather than lacZ, are also expressed exclusively in the nervous system and specifically rescue the neurogenic but not hematopoietic skeletal muscle and lens defects in Rb+/− embryos (7, 18, 27). We previously thought that the expression pattern of these Rb minigenes reflected the differential stability in different tissues of the human growth hormone cassette at the 3′-end of the Rb minigene (Fig. 1a). The present study indicates that the Rb promoter dictates this expression pattern. Since the Rb promoter directs expression exclusively to the nervous system, the mgRb-Rb+/− fetuses can be viewed as null for Rb in tissues other than the nervous system. As the Rb minigene can rescue the mid-gestation death of Rb+/− embryos, but is only expressed in the nervous system but not the hematopoietic system, it is tempting to speculate that Rb+/− embryos die at E13.5–E14.5 due to a neurogenic rather than hematopoietic deficiency.

In addition to the 4.5-kb Rb minigene (Fig. 1a), another Rb minigene capable of partially suppressing the Rb+/− defect contains a 1.3-kb mouse Rb promoter region and the first intron of Rb (7). Since this minigene could also only rescue the
Rb−/− neurogenic defect, it is unlikely that the regulatory elements required for expression of Rb in liver, lens, and muscles are located in the first intron. Thus, the HS sites detected in the first intron of Rb (Fig. 4, b and c) may not be important for expression in these tissues. To identify the regulatory elements required for expression of Rb during hematopoiesis, skeletal myogenesis, and lens development, large DNA segments 20–40 kb upstream of the Rb CP, the 3′-UTR, and downstream sequences will have to be subject to DNase I HS and transgenic analysis.

In contrast to the mouse minigenes, which only partially rescue the Rb−/− embryonic defect, a human Rb minigene consisting of a similar promoter DNA segment used in our study can completely rescue the Rb defect (16, 20). The discrepancy between these observations is not clear but may be attributed to different 3′-UTR used in the two studies or to the widespread, deregulated, expression of the human Rb transgene in some transgenic lines (Fig. 6, j–m), which might be sufficient for phenotypic rescue of the Rb−/− defect. Recently, transgenic mice expressing N-terminal deletions of Rb under control of the human Rb promoter were found to rescue the neurogenic but not skeletal or hematopoietic defects of Rb−/− mutant embryos (16, 20), yielding a phenotype similar to the mgRb:Rb−/− fetuses. It would be interesting to determine the expression of the N-terminal Rb mutants in the liver and muscles relative to the nervous system.

It is somehow puzzling that the highly conserved 5′ region, 5′-CR, is not involved in transcriptional activation as determined by transgenic and DNase I hypersensitive analyses. The 5′-CR may, however, communicate with upstream or downstream elements and assist in expression of Rb in some specific cell lines or adult tissues. We delineated the region required for mouse Rb transgene expression during neurogenesis to 250 bp upstream of the transcription starting site. This region contains several consensus-binding sites including an E2F site that functions as a negative element in vitro (23). This E2F site may allow autoregulation by Rb or cross-regulation by p107 or specific mutations has been implicated in the initiation of retinoblastoma (19, 30). Our results suggest that perhaps similar alterations may also suppress transcription of the Rb gene in other neuronal tissues. Although several transcription factors were shown to bind the Rb core promoter in certain tissues, the neurogenic factors that control the expression of Rb during development are not known. Future experiments should allow us to identify the cis-elements and the corresponding factors that control Rb expression in the retina, the central, and peripheral nervous systems and study their effects on expression of Rb in the contexts of normal development as well as neoplastic transformation.

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