Enhancer-Dependent, Locus-Wide Regulation of the Imprinted Mouse Insulin-Like Growth Factor II Gene[1]

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The mouse insulin-like growth factor II (IGF-II) gene is subject to parental imprinting and is predominantly expressed from the paternal chromosome. This allele-specific expression is modified further by cell type, developmental stage, and growth conditions. We show that the ratio of the three major IGF-II mRNAs, each produced from a distinct promoter, is consistent in a variety of tissues and cells representing different modes and phases of the complex regulation. Nuclear run-on assays show that the major changes in total IGF-II mRNA level occur at the level of transcription. Moreover, a targeted disruption of the endoderm-specific enhancers, located 90 kb away from the gene, affects all promoters. The dependency of the promoters on distal enhancers is also shown by transgenesis experiments. Our findings suggest that enhancer-dependent, locus-wide mechanisms play a major role in the coordinate regulation of the multiple IGF-II promoters.

Key words: enhancer, genomic imprinting, insulin-like growth factor II, promoter usage, transgenic mouse.

Insulin-like growth factor II (IGF-II) is a mitogenic polypeptide which plays an important part in embryonic growth and development (1, 2). The mouse gene coding for this growth factor is subject to complex regulation (3-9). First, the IGF-II gene is subject to parental imprinting: in most tissues, the gene is predominantly expressed from the paternal allele, while only a low level of expression is detectable from the maternal allele (3, 4). The only tissues in which both parental alleles are equally active are the choroid plexus and leptomeninges (3). Second, the gene is actively expressed in a wide array of mesodermal tissues, but in only a few endodermal and ectodermal derivatives (5). Third, IGF-II becomes down-regulated during the neonatal period in most tissues although high levels of expression continue to adulthood in the choroid plexus and leptomeninges (6, 7). Fourth, in cultured embryonic fibroblast cells, the IGF-II mRNA level is increased upon growth arrest by contact inhibition or by serum starvation (8, 9). Thus, expression of IGF-II is dependent on parental

origin, cell type, developmental stage, and growth conditions.

The mouse IGF-II gene spans 11 kb and consists of six exons (E1-6, see Fig. 1) (10-12) although a recent study revealed the presence of extra upstream exons which code for multiple minor transcripts (13). The major 5′ exons, E1-3, are transcribed from individual adjacent promoters 1 to 3 (P1-3), respectively, and contain alternate 5′ untranslated regions. These exons are each spliced onto E4. E4-6 are contained in all major mRNAs and encode the 180 amino acid IGF-II precursor polypeptide and the 3′ untranslated region. Sequence analysis of the genomic locus revealed that the three major promoters have distinct structural features (10-12). The most 5′ promoter, P1, has only a low level of activity and contains multiple transcription initiation sites without TATA- or CAAT-like sequences. P2 is a CpG-rich island with a TATA box, a CAAT-like box and putative SP-1 binding sites. The most 3′ promoter, P3, has the highest activity and contains a TATA-like box and putative SP-1 binding sites. This promoter can be transactivated by AP-1 in a transient expression assay (14). Despite all these distinct characteristics, the tissue-specific imprinting of the IGF-II promoters is coordinately regulated in rats (15). Similar observations were made on the corresponding promoters of the human gene although an extra promoter specific to this species behaves in a different manner (16-18).

In the present study, we asked whether the ratio of the activities of the three IGF-II promoters is maintained under various conditions. We used a sensitive RT-PCR assay that enables simultaneous amplification of the three

promoter-specific mRNAs and compared their ratios in various tissues and cells. We also carried out nuclear run-on assays to examine whether the major changes in amount of IGF-II mRNAs occur at the level of transcription. Based on the results, together with the data obtained with transgenic mice and mice harboring a disruption in the endoderm-specific enhancers, we suggest that the activities of the three IGF-II promoters are coordinately regulated by locus-wide mechanisms that are dependent on the action of distal enhancers.

EXPERIMENTAL PROCEDURES

Materials—Adult mouse organs were dissected out from C57BL/6 females. Normal embryos were obtained by crossing C57BL/6 females with CBA males. Embryos maternally disomic for chromosome 7 (MatDi7) were obtained by crossing T9Hc/+c females with T9H+/++ males (19), which were kind gifts from B.M. Cattanach and C.V. Beechey. A lack of eye pigment due to the c alleles at the *albino* locus was used to identify disomic embryos. Embryonic fibroblast cultures were derived from fetuses at embryonic day 14.5 (e14.5) as described (8). NCl 1 is a ring-cloned embryonic fibroblast line derived from a normal littermate in a T9H intercross (8). Cells were cultured in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, and 10% bovine calf serum or fetal bovine serum. RNA samples from mice with a targeted disruption of the endoderm-specific enhancers (20) were a kind gift from S.M. Tilghman.

Isolation of RNA—Total RNA was isolated from adult organs, decapitated embryos (e10.5-15.5), and placentae by the method of Chirgwin *et al.* (21). Embryos were decapitated because the biallelic expression of IGF-II in the brain might affect measurement of maternal IGF-II expression in the rest of the body. Total RNA from earlier embryos (e8.5) and cultured cells was obtained by the method of Chomczynski and Sacchi (22).

Quantitative RT-PCR—Total RNA (2.5 μg) was mixed with 150 ng of random hexamers and the volume was adjusted to 17.5 μl. The mixture was incubated at 65°C for 5 min and then at room temperature for 10 min. The samples were brought into the RT mixture (final volume 25 μl) containing 10 units of Molony murine leukemia virus reverse transcriptase (Stratagene). RT reaction was carried out at 37°C for 1 h. A proportion of the products equivalent to 200 ng of total RNA was subjected to PCR amplification. The PCR mixture contained 160 ng each of the sense and antisense primers and 1 unit of Taq DNA polymerase (Promega). Each PCR cycle consisted of 1 min at 93°C, 1 min at 65°C, and 1 min at 72°C. The cycle number appropriate for quantitation was determined for each sample based on the amplification curve obtained in pilot experiments. β-Actin mRNA was coamplified and used as an internal control. Oligonucleotide primers are: OP1 (E3, sense), 5'-AGCGGCCTCCTTACCCAACTTCAG-3'; OP3 (E2, sense), 5'-AGAAGGGCCTTCGCCCGCTGTT-3'; OP7 (E1, sense), 5'-GTCTTCCAACGGACTGGGCGTTG-3'; OP8 (E4/E5 junction, antisense), 5'-AAGGCCTGCTGAA-GTAGAAGCCG-3'; OP9 (β-actin, sense), 5'-GCTGTGCT-ATGTTGCTCTAGACTTC-3'; OP10 (β-actin, antisense), 5'-CTCAGTAACAGTCCGCCTAGAAGC-3'.

Southern Blotting—Products from RT-PCR were electro-

phoresed on a 1% agarose/3% Nusieve agarose gel or a 2.5% agarose gel in a Tris-borate/EDTA buffer, transferred to a nylon membrane (Hybond-N+, Amersham) with 0.4 N NaOH, and UV-cross-linked (UV Stratalinker 2400, Stratagene). The membrane was hybridized with end-labeled OP8 (for IGF-II) or OP9 (for β-actin) in a solution described by Church and Gilbert (23). Washing was performed in a solution containing 300 mM NaCl, 30 mM sodium citrate, pH 7.0, and 0.1% sodium dodecyl sulfate at 65°C. Radioactivity of each band was measured on FUJIX BAS 2000 (Fuji Photo Film). The results were presented as average plus/minus standard deviation of three independent RT-PCR assays.

Run-On Transcription—Nuclei from tissues and embryos were isolated as described by Marzluff and Huang (24) except that 0.1 mM phenylmethylsulfonyl fluoride was added. Nuclei from cultured cells were isolated according to Greenberg and Ziff (25) except that 0.1 mM $CaCl_2$ was included in the lysis buffer. Run-on reaction was performed with $1-3\times10^7$ nuclei in 150 μl of 17 mM Tris-HCl, pH 8.0, 30% glycerol, 5 mM $MgCl_2$, 150 mM KCl, 2 mM DTT, 0.5 mM each of ATP, CTP, and GTP, and 200 μCi of [α- ^{32}P]UTP (400 Ci/mmol) at 37°C for 15 min. Whenever possible, the number of cell nuclei was matched between the samples to be compared. The reaction was terminated by addition of 2 ml of 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 4 M guanidium thiocyanate, and 2% N-lauroylsarcosinate, and labeled RNA was isolated as above. The RNA was hybridized to DNA probes devoid of vector sequences that had been denatured and fixed onto a nylon membrane (Hybond-N, Amersham). The amount of each probe corresponded to 10 μg of the plasmid. Hybridization and washing followed the protocol of Kou and Rotwein (26) except that 10 μg/ml of RNase A was used to remove unhybridized, excess RNA. The membranes were exposed to an imaging plate and this was analyzed on a FUJIX BAS 2000.

Transgenic Mice—DNA fragments to be injected were liberated from vector sequences by restriction digestion, electrophoresed in an agarose gel, and recovered by electro-elution in dialysis tubing. Fertilized one-cell eggs were recovered from (C57BL/6 $\times$ C3H)F1 intercrosses, microinjected with about 200 copies of the transgene fragments, cultured overnight, and transferred to the oviducts of pseudopregnant ICR females at the two-cell stage. Embryos were collected at e11.5 and stained for β-galactosidase activity by a 1-h fixation at 4°C in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, and 0.02% NP-40, and an overnight staining at 37°C in PBS containing 0.4 mg/ml X-gal, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, and 1 mM $MgCl_2$. Placental DNA was subjected to PCR to check for the presence of the injected DNA. Primers used are: HOP1 (intron 3, sense), 5'-CCTGGGGTCCATTGAATTTCTC-3'; HOP2 (β-galactosidase, antisense), 5'-TTTGAGGGGACG-ACGACAGTAT-3'.

RESULTS

Outline of Mouse IGF-II Regulation—The overall structure of the mouse IGF-II gene and the major mRNAs produced from its three promoters are depicted in Fig. 1A. In this study, we attempted to compare the abundance of the three mRNA species directly, not only in tissues where

IGF-II is actively transcribed, but also in those where the activity is very low. We devised a sensitive RT-PCR assay that utilizes an antisense primer (OP8) hybridizing to all mRNAs and three sense primers (OP7, 3, and 1) specific to the respective mRNAs (Fig. 1B). A mixture of all these primers successfully amplified the three mRNA species from whole embryos at e15.5 (Fig. 1C). The relative abundance of the three products was consistent with the reported strength of the promoters (P3 > P2 > P1) (*11*).

The complex regulation of IGF-II was outlined by limited cycles of the PCR as illustrated in Fig. 2 (see legend for exact cycle numbers). First, the gene is abundantly expressed in whole embryos and embryonic liver at e12.5, but the mRNAs are barely detectable, if present, in adult organs except brain, which contains the choroid plexus and leptomeninges. Second, MatDi7 embryos, which have two repressed maternal copies of IGF-II (*27*), display very low expression while their normal littermates contain high levels of the mRNAs. Third, in an embryonic fibroblast line NCl 1, the gene is highly expressed in the confluent phase but repressed in the growing phase. These results confirmed the various previously reported modes of regulation (*3–9*).

Ratio of the Three IGF-II mRNAs—We next wished to measure the ratio of the three mRNAs in various tissues and cells, including those where IGF-II levels are very low. Preliminary experiments indicated that the three mRNAs are indeed detectable in all tissues if the PCR cycle number is increased. For example, decapitated MatDi7 embryos gave only a faint signal after 19 cycles of PCR (Fig. 2), but all bands became clearly detectable when PCR was continued for more than 25 cycles (Fig. 3A, left). To determine the mRNA ratio, however, it is important that the three mRNAs are amplified in proportion up to the point where signals are measured. Therefore, for each sample, intensities of the three bands were plotted against cycle number and the number most appropriate for quantitation was determined based on the curves (Fig. 3A, right and data not shown).

In addition to the ratios of the three mRNAs, we also compared the levels of P3 mRNA (the most abundant IGF-II mRNA species) between tissues and cells. For example, the P3 mRNA level in adult brain was compared with that in e15.5 whole embryos (Fig. 3B). First, P3 mRNA was amplified for 19 cycles (amplification was colinear up to this point) using the primer pair OP8 and 1. The products from this sample were used to make a series of dilutions and their signals were measured to obtain a standard curve. The degree of dilution that gives the signal corresponding to that of adult brain was then determined
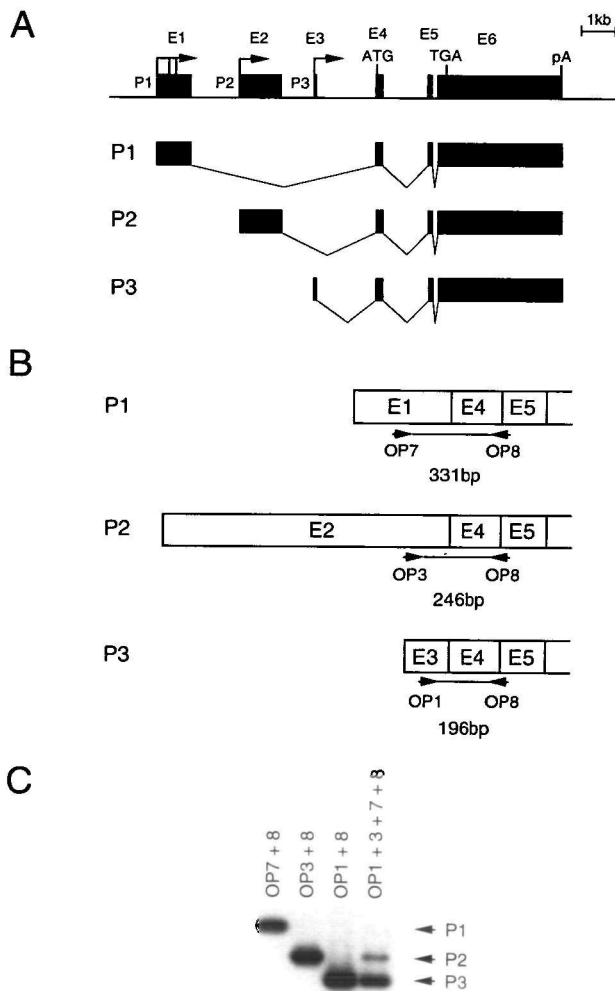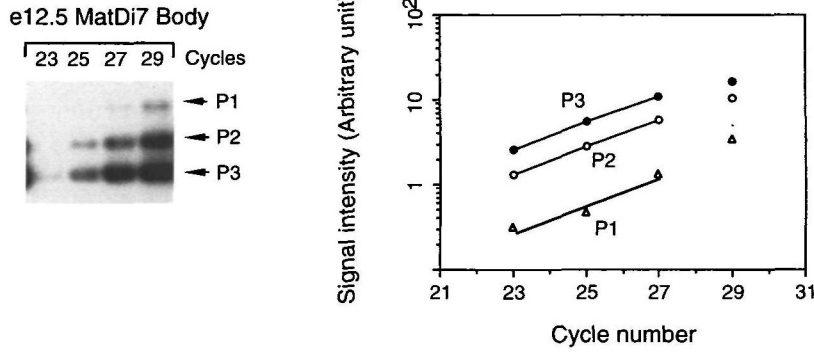

Fig. 1. **Promoter-specific mRNAs of mouse IGF-II and design of RT-PCR.** A, structure of the mouse IGF-II gene. The positions of six exons (E1-6, solid boxes), three promoters (P1-3), and transcription start sites (arrows) are indicated. ATG, TGA, and pA denote the initiation codon, termination codon, and major polyadenylation site, respectively. Three major transcripts derived from the three promoters are shown below. B, positions of the oligonucleotide primers for RT-PCR and sizes of the expected products. C, autoradiograph of a test RT-PCR. Total RNA from e15.5 embryos was used as templates and PCR was done for 30 cycles. The products specific to respective promoters (P1-3) were detected by hybridization with end-labeled OP8.
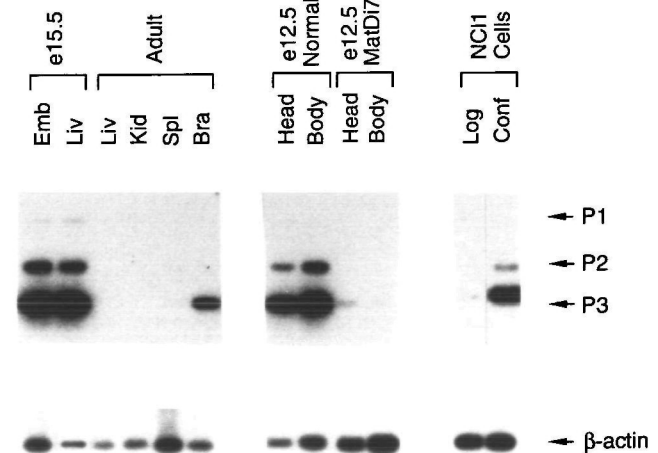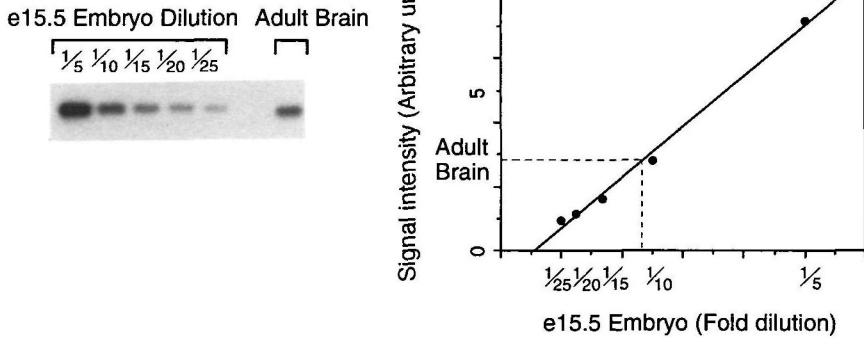
Fig. 2. **Regulation of the mouse IGF-II mRNAs.** Expression of the IGF-II gene is subject to various kinds of regulation dependent on developmental stage, tissue or cell type, parental origin, and growth condition. The three IGF-II mRNAs were coamplified for 25 cycles (NCl 1 cells) or 19 cycles (other samples). β-Actin mRNA was amplified for 19 cycles. Pilot experiments indicated that the proportion of the three RT-PCR products was maintained colinearly up to these cycle numbers (data not shown). Emb, embryo; Liv, liver; Kid, kidney; Spl, spleen; Bra, brain; Log, logarithmic phase; Conf, confluent phase.
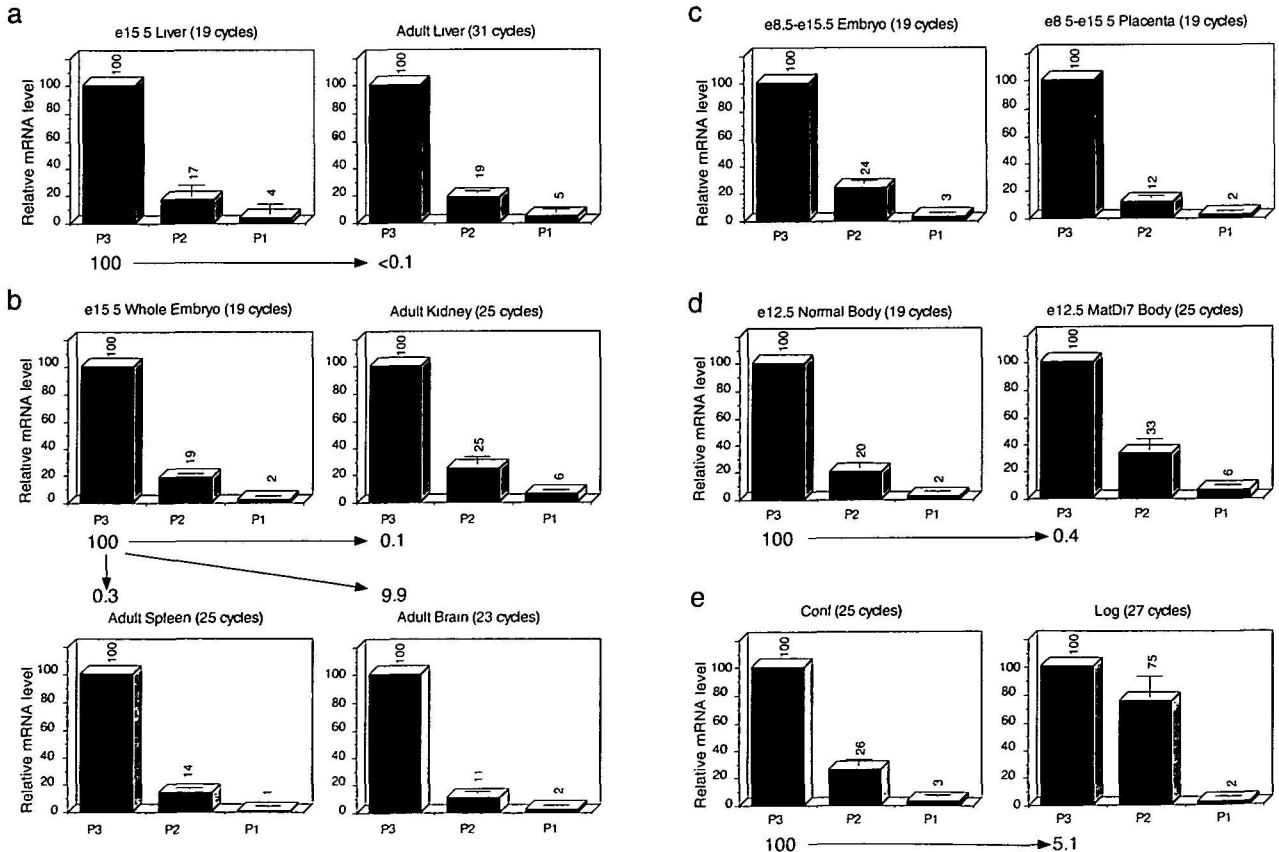
A

e12.5 MatDi7 Body

B

C

using the curve. When the values were normalized to β-actin levels and the average of three repeated experiments was taken, the P3 mRNA level in adult brain relative to that in e15.5 embryos was 9.9/100. This was close to the value obtained by Northern blotting (12.8/100, data not shown), verifying the accuracy of the assay.

Figure 3C summarizes the results obtained with various samples. For each sample, both the P1 and the P2 mRNA levels are presented relative to the P3 mRNA level (by setting the P3 mRNA level as 100). The abundance of P3 mRNA in a given sample relative to that in another is shown below each graph (by setting the higher level as 100). First, the developmental down-regulation of IGF-II in the liver does not influence the ratio of the three mRNAs, although the overall level (represented by the level of P3 mRNA) differs more than 10^3-fold (Fig. 3C, panel a). Second, although the ratio can vary to some extent in different tissues (panels b and c), such differences are rather small if the large differences in overall expression are considered (up to 1,000-fold). The tissue-specific differences include a 1.6-fold increase in the P2/P3 mRNA ratio in the embryo proper compared with the placenta. The P1/P3 mRNA ratio is even more variable (up to 6-fold), for example, between adult spleen and kidney. Third, although the P2/P3 and P1/P3 mRNA ratios are 1.5 and 3-fold higher, respectively, in MatDi7 embryos than in controls, the overall level differs 250 fold (panel d). Lastly, the P2/P3 mRNA ratio is 3-fold higher in growing cells than in confluent cells (panel e) but, again, the difference is rather small since the confluent cells contain 20-fold more mRNAs than the growing cells. In all cases, the order of the abundance of the three mRNAs (P3 > P2 > P1) is maintained. (However, neonatal liver is an exception: see Fig. 5). These results collectively indicate that, although there exists mRNA-specific regulation depending on cell type, parental origin, and growth condition, the ratio of the three IGF-II mRNAs is rather constant considering the large differences in total expression level.

Run-On Assay—The consistency in IGF-II mRNA ratio can be explained either by coordinated transcription from the three promoters or through a shared mRNA sequence affecting their stability. Therefore, we next examined whether the major changes in IGF-II expression occur at the level of transcription. Three probes devoid of repetitive sequences were isolated from the E6 region for nuclear run-on assays (Fig. 4A). Nuclei isolated from tissues and cells were allowed to continue transcriptional elongation *in vitro* and nascent RNAs were purified and hybridized with the probes. The results demonstrated that both the allele-specific and stage-specific IGF-II expression or repression mainly occur at the level of transcription (Fig. 4B). Likewise, the arrest-specific up-regulation in NCl 1 cells results from an increased rate of transcription although the overall transcription level is low (Fig. 4B). Upon addition of α-amanitin to the reaction mixtures at a final concentration of 5 μg/ml, all signals disappeared (data not shown), verifying that RNA polymerase II accounts for the RNA synthesis. We conclude that the major changes in IGF-II expression occur at the level of transcription, although this does not necessarily exclude the involvement of post-transcriptional mechanisms.

Promoter Usage in Enhancer Deletion Mice—It was previously reported that a targeted deletion of the two

endoderm-specific enhancers located 90 kb downstream of the IGF-II gene greatly affects the expression of this gene (20). These enhancers, originally identified as regulatory regions for the neighboring H19 gene, are known to be involved in the opposite imprinting of H19 and IGF-II (20, 28). To ask whether the enhancer deletion influences the promoter usage of IGF-II, we examined the neonatal tissues from deletion homozygotes by means of the above RT-PCR (Fig. 5). Quantitation of P3 mRNA showed that its levels in the liver and gut are reduced to 4.9 and 15.3%, respectively, in deletion mice, compared to their normal littermates. When the ratios of the three mRNAs are compared between the deletion mice and controls, the differences are not significant in the liver (Fig. 5A). This suggests that the enhancers influence all three promoters to similar extents. In the gut of deletion homozygotes, however, the P2/P3 ratio is 3-fold higher than in normal gut (Fig. 5B). This could arise from an unequal action of the enhancers on the different promoters, but it is also possible that P2 activity is slightly higher in non-endodermal cells, which are unaffected by the enhancer deletion.

Enhancer-Dependent Expression of IGF-II Transgenes—The experiments with knockout mice clearly showed that expression of IGF-II is dependent on the distal enhancers, at least in organs rich in endodermal cells (20 and this study). Although no other enhancers have been reported for IGF-II, it is possible that expression in other tissues may also be dependent on heretofore unidentified enhancers. To test this idea, we produced mice carrying a transgene construct containing the P2/P3 region of IGF-II fused with a bacterial β-galactosidase (*lacZ*) reporter gene (Fig. 6A). Three independent transgenic embryos were
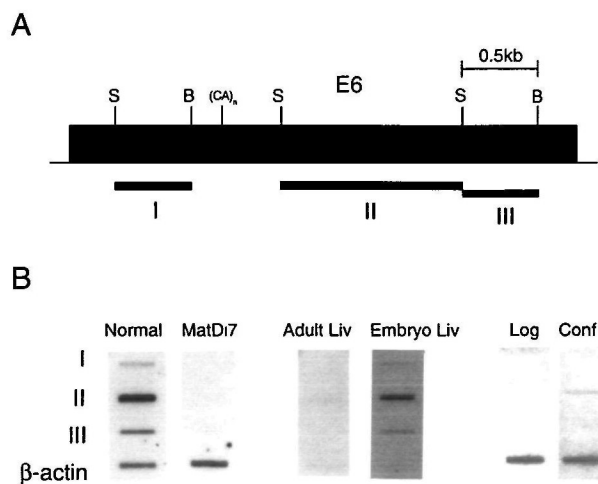
Fig. 4. **Transcriptional activity of the IGF-II gene assayed by run-on transcription.** A, map of the E6 region and the locations of probes I–III. A microsatellite repeat is indicated by $(CA)_n$. S, *SacI*; B, *Bam*HI. B, results obtained by the nuclear run-on assay. The numbers of nuclei used were 3×10^7 for normal embryos, MatDi7 embryos, and embryonic liver, and 1×10^7 for adult liver, logarithmic cells, and confluent cells. The amounts of the probes fixed on the membrane were 1.67, 2.86, 1.83, and 3.02 μg for I (0.6 kb), II (1.2 kb), III (0.67 kb), and β-actin (1.3 kb), respectively, each corresponding to 10 μg of the plasmid. The exposure time was adjusted to give comparable β-actin control signals. The probe for β-actin was a 1.3-kb cDNA fragment. Liv, liver; Log, logarithmic phase; Conf, confluent phase.
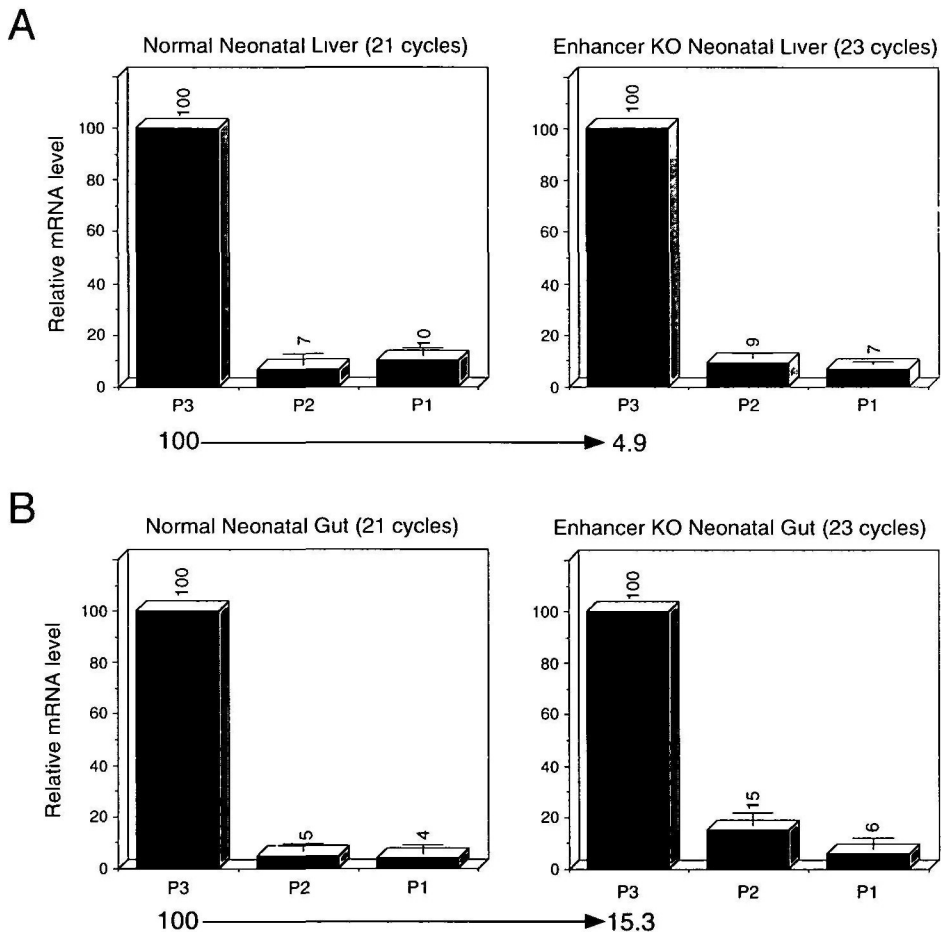
Fig. 5. Ratio of the three mRNAs in enhancer deletion mice. RNAs from neonatal liver and gut of normal and knockout mice were analyzed as in Fig. 3. The results are expressed as in Fig. 3C.
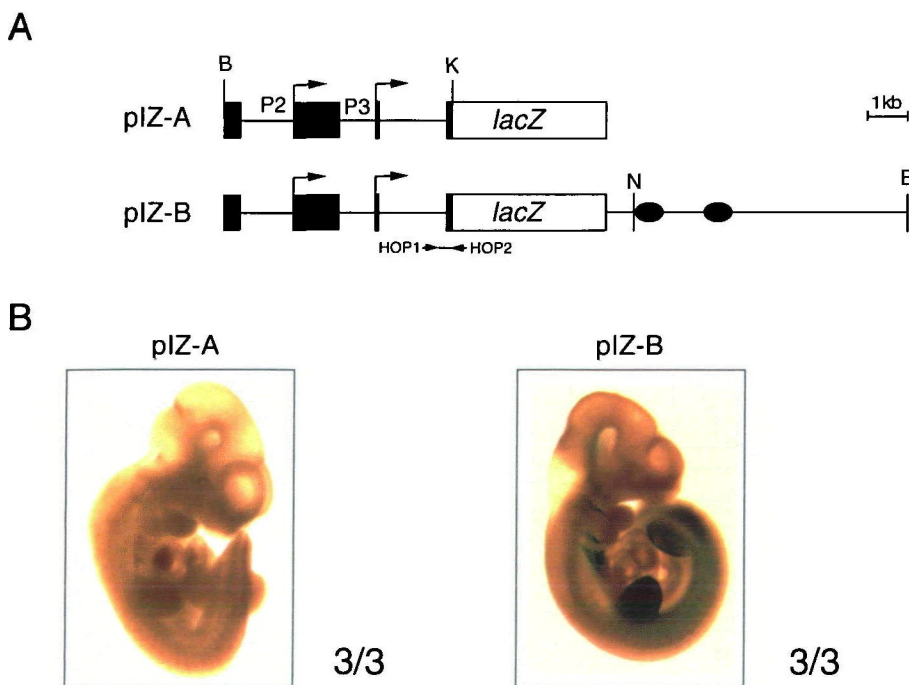

Fig. 6. Dependency of IGF-II transgenes on distal enhancers. A, structure of the IGF-II–*lacZ* fusion gene with (*pIZ-B*) or without (*pIZ-A*) the endoderm-specific H19 enhancers. A 5.6-kb *Bam*HI-*Kpn*I fragment containing both P1 and P2 was linked to the bacterial β-galactosidase gene. The two enhancers were provided as a 6-kb *Nsi*I-*Bam*HI fragment. Filled circles represent the enhancers. Locations of the PCR primers used to identify transgenic embryos are shown below the constructs. B, *Bam*HI; K, *Kpn*I; N, *Nsi*I. B, β-galactosidase staining of the transgenic embryos. Embryos were collected at e11.5, fixed and stained, and observed under a dissecting microscope. None of the three embryos carrying pIZ-A showed staining while all three embryos containing pIZ-B showed a specific pattern, not only in endodermal tissues, but also in some mesodermal and ectodermal tissues.

obtained, none of which displayed β-galactosidase activity in any tissue (Fig. 6B, left). By contrast, when a 6-kb fragment containing the endoderm-specific enhancers was added to the fusion gene, staining was observed in all three

embryos obtained (Fig. 6B, right). These results strongly support the idea that the IGF-II promoters are intrinsically incapable of driving a high rate of transcription in any tissue and their expression relies on distal enhancers. The tissues stained with this construct include the liver, epithelial layer of gut, sclerotome, neural tube, and the placodes of cranial nerves VII, IX, and X, suggesting that the activity of the two distal enhancers may not be restricted to endodermal tissues.

DISCUSSION

The mouse IGF-II gene is known to be subject to complex regulation dependent on parental origin, cell type, developmental stage, and growth conditions (3–9). We have shown by quantitative RT-PCR that the ratios of the three promoter-specific mRNAs of IGF-II are relatively unaffected by these various factors, in spite of the large differences in overall expression level concomitant with the regulation. Run-on transcription assays confirmed that the complex regulation mainly occurs at the level of transcription. These results indicate that the three promoters are coordinately regulated irrespective of the mode of regulation. Since the three promoters are structurally unrelated to each other (10–12), our findings suggest a role for locus-wide mechanisms in IGF-II expression. However, we note that there exist limited but specific alterations in mRNA ratios under certain conditions. This suggests that promoter-specific factors are also involved in IGF-II regulation, perhaps for fine tuning of transcription.

A previous report by Leighton *et al.* showed that a targeted deletion of the two endoderm-specific enhancers of a linked H19 gene greatly affects the expression of IGF-II (20). Therefore, it seemed likely that distal enhancers provide a basis for the locus-wide regulation. Such a model was previously proposed by Pedone *et al.* based on their observation that the rat IGF-II promoters are coordinately regulated by imprinting (15). We tested this possibility by analyzing the above deletion mice and showed that the disruption of the endoderm-specific enhancers affects all IGF-II promoters. Dependency of the promoters on distal enhancers in other tissues was demonstrated by transgenesis experiments, which showed that expression from P2 and P3 is undetectable in any tissue in the absence of the enhancers. This is in agreement with the previous report that a 30-kb rat IGF-II transgene showed either no expression or various unrelated expression patterns caused by position effects (29). By contrast, a transgene linked with the endoderm-specific enhancers reproducibly displayed expression in endodermal tissues such as the liver and gut, and in some mesodermal and ectodermal derivatives such as sclerotome, neural tube and the placodes of cranial nerves. Since both the endogenous IGF-II and H19 genes are expressed in many other tissues, there must be additional tissue-specific enhancers in this genomic region.

The above findings collectively suggest that the mouse IGF-II gene is mainly controlled by enhancer-dependent, locus-wide mechanisms. Its tissue- and stage-specific expression patterns are probably determined by the combinations of specific regulatory factors that bind to the distal enhancers, while those binding to the individual promoters determine their basal activities. This provides an efficient way of regulating multiple promoters in a coordinate

fashion. Furthermore, our results are concordant with the previous observations on the other modes of regulation. For example, the imprinted expression of IGF-II in endodermal tissues probably occurs through competition by this gene and the H19 gene for the endoderm-specific enhancers, as shown by gene knockout experiments (20, 28). It is possible that its imprinting in other tissues is also dependent on as yet unidentified distal enhancers. Another example is the up-regulation of both the IGF-II and H19 genes upon growth arrest (9). This also predicts the presence of a shared distal control element responsible for the growth-dependent regulation.

At present the long-range mechanism whereby distal IGF-II enhancers control the entire IGF-II locus remains speculative. One possibility is that the distal enhancers alter the higher-order chromatin structure of the entire locus. However, such changes were undetectable between parental chromosomes (4) or in the liver at different developmental stages (H.S., unpublished observations), when DNase I hypersensitivity was used as a marker. A second possibility is that regulatory complexes formed at the distal enhancers act together to interact directly and alternately with the three IGF-II promoters, perhaps through a stochastic looping mechanism, as proposed for the β-globin locus control region (30). In this case, a large enhancer holocomplex may be capable of interacting with only one promoter at a time, but with the frequency and duration specific to the respective promoters. Other possibilities include the changes in replication timing or superhelical density of DNA, which may also be influenced by the action of the distal enhancers. Further studies are needed to distinguish between these possibilities to understand the precise mechanisms that control the complex regulation of the IGF-II gene.

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