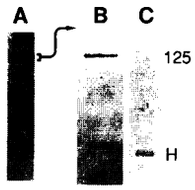

Fig. 5. Protein complex formation of hemolin with a 125-kD protein. The proteins were separated on a 10% polyacrylamide gel under nondenaturing conditions at pH 4, and visualized by Coomassie brilliant blue. Protein immunoblots were prepared (*27*) and tested with antiserum to hemolin that has been purified on a hemolin affinity column (*12*). Blots were incubated at 4°C overnight in an antibody solution (120 μg protein/ml), diluted 1:1000 in a buffer containing 0.5% bovine serum albumin, 150 mM NaCl, and 10 mM Tris-HCl (pH 8). After being washed with 0.02% Tween 20 in 0.9% NaCl the blots were incubated with alkaline phosphatase conjugated to the $F(ab')_2$ fragment of goat antibodies directed against rabbit Ig (sigma), and developed with a solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. (**A**) Native gel electrophoresis of protein extracts eluted from the bacterial surface after 2 min. (**B**) Separation of the major band (A) by SDS-PAGE, stained with Coomassie brilliant blue. (**C**) Protein immunoblot of (B) with antiserum against hemolin.

and it is strongly induced after bacterial infection. Finally, hemolin binds to surface structures shared by many bacteria, where it forms a complex with another hemolymph protein, which might constitute an important part of the primary immune response.

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Dominant Negative Regulation of the Mouse α-Fetoprotein Gene in Adult Liver

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Transcription of the mouse α-fetoprotein gene is activated in the developing fetal liver and gut and repressed in both tissues shortly after birth. With germline transformation in mice, a cis-acting element was identified upstream of the transcription initiation site of the α-fetoprotein gene that was responsible for repression of the gene in adult liver. This negative element acts as a repressor in a position-dependent manner.

THE APPROPRIATE DEVELOPMENT OF a multicellular organism requires the expression of genes at the correct time and in the correct cell type. Expression of certain genes must also be repressed at a later stage of development, once their transcripts are no longer required by the organism. The α-fetoprotein (AFP) is activated in the embryonic yolk sac, the fetal liver, and, to a lesser extent, the fetal gut, but is subsequently repressed in both neonatal liver and gut (*1–3*). The decrease in liver AFP mRNA occurs primarily at the level of transcription (*3*).

Two opposing models can be proposed to explain postnatal repression. In one instance, the positive factors necessary for AFP transcription are inactivated after birth, thereby leading to a decrease in AFP mRNA. Alternatively, these factors remain unchanged in concentration, but are no longer able to activate transcription of the gene, due to the presence of a dominant repressor. This is the case for the two silent mating type loci in yeast (*4–6*). Genetic experiments in mice have identified at least one locus (termed *raf*, for regulation of AFP) that might encode such a repressor (*2, 7, 8*).

One way to discriminate between these two models is to ask whether the sequences that are necessary for both positive and negative regulation of the AFP gene coincide, which must be the case in the first model but need not be the case in the second. At least four elements that participate in high-level transcription of the AFP gene have been identified by transfection into tissue culture cells (*9–14*). In the mouse gene, these include a proximal promoter in the first 250 base pairs (bp) upstream of the AFP cap site, as well as three enhancers located upstream of the site of transcription (+1 bp) at −2.5 kilobases (kb), −5.0 kb, and −6.5 kb (*9, 10, 14*). In transgenic mice, these four elements were sufficient to direct high-level expression of a reporter gene in all chromosomal locations into which the gene was integrated (*15*). In addition, the reporter minigene was transcriptionally repressed after birth in a manner indistinguishable from that of the endogenous gene.

As a first test of the two models to explain repression, we established that the AFP enhancers play no obligatory role in the postnatal repression of the gene. The enhancers were used to direct transcription of the evolutionarily related albumin gene, which normally does not undergo repression. No repression in adult livers of transgenic mice was observed. On the other hand, the reciprocal construct that consisted of the albumin gene enhancer upstream of the AFP promoter and minigene exhibited appropriate repression after birth (*16*). These results argued that the enhancers were not responsible for repression, and we focused our attention on the region proximal to the AFP gene itself.

Three constructs bearing the AFP enhancers, an internally deleted AFP minigene (*15*), and different amounts of DNA between −838 and −118 bp were generated

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for microinjection into mouse zygotes. The three deletions removed nucleotides from −604 to −838 (Δ1), −250 to −838 (Δ3), and −118 to −838 bp (Δ7) (Fig. 1A). In cultured cells, these three deletions do not affect the rate of transcription of the AFP minigene when assayed in the presence of the three enhancers (*9*). In the absence of the three enhancers, however, transcription was decreased significantly with the largest deletion, Δ7, due to the loss of a binding site for the liver-specific transcription factor, hepatocyte nuclear factor-1 (HNF-1) (*17*).

All three constructs were expressed in the livers of transgenic fetuses at concentrations comparable to that of the endogenous AFP mRNA, as indicated by the presence of a 600-bp transcript identified by Northern (RNA) blot analysis (Fig. 1B). One exception was line P1, where the concentrations were ~50% of the endogenous concentrations. The expression of the minigene in the fetal gut was far more variable, depending on the construct tested (Fig. 1B). With Δ7, no expression in the gut was observed. In contrast, expression of the AFP minigene in the fetal gut was relatively high for Δ3 and Δ1, consistent with our observations with the intact 5′ flanking domain (*15*). The tissue-specificity of expression was also maintained, with high concentrations of mRNA observed in the yolk sac and fetal liver, and no expression in the fetal brain.

In the adult, some degree of maintenance of expression of the AFP minigene was observed in liver with all three constructs (Fig. 2). With approximately equal amounts of poly(A)$^+$ RNA from the neonatal and adult stage livers, a decrease of the AFP minigene transcript was observed with two independent lines that harbored Δ1. This decrease was, however, significantly less than that of the endogenous mRNA. The adult expression was even more striking with animals carrying Δ3, where no decrease in AFP minigene transcripts was observed. In the case of Δ7, the pattern was less consistent, as the mRNA transcripts from the minigene declined in adult liver in two out of three lines tested. This is likely the consequence of deletion of the distal HNF-1 site, which may be important for transcription in the adult. Nevertheless, in all three lines perturbation in the repression of the mRNA was evident.

These results suggest the presence of an important element, located between and distinct from the enhancers and promoter of the AFP gene, which is required for the repression of its transcription in the adult liver. The partial derepression of the Δ1 construct and the essentially complete derepression of Δ3 suggest that this element resides between 250 and 838 bp upstream of the cap site of the gene. Without this sequence, the gene was transcribed constitutively, indicating that all of the factors necessary for its transcription were present in the adult liver.

The Δ1 and Δ3 deletions of the repressor site had less profound effects on repression in adult gut (Fig. 3). In the Δ1 line G2, the minigene transcript was barely detectable in the adult gut, in contrast to the liver, where it was easily detectable. Likewise, in the Δ3 line F2, expression of minigene mRNA in adult liver was significantly higher than in the adult gut (Figs. 2 and 3). This tissue difference may be explained by recent in situ hybridization studies (*18*), which showed that the reduction in AFP mRNA in the adult gut is a consequence of a reduction in the number of cells that express the mRNA, rather than a uniform reduction in mRNA in all gastrointestinal cells, as is the case for adult liver (*19, 20*).

The observed absence of postnatal repression (Fig. 1) could be the incidental consequence of moving the AFP enhancers closer to the promoter, rather than the removal of a dominant negative element. To ask whether there is, in fact, a negative regulatory element between the AFP promoter and enhancers, a fragment that spanned from −1010 to −250 bp was subcloned into a site at −600 bp upstream of the mouse albumin gene promoter (ALB_EALB.2, Fig. 4A). This promoter was fused to a structural minigene for albumin (*16*) that consisted of the first two and the last exons of the authentic albumin gene. Upstream of the repressor site was inserted a 3-kb fragment that contained the albumin gene enhancer (*21*). This construct allowed us to effectively test whether the negative element could function within the context of a gene that is normally transcribed at high rates in the adult liver (*3*).

As a control for this experiment, the albumin minigene was tested without the AFP repressor element (ALB_EALB.1, Fig. 4A). Six lines were generated, four of which

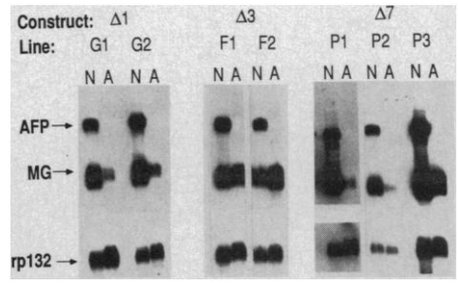

Fig. 2. Developmental regulation of the AFP minigene in the liver. Progeny obtained after mating transgenic founders to CD1 [Crl:CD-1^R (ICR)BR Swiss] mice were sacrificed between day 16 of gestation and day 1 after birth for neonates (N), and around 5 weeks after birth for adults (A). Poly(A)$^+$ RNA's from livers were analyzed as described in Fig. 1, legend. Each blot was hybridized to the AFP-specific probe, stripped, and then hybridized to a probe specific for the ribosomal protein, L32 (*32*), to quantify the amount of RNA loaded for each sample.

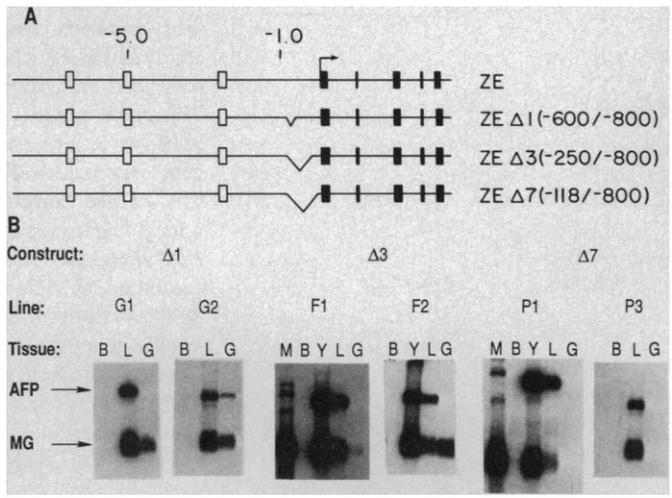

Fig. 1. Fetal expression of the AFP minigene bearing various deletions of DNA in the 5′ flanking region. (**A**) A schematic representation of the three constructs injected into mouse zygotes. Bal 31 was used to construct three deletions between −1 kb and the cap site of the AFP gene as reported (*9, 29*). A 7.5-kb Xba I fragment that contained the three AFP enhancers (open rectangles) was cloned upstream of the Bal 31–derived endpoints at −604 (Δ1), −250 (Δ3), and −118 (Δ7) bp relative to the start of transcription at +1. The AFP minigene contained the first three exons fused within introns 3 and 13 to exons 14 and 15 (filled rectangles). Each linearized fragment was freed of vector DNA, purified on a CsCl gradient, and injected at a concentration of 4 ng/μl into F2 oocytes from C57BL/6 × SJL/J parents (*15*). (**B**) Total RNA extracted from several tissues of day e17-19 F1 progeny of transgenic founders by the LiCl/urea procedure (*30*). Poly(A)$^+$ RNA's were selected (*31*) from yolk sac (Y), liver (L), gut (G), and brain (B) and were subjected to electrophoresis on denaturing formaldehyde agarose gels. After blotting onto nitrocellulose, the RNA's were hybridized with an AFP first exon probe. Results are shown for two independent lines that contained each of the three constructs in (**A**). Transcripts corresponding to the endogenous AFP gene (AFP) and the minigene (MG) are shown with arrows. M corresponds to a lane in which the 600-nucleotide minigene transcript was derived from transfection into HepG2 tissue culture cells (*9, 10*). DNA copy numbers for the different lines were approximately G1, 20; G2, 10; F1, 30; F2, 80; P1, 40; P2, 10; P3, 50.

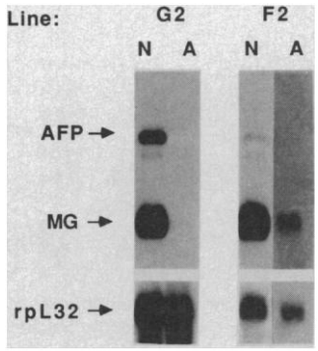

Fig. 3. Developmental regulation of the AFP minigene in the gut. Poly(A)$^+$ RNA was prepared from the guts of neonatal (N) and adult (A) transgenic animals that carried Δ1 and Δ3 (G2, and F2, respectively). The RNA's were analyzed as described in the legends to Figs. 1 and 2.

expressed the albumin minigene at birth. This frequency of transgenic animals that express the transgene is consistent with studies that utilized albumin gene regulatory regions (*16, 21, 22*) and reflects the relatively weak activity of the albumin gene enhancer as compared to the three intergenic AFP enhancers. Two examples of transgenic ALB$_E$ALB.1 mice are shown (Fig. 4B). Expression of the albumin minigene was low in neonates and either remained unchanged or was increased in adult liver.

Eight lines of mice were generated with the AFP repressor inserted between the albumin enhancer and the rest of the gene (ALB$_E$ALB.2). None of the three lines generated in which the repressor was in the 3′ to 5′ orientation expressed the albumin minigene mRNA even at birth, and, therefore, the activity of the repressor could not be tested. However, of the five lines generated in which the repressor site was in its 5′ to 3′ orientation, two expressed the minigene at birth and, in both instances, extinguished expression in adult liver. Thus the repressor site can function within the context of a heterologous gene, in at least one orientation.

To investigate the position dependence, the repressor element was placed in a 5′ to 3′ orientation upstream of the albumin enhancer (ALB$_E$ALB.3; Fig. 4A). Nine lines were generated, three of which expressed the minigene at birth. Analysis of lines U16 and U48, as well as line U53 (*23*), revealed that the albumin minigene mRNA increased markedly after birth, to concentrations that approached those of endogenous albumin mRNA (Fig. 4B). Thus the negative effect of the repressor displayed a position dependence.

Two distinct kinds of negative regulatory elements exist in eukaryotes. In the yeast *Saccharomyces cerevisiae*, distinctions have been made between silencers (*4–6*), which act in a position- and orientation-independent manner, and repressors (*24*), which display position dependence. Repressor elements function most effectively between a promoter and an upstream activating sequence. In mammalian genes, a negative regulatory element in the T cell receptor α locus functions in a position-independent manner, thereby qualifying as a silencer (*25*). In contrast, a negative element upstream of the rat growth hormone gene exhibits maximal activity only when placed between the promoter and the Rous sarcoma virus enhancer (*26*).

The negative element in the AFP gene falls in the repressor class (Fig. 4). That is, albumin transcription was repressed in a stage-specific manner when the repressor was between the gene promoter and enhancer, but not when it was placed upstream of the enhancer. Position dependence may, in fact, be essential for the repressor in order to achieve its selective effects on the AFP gene. The albumin gene lies just 34 kb upstream of the AFP gene (Fig. 4A). We have argued on the basis of transgenic mouse experiments (*16*) that the three AFP enhancers between the two genes activate transcription of both genes in a bidirectional manner (*16*). A silencer in the locus would probably depress transcription of both genes. However, by situating a repressor between the enhancers and AFP promoter, its gene-specific activity is ensured.

This report describes the first mammalian repressor to have functional significance in vivo and the first whose developmental specificity has been established. The use of negative regulatory elements to suppress transcription in development has been demonstrated for several genes in *Drosophila melanogaster*. For example, timing of chorion gene expression (*27*) and the spatial expression of the homeobox gene *zen* (*28*) are under negative regulation. Likewise, the AFP repressor displays a striking temporal specificity, as it had no effect on either the AFP or albumin gene before birth. This implies that its action is mediated by proteins that are activated or accumulate after birth.

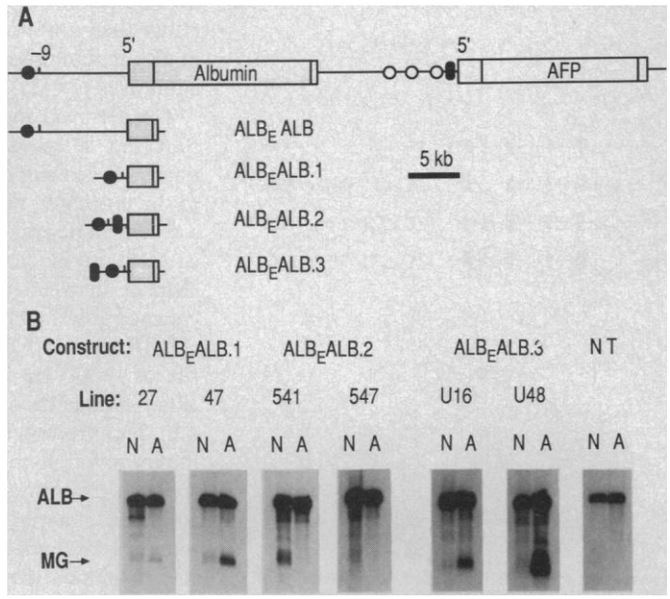

Fig. 4. Effect of the AFP gene repressor on the developmental regulation of an albumin minigene in the liver. (**A**) The top line represents the structure of the AFP-albumin gene loci, with the albumin enhancer between −12 and −9 kb (closed circle), the three intergenic enhancers (open circles), and the AFP repressor (hatched oval) as indicated. ALB$_E$ALB (*16*) was generated by an internal deletion within the albumin gene which removed exons 3 to 14, leaving a chimeric intron that consisted of parts of introns 2 and 14. It included, as well, 12 kb of 5′ flanking DNA. In ALB$_E$ALB.1, the flanking region of the albumin gene between a Bam HI site at −9 kb and an Ava I site at −0.6 kb was deleted. ALB$_E$ALB.2 contained an insertion of the AFP repressor (defined as a Bam HI fragment that spanned −1010 to −250 bp) into the Bam HI site at −0.6 kb of ALB$_E$ALB.1. Finally, in ALB$_E$ALB.3 the AFP repressor Bam HI fragment was cloned upstream of ALB$_E$ALB.1. (**B**) Progeny obtained after mating transgenic founders to CD1 mice. The progeny were killed either shortly after birth (N) or at 5 weeks of age (A). Poly(A)$^+$ RNA from livers were analyzed by Northern (RNA) blot analysis with a hybridization probe derived from the first exon of the authentic albumin gene (*16*). MG, the migration of the albumin minigene transcript; ALB, authentic albumin mRNA; NT, an example of RNA analysis of a nontransgenic mouse. The number of transgene copies in the lines were approximately as follows: Line 27, 5; 47, 5; 541, 10; 547, 15; U16, 5; U48, 5.

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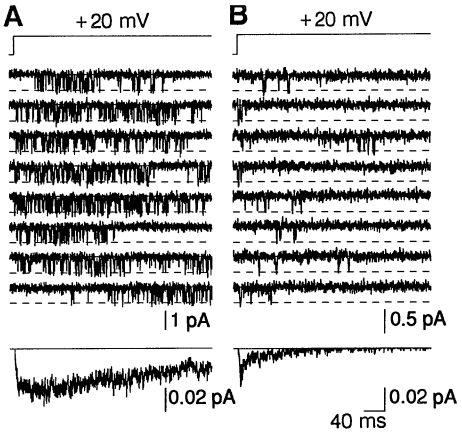

Fig. 1. Differences in single-channel gating with (**A**) Ba^{2+} and (**B**) Ca^{2+} as charge-carrier. (Top) Voltage protocol from holding potential of −50 mV. (Center) Representative traces from patches with a single Ca^{2+} channel. Unitary currents were low pass–filtered at (A) 2 kHz and (B) 0.8 kHz (*12*). (Bottom) Ensemble average of unitary current records. [(A) patch rle57; (B) patch r2es46.]

Calcium-Sensitive Inactivation in the Gating of Single Calcium Channels

DAVID T. YUE,* PETER H. BACKX, JOHN P. IMREDY

Voltage-activated calcium channels open and close, or gate, according to molecular transition rates that are regulated by transmembrane voltage and neurotransmitters. Here evidence for the control of gating by calcium was found in electrophysiological records of single, L-type calcium channels in heart cells. Conditional open probability analysis revealed that calcium entry during the opening of a single channel produces alterations in gating transition rates that evolve over the course of hundreds of milliseconds. Such alteration of calcium-channel gating by entry of a favored permeant ion provides a mechanism for the short-term modulation of single-ion channels.

CALCIUM CHANNELS FORM A VITAL link in the transduction of extracellular signals to numerous physiological processes, which include neurotransmitter release (*1*) and contraction (*2, 3*). To appreciate how Ca^{2+} channels function in this capacity, a rigorous understanding of their gating properties is necessary. Yet fundamental questions about Ca^{2+}-channel gating remain unanswered, in large part because of the complexities of Ca^{2+}-facilitated inactivation, a prominent feature of many Ca^{2+} channels whereby Ca^{2+} entry through the channel pore appears to speed inactivation (*4–7*). Conflicting viewpoints on this property arise from studies of macroscopic and single-channel Ca^{2+} currents. Macroscopic data hint that gating transition rates change slowly according to prior channel activity (*5*) and that elevations of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) predominate in modulating inactivation (*8*). In contrast, single-channel records have so far failed to reveal alterations in gating by bursts of Ca^{2+} entry (*9*) or by tonic elevations of $[Ca^{2+}]_i$ (*10*). These discrepancies leave open elementary questions about the very class of gating models and mechanisms required for Ca^{2+}-channel gating. This report aims to clarify some of these unresolved questions.

There are differences in the pattern of gating that result when Ba^{2+} substitutes for Ca^{2+} as the charge-carrier through L-type Ca^{2+} channels (Fig. 1) (*11*). With Ba^{2+}, the majority of records containing channel activity exhibit tightly clustered openings that often continue to the end of records (Fig. 1A). The ensemble average of the unitary Ba^{2+} currents declines very slowly, as expected without enhanced inactivation by Ca^{2+} ions (Fig. 1A, bottom). Although gating is similar when other permeant ions such as Sr^{2+} (*7, 12*) or Na^+ (*13*) are used in place of Ba^{2+}, a different picture emerges when Ca^{2+} carries the current: not only are unitary currents (*i*) smaller (−0.34 versus −0.85 pA), but openings occur less frequently and are rare by the end of each record (Fig. 1B). Accordingly, the ensemble average is smaller and declines faster (Fig. 1B, bottom). Although the overall nature of single-channel gating is unique with Ca^{2+} as the charge-carrier [but see (*14*)], these results could be explained by either of two schemes: Ca^{2+} entry could be producing slow changes in gating transition rates, as macroscopic studies (*5*) suggest; or the enhancement of inactivation might be occurring almost instantaneously with the passage of Ca^{2+} ions into the channel pore, consistent with prior single-channel reports (*9, 10*). The latter case would yield transition rates that appear to be invariant on the time scale of patch-clamp records.

To distinguish between these possibilities, we used conditional open probability analysis (COPA), developed by Sigworth (*15*) for macroscopic currents. Here single-channel records are used to calculate the conditional open probability $P_{oo}(t, t_j)$, defined as the chance that a channel is open at time t if it is known to be open at time t_j (*16*). Two properties of $P_{oo}(t, t_j)$ are relevant. (i) If rate constants remain constant ("homogeneous" process) and there is a single open state, then the decay of $P_{oo}(t, t_j)$ with increasing t ($t \geq t_j$) follows an invariant time course, independent of the choice of t_j (*17*). (ii) If Ca^{2+} entry progressively alters rate constants ("inhomogeneous" process), then the decay of $P_{oo}(t, t_j)$ should evolve with increasing t_j. Because a single open state predominates here (*16*), COPA can distinguish between homogeneous and inhomogeneous gating of the Ca^{2+} channel.

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