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The regulation of α-foetoprotein minigene expression in the germline of mice

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INTRODUCTION

Mammalian development is a complex process involving the coordinated activation and suppression of gene expression. Once differentiated, cells must also maintain the ability to respond to signals that alter the timing and extent of cell-specific transcription. To understand these processes demands an elucidation of the nature of the DNA control sequences associated with regulated genes and the number and nature of trans-acting factors that interact with those sequences. The ability to introduce cloned genes into the germline of mice offers an opportunity to study DNA sequences that control both developmental and tissue-specific gene regulation. We have chosen to investigate the nature of these control sequences in the cloned murine α-foetoprotein (AFP) gene (Krumlauf, Hammer, Tilghman & Brinster, 1985).

The AFP gene forms a small multigene family with the evolutionarily related serum albumin gene (Gorin *et al.* 1980; Ingram, Scott & Tilghman, 1981). In mice, both the AFP and albumin genes reside on chromosome 5 and are coactivated during embryonic development in the visceral endoderm of the yolk sac and several days later in foetal liver and the gastrointestinal tract (Tilghman & Belayew, 1982; Young & Tilghman, 1984). Shortly after birth, expression of the AFP gene in both the liver and gut is turned off with the rate of transcription in the liver reduced by more than 10 000-fold (Tilghman & Belayew, 1982). In contrast, albumin gene expression remains high in the liver and declines only in the gut. This decline in AFP expression in neonatal life is under genetic control by at least one trans-acting gene, termed *raf*, which ultimately determines the adult basal level of AFP mRNA (Belayew & Tilghman, 1982; Olsson, Lindahl & Ruoslahti, 1977).

Information on the nature of the DNA sequences that regulate AFP expression was provided by the demonstration that a transfected AFP minigene was activated

Key words: transgenic mice, AFP gene, gene regulation, X-inactivation, mouse, germline, α-foetoprotein.

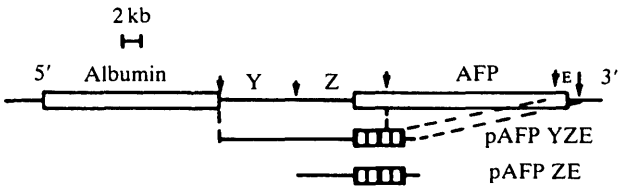

Fig. 1. Mouse AFP minigenes. The AFP minigenes YZE and ZE were prepared using cloned Y, Z and E EcoR1 restriction fragments indicated by the arrows. YZE and ZE contain 14 and 7 kb of 5′ flanking DNA, respectively. Both linearized genes contain fragments of the cloning vector, pBR 322.

during differentiation of F9 embryonal carcinoma cells into visceral endoderm (Scott, Vogt, Croke & Tilghman, 1984). This established that the introduced DNA which included the entire 14 kb intergenic DNA between the 3′ end of the albumin gene and the 5′ end of the AFP gene (Fig. 1) contained sufficient information to confer tissue-specific expression. However, this system's usefulness is limited as the importance of these sequences in the specificity and timing of expression of the gene in other tissues cannot be determined. Consequently, we have investigated the expression of modified cloned murine AFP genes introduced into the germline of mice.

TISSUE SPECIFICITY OF AFP MINIGENE EXPRESSION

Two cloned AFP minigenes that differ only in amount of 5′ flanking DNA were introduced into mice by microinjection of eggs. The YZE construct had 14 kb of 5′ flanking DNA while the ZE had 7 kb of 5′ flanking sequence (Fig. 1). These

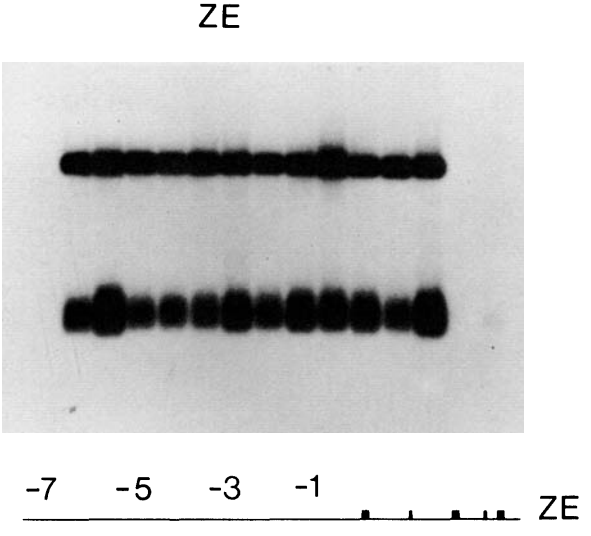

Fig. 2. Expression of ZE AFP minigene without vector sequences in yolk sacs of transgenic mice. Foetuses were sacrificed on day 19 and yolk sac poly(A)$^+$ RNA was analysed by hybridization for expression of AFP mRNA (upper band) and ZE mRNA (lower band).

constructs were composed of the first three and the last two coding blocks of the AFP gene and when introduced into cells produce a readily distinguishable transcript of 600 nucleotides.

Foetuses were sacrificed on day 19 of gestation and transgenic animals were identified by southern blotting (Fig. 2). Poly(A)$^+$ mRNA was then isolated from the yolk sacs of 34 transgenic foetuses. Of these, fifteen transgenic foetuses harbouring either YZE or ZE genes had detectable levels of minigene mRNA in yolk sac. Among the animals, expression in yolk sac was variable, ranging between 0·1 % to 25 % of endogenous AFP mRNA. All foetuses were dissected into liver, gut, kidney and heart to examine the extent and specificity of minigene expression. AFP minigene transcripts were detectable in foetal liver and to a lesser extent gut but not in other tissues. The levels of expression of the AFP minigene varied between animals and was lower than that observed for the endogenous AFP gene. Taken together these data indicated that the DNA sequences within 7 kb of 5′ flanking DNA were sufficient to provide tissue-specific expression of a minigene.

Interference of vector DNA with expression of genes introduced into the germline of mice has been demonstrated for the human β globin gene (Townes *et al.* 1985), a hybrid mouse/human β globin gene (Chada *et al.* 1985) and the mouse metallothionein-human growth hormone gene (Hammer, Brinster & Palmiter, 1985). To determine if the low and variable levels of the AFP expression in transgenic mice was the result of a similar vector phenomenon we introduced AFP constructs without vector into eggs and examined the resulting 12 transgenic mice for expression of AFP. The levels of minigene mRNA in the yolk sac of transgenic foetuses were very high, approaching endogenous levels. Thus, the low level of expression of the AFP minigene in mice is due to the presence of prokaryotic vector sequences.

Improving the levels of expression of the AFP gene in mice to near endogenous levels will greatly facilitate more precise mapping studies of the cis-acting regulatory elements in the 7 kb of 5′ flanking sequences. Information on the regulatory elements within this flanking area has recently been provided by a transient expression assay following the introduction of AFP genes into human hepatoma cell lines. Three regulatory elements spanning this 7 kb distance have been identified and found to function in an orientation- and position-independent manner that is typical of enhancers (Godbout, Ingram & Tilghman, 1986). Experiments are in progress to determine the importance of these elements to the tissue specificity and developmental regulation of AFP expression in mice.

DEVELOPMENTAL REGULATION OF AFP GENE EXPRESSION

To test whether the ZE construct contained sufficient flanking sequences for appropriate developmental regulation in neonatal liver we bred founder mice and confirmed that the tissue-specific pattern of expression was stably inherited. F_1 animals were then bred and progeny sacrificed at 3, 7, 14 and 28 days after birth, and expression of AFP in the livers of transgenic mice examined. As shown for one

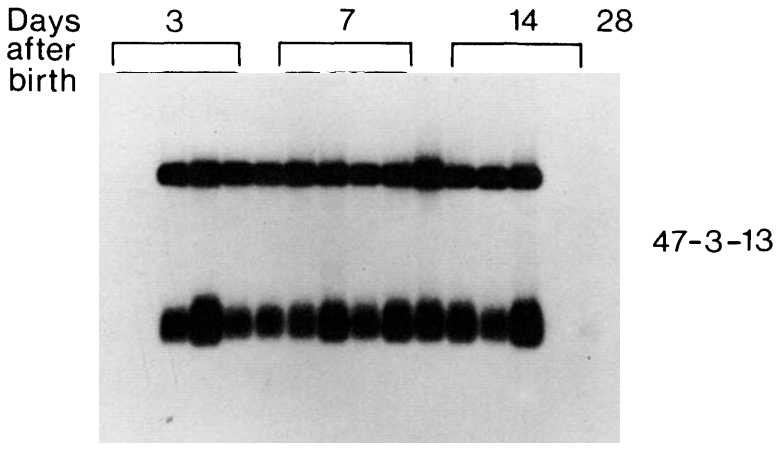

Fig. 3. Developmental regulation of AFP minigene mRNA levels in liver. Transgenic male 47-3-13 was bred and progeny sacrificed at 3, 7 and 14 days after birth. Poly(A)$^+$ mRNA was isolated from the livers of transgenic foetuses and hybridized with a probe homologous to the first AFP coding block. Upper bands are endogenous AFP and lower bands are from the ZE minigene.

line (Fig. 3) the minigene and authentic AFP mRNA levels decrease over 100-fold between days 3 and 14 and were not detectable by day 28. This parallel decline in endogenous and minigene mRNA suggests that 7 kb of 5′ flanking sequences in the ZE construct contain sequences required for postnatal transcriptional suppression of liver expression.

DIFFERENTIAL EXPRESSION OF AN AFP MINIGENE ON THE INACTIVE X CHROMOSOME

The introduction of genes into the germline of mice provides an opportunity to investigate the effects of chromosomal position on gene expression. Because genes on the paternally derived X chromosome are preferentially inactivated in the extraembryonic endoderm we investigated whether a line of transgenic mice (47-3-13) bearing an X-linked AFP gene would also exhibit X-inactivation (Krumlauf *et al.* 1986).

In this line of mice the X-linked AFP gene was expressed on both the paternal and maternal X chromosome in yolk sac, being at most two- to threefold higher when on the maternal X chromosome. This continued expression was not attributable to the failure of the X to inactivate since in female progeny derived from matings of transgenic males to female congenic mice bearing the *pgk-1*^{a} and *hprt-*^{a} alleles only the maternally derived isozymes were detectable in yolk sac. Thus, the paternally derived AFP gene escapes normal inactivation in visceral endoderm and the escape is not the result of the autosomal gene interfering with normal inactivation of the entire chromosome.

While the X-linked AFP gene escaped inactivation in extraembryonic visceral endoderm it was not able to escape inactivation on a nonvisceral endoderm-derived inactive X (Krumlauf *et al.* 1986). Thus, the transcriptional activity of the minigene on the inactive X chromosome is specific to the state of that inactive X and not due to the integration of the minigene into a region that normally escapes inactivation.

CONCLUSIONS

We have investigated the cis-acting DNA sequences that control the tissue specificity and developmental regulation of AFP expression by introducing cloned minigenes into the germline of mice. Introduced AFP genes are expressed in visceral endoderm of yolk sac and foetal liver and gut, and the time course of the decline in neonatal liver of AFP minigene expression parallels the repression of authentic liver AFP mRNA. Thus, 7 kb of AFP 5′ flanking DNA is sufficient to direct qualitatively a pattern of expression identical to the endogenous gene. In addition, these sequences not only direct expression to the appropriate tissues but also respond to developmental signals that modify its pattern of expression. Increasing the levels of AFP expression in transgenic mice by removing vector DNA will greatly facilitate mapping studies on the cis-acting DNA sequences that control expression. Transient expression assays of AFP genes in human hepatoma cell lines indicate that the 7 kb of 5′ flanking DNA contains multiple regulatory elements which function as enhancers. The role and importance of these elements in regulating the tissue specificity and developmental timing of AFP expression in mice is under investigation.

A line of transgenic mice bearing an X-linked AFP minigene has been used to investigate the nature of X-inactivation. The X-linked autosomal gene escapes inactivation on visceral endoderm but not in foetal liver. Thus, the transcriptional activity of the AFP gene on the inactive X chromosome is dependent on the tissue in which it resides and most probably reflects differences in the nature of the maintenance of the inactive state of the extraembryonic and embryonic X chromosomes.

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