Analysis of transcriptional regulatory regions *in vivo*

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ABSTRACT **Understanding the transcriptional regulation of development and tissue-specific gene expression is a central goal of modern biology. Although the analysis of gene transcription in transfected cultured cells has been essential in establishing many key aspects of this gene control, only analysis in animals can determine developmental timing and cell-specificity of expression within a complex organ and in all the tissues of an animal. The advent of transgenesis made *in vivo* studies possible. A summary of the *in vivo* regulatory properties of the pancreas-specific transcriptional enhancer of the rat elastase 1 gene *(ELA1)* and the role individual elements in this enhancer play in directing high level, cell-specific transcription illustrates the nature, revelations and limitations of transgenic analysis.**

KEY WORDS: *transcriptional enhancer, promoter, transgenic, pancreas, elastase*

There are several inherent advantages of transgenic technology for the analysis of transcriptional regulatory sequences. First and foremost, the stably integrated transgene has the potential to act *in vivo* as a normal chromosomal locus subject to all influences of animal development and physiology. This can be an important distinction, because results from transfection of cultured cells are often not mirrored in studies of transgenic animals (e.g., Babiss *et al.,* 1987; Distel *et al.,* 1987; Pinkert *et al.,* 1987; Swift *et al.,* 1989; Ross *et al.,* 1990; Goldhamer *et al.,* 1992).

Second, animals can be obtained with an identical transgene at the same chromosomal site in every cell, so that a comprehensive analysis of cell- and organ-specific transcription is limited only by the technology to detect expression of the transgene. Appropriate differentiated lines for transfection analysis of gene transcription are available for only a few cell types, and assembling a collection of cell lines for genes that are widely expressed is not generally feasible.

Third, the transgene can be modified by recombinant DNA techniques before introduction into mice to examine the properties of control regions and the specific functions of their subdomains. Such modifications have revealed that diverse, yet tissue-specific, transcription can be the simple sum of the activities of individual subdomains capable of acting independently (e.g., Ornitz *et al.,* 1987; Arnone and Davidson, 1997).

Fourth, stable inheritance of the transgene and the phenotype it confers ensures a reliable source of animals with faithful transgene expression through the establishment of lines. Consequently the properties of regulatory regions can be examined during embryonic development as well as in adult animals.

Fifth, the ability to direct the expression of virtually any gene in a temporal and site-specific manner provides a powerful means to investigate the effects of aberrant gene expression on the development and physiology of an animal. This approach is now possible because of the availability of an extensive collection of transcriptional regulatory sequences whose efficacy has been proven in transgenic animals. This approach has led to the creation of a wide variety of animal models of human diseases (Kappel *et al.,* 1994). New regulatory strategies allow the experimental control of transcription to limit the onset and duration of the expression of a transgene (e.g., Efrat *et al.,* 1995). This approach relies on the use of a small molecule effector that controls the activity of a transgene encoding a novel transcription factor that binds to and thereby controls the activity of the modified promoter of a second transgene (for a review, Saez *et al.,* 1997).

Transgenesis, as commonly practised by pronuclear microinjection, has several inherent disadvantages as well. First, to help ensure an acceptable proportion of transgenic pups, up to several hundred molecules of the transgene generally are injected into a single egg. Therefore, most transgenic mice contain multiple integrated transgene copies per cell. The number of copies can range from a fraction of a complete copy up to hundreds. Because the level of expression is often affected by the number of copies, the inability to control the number of integrated transgenes causes variation in the level of expression among independent founder mice (and between lines derived from them) bearing the same transgenic construct. Quantitative analysis of control regions that affect transcription must compensate for the presence of multiple transgene copies and the potential effects of

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reiteration on chromatin structure of the entire array (discussed below).

Second, integration occurs, if not randomly, at any of a vast number of genomic sites. The likelihood of insertion at the homologous mouse gene or at the same site in two independently derived founder mice is vanishingly small. Each integration site may impose novel regulation on the transgene (e.g., al-Shawi et al., 1990). Chromosomal positions may be permissive, neutral, or inhibitory. Some sites may activate a transgene in an unexpected and inappropriate tissue or cell; other sites may suppress expression. In these instances the extent of expression may be the sum of that specified by transgenic regulatory elements and that specified by a regulatory region (positive or negative) near the integration site. Consequently, position effects can cause quantitatively and qualitatively different expression of the same transgenic construct in two independently derived animals even though transgene copy number may be identical.

When multiple copies integrate, they generally do so at the same site as a head-to-tail tandem repeat. Consequently, all transgene copies are subject to the same chromosomal position effects. The head-to-tail organization of tandem transgenes suggests that the injected transgenes first circularize, then recruit additional copies into a growing circle by homologous recombination until integration occurs (Brinster et al., 1985), probably at a double-stranded chromosome break and perhaps frequently within a replication bubble (Wilkie and Palmiter, 1987). Occasionally, integration occurs at more than one chromosomal location and the multiple loci, with different numbers of transgene copies, may express the same transgene construct differently (MacDonald et al., 1986a), presumably due to different position effects at the two integration sites. Founder mice with multiple integration sites are identified only if bred to detect independent segregation of the transgenic loci.

Third, multicopy arrays of transgenes are inherently susceptible to transcriptional silencing (Davis and MacDonald, 1988; Dorer, 1997). The nature of the silencing is frequently evidenced in two ways. First, loci with large numbers of transgenes generally are not expressed at levels higher than loci with fewer copies of the same transgene. Second, the fraction of expressing cells often decreases with increasing copy numbers, which gives rise to a variegated pattern of expression. The latter appears to be the cause of the former. The probability of silencing is affected by the site of chromosomal integration (Dobie et al., 1997) and grows with increasing numbers of tandem gene copies (Robertson et al., 1995). Consequently, the rates of silencing of different integrants of the same transgene are not equal. For loci with numerous copies the effects of silencing can make strong transcriptional control regions appear weak.

Fourth, if integration occurs before the first zygotic cleavage, all cells bear the transgene. However, if integration occurs later, the mice may be mosaic. One extensive study of a wide variety of transgenes showed that mosiacism may average 30% (Wilkie et al., 1986). Therefore, studies of transgene expression that utilize founder mice or embryos, rather than lines, are subject to the additional variable of mosaicism. The only reliable way to identify mosaic animals is by examining progeny. Nonmosaic mice are obtained in the next generation, although mosaicism generally extends to the germ cells and the fraction of transgenic progeny may be much below the one-half expected for nonmosaic hemizygous parents.

As a result of these uncontrollable variables, each independently derived transgenic animal bearing the same transgenic construct is unique: the number of copies, the site of integration, differential silencing, and possible mosaicism in founder mice all affect the level and occasionally the specificity of expression of the transgene. For the analysis of the strength and specificity of tissue-specific transcriptional regulatory regions, this variability is a major drawback and results must be compared for a number of founder mice (or lines derived from independent founders) bearing the same transgene.

The problems in comparing the transcriptional activity of different transgenes due to multiple transgene copies integrated at random sites with variable amounts of silencing can be overcome by the site-specific integration of single-copy transgenes through gene targeting. This technique involves homologous integration in embryonic stem cells followed by generation of transgenic mice through the introduction of the embryonic stem cells into blastocysts, and subsequent breeding of the resultant chimeric mice to establish transgenic lines. For example, Bronson et al. (1996) compared the strength of two related promoters by integrating them into the identical locus. Their finding that multiple independently isolated integrants of each particular promoter had identical levels of expression, but the expression levels for the two promoters differed, validates this approach. The target for integration, in this case the hypoxanthine phosphoribosyl transferase locus, does not need to be the endogenous locus of the gene from which the control

Transgene copy no.	Penetrance	Expression mRNAs/cell (% endogenous)
2-250	8 of 8	2,400-49,000 (25-500)
9-51	4 of 4	1,900-4,100 (20-40)
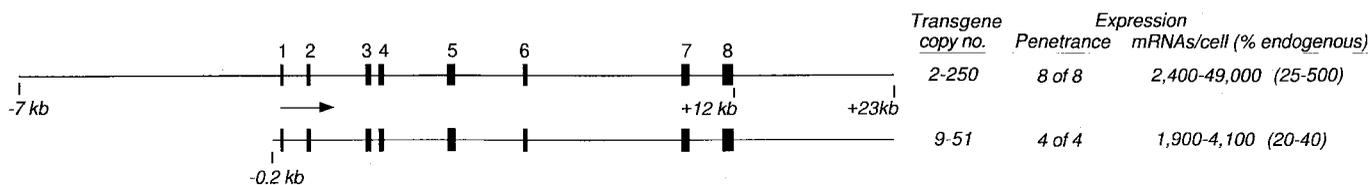
Fig. 1. Expression of rat *ELA1* transgenes in mice. *The two ELA1 transgenes have 12 kb of intronic and exonic sequences and nearly 5 kb of 3' flanking DNA. The long (23 kb) transgene has 7 kb and the short (17 kb) transgene has 205bp of 5' flanking DNA. The range of transgene copy numbers are for the eight lines bearing the long and the four founders bearing the short transgenes. Expression penetrance is the number of expressors out of the total number of founders/lines assayed. Expression level is the number of rat ELA1 mRNA molecules per pancreatic cell. The mean numbers of mRNAs/cell for the long and short transgenes were 31,000 and 3,800, respectively, compared to approximately 10,000 mRNAs per cell for ELA1 in rat pancreas. Rat ELA1 transcripts were quantified and distinguished from the endogenous mouse transcripts with a hybridization probe derived from the 3' untranslated region of the rat mRNA.*

region is derived. Thus, a single basic targeting vector can be used to accept regulatory cassettes for different genes, thereby obviating the need to create vectors with different homologous recombination sequences for each gene. Transcriptional regulatory regions also can be tested within the context of the endogenous gene locus. For example, by deleting the Igκ 3' enhancer *in situ*, Gorman *et al.* (1996) demonstrated that this enhancer is not required for normal κ expression in activated T cells, although it is critical for establishing the normal ratio of κ to λ. These experimental variations enhance the value of transgenesis as a tool for the analysis of gene function within the context of complex mammalian physiology and embryonic development.

Cell-specific transcription

Initial attempts to reproduce cell-type specific expression by transgenesis in mammals were thwarted by the unsuspected complexity of metazoan transcriptional regulatory regions and the great molecular distance these regions may reside from the transcribed gene. For example, an 11 kb rabbit β-globin transgene including 4 kb 5' and 4 kb 3' flanking DNA was not expressed in mouse erythroid cells (Lacy *et al.,* 1983). Rabbit β-globin transcripts, however, were detected at low levels in inappropriate tissues, such as brain, liver, kidney, lung, muscle and testes. Similarly, transgenes of rat and human growth hormone and chicken transferrin with limited flanking DNA were not expressed properly (McKnight *et al.,* 1983; Wagner *et al.,* 1983; Hammer *et al.,* 1984). Low expression penetrance (the fraction of nonmosaic founder animals that express the transgene) and low amounts of (or absent) mRNA are diagnostic for transgenes missing crucial regulatory sequences. In addition, incomplete transgenes are frequently expressed in inappropriate organs, often at levels as high or higher than in the appropriate organ, due to a susceptibility to endogenous control regions present at the chromosomal integration site. Subsequently, crucial transcriptional control regions were discovered distant from the short β-globin and human growth hormone (Jones *et al.,* 1995) transgenes initially analyzed.

The first demonstrations of highly selective, organ- and cell-specific expression of transgenes that recapitulated the expression of the gene in its normal locus were for genes with all crucial transcriptional control regions close to the structural genes. Thus, effective erythroid expression of a rearranged immunoglobulin κ light chain transgene was demonstrated in 1983 (Brinster *et al.,* 1983) and pancreas-specific expression of a pancreatic elastase 1 *(ELA1)* transgene shortly thereafter (Swift *et al.,* 1984b). mRNA from the κ transgene accounted for a full third of the total 9,000 κ mRNA molecules in an average spleen cell from transgenic mice. Subsequent analyses showed that the transgenic κ mRNA was present selectively in B lymphocytes, not in T lymphocytes or any of the seven nonlymphoid tissues examined (Storb *et al.,* 1984). This rigorous cell-specificity mimicked the expression of the endogenous gene. It is now known that the 15 kb κ transgene contained three important transcriptional elements –the Vκ promoter with its transcriptional start site, an enhancer in the intron between the Jκ region and the Cκ constant region exon, and a second, stronger enhancer 9 kb downstream of the Cκ exon (Picard and Schaffner, 1984; Queen and Stafford, 1984; Meyer *et al.,* 1990). Either enhancer is effective and together they can act synergistically (Fulton and Van Ness, 1993).
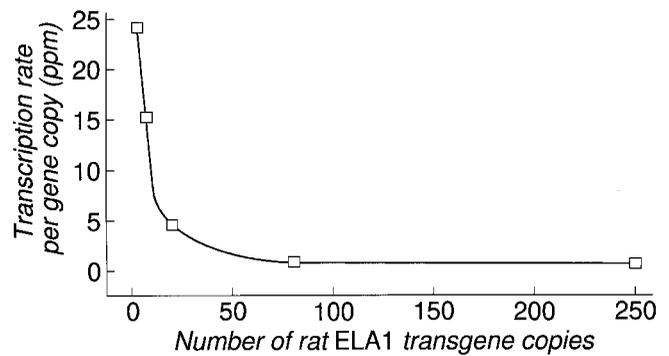
Fig. 2. The rate of transcription per transgene copy decreases with increasing numbers of tandem transgenes in an array. *The rates of transcription for ELA1 transgenic loci were measured in vitro by run-off transcription with nuclei isolated from the pancreas of transgenic mouse lines bearing from 2 to approximately 250 transgenes. The transcription rates are expressed as parts per million of the RNA synthesized. Redrawn from Davis and MacDonald (1988).*

The transcriptional control elements of the *ELA1* gene are even more compact: all the information for characteristic high level expression selectively in pancreatic cells is confined to a gene-proximal region from the transcriptional start to 200 nucleotides upstream. The properties of the *ELA1* transcriptional control regions are the subject of the rest of this review.

Transgenic analysis of *ELA1* transcription

ELA1 encodes a representative member of the group of digestive hydrolytic enzymes that is expressed to high levels selectively in the acinar cells of the exocrine pancreas. The mRNAs for the approximately 18 secretory enzymes comprise more than 80% of the total mRNA of the gland (Harding *et al.,* 1977). The average rat acinar cell has approximately 10,000

TABLE 1

EXPRESSION LEVELS OF *ELA1* TRANSGENES FOR LOW COPY NUMBER ANIMALS

Gene or transgene	Average mRNAs/ cell/copy	SEM	No. of founders or lines	Range of copy number
endogenous *ELA1*	5,000			2
23 kb *ELA1*	4,800	1,300	4	1 - 9
4.5*ELA1*.hGH	3,100	980	6	1 - 6
0.5*ELA1*.hGH	5,700	2,500	3	1 - 4
0.2*ELA1*.hGH	7,800	2,900	4	2 - 4
134*ELA1*.hGH	14,000	2,200	2	4 - 7

	Penetrance	Pancreas	Non-pancreas
	6 of 8	++	-
	5 of 7	++	-
	26 of 29	++	-
	0 of 9	-	-
	0 of 12	-	-
	7 of 7	++	-
in the first hGH intron	8 of 10	+	-
with the hGH promoter	2 of 3	++	-
	4 of 4	++	-
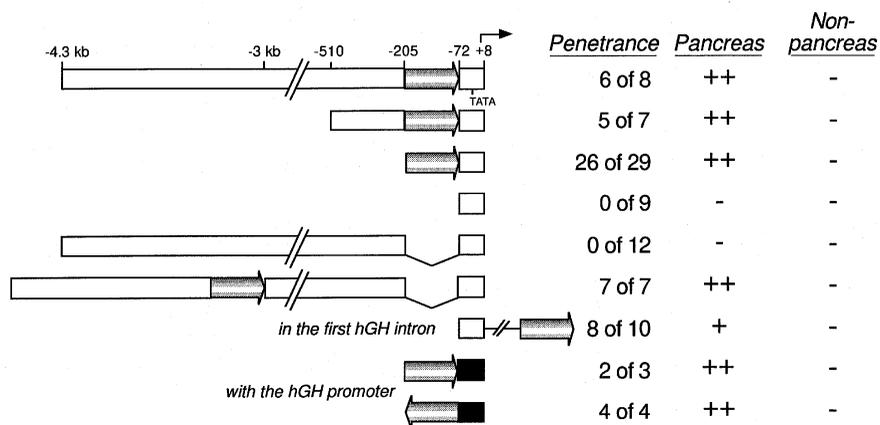
Fig. 3. Location and properties of the *ELA1* transcriptional enhancer. *Penetrance is the number of independent founder mice that expressed the transgene out of the total number analyzed. The absence of expression (-) indicates that the level of transgenic hGH mRNA was below detection (about 10 hGH mRNA molecules/cell). ++ indicates high expression (average mRNAs/cell between 10,000 and 75,000 for the different constructs), comparable to the level of ELA1 mRNA (10,000-20,000 mRNAs/cell). + indicates moderate expression (an average of 4,000 mRNAs/cell for this transgene). Extra-pancreatic expression was examined in intestine, kidney, liver, parotid, spleen, submaxillary, and testes or ovary.*

ELA1 mRNA molecules. *ELA1* is generally not detectable in other organs, with the exception of other parts of the gut (intestine and stomach) with mRNA levels about 100-fold lower than pancreatic levels (Rose *et al.*, 1994).

Rat *ELA1* transgenes bearing 7 kb of 5' and 4 kb of 3' flanking DNA were expressed in a manner indistinguishable from the endogenous gene (Swift *et al.*, 1984b; MacDonald *et al.*, 1986b). Because expression of the endogenous gene is strong and highly pancreas-specific, it was straightforward to demonstrate rigorously the specificity of the transgene. mRNA levels from the transgenes equalled or exceeded that of the endogenous gene (Fig. 1). Transgenic mRNA levels varied with copy number and ranged from 25 to 500% the level of *ELA1* mRNA in rat pancreas. Strikingly, two tandem copies of the transgene produced a level of mRNA equal to that of the two endogenous alleles. Expression was organ-specific: no significant expression was detected in the seven non-pancreatic organs examined. Expression was reproducible: all eight transgenic lines had high mRNA levels selectively in the pancreas.

The high pancreatic expression of a second rat *ELA1* transgene with only 205bp of 5' flanking DNA (Fig. 1) suggested that the organ-specific control sequences were indeed short and immedi-ately adjacent to the transcriptional start site, or possibly within the transcription unit (Ornitz *et al.*, 1985).

Transcriptional run-on experiments with nuclei isolated from the liver and pancreas of transgenic mice verified that the pancreas-specific expression was determined transcriptionally (MacDonald *et al.*, 1986a). Moreover, all the steps in transcription, RNA processing, and translation occurred correctly and the rat elastase I protein was secreted normally by the transgenic mouse pancreas.

Attenuated expression of tandemly repeated transgenes

It was recognized early that tandem arrays of transgenes had unusual properties (Davis and MacDonald, 1988). Although small numbers of tandem *ELA1* transgenes were transcribed efficiently, the overall rate of transcription, measured in isolated nuclei, remained nearly constant with increasing numbers of tandem *ELA1* transgenes, so that the transcription rate per copy decreased dramatically (Fig. 2). The attenuated transcription was not due to titration of an essential transcription factor(s), because the endogenous gene and unlinked transgenic loci were unaffected. We proposed that the creation of tandem repeats of

Fig. 4. Pancreatic expression of the hGH fusion transgene with 0.5 kb of *ELA1* 5' DNA is limited to acinar cells. *Intense immunofluorescent staining of the hGH reporter protein is observed for most acinar cells. hGH is not detected in cells of islets of Langerhans (i), exocrine ducts (d), connective tissue (c) and blood vessels (not shown). Immunostaining of cross sections of acini reveal that hGH is restricted to the apical regions of the cells, coincident with the localization of the secretory (zymogen) granules. The lumen of an intralobular duct (d), which collects and channels acinar cell secretions, contains hGH. Therefore, the endocrine protein hGH appears to be packaged effectively with the exocrine secretory enzymes, transported to the apical lumen, and secreted. From (MacDonald et al., 1986b), with permission.*
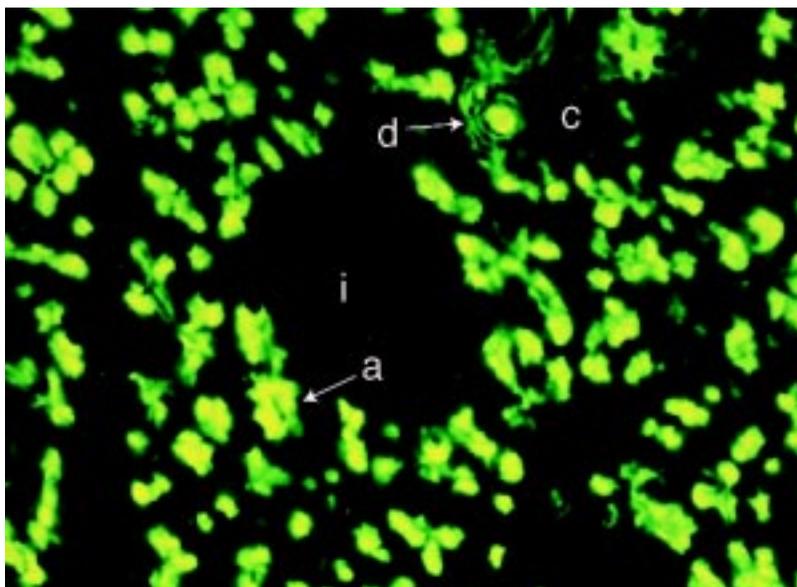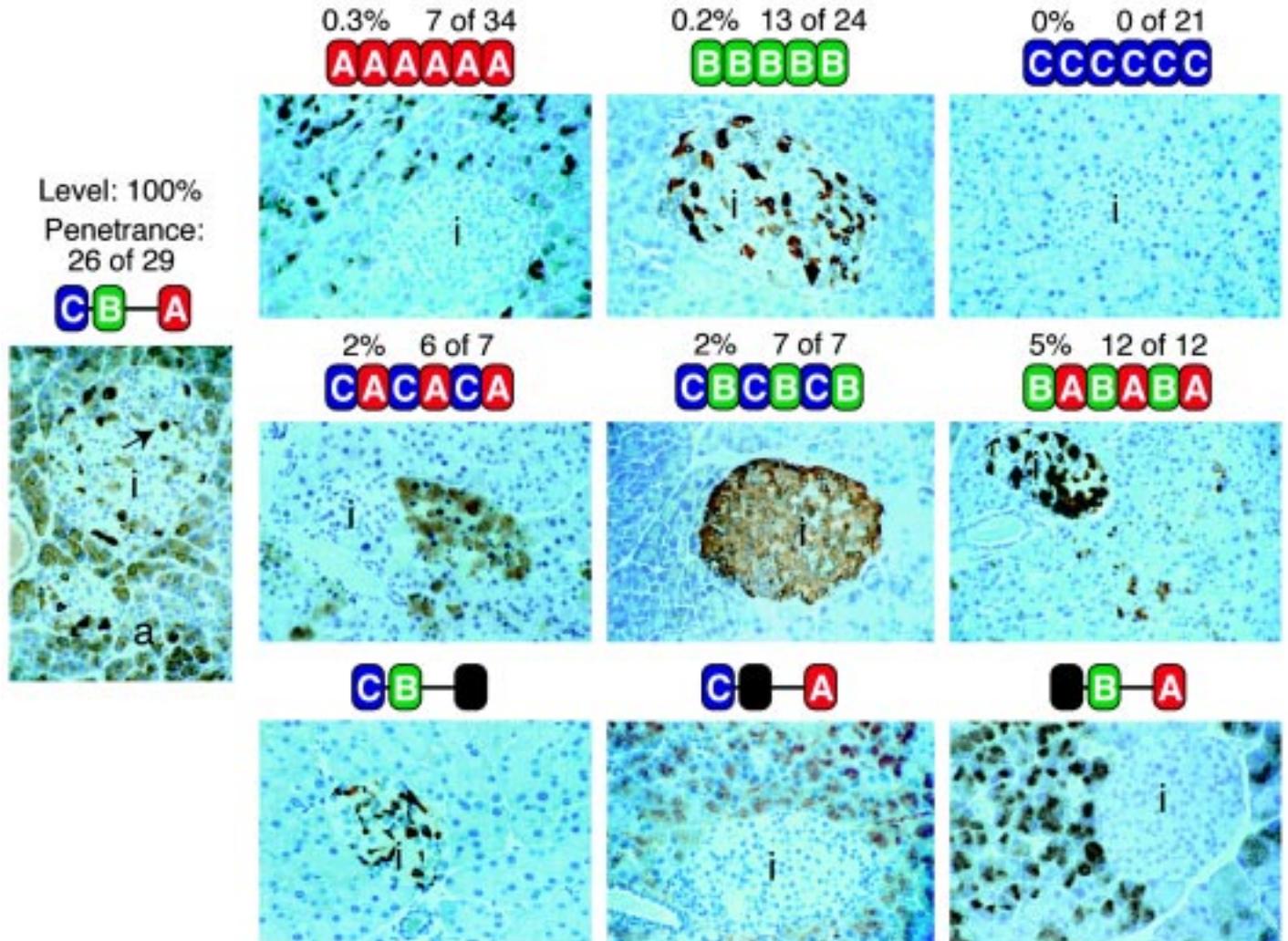
Fig. 5. The three elements of the *ELA1* enhancer display distinct and different transcriptional activities in transgenic mice. *Cell-type specific expression is detected by immunostaining for the product of the hGH reporter gene. The majority (approximately 80%) of pancreatic tissue is acinar (a); 1-2% is islets (i).* **Leftmost panel:** *In the absence of the upstream repressor region, the three element enhancer directs expression to acinar cells and to a subset of β-cells of islets.* **Top panels:** *Synthetic enhancers comprising homomultimeric repeats of the A element and the B element direct expression to acinar and islet cells, respectively, whereas the repeat of the C element is inactive.* **Middle panels:** *Addition of the C element to repeats of the A or B elements increases the level of transgenic expression about 10-fold without altering the cell-type specificity of the homomultimeric A or B elements. The A+B heteromultimer gives the simple sum of the A and B cell-specifities.* **Bottom panels:** *Mutational inactivation of each element of the enhancer affects the expression pattern in a characteristic manner predicted by the activities of the homo- and hetero-multimeric repeats. Mutation of the A element eliminates acinar expression; mutation of the B element eliminates islet expression; mutation of the C element eliminates islet but not acinar expression. Assembled from the data of (Kruse et al., 1993, 1995) and unpublished.*

otherwise single copy genes reduces the efficiency of transcription of genes within the array.

Copy-number dependent silencing of transgenes has since been observed in *Drosophila*, plants, and mice (Dorer, 1997). Reduced transcription rates measured in a population of cells may be due to a smaller fraction of active genes in an array or a smaller fraction of cells in which the array is active. The observation that transgene expression often is limited to discrete tissue sectors (variegated expression) suggests that decreased transcriptional efficiency is due to silencing the entire array. Thus, decreased rates of transcription observed for transgenic arrays with greater

numbers of *ELA1* genes is probably due to an increasing probability of inactivation of the entire array in some cells of the population.

The extent of silencing is affected by integration site as well as the number of tandem transgene copies (Dobie *et al.,* 1997; Dorer, 1997). Integration near or within heterochromatin enhances silencing. The observations for tandemly arranged transgenes suggest that pairing between repeat units induces the formation of heterochromatin throughout the transgenic array (Dorer and Henikoff, 1994; Wolffe, 1998). By *in vivo* manipulation of the number of transgene copies in an array, Garrick and colleagues (Garrick *et al.,* 1998) demonstrated directly that the number of copies affected

the probability that the entire array would be silenced. Cre recombinase was used to delete *loxP*-flanked α-globin-lacZ transgene copies from two different transgenic loci, each originally containing more than 100 tandem copies. These two high copy loci were generally silent; less than 1% of erythroid cells had an active transgenic locus. Reduction of transgene copies to 5 or 1 increased the number of expressing cells in the population 200- to 1,000-fold. The transgenes in a low copy array were undermethylated and in an open chromatin structure compared to those in the high copy array at the same genomic location. Hypermethylation and hetero-chromatin are hallmarks of inactive genes. One conclusion from these studies is that arrays with less than about 10 copies best reflect the inherent transcriptional strength of a transgene. These experiments also demonstrate that many silent, high copy-number integrants may be rescued by Cre recombinase treatment, if a single *loxP* site is included in the transgenic construct.

Defining the regulatory strategy of the pancreatic *elastase I* gene

The transcriptional regulatory information for pancreatic acinar expression of *ELA1* is present in the 5' flanking region within 4.2 kb of the transcriptional start (Ornitz *et al.,* 1985). Fusion transgenes containing the *ELA1* region from -4.2 kb to +8bp linked to the structural gene for the human growth hormone (hGH) were routinely expressed selectively and to high levels in the pancreas (Fig. 3).

Immunofluorescent localization of the hGH reporter protein in these and similar transgenic mice bearing hGH fusion transgenes with 0.5 kb of *ELA1* flanking DNA (Fig. 4) showed that nearly all acinar cells expressed the transgenes. This result is consistent with the synthesis of the complete complement of digestive enzymes, includ-ing elastase 1, in each acinar cell (Mroz and Luchene, 1986). Even though immunodetection of hGH is very sensitive, hGH protein could not be detected in the other pancreatic cell types (cells of islets, ducts, connective tissue, or blood vessels) that do not express the endog-enous *elastase I* gene. Therefore, the 5' proximal gene region contains the regulatory sequences for both the characteristic high level pancreatic expression and the single cell-type specificity of *ELA1*.

Because strong control regions better overcome repressive chro-mosomal position effects, the fraction of founder mice that express a particular transgenic construct can be used as one measure of the strength of control regions contained within the construct. The high expression penetrance of the 4.2*ELA1*.hGH fusion transgene –six of the eight independent founders expressed– indicates that the tran-scriptional control sequences in the 4.2 kb of *ELA1* flanking DNA are robust. The relative levels of transgenic mRNA can also be used to judge the strength of transcriptional control regions if certain precau-tions are met and conclusions are tempered. To adjust for the effect of the number of transgene copies in an array, the level of expression can be expressed per transgene copy. By this measure, transgenic hGH mRNA in the pancreas averaged 3,100 mRNAs/cell/copy (±1,000 SEM) for the six founders with 1-6 transgene copies (Table 1). This is comparable to the average of 4,800/cell/copy for the low copy-number 23 kb-*ELA1* transgenic animals (1-9 transgene copies) and 5,000 for each endogenous rat gene.

Two assumptions, both arguable, were made for these compari-sons: that expression in low copy number animals is more repre-sentative than that of high copy number animals and that the levels of two different transgenic mRNAs (*ELA1* and hGH) can be used

accurately to compare relative transcription rates. Only animals with less than ten transgenes were in the comparison, because increas-ing numbers of transgenes in an array increases the probability that the entire array will be silenced (discussed above), thereby disproportionally decreasing the transcription rates of large arrays. This assumption may indeed be tenable, because for loci with less than 10 transgene copies there is a clear copy number dependence for mRNA levels of both the intact *ELA1* and the *ELA1*-hGH fusion transgenes, as judged by acceptable standard errors of the mean (Table 1). Loci with more than about 10 copies have exceedingly variable levels of mRNA/copy. One conclusion from the expression of low copy number loci is that the *ELA1* transcriptional control regions, though close to and regulating only the *ELA1* gene, have the copy-number dependent property generally associated with a locus control region (Grosveld *et al.,* 1987).

The use of steady state levels of the two different mRNAs to compare the relative transcriptional efficiencies of the *ELA1* and hGH reporter genes also may be justified, because the major difference should be mRNA stability and both hGH and *ELA1* mRNAs are long lived. To validate such a comparison, it would be necessary to compare mRNA levels from the two reporters linked to the identical transcriptional control region. Similar (though non-identical) *ELA1* 5' flanking regions do indeed yield nearly equivalent transgenic mRNA levels (Table 1). The 23 kb *ELA1* transgene (Fig. 1) contains 7 kb of 5' flanking DNA, whereas the 4.2*ELA1*.hGH transgene has 4.2 kb. These two flanking regions appear to be functionally equivalent, because most, if not all, of the transcriptional control information is in the 0.5 kb immediately upstream of the transcriptional start site (see below).

The minimal pancreas-specific information is only about 100 base pairs long

Stepwise deletion of the *ELA1* flanking region from -4.2 kbp down to -71bp has defined the region necessary and sufficient for pancre-atic acinar specific transcription of hGH fusion transgenes (Ornitz *et al.,* 1985; Hammer *et al.,* 1987). Removal of -4.2 kbp to -205bp had no readily discernible effect on expression penetrance, transgene mRNA levels or organ-specificity (Fig. 3). 26 of 29 independent founder mice expressed the fusion transgene and the average hGH mRNA level per transgene copy was 7,800 mRNAs/cell for the four low copy founders analyzed (0.2*ELA1*.hGH; Table 1). Fusion transgenes with 0.5 kb of 5' ELA1 sequences had similar expression penetrance (5 of 7) and level (0.5*ELA1*.hGH; Table 1). Deletion to -71bp abolished expression: none of the nine founders had detect-able hGH mRNA in the pancreas or the seven nonpancreatic organs examined (Fig. 3).

The results of these experiments showed that the region from -205 to -72 is important for pancreatic expression *in vivo*. Deletion of this 134bp region from a fusion transgene containing the remainder of the -4.2 kb flanking sequence also eliminated expression (Fig. 3). Therefore, within the context of the extensive *ELA1* 5' flanking sequence, this region appears necessary for transcription. These results, however, do not exclude the possibility that redundant transcriptional control sequences are present within or downstream of the *ELA1* gene.

All of the constructs used in these experiments retained the *ELA1* promoter elements nearby the transcriptional start site. This *ELA1* region from -71 to +8 (or -92 to +8) has the properties of a nonspecific minimal promoter; it is inactive in transgenic mice or transfected cells,

Fig. 6. Summary of the functional elements of the *ELA1* promoter region. *The minimal promoter region, the three elements of the enhancer and the repressor are shown with their known or presumed DNA binding transcription factors shown for acinar cells (above) and β-cells (below). The identities of the factors bound to the C element and the repressor region are not known.*
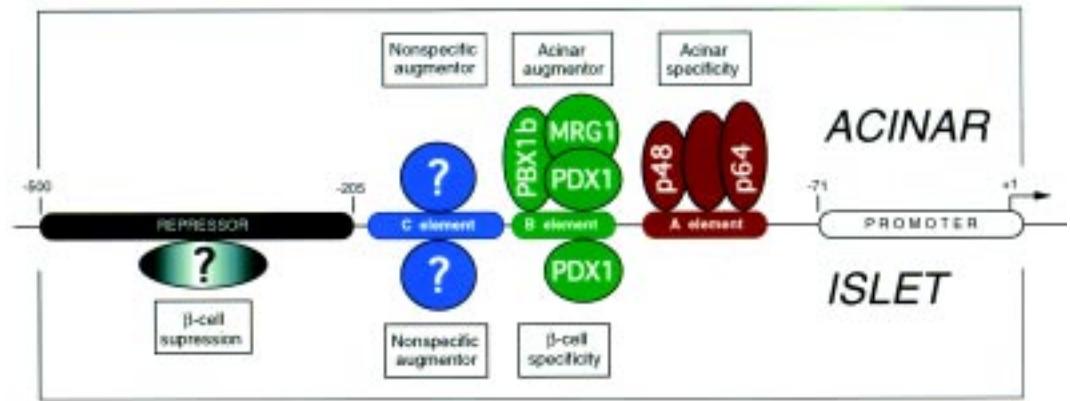
but can be activated by its cognate control region or by heterologous enhancers (Ornitz *et al.,* 1985; Hammer *et al.,* 1987). To determine whether the 134bp control region contained all the necessary organ-specific information, a fusion transgene containing this region linked to the hGH reporter gene with its minimal promoter intact (out to -83) was tested in mice. The -83bp version of the hGH reporter gene, without any additional regulatory information, is inactive (Ornitz *et al.,* 1987). With the addition of the -205 to -72 *ELA1* region, however, the -83bp hGH reporter gene was activated selectively in the pancreas of two of the three founders tested. Transgenic mRNA levels (134*ELA1*.hGH; Table 1) were even higher than the nearly identical construct with the *ELA1* minimal promoter region. Therefore, the 134bp *ELA1* region (from -205 to -72) appears to be necessary and sufficient for highly specific pancreatic expression.

The 134bp control region acts as a transcriptional enhancer in animals

The ability of the 134 bp region to activate the minimal hGH gene promoter in mice suggested that it may have the properties of a transcriptional enhancer. Although enhancers had been characterized by transfection into cultured cells, it was important to show whether enhancers have the same properties when integrated into a chromosome of an animal and exhibit cell- and stage-specificity during complex mammalian development. We demonstrated (Hammer *et al.,* 1987) that the 134bp fragment activated transcription from the correct start site (i) independent of orientation, (ii) with a heterologous promoter, (iii) at a distance far upstream, and (iv) from a position downstream of the transcriptional start (Fig. 3). Thus, the control region, in either orientation, directed pancreas-specific activation of its own promoter or the hGH gene promoter. Activation was not affected by moving the 134bp fragment 3 kb upstream. Placing it within the first intron of the hGH reporter gene had only a modest effect on mRNA level (about 5-fold for low copy transgenics) without affecting organ-specificity.

A DNAse I hypersensitive site characteristic of a regulatory complex resides at the enhancer in chromatin isolated from pancreatic nuclei but not from hepatic nuclei. This pancreas-specific site remained associated with the enhancer when it was linked to the hGH promoter, repositioned 3 kb upstream, or placed downstream of the transcriptional start at a position in the first intron of the hGH gene (Hammer *et al.,* 1987). The invariant coincidence of the hypersensitive site and the enhancer demonstrated that the 134bp regulatory domain has all the sequence information to

initiate the formation of a pancreas-specific transcription complex.

The *ELA1* enhancer also contains the regulatory information for the correct timing of transcriptional activation during embryogenesis (Hammer *et al.,* 1987). The expression of transgenes containing only the 134bp enhancer fused to the hGH gene and promoter was monitored by hGH immunostaining at various stages of pancreogenesis. hGH was just barely detectable in the epithelial cells of the pancreatic rudiment at embryonic day 14, coincident with the normal appearance of the digestive enzymes, including elastase 1, in these cells. At days 16 and 17, hGH immunostaining increased in parallel with the accumulation of the digestive enzymes and the number of acini.

It is surprising still that such a short DNA sequence has all the regulatory information to assemble an organ-specific transcription complex, alter chromatin architecture, and activate a nonspecific promoter to high levels at the proper time during development within a complex mammalian genome.

Acinar specific transcription requires a repressor region

Although the regulatory information necessary and sufficient for pancreatic transcription is contained in the 134bp enhancer, this region alone does not confer complete acinar cell specificity (Kruse *et al.,* 1993). Pancreata of mice bearing hGH fusion transgenes with only 0.2 kb of *ELA1* 5' flanking DNA have hGH immunostaining throughout pancreatic acinar tissue and also in a subset (about 10%) of the β-cells of the islets of Langerhans (e.g., see the leftmost panel of Fig. 5). Neither the endogenous *ELA1* gene nor transgenes with longer *ELA1* 5' flanking DNA (Ornitz *et al.,* 1985) are expressed in islets. The aberrant islet expression is directed by a single transcriptional element within the 134bp enhancer (see below). When the 300bp immediately upstream of the enhancer is added to transgenes containing the enhancer, islet cell expression is suppressed without noticeably affecting acinar cell expression. Therefore, the *ELA1* enhancer directs expression to both endocrine and exocrine tissues of the pancreas, and a repressor region imposes acinar specificity.

The roles of individual enhancer elements in animals

The effects of linker-scanning mutations showed that the 134bp enhancer comprises three mutation-sensitive elements (see Fig. 6): A (-96 to -115), B (-147 to -158) and C (-166 to -195) (Rose *et*

al., 1994). Mutation of any one element inactivated the enhancer in both transient and stable transfections of cultured pancreatic acinar cell lines (Swift *et al.,* 1989; Rose *et al.,* 1994). However, mutation of either the B or the C element has little effect on transgenic mRNA levels in mice; mutation of both is required to inactivate the enhancer (Swift *et al.,* 1989). Therefore, each element is essential in transfected cells in culture but B and C are somehow redundant in transgenic animals. We (Swift *et al.,* 1989) attributed this difference to a more differentiated state of acinar cells of pancreatic tissue than tumor cell lines in culture. We proposed that acinar cells *in situ* have a transcription factor activity that compensates for the loss of function of any one *ELA1* enhancer element, and that one consequence of the dedifferentiation of the acinar tumor cells is the loss of this activity. Consequently, the activity of the enhancer in transfected cells is susceptible to the inactivation of any one element, but not in transgenic animals.

The ability of any two elements to direct pancreas-specific transcription in mice revealed that at least two of the three enhancer elements must contain information for pancreatic expression. To investigate the contribution of each element to the regulatory properties of the enhancer, the expression of hGH fusion transgenes driven by synthetic homomultimeric repeats of each element was examined in mice (Kruse *et al.,* 1993; Rose *et al.,* 1994). Although artificial, homomultimerization accommodates the need for more than a single *ELA1* enhancer element for expression in animals (see above) and the efficacy of this strategy has been proven in numerous studies with transfected cells. Indeed, homomultimers of the A or B elements directed highly specific expression of reporter transgenes.

The A element

Six tandem copies of the 26-bp A element activated transcription in pancreatic acinar cells of transgenic mice (Fig. 5). The presence of hGH reporter protein was not detected in other pancreatic cell types in several independent lines examined. Pancreatic expression was independent of the nature of the minimal promoter, because the *ELA1* and the HSV tk promoters were equally responsive (Rose *et al.,* 1994). These results showed that a single element is capable of directing highly selective transcription in animals. However, expression penetrance and transgenic mRNA levels for the A multimer were much lower than those for the three element enhancer. Consequently, other enhancer elements are required to ensure effective levels of gene activation.

ELA1 expression is not exclusively pancreatic; its mRNA is present in stomach and parts of the intestine at about 1% the level in the pancreas. This characteristic gastro-enteropancreatic expression of elastase and other pancreatic digestive enzymes (Ceci *et al.,* 1991; Davis *et al.,* 1992) is recapitulated by transgenes driven by the *ELA1* enhancer and by the homomultimeric A element (Rose *et al.,* 1994). Therefore, the A element alone is capable of directing expression appropriately to both pancreatic and non-pancreatic sites.

The B element

Five or six tandem copies of a 21-bp B element activated transgene expression selectively in the pancreas (Fig. 5); transgenic mRNA was not detected for the 21 different nonpancreatic organs examined for several independent lines with either the *ELA1* and

HSV tk promoters (Kruse *et al.,* 1993). In contrast to expectations, however, the transgenes were not expressed in acinar cells, but rather exclusively in a subset of islet β-cells (Fig. 5). Selective hGH immunostaining of islets was observed for all four independent lines examined. The pattern of intense hGH staining in a fraction of the cells of the islet is similar to the islet expression of the three element enhancer in the absence of the 300bp repressor region described above.

It appeared that the islet cell activity of the three-element enhancer was derived from the B element and the acinar activity from the A element. As a first test of these activities, we examined the expression of a mixed $(AB)_3$ multimer (Kruse *et al.,* 1995). In contrast to the partial penetrance of the A and B homomultimers, all 12 founders expressed the AB transgene in the pancreas. The immunostaining pattern for the transgenic hGH reporter was the combination of the islet-specificity of the B element and the acinar specificity of the A element (Fig. 5). In addition, the average transgenic mRNA levels were 20-fold greater that those for the homomultimers. The high expression and dual-specificity of the mixed AB multimer recapitulates the expression properties of the three-element enhancer.

The C element

Transgenes driven by a six-copy repeat of the 32-bp C element were inactive in mice (Fig. 5). There was no expression in the pancreas of the 21 founders examined, and none in the 18 different nonpancreatic organs examined for two lines (Kruse *et al.,* 1995). However, it is clear from the effects of mutating the C element that this element plays a role in acinar cell transcription in both transfected acinar cells and in transgenic mice (see above). The nature of this role becomes evident in the expression properties of transgenes driven by mixed multimers of C with either B or A. A three-fold repeat of CA increased transgenic hGH mRNA levels approximately 10-fold over the A repeat without altering the acinar specificity of the A element (Fig. 5). Similarly, a three-fold CB repeat increased mRNA levels nearly an order of magnitude over the B repeat without altering the β-cell specificity of the B element. Thus, the C element augments the activity of the A and B elements in the enhancer without affecting their cell-specificity.

From the transgenic regulatory properties of the individual and pairwise combinations of the three elements, it is possible to propose a simple model for the role of each in the enhancer (Fig. 6). The A element appears to be the sole positively acting element for acinar cell expression and plays no role in β-cells. The B element is responsible for activating transgene expression in β-cells, whereas in acinar cells it augments the activity of the A element. The C element does not activate a transgene on its own and provides no organ- or cell-specific information; its action is to augment the activity of the A element in acinar cells and the B element in islet cells. Finally, the repressor region immediately upstream of the *ELA1* enhancer suppresses the islet activity of the B element without silencing acinar expression. The integration of these cell-specific, augmenting, and negative activities makes a potent enhancer that activates transcription to high levels selectively in pancreatic acinar cells and to lower levels in other selected sites of the gut.

The effects of directed mutations in the enhancer confirmed the regulatory properties proposed for the three elements (Fig. 5, bottom). Mutational inactivation of the A element abolished acinar

expression of transgenes without affecting β-cell expression. Thus, in the context of the enhancer the A element directs acinar expression, and a single copy of B together with C is sufficient for islet activity. Conversely, inactivation of the B element abolished β-cell expression without affecting acinar expression. Consistent with a solely augmenting function for the C element, inactivation of C eliminates β-cell expression, but does not affect acinar expression. Thus, without a role for the A element in β-cells and in the absence of C, the single B element is insufficient for transcription in mice. In the absence of the C element, however, B-element augmentation of A-element activity in acinar cells is sufficient. The results of these experiments clearly define discrete roles for the three transcriptional elements of the minimal *ELA1* enhancer *in vivo*. They show that the enhancer is required for transcription and that the A, B and C elements are crucial for the activity of the enhancer.

Transcriptional enhancers affect the probability of transcriptional activation

In vivo analysis of enhancer function by deleting enhancer subdomains, or reconstructing synthetic enhancers reveals quantitative as well as qualitative properties (Rose *et al.,* 1994; Kruse *et al.,* 1995). Decreased expression of transgenes with partial enhancers is manifested in two ways. First, expression penetrance decreases dramatically: relatively few founder animals express transgenes with crippled enhancers. The property of expression, however, is heritable; therefore lines derived from expressing founders give reliable transgenic activity.

Second, transgenic mRNA levels tend to be extremely low when the entire mRNA population of an organ is sampled. Expression in individual cells, however, can be as high as transgenes with an unaltered enhancer. The decreased organ-wide expression is generally due to increased variegation: fewer cells in the organ express the transgene. Thus, entire transgene arrays are silent more frequently, due either to increased rates of silencing (Walters *et al.,* 1996), or to less effective initiation of an active array. Because the predominant position effect for random integration appears to be repression, an important enhancer function is to counteract this negative effect of integration sites. The stepwise removal of elements can make an enhancer less effective at opposing the repressive effects of chromatin. Another manifestation of this effect is a greater range of expression levels in independent founders for the same construct as the strength of an enhancer is decreased, consistent with the idea that weak enhancers are much more susceptible to chromosomal position effects.

These observations are consistent with a binary mechanism in which an enhancer affects the probability that its promoter will be active (on versus off) (Moreau *et al.,* 1981; Reeder, 1984; Weintraub, 1988; Walters *et al.,* 1995). It generally has been assumed that enhancers increase transcription by increasing the rate that transcription initiates at an active promoter. However, most of the experiments tendered to support this conclusion were based on the analysis of expression in whole cell populations. Such analysis cannot distinguish between a change in all expressing cells or a change in the number of expressing cells (or some combination). Indeed, when enhancer effects were analyzed for individual cells, it was observed that an enhancer increased the proportion of expressing cells in a population without increasing the level of expression in each cell (Kruse *et al.,* 1993; Walters *et al.,* 1995).

In the binary model of enhancer action, the rate that RNA polymerase molecules load and begin transcription on an active promoter is an inherent property of the promoter. A distant enhancer acting on that promoter has no affect this inherent rate; rather it affects the likelihood that the promoter will be "on". Once the promoter is in an active state, the rate of transcriptional initiation is independent of the enhancer. The presence of an enhancer, however, dramatically decreases the rate of silencing of an active promoter (Walters *et al.,* 1996).

The binary activity of enhancers in transgenic mice is observed for the synthetic B-element enhancer (Kruse *et al.,* 1993). Five tandem repeats of the 21bp B element activate transgenes in only 13 of 24 independent founders (Fig. 5). Thus, the B element homomultimer was about half as effective as the 3-element enhancer. The expression penetrance of the A element homomultimer was even lower (7 of 34). Adding the C element boosts expression penetrance to 100% and increases the level of transgenic mRNA 10-fold. This increased mRNA is not due to an increase in the level for each cell; rather the number of expressing cells increases about 10-fold (Fig. 5). The role of the augmenting activity of the C element may be to increase the probability that the promoter will be active by helping ensure an open chromatin environment.

Recent experimental results from Martin and colleagues (Walters *et al.,*1995) indicate that metallothionein and β-globin transcriptional enhancers operate by counteracting an active repression process mediated by chromatin. By counting the number of expressing cells in a stably transfected population, they showed that transgenic loci without an enhancer had a much lower proportion of expressing cells than loci with an enhancer, but that the level of the transgenic product in individual expressing cells was independent of the enhancer. The percentage of expressing cells decreased during extended culture for loci with or without an enhancer. However, the rate of this silencing was much slower for transgenic loci with an enhancer. In this regard it appears that the enhancer establishes and then maintains a nonrepressing chromatin environment that permits the activity of a linked promoter.

Pancreatic transcriptional factors that mediate the *in vivo* activities

The A and B elements bind pancreas-specific transcription factor complexes. The C element binds nuclear proteins that are similar in a variety of pancreatic and nonpancreatic cell lines, consistent with its cell-nonspecific, augmenting activity.

A-like elements are present in the 5' proximal regions of all twelve murine pancreatic digestive enzyme genes sequenced so far (Swift *et al.,* 1984a; Boulet *et al.,* 1986; Cockell *et al.,* 1989). When tested in context, this element is required for acinar transcription (Kruse *et al.,* 1988; Cockell *et al.,* 1989; Meister *et al.,* 1989) and binds an acinar specific nuclear protein complex (Cockell *et al.,* 1989; Howard *et al.,* 1989; Weinrich *et al.,* 1991; Rose *et al.,* 1994). These and other experiments indicate that the A element is the principal, if not the sole, mediator of acinar cell-specificity of the pancreatic transcriptional enhancers.

Each A element is bipartite, containing an E-box (CACCTG consensus) and a box A (TTTCCC consensus) separated by one (or from amylase genes, two) DNA helical turn. The acinar specific transcription factor PTF1 mediates the acinar specific activity of the

A element (Fig. 6) (Cockell *et al.,* 1989; Rose *et al.,* 1994). PTF1 is a complex containing a 48 kDa bHLH protein that binds the E-box and a 64 kDa protein of unknown properties that binds box A (Krapp *et al.,* 1996; Roux *et al.,* 1989). The p48 subunit, most closely related to twist-like proteins, is pancreas-specific and appears to determine the acinar cell-specificity of the PTF1 complex. Mutation of either the E or A boxes prevents PTF1 binding and inactivates the A element. The E and A boxes cooperatively bind the acinar specific PTF1 complex, are conserved among acinar specific genes, and are crucial for *ELA1* enhancer activity.

The distinct β- and acinar cell activities of the B element are mediated by different forms of the pancreas/duodenum-specific homeodomain protein PDX1 (Fig. 6) (Swift *et al.,* 1998). The acinar activity is due to the binding of a trimeric complex containing two additional homeodomain proteins, PBX1b and MRG1, in addition to PDX1. Mutations in the PDX1 half-site or in the PBX1b half-site of the B element abolish the augmenting function of the B element required for enhancer activity in transfected acinar cell lines. PBX1b and MRG1 are absent from β-cell lines, and PDX1 binds the B element as a monomer. The ability of the B element repeat to activate transcription of a promoter in β-cells is due to the monomer form of PDX1, as mutations of the PBX1b half-site have no effect.

PDX1 is required for the formation of both the endocrine and exocrine pancreas. Mice with both alleles inactivated are born without a pancreas (Jonsson *et al.,* 1994; Offield *et al.,* 1996). Similarly, a homozygous mutation that creates an inactive, truncated form of PDX1 is associated with pancreatic agenesis in a human patient (Stoffers *et al.,* 1997). PDX1 is present in differentiating pancreatic epithelial cells that co-express amylase as well as those that express insulin (Guz *et al.,* 1995). PDX1 persists at high levels in the insulin-expressing β-cells of the adult pancreas, consistent with its role in the insulin promoter, but only at a low level in mature acinar cells. PDX1 was identified as a transcription factor for the insulin promoter (Ohlsson *et al.,* 1993), where it binds and functions without PBX1 and MRG1. The discovery of different forms of PDX1 in β- and acinar cells led to the suggestion that each form may activate a different set of target genes as part of the program that distinguishes the endocrine and exocrine cell lineages that arise from a common pool of precursor cells during pancreogenesis (Swift *et al.,* 1998). Although PDX1 is required for the formation of acinar cells and for the activity of the *ELA1* enhancer in transfected acinar cell lines, its role in *ELA1* transcription in mature acinar cells *in situ* has not been firmly established.

Analysis of the human *ELA1* gene corroborates the roles that the A, B and C enhancer elements, PTF1 and PDX1 play in *ELA1* transcription. Although the human gene encodes a potentially functional mRNA and enzyme, it is transcriptionally silent in the pancreas (Tani *et al.,* 1987; Kawashima *et al.,* 1992). The 5' proximal region corresponding to the enhancer/promoter of the rat *ELA1* is less conserved than the transcribed region. Thirteen of the 34 nucleotide differences between the human and rat enhancers are in the three enhancer elements. When tested by transfection into cultured pancreatic acinar cell lines the human enhancer was inactive (Rose and MacDonald, 1997). Alteration of the 13 variant nucleotides in the three rat enhancer elements to the human sequence inactivated the rat enhancer in transfected pancreatic acinar cells. Conversely, alteration of these 13 nucleotides in the human elements to the rat sequence was sufficient to activate the human enhancer. A single nucleotide difference in the E-box of the A element abolishes PTF1 binding, which is required for enhancer activity in the acinar cell lines. Similarly, a single difference in the PDX1 half-site of human B element prevents the binding of the PDX1-PBX1b-MRG1 complex, which also is required for enhancer activity in the acinar cell lines. Therefore, the transcriptional silence of human *ELA1* appears due to mutations in the three enhancer elements which prevent binding of crucial organ-specific transcription factors.

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