Limited transcription of rat elastase I transgene repeats in transgenic mice

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The rat elastase I (EI) regulatory region is an enhancer that directs efficient pancreas-specific transcription of linked genes integrated in the chromosomes of transgenic mice. However, with increasing numbers of tandemly repeated EI transgenes, the transcription rate per gene decreased. This decrease was not due to a titration of essential transcription factors, because transgenic mice with as many as 250 copies of the rat EI enhancer continued transcription of the endogenous mouse EI gene at its normal rate. Furthermore, when transgenic mice bearing 250 EI genes and mice bearing 7 copies of an EI enhancer-directed human growth hormone (hGH) gene were mated to produce mice with two unlinked arrays of EI enhancer-driven transgenes, each array was transcribed at the same rate as in mice bearing each array separately. Long tandem arrays of transgenes may inhibit efficient transcription despite the presence of ample amounts of essential transcription factors. Although the transcription rate of the mouse EI gene was not affected by the presence of large numbers of transgenes, the amounts of mouse elastase I and elastase II mRNAs were decreased in the presence of high levels of transgene mRNA, indicating an adjustment of the cell mRNA population.

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The regulatory element that specifies pancreas-specific expression of the rat elastase I (EI) gene is a cell-type specific enhancer located between -205 and -72 relative to the start site of transcription (Hammer et al. 1987). In transgenic mice this 134-bp rat EI enhancer region is sufficient to direct high levels of pancreas-specific transcription of a linked human growth hormone (hGH) gene in an orientation-, distance-, and position-independent manner. The EI enhancer, driving the hGH gene, generates a pancreas-specific DNase I hypersensitive site in chromatin of transgenic mice (Ornitz et al. 1985b; Hammer et al. 1987). This chromatin hypersensitive site follows the EI enhancer when the enhancer is moved far (3 kb) upstream of its normal position near the transcription start site, or when it is moved to a site within the hGH gene. This coincidence of position of the EI enhancer and the chromatin hypersensitive site demonstrates that the DNA sequence of the enhancer is responsible for the binding of regulatory proteins in a pancreas-specific manner to form an active transcription complex. In vitro footprint analysis by DNase I protection indicates that nuclear proteins bind to nine discrete sites within the EI enhancer/promoter domain (Kruse et al. 1988), including a DNA sequence element that is conserved among highly expressed genes of the rat exocrine pancreas (Swift et al. 1984b; Boulet et al. 1986). The conservation of this sequence element extends to the homologous mouse genes (Stevenson et al. 1986) and may be one site for the binding of cell-specific transcrip-

tion factors. The efficient pancreas-specific transcription of the rat transgene (Ornitz et al. 1985b; MacDonald et al. 1986) and the presence of enhancer-generated chromatin hypersensitive sites in transgenic mice demonstrate that mouse regulatory factors recognize and bind the rat EI enhancer regulatory elements.

In eight transgenic mouse lines, rat EI transgenes were integrated at single loci as tandemly repeated head-to-tail arrays of 2–250 copies (MacDonald et al. 1986). Progeny that inherit the integrated transgenes continue to express them at the level characteristic of the founder mouse for each line. We initially proposed that the EI mRNA levels correlated with the number of transgenes (Swift et al. 1984a). Indeed, at low numbers of transgenes, mouse lines with more EI transgenes tend to have higher levels of rat EI mRNA in the pancreas. However, examination of additional transgenic mice demonstrated that with higher numbers of EI transgenes (nine copies or greater), pancreatic levels of rat EI mRNA do not increase above a maximum of about five times the level in normal rats (MacDonald et al. 1986).

We investigated the limited expression of large numbers of rat EI transgenes in the pancreas of transgenic mice. Because multiple enhancer copies can compete for the binding of shared *trans*-acting transcription factors (Scholer and Gruss 1984; Mercola et al. 1985; Scholer et al. 1986), one simple model is that the necessary transcription factors become titrated by the presence of multiple rat EI transgenes. We demonstrate that

multiple rat EI enhancers do not compete with the endogenous mouse EI genes or with other unlinked rat EI transgenes for a limiting supply of transcription factors. Rather, it appears that the rat EI transgenes are transcribed inherently inefficiently as tandem repeats and the inefficiency becomes manifest with greater numbers of tandem gene copies.

Results

Transgenic mouse lines with 2, 7, 9, 20, 40, 80, 100, and 250 integrated copies of the rat EI gene were obtained previously (Swift et al. 1984a) by microinjection of the rat EI gene into fertilized mouse eggs. Each line of transgenic mice bears a rat EI gene construct with 7 kb of 5'-flanking sequences, 11 kb of structural gene sequences, 5 kb of 3'-flanking sequences and 3.5 kb of pBR322 (Fig. 1). The lines of mice differ in the number of head-to-tail tandem repeats of the rat transgene (MacDonald et al. 1986). All the mice except the 100-copy mice have the transgenes integrated at a single locus; the 100-copy mice have two transgenic loci of 20 and 80 copies which segregate independently to give progeny of 20, 80, or 100 copies. Because microinjected DNA becomes integrated, if not randomly, at any of a large number of chromosomal positions (Palmiter and Brinster 1986), each line has a different integration site of the transgenes in the mouse genome. All eight lines of mice stably inherit the EI transgenes and the high-level, pancreas-specific expression characteristic of the founding (G_0) mouse of each line (MacDonald et al. 1986).

Rat EI mRNA levels become limited in transgenic mice

With increasing numbers of transgenes the level of rat EI mRNA in the pancreas of transgenic mice increases initially, then reaches a maximum at 40,000–50,000 mRNAs/cell (Fig. 2). Transgenic mice with two rat EI transgenes express approximately the same level of rat

EI mRNA as the two endogenous genes in normal rats (~10,000 EI mRNAs/cell). In mice with seven transgenes, pancreatic rat EI mRNA levels are increased about three-fold. Therefore, transgenic mice with low copy numbers of tandemly repeated transgenes express rat EI as if each transgene in mouse were about as active as each EI gene in the rat. However, pancreatic levels of rat EI mRNA in mice with 9, 20, 40, 80, or 250 tandemly repeated transgenes are only about two- to fivefold higher than rat (Fig. 2). These results demonstrate that as the number of tandemly repeated rat EI transgenes increases to greater than about seven copies, expression does not increase above a level approximately five times that of normal rat.

The plateau pattern cannot be explained by differential chromosomal position effects, because such effects should be varied, rather than fit a pattern related to transgene copy number. Moreover, the rat EI transgene appears insensitive to large position effects, since all eight independent transgenic lines obtained express the integrated genes; none had silent loci or high aberrant expression in nonpancreatic tissues (Swift et al. 1984a; MacDonald et al. 1986). The small differences among the averaged values at the plateau level (Fig. 2), however, may be ascribed to effects of chromosomal position.

Rat EI expression is limited at the transcriptional level

To determine whether the expression of the large numbers of tandem transgenes is limited transcriptionally, transcription rates were measured for several transgenic mouse lines with different numbers of transgenes. Pancreatic nuclei were isolated and nascent transcripts initiated *in vivo* were elongated *in vitro* in the presence of radiolabeled ribonucleotides. The transcriptional activity of rat EI transgenes and the endogenous mouse EI genes in these nuclear run-on assays were quantified by hybridizing the labeled transcripts to cloned rat and mouse EI cDNAs. Although the rat and mouse EI mRNAs are closely related and readily cross-hybridize,

Figure 1. Organization of the tandem repeats of the rat elastase I transgene in transgenic mice. The rat EI transgene contained 7 kb of 5'-flanking sequence, 11 kb of structural gene sequence including 8 exons (filled boxes), 5 kb of 3'-flanking sequence, and 3.5 kb of pBR322 sequence (open rectangles) integrated into chromosomal DNA (striped boxes). The positions of the -205 to -72 enhancer region, containing the conserved element centered at -105, and the TATA box are shown. The rat EI-specific cDNA probe used to quantify rat EI transcripts was derived from the 3' untranslated region of exon 8 as indicated.

Figure 2. Rat EI mRNA levels in normal rats and transgenic mouse lines bearing different numbers of rat EI transgenes. Rat EI mRNA was quantified by solution hybridization with a rat EI-specific oligonucleotide probe to total pancreatic RNA samples (see Methods). The error bars represent the standard deviation from the mean, the numbers above the error bars indicate the number of animals assayed independently for each determination.

the transcripts of each could be distinguished with cDNA probes comprising the 3' untranslated regions of each mRNA, provided stringent washes were performed after hybridization (see Methods).

Figure 3 shows the results of a nuclear run-on experiment using pancreatic nuclei isolated from transgenic mice with 2 and 250 rat EI transgenes. The total rate of transcription for the 2 transgenes (47 ppm) is nearly that for the 250 transgenes (59 ppm). The results imply either that only 2 or 3 of the 250 transgenes are transcriptionally active at the rate of each of the 2 transgenes in the 2-copy mouse or that many of the 250 transgenes are transcriptionally active, but each at a greatly reduced rate. If all 250 transgenes were active, the rate per gene would be approximately 100-fold lower than for the 2-copy locus.

The transcription rate per gene (transcriptional efficiency) decreases with increasing numbers of transgenes for mice bearing 2, 7, 20, 80, and 250 rat EI tandemly

repeated transgenes (Fig. 4). The progressive decrease suggests that the limited transcription is a property of tandem transgenes and not an effect of chromosomal position. The results demonstrate that the limit of mRNA accumulation in the pancreas of rat EI transgenic mice is due to limits in the transcription of increasing numbers of repeated transgenes.

Endogenous mouse EI gene expression is unaffected by increasing numbers of rat EI transgenes

Enhancer action requires the binding of sequence-specific *trans*-acting factors (Dyan and Tjian 1985; Maniatis et al. 1987). Transfection experiments in cell culture indicate that large numbers of enhancers can titrate a limiting amount of a freely diffusible transcription factor, causing decreased transcription of an enhancer-linked test gene (Scholer and Gruss 1984; Mercola et al. 1985; Scholer et al. 1986). Therefore, large numbers of enhancers of multiple repeated rat EI transgenes might titrate a limiting amount of one or more DNA-binding transcription factors. A limiting amount of one or more transcription factors that could support the transcription of only a few EI genes might explain the low transcription rates of the large transgene repeats. This model would predict that multiple rat transgenes should also compete with the endogenous mouse EI gene for transcription factors. In different mouse lines, as rat EI transgene copy number increases, competition for factors should decrease the mRNA level and the transcription rate of the endogenous EI gene.

To test this prediction, we measured rat EI and endogenous mouse EI mRNA levels in the pancreas of normal mice and transgenic mice by solution hybridization using synthetic oligonucleotide probes that discriminate between the two related mRNAs. Even in transgenic mice which express rat EI transgenes to high levels, endogenous mouse EI message levels decrease only slightly compared to levels in control mice (Fig. 5). At most, endogenous mouse EI mRNA levels decrease about one-third at high rat EI mRNA levels. Compared with the

Figure 3. Relative transcription rates of rat EI transgenes in transgenic mice bearing 2 and 250 rat EI genes. Pancreatic nuclei from transgenic mice bearing 2 or 250 rat EI genes were isolated and endogenous RNA polymerases were allowed to elongate nascent RNA transcripts as described in Methods. ^{32}P -labeled RNA transcripts were hybridized to immobilized rat EI cDNA plasmid DNA with wash conditions that discriminate rat and mouse EI transcripts. The slope from the plot of input ^{32}P -labeled RNA versus hybridized RNA yields the relative transcription rate expressed as parts per million of the total RNA synthesized.

Figure 4. Relative transcription rates per rat EI gene copy in transgenic mouse lines. Pancreatic nuclei from transgenic mice bearing 2, 7, 20, 80, and 250 rat EI genes were used in nuclear run-on transcription assays as described in Methods. The rat EI transcription rate of each mouse was divided by the number of rat EI gene copies present to give the average transcription rate per gene copy (ppm/copy).

dramatic limitation of rat EI mRNA levels, the modest effect on mouse EI mRNA levels is inconsistent with the competition for a diffusible factor equally available to the mouse and rat EI genes. Moreover, this effect is not correlated with the number of transgenes, which would be expected if the decrease of mRNA reflected lower transcription due to titration of a necessary transcription factor. Rather, the reciprocal correlation between rat and mouse EI mRNA levels (Fig. 5) suggests that the mouse EI mRNA levels may be reduced by a post-transcriptional mechanism.

To measure the effects on endogenous EI transcription directly, the transcription rates of endogenous mouse EI genes were measured by nuclear run-on assays. Transcription rates of the endogenous mouse EI genes in mice with 2, 7, 20, 80, or 250 transgenes were not consistently lowered in mice with larger numbers of transgenes (Fig. 6). Therefore, rat EI transgenes have no systematic effect on endogenous mouse EI gene transcription. By this criterion there is no detectable titration of a shared *trans*-acting transcription factor in mice with large numbers of rat EI transgenes. The rat EI transgenes also had no reproducible effect on the rates of pancreatic transcription for β -actin and gene 85 (Schutzbank et al. 1982), another mouse gene expressed in many, if not all, tissues (data not shown).

Unlinked transgenic loci are expressed independently

If the mouse EI enhancer has a higher affinity than the rat EI enhancer for a titratable mouse transcription factor, the transgenic rat EI enhancers may be unable to compete effectively with the endogenous mouse EI genes. However, rat EI transgenes may still compete with one another for a small amount of the remaining factor, limiting expression of the rat transgenes only. This model predicts that if a second set of competing rat EI enhancers were introduced into a transgenic mouse

bearing rat EI transgenes, the two sets of EI enhancers would compete for a limiting factor and transcription from each set should be depressed. For the second set of transgenes, we used the elastase I–human growth hormone (EI–hGH) fusion gene, which bears 0.5 kb of the rat EI gene 5′-flanking region fused to the hGH gene coding region and 3′-flanking region (Ornitz et al. 1985a). In mice the EI–hGH fusion genes express high levels of hGH mRNA selectively in the acinar cells of the pancreas. Expression of both sets of transgenes can be monitored independently by assaying for rat EI and hGH mRNA sequences.

Transgenic mice with 250 rat EI transgenes were mated to transgenic mice with 7 rat EI–hGH fusion transgenes (line 43-5; Ornitz et al. 1985b). Progeny with four transgenic genotypes were obtained—those with: (1) no transgenes, (2) 7 EI–hGH transgenes, (3) 250 rat EI transgenes, and (4) 7 EI–hGH plus 250 rat EI transgenes. The four genotypes were identified by tail DNA dot blot analysis.

Transcription rates for endogenous mouse EI, rat EI and EI–hGH genes were measured for pairs of animals of each of the four genotypes. To compare transcription rates accurately, we isolated nuclei from transgenic littermates concurrently and then performed the *in vitro* transcription assays concurrently. Mice with both transgenic loci (Fig. 7, lower right panel) transcribe the rat EI transgenes at 308 ppm and the hGH transgenes at 476 ppm. These transcription rates are similar to the rates in mice with either the 250 rat EI transgene locus alone (412 ppm; Fig. 7, upper right) or in mice with the 7 copy hGH fusion gene locus alone (502 ppm; Fig. 7, lower left). Therefore, there was no inhibitory effect on the transcription of either set of transgenes by the presence of the other. In this experiment, transcription of the endogenous mouse EI in transgenic mice (average of 61

Figure 5. Response of mouse EI mRNA levels to the expression of rat EI transgenes in transgenic mice. Mouse and rat EI mRNA levels (molecules per cell) were quantified by solution hybridization with a mouse-specific or rat-specific oligonucleotide probe to total pancreatic RNA samples in transgenic mice bearing 2, 7, 9, 20, 40, 80, 100, and 250 rat EI genes. Each point represents determinations of mouse and rat mRNA levels from three concentrations of total RNA in duplicate from each animal as described in Methods. The zero rat EI mRNA points represent non-transgenic mice.

Figure 6. Relative transcription rates of endogenous mouse elastase I genes in different lines of transgenic mice. Mouse elastase I transcription rates were measured for pancreatic nuclei from mice and transgenic mice bearing 2, 7, 20, 80, and 250 rat EI transgenes by nuclear run-on transcription assays. The transcription rates (in ppm) were derived from plots of input RNA versus hybridized RNA as described in Methods and in the legend to Fig. 3. Error bars represent standard deviation from the mean transcription rate for two mice from each line (except for the two rat EI copy line in which only one transgenic mouse pancreas was assayed).

ppm) and control mice (82 ppm; Fig. 7, upper left) was similar.

The levels of rat EI and hGH mRNAs (Table 1) reinforce the results of the transcription studies. Pancreatic levels of mRNA in mice with both transgenic loci were 20,000 rat EI mRNAs/cell and 260,000 hGH mRNAs/cell. The mRNA levels for either transgene alone were largely unchanged (20,000 rat EI mRNAs/cell or 300,000 hGH mRNAs/cell). These results and the analysis of transcription rates (above) indicated that there is no competition for transcription factors between rat EI enhancers of unlinked transgenic loci. Since the expression of transgenes can be increased by increasing the number of transgenic loci, the transcriptional limit of transgene expression appears to be at each single transgenic locus, possibly due to the tandemly repeated organization of the genes.

The mouse EI mRNA level decreases slightly for mice with EI or EI-hGH transgenes (Table 1). A similar effect was suggested previously for mice with widely different numbers of EI transgenes (Fig. 5). The magnitude of the effect appears to be correlated with the level of transgene mRNA (either hGH or rat EI), rather than transgene number. Thus, the effect is greater with seven EI-hGH transgenes, which produce an exceptionally high level of hGH transgene mRNA, than with 250 EI transgenes, which give a lower transgene mRNA accumulation. The effect of the presence of both sets of transgenes is nearly additive. In a separate experiment we determined the variation of endogenous EI mRNA levels due to age, sex, and strain differences in the mouse lines used to generate the transgenic mice. Pancreatic EI mRNA levels were measured for two SJL males, a C57 male and female, and two males and two females from two independent C57 $\times$ SJL crosses. The eight mice tested ranged

between 1.7 and 7.3 months of age. The average EI mRNA level was 54,700 molecules/cell with a standard deviation of only ± 6400 , far smaller than the effect due to the presence of transgenes.

Discussion

We investigated the mechanisms that limit the high-level, pancreas-specific expression of tandemly repeated rat EI transgenes in transgenic mice. A simple model proposes that the level of transcription factors necessary for EI gene transcription becomes limiting with large numbers of rat EI transgenes. Consistent with this model is the observation that a limiting step of rat EI transgene expression is transcription. Rat EI transgene transcription does not increase with increasing numbers of tandemly repeated transgenes; rather the efficiency of transcription per transgene decreases steadily with increasing numbers of transgenes to a rate per gene in 250-copy mice that is 100-fold lower than in 2-copy mice. The model predicts that the titration of a freely diffusible transcription factor by multiple rat EI transgenes would inhibit the expression of the unlinked endogenous mouse EI genes. However, the transcription rate of the endogenous mouse EI genes varied less than threefold in a series of transgenic mouse lines with a 120-fold variation in the number of rat EI transgenes. Furthermore, this threefold variation did not correlate with increasing numbers of transgenes. The titration model also predicts that two sets of transgenes under control by the same enhancer would compete for a shared transcription factor if it were present in limiting amounts. However, the expression of either of two EI transgene loci, introduced by mating mice bearing two different EI enhancer-driven transgenes, was not affected by the presence of the other. The highly expressed pancreatic genes for the secretory enzymes share an enhancer consensus sequence (Swift et al. 1984b; Boulet et al. 1986), and therefore may share common transcription factors. The need to express many pancreas-specific genes at high levels may require large amounts of transcription factors which makes titration unlikely with the number of gene copies that were attained in these transgenic mice.

An alternative model proposes that the organization of rat EI genes into tandem repeats reduces the efficiency of transcription of genes within the repeat. Increasing the number of tandem copies within a repeat does not increase the total level of transcription. This model allows that two independent EI transgene loci would be expressed independently of each other. Indeed, EI-hGH transgenes and EI transgenes present at different chromosomal sites are expressed additively. These results are consistent with the inhibition of transcription by extended tandem repetition of the EI genes. Because each transgene copy within a repeat is identical, it is not possible to determine whether a few genes (e.g., at the ends or in the middle of the repeat) are maximally active, or all (or most) genes are equally active, but at a much reduced rate. The inefficient transcription may be due to

Figure 7. Relative transcription rates for rat EI, mouse EI, and hGH genes in mice and transgenic mice. Pancreatic nuclei from mice and transgenic mice bearing 250 rat EI transgenes, 7 EI-hGH transgenes, or both were used in nuclear run-on transcription assays to determine the transcription rates for rat EI ($\square$), mouse EI ($\blacksquare$), and hGH ($\circ$) genes. The transcription rates (ppm) were derived from the slopes of the lines. The low apparent transcription of rat EI in mice without transgenes (indicated in parentheses) was due to cross-hybridization with the mouse EI transcripts.

the inability of more than a few genes within a limited chromatin domain to adopt a transcriptionally active structure on the nuclear scaffold (Gasser and Laemmli 1987). However, other explanations are possible. For example, the inefficient expression of two tandem α -globin genes has been proposed to be due to readthrough transcription, because the insertion of transcriptional stop signals between the genes increased expression manyfold (Proudfoot 1986). Two pancreatic genes, α -amylase and elastase II, are terminated within 2–4 kb of their poly(A) addition sites (Hagenbuchle et al. 1984; Stevenson et al. 1986); therefore, the 5 kb of EI gene 3'-flanking sequences present in the transgene may be expected to harbor transcriptional stop sequences as well. However, we cannot exclude readthrough transcription as the cause of the inefficient transcription of the tandemly linked EI transgenes.

Naturally occurring gene repeats are expressed efficiently, probably due to additional elaborate mechanisms that optimize their transcription. For example, the genes for the large ribosomal RNAs are transcribed by a unique dedicated RNA polymerase and are seques-

tered in a specialized nucleolar structure that may optimize access by rRNA gene transcription factors and RNA polymerase I. Each rRNA gene of *Xenopus laevis* contains multiple enhancer and promoter elements that augment transcription (for review, see Reeder 1984). Furthermore, each gene has its principal termination site about one polymerase-diameter upstream of the promoter of the next gene in the repeat, so that terminated polymerase may be optimally positioned for reinitiation at the next transcription start site without being released from the chromatin template (McStay and Reeder 1986). If polymerase I is passed on from one transcription unit to the next in this manner, the potentially rate-limiting step of polymerase reassociation with each promoter of the repeat is eliminated.

Genes such as DHFR and CAD can be amplified in response to drug selection and give increased levels of the corresponding mRNAs and proteins. However, the increase in mRNA and protein does not exactly correspond to the increased number of gene copies (Wahl et al. 1979; Lewis et al. 1982; Zieg et al. 1983). Moreover, linked genes that are amplified to the same extent as the

Table 1. Decreased levels of mouse elastase I mRNA in the presence of rat elastase I and hGH mRNAs in the pancreas of transgenic mice

Transgenic genotype	mRNA levels (molecules per cell $\times 10^{-3}$)		
	mouse EI	rat EI	hGH
Control mouse 1	51	— ^a	—
Control mouse 2	65	—	—
250 rat EI genes	38	20	—
7 hGH genes 1	35	—	300
7 hGH genes 2	32	—	300
250 Rat EI genes + 7 hGH genes 1	26	19	270
250 Rat EI genes + 7 hGH genes 2	28	22	250

The mice in this table are the same for which transcription rates were determined in Fig. 7 and are littermates except for the two control mice. The control mice were littermates from a C57 $\times$ SJL cross.

^a Dashes indicate that this level was not assayed in this experiment, but previous solution hybridization analyses show that rat EI- or hGH-specific oligonucleotide probes do not hybridize to total pancreatic RNA from mice (see Methods).

selected gene are expressed at lower levels (Ringold et al. 1981) or not at all (Collins and Groudine 1983). Therefore, in the absence of selection for increased expression, the tandem amplification of a gene does not necessarily lead to increased expression. Single-copy genes such as rat EI, DHFR, and CAD genes probably lack the specific mechanisms that genes normally present as repeats have acquired to optimize transcription.

Rat EI transgene expression in the pancreas appears to reduce the level of endogenous mouse EI mRNA slightly. The extent of the decrease cannot be correlated with the number of transgene copies, but may correlate with the level of transgene mRNA. The effect is not specific to rat EI transgene mRNA, because high pancreatic levels of hGH transgene mRNA also reduce mouse EI mRNA levels and the effects of coexpression of rat EI and hGH transgenes are nearly additive. Moreover, the endogenous EI is not the only mRNA affected: The level of the mRNA for another pancreas-specific secretory enzyme, elastase II, also decreases in transgenic mice expressing rat EI mRNA (B. Davis and R. MacDonald, unpubl.). Because endogenous mRNA reduction is observed in the absence of a concomitant effect on the rate of endogenous gene transcription, the causes are likely to be post-transcriptional.

Based upon the mRNA content per cell (0.6 pg) and the average size of pancreatic mRNAs (about 1200 nucleotides), we estimate that there are approximately 10^6 mRNA molecules in an average pancreatic acinar cell. Therefore, foreign mRNA levels up to 300,000 per cell would represent a significant increase. It may be that the high levels of additional (foreign) mRNAs cause a compensatory decrease of other cellular mRNAs by as yet unknown mechanisms. Cells may limit the total number of mRNA molecules or, conversely, may be unable to compensate by increasing the level of other cell

constituents needed to accommodate larger numbers of mRNAs. For example, ribosomes may become limiting and excess mRNAs not in polysomes may turn over more rapidly. Similarly, when additional foreign mRNAs encode secretory proteins, the number of docking sites on the rough endoplasmic reticulum for signal recognition particles (SRPs) may be limited and the SRP-stalled secretory mRNAs may be labile.

One conclusion that can be drawn from the poor transcriptional efficiency of tandem transgenes is that strategies to increase expression of transgenic products should emphasize the number of chromosomal loci of transgenes rather than the absolute number of transgenes present in repeats. In addition, although EI-enhancer driven transgenes can produce very high mRNA levels, the number of foreign mRNAs per cell certainly has a limit. The limit will be reached in part because cells appear unable to accommodate significant additional mRNA molecules, and exceedingly high levels of foreign mRNA may reduce endogenous mRNAs until crucial cell functions become limiting.

Methods

The transgenic mouse lines bearing rat EI genes and EI-hGH fusion genes used in this study were originally described by Swift et al. (1984a) and Ornitz et al. (1985a). The rat EI transgene contains 7 kb of 5'-flanking sequence, 11 kb of structural gene sequence, 5 kb of 3'-flanking sequence, and 3.5 kb of pBR322 sequence. The EI-hGH fusion gene contains 0.5 kb of rat EI 5'-flanking sequence fused to the entire 2.3-kb hGH gene without plasmid vector sequences. The lines of transgenic mice were propagated by mating male transgenic mice to female C57 $\times$ SJL mice. Transgenic mice bearing both rat EI and EI-hGH transgenes were obtained by mating a male transgenic mouse bearing 250 rat EI genes to a female transgenic mouse bearing 7 EI-hGH fusion genes. Transgenic progeny were identified by quantitative tail DNA dot blot analysis (Brinster et al. 1985) with ³²P-labeled, nick-translated pBR322 for the rat EI transgene or the 2.3-kb ³²P-labeled, nick-translated hGH fragment for the EI-hGH transgene.

Quantification of mRNA levels by solution hybridization

Total pancreatic and liver RNA was isolated by the guanidinium thiocyanate method (Chirgwin et al. 1979; MacDonald et al. 1987). For animals from which both transcription rates and specific RNA levels were required, nine-tenths of the tissue was used for isolation of nuclei for run-on transcription assays and one-tenth of the tissue was used for isolation of RNA to measure mRNA levels by solution hybridization analysis. The concentrations of specific mRNAs were quantified by solution hybridization with message-specific oligonucleotide probes. The conditions were as described by Durnam and Palmiter (1983), except that for rat EI mRNA annealing was done at 42°C plus 10% formamide and S1 nuclease digestion was at 42°C; for mouse EI and hGH mRNAs, annealing and S1 nuclease digestion were at 37°C. Oligonucleotides 20–24 nucleotides long that were designed to hybridize specifically to either rat EI, mouse EI, or hGH mRNAs were synthesized by solid-phase phosphoramidite chemistry with an Applied Biosystem 380A DNA Synthesizer. The 20-mer mouse-specific EI oligonucleotide probe (GtTCAGTagTCtGATtTGcT) was complementary to nucleotides 1041–1060 within the 3' untranslated region of the mouse EI mRNA (B.P. Davis and R.J. MacDonald, unpubl.)

and contained six differences with the rat EI mRNA (the positions of the differences are indicated by lowercase letters, above). The 24-mer rat-specific EI oligonucleotide probe (GCtAGGcaCCATCtTGGGGAGGCC) was complementary to nucleotides 842–865 within the 3' untranslated region of the rat EI mRNA (MacDonald et al. 1982) and contained four differences with the mouse EI mRNA. Under the solution hybridization conditions described above, there was no detectable cross-hybridization of the species-specific EI oligonucleotide probes with the EI mRNA of the other species. The 24-mer hGH-specific oligonucleotide probe (GGTTGGGAAGGCACTGCCCTCTTG) was complementary to the hGH mRNA second exon at nucleotides 126–149 of the mRNA (Seeburg 1982). There was no detectable cross-hybridization of the mRNA-specific probes to any other RNAs in mouse and rat pancreas.

To determine the number of specific mRNA molecules in the total RNA sample, the hybridization of an mRNA-specific oligonucleotide probe to known amounts of total RNA samples was compared to the hybridization of the probe to known amounts of single-stranded recombinant M13 phage bearing inserts of the appropriate mRNA sequence. Recombinant phage contained the rat EI mRNA 3' untranslated region, the mouse EI mRNA 3' untranslated region, or the entire hGH gene sequence. Solution hybridization assays for each specific mRNA were performed with three concentrations of total pancreatic RNA in duplicate for each animal. The number of specific mRNA molecules per cell was calculated assuming that an average mouse pancreatic cell contains 60 pg of total RNA (Swift et al. 1984a).

Nuclear run-on transcription

Pancreatic nuclei were isolated according to Mulvihill and Palmiter (1977) with the following modifications. Buffers NA and NB contained 10 U/ml RNasin (Promega Biotec) and 1 mM EGTA. The tissue was dissected from mice or rats and quickly homogenized at low speed for 10 sec with a small Tisumizer (Tekmar) probe in 10 ml of NA buffer per gram of tissue. The yield of nuclei was determined by measuring nuclear DNA with the fluorometric method of Labarca and Paigen (1980). To obtain reproducible transcription rates for comparison among mice, nuclei were isolated simultaneously from all mice involved in a comparison.

Nascent RNA transcripts in isolated pancreatic nuclei were extended by incubating approximately 10^7 nuclei at 26°C for 45 min in 100 μ l of reaction mix containing [α - 32 P]UTP as described by McKnight and Palmiter (1979). Under these conditions the nascent RNA transcripts were extended 100–200 nucleotides (data not shown).

The following recombinant plasmids bearing cDNA inserts were used to detect gene-specific run-on transcripts from isolated nuclei: pREI3', which contained the 3' untranslated region of rat EI mRNA (nucleotides 875–1109 plus 11 poly(A) residues) in pBR322 (Swift et al. 1984a); pMEI3', which contained the 3' untranslated region of mouse EI mRNA (nucleotides 830–1109 plus 25 poly(A) residues) in pUC9 (B.P. Davis and R.J. MacDonald, unpubl.); clone 85, which contained 1.4 kb of cDNA of a mouse mRNA expressed in all tissues (in pBR322; a gift from R. Robinson, University of Texas Southwestern Medical Center) (Schutzbank et al. 1982); and pA1, which contained 1.8 kb of cDNA of chick actin mRNA in pBR322 (Cleveland et al. 1980). pBR322 was used to monitor background hybridization.

Recombinant plasmids propagated in HB101 or DH5 α *E. coli* strains (BRL) were isolated by the alkaline lysis method of

Maniatis et al. (1982). The plasmids were bound to 8-mm nitrocellulose discs according to Farnham and Schimke (1985). All filters were prepared the day of the hybridization and equilibrated in hybridization solution before hybridization with radiolabeled RNA. Preliminary experiments demonstrated that 4 μ g of DNA on each filter was in excess over hybridizable transcripts.

Hybridization The radiolabeled RNA was dissolved and then filtered through 0.2 μ m nylon filters (Schleicher & Schuell) in a hybridization solution containing 500 mM NaCl, 50 mM PIPES (pH 7.0), 33% formamide, 0.4% SDS, 2 mM EDTA, and 10 μ g/ml poly(A) (McKnight and Palmiter 1979). The labeled RNA from the nuclei of a single pancreas was divided among 5-ml polypropylene tubes, each containing as many as three filters with different recombinant cDNA plasmids bound. To enhance accuracy, transcription rates were measured in triplicate by dispensing the 32 P transcripts into three hybridization tubes in the ratio 1 : 2 : 4. The transcription rates then were calculated from the slope of the plot of cpm hybridized versus input cpm in the hybridization mixes. Because the mouse and rat EI filter-bound cDNA probes are 70% homologous, they compete for both EI transcripts, which are present in limiting amounts; therefore, to quantify mouse and rat EI transcription rates accurately, the mouse and rat EI cDNA probe filters were hybridized to radiolabeled run-on transcripts separately. The final hybridization solution of 100 μ l was overlaid with three drops of paraffin oil and filters were hybridized at 45°C with agitation for 3 days.

A 3 H-labeled RNA transcript of the mouse EI mRNA 3' untranslated region was included in all hybridization experiments to monitor differences in the extent of hybridization between samples within the same experiment and among different experiments. The 3 H-labeled RNA transcript was generated by T7 RNA polymerase transcription of a recombinant pGEM-2 plasmid (Promega Biotec) which contained the 3' untranslated cDNA region of the mouse EI mRNA. Hybridization of the 3 H-labeled RNA to the mouse EI cDNA probe varied from 30% to 60% between different experiments, but differences in hybridization efficiency between samples within a single experiment varied by less than 10%. Filter-bound recombinant plasmid DNAs for chick β -actin mRNA and mouse clone 85 mRNA were included in each hybridization experiment to monitor effects on the transcription rates of genes that were not pancreas specific.

After hybridization, stringent washes were required to distinguish the related rat and mouse elastase I transcripts. The three filters in each tube were washed twice quickly at room temperature with 2 ml of buffer A [10 mM Tris-Cl (pH 7), 300 mM NaCl, 2 mM EDTA] plus 0.1% SDS, then with 2 ml of buffer A plus 0.1% SDS at 45°C with agitation for 1 hr. The filters were washed once quickly at room temperature with 2 ml of buffer A and then with agitation at 45°C in 2 ml of buffer A for 30 min. The filters then were incubated for 30 min at 37°C in 2 ml of buffer A containing 10 μ g/ml RNase A and 1 μ g/ml T1 RNase. The RNase-treated filters were washed twice quickly in 2 ml of 0.1 $\times$ SSC, with 0.5% SDS and 0.1% sodium pyrophosphate, twice for 30 min each in 2 ml of the above solution at 65°C with occasional agitation, then twice for 30 min each in 2 ml of 0.1 $\times$ SSC at 65°C with occasional agitation. Cross-hybridization of the 3 H-labeled mouse EI RNA to the rat EI cDNA probe varied between experiments from 0% to 10%, indicating that the hybridization and wash conditions generally were stringent enough to distinguish rat and mouse EI transcripts.

Quantification of transcription rates The radioactivity bound

to the filters was measured by scintillation counting according to Farnham and Schimke (1985). Transcription rates (ppm) were calculated as ^{32}P -cpm hybridized to the cDNA probe minus ^{32}P -cpm hybridized to pBR322 (background), divided by the total ^{32}P -cpm in the hybridization solution. Because the lengths of the cDNA inserts in the recombinant probes varied, the transcription rates were standardized as ppm/kb.

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