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Introns increase transcriptional efficiency in transgenic mice

(intervening sequences/gene expression/gene transfer)

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ABSTRACT **Experiments were designed to test the effect of introns on gene expression in transgenic mice. Four different pairs of gene constructs, which were identical except that one member of each pair lacked all introns, were compared for expression of mRNA after introduction into the murine germ line by microinjection of fertilized eggs. The expression of two chimeric genes, made by fusing either the mouse metallothionein I or the rat elastase 1 promoter/enhancer to the rat growth hormone gene, was assayed in fetal liver or pancreas, respectively, while two natural genes, an oligonucleotide-marked mouse metallothionein I gene and the human β-globin gene, were assayed in fetal liver. In each case there was, on average, 10- to 100-fold more mRNA produced from the intron-containing construct. Moreover, mRNA levels were proportional to the relative rates of transcription that were measured in isolated nuclei. However, when the expression of the two mouse metallothionein I gene-based constructs was tested after transfection into cultured cells, little difference was observed. These observations suggest that introns play a role in facilitating transcription of microinjected genes and that this effect may be manifest only on genes exposed to developmental influences.**

Most mammalian genes coding for mRNA are interrupted by noncoding sequences known as introns, many of which are larger than the exons; thus, the entire gene may span tens or even hundreds of kilobases (1). For some genes, introns clearly separate functional or structural domains of the proteins encoded by the exons. This observation, and the fact that similar domains can be found within different proteins, has led to the suggestion that one function of introns may be to accelerate the evolution of proteins with different properties (2, 3). Introns also allow differential joining of exons during splicing, which can result in the synthesis of variant proteins with new properties (4, 5).

The possibility that introns may be necessary for efficient processing and transport of mRNA to the cytoplasm has been examined. Hamer and Leder (6) showed that a series of simian virus 40 viruses that contained various combinations of simian virus 40 and mouse β-globin splice sites produced stable mRNAs only if at least one splice site, derived from either the virus or β-globin, was retained. In another experiment, a simian virus 40 mutant missing a late gene intron failed to produce stable transcripts but could be rescued by addition of an heterologous intron (7, 8). These early observations suggested that splicing was obligatory for mRNA accumulation in the cytoplasm. As a consequence, the first-generation cDNA expression vectors usually included a heterologous intron in addition to promoter and polyadenylylation sequences (9). Subsequently, it was discovered that deletion of introns does not always result in loss of mRNA production. For instance, several viral genes, including those for E1A protein (10) and polyoma large tumor (T) antigen (11) function without introns. Likewise, the genes for bean phaseolin (12), yeast actin (13), and chicken thymidine kinase (14) produce approximately equal amounts of mRNA regardless of whether introns are present. These experiments, and the fact that many second-generation cDNA expression vectors lack introns yet allow good expression, suggest that only in a few special cases are introns necessary for mRNA accumulation, at least when tested in culture cells.

The ability to redirect expression of genes to various cell types in transgenic animals by combining the regulatory elements of one gene with the coding region of another has facilitated a number of unique experimental approaches to developmental, physiological, and pathological processes (15, 16). Because of the greater availability of cDNAs and because some natural genes are too large to manipulate conveniently, many chimeric genes based on cDNA expression vectors have been constructed. Although many worked well when tested in cell culture and some have expressed in transgenic mice, our experience is that there are a large number of cDNA-based constructs that are not expressed or are expressed poorly in transgenic mice (ref. 16; and our unpublished observations). Furthermore, addition of heterologous introns to cDNA constructs usually did not rescue their expression. Because many of these experiments were not well controlled, we tested several different genes with and without their introns, but with identical 5′ and 3′ flanking regions, to ascertain whether introns have a consistent effect on expression of genes in transgenic mice.

MATERIALS AND METHODS

Gene Constructs. The MTrGH gene construct (Fig. 1) has 1.8 kilobases (kb) of the 5′ flanking sequence of the gene for mouse metallothionein I (mMT-I) (dashed line) fused at the *Bgl* II site (position +64) to the *Xho* I site of the gene for rat growth hormone (rGH) (17, 18). The 6.8-kb *Eco*RI–*Bam*HI fragment was isolated for microinjection. The ErGH gene construct has 4.5 kb of the 5′ flanking region of the gene for rat elastase I (dashed line) fused at an artificial *Xho* I site (position +10) to the *Xho* I site of the gene for rGH (19). The 8.5-kb *Hin*dIII fragment was isolated for microinjection. For transcription-rate measurements, a plasmid containing the rGH sequences between *Xho* I–*Xba* I and *Ava* I–*Nsi* I was constructed (solid bars beneath the ErGH construct), thereby eliminating the repeat sequences that lie in intron B. The mMT-I gene (17, 20) was marked by insertion of a pair of oligonucleotides (the mRNA strand sequence is 5′

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Abbreviations: rGH, rat growth hormone; mMT-I, mouse metallothionein I; BHK cells, baby hamster kidney cells.

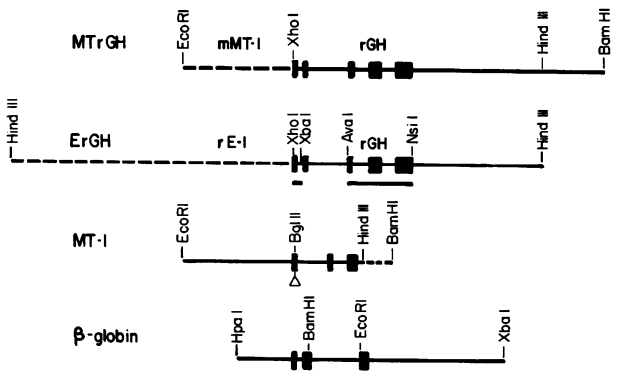

FIG. 1. Diagram of gene constructs injected into fertilized mouse eggs. The intronic version of each of the genes is shown, drawn approximately to scale; the intronless versions are the same except that cDNA sequences were substituted between the first and last exons.

CACGGTTGACTAAGCTA) into the *Bgl* II site (position +64). The *Eco*RI–*Bam*HI fragment that included 350 base pairs (bp) of pBR322 (dashed line) was microinjected. The human β-globin gene is included on a *Hpa* I (−815)–*Xba* I (+3300) fragment that was microinjected (21). Transgenic mice were identified by dot hybridization using nick-translated probes: for rGH, a 385-bp *Xho* I–*Pvu* II fragment that includes most of exons 1 and 2 and intron A was used; for mMT-I, pBR322 was used; and for β-globin, the *Hpa* I–*Xba* I fragment was used.

Transgenic Mice. Each of the genes shown in Fig. 1 (with or without introns) was microinjected into fertilized mouse eggs, and transgenic fetuses or mice were identified by dot hybridization (22). Total nucleic acids were isolated by the $NaDodSO_4$/proteinase K method (23). The amount of mRNA in liver or pancreas was measured by solution hybridization with oligonucleotides complementary to rGH mRNA (5′ GCATTGGCAAACAGACTGGACAAGGGCATG), the marked mMT-I mRNA (5′ GATCTAGCTTAGTCAACCGTG), or human β-globin mRNA (5′ CCACAGGGCAGTAACGGCAGA) as described (24). Single-stranded phage M13 clones carrying the complementary sequence were used as hybridization standards. mRNA molecules per cell were calculated after determining the fraction of total nucleic acid that was DNA: day 16 fetal liver, 50%; day 15 fetal pancreas, 33%; and week 6 adult pancreas, 11% (25).

Transcription "Run On" Experiments. Nuclei were isolated (26) and used to measure the relative rate of transcription essentially as described (27). Nuclei (100 μg of DNA) were incubated in 100 μl in the presence of transcription buffer containing 100 μCi (1 Ci = 37 GBq) of [^{32}P]UTP for 45 min at 26°C. RNA was isolated as described (27), and three different quantities (7.5, 15, and 30 μl containing 7–13 × 10^4 cpm/μl) were hybridized to immobilized filter disc containing 1.2 μg of rGH in pUC (see Fig. 1), mouse albumin cDNA in pBR322, mMT-I, and pUC19 for 48 hr at 45°C, washed extensively, treated with RNases A and T, and then assayed (27). The relative rate of transcription was calculated as cpm specifically bound per 10^6 cpm of input RNA and is expressed as ppm. Because the rGH plasmid has 1.17 kb of DNA that could hybridize to the MTrGH transcripts but only 0.63 kb of that would be present in intronless transcripts, the latter values were multiplied by 1.85 for comparison. The albumin cDNA has a 1.3-kb insert. The average rate of mMT-I transcription (1.1 kb of hybridizable sequence) was 69 ppm for mice 1–3 and 33 ppm for mice 4–6 (not shown).

Tissue Culture Transfection. Plasmids containing the mMT-I or MTrGH genes with or without introns (10 μg) were mixed with 8 μg of carrier herring sperm DNA and 2 μg of thymidine kinase–neomycin-resistance gene as a selectable gene, precipitated with calcium phosphate, and added to semiconfluent cultures of baby hamster kidney (BHK) cells on 100-mm Petri dishes in 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum (28). After 6 hr, the medium was changed, and the cells were allowed to recover for 24 hr before G418 was added (750 μg/ml) to select for cells that had stably incorporated the foreign genes. After 10 days, when all nontransfected cells had died and the plates were nearly confluent with stably transformed cells, the cells were split onto two plates. One plate served as a control, whereas 100 μM $ZnSO_4$ was added to the other to induce the mMT-I gene promoter. The cells were harvested 8 hr later, and total nucleic acid was isolated for solution hybridization analysis of mMT-I and rGH mRNA levels as described above. Each transfection was performed in triplicate, and the results represent the means ± SEM. The amount of foreign DNA taken up by the cell populations was estimated by quantitative dot hybridization using nick-translated pUC sequences as a probe. These DNA values varied by <2-fold compared with the cells transfected with genes with or without introns.

RESULTS

Effect of Introns on MTrGH Expression in Fetal Liver. The introns were removed from a genomic clone of the rGH gene by substituting cDNA sequences between the first and last exons. Then the entire gene and 3′ flanking sequences were fused to the mMT-I gene promoter and its 5′ flanking sequences (Fig. 1). Fig. 2*A* shows that 9 of 11 transgenic samples from mice with the normal gene had significant rGH mRNA levels, whereas only 7 of 15 of the intronless samples had detectable mRNA. Furthermore, comparison of the amount of rGH mRNA in the two sets of data revealed 6-fold more mRNA in the intron-containing samples than in the intronless ones. Thus, both the frequency of expression and the average level of mRNA accumulation were depressed with the intronless construct; the product of these two values represents the overall efficiency of expression, which for this pair of constructs differed by about 10-fold as summarized in Table 1. As a control for possible differences in mRNA degradation during sample preparation, we quantitated the amount of endogenous mMT-I mRNA and found no significant difference in samples from the two constructs (data not shown). Furthermore, there was no obvious difference in gene copy number in the samples from these two constructs or any of the other pairs described below (data not shown).

The endogenous mMT-I gene is inducible by heavy metals (29), and various mMT-I fusion genes in transgenic mice are inducible by zinc or cadmium (23, 30). Thus, in repeating the experiment, we placed the foster mothers on a zinc diet to induce both maternal and fetal mMT-I gene expression. The data from the zinc-treated fetal liver samples (Fig. 2*B*) are similar to untreated samples except that the average amount of rGH mRNA is 2-fold higher. In this experiment, 7 of 9 of the transgenic samples containing the normal gene had high levels of rGH mRNA, whereas only 4 of 20 samples from the intronless construct had rGH mRNA, and the average level of mRNA in samples where it was detected was much lower. The overall efficiency of expression of the intronless construct was only 1% of the normal gene (Table 1).

To determine whether the difference in accumulation of rGH mRNA from the two constructs was due to a difference in the rate of transcription or the rate of mRNA degradation, transcription "run-on" experiments were performed with nuclei isolated from three fetal liver samples (from the experiment shown in Fig. 2*B*) of mice with either the intron

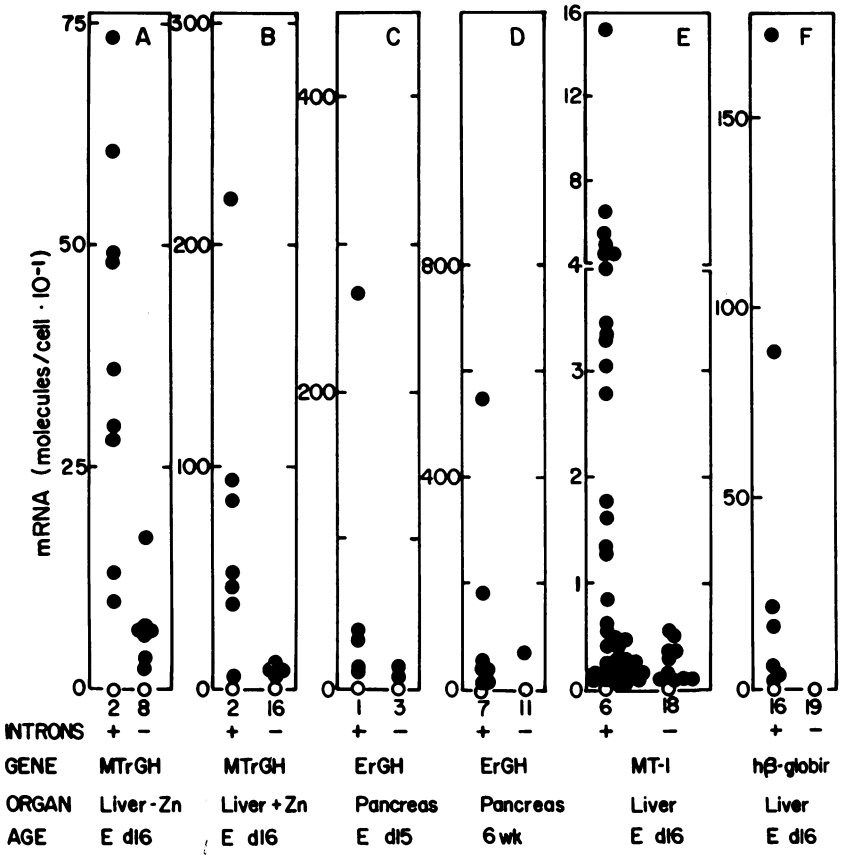

FIG. 2. Effect of introns on transgene expression. The number of samples that gave no detectable hybridization signal (<1 to 10 mRNA molecules per cell, depending on specific activity of the probe and the amount of nucleic acid used) are indicated by the numbers below each scatter diagram. In *B*, the foster mothers were reared on a diet that included 25 mM $ZnSO_4$ in the water.

or intronless constructs. The relative rate of transcription paralleled the relative mRNA abundance in all samples (Fig. 3). Of particular significance, the relative rates of transcription were low for the three intronless samples; thus, the presence of introns has a primary influence on transcription, not on mRNA stability. Because the plasmid used to measure rGH transcripts was derived primarily from the last three exons of the gene, we cannot eliminate the possibility that transcriptional attenuation occurs upstream of exon 3. As an internal control in this experiment, we measured the relative rate of albumin transcription; it was marginally higher (1.3-fold) in the intron-containing samples (Fig. 3) as were the rates of endogenous mMT-I gene transcription (2-fold) and mMT-I mRNA accumulation (1.3-fold). We conclude that the relative rate of transcription (either initiation or elongation beyond exon 3) is increased when transgenes containing introns are introduced into mice.

Effect of Introns on ErGH Gene Expression in the Pancreas. In the previous experiments, a promoter was used that is expressed in many different cell types. We also tested the cell-specific elastase I gene promoter/enhancer (19) fused to the rGH gene constructs with and without introns. The accumulation of rGH mRNA in fetal (day 14 or 15) and adult (week 6) pancreases of transgenic mice made with the two constructs are shown in Fig. 2 *C* and *D*. The patterns of expression are similar to the fetal liver data. When the two sets of data (Fig. 2 *C* and *D*) were combined, rGH mRNA was detected in 12 of 20 samples from the intron-containing construct but in only 3 of 17 samples from the intronless construct. Moreover, the average level of rGH mRNA was

Table 1. Effect of introns on gene expression in transgenic mice

Construct	Fig. 2 panel	Introns present	Tissue (age)*	Frequency of expression	Average mRNA concentration,† molecules per cell	Overall efficiency†
MTrGH	*A*	+	Liver (E16) −Zn	9 / 11	365	300
		−	Liver (E16) −Zn	7 / 15	63	29
	B	+	Liver (E16) +Zn	7 / 9	770	599
		−	Liver (E16) +Zn	4 / 20	30	6
ElrGH	*C*	+	Pancreas (E15)	5 / 6	715	595
		−	Pancreas (E15)	2 / 5	110	44
	D	+	Pancreas (6 wk)	7 / 14	1365	680
		−	Pancreas (6 wk)	1 / 12	720	60
MT-I	*E*	+	Liver (E16)	38 / 44	19.3	16.7
		−	Liver (E16)	10 / 28	2.6	0.94
hβG	*F*	+	Liver (E16)	7 / 23	442	134
		−	Liver (E16)	0 / 19	0	0.0

Summary of data from Fig. 2.
*E16 and E15, embryonic day 16 and 15.
†The average mRNA concentration is based on those mice that had detectable levels of mRNA (solid symbols in Fig. 2), whereas overall efficiency is the product of the average mRNA concentration and the frequency of expression. All paired groups showed significant differences ($P < 0.05$) by the Mann–Whitney *U* test except for group *C*, which was $P < 0.1$.

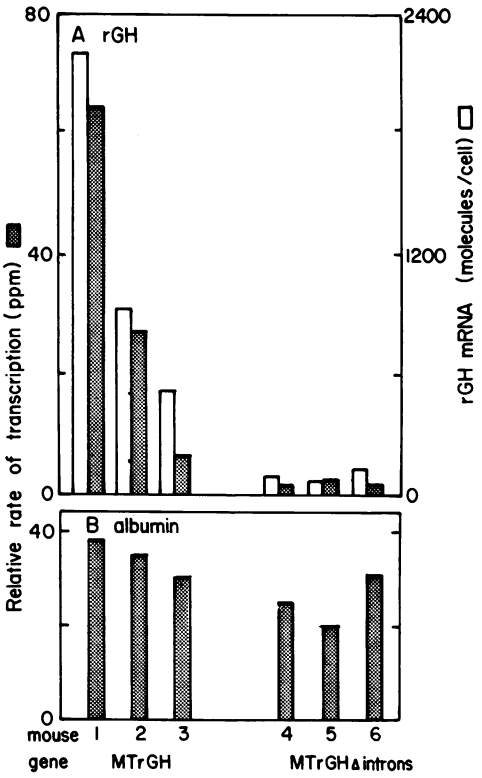

FIG. 3. Effect of introns on the rate of transcription of the MTrGH gene in transgenic mice. Fetal livers from three transgenic mice bearing the MTrGH gene with introns (mice 1–3) and three without introns (mice 4–6) were used for the determination of rGH mRNA (open histograms). (*A*) Relative rate of rGH gene transcription and rGH mRNA abundance. (*B*) Relative rate of albumin gene transcription.

lower in samples containing the intronless construct. Thus, expression in two different tissues with two different promoters reveals the same phenomenon.

No Effect of Introns on Expression of Constructs Transfected into Cultured Cells. The MTrGH constructs with and without introns were also transfected into BHK cells. Fig. 4*B* shows that there was no significant difference in either the uninduced or induced levels of rGH mRNA measured after transfection with either construct. Quantitative dot hybridizations of DNA from the transfected cells indicated no significant difference in the average number of integrated gene copies with the two constructs (data not shown). Thus, in contrast to the results obtained in transgenic mice, MTrGH gene expression in cultured cells seems to be insensitive to the presence of introns. Although it may be significant that the cells were selected for expression whereas the mice were not, there is always a significant effect of introns on transgene expression even when one compares only those mice that express the gene (Table 1).

Effect of Introns on Expression of the mMT-I Gene in Fetal Liver. In the previous experiments, we tested two different chimeric genes. In these experiments we tested a marked natural gene. The data (Fig. 2*E*) closely resemble those obtained with the chimeric constructs; both the frequency of expression and the accumulation of mMT-I mRNA were much lower with the intronless construct compared with results with the intact gene. Only 10 of 28 mice with the intronless construct gave detectable mMT-I mRNA compared to 38 of 44 with the intron-containing construct, and there was less mRNA in the samples containing the intronless construct by a factor, on average, of 7.5, as summarized in Table 1.

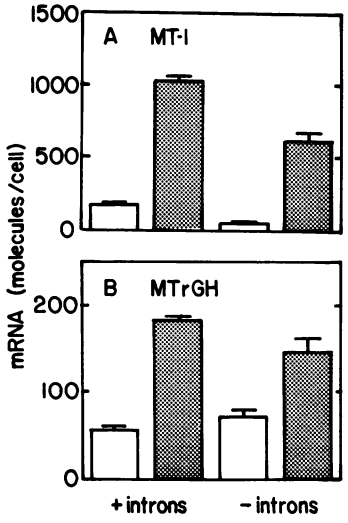

FIG. 4. Effect of introns on expression of mMT-I and MTrGH genes transfected into tissue culture cells. □, Control; ▨, induced with $ZnSO_4$.

These two constructs were also tested by transfection into BHK cells (Fig. 4*A*). In the absence of induction, there was less mMT-I mRNA from the intronless construct; however, after induction there was only a 1.4-fold difference in the amount of mMT-I mRNA accumulation. These differences are small compared to the differences observed in transgenic mice. Thus, the results support the previous conclusion obtained with MTrGH that introns have little influence on expression of a gene when stably integrated into chromosomes of cultured cells.

Effect of Introns on Globin Gene Expression. We also tested the effect of introns on the expression of the human β-globin gene in transgenic mice. Normal and intronless human β-globin gene constructs (Fig. 1) were microinjected into fertilized eggs, and day 16 fetal livers were isolated for quantitation of human β-globin mRNA. Fig. 2*F* shows that in the absence of introns there was no expression in any of 19 transgenic mice, whereas 7 of 23 of the samples containing the intact gene had measurable amounts of human β-globin mRNA.

DISCUSSION

The general conclusion from these studies with matched pairs of genes with or without introns is that introns improve transcriptional efficiency 10- to 100-fold in transgenic mice, but they have little effect on expression when transfected into cultured cells. Both the number of transgenic mice that have detectable levels of expression and the average level of mRNA expression are consistently depressed in the absence of introns (Table 1).

At the outset, it seemed reasonable to expect that introns might have an important effect on mRNA processing. This notion predicts that nuclear mRNA precursors are relatively unstable and that association of the precursors with "spliceosomes" would lead to nuclear stabilization and/or more efficient transport into the cytoplasm. This model suggests that the average rate of transcription would be comparable regardless of the presence of introns, but the accumulation of mRNA would differ. However, the nuclear "run-on" experiments presented in Fig. 3 indicate that mRNA abundance is proportional to the relative rate of transcription. Furthermore, one might expect an effect of introns on some basic aspect of RNA processing or transport to be revealed after transfection into tissue culture cells, contrary to what was observed. Although tissue-specific factors may affect

splicing, this is not a likely explanation for these results because the rGH gene was not assayed in the cell type in which this gene is normally expressed either *in vivo* or in culture. These observations lead us to suspect that a major effect of introns, when assayed in transgenic mice, is on some aspect of transcription rather than RNA processing.

Transcriptional enhancers can function in a position- and orientation-independent manner (31); they function when placed within introns (32), and they naturally reside within the introns of some genes (33, 34). Therefore, one might imagine that each of the genes we chose to study has one or more previously unidentified enhancers in its introns. Indeed, our previous analysis of the human β-globin gene implicated the presence of important enhancer-like elements near the boundary of intron B/exon 3 as well as 3′ of the structural gene (35). Similar, all-or-none expression results were obtained by infection of murine erythroleukemia cells with retroviruses containing the human β-globin gene with or without introns (M. A. Bender, A. D. Miller, and R.E.G., unpublished data). Thus, the observation presented here, that the intronless human β-globin gene is not expressed at all, is most easily interpreted as the loss of an essential enhancer element.

By extension of the enhancer argument, one might postulate that the rGH gene and the mMT-I gene also have important control elements within their introns. Several observations suggest that this is unlikely. First, the 5′ flanking regions of both mMT-I and rGH genes have been shown to confer proper tissue-specific expression and/or regulation to heterologous reporter genes (29, 30, 36–38). Second, because the rGH gene was assayed in two cell types that do not normally express it (hepatocytes and pancreatic acinar cells) and introns had a positive effect in each, it seems unlikely that a tissue-specific enhancer can be involved. Third, if a general enhancer-like element were present in the introns, then the introns might be expected to affect expression after transfection in cultured cells. The experiments in BHK cells do not support this latter view, although this cell line, and perhaps all transformed cell lines, may have alternative mechanisms of stimulating transcription that are independent of general enhancers. Thus, it might be informative to test the effect of introns after introduction of genes into primary cell lines.

Another more provocative possibility is that introns contain DNA sequences that are recognized at some stage during development but are not required after transfection into established cell lines. If this hypothesis is correct, then one might expect to find conserved recognition sequences within introns.

A mechanistically different possibility is that introns (or exons) contain sequences that are important for phasing nucleosomes relative to important promoter elements (39, 40). According to this hypothesis, the position and orientation of introns would be critical, a property that distinguishes this idea from those invoking enhancer-like properties to introns. One might explain why introns have little effect after transfection into cultured cells by suggesting that critical phasing of nucleosomes is established during development or that nucleosomes in cultured cells are not phased as rigidly as they are *in vivo* (41).

Clearly, introns have an important influence on the function of genes introduced into the germ line of mice by microinjection. The results suggest that they may help maintain transcriptional activity during development. Aside from the practical aspect of considering their presence during the preparation of gene constructs destined for expression in transgenic animals, these results provide new insight into the role of intron sequences during development and evolution.

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