A 3' enhancer contributes to the stage-specific expression of the human β -globin gene

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The human β -globin and ϵ -globin genes are expressed at different stages of human development and also show distinct temporal patterns of expression when transferred into the mouse germ line. In transgenic mice, the β -globin gene is expressed only in fetal and adult erythroid cells, whereas the ϵ -globin gene is active only in embryonic erythroid cells. Previous experiments suggested that β -globin 3' sequences were important for expression in mouse fetal and adult erythroid cells, and in this paper we directly demonstrate the presence of an enhancer in the 3'-flanking region of the gene. First, deletion of sequences between 605 and 895 bp, 3' to the poly(A) site, results in a 10-fold reduction in the average level of expression of the β -globin gene in transgenic mouse fetal livers. Second, a DNA fragment including β -globin 3'-flanking sequences [425–1480 bp from the poly(A) site], in either orientation, activates transcription from the otherwise silent ϵ -globin promoter in the mouse fetal liver; DNA sequences between 150 and 730 bp or between 920 and 1680 bp, 3' to the β -globin gene, are inactive by this assay. Together, these experiments identify an enhancer, in the region approximately 600–900 bp, 3' to the β -globin poly(A) site, which contributes to the differential stage-specific expression of the β -globin and ϵ -globin genes.

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The mechanisms by which different members of the β -globin gene family are sequentially expressed during mammalian development represent a complex problem of long-standing interest (Collins and Weissman 1984). In humans, the ϵ -globin gene is an "embryonic" gene expressed early in gestation, the "fetal" ϵ -globin and Λ -globin genes are active during the remainder of gestation, and the "adult" δ -globin and β -globin genes are expressed primarily after birth. In attempting to understand the regulation of this gene family, an important problem is the identification of *cis*-acting regulatory elements that presumably interact with different *trans*-acting factors in erythroid cells at different stages of development. One way to address this question is to microinject exogenous globin genes into fertilized mouse eggs (Gordon et al. 1980; Brinster et al. 1981; Costantini and Lacy 1981; E. Wagner et al. 1981; T. Wagner et al. 1981) and analyze their expression during the development of the resulting transgenic mice.

When the human adult β -globin gene (Costantini et al. 1985; Townes et al. 1985) or a 5' mouse/3' human hybrid β -globin gene (Chada et al. 1985; Magram et al. 1985) was introduced into the mouse germ line, it was expressed specifically in erythroid tissues at the fetal stage (fetal liver) and in the adult (bone marrow and peripheral blood) but not in the embryonic (yolk sac-derived) erythroid cells. This paralleled the expression of

the homologous mouse β -globin genes, β^{maj} and β^{min} , which are expressed at the fetal as well as the adult stages, and it therefore demonstrated appropriate developmental regulation of the transgene. The human ϵ -globin (Costantini et al. 1985; Chada et al. 1986) or Λ -globin gene (Kollias et al. 1986; Trudel et al. 1987b) was expressed only in the embryonic erythroid cells of transgenic mice and not in the fetal liver or the adult erythroid tissues. Although this differed from the fetal pattern of expression of the γ -globin genes during human development, it resembled the embryonic stage-specific expression of their murine homolog, the $\beta H1$ -globin gene (Hill et al. 1984) and suggested that human γ -globin transgenes were coordinately regulated with the endogenous $\beta H1$ gene during mouse development (Costantini et al. 1985; Chada et al. 1986; Kollias et al. 1986). These studies suggested that the *cis*-acting elements responsible for the differential expression of the human β -globin and γ -globin genes could be analyzed, using the transgenic mouse as an experimental system.

As a first step in this analysis, we studied the expression of a series of hybrid globin genes in which different upstream, intragenic, or downstream regions were contributed by the β -globin gene, where as the remainder of each gene consisted of the ϵ -globin sequences. These experiments indicated that β -globin sequences downstream from the *EcoRI* site in exon III (Fig. 1C) were nec-

Human β -globin enhancer

Figure 1. (See following page for legend.)

essary and sufficient for the expression of a 5'^G-γ/3'β hybrid gene in fetal liver and in adult erythroid tissues (Trudel et al. 1987b). Several other observations also suggested the possibility of a regulatory element in the 3'-flanking region of the human β-globin gene. First, an enhancer in the 3'-flanking region of the chicken adult β-globin gene had been reported (Choi and Engel 1986; Hesse et al. 1986). Second, the human β-globin gene with 560 bp of 3'-flanking DNA was expressed in a series of transgenic mice (F.C., K. Chada, and J. Magram, unpubl.) at much lower levels than the same gene with 1680 or 3500 bp of 3'-flanking DNA (Costantini et al. 1985; Townes et al. 1985), suggesting that sequences between 560 and 1680 bp from the poly(A) site were required for efficient expression. Third, a DNase I hypersensitive site had been described approximately 800 bp 3' to the β-globin gene in human erythroid cells (Groudine et al. 1983), and in transgenic mice (Radice and Costantini 1986), which was consistent with the interaction of *trans*-acting factors with DNA sequences in this region.

In this paper, we provide direct evidence for a 3' enhancer that contributes to the stage-specific expression of the human β-globin gene. We show by truncation experiments that a region approximately 600–900 bp, 3' to the poly(A) site, is required for efficient expression of the β-globin gene in transgenic mouse fetal livers. Furthermore, we show that a DNA fragment including this enhancer region can activate transcription from the otherwise silent ^G-γ-globin promoter in mouse fetal erythroid tissues.

Expression of β-globin genes with truncated 3'-flanking sequences

As one test for a positive regulatory element downstream from the human β-globin gene, we analyzed the expression of four DNA fragments containing the entire gene, together with 2.7 kb of 5'-flanking DNA, but with different lengths of 3'-flanking DNA (Fig. 1). Each fragment was purified from plasmid vector sequences, microinjected into fertilized mouse eggs, and the eggs reimplanted into pseudopregnant foster mothers. Rather than allowing the transgenic mice to come to term and breeding them to obtain F₁ transgenic offspring, as in previous studies, we terminated the pregnancies at 16.5 or 17.5 days of gestation and analyzed the fetuses for the expression of the transgene (Hammer et al. 1987). Although this shortcut allowed us to analyze each transgenic mouse at only the fetal stage of development, it

drastically reduced the time required to complete such a study. As the pattern of globin gene expression is similar in fetal and adult erythroid tissues, with respect to endogenous globin genes (Russell 1979) as well as globin transgenes (Costantini et al. 1985; Magram et al. 1985; Chada et al. 1986), it is sufficient for most purposes to measure expression at either the fetal or adult stages.

The liver was dissected from each fetus carrying one or more intact copies of the transgene (determined by Southern blot analysis; data not shown), total RNA was isolated, and the level of human β-globin mRNA was measured by an RNase protection assay with a 5' probe (Fig. 1; Table 1). When the β-globin gene was introduced with either 895 or 1200 bp of 3'-flanking DNA, a majority of transgenic fetal livers contained relatively high levels of human β-globin mRNA. The levels of expression of these genes were similar to those of the β-globin gene with 3500 bp of 3'-flanking DNA (Costantini et al. 1985; F. Costantini, unpubl.). Therefore, our data provide no evidence for positive regulatory elements between 895 and 3500 bp, 3' to the poly(A) site.

Deletion of all but 605 or 285 bp, 3' to the poly(A) site, however, resulted in a clearly lower distribution of expression levels (Table 1). When the average levels of β-globin mRNA in all DNA-positive fetal livers are calculated, the genes with 605 and 895 bp of 3'-flanking sequence (for which the most data were obtained) show a 10-fold difference in expression. All of the fetal liver RNAs contained equivalent levels of endogenous mouse β-globin mRNA, as shown by cross-hybridization with the human β-globin probe (see bands at bottom of autoradiogram in Fig. 1A), providing an internal control. These results indicate that a positive regulatory element active in mouse fetal erythroid cells resides, at least in part, between 605 and 895 bp, 3' to the human β-globin poly(A) site.

We also examined RNAs from brain and kidney of several of the transgenic fetuses, to determine whether the 3'-flanking sequences were required for the tissue-specific expression of the β-globin gene. In two fetuses expressing the 895-bp fragment, the levels of human β-globin mRNA in kidney and brain were 8% and 2–3%, respectively, of the level in the liver, as determined by densitometry of autoradiograms. These levels are consistent with the usual degree of blood contamination in nonerythroid tissues (see Fig. 2C and legend). In the one fetus expressing the 285-bp fragment and two of those expressing the 605-bp fragment, the level of human β-globin mRNA in kidney was 30–40% of the level in liver, and the level in brain was 3–10% of the level in

Figure 1. Expression of human β-globin genes containing 285, 605, 895, or 1200 bp of 3'-flanking sequences. (A) Detection of human β-globin mRNA in transgenic fetal liver RNAs by 5'-RNase protection analysis. Each lane represents a different transgenic fetus. (HB, lanes 1–4) 0.23, 2.3, 23, and 230 ng, respectively, of human reticulocyte total RNA. (m) Molecular weight markers. (B) Detection of human β-globin mRNA in liver (L), brain (B), and kidney (K) of transgenic fetuses by 3'-RNase protection analysis. (HB, lanes 1–3) 0.23, 2.3, and 23 ng, respectively, of human reticulocyte RNA; (lane 4) yeast RNA; (lane 5) control mouse fetal liver RNA. (C) DNA fragments used for microinjection; restriction map of the β-globin gene. The *Hind*III site is 2571 bp from the cap site (→). (p) Probes; (pf) protected fragments (131 nucleotides for 5' probe; 212 nucleotides for 3' probe). In A, 3 μg of RNA from each fetal liver was used for hybridization. In B, 1 μg (fetus 895-6), 1.5 μg (fetuses 285-2 and 895-3), or 5 μg fetuses (605-2 and 605-9) was used for hybridization. The level of human β-globin mRNA in the human reticulocyte RNA was 10 pg/ng total RNA (Chada et al. 1985).

Table 1. Expression in transgenic fetal livers of β -globin genes with varying amounts of 3' flanking DNA

3'-flanking DNA (bp)	Fetus no.	Transgene copy no.	mRNA ^a (pg/ μ g total RNA)
285	6	10	0
	5	40	0
	4	8	0
	3	1	0
	1	13	0
	2	25	150 (45,5)*
605	7	0.5	0
	6	0.5	0
	1	57	0
	5	20	1
	3	2.5	2
	4	40	3
	2	7	10 (4,1)
	9	51	30 (9,1)
	8	28	80
895	7	5	0
	8	0.8	4
	9	6	8
	10	11	20
	4	60	50
	1	6	50
	5	10	150
	3	17	200 (16,4)
	2	43	200
	6	46	700 (56,18)
1200	6	3	0
	5	1.5	0
	2	14	50
	1	12	50
	4	15	100
	3	8	150

^a mRNA levels were determined by comparing the hybridization signals obtained with fetal RNAs vs. human reticulocyte RNA, as shown in Fig. 1. The level of endogenous β -globin mRNA in mouse fetal liver is approximately 1000 pg/ μ g total RNA (Chada et al. 1985; Magram et al. 1985). (*) Expression in fetal kidney, brain.

liver. Therefore, the β -globin gene with only 285 or 605 bp of 3'-flanking sequence is still expressed preferentially in erythroid tissues, in those few fetuses in which it is expressed at all.

Activation of the ϵ -globin gene in mouse fetal livers by β -globin 3'-flanking sequences

The positive influence of 3'-flanking sequences on β -globin mRNA levels indicated that a 3' element (or elements) was capable of enhancing transcription from the distant β -globin promoter. As an independent assay for an enhancer in this region, we examined the ability of β -globin 3'-flanking sequences to activate transcription of the human ϵ -globin gene in the mouse fetal liver. Although the ϵ -globin gene is a "fetal" gene in humans, it is expressed only in yolk-sac-derived embryonic blood cells and not fetal liver erythroid cells in transgenic mice (Costantini et al. 1985; Chada et al. 1986). If the

β -globin 3' element could enhance transcription from the β -globin promoter in response to *trans*-acting factors in mouse fetal or adult erythroid cells, it might be also able to activate the ϵ -globin promoter in these cells.

Three overlapping DNA fragments containing β -globin 3'-flanking sequences were inserted at the *Bgl*II site, 1.6 kb upstream from the ϵ -globin gene (Fig. 2D). These constructs (with plasmid sequences removed) were injected into fertilized mouse eggs, the fetuses were recovered at 16.5 or 17.5 days of gestation, and transgenic fetuses were identified by Southern blot analysis (data not shown). Total RNA was isolated from the livers of DNA-positive fetuses, and ϵ -globin mRNA was measured using a 5'-RNase protection assay. The results (Fig. 2; Table 2) showed that the "a" fragment, in either orientation, caused expression of the ϵ -globin gene in the majority of transgenic fetal livers. This confirms the presence of an enhancer in the 3'-flanking region of the β -globin gene, which can activate transcription from a distant promoter in mouse fetal liver cells. In contrast, the ϵ -globin gene joined to either the "b" or "c" fragment showed no expression in nearly all transgenic fetuses (9/10 and 18/19, respectively) and only barely detectable levels of expression in two cases. These results, taken together with the results of the 3'-truncation experiments, allow us to localize the enhancer element(s) to a certain degree (see Discussion).

ϵ -Globin expression, under the influence of the β -globin enhancer, was tissue-specific, in most cases. In six fetuses, the levels of ϵ -globin mRNA in kidney were 4–20% of the levels in liver, and in brain 1–5% of the levels in liver (Fig. 2B; Table 2); these levels of globin mRNA in nonerythroid tissues appear to be largely due to blood contamination (Fig. 2C). In two fetuses (a'1 and a5), however, the level of ϵ -globin mRNA in kidney was similar to that in liver and probably represented expression of the ϵ -globin gene in an inappropriate tissue. The levels of ϵ -globin mRNA in the transgenic fetal livers were generally lower than those of β -globin transgenes, including the 3'-enhancer region (Table 1). This could suggest that (1) the β -globin 3' enhancer may interact more efficiently with the β -globin than the ϵ -globin promoter; (2) ϵ -globin expression may be negatively regulated by an independent mechanism in mouse fetal erythroid cells; or (3) possible internal or upstream regulatory elements (see Discussion) may contribute to the higher level of expression of β -globin transgenes.

Discussion

The human β -globin and ϵ -globin genes are expressed in erythroid cells at different stages of human development, and they retain the ability to be regulated temporally when introduced into the mouse germ line. In this study, we have used the transgenic mouse system to identify an enhancer that appears to be responsible, at least in part, for the stage-specific expression of the human β -globin gene. In one experiment, we examined the expression of the β -globin gene in transgenic mouse fetal livers after successive truncation of 3'-flanking se-

Figure 2. (See facing page for legend.)

Table 2. Expression in transgenic fetal livers of the ζ -globin gene attached to 3' β -globin fragments

β -globin 3' fragment					
a (<i>Bst</i> NI– <i>Bst</i> NI)		b (<i>Hph</i> I– <i>Mst</i> II)		c (<i>Ava</i> II– <i>Xba</i> I)	
fetus	γ mRNA	fetus	γ mRNA	fetus	γ mRNA
a'2	28 (2, 0.4)*	b8	0.2	c8	0.1
a'1	28 (56, 2)	b1	0	c1	0
a4	2.4 (0.1, 0.02)	b2	0	c2	0
a8	2 (0.2, 0.1)	b3	0	c3	0
a3	1.6 (0.05, 0.01)	b4	0	c4	0
a5	0.8 (0.4, 0)	b5	0	c5	0
a'5	0.6 (0.1, 0)	b6	0	c6	0
a'3	0.4	b7	0	c7	0
a'4	0.3 (0, 0)	b9	0	c9	0
a1	0	b10	0	c10	0
a2	0			c11	0
a6	0			c12	0
a7	0			c13	0
				c14	0
				c15	0
				c16	0
				c17	0
				c18	0
				c19	0

mRNA levels (pg/ μ g total RNA) are from Fig. 2 (and data not shown) and were determined using K562 cell RNA as a hybridization standard. Zero (0) indicates less than 0.1 pg/ μ g or undetectable level of ζ -globin mRNA. Fetuses labeled a' carried the β -globin 3' fragment in the opposite orientation with respect to the ζ -globin gene; in all other cases, the β -globin 3' fragment was in the same orientation. (*) Expression in fetal kidney, brain.

quences. In a second experiment, we tested the ability of β -globin 3'-flanking sequences to activate transcription from the ζ -globin promoter in the fetal liver.

The most definitive evidence for a 3' β -globin enhancer was provided by the latter experiment. A DNA fragment containing only 3'-flanking sequences from the β -globin gene [425–1480 bp from the poly(A) site] was capable, in either orientation, of activating transcription of the ζ -globin gene in the mouse fetal liver, where this gene normally fails to be expressed. Previous experiments had shown that hybrid 5' ζ /3' β -globin genes, containing β -globin intragenic as well as 3'-flanking sequences, were expressed in fetal and adult mouse erythroid tissues (Trudel et al. 1987a). Although this result raised the possibility of a 3' β -globin enhancer, other interpretations (e.g., post-transcriptional

effects due to β -globin sequences in the mRNA transcription unit) could not be eliminated. In the present experiment, the ζ -globin transcription unit was left intact, and, therefore, the β -globin 3' sequences must have stimulated ζ -globin expression at the transcription level.

Although it is not feasible to define enhancer elements precisely using transgenic mice as an assay system, our experiments allow us to delimit the β -globin 3'-enhancer region to a certain extent. The failure of DNA fragments b and c (Fig. 2d) to activate ζ -globin transcription indicated that either (1) an essential enhancer element is located between positions 730 and 920, or (2) more than one sequence element is required for enhancer function, and the a fragment, but not the b or c fragment, contains all of them. Complementary in-

Figure 2. A β -globin 3' element, in either orientation, activates the ζ -globin gene in transgenic mouse fetal livers. (A) Detection of human ζ -globin mRNA in fetal livers, detected by 5'-RNase protection analysis. Each lane represents a different transgenic fetus. Fragments a, b, or c (panel D) were ligated to the ζ -globin gene (*Bgl*II–*Sph*I fragment) in the same 5' to 3' orientation; (a') fragment a in opposite orientation with respect to the ζ -globin gene; (K562) 2.4 μ g (lane 1) and 0.8 μ g (lane 2) of total RNA from hemin-induced human K562 cells (Lozzio and Lozzio 1975); (m) molecular weight markers. (B) Detection of ζ -globin mRNA in liver (L), kidney (K), and brain (B) of three transgenic fetuses. (Lanes 1–4) 1.6, 0.32, 0.064, and 0.016 μ g, respectively, of K562 cell RNA. (C) Detection of endogenous mouse β -globin mRNA in 0.4 μ g of RNA from liver, kidney, and brain of transgenic fetus a3, using a 3' RNA probe derived from the mouse β^{maj} gene. The levels of endogenous β^{maj} -globin mRNA in kidney and brain varied from fetus to fetus but were lower than 10% (kidney) or 5% (brain) of the level in liver. (D) Restriction map of the β -globin 3'-flanking region, showing the DNA fragments used in constructions. The a (*Bst*NI–*Bst*NI), b (*Hph*I–*Mst*II), or c fragment (*Ava*II–*Xba*I) was inserted in front of a *Bgl*II–*Sph*I fragment containing the ζ -globin gene. A DNA fragment containing the complete ζ -globin gene, together with the β -globin 3' fragment, was purified, and transgenic fetuses were generated and analyzed as described in Methods (4 μ g of RNA from each fetal tissue was used for each analysis). (p) RNA probe; (pF) 147 nucleotide protected fragment. The RNA probe was an *Acc*I–*Bst*EII fragment of the ζ -globin gene, which hybridizes with ζ -globin mRNA. The level of ζ -globin mRNA in K562 cell total RNA was 16 pg/ μ g total RNA (Chada et al. 1986).

formation on the location of enhancer elements was provided by the β -globin 3' truncation experiments (Fig. 1; Table 1); removal of sequences between 605 and 895 bp, 3' to the poly(A) site, resulted in a 10-fold reduction in the average level of expression of the β -globin gene. Therefore, elements that stimulate β -globin gene expression are located between 605 and 895 bp from the poly(A) site. Consistent with both of these estimates is the location of an erythroid cell-specific DNase I hypersensitive site, which maps at approximately 800 bp, 3' to the poly(A) site (Groudine et al. 1983; Radice and Costantini 1986). Taken together, these data suggest that a factor or factors present in mouse fetal and adult erythroid cells are capable of acting at a site approximately 600–900 bp from the β -globin poly(A) site and stimulating transcription from the β -globin promoter. Presumably, the normal role of these factors is to stimulate transcription of one or both of the mouse fetal/adult β -globin genes, β^{maj} and β^{min} . However, the locations of enhancers associated with the mouse β -globin genes have not been determined.

When the β -globin 3'-enhancer region is placed close to the γ -globin gene, it is also capable of activating transcription from the γ -globin promoter in mouse fetal livers, and similar studies (T. Townes and F. Grosfeld, pers. comm.) have shown that it can activate the human γ -globin gene as well. In its normal chromosomal context, however, this enhancer is located approximately 2 kb downstream from the β -globin promoter but 24 and 30 kb downstream from the γ -globin and γ -globin promoters, respectively. Thus, it is likely to preferentially stimulate the β -globin gene and, therefore, contribute to its stage-specific expression during human development.

Whether the β -globin 3' enhancer is a cell type-specific enhancer (Serfling et al. 1985) and is responsible for the erythroid tissue-specific expression of the β -globin gene is not yet clear. Although the enhancer activates γ -globin gene expression in an erythroid tissue-specific manner in transgenic mouse fetuses, it is possible that other regulatory elements associated with the γ -globin gene may restrict the expression of this hybrid construct to erythroid cells. The ability of the β -globin 3' enhancer to direct erythroid cell-specific expression of other genes is currently being tested.

In either case, it appears that the enhancer defined by our studies is not the sole regulatory element associated with the β -globin gene. First, β -globin genes lacking the 3'-enhancer region were still expressed preferentially in erythroid tissue in the few transgenic mice in which they were expressed. This suggests that upstream or intragenic elements may contribute to the tissue-specific expression of the gene [although several studies have failed to demonstrate a role for upstream β -globin sequences in tissue-specific or stage-specific expression in transgenic mice (Townes et al. 1985; Trudel et al. 1987a)]. Second, β -globin genes lacking the 3'-enhancer region can be expressed in an inducible manner during the *in vitro* differentiation of murine erythroleukemia cells (Chao et al. 1983; Charnay et al. 1984). Third, even

when the 3' enhancer is included, the levels of expression of β -globin transgenes rarely reach those of endogenous β -globin genes in their normal chromosomal context, suggesting an additional role for more distant sequences in the β -globin gene cluster. These observations indicate that the 3' enhancer identified by our studies is one of several regulatory elements that interacts to determine the pattern of β -globin expression during development.

Methods

DNA constructions

Deletions of β -globin 3'-flanking sequences were generated by *Bal31* digestion from the *Xba*I site (Fig. 1), followed by addition of an *Xho*I linker and religation. Four clones were chosen containing 285 ± 10 , 605 ± 20 , 895 ± 10 , and 1200 ± 25 bp of 3'-flanking DNA, as determined by restriction mapping. The DNA fragments were excised as *Hind*III–*Xho*I fragments and purified by agarose gel electrophoresis.

The γ -globin constructions were produced by inserting a *Bgl*III–*Sph*I fragment, including the gene, into the *Bam*HI and *Sph*I sites of pUC18. β -globin fragments a, b, and c (Fig. 2) were inserted into a *Sma*I site upstream from the γ -globin fragment, and the pUC18 vector was removed by digestion with *Kpn*I and *Sph*I.

Production of transgenic mice

DNA fragments were purified on CsCl gradients and microinjected into fertilized mouse eggs, obtained from (C57BL6/J $\times$ CBA/J) F₁ females mated with males of the same F₁ hybrid strain, as described (Hogan et al. 1986). The microinjected eggs were implanted into the oviducts of pseudopregnant foster mothers, which were sacrificed at 16.5 or 17.5 days of gestation. The fetuses were frozen in liquid nitrogen and stored at -135°C , and DNA was isolated from the placenta or part of the fetus. DNAs were analyzed by Southern blot hybridization, and fetuses carrying intact copies of the transgene were used for RNA analysis. Transgene copy number (per diploid genome) was measured by dot blot hybridization with a probe for the human β -globin gene, after measuring the DNA concentration by a fluorescence method (Thomas and Farquhar 1978). Human genomic DNA and the cloned human β -globin gene were used as standards.

Analysis of globin mRNAs

Transgenic fetuses were thawed in phosphate-buffered saline at room temperature, and the livers, kidneys, and brains were rapidly dissected out. The tissues were homogenized in LiCl/urea with a Brinkman Polytron homogenizer, and total RNA was isolated (Auffray et al. 1980). mRNA levels were measured by RNase protection analysis, as described by Melton et al. (1984), using the probes described in the legends to Figures 1 and 2. RNA samples were hybridized with 4–9 ng of ^{32}P -labeled RNA probe in 80% formamide buffer for 16 hr at 45°C . Hybrids were treated with RNase A and T1 and analyzed by electrophoresis on a urea/polyacrylamide gel.

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