

Tissue-specific expression of a *FMR1*/β-galactosidase fusion gene in transgenic mice

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Fragile X syndrome is one of the most common genetic causes of mental retardation, yet the mechanisms controlling expression of the fragile X mental retardation gene *FMR1* are poorly understood. To identify sequences regulating *FMR1* transcription, transgenic mouse lines were established using a fusion gene consisting of an *E.coli* β-galactosidase reporter gene (*lacZ*) linked to a 2.8 kb fragment spanning the 5'-region of *FMR1*. Five transgenic mouse lines showed *lacZ* expression in brain, in particular in neurons of the hippocampus and the granular layer of the cerebellum. Expression of the reporter gene was also detected in Leydig cells and spermatogonia in the testis, in many epithelia of adult mice, and in the two other steroidogenic cell types, adrenal cortex cells and ovarian follicle cells. Embryonic tissues which showed strong activity of the reporter gene included the telencephalon, the genital ridge, and the notochord. This expression pattern closely resembles the endogenous one, indicating that the 5' *FMR1* gene promoter region used in this study contains most *cis*-acting elements regulating *FMR1* transcription.

INTRODUCTION

Fragile X syndrome, an X-linked inherited disease and the most common form of monogenic inherited mental retardation, is mainly due to a block in the transcription of the fragile X mental retardation 1 gene (*FMR1*). Other clinical features of the fragile X syndrome are postpubertal macroorchidism in male patients, a longish face, large ears and several symptoms reminiscent of a connective tissue dysplasia (1). The only neuroanatomic alteration reported in the brains of affected patients is a reduction in size of the posterior vermis of the cerebellum (2). A characteristic feature of the pathogenesis is the expansion of a CGG-trinucleotide repeat, located upstream of the translation initiation site in the first exon of *FMR1*, and a concomitant cytosine methylation of the CpG island encompassing the 5'-end of this gene (3,4). It has been postulated that this erroneous methylation of the putative promoter region results in the inhibition of transcription of the *FMR1* gene, and that the absence of the *FMR1* gene product leads to the various disease symptoms (3,4). The causative role of the *FMR1* gene for fragile X syndrome has been confirmed by the description of patients suffering from mental retardation similar to the fragile X syndrome who carried deletions in this gene (5–7). Moreover, *FMR1* mRNA and

protein were found to be strongly reduced or absent in cells and tissues derived from fragile X syndrome patients (8–11).

FMR1 consists of 17 exons spanning 38 kb of chromosome Xq27.3, and the corresponding 4.8 kb transcript is subjected to alternative splicing resulting in several isoforms of the protein, including the most abundant 69 kDa isoform (12–14). The *FMR1* gene product is an RNA-binding protein that binds to various sequences with different affinities. It has been estimated that about 4% of all mRNAs expressed in the fetal human brain are bound by the *FMR1* protein (15,16). Indeed, in a mentally retarded male patient with the symptoms of fragile X syndrome, no CGG-repeat amplification or methylation in *FMR1* was found, however, a point mutation was identified in *FMR1* which inhibited binding of the *FMR1* protein to RNA (17,18). Recently, an animal model for the fragile X syndrome has been generated by targeted inactivation of the murine *Fmr1* gene in embryonic stem cells. The resultant knockout mice did not express the *FMR1* protein and showed increased testicular weight, learning deficits, and hyperactivity (19).

FMR1 mRNA has been detected by reverse transcriptase-mediated PCR and Northern blot analyses in a variety of tissues including fetal brain, liver and pancreas, and adult

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brain, testis and lymphocytes (9,14,20). *In situ* hybridization studies localized endogenous human and murine mRNA in multiple tissues of pre- and postnatal origin. Postnatally *FMR1* transcripts were observed in the central nervous system, in particular in the hippocampus and the cerebellum, and also in the testis (21,22). Highest *FMR1* protein expression was observed in neurons as confirmed by immunostaining (8,11).

More information about the regulation of developmental and tissue-specific transcription of *FMR1* will contribute to our understanding of the pleiotropic phenotype of the fragile X syndrome and of the molecular mechanisms leading to transcriptional silencing of *FMR1* in affected patients. An established procedure to study the regulation of gene expression in an organism is the generation of transgenic mouse lines using a DNA construct consisting of a reporter gene linked to the relevant regulatory fragment (23). In an attempt to define *cis*-acting sequences regulating expression of *FMR1*, we generated five transgenic mouse lines by introducing a fusion gene consisting of the putative 5'-regulatory region of *FMR1* fused to *lacZ* into the mouse genome and analyzed expression of the reporter gene in developing and adult mice.

RESULTS

Generation of transgenic mice expressing a *FMR1/lacZ* fusion gene

The fusion gene *FMR1/lacZ* contains a fragment from the upstream region of *FMR1* linked to the *lacZ* reporter gene (Fig. 1). The 2830 bp *FMR1* sequence includes the 5'-region of the CpG island and ends 18 bp upstream of the translation initiation codon. As a major transcription start site of *FMR1* has been reported to lie about 320 bp upstream of the translation initiation codon, approximately 300 bp of 5'-untranslated sequences are therefore included (12). To direct the reporter gene product to the nucleus and thereby increase the resolution of the histological analysis, we used a reporter gene containing the nuclear localization signal from the large T antigen of simian virus 40 (SV40) at the N-terminus of β -galactosidase (24). Injection of the fusion gene into zygote pronuclei resulted in 43 newborn mice from which six were identified to be transgenic by Southern blot analysis of tail DNA using a *lacZ* probe. The six independent mouse lines and the approximate copy number of the transgene (in parentheses) within these lines were: 22 [1-5], 35 [1-5], 38 [5-20], 41 [50-100], 42 [50-100], and 43 [1-5]. Mouse line 38 did not give rise to transgenic offspring and was excluded from further analysis.

Transgene expression in the central nervous system

The regions of the brain expressing the reporter gene were identified by overnight whole-mount staining of perfusion-fixed brains with Bluo-Gal, a substrate for β -galactosidase, followed by paraffin-embedding and sectioning, or by staining of frozen sections (23). The expression level was estimated from staining intensity. Varying concentrations of the reporter gene product were observed in different parts of the brain (Fig. 2a-c; summarized in Table 1). Although the expression level was variable between the different transgenic mouse lines, the expression pattern between the five lines was very similar (Tables 1 and 2). This implies that the observed pattern was due to intrinsic properties of the *FMR1* promoter rather

than to positional effects after integration of the transgene. Both the expression pattern and relative expression level in tissues were similar or identical between mice of the same line. The hippocampus and the granular layer of the cerebellum showed the strongest signals (Fig. 2a, b). In brain slices of perfused mice, the hippocampus showed intense staining as early as two hours after incubation, while in other parts of the brain little or no staining was visible (Fig. 2a). Nevertheless, overnight staining revealed a lower level of *lacZ* expression in other neurons, e.g. in the bulbos olfactorius (Fig. 2c). Staining was also observed in the retinal bipolar neuronal layer and in the nervus vagus in the oesophagus (not shown).

Transgene expression in other adult tissues

The *lacZ* expression was analyzed in testicular and ovarian tissues (Fig. 3; summarized in Table 2). When visualized by polarized light, the sensitivity of the *lacZ* assay is increased and allows a detailed histological analysis of the expression pattern (25). Figures 2f,g and 3d,e show the same tissue specimens in bright field and in dark field with polarized light, respectively, thus illustrating the increase in sensitivity. In testis, expression was detected in the interstitial Leydig cells (Fig. 2f,g). Staining of spermatogonia was strong near the surface of whole-mount stained testis and in frozen sections (Table 2), and the seemingly exclusive staining of Leydig cells in Figure 2g results from better diffusion of β -galactosidase substrate in the interstitium compared to diffusion into the seminiferous tubuli. In female mice, staining was observed in granulosa cells of the ovarian follicles (Fig. 2e). *LacZ* activity was found only in a subgroup of cells at later stages of follicle development, suggesting that *FMR1* might be expressed in a regulated manner during follicle maturation. The oocytes did not show significant staining. Heart, liver, skeletal and smooth muscle did not show β -galactosidase activity (summarized in Table 2). Staining was observed in a number of epithelia, most prominently in epithelia lining the organs of the urogenital system, e.g. urethra, vas deferens and epididymis (Fig. 2d), and to a lesser degree in the endothelia of the blood vessels and the epithelia of Bowman's capsule in the kidney. Other epithelia expressing *lacZ* were the dermis, the epithelia of hair follicles, and epithelia in the salivary glands and the trachea.

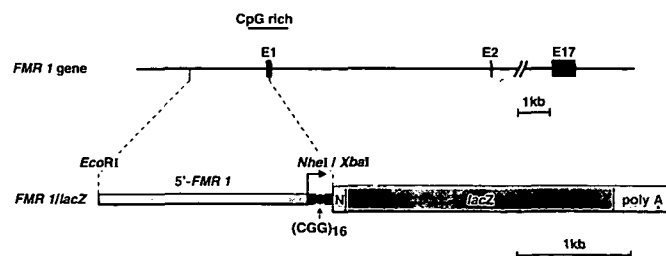


Figure 1. Structure of the 5'-end of the *FMR1* gene and of the *FMR1/lacZ* fusion gene injected into mouse oocytes. The restriction sites used for the construction of the fusion gene are indicated. The narrow part of the construct is the 5'-fragment of the human *FMR1* gene. The CpG island, the approximate transcription start site (arrow), and the CGG-repeat (sphere) are marked, as well as the nuclear localization signal (N) and the polyadenylation signal (polyA) from SV40 adjacent to the *E.coli lacZ* gene.

Transgene activity during embryonic development

Expression of the *FMR1/lacZ* fusion gene was assayed in transgenic embryos from line 22 at midgestation (Figs 3 and 4; summarized in Table 3). In an 11.5 day old embryo, expression of the transgene was more widespread than in adult

tissues although not ubiquitous. A strong specific signal was observed in the notochord, preceding the *lacZ* expression in developing intervertebral discs, which contain cells invaginated from the notochord (Fig. 3c). Expression in cells of the developing nervous system was also specifically regulated:

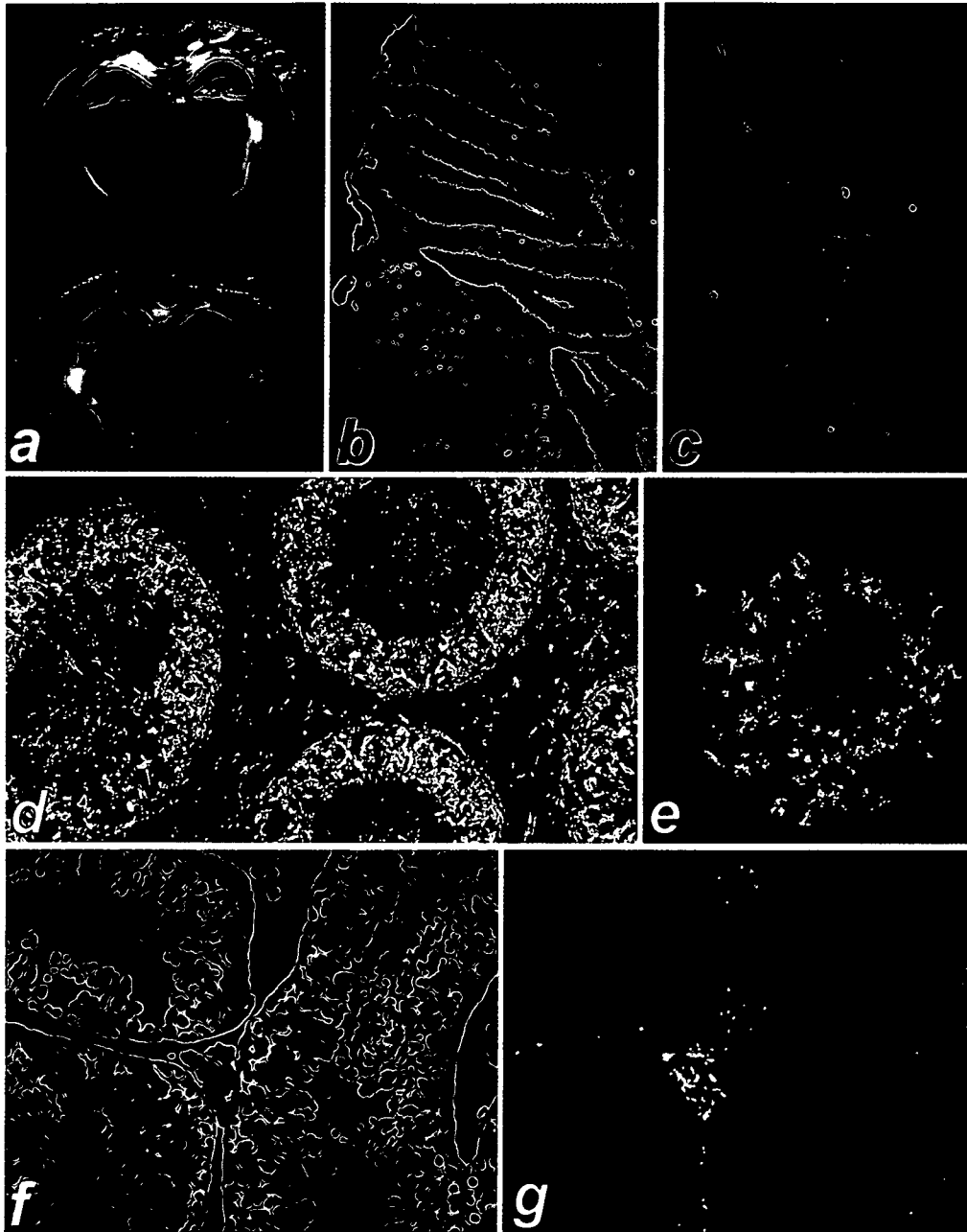


Figure 2. Expression of the *lacZ* reporter gene in tissues of adult transgenic mice. Using Bluo-Gal as chromogenic β -galactosidase substrate, the reaction product can either be visualized as blue stain in normal light (a, b, c, and f), or as a bright stain in polarized light (d, e, and g). (a) The frontal brain section in a 10 week old mouse (line 22) showed intense *lacZ* activity of the hippocampus after staining for two hours. (b) The cerebellum revealed *lacZ* expression in the granular layer, as well as in singular cells in the molecular layer (frozen section, line 41). (c) A frontal section of the bulbous olfactorius showed tissue-specific *lacZ* expression in a cone-shaped layer of neurons (frozen section, line 43). (d) The β -galactosidase staining of the epididymis revealed strong *lacZ* expression in the epithelium. The sperm cells in the lumen of the epididymal tubules show also some expression of the reporter gene (paraffin embedded section after whole-mount staining, line 35). (e) In an ovary, *lacZ* expression was visible in a subgroup of follicle cells, but not in the oocyte. Adjacent follicles in earlier stages of the ovarian cycle did not show *lacZ* activity (paraffin embedded section after whole-mount staining, line 41). (f) The same testis section in bright field (paraffin embedded section after whole-mount staining, line 43). (g) Testis section in polarized light, showing *lacZ* expression in a group of Leydig cells.

Table 1. Expression of the *lacZ* reporter gene in brain tissues of the five transgenic mouse lines

Tissue	Line 22	Line 35	Line 41	Line 42	Line 43
Bulbus olfactorius	++				++
Caudate putamen					++
Cerebellum (granular layer)	++++	+++	+++		++++
Corpus callosum	-	-	-	-	-
Choroid plexus	++	++	++		++
Hippocampus	++++		+++		++++
Neocortex (neurons of the pyramidal layer)	++	++	++		++
Retina (neuronal layer)	++			-	++

The estimation of staining intensities was done qualitatively, using four categories: '+' : single cells visualized with polarized light; '++' : majority of all cells or strong staining of single cells with polarized light, weak staining in bright field; '+++': staining in a tissue in bright field; '++++': strong staining in bright field

Table 2. Expression of *lacZ* reporter gene in different tissues of the five transgenic mouse lines

Tissue	Line 22	Line 35	Line 41	Line 42	Line 43
Copy number of the transgene (est.)	1-5	1-5	50-100	50-100	1-5
Adrenal gland	+	+	+		
Bone osteoclasts	+	+	-	-	
Epidermis	++				++
Epididymis epithelium	++++	++++	++	++	++++
Hair follicle	++				++
Heart muscle	-	-		-	-
Intestinal epithelium		-	-	-	+
Kidney (Bowman's Capsule)	+	+	+	+	+
Liver parenchyma cells	-	±	-	-	±
Lung		++			
Oesophagus	-		-	-	-
Oocytes	-			-	
Ovary (granulosa cells of follicles)	+		+	+	
Salivary gland	++		++	++	++
Skeletal muscle	-	-	-	-	-
Smooth muscle	-	-	-	-	-
Spleen	++	++			++
Testis: Spermatogonia ++	++		+	++	
Testis: Sperm	+	+		+	+
Testis: Leydig cells	++	++		+	++
Testis: Sertoli cells	-	-	-	-	-
Trachea epithelium	++		++	++	
Urethra epithelium	++++		++	++	+++
Uterus			-	-	

The estimation of staining intensities was as described in the legend for Table 1

particularly strong expression was detected in the telencephalon, whereas other structures, e.g. the neural tube and the dorsal root ganglia, showed weak or no *lacZ* activity (Fig. 3a-c). A strong *lacZ* signal was also detected in different developing organs, e.g., the pericardium and the tongue (Fig. 3d, e), and in particular in the genital ridge (Fig. 4).

DISCUSSION

After introduction of the *FMR1/lacZ* fusion gene into the mouse germline, similar expression patterns of the reporter gene β -galactosidase were found in a number of tissues of transgenic offspring from five independent founders. We observed that the tissue-specificity and the level of transgene expression were regulated similarly to the endogenous gene: in the brain, the neurons of the hippocampus and the cerebellum showed the highest β -galactosidase activity. This pattern resembles transcription of the endogenous gene found in the

brain of human fetuses (21) and adult mice (22). Since the hippocampus is involved in establishing memory, it is tempting to speculate that the absence of *FMR1* protein in hippocampal neurons might cause some of the symptoms of mental retardation observed in fragile X syndrome patients. In addition, strong expression of the transgene occurs in the cerebellum. Significantly, the only neuroanatomical alteration found in affected individuals so far is a reduction in size of the posterior vermis of the cerebellum (2). Different methods were used for tissue preparation and staining, resulting in a broad range of detectable staining intensities, and most tissues were analyzed with several techniques. In particular, polarized light darkfield imaging increased sensitivity in paraffin sections approximately 10-fold compared to bright field, and paraffin embedding and frozen sections required sections of different thickness also affecting staining intensities.

During fetal development, transcription of the human and mouse gene has been reported to be more widespread than in

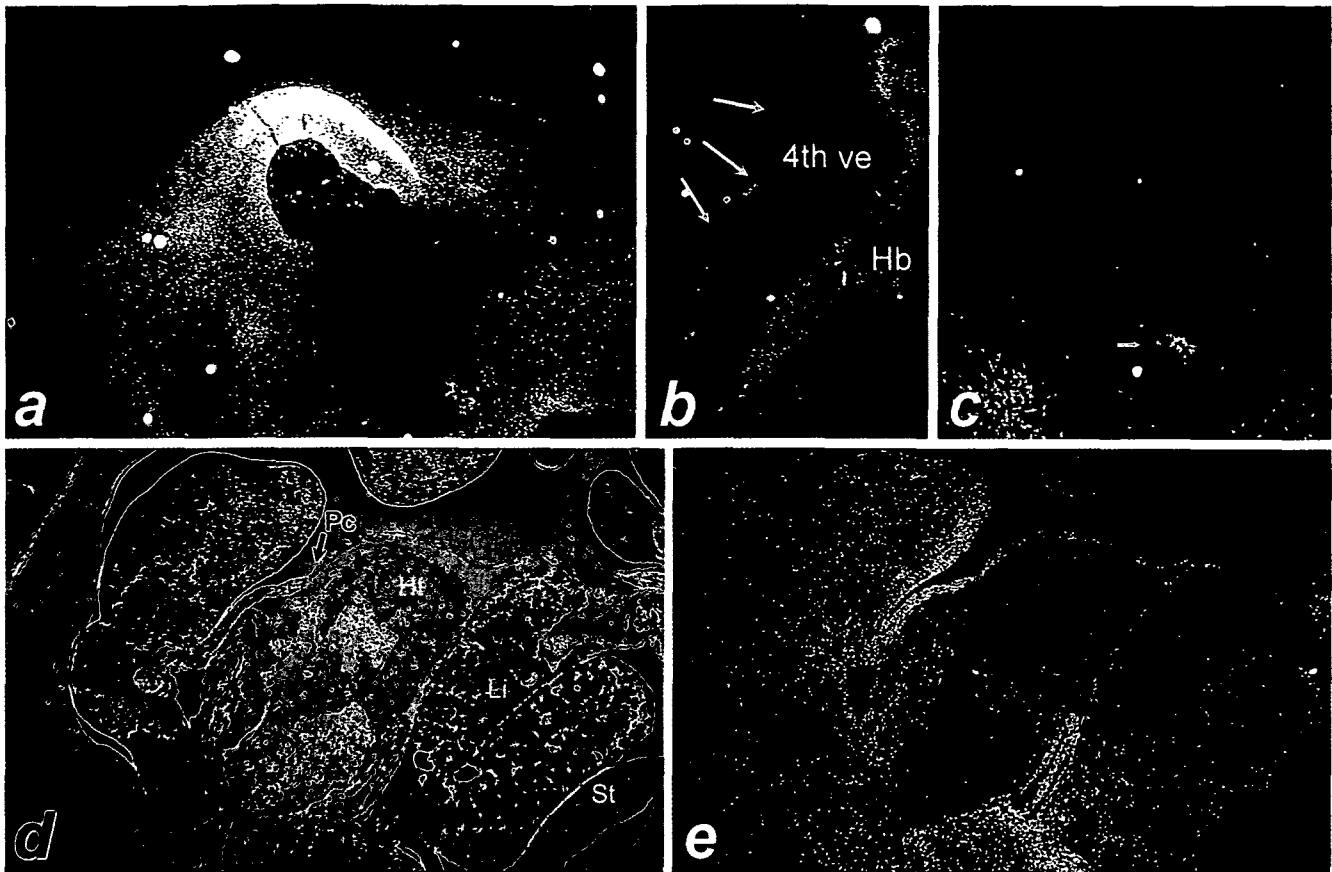


Figure 3. Expression of *FMR1/lacZ* in embryonic tissues at embryonic day 11.5 (mouse line 22). (a) Longitudinal section through the head of a whole mount stained, paraffin-embedded embryo, photographed in polarized light. Intense staining of the telencephalon is visible. (b) The same section as in (a) shows *lacZ* expression in the hindbrain (Hb) adjacent to the fourth ventricle (4th ve). The arrows point to β -galactosidase activity in the roof of the 4th ventricle. (c) Neural tube, notochord, and spinal ganglia in polarized light (transverse section). No staining is visible in the cells of the neural tube, the neural crest and the spinal ganglia. *LacZ* expression is specifically restricted to the notochord (arrow). Some staining is also found in the mesoderm of the somites (lower left). (d) A longitudinal section of the thorax, showing the developing tongue, the pericardium (Pc), the atrioventricular junction of the heart (Ht), the liver (Li), and the stomach (St). (e) The same section as in (d) in polarized light shows particularly strong staining in the tongue, the pericardium, and mesenchymal tissues below the heart and liver.

adult tissues, but the transcription pattern has been controversial (22,26). In an 11.5 day old embryo, we observed many of the features described for endogenous *Fmr1* transcription, most notably the enhanced expression at the genital ridge and in the telencephalon revealing tissue specific expression of the transgene. In addition, *lacZ* gene product was found in developing organs such as the pericardium, which do not express the gene in adults.

In testis, expression of the transgene was found in different stages of spermatogenesis and in the interstitial Leydig cells. Expression in spermatogonia was enhanced, but in whole-mount stained testes the *lacZ* activity seemed to be weak in those tubuli not directly exposed to the staining solution. This observation might be explained by a reduced diffusion rate of the substrate into the tubuli. No transgene expression was found in Sertoli cells, and no differences in *lacZ* activity have yet been observed between different seminiferous tubuli. We detected a weak *lacZ* signal in mature sperm of transgenic mice. However, this signal may have resulted from remnants of β -galactosidase expressed during earlier stages of spermatogenesis rather than from transgene expression in mature sperm.

The testicular expression pattern of endogenous *Fmr1* showed enhanced gene transcription in the periphery of seminiferous tubules, which was either located to Sertoli cells (22) or to spermatogonia (26). Different tubuli showed different levels of *Fmr1* transcription (22,26). The *FMR1* protein was identified in human spermatogonia, but not in sperm, by immunostaining (8). Transcription or expression of the endogenous gene in interstitial cells was not observed (8,22,26). *Fmr1* transcription was found in ovarian follicle cells in one study (22), but this result was not confirmed in another publication (26). Our transgenic animals showed *lacZ* activity in ovarian follicle cells. In conclusion, the expression of the *FMR1/lacZ* transgene is observed in several cell types in genital organs, where the transcription pattern of the endogenous gene is either a matter of debate, or has not been observed yet. Enhanced *lacZ* expression was found in the three cell types synthesizing androgens, namely Leydig cells, ovarian follicle cells and adrenal cortex cells (27). It is tempting to speculate that the *FMR1* protein has a function in steroid synthesis, and that its absence might affect the function of the peritubular Leydig cells. The macroorchidism of fragile X syndrome patients

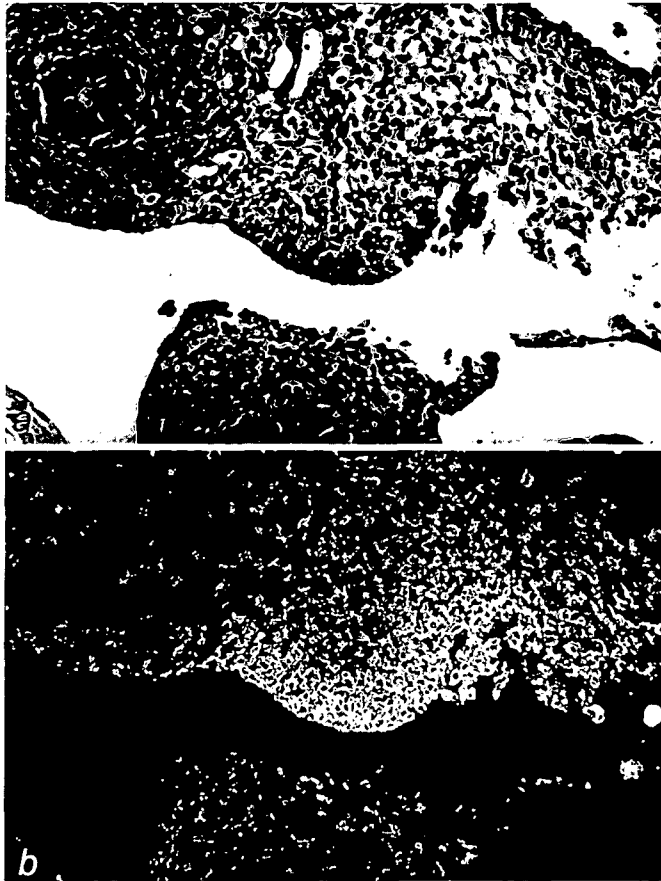


Figure 4. Expression of the *FMR1/lacZ* fusion gene in the genital ridge of the murine embryo at embryonic day 11.5 (line 22). (a) Longitudinal section in normal light. (b) The same section in polarized light showing strong β -galactosidase activity in the cells of the genital ridge.

Table 3. Expression pattern of the reporter gene in embryonic tissue of mouse line 22

Tissue	Line 22
Telencephalon	++++
Diencephalon	++
Midbrain	++
Pons ++	
Medulla	+
Thoracic neural tube	-
Notochord	++
Genital ridge	+++
Heart	++
Liver	+
Otic vesicle	++
Spinal ganglia	-

seems to result from an increase in peritubular tissue (28,29), although in the enlarged testis of the *Fmr1* null mutant mice no apparent changes in cell number or size have been detected (19). It remains to be elucidated, whether the *FMR1* protein plays a physiological role in the Leydig cells as suggested by the transgene expression. Another discrepancy between the

expression patterns of the transgene and the endogenous *Fmr1* was a lack of *lacZ* signal in the oesophagus epithelium of adult transgenic mice (11). In summary, these differences suggest that some regulatory sequences might be missing from the 2.8 kb 5'-sequence of *FMR1* used in our construct. We also observed that the expression level of the transgene is not directly related to its copy number, suggesting that our transgene does not contain a locus control region (30). Indeed, the mouse lines carrying the highest copy number of the transgene showed relative low expression level. This phenomenon might result from inactivation of long strands of tandem repeats of transgenes (31). Additional regulatory elements might be located in the 9.9 kb first intron of *FMR1*, which contains the 3' part of the CpG island excluded from the transgene. For example, enhancer sequences have been identified in the first and second introns of the gene for the intermediate filament nestin, directing expression to differentiating muscle cells and neural precursors, respectively (32). Independent regulatory elements acting on a CpG-rich promoter have also been identified in the *Thyl* gene. Expression of fusion genes in transgenic mice was more tissue-specific, when sequences located downstream from the transcriptional initiation site were included (33). In contrast, the CpG-rich promoter of the rat calmodulin gene II was active in neurons and testis of transgenic mice generated with a construct harboring 294 bp of 5'-upstream sequence plus 68 bp of 5'-untranslated leader sequence of calmodulin (34,35). Taken together, the similarity observed between the expression pattern of the transgene and the endogenous gene in mouse and in human strongly suggests that not all, but most of the *cis*-regulatory sequences of *FMR1* are present within our construct. This notion is further supported by the similar expression patterns between the independent transgenic mouse lines.

The mechanism(s) which regulate *FMR1* transcription are not yet understood. A patient with the clinical phenotype of the fragile X syndrome carrying a 1.6 kb deletion upstream and adjacent to the CGG repeat of *FMR1* confirms that these sequences are essential for the expression of the gene (6). It is also known that CpG-rich promoters are present in active chromatin (36), and can be activated by constitutively bound transcription factors in response to multiple stimuli in a cell-specific manner in various cell types including neurons (37). In the case of *FMR1*, no factors binding to the promoter region have been described so far. However, several factors were found, which bind to the CGG-repeat of *FMR1*, but the significance of these protein-DNA interactions for *FMR1* gene expression remains to be elucidated (38). In preliminary experiments, we found binding sites for putative transcription factors in the *FMR1* promoter region by gel mobility shift assays (M.H., unpublished observations). Mutagenesis of these binding sites will help to identify functional regulatory sequences in *FMR1*. Further characterization of elements controlling *FMR1* gene expression using transgenic model systems like the one described here may contribute to the understanding of the molecular mechanisms of the fragile X syndrome of mental retardation.

MATERIALS AND METHODS

Cloning of the fusion gene

pNL(Not2X) is a derivative of the pNL plasmid that expresses the *E.coli lacZ* gene with a nuclear localization signal of the SV40 large T antigen at the

N-terminus of the β -galactosidase and an SV40 polyadenylation signal downstream of the open reading frame (24). The 5.1 kb *EcoRI*-fragment from the genomic *FMR1* clone pE5.1 containing 16 CGG-triplets (39; a gift of Dr D. Nelson, Houston, USA) was blunt ended with Klenow DNA polymerase and recut with *NheI*, 18 bp upstream of the ATG codon of the *FMR1* open reading frame (12). Before ligation with the *EcoRI*-*NheI*-fragment of *FMR1*, pNL(Not2X) was cut with *SphI*, blunt ended with Klenow DNA polymerase, and digested with *XbaI* in the pNL(Not2X) polylinker.

Generation and identification of transgenic mice

The fusion gene was excised by *NotI* restriction digestion, separated from vector-derived sequences by agarose gel electrophoresis and purified by DNA extraction with Prep-A-Gene (Biorad) and filtration through a Millipore filter (pore size 0.45 μ m). The isolated fragment was injected into the pronuclei of fertilized oocytes of B6C3F1 heterozygote donor mice, which were transferred into foster mothers. Transgenic animals were identified by Southern blotting of *HindIII*-digested tail DNA using the *E.coli lacZ* gene as a probe. Transgenic founder mice were allowed to mate overnight with nontransgenic animals. Gestational days were counted from the presence of a vaginal plug in the morning (embryonic day 0.5).

Analysis of *lacZ* expression

Animals were anesthetized by inhalation of Methoxyflurane (Metofane, Arovet AG), and perfused through the left ventricle with PBS containing 5 IU/ml heparin, followed by 4% PBS-buffered paraformaldehyde (23). Perfusion-fixed tissues were directly stained for 12–48 h at 37°C in PBS with 5 mM $K_3Fe(III)(CN)_6$, 5 mM $K_4Fe(II)(CN)_6$, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium-deoxycholate, and 1 mg/ml Bluo-Gal (Life Technologies; 100 mg/ml dissolved in dimethylformamide). The stained tissues were postfixed in 4% PBS-buffered paraformaldehyde, embedded in paraffin, sectioned in 5 μ m sections and counterstained with hematoxylin. Frozen sections were also made from tissues of perfused animals, which were frozen in isopentane (Fluka). Sections of 15–20 μ m were mounted on glass slides, fixed for 5 min in 4% PBS-buffered paraformaldehyde, washed for 5 min in PBS, and stained under identical conditions as whole tissues (23). After staining the sections were washed 10 min in PBS, 10 min in water, covered with Crystal Mount (Biomed) and analyzed under bright field and polarized light. The embryos were fixed in PBS containing 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, and 0.02% NP-40, stained, sectioned and analyzed as described above.

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