Biochemical and Biophysical Research Communications **290,** 53–61 (2002)
doi:10.1006/bbrc.2001.6162, available online at http://www.idealibrary.com on IDEAL®

Effect of the Rabbit αs1-Casein Gene Distal Enhancer on the Expression of a Reporter Gene *in Vitro* and *in Vivo*

Thais Pantano,*[,1] Geneviève Jolivet,† Sonia Prince,† Celeste Menck-Le Bourhis,* Caroline Maeder,† Céline Viglietta,† Sylvie Rival,† and Louis-Marie Houdebine†

**Bioprotein Technologies, 63 Boulevard Massena, 75013, Paris, France; and †Unité de Biologie du Développement et Biotechnologies, Institut National de la Recherche Agronomique (INRA), 78352 Jouy-en-Josas, France*

Received November 26, 2001

Several gene constructs containing the firefly luciferase gene and the herpes simplex virus thymidine kinase gene promoter (TK) were used to evaluate the transcriptional activity of the distal enhancer (−3442, −3285) of the rabbit αs1-casein gene. Six copies of the enhancer (6i) were added upstream of the TK–luciferase construct in the presence or absence of the chicken β-globin 5′HS4 insulator. The activity of the constructs was tested by transient transfection in CHO cells and in rabbit primary mammary cell cultured on plastic or on floating collagen. Constructs were also tested in stably transfected mouse mammary HC11 cells. In all cell types the multimerized αs1-casein enhancer strongly stimulated luciferase gene expression in the presence of lactogenic hormones. It was also sensitive to the extracellular matrix in rabbit primary mammary cells. The constructs were used to generate transgenic mice. The 6i TK transgenic animals expressed the luciferase gene at very low levels irrespectively of the physiological state. No preferential expression in the mammary gland was observed. Addition of 5′HS4 insulator to the 6i TK construct did not prevent silencing in most of the transgenic lines. However, two lines expressed high luciferase levels specifically in the mammary gland. Our data suggest that 6i may confer, when insulated properly, a higher and mammary-specific expression to the TK promoter.

Key Words: **enhancer; multimerization; transgene; rabbit αs1-casein; transfection; rabbit primary mammary cells; insulator.**

Increasing gene transcription using a multimerized enhancer is a strategy that was previously proposed (1, 2). The presence of multiple binding sites enhances the concentration of transcription factors in the promoter region which would increase the probability of the transcription event (1, 3). Such a strategy is especially attractive when the enhancer is regulated hormonally, developmentally or is tissue specific, since transcription can occur in precisely defined conditions. Hoag and collaborators (1) showed that the activity of the human Factor IX proximal promoter in transfected cells was increased 20-fold by adding a multimerized enhancer from the homologous promoter. This multimer exhibited an enhancer activity in liver cells but not in mammary and muscle cell lines, exhibiting a high degree of tissue specificity.

Several milk protein genes contain a distal enhancer whose transcriptional activity is regulated by lactogenic hormones and the extracellular matrix (see reviews (4, 5). These distal enhancers were described in the bovine (6) and human β-casein gene (7), in the rat whey acidic protein (WAP) gene (8) and in the rabbit αs1-casein gene (9). They contain binding sites for several transcription factors such as the signal transducer and activator of transcription 5 (STAT5), the CCAAT enhancer binding protein (C/EBP), the nuclear factor (NF-I), the glucocorticoid receptor (GR), the E26-specific sequence factor (Ets) and the octamer binding transcription factor (Oct-1) (4, 5). The importance of these binding sites in distal enhancers was highlighted by the inactivation of transcription factors or by the mutation of their binding sites. Maximum stimulation by the distal enhancer was achieved only when all the binding sites were functional with their transcription factors present and interacting (10–12).

The distal enhancer of the rabbit αs1-casein gene located between nucleotides −3442 and −3285 upstream of the transcription start point is a good candidate for testing whether its addition in a multimerized form can increase the transcriptional efficiency and the mammary specificity of a reporter gene. A previous study showed that this enhancer has the ability to increase the prolactin-dependent transcriptional activity of the rabbit αs1-casein minimal promoter in trans-

[1] To whom correspondence should be addressed. Fax: + 33 (0)1 34 65 25 21. E-mail: pantano@jouy.inra.fr.

0006-291X/02 $35.00

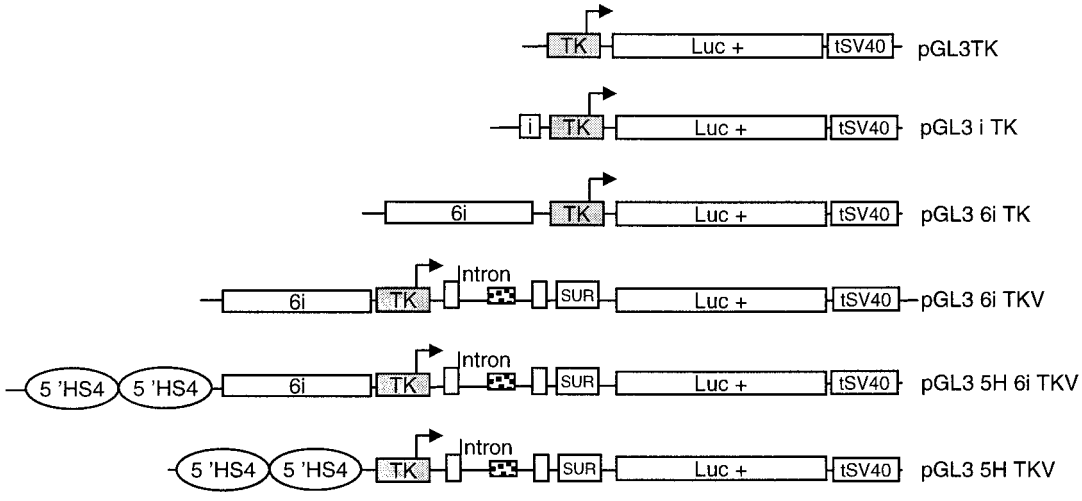

FIG. 1. Structure of the pGL3TK-based plasmids. TK: promoter region from the TK promoter (nt −109 to nt +55). Luc+: luciferase cDNA. tSV40: SV40 late terminator. i and 6i: one or six copies of the rabbit αs1-casein distal enhancer (nt −3442 to −3285). Intron: VP1 intron associated to the MMTV promoter fragment (dotted box) and SUR. 5′HS4: 5′HS4 insulator element from the chicken β-globin gene.

fected CHO cells (13). This enhancer presents four distinct nuclear protein-binding sites (13), of which a STAT5 and a C/EBP binding site were characterized (11, 14). The presence of all binding sites is necessary for maximal transcriptional activity of this enhancer (11). In the present study, we evaluated the effect of the multimerized αs1-casein distal enhancer using transfected cells and transgenic mice. For this purpose, a weak and non-hormonally inducible promoter, the Herpes simplex virus thymidine kinase (TK) gene promoter, was used.

MATERIALS AND METHODS

Plasmid constructs. The rabbit αs1-casein distal enhancer named "i" (for "insert") and encompassing the −3442, −3285 region was obtained by PCR as previously described (11) and cloned in the *Bam*HI site of pBluescript after blunting (pBSi plasmid). Cloning the enhancer reconstituted a *Bgl*II site at its 5′P- and 3′OH ends. A multimer of i was obtained as follows: i was excised from pBSi by *Bgl*II digestion and cloned into the *Bam*HI site of pBluescript. A first plasmid containing three copies of i inserted in the same direction was isolated giving the plasmid pBS 3i. The three copies were then released by *Sma*I and *Spe*I digestion of pBS 3i, filled in, and cloned into the *Sma*I site of pBS 3i. Sequencing assessed the direction and integrity of the multimer. The pBS6i plasmid, which encompassed 6 multimerized copies of i in the same direction, was thus obtained. A series of luciferase-expressing plasmids was prepared from the pGL3 basic vector (Promega) (Fig. 1). The TK promoter sequence, located between nucleotides (−109, +55) respectively from the transcription starting point, was excised from pTKM (kindly provided by Dr. Herbomel) by digestion with *Xba*I and *Bgl*II. Its extremities were filled in and the promoter cloned into the pGL3 basic vector digested by *Bgl*II and *Nhe*I with the extremities also filled in. The resulting plasmid was named pGL3TK. The distal enhancer i excised from pBSi by BglII digestion was cloned upstream of the TK promoter into the Asp718 blunt ended site of pGL3TK generating the pGL3 i TK plasmid. The six-multimerized fragment containing 6i was obtained from pBS6i by *Eco*RV and *Spe*I digestion and cloned into the blunt ended Asp718 site of pGL3TK to generate the pGL3 6i TK plasmid. The VP1 intron from the SV40 late gene containing in its *Eco*RV site the MMTV (mouse mammary tumor virus) promoter devoid of its transcription start site (15) was added to the vectors together with the SUR (SV40 5′UTR region). The SUR is an association of the 5′UTR from SV40 early genes and the U3R region from the HTLV-I virus. It stimulates the transcription and transduction (16). SUR was mutated to eliminate a splicing donor site which considerably reduces luciferase gene expression (17 and unpublished data). The fragment containing the VP1 intron, the MMTV promoter fragment and the mutated SUR was amplified by PCR (high fidelity PCR, Roche) from the p42 plasmid (15). The amplified DNA fragment was subcloned into the pGEMT easy plasmid (Promega), recovered by digestion with *Not*I, blunt ended and cloned into the *Hin*dIII blunt site of pGL3 TK and pGL3 6i TK, resulting in the pGL3 TKV and pGL3 6i TKV plasmids. Two copies of the 5′HS4 insulator in tandem from the chicken β-globin locus (kindly provided by Dr. G. Felsenfeld) were cloned into the *Sma*I site of the pGL3 6i TKV vector and into the Asp718 site of the pGL3TKV vector. The resulting plasmids were respectively pGL3 5H 6i TKV and pGL3TK 5H TKV.

Cell culture, transfection, and hormonal induction. CHO-K1 (immortalized Chinese hamster ovary cell line) cells were grown as previously described (18). The day before transfection, cells were seeded in 35-mm-diameter culture dishes. Transfection was performed using 1 μg of a plasmid encoding for the rabbit mammary prolactin receptor (19), 1 μg of the pCH110 plasmid (Pharmacia) and 0.5 μg of luciferase-expressing plasmids, plus 3.6 equivalents of Exgen500 (Euromedex) in a DMEM/F12 (Eurobio) serum-free medium according to the manufacturer's recommendations. Four hours later, the transfection medium was removed and the cells were cultured in a modified GC3 serum-free medium supplemented with bovine insulin (InVitrogen) (1 μg/ml), cortisol (Sigma) (10 ng/ml) with ovine prolactin (+PRL), 1 μg/ml, or without (−PRL) (18). Each luciferase-expressing plasmid was transfected independently in six separate dishes (three +PRL and three −PRL). Forty hours later, cells were assayed for luciferase activity as described below. Rabbit primary mammary cells were prepared from 14- to 16-day pregnant rabbit mammary gland as previously described (14). Isolated mammary cells were seeded on 35-mm dishes directly on plastic or on rat tail collagen. The following day, luciferase-expressing plasmids were transfected using Lipofectin (InVitrogen) according to the manufacturer's recommendations. Cells were incubated overnight in the presence of plasmid–Lipofectin mixture in OPTIMEM medium (In-

Vitrogen): 4 μg of luciferase-expressing plasmid and 1 μg of the pCMV-βgal, a plasmid expressing the β-galactosidase gene under the control of the CMV promoter (Pharmacia). The following morning, after discarding the overnight medium, a fresh differentiation medium containing DMEM/F12 supplemented with 2% UltroSer (a steroid-free substitute of serum, Biosepra), 1 μg/ml insulin, 100 ng/ml cortisol, with or without PRL (1 μg/ml), was added. Collagen was detached from the dishes and allowed to float. Luciferase and β-galactosidase assays were performed 48 h later as described below. Whenever possible, transfection efficiency was assessed by the cotransfection of a reporter plasmid encompassing the β-galactosidase gene: pCH110 for CHO cells and pCMV-βgal for primary mammary cells. The pCMV-βgal plasmid was chosen for primary mammary cells instead of pCH110 because CMV promoter is more efficient than the SV40 promoter of pCH110. However, the presence of pCH110 and pCMV-βgal dramatically disturbed the expression of plasmids containing the SUR element, regardless of the hormonal treatment (data not shown). Consequently, pCH110 and pCMV-βgal were not cotransfected with plasmids containing the SUR element. In this case, multiple transfections were carried out and luciferase activity (RLU) for each plasmid was referred to the value obtained with a reference plasmid (p GL3TK) as described in the legends of figures. HC11 cells, subcloned from the spontaneously immortalized mouse mammary cell line COMMA1D (20), were grown in RPMI medium (RPMI 1640 (Eurobio)) supplemented with 10% of heat inactivated fetal bovine serum (FBS, Sigma), glutamine (300 μg/ml), insulin (5 μg/ml) and Epidermal Growth Factor (InVitrogen) (10 ng/ml) as previously described (20). Three independent transfections were performed for each luciferase-expressing plasmid. The luciferase–plasmids (10 μg) were cotransfected with the pRSVneo plasmid (1 μg). Transfection and selection of the HC11 stably transfected cells by Geneticin (G418, InVitrogen), 150 μg/ml were performed as previously described (15). The resulting pools of cells were grown to confluence. After 5 days of confluence, cells were cultured in a serum-free medium: RPMI (Eurobio) without SVF and EGF and supplemented with transferrin and nonessential amino acids. Twenty-four hours later, cells were hormonally stimulated with one of the hormonal combinations: insulin alone (I) 5 μg/ml, I plus cortisol (F) 10^{-6} M; I + PRL 1 μg/ml or (I + F + P) in a serum-free RPMI medium during 48 h. The luciferase values were normalized to the protein content of each extract using the Bradford method (Bio-Rad).

Luciferase and β-galactosidase assays. Cells grown directly on plastic were washed twice with cold PBS, harvested and lysed in a glycylglycine buffer (21) by pottering. Cells grown on collagen were collected after complete collagenase digestion of collagen (1 mg collagenase type IV, Sigma) in 1 ml of PBS, pelleted and lysed in a glycylglycine buffer by pottering. Luciferase activity was assayed as described (21, 22), using a Lumat LB 9501 photometer (Berthold Instruments). β-Galactosidase and luciferase activities were evaluated using aliquots from the same cellular extracts. βgal activity was measured as previously described (11).

Transgenic mice. Three constructs were used for generating transgenic mice: 6i TK, 5′ TKV and 5′ 6iTKV. Linearization of the plasmid was obtained after digestion. The 6i TK insert was obtained after digestion of pGL3 6i TK with *Sma*I and *Bam*HI. The 5H TK V and 5H 6i TKV inserts were obtained after digestion of the pGL3 5H TK V and pGL3 5H 6i TK V with *Bam*HI. Inserts were purified using Glassmilk (Bio 101). The recovered DNA was diluted in a 5 mM Tris–HCl, 0.1 mM EDTA, pH 7.4, solution. Transgenic mice were generated by standard DNA microinjection procedures (23). Embryos from C57-Bl/CBA F1 mice were microinjected with a solution containing 2 ng/μl of luciferase-expressing plasmids.

Identification of transgenic animals. Tail genomic DNA was extracted as described before (24). Founders and progeny were screened by PCR using a set of primers located inside the luciferase gene (22).

Luciferase assay in tissue samples. Tissue from adult transgenic F1 males, nonlactating females and lactating females from day 10 to day 12 of lactation was analyzed for luciferase gene expression. Besides the mammary gland, ovary, kidney, liver and brain were collected. Fragments of fresh organs were ground with a potter in 50–200 μl lysis buffer solution (21). Five microliters of the organ extract was assayed for luciferase activity as described above. At least two lactating F1 females were analyzed for each transgenic line. An internal control was included in all the luciferase assays using purified luciferase (Sigma). The luciferase values were normalized to the protein content of each extract using the Bradford method (Bio-Rad).

Statistical analysis. Whenever possible statistical analysis was performed using the Student paired *t* test.

RESULTS

Multimerized i (6i) Is a Strong PRL-Dependent Enhancer in CHO, HC11, and Rabbit Primary Mammary Cells

The effects of the multimerized enhancer 6i were evaluated in three types of transfected cells: CHO cells transfected with a plasmid encoding for the prolactin receptor, rabbit primary mammary cells and HC11 cells. These cellular types were chosen because they are expected to provide different and complementary information about the 6i effect. Addition of one copy of the i element did not stimulate the TK promoter upon prolactin addition in any of the cell types (Figs. 2A–2C). In contrast, the presence of 6i did increase luciferase levels after prolactin stimulation. The induction was 9.7-fold in CHO cells and 6.8-fold in primary mammary cells. In primary mammary cells, the 6i effect was detected only when cells were cultured on collagen, indicating that both PRL and the extracellular matrix regulate pGL3 6i TK expression. In stably transfected HC11 cells, addition of 6i resulted in a 9.1-fold induction by cortisol + PRL. This value was statistically different to that obtained with pGL3 TK (1.2-fold) and pGL3 i TK (2.4-fold) with a 9.2 and 8% statistic significance level, respectively. It is worth to mention that the maximum expression level of the different luciferase-expressing plasmids under prolactin stimulation was as high as the expression levels obtained with the RSV luc plasmid (data not shown). Taken together, these results show that the 6i multimer can stimulate the TK promoter and render the constructs sensitive to prolactin and the extracellular matrix in transfected cells.

When Associated to the TK pRomoter, 6i Enhancer Does Not Enhance Gene Expression in Vivo

The strong prolactin stimulation obtained in transfected cells after addition of 6i upstream of the TK promoter led us to ask whether the same effect would be observed *in vivo,* in transgenic animals. Five transgenic lines were obtained with the 6i TK construct. Luciferase mRNA was not detectable by Northern blot

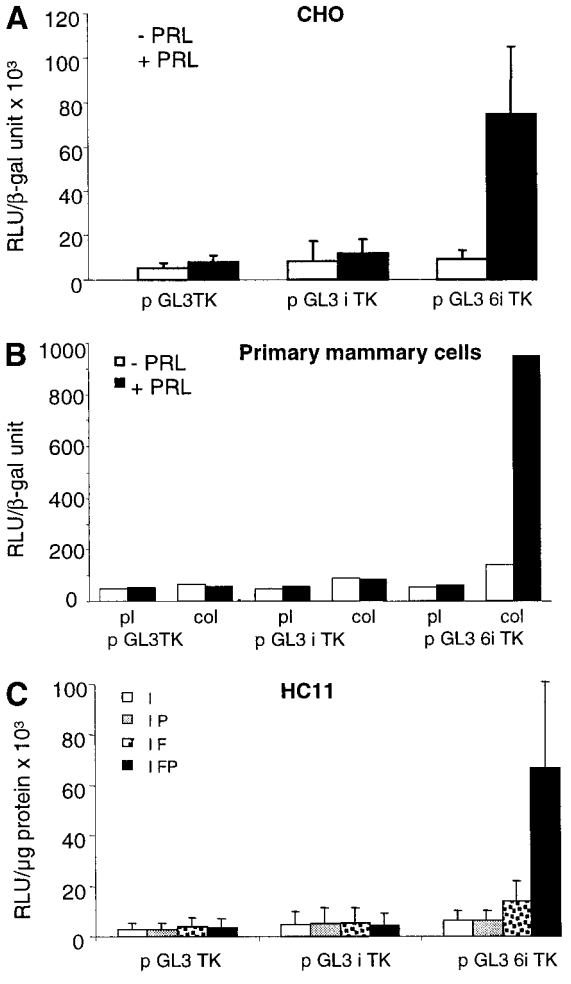

FIG. 2. Effect of 6i enhancer in CHO, HC11 and rabbit primary mammary cells. (A) CHO cells were transiently transfected as described under Materials and Methods. Luciferase activity was normalized using β-galactosidase activity. Cells were treated with insulin and cortisol (−PRL) or insulin, cortisol, and prolactin (+PRL) for 48 h. Values are shown as the mean ± SEM of at least 9 independent transfections. (B) Transfection of rabbit primary mammary cells. Within each experiment, luciferase-expressing plasmids were transfected on twelve 35-mm dishes (six with cells cultured on plastic and six on collagen). Under each culture condition (plastic (pl) or collagen (col)), three dishes were treated with insulin and cortisol (−PRL) and three dishes with insulin, cortisol and PRL (+PRL), for 48 h. Luciferase activity was normalized using β-galactosidase activity. Here a representative profile of the mean values obtained within one experiment is given. This profile was obtained with six independent experiments. (C) HC11 cells were stably transfected with luciferase-expressing plasmids as described under Materials and Methods. The pools of stable transfectants were treated hormonally: insulin (I), insulin and prolactin (IP), insulin and cortisol (IF), and insulin, cortisol, and prolactin (IFP). Luciferase activity was corrected to the amount of proteins in each cellular extract. Results are the mean ± SEM of three independent transfections.

in tissues tested from the five transgenic lines (data not shown). Luciferase activity was hardly detected in mammary gland of lactating mice. Mean levels ranged from 0.8 to 26.1 relative luciferase units (RLU)/μg protein (Fig. 3A). During lactation transgene expression was also measured in ovary, liver, spleen and brain with average levels ranging between 0.3 to 15.0 RLU/μg protein (Fig. 3B). These data suggest that 6i is able neither to stimulate the TK promoter nor to confer tissue specificity to this promoter in the mammary gland of lactating mice.

Effect of the Association of 6i TK with the 5′HS4 Element, VP1 Intron, MMTV Promoter Fragment, and SUR on Luciferase Gene Expression in Transfected Cells

The poor activity of the 6i TK luciferase construct *in vivo* is inconsistent with the results obtained with transfected cells. One possible explanation is the absence of intron and insulator in the 6i TK construct resulting in the inability of the construct to prevent transgene extinction *in vivo*. These elements may be necessary *in vivo* but not in transfected cells. To verify these hypotheses, the 5′HS4 insulator, the VP1 intron, the MMTV promoter fragment and the SUR transcription stimulator were added to the 6i TK luciferase construct. The 5′HS4 fragment is an element derived from the β-globin chicken locus control region (LCR). Its insulating property has been

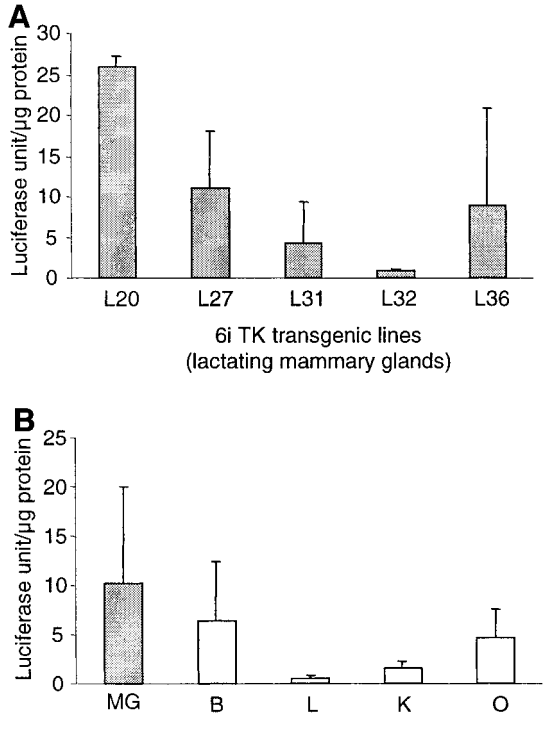

FIG. 3. Luciferase activity in 6i TK transgenic animals. Five lines (L) were generated with the 6i TK construct. (A) Average luciferase levels ± SEM in the mammary gland of lactating females. At least two animals were analyzed per transgenic line. Luciferase activity was corrected to the amount of proteins in each tissue extract. (B) Average luciferase levels ± SEM in different tissues from lactating females. Result for each tissue is the mean of luciferase activity in the five transgenic lines.

described in transfected cells (25, 26), in cells infected *in vivo* by retroviral vectors (27) and in transgenic animals (25, 28–31). In transfected cells, the presence of introns in a construct is often not mandatory (15). The same is generally not true *in vivo,* in transgenic animals. The presence of at least one intron is generally essential for a transgene to be expressed (32). The luciferase gene employed in this study is an intronless gene. The VP1 intron together with the MMTV promoter fragment and SUR are able to enhance the transcription of cDNA *in vivo* (15). We therefore added these three elements and the 5′HS4 region to the pGL3 TK and pGL3 6i TK plasmids resulting in the pGL3 5H 6i TK V, pGL3 6i TKV and pGL3 5H TKV plasmids. The activity of these constructs was firstly tested in transfected cells and later in transgenic animals. In CHO cells, addition of 5′HS4, VP1 intron, MMTV promoter fragment and SUR did not modify the transient expression level under prolactin stimulation (Fig. 4A). In primary rabbit mammary cells (Fig. 4B), the luciferase basal expression level (−PRL) was higher with constructs containing the VP1 intron, MMTV promoter fragment and SUR (pGL3 5H TKV, pGL3 6i TKV, pGL3 5H 6i TKV). This can be due to the presence of numerous GR binding sites within the fragment of the MMTV promoter. Prolactin stimulated luciferase expression levels in all transfected cells cultured on collagen and transfected with plasmids containing the 6i multimer. However, the prolactin fold level (−PRL/+PRL) was lower in constructs containing 5′HS4 (compare pGL3 6i TKV and pGL3 5H 6i TKV transfections) suggesting that 5′HS4 interferes with the 6i enhancer activity. Such an enhancer-blocking activity was observed when erythroid cells were transiently transfected with a construct containing 5′HS4 (33). In the HC11 stably transfected cells (Fig. 4C), addition of the VP1 intron, MMTV promoter fragment and SUR increased the luciferase basal levels about 20-fold: compare pGL3 6i TK (6278 RLU/μg protein) and pGL3 6i TKV (139496 RLU/μg protein). In these cells, the presence of 5′HS4 significantly enhanced luciferase gene expression under glucocorticoid and prolactin stimulation: 139,496 RLU/μg protein without the insulator (pGL3 6i TKV) and 250,723 RLU/μg protein with the insulator (pGL3 5H 6i TKV). The prolactin-fold stimulation (IFP/IF) was the same for the pGL3 6i TK, pGL3 6i TKV and pGL3 5H 6i TKV constructs, indicating that neither the VP1 intron, MMTV promoter fragment and SUR nor 5′HS4 are sensitive to prolactin. Thus it is clear that in HC11 stably transfected cells, the presence of the 5′HS4, VP1 intron, MMTV promoter fragment and SUR increases expression levels without interfering with induction by prolactin.

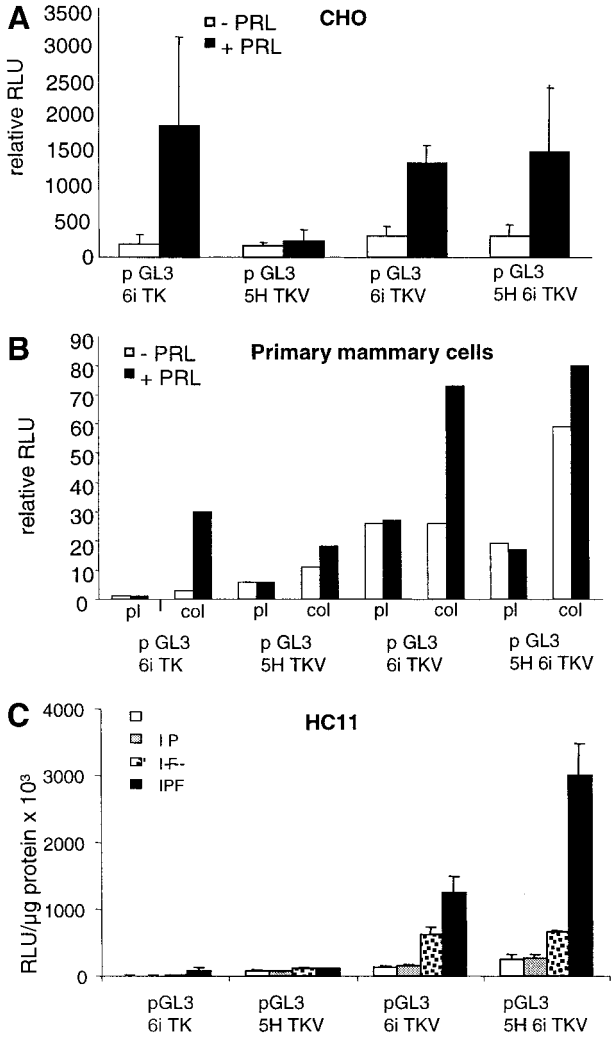

FIG. 4. Influence of the 5′HS4, the VP1 intron, MMTV promoter fragment and SUR on TK promoter activity in transiently and stably transfected cells. (A) CHO cells were transfected as described under Materials and Methods. The relative RLUs are the mean ± SEM of the luciferase levels (RLUs) normalized to the control pGL3 TK (−PRL). At least 9 independent transfections were performed. (B) Primary rabbit mammary cells were cultured on collagen or plastic, transfected and hormonally induced as described in the legend to Fig. 2B. Luciferase activity was normalized to the luciferase activity obtained in cells transfected in the same experiment with the control, pGL3 TK (−PRL). This figure is representative of three experiments. (C) HC11 cells were stably transfected with luciferase-expressing plasmids according to the protocol described under Materials and Methods. The pools of stable transfectants were treated with insulin (I), insulin and prolactin (IP), insulin and cortisol (IF), and insulin, cortisol, and prolactin (IFP). Results are the mean ± SEM of three independent transfections.

Effect of the Association of 6i TK with the 5′HS4 Element, VP1 Intron, MMTV Promoter Fragment, and SUR on Luciferase Gene Expression in Transgenic Animals

The pGL3 5H 6i TKV construct and its control, pGL3 5H TKV, were tested *in vivo.* Nine lines of transgenic

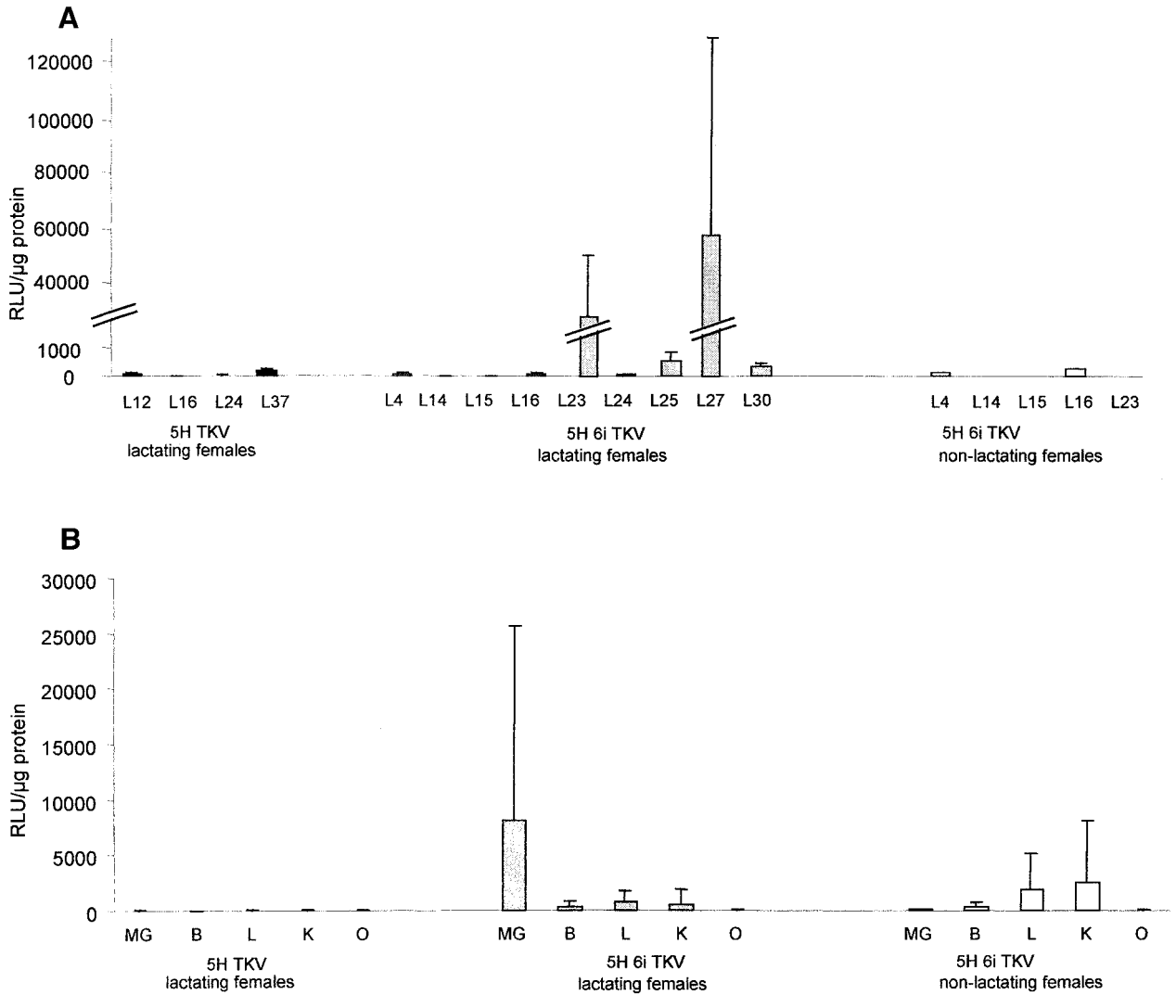

FIG. 5. Luciferase expression levels in 5H 6i TKV and 5H TKV transgenic lines (L). (A) Luciferase mean levels ± SEM in the mammary gland from lactating and nonlactating females. (B) Luciferase average levels ± SEM in different tissues from lactating and nonlactating animals: MG, mammary gland; B, brain; K, kidney; O, ovary; L, liver. For each tissue the presented value is the mean of luciferase activity in the different transgenic lines.

mice were obtained with 5H 6i TKV construct and four with 5H TKV construct. Surprisingly, the luciferase levels varied greatly (0.5 to 51012 RLU/μg protein) (Fig. 5A). Besides the high luciferase activity detected in lactating mammary glands from lines L23 and L27, luciferase levels were hardly detectable in the other seven lines. Consequently, the luciferase mean level in mammary gland from lactating 5H 6i TKV animals did not differ statistically from that observed in the mammary gland from 5H TKV lactating females (0.6 to 143.2 RLU/μg protein), 6i TK lactating females (0.8 and 26.1 RLU/μg protein) and from 5H 6i TKV nonlactating females (1.6 to 178 RLU/μg protein (Figs. 3A and 5A). Ovary, liver, kidney, and brain were also analyzed and no significant difference was observed among lactating and nonlactating animals and among the 5H 6i TKV, 5H TKV, and TK6i lines (Figs. 5B and 3B). The expression of the luciferase gene was not significantly higher in the mammary gland compared to other tissues except for lines L23 and L27. These results suggest that addition of the 5′HS4, VP1intron, MMTV promoter fragment and SUR to the 6iTK construct did not allow 6i to amplify significantly the transgene expression in most lines.

DISCUSSION

The rabbit αs1-casein gene contains in its distal promoter an enhancer that is able to stimulate the activity of the minimal αs1-casein promoter in the presence of prolactin (11, 13). In the present paper, we studied the transcriptional efficiency of the multimer of this en-

hancer associated with a weak and hormonally nonregulated promoter: the TK promoter (34). Our results revealed that the multimerized element of the i element (6i) was, in transfected cells, able to greatly enhance the TK promoter activity and render the constructs sensitive to both prolactin and the extracellular matrix. Unexpectedly, the enhancer activity of 6i was hardly observed *in vivo* in transgenic animals.

The TK promoter was previously assayed in transfected cells associated with various enhancers. Li *et al.* (8) and Winklehner-Jennewein *et al.* (7) stimulated and rendered the constructs sensitive to prolactin using, respectively, the HSS1 region from the rat whey acidic protein gene promoter and the enhancer from the human β-casein gene. Sassi *et al.* (35) using the glucocorticoid responsive elements (GRE) from the rat tyrosine kinase gene, rendered the constructs containing the TK promoter sensitive to glucocorticoids. Sotiropoulos *et al.* (36) associated the TK promoter with a six-copies multimer of STAT5 (LHRE element). The multimer exhibited a prolactin sensitivity *in vitro.* Here we demonstrate that 6i is able to render the TK promoter sensitive to hormones as observed with other enhancers.

One copy of the i element was not able to stimulate the TK promoter in any of the cell types. Sax *et al.* (37) associated one copy of the 18bp mouse α-A crystallin gene enhancer with the pTKCAT plasmid. One copy did not stimulate the TK promoter in several cell types. Increasing the copy number of mouse α A-crystallin to 2, 3 and 4 copies resulted in an increase in CAT activity. Four copies gave the best stimulation. According to Schaffner *et al.* (3), the presence of a larger number of binding sites in the enhancer would facilitate the formation of a functional complex by protein–protein contacts. The results with i TK and 6i TK confirm the observations reported above: increasing the copy number of the i element (6i) made it possible to strongly stimulate the TK promoter. Contrary to our data, James *et al.* (2) did not observe an enhancer effect on the β-lactoglobulin promoter when a multimer of this promoter was inserted upstream of the proximal promoter. The authors suggest that the combination of binding sites in the β-lactoglobulin promoter may not be ideal. Besides, it is worth to consider that in contrast to our work, a larger fragment (376 bp) was employed and longer sequences may contain binding sites for inhibitor factors.

The 6i TK transgenic animals very poorly expressed the luciferase gene. No difference in luciferase activity was observed between nonlactating or lactating females. The luciferase level in the mammary gland was similar to those found in kidney, brain, liver and ovary indicating that no stimulation or specificity occurred at the mammary gland level. This is in marked contrast with the *in vitro* studies where 6i increased the expression of the reporter gene in the presence of prolactin. In transgenic animals, the TK promoter alone is poorly active and unable to avoid the chromatin position effects (34). Sassi *et al.* (35) reported that a glucocorticoid-dependent enhancer stimulated manyfold the TK promoter *in vitro* but not *in vivo.* Cassard-Doulcier *et al.* (38) produced transgenic animals with the TK CAT construct. Only one line expressed CAT weakly in several tissues while all the other lines were silent. Association of the TK CAT construct with the enhancer from the UCP-1 gene did not increase the number of transgenic lines expressing the CAT gene. Nelson *et al.* (39) studied the androgen-specific enhancer of the mouse Slp gene associated with the TK CAT construct in transgenic animals. When a 160-bp fragment of the enhancer was placed upstream of the TK CAT gene reporter, 4 out of 6 transgenic lines expressed the transgene. When a 120-bp region of the enhancer was associated with the TK CAT construct, only 1/11 lines expressed the CAT gene. In these three studies, the authors suggested that the association of the TK gene promoter and enhancers may not contain all the elements necessary for transgene expression in any integrated site.

Our observations together with those previously reported lead us to formulate two hypotheses: (a) 6i does not harbor essential regulatory elements to stimulate the TK promoter *in vivo.* (b) 6i contains regulatory elements but the transgene is strongly extinguished when integrated in the chromatin. To tentatively reduce transgene silencing and to increase transfection efficiency, the 5′HS4 insulator, the VP1 intron, a fragment from the MMTV promoter and the SUR were added to the 6i TK construct. Addition of VP1 intron, MMTV promoter fragment and SUR improved luciferase transcription in primary mammary cells and HC11 cells in agreement with a previous study (15). In HC11 stably transfected cells, addition of 5′HS4 increased the luciferase expression level. This observation is consistent with other studies in which different stably transfected cells and promoters were used (25, 26, 29, 33).

Association of the 5′HS4 insulator, VP1 intron, MMTV promoter fragment and SUR with the 6i TK construct did not increase significantly luciferase activity in most transgenic lines. When the 5′HS4 insulator was associated with the human EF1α ubiquitous promoter (28), the whey acidic protein mammary-specific promoter (29), a liver-specific promoter (31), a tyrosinase minigene (40), and a retrovirus promoter (27), an insulator effect was observed. All mouse lines expressed the transgene and higher gene expression levels were obtained. This insulating activity is believed to be due to 5′HS4 ability to favor histone acetylation in the transgene area preventing the formation of inactive chromatin (41). However, 5′HS4 is not able to completely protect transgenes in all chromatin integration sites (42). Indeed, variegation of *DAF* and

CD59 cDNA expression (28) was observed in transgenic rabbits (unpublished data) despite the presence of 5′HS4. The high variability observed among the 5H 6iTKV transgenic lines led us to suppose that an incomplete insulation by 5′HS4 occurred.

The low luciferase activity in most 5H 6i TKV transgenic lines may be due either to the silencing of the TK promoter even in the presence of 5′HS4 or to the weakness of this promoter whose efficiency cannot be improved even in the presence of additional regulatory elements. The TK promoter is rich in CpG dinucleotides. Different assays in transfected cells and *in vivo*, in *Xenopus laevis* oocytes, showed a considerable decrease in transcription when the CpGs in the TK promoter are methylated (43, 44). According to Montoliu *et al.* (45), the probability of silencing of a transgene can be increased by one of the following events: transgene integration in tandem, absence of insulator sequences or presence of CpG motives causing the transgene to become methylated. The lacZ gene, like the TK promoter, is rich in CpG nucleotides and poor in SpI sites. In transgenic animals, variegation of lacZ expression due to methylation of CpG dinucleotides has often been observed. Cohen-Tannoudji *et al.* (46) reported that the epigenetic phenomena of demethylation followed by the *de novo* methylation that occurs in the embryo during the pre-implantation period can result in preferential methylation of the transgenes rich in CpGs like the *lacZ* gene. The 5′HS4 insulator prevents transgene methylation indirectly (26, 41). When associated with the human α-EF1 promoter and its first intron, 5′HS4 did not completely prevented the variegation effect (unpublished data). The α-EF1 promoter and intron have a high CpG content. This example suggests that the efficiency of 5′HS4 can to some extent be dependent on the CpG content in transgenes. The presence of exogenous enhancers and insulators may not be sufficient to prevent the silencing of a transgene. The data in the present study shows that, 5′HS4 has not an appropriate insulating activity when associated to the TK promoter indicating that, although this insulator presents a broad spectrum of activity, it may not be efficient in all cases.

Accumulating evidence suggests that enhancers act by reducing the silencing and variegation of gene expression (47). This hypothesis implies that enhancers may contribute in some way to the insulating action. Our study shows that the rabbit αs1-casein enhancer, even in a multimerized form and associated with the 5′HS4 insulator, was unable to isolate the transgenes containing the TK promoter. The lactogenic-hormone dependent enhancer activity of this multimer is clear in transfected cells. In transgenic animals this enhancer activity was observed only in the presence of the 5′HS4 insulator and in the mammary gland of only two lactating transgenic lines. More data is necessary to better conclude about the multimer enhancer activity *in vivo*. Association of this multimer to a promoter less sensitive to extinction effects would help to solve this issue.

ACKNOWLEDGMENTS

We thank Dr. D. Vaiman for helping in the statistical analysis, Dr. J. Attal and Dr. D. Thepot for their helpful discussion, and all the animal house staff, for animal care. This work was cofinanced by Bioprotein Technologies and INRA (Institut National de la Recherche Agronomique).

REFERENCES

1. Hoag, H., Gore, J., Barry, D., and Mueller, C. (1999) *Gene Ther.* **6,** 1584–1589.
2. James, R. M., Neil, C., Webster, J., Roos, S., Clark, A. J., and Whitelaw, C. B. (2000) *Biochem. Biophys. Res. Commun.* **272,** 284–289.
3. Schaffner, G., Schirm, S., Muller-Baden, B., Weber, F., and Schaffner, W. (1988) *J. Mol. Biol.* **201,** 81–90.
4. Rosen, J. M., Wyszomierski, S. L., and Hadsell, D. (1999) *Annu. Rev. Nutr.* **19,** 407–436.
5. Rosen, J. M., Zahnow, C., Kazansky, A., and Raught, B. (1998) *Biochem. Soc. Symp.* **63,** 101–113.
6. Schmidhauser, C., Casperson, G. F., Myers, C. A., Sanzo, K. T., Bolten, S., and Bissell, M. J. (1992) *Mol. Biol. Cell* **3,** 699–709.
7. Winklehner-Jennewein, P., Geymayer, S., Lechner, J., Welte, T., Hansson, L., Geley, S., and Doppler, W. (1998) *Gene* **217,** 127–139.
8. Li, S., and Rosen, J. M. (1994) *Mol. Endocrinol.* **8,** 1328–1335.
9. Pierre, S., Jolivet, G., Devinoy, E., Theron, M. C., Malienou-N'Gassa, R., Puissant, C., and Houdebine, L. M. (1992) *Mol. Cell. Endocrinol.* **87,** 147–156.
10. Wyszomierski, S. L., and Rosen, J. M. (2001) *Mol. Endocrinol.* **15,** 228–240.
11. Jolivet, G., L'Hotte, C., Pierre, S., Tourkine, N., and Houdebine, L. M. (1996) *FEBS Lett.* **389,** 257–262.
12. Schmitt-Ney, M., Doppler, W., Ball, R. K., and Groner, B. (1991) *Mol. Cell. Biol.* **11,** 3745–3755.
13. Pierre, S., Jolivet, G., Devinoy, E., and Houdebine, L. M. (1994) *Mol. Endocrinol.* **8,** 1720–1730.
14. Jolivet, G., Meusnier, C., Chaumaz, G., and Houdebine, L. M. (2001) *J. Cell. Biochem.* **82,** 371–386.
15. Petitclerc, D., Attal, J., Theron, M. C., Bearzotti, M., Bolifraud, P., Kann, G., Stinnakre, M. G., Pointu, H., Puissant, C., and Houdebine, L. M. (1995) *J. Biotechnol.* **40,** 169–178.
16. Attal, J., Theron, M. C., Rival, S., Puissant, C., and Houdebine, L. M. (2000) *Mol. Biol. Rep.* **27,** 21–26.
17. Attal, J., Theron, M. C., Kann, G., Bolifraud, P., Puissant, C., and Houdebine, L. M. (2000) *J. Biotechnol.* **77,** 179–189.
18. Lesueur, L., Edery, M., Ali, S., Paly, J., Kelly, P. A., and Djiane, J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 824–828.
19. Edery, M., Jolicoeur, C., Levi-Meyrueis, C., Dusanter-Fourt, I., Petridou, B., Boutin, J. M., Lesueur, L., Kelly, P. A., and Djiane, J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2112–2116.
20. Ball, R. K., Friis, R. R., Schoenenberger, C. A., Doppler, W., and Groner, B. (1988) *EMBO J.* **7,** 2089–2095.
21. de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987) *Mol. Cell Biol.* **7,** 725–737.
22. Menck, M., Mercier, Y., Campion, E., Lobo, R. B., Heyman, Y.,

Renard, J. P., and Thompson, E. M. (1998) *Transgenic Res.* **7,** 331–341.

23. Hogan, B., Constantini, F., and Lacy, E. (1986) Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. Attal, J., Cajero-Juarez, M., Petitclerc, D., Theron, M. C., Stinnakre, M. G., Bearzotti, M., Kann, G., and Houdebine, L. M. (1995) *Mol. Biol. Rep.* **22,** 37–46.
25. Chung, J. H., Whiteley, M., and Felsenfeld, G. (1993) *Cell* **74,** 505–514.
26. Pikaart, M. J., Recillas-Targa, F., and Felsenfeld, G. (1998) *Genes Dev.* **12,** 2852–2862.
27. Emery, D. W., Yannaki, E., Tubb, J., and Stamatoyannopoulos, G. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 9150–9155.
28. Taboit-Dameron, F., Malassagne, B., Viglietta, C., Puissant, C., Leroux-Coyau, M., Chereau, C., Attal, J., Weill, B., and Houdebine, L. M. (1999) *Transgenic Res.* **8,** 223–235.
29. Rival, S., Pantano, T., Viglietta, C., Maeder, C., Prince, S., Attal, J., Jolivet, G., and Houdebine, L. M. (2001) Submitted.
30. Soulier, S., Lepourry, L., Stinnakre, M. G., Langley, B., L'Huillier, P. J., Paly, J., Djiane, J., Mercier, J. C., and Vilotte, J. L. (1999) *Transgenic Res.* **8,** 23–31.
31. Wang, Y., DeMayo, F. J., Tsai, S. Y., and O'Malley, B. W. (1997) *Nat. Biotechnol.* **15,** 239–243.
32. Palmiter, R. D., Sandgren, E. P., Avarbock, M. R., Allen, D. D., and Brinster, R. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 478–482.
33. Recillas-Targa, F., Bell, A. C., and Felsenfeld, G. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 14354–14359.
34. Allen, N. D., Cran, D. G., Barton, S. C., Hettle, S., Reik, W., and Surani, M. A. (1988) *Nature* **333,** 852–855.
35. Sassi, H., Fromont-Racine, M., Grange, T., and Pictet, R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7197–7201.
36. Sotiropoulos, A., Moutoussamy, S., Renaudie, F., Clauss, M., Kayser, C., Gouilleux, F., Kelly, P. A., and Finidori, J. (1996) *Mol. Endocrinol.* **10,** 998–1009.
37. Sax, C. M., Klement, J. F., and Piatigorsky, J. (1990) *Mol. Cell. Biol.* **10,** 6813–6816.
38. Cassard-Doulcier, A. M., Gelly, C., Bouillaud, F., and Ricquier, D. (1998) *Biochem. J.* **333,** 243–246.
39. Nelson, S. A., and Robins, D. M. (1997) *Mol. Cell. Endocrinol.* **133,** 89–97.
40. Potts, W., Tucker, D., Wood, H., and Martin, C. (2000) *Biochem. Biophys. Res. Commun.* **273,** 1015–1018.
41. Bell, A. C., and Felsenfeld, G. (1999) *Curr. Opin. Genet. Dev.* **9,** 191–198.
42. Walters, M. C., Fiering, S., Bouhassira, E. E., Scalzo, D., Goeke, S., Magis, W., Garrick, D., Whitelaw, E., and Martin, D. I. (1999) *Mol. Cell. Biol.* **19,** 3714–3726.
43. Muiznieks, I., and Doerfler, W. (1994) *FEBS Lett.* **344,** 251–254.
44. Ben-Hattar, J., Beard, P., and Jiricny, J. (1989) *Nucleic Acids Res.* **17,** 10179–10190.
45. Montoliu, L., Chavez, S., and Vidal, M. (2000) *Transgenic Res.* **9,** 237–239.
46. Cohen-Tannoudji, M., Babinet, C., and Morello, D. (2000) *Transgenic Res.* **9,** 233–235.
47. Martin, D. I. (2001) *Trends Genet.* **17,** 444–448.