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Effect of DNA Loop Anchorage Regions (LARs) and Microinjection Timing on Expression of β-Galactosidase Gene Injected Into One-Cell Rabbit Embryos

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Abstract The conditions favoring expression of a reporter gene microinjected into a male pronucleus of naturally ovulated and fertilized rabbit eggs have been studied. Injection of the reporter gene during S phase of the cell-cycle allows the highest level of expression of the gene. Incorporation of DNA loop anchorage regions (LARs) into constructs upstream and/or downstream of the reporter gene significantly increased the efficiency of expression. In all cases the expression of the microinjected gene started after a period of transcriptional quiescence, i.e., together with the expression of the host genome. Correct targeting of microinjected constructs within the nuclei via interaction of LAR elements with receptor sites on the nucleoskeleton may facilitate expression of injected DNA constructs as well as their integration into host cell DNA. J. Cell. Biochem. 92: 1171–1179, 2004. © 2004 Wiley-Liss, Inc.

Key words: transgenesis; cell-cycle; DNA loop anchorage regions; transcription; integration

Despite recent developments in the field of sperm-mediated gene transfer [Perry, 2000], microinjection remains a tool of choice for introduction of new genetic information into mammalian oocytes and embryos. This method has an enormous potential for production of transgenic animals. Although the techniques and equipment used for the microinjection have significantly improved during the past years, the efficiency of expression and integration of foreign DNA in the host genome remains unsatisfactory. The success rate in experiments with large animals, such as cows, is particularly low, only approximately 0.2% of injected embryos develop eventually into primary transgenics. Hence, it is important to understand the molecular mechanisms underlying the integration of injected DNA into host genome and select the conditions that favor the expression of DNA in the host genome. An increase in efficiency of the microinjection would permit to significantly decrease the cost of production of transgenic animals.

The exact nature of molecular mechanisms ensuring integration of foreign genes into the host cell DNA remains largely unknown. The efficiency of integration of exogenous DNA depends on many factors, including its location in the cell, its topological state, and the timing of its introduction into the embryo [Brinster et al., 1985; Bishop, 1996]. Higher integration efficiencies are observed upon injection of linear versus circular DNA, and it is not surprising that injection of exogenous DNA into the cytoplasm leads to lower frequency of integration events than injection into the pronuclei. The male pronucleus constitutes a better target for microinjection as compared to a female pronucleus [Brinster et al., 1985]. This may

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reflect an increased accessibility of male pronucleus DNA during substitution of protamines by histones. Fusion of the sperm with the oocyte causes a profound rearrangement of its nuclear architecture [Wright, 1999]. Sperm-specific nuclear protamines are replaced by histones during the first hours after fertilization. The S phase begins after chromatin remodeling [Demeret et al., 2001]. Some data suggest that exogenous DNA integration into genome occurs preferentially during replication of host cell DNA, i.e., during the S phase of the cell-cycle [Wong and Capecchi, 1987; Russell et al., 1994]. Hence, the timing of microinjection may affect the percentage of "productive" injections. In the present study we have addressed the question experimentally. A linearized construct containing a reporter gene (β-galactosidase, *lacZ*) expressed under the control of Rous Sarcoma Virus (RSV) promoter was microinjected into the male pronucleus of naturally fertilized rabbit eggs at different times after insemination. The embryos expressing β-galactosidase were obtained only when microinjection was carried out during S-phase of cell-cycle.

An additional challenge in the gene transfer experiments is a silencing of integrated transgene constructs. Indeed, the ratio between the number of integrated exogenous gene copies and the level of gene expression is rarely linear [Martin and Whitelaw, 1996; Henikoff, 1998]. It is known that the context of chromatin is generally repressive and does not favor gene expression since over 90% of the human DNA does not code for any genes (junk DNA), and only 10–30% of the genes are active in a cell at one time. Moreover, integration of linearized DNA often occurs in tandem arrays, which tend to form heterochromatin thus repressing transcription.

DNA in nuclei has several levels of compaction. Nucleosomal arrays are folded into 30 nm fibers, and they are further compacted to form the DNA loop domains [Vassetzky et al., 2000a]. These loop domains can be visualized by extraction of histones from the isolated nuclei or metaphase chromosomes where the loops are anchored to the proteinaceous nucleoskeleton, also called nuclear matrix or scaffold. DNA loops are attached to the nuclear matrix via loop anchorage regions (LARs) [Razin, 1996; Vassetzky et al., 2000a].

LARs are complex structures that may include MARs (genomic elements capable of interacting in vitro in a specific fashion with isolated nuclear matrix), topoisomerase II binding sites and other sequence motifs [Iarovaia et al., 1996; Razin, 1996; Vassetzky et al., 2000a; Razin, 2001]. LARs can be mapped by topoisomerase II-mediated DNA loop excision approach and are usually several Kbp long, in contrast to relatively short AT-rich MARs [Iarovaia et al., 1996].

LARs render the loops topologically and spatially isolated, and can insulate the genes within the loop domain from the repression mediated by various chromatin states. Hence, the utilization of LARs as artificial border elements in the transgenic delivery constructs could protect a transgene from the influence of surrounding (hetero)chromatin and hence enhance its transcription. An additional advantage of LARs is that they target DNA to the nucleoskeleton where transcription takes place [Pombo et al., 1999]. We used a combination of a LAR from the chicken α-globin gene domain [Kalandadze et al., 1990] with the reporter gene to test the expression from such constructs integrated into the genome. We show that the percentage of productive injections significantly increases upon incorporation of a DNA loop anchorage region into the constructs. In this case, the injection timing also remains crucial in obtaining a high level of expression of the integrated construct since most of the gene expression was detected in the embryos microinjected during the S phase.

MATERIALS AND METHODS

Gene Constructs

The basic pRSV-LacZ plasmid was constructed on the basis of the pUC19 vector. An RSV promoter was inserted at the 5′ side of the bacterial *LacZ* gene and a polyA signal from bovine growth hormone gene was inserted at the 3′ side of the gene to achieve correct polyadenylation of the LacZ mRNA [Gogolevskii et al., 1991]. Two other constructs contained either one LAR (DNA loop anchorage region) inserted upstream to the RSV promoter or two LARs flanking the whole gene cassette (Fig. 1A). The 1.7 kb LAR from the chicken domain of α-globin genes was excised by Hind III from the pUC-alpha62 plasmid [Kalandadze et al., 1990] and inserted into the Hind III site of the pRSV-LacZ. The second LAR was blunted and inserted into the Kpn I site of the pRSV-LacZ plasmid.

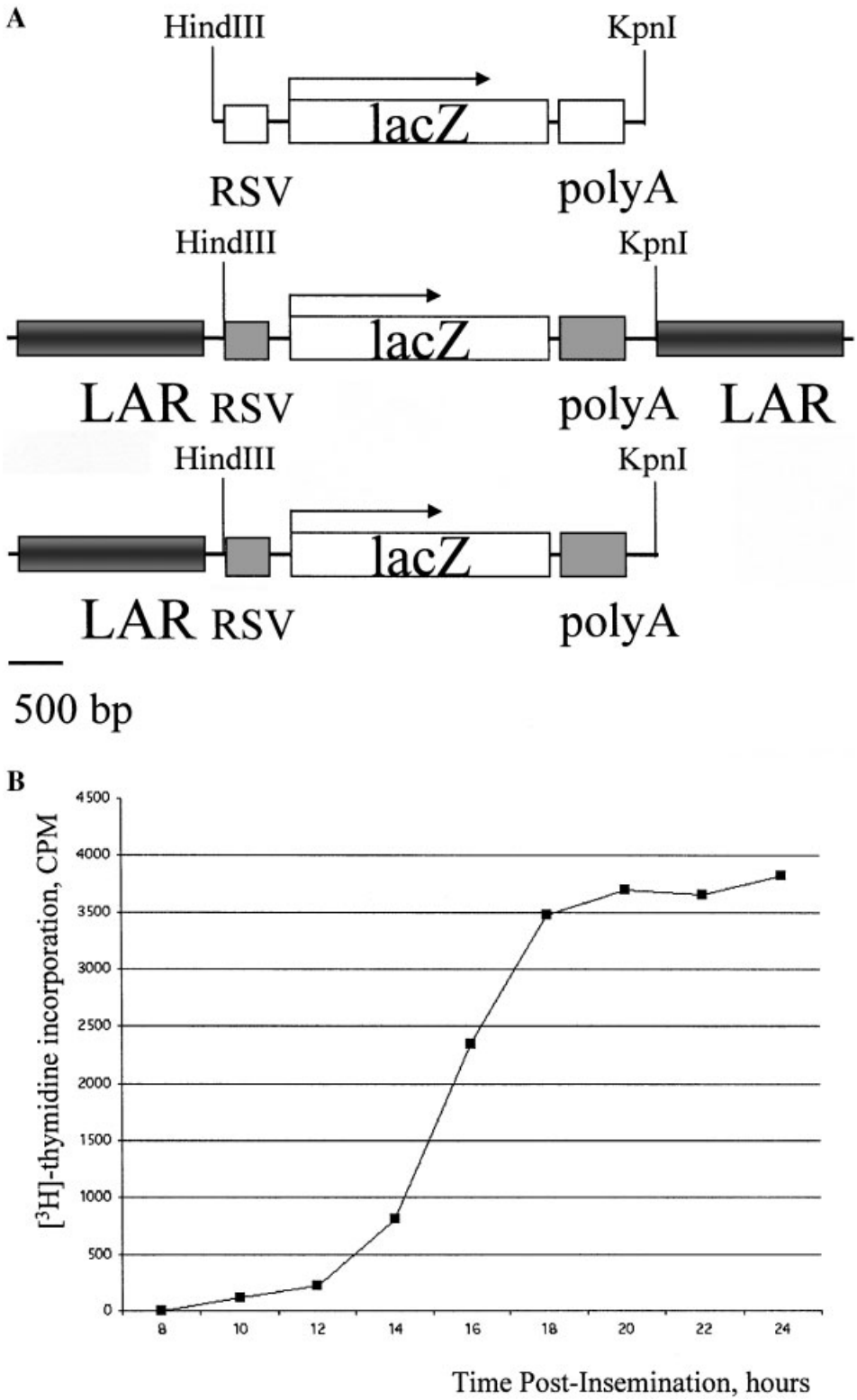

Fig. 1. **A**: Gene constructs used. LacZ, beta-galactosidase gene; RSV, Rous Sarcoma Virus (RSV); poly A, poly A signal from bovine growth hormone gene; LAR, a loop attachment region from the chicken α-globin gene domain. **B**: Time course of ^{3}H-thymidine incorporation into fertilized rabbit eggs. The eggs were washed out by an excess of Hanks solution from ovaries of rabbits 8 h after natural insemination and further incubated in the ^{3}H-thymidine-containing Hanks medium. Five eggs were taken per time point.

Before microinjection all constructs were linearized and prokaryotic vector sequences were cut out. DNA was purified using the Qiagen gel extraction kit.

Preparation of Fertilized Rabbit Eggs and Microinjection

The eggs were washed out by an excess of Hanks solution from ovaries of rabbits 8, 14, or 19 h after natural insemination. Microinjection of DNA constructs and subsequent cultivation of embryos was carried out in a medium 199 (Flow Labs., Irvine, Scotland) supplemented with bovine serum albumin (fraction V, BSA) to a final concentration of 3 mg/ml. The DNA constructs diluted in 0.1 mm EDTA, 10 mM Tris, pH 7.4 were microinjected into male pronuclei of one-cell embryos using a Narishige microinjector (Narishige Scientific Instrument Lab., Tokyo, Japan) with an needle with an outer diameter of 1.5 μm (Fig. 2). The microinjection was monitored using an inverted microscope Axiovert 35 (Carl Zeiss, Jena, Germany). Approximately 4 pl of DNA solution containing 400–800 copies of a linear construct was injected in a standard experiment. After injection, the embryos were incubated for 28–48 h at 38.5°C in medium 199 supplemented with BSA. Two independent series of injections were carried out for each experiment.

β-Galactosidase Detection

The embryos were washed three times with PBS buffer (pH 7.2) and then fixed in a 4% solution of paraformaldehyde in PBS. Fixation was carried out for 20 min at 4°C. After fixation, the embryos were washed with PBS for 30 min at 4°C. The embryos were transferred into PBS supplemented with 0.4 mg/ml X-gal, 4 mM $K3Fe(CN)_6$, 4 mM $K4Fe(CN)_6$, and 2 mM $MgCl_2$. The percentage of stained (i.e., containing active β-galactosidase) embryos was calculated microscopically after incubation for 14–16 h at 30°C.

RESULTS AND DISCUSSION

Microinjection of the β-Galactosidase Gene at Different Stages of the Cell-Cycle Does not Affect the Cleavage of Rabbit Embryos

Fertilization causes a profound rearrangement of sperm chromatin: sperm-specific nuclear proteins are replaced by histones during the first hours post-fertilization. The S phase begins after the chromatin remodeling is over, and in naturally fertilized rabbit eggs it usually starts between 14 and 17 h after insemination [Szollosi, 1966]. We have checked the time course of the cell-cycle by following ^3H thymidine incorporation into DNA of fertilized eggs. Indeed, the beginning of the S-phase was detected between 13 and 18 h after the insemination (Fig. 1B).

Injection timing is an important factor in obtaining a high efficiency of exogenous DNA integration into the genome and its subsequent expression. However, it was reported that the survival of the injected embryos also depends on the injection timing. We then tried to evaluate the embryo cleavage pattern after the injection of β-galactosidase gene constructs at different stages of the cell-cycle. The injection of the embryos was carried out into the male pronu-

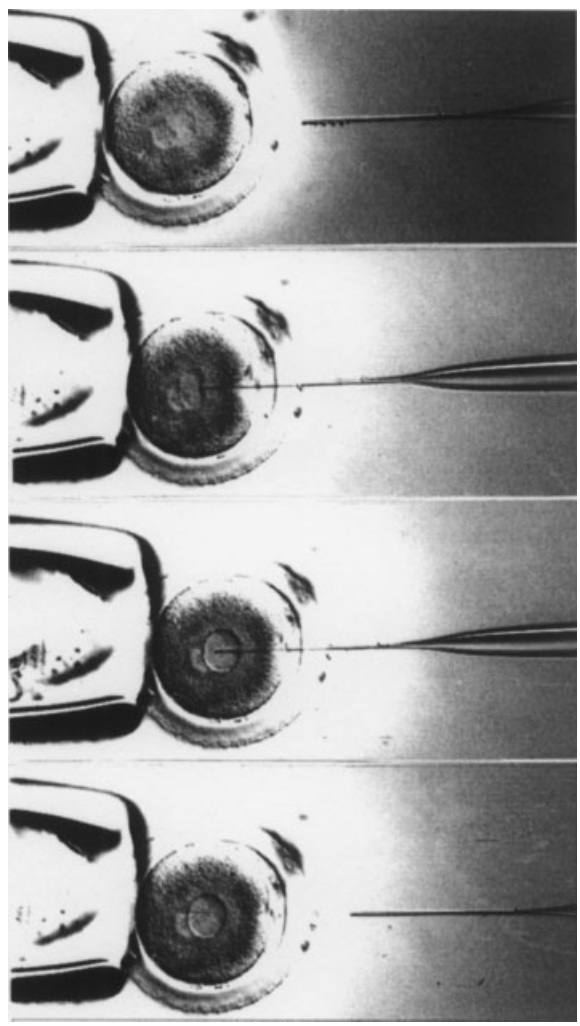

Fig. 2. Microinjection of a fertilized rabbit oocyte.

cleus (Fig. 2) as this seems to give a higher level of gene integration and expression [Brinster et al., 1985]. We have checked the embryo survival rate after injecting the one-cell rabbit embryos at different times post-insemination. The survival was estimated by direct microscopic observation.

Contrary to the published data on survival of bovine embryos [Gagne et al., 1995], no significant difference was observed between the embryos injected in S and G_2 phases. The embryos injected during the S-phase have a slightly lower survival rate than the control embryos or those injected during the G_2 phase, suggesting that the rabbit embryo microinjection during the S-phase does not cause significant damage to the embryo (Fig. 3). This discrepancy with the published data may be due to the differences in the microinjection procedure and the fragility of the bovine embryos.

Microinjection of the β-Galactosidase Gene at the S Phase-of the Cell-Cycle Allows Expression of the Reporter Gene

The gene constructs containing the β-galactosidase gene under the control of the RSV promoter (RSV-LacZ) were injected into one-cell rabbit embryos either at the S (14.5–17 h after insemination) or G_2 (18–19.5 h after insemination) phases of the cell-cycle. Rabbit embryos were allowed to develop for another 28–48 h after the injection, and the β-galactosidase expression was estimated by X-gal staining. No β-galactosidase expression was detected 28 h after the injection (4-cell embryos) in either S or G_2-phase injected embryos. However, approximately 5% of the embryos injected during the S-phase of the cell-cycle were found to express β-galactosidase 48 h after the injection (8 and 16-cell stage). No β-galactosidase expression was detected 48 h post-injection in the embryos injected at the G_2 phase (18–19.5 h after insemination). Interestingly, in 70% embryos, the expression of the gene was mosaic, i.e., confined to one or several blastomers (Fig. 4C), while strong and uniform expression was observed in 30% embryos (Fig. 4B). In the case of microinjection of murine embryos, a symmetrical expression pattern of β-galactosidase was observed in mosaic transgenic embryos [Stevens et al., 1989]. This is not the case in the rabbit embryos: no specific expression pattern was observed. This may suggest that either the reporter gene integration occurred during the second cell-cycle, or that the regulation of gene expression differs between the blastomers. Alternately, the transcription may be completely inhibited in some blastomers at the 8–16 blastomer stage [Telford et al., 1990].

Hence, we have shown that the expression of the microinjected *lacZ* gene varies in relation to the timing of microinjection. Cells microinjected

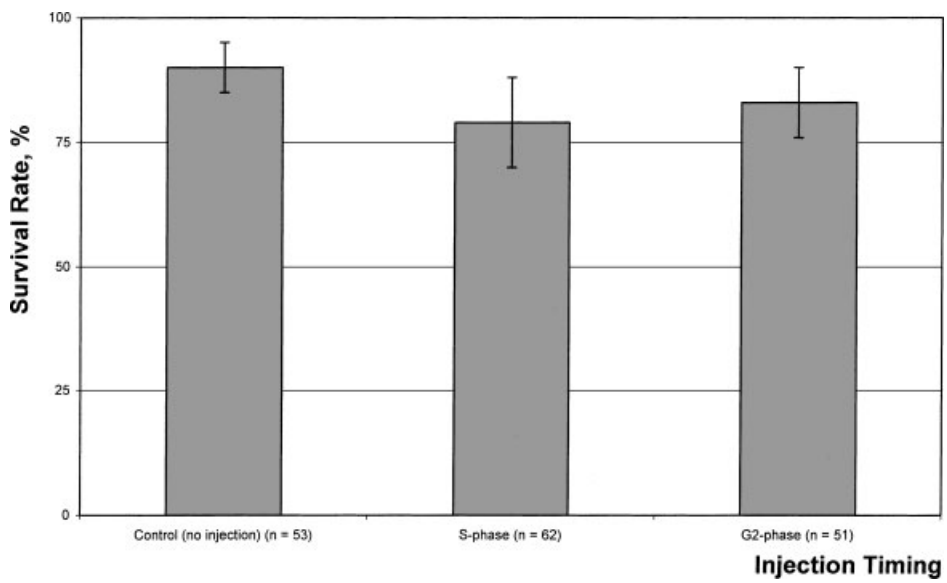

Fig. 3. Effect of DNA microinjection into fertilized rabbit oocytes at different post-insemination times on survival of early embryos. Results of two independent experiments are presented. N indicates an average number of fertilized oocytes injected.

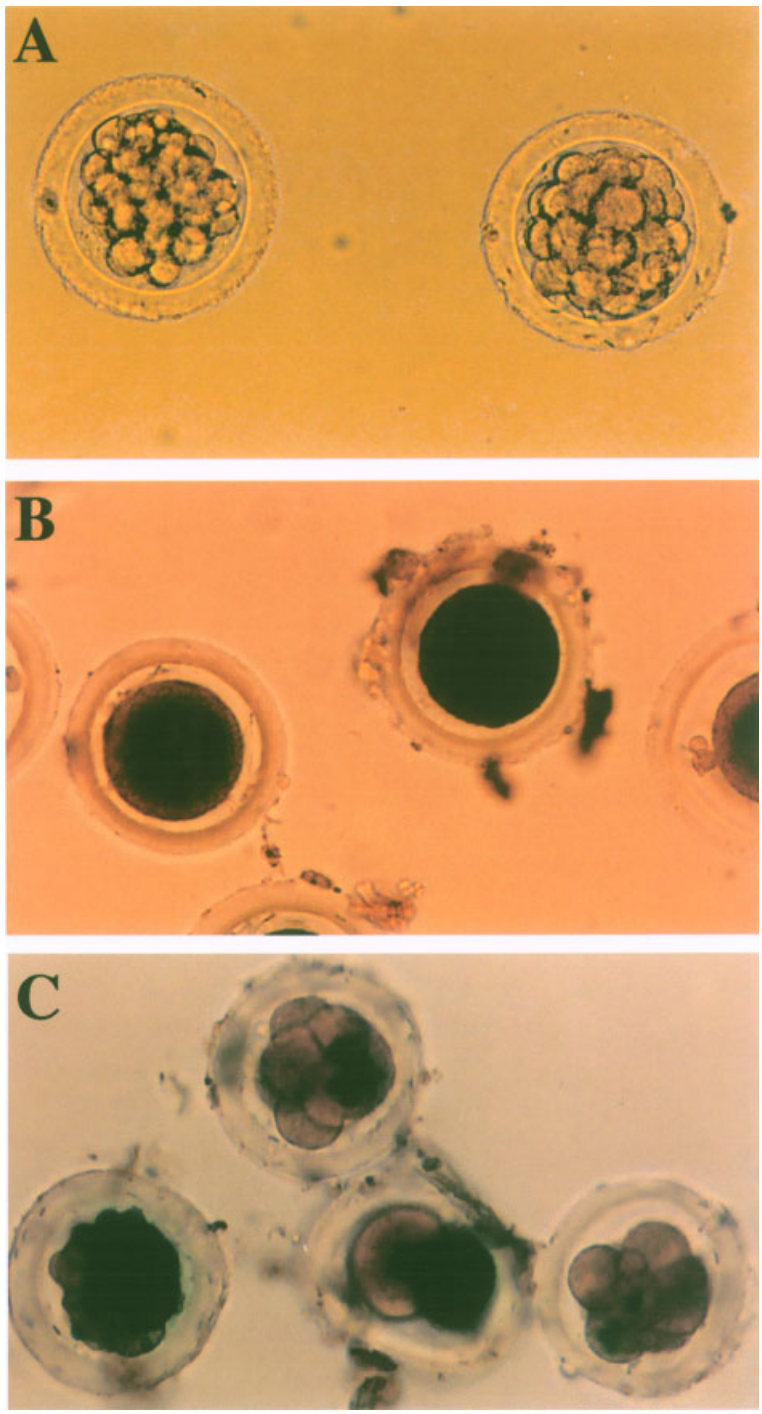

Fig. 4. Expression of β-galactosidase in rabbit embryos 48 h after the microinjection at different phases of cell-cycle. **A**: Mock injection. **B**: Embryos expressing β-galactosidase 48 h postinjection. **C**: Mosaic expression of β-galactosidase 48 h postinjection. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

during the S-phase express β-galactosidase, while no expression was detected in cells injected at the G_2 phase. One disadvantage of the linearized vector sequences is their inefficient integration into the host genome, and the integration may possibly be enhanced, i.e., via homologous recombination, during the S phase.

LARs Enhance the Reporter Gene Expression in Microinjected Rabbit Embryos

Silencing of integrated transgene constructs is a major problem in gene transfer experiments. Few, if any of the integrated exogenous gene copies are expressed at the level of corresponding endogenous genes or circular plasmids. The context of chromatin is generally repressive and does not favor gene expression. Integration of linearized DNA into genome often occurs in a form of tandem arrays which favors the formation of heterochromatin, thus repressing transcription [Martin and Whitelaw, 1996; Henikoff, 1998]. In the nucleus, DNA loops domains are attached to the nuclear matrix via LARs [Vassetzky et al., 2000a]. The LARs render the loops topologically and spatially isolated, and can insulate the genes within the loop domain from the repression mediated by various chromatin states. We used a combination of a LAR from the chicken α-globin gene domain [Kalandadze et al., 1990] with the reporter gene to test the expression from such constructs integrated into the genome. This chicken LAR was shown to be functional in mammalian cells of different species, including rabbit cells. Microinjection of the reporter gene with the LAR located either upstream to or flanking the *lacZ* gene leads to a significant increase in the percentage of productive injections (Fig. 5). In this case, the injection timing also remains crucial in obtaining a high level of expression of the integrated construct since most of the gene expression was detected in the embryos microinjected during the S phase.

DNA LARs define the borders of active genomic domains. They insulate the domain from the inhibitory effect of the surrounding chromatin [Allen et al., 2000]. When exogenous DNA is microinjected into the pronuclei of zygotes, its integration into any genomic domain seems to be equally probable. In differentiating cells, only genes that happen to integrate into domains active in a given cell type or at a given stage will be expressed. A logical way to solve the problem of position effects is to create artificial mini-domains including a reporter gene, a block of regulatory sequences, and the domain borders. Several nuclear scaffold/matrix attachment regions (SARs/MARs) have been tested for the ability to insulate transgenes from suppressing effects of the host chromatin domain. In some experiments it was observed that MARs, when placed upstream and downstream to the transgene, suppressed (in some cases only partially) the position

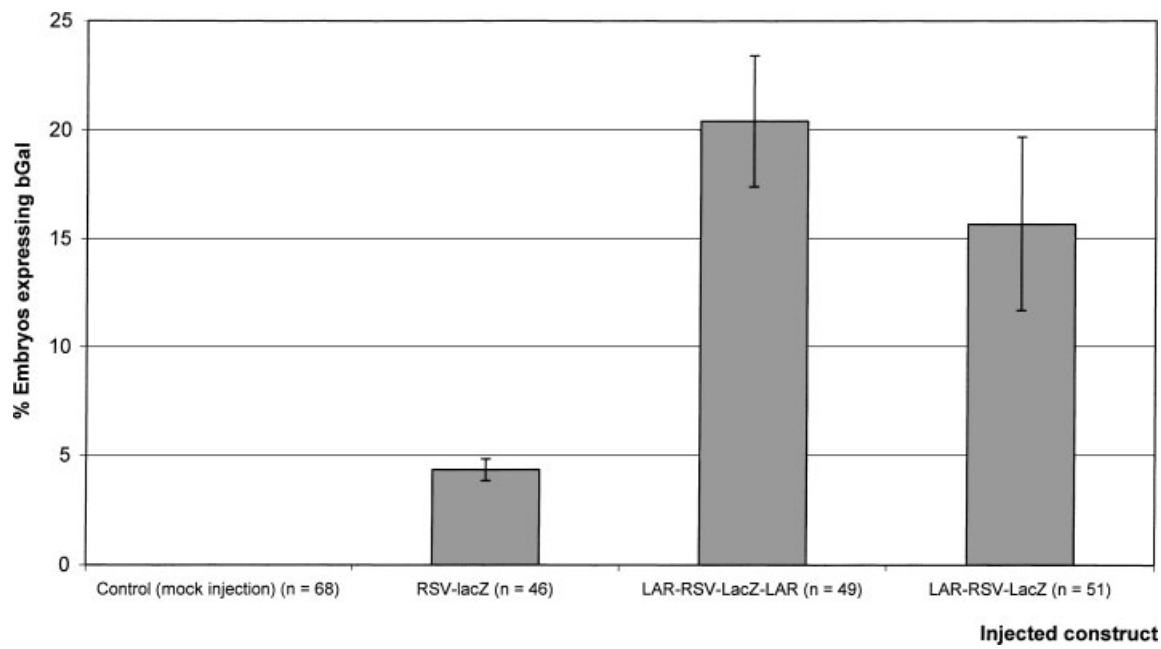

Fig. 5. Effect of flanking LARs on β-galactosidase expression 48 h after microinjection of DNA into fertilized rabbit oocytes. The constructs used are shown in Figure 1. Results of two independent experiments are presented. N indicates an average number of fertilized oocytes injected.

variegation effects [Allen et al., 2000]. In contrast to the stimulation of gene expression by enhancers, which may be observed in a transient assay (i.e., when transcription of episomal genes is studied), MARs stimulate gene expression only after stable integration of corresponding constructs into the host-cell genome. In our experiments, we inserted a LAR from the chicken α-globin gene domain upstream and downstream of the reporter gene. An approximately ten-fold increase in the efficiency of the β-galactosidase expression was observed as compared to the control (pRSV-lacZ; Fig. 5). Interestingly, a similar enhancement of the expression level was observed when the chicken globin LAR was inserted only upstream of the gene. This may be due to integration of the construct in the form of a concatemer, and in this case at least one reporter gene would be flanked by two LARs.

Indeed, tandem integration of the construct containing the reporter gene and one LAR was studied in the transgenic rabbits containing the integrated construct and in stably transfected primary rabbit fibroblast by Southern blotting and PCR. In both cases, we have detected several tandemly integrated copies (Goldman et al., in preparation). Our data are in agreement with the published results on the effect of MARs on expression of reporter genes in preimplantation mouse embryos [Gutierrez-Adan and Pintado, 2000].

Another property of MARs is their ability to target DNA to the nucleoskeleton compartment where active transcription and replication take place [Vassetzky et al., 2000b] and MARs may potentially facilitate integration by bringing the injected DNA in contact with the replication machinery, thus favoring integration of the constructs or their transcription. Indeed, the plasmid constructs containing the α-globin LAR are preferentially located within the nuclear matrix compartment when transfected into primary rabbit fibroblasts (data not shown).

The α-globin LAR contains an internal MAR [Razin et al., 1991], thus its effect on transcription could in principle be explained just by the ability of the internal MAR to associate with the nuclear matrix. On the other hand, the attempts to use the subclones of the α-globin LAR led to a drastic reduction in the ability of the injected constructs to enhance transcription (Goldman et al., in preparation).

Interestingly, in the case of LAR-flanked construct, the injection timing also remains crucial in obtaining a high level of expression since most of the gene expression was detected in the embryos microinjected during the S phase. Further research will be aimed to find an optimal combination of insulators/enhancers and the injection timing to obtain the maximal efficiency of gene transfer into rabbits.

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