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# Dicistronic targeting constructs: Reporters and modifiers of mammalian gene expression

**(differentiation-inhibiting activity/leukemia-inhibitory factor/transcription factor Oct-4/embryonic stem cells/homologous recombination)**

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**ABSTRACT** **To investigate the activity of candidate regulatory molecules in mammalian embryogenesis, we have developed a general strategy for modifying and reporting resident chromosomal gene expression. The picornaviral internal ribosome-entry site was incorporated into gene targeting constructs to provide cap-independent translation of a selectable marker from fusion transcripts generated following homologous recombination. These promoterless constructs were highly efficient and have been used both to inactivate the stem-cell-specific transcription factor Oct-4 and to introduce a quantitative regulatory modification into the gene for a stem-cell maintenance factor, differentiation-inhibiting activity. In addition, the inclusion of a β-galactosidase reporter gene in the constructs enabled accurate and sensitive detection of cellular sites of transcription. This has allowed visualization of putative "stem-cell niches" in which sources of elevated expression of differentiation-inhibiting activity were localized to the differentiated cells surrounding colonies of stem cells.**

The ability to introduce predetermined mutations into the mouse germ line via homologous recombination in embryonic stem (ES) cells marks a major advance for the application of reverse genetics in mammalian biology (1–3). Gene targeting is currently widely employed to generate null alleles or otherwise mutate genes of interest. However, realization of the full potential of this approach requires the development of systems which facilitate the introduction and monitoring of precise qualitative and quantitative manipulations of chromosomal gene expression.

Homologous recombination has been employed to introduce a reporter such as the *Escherichia coli lacZ* gene into a locus (4, 5). In principle this provides a means of obtaining accurate gene expression profiles with cellular resolution. Previous approaches, however, have been compromised by the requirement to include an exogenous promoter in the targeting constructs in order to drive expression of a selectable marker. For maximum fidelity, the reporter should be integrated without addition of any exogenous transcriptional control elements. We have investigated the potential utility of the picornaviral internal ribosome-entry site (IRES) to achieve this end. The IRES is a sequence of some 500 nt which acts as a ribosome binding site and permits the effective internal initiation of translation in mammalian cells (6–9). Preliminary transfection experiments established that the IRES of encephalomyocarditis virus (EMCV) (10) functioned efficiently in undifferentiated ES cells (data not shown). We therefore incorporated the EMCV IRES into promoterless targeting vectors as depicted in Fig. 1. Exploitation of the IRES offers an advantage over previous promoter-trap or gene-fusion methods (11) in that when it is integrated into a transcriptionally active gene, production of functional selectable marker and/or reporter protein is expected to be independent of context in the fusion transcript. Thus IRES-mediated translation is significantly more flexible and efficient than strategies reliant upon the production of an active fusion protein or upon reinitiation of translation (stop–start) (12). Moreover, the functionality of the IRES regardless of translation frame or of location in coding or noncoding sequences should greatly simplify the design and construction of targeting traps.

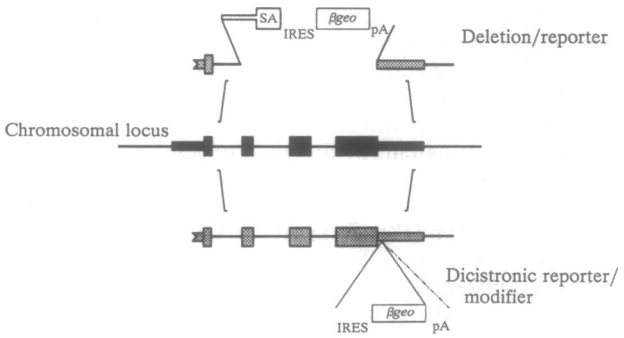


FIG. 1. Applications of the IRES–*βgeo* cassette in gene targeting. Constructs can be designed either to delete all or part of a gene while incorporating the *lacZ* reporter or to append the reporter with or without modification of the intact gene. Thin lines indicate intergenic or intronic sequences, intermediate lines indicate 5′ and 3′ untranslated regions (UTRs), and thick lines designate coding sequences. SA, *en-2* splice acceptor; pA, simian virus 40 (SV40) polyadenylylation sequence.

A major potential application of this approach lies in combination of the IRES with the *lacZ–neo*$^R$ fusion gene *βgeo* (13). The latter provides for cointegration of a histochemically detectable reporter along with a selectable marker by encoding a fusion protein which both possesses enzymatic β-galactosidase activity and confers resistance to the selection agent G418. Promoterless insertion of *βgeo* into an active locus with an IRES vector therefore allows for selection of transfectants while intimately coupling expression of the reporter with endogenous transcriptional and posttranscriptional cis-regulatory sequences. Constructs can be conveniently designed to report either normal gene expression by simple insertion of the IRES–*βgeo* cassette or

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Abbreviations: DIA/LIF, differentiation-inhibiting activity/leukemia-inhibitory factor; EMCV, encephalomyocarditis virus; ES cell, embryonic stem cell; IRES, internal ribosome-entry site; SV40, simian virus 40; UTR, untranslated region.

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altered expression arising from defined modification of the locus.

We have employed this strategy to examine the expression and modify the activity of two genes implicated in the control of self-renewal in ES cells and in the early mouse embryo, the cytokine differentiation-inhibiting activity/leukemia-inhibitory factor (DIA/LIF) and the POU-domain transcription factor Oct-4.

## METHODS

**ES Cell Culture and Manipulation.** ES cells were routinely maintained (14) in the absence of feeder cells in medium supplemented with murine DIA/LIF. The germ-line competent cell line CGR8 was established from strain 129 mouse embryos by published procedures (15). Aggregation chimeras were produced between ES cells and outbred MF1 embryos by a modification of the method of Wood *et al.* (16) in which coculture is performed in hanging drops. For germ-line transmission, chimeras were produced by blastocyst injection (15). For isolation of homologous recombinants, $10^8$ cells were electroporated with 150 $\mu$g of linearized plasmid at 0.8 kV and 3 $\mu$F in a 0.4-cm cuvette, then selected in the presence of G418 (175 $\mu$g/ml). Genomic DNA was prepared in agarose plugs (17) from 24-well plate cultures while duplicate plates were stored frozen (18). To assay DIA/LIF production, ES cells were induced to differentiate by incubation with 6 mM 3-methoxybenzamide (14) and conditioned medium was harvested and assayed for the ability to inhibit ES cell differentiation (14). The assay was rendered specific for DIA/LIF by inclusion of a neutralizing polyclonal antiserum raised against murine DIA/LIF (A.S., unpublished work). Histochemical staining for $\beta$-galactosidase was carried out with 5-bromo-4-chloro-3-indolyl $\beta$-D-galactopyranoside (19), and fluorescent staining was performed with DetectaGene Green (Molecular Probes) according to the manufacturer's instructions.

**Plasmid Construction.** DNA manipulations were carried out by standard procedures (20). The IRES is a 594-bp sequence from the 5′ UTR of EMCV mRNA which has been modified by mutagenesis of the native initiation codon (10). Translation is initiated at an ATG codon which lies 9 bp 3′ of the normal start site and forms part of an *Nco* I cloning site. Full details of vector construction will be given elsewhere. Briefly, the IRES–*βgeo* cassette was constructed by ligating a 5′ fragment of the EMCV IRES–*lacZ* fusion (10) to 3′ *lacZ–neo*$^R$ sequence of the *βgeo* gene fusion (13). The pGTIRES*β*geopA plasmid was then generated by 5′ ligation of the *en-2* splice acceptor (21) and 3′ ligation of the SV40 polyadenylylation sequence. Replacement-type targeting constructs were prepared from genomic clones isolated from a strain 129 library. DIA/LIF targeting constructs were generated within a 7-kb fragment extending from a *Sac* II site between the alternative first exons (22) to a *Hin*dIII site 3′ of the gene. The DIA–*βgeo* construct was prepared by isolation of the IRES–*βgeo* cassette as an *Xba* I fragment and ligation into the *Xba* I site which overlaps the DIA/LIF stop codon. To generate the DIA–*βgeo*pA construct, a 1.2-kb *Bam*HI fragment containing 3′ *βgeo* sequence and SV40 polyadenylylation sequences was isolated from pGTIRES*β*geopA and ligated into the *Bam*HI-digested DIA–*βgeo* construct. This results in insertion of the 200 bp of SV40 sequence in place of a 400-bp fragment of DIA/LIF 3′ UTR. The Oct-4 targeting construct contained 1.6 kb of 5′ homology, extending from a *Hin*dIII site within the first exon to an *Xho* I site in the first intron, and 4.3 kb of 3′ homology, extending from the *Nar* I site 3′ of the polyadenylylation sequence to a *Hin*dIII site.

**DNA and RNA Hybridization Analyses.** Hybridizations were performed on nylon membranes according to standard procedures (20) using $^{32}$P-labeled probes generated by random priming or, where specified, by *in vitro* transcription with T7 polymerase. Homologous recombinants were characterized with probes from both 5′ and 3′ flanking sequences. Whole-mount *in situ* hybridization with digoxigenin-labeled Oct-4 antisense RNA (23) was performed essentially as described (24).

## RESULTS AND DISCUSSION

DIA/LIF is a pleiotropic cytokine which suppresses the differentiation of ES cells and has a range of activities on other stem cells and differentiated cell types *in vitro* and *in vivo* (25–29). An essential requirement for maternal expression of DIA/LIF has been revealed by gene knock-out experiments (30) as have roles in hemopoiesis and thymocyte maturation (31). With the exception of the endometrial glands in the uterus (29, 32), however, cellular sources of DIA/LIF transcription have not been defined. Although transcripts are demonstrable in many embryonic and adult tissues by RNase protection (33), the levels are extremely low and at the limits of detection by *in situ* hybridization. It has been shown, though, that the production of DIA/LIF by a variety of cell types can be modulated by cytokines and hormones (34). To investigate the specificity and control of DIA/LIF expression more effectively *in vitro* and *in vivo*, we sought to integrate a *lacZ* reporter into the chromosomal locus. As the steady-state level of DIA/LIF mRNA in ES cells is <10 copies per cell (33, 34), this also provided a stern test of the general utility of IRES targeting vectors. Targeting vectors were constructed by introduction of the IRES–*βgeo* module at the *Xba* I site which overlaps the stop codon (Fig. 2*A*). The entire coding sequence and the stop codon thus remained intact and intron sequences were unaltered. Two constructs were prepared, DIA–*βgeo* and DIA–*βgeo*pA, which differed by inclusion of the SV40 polyadenylylation signal 3′ of the *βgeo* sequence. The fusion transcript generated by homologous recombination with the former construct utilizes the endogenous 3′ UTR and polyadenylylation signal of the DIA/LIF gene, whereas the DIA–*βgeo*pA construct gives rise to a truncated transcript lacking these sequences. Comparison of the efficacy of these two constructs was anticipated to yield information on the significance of the 3′ UTR of DIA/LIF mRNA. This 3.5-kb sequence contains several copies of the AUUUA motif implicated in turnover of short-lived transcripts (35). Therefore the truncating construct was expected to yield higher levels of fusion transcript and hence greater resistance to G418 and higher $\beta$-galactosidase activity than the nontruncating UTR capture construct.

In contrast to DIA/LIF, both mRNA and protein for the octamer-binding transcription factor Oct-4 (also known as Oct-3; ref. 36) are relatively abundant in ES cells (23, 37, 38). Oct-4 is also found in oocytes, pluripotential early embryo cells, and primordial germ cells (36). The association of Oct-4 with pluripotency is strengthened by its rapid down-regulation during differentiation (38, 39). An IRES–*βgeo* vector was designed both to generate a null allele and to introduce an expression marker into the Oct-4 locus (Fig. 1). The latter could facilitate the detection of hitherto unidentified sites of Oct-4 expression. The POU-specific domain and the homeodomain coding sequences in exons 2–5 were deleted and replaced by the IRES–*βgeo*pA module (Fig. 2*B*). Since the 5′ arm of homology ended within the first intron, the *en-2* splice acceptor sequence (21) was included 5′ to the IRES in order to facilitate productive splicing from exon 1 after homologous recombination.

Following electroporation and selection in medium with G418, individual clones were analyzed by Southern hybridization with both 5′ and 3′ flanking probes to detect replacement targeting events (Fig. 2) and with internal probes to monitor for multiple integrations. The frequencies of homologous recombination obtained with the three constructs are

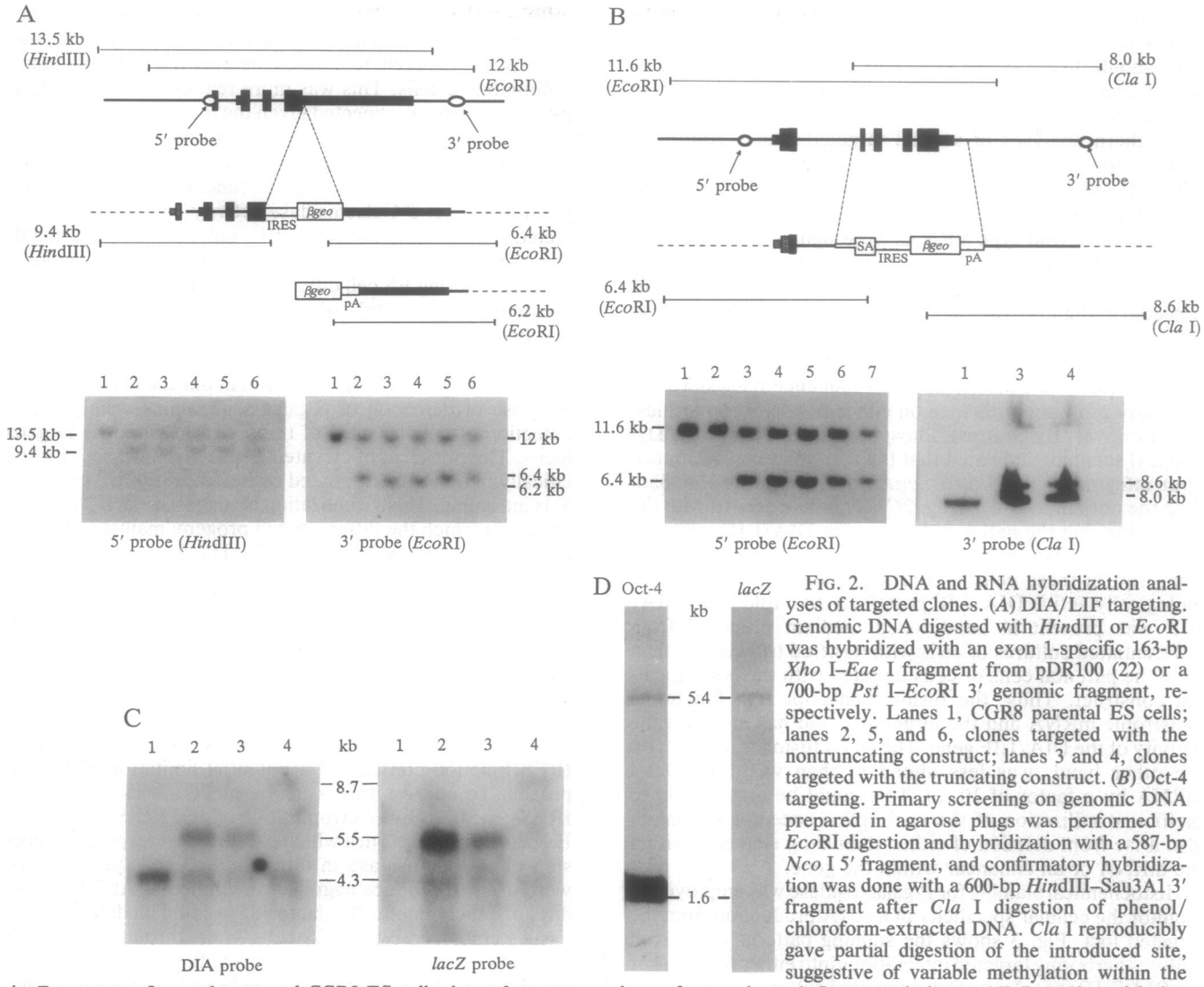


FIG. 2. DNA and RNA hybridization analyses of targeted clones. (*A*) DIA/LIF targeting. Genomic DNA digested with *Hin*dIII or *Eco*RI was hybridized with an exon 1-specific 163-bp *Xho* I–*Eae* I fragment from pDR100 (22) or a 700-bp *Pst* I–*Eco*RI 3′ genomic fragment, respectively. Lanes 1, CGR8 parental ES cells; lanes 2, 5, and 6, clones targeted with the nontruncating construct; lanes 3 and 4, clones targeted with the truncating construct. (*B*) Oct-4 targeting. Primary screening on genomic DNA prepared in agarose plugs was performed by *Eco*RI digestion and hybridization with a 587-bp *Nco* I 5′ fragment, and confirmatory hybridization was done with a 600-bp *Hin*dIII–Sau3A1 3′ fragment after *Cla* I digestion of phenol/chloroform-extracted DNA. *Cla* I reproducibly gave partial digestion of the introduced site, suggestive of variable methylation within the *lacZ* sequence. Lanes 1, parental CGR8 ES cells; lanes 2, nontargeted transfectant; lanes 3–7, targeted clones. (*C*) Detection of fusion transcripts in ES cell clones with targeted integrations at the DIA/LIF locus. To increase the level of DIA expression, ES cells were induced to differentiate by exposure to 1 μM retinoic acid. RNA was prepared after 4 days, applied to a formaldehyde/agarose gel, and transferred to nylon membrane. The filter was hybridized with random-primer labeled 4.08-kb *Xba* I fragment of *βgeo*, then stripped and rehybridized with a 650-bp DIA/LIF coding-sequence RNA probe (34). Lane 1, parental CGR8 cells; lanes 2 and 3, cells targeted with the truncating construct; lane 4, cells targeted with the nontruncating construct. (*D*) Detection of fusion transcript in Oct-4-targeted ES cells. Total RNA was prepared from an undifferentiated culture and 10 μg was subjected to Northern hybridization sequentially with random-primed Oct-4 and *lacZ* probes. The Oct-4 probe was a 408-bp *Nco* I–*Pst* I 5′ cDNA fragment which contains only 24 bp of exon 2 and should therefore give equivalent hybridization to wild-type and fusion transcripts, whereas the *lacZ* probe was an 800-bp fragment of 5′ coding sequence.

presented in Table 1. Replacement events were observed with all three vectors. A particularly high frequency was reproducibly obtained at the Oct-4 locus. This may reflect the high expression level of this gene in ES cells, in addition to the contributions of isogenic DNA (40) and the enrichment afforded by a promoterless construct (11). Targeting of DIA/LIF with the pA-containing construct was also efficient. The isolation of targeted clones at the DIA/LIF locus establishes that IRES-mediated translation is applicable to genes expressed at very low levels in ES cells. In fact, these results confirm unambiguously that DIA/LIF is transcribed in undifferentiated ES cells, contrary to a previous report (41). Somewhat surprisingly, the frequency of targeted events with the UTR capture construct was lower by a factor of 10, and these clones did not exhibit the restriction pattern produced by a single simple replacement event. Instead, hybridization with a *lacZ* probe indicated the presence of additional independent insertions in two clones and of a multimeric integration at the DIA/LIF locus in a third (clone D70). The reduced frequency of isolating homologous recombinants and the incidence of anomalous integrations are indicative of insufficient expression of $neo^R$ (42). This is consistent with a destabilizing effect of the DIA/LIF 3′ UTR causing a reduction in the steady-state level of fusion transcript and a corresponding decrease in the level of neomycin phosphotransferase below the threshold required to give G418 resistance. Significantly, the IRES configuration used in these studies may not be optimal for translation of the 3′ cistron. The precise location of the ATG relative to the 3′ end of the IRES has a major effect on translational efficiency (43).

Table 1. Frequency of isolation of homologous recombinants

| Construct | Cell line | No. of colonies screened | No. positive | Percent positive |
|---|---|---|---|---|
| Oct-4–*βgeo*pA | CGR8 | 51 | 44 | 86 |
| | E14TG2a | 10 | 7 | 70 |
| | D1C2 | 30 | 21 | 70 |
| DIA–*βgeo*pA | CGR8 | 79 | 21 | 26 |
| DIA–*βgeo* | CGR8 | 109 | 3 | 2.7 |

It would therefore appear that production of *βgeo* could be increased severalfold over that achieved in the present study. This should increase the ability to isolate recombinants in poorly expressed genes and also enhance the sensitivity of the *lacZ* reporter.

Northern analyses of several targeted clones confirmed that all contained fusion transcripts of the predicted sizes (Fig. 2 *C* and *D*) which hybridized to both *lacZ* and DIA/LIF or Oct-4 probes. The steady-state levels of the fusions relative to the wild-type mRNAs were significantly different, however. The transcript generated by nontruncating insertion of IRES–*βgeo* into the DIA/LIF gene in clone D70 was detected in slightly lower amounts than the normal transcript, although the apparent difference may simply reflect partial degradation or poorer transfer of the larger fusion transcript. This indicates that the IRES–*βgeo* sequence itself does not have any profound influence on either transcription or message turnover. By contrast, PhosphorImager (Molecular Dynamics) scanning indicated that the fusion species produced upon integration of IRES–*βgeo*pA was 5-fold more abundant than the normal message (Fig. 2*C*). This is in accord with the suggestion that the native 3′ UTR of the DIA/LIF gene acts to reduce mRNA half-life. The increased level of fusion transcript in these cells was reflected in the production of biologically active DIA/LIF protein; 3- to 6-fold more DIA/LIF was present in conditioned medium prepared from differentiated cultures of cells with targeted truncations than from the parental cells or cells targeted with the nontruncating construct. Thus, the fusion transcript is a functional dicistronic mRNA and the targeting event has modified the activity of the DIA/LIF gene. The Oct-4 fusion transcript, on the other hand, was less abundant than wild-type Oct-4 mRNA by a factor of 10–20. This could be attributable to inefficient utilization of the *en-2* splice acceptor but might also arise from deletion of either stabilizing elements within the mRNA or an enhancer within the gene.

Histochemical staining for β-galactosidase was employed to monitor the cellular specificity of mRNA production from the targeted loci. Fig. 3 shows the staining patterns of ES cell cultures harboring targeted integrations into either the Oct-4 or the DIA/LIF gene. In the Oct-4-targeted cultures, activity was restricted to undifferentiated ES cells and was not detectable in differentiated cells, mirroring the expression of Oct-4 mRNA and protein (36). The DIA/LIF integrants did not show visible staining in undifferentiated cells, but β-galactosidase activity was apparent to different degrees in subpopulations of differentiated cells. This was more readily detected with the pA truncations, as expected given the increased expression of this fusion transcript. The absence of staining in undifferentiated cells reflects the limits of sensitivity of the histochemical assay, since these cells do express functional neomycin phosphotransferase. β-Galactosidase activity could in fact be detected in undifferentiated DIA/LIF-targeted cells by using the more sensitive fluorescent substrate. Enhanced expression of DIA/LIF during ES cell differentiation has been documented previously and postulated to play a role in the feedback regulation of stem-cell renewal (34), but the low levels of transcript have proved to be beyond the limits of reliable localization by *in situ* hybridization in our hands. In contrast, the ease of detection of β-galactosidase has enabled ready detection of cellular sites of transcription from the targeted locus. The presence of elevated β-galactosidase activity specifically in those differentiated cells adjacent to ES cells (Fig. 3) is intriguing. This visualization is suggestive of a "stem-cell niche" in which the differentiated progeny maintain the stem cells via production of DIA/LIF. The latter may even be induced by a cytokine released by the ES cells themselves (34). This evidence is consistent with the proposition that local regulation of DIA/LIF expression may be an important component of the developmental and homeostatic mechanisms which govern stem-cell fate (34, 44).

The *in vitro* studies illustrate the potential of the dicistronic targeting strategy for the resolution of cellular profiles of gene expression. However, the possibility of tissue specificity of IRES function constituted a potential limitation of this approach. To address this issue we made a series of random IRES gene traps by electroporation of pGTIRES*β*geopA into ES cells. Several clones which exhibited widespread expression of β-galactosidase in differentiated cell types *in vitro* were used to produce aggregation chimeras. At 7.5 and 8.5 days of development, β-galactosidase could be detected in all tissues colonized by the ES cells—i.e., throughout the embryo and in the amnion and visceral yolk sac (Fig. 4 and data not shown). These gene traps have been transmitted through the germ line, confirming that the presence of the IRES is compatible with functional gametogenesis, and preliminary

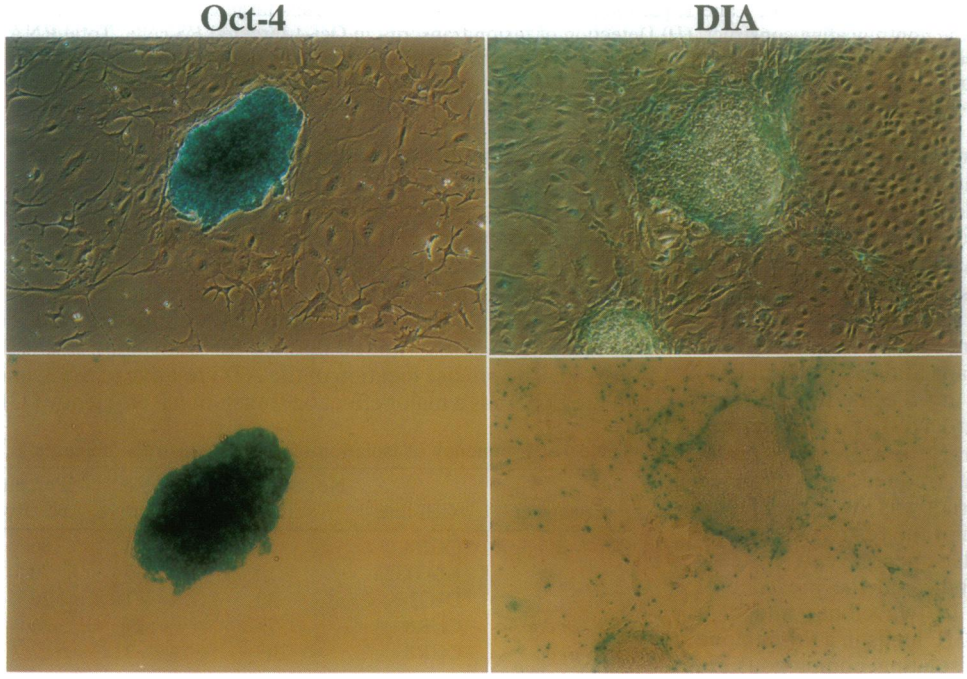


FIG. 3. β-Galactosidase reporter expression in targeted ES cell cultures. Mixed cultures of ES cells containing both undifferentiated and differentiated cells were stained for β-galactosidase. Samples were photographed under phase-contrast (*Upper*) and bright-field (*Lower*) optics. In the Oct-4-targeted culture (*Left*), staining is confined to the phenotypically undifferentiated cells. In the DIA–IRES–*β*geopA-targeted culture (*Right*), staining is not evident in undifferentiated cells but is detectable at various levels in differentiated cell types. Staining is most intense in those cells adjacent to undifferentiated colonies.

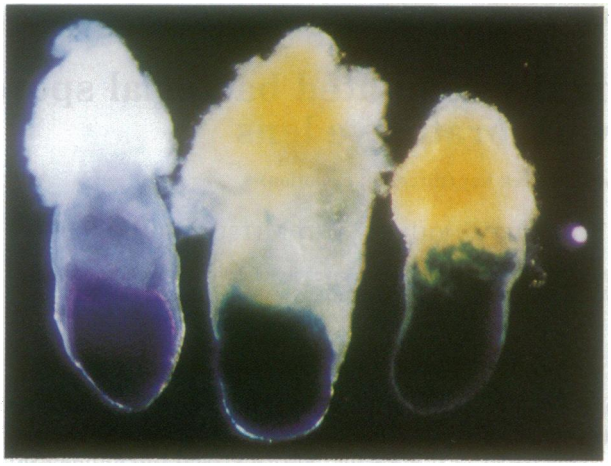

FIG. 4. β-Galactosidase reporter expression from the Oct-4 locus in chimeric embryos matches expression of the endogenous Oct-4 gene. Representative 7.5-day mouse embryos are shown. (*Left*) Detection of Oct-4 mRNA in nonmanipulated embryo by *in situ* hybridization. (*Center*) β-Galactosidase staining of aggregation chimera produced with Oct-4-targeted cells, showing restriction of enzymatic activity to the primitive ectoderm. (*Right*) Widespread β-galactosidase staining of aggregation chimera produced with ES cells containing a random gene-trap insertion of pGTIRES-βgeopA.

analyses on the heterozygotes indicate that the IRES is functional in a wide variety of embryonic and adult tissues.

Aggregation chimeras have also been produced with the Oct-4-targeted cells. The staining pattern of such embryos at 7.5 days is shown in Fig. 4 along with a whole-mount *in situ* hybridization to Oct-4 mRNA in wild-type embryos. This comparison shows that the tissue-specific distribution of Oct-4 mRNA is accurately reflected by the β-galactosidase expression pattern.

The experiments reported here establish that the use of IRES targeting is a powerful means of reporting and modifying mammalian gene expression. Further, it is apparent that nondisruptive integration of an IRES-linked marker into a 3′ UTR provides a convenient means for introducing subtle mutations into a gene. Moreover, the IRES strategy is not limited to modification of endogenous genes and the introduction of reporters but is equally applicable to the controlled expression of transgenes. The desired specificity and levels of transgene expression could be ensured by the use of IRES-mediated translation either in genomic constructs for pronuclear injection or following homologous integration into an appropriate locus. The latter could be achieved by the construction of polycistronic vectors containing two IRES elements (9). Alternatively, sequential rounds of homologous replacement (45) or targeting followed by recombinational deletion of the selectable marker (46) could be employed to introduce an IRES expression cassette with minimal disruption into any genes which are not expressed in ES cells. In general, therefore, the flexibility and utility of IRES-mediated translation seem likely to find widespread application in transgenic research.

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