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Use of an internal ribosome entry site for bicistronic expression of Cre recombinase or rtTA transactivator

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ABSTRACT

Conditional gene targeting depends on tissue and time specificity of recombination events. Endogenous promoters are often used to drive various transgenic constructs. To avoid the problems associated with reconstituting a specific expression pattern in transgenic animals by this method, we tested the internal ribosome entry site of the encephalomyocarditis virus, to enable linkage of the Cre recombinase or rtTA transactivator to 3′ untranslated ends of endogenous genes. Here we report that these constructs function effectively in COS cells. The data suggest that these cassettes will be appropriate for 3′ targeting of mouse genes.

In conditional gene targeting, a recombinase protein acts on its artificially incorporated recombination elements to delete or invert a selected part of the genomic DNA. When the recombinase is expressed inducibly or in a tissue restricted pattern, the genomic recombination will take place in a restricted set of cells at a specific time point (1,2). The most widely used recombination system is Cre/loxP from phage P1 (3), but also the yeast Flp/frt system has been shown to work (4). Inducible gene expression can be achieved in several ways in the mouse. The best characterised system is the tetracycline transactivator tTA, where the tetracycline repressor tetR from transposone Tn10 is fused to the transcriptional activating domain VP16 of herpes simplex virus (5). Mutations of four amino acids in the tetR domain of tTA lead to a reversed transactivator, rtTA, in which the addition of tetracycline analogues results in induction instead of repression of transcription from a minimal promoter containing tetracycline responsive operons (6). Both tTA and rtTA have been shown to work in transgenic mice (7) as well as by viral delivery to adult animals (8). Inducible knockouts can then be made by breeding a mouse having a desired target flanked by loxP sequences, to a double transgene carrying a tetO driven Cre and the tTA or rtTA transactivator and induce the activity with the tetracycline analogue doxycycline (9). If the transactivator expression is patterned an inducible tissue-specific knockout may result.

To obtain the necessary tissue restricted expression of Cre or a tetracycline responsive transactivator, the two major approaches have been to use viral expression systems or transgenes. The drawbacks of the viral systems are problems in stable viral gene delivery and that viral infection has to be repeated in every mouse. This introduces the probability that expression will not be uniform. The transgenic approach is limited by difficulties in obtaining the normal expression pattern from a promoter lacking some of its additional enhancer elements. There may be ectopic expression and considerable variation between different founder lines, attributed to differences in integration site and copy number. Another approach taken is to knock-in the Cre recombinase gene into an endogenous gene (10). This results in a correct expression pattern, but also in a knockout of the targeted gene. In some cases this may not be a problem, but phenotypes can be found in most heterozygous knockouts and it may be difficult to identify mild phenotypic effects of a tissue-specific conditional knockout in the background of the heterozygous knockout. By applying an internal ribosome entry site (IRES) it is possible to circumvent the problems with transgenic variability and to knock-in a cDNA without destroying the targeted gene (11). Our aim is to use the encephalomyocarditis virus (EMCV) IRES to link expression of Cre recombinase and rtTA transcriptional activator to that of endogenous genes, thus obtaining restricted expression in the same pattern as that of the endogenous genes.

The EMCV IRES is a type 2 IRES shown to work efficiently in vertebrate cells as well as in transgenic mice (12,13). This sequence seems to be more widely functional than IRESs from the type 1 family (14), but it is still unclear if it works in all types of vertebrate cells. We fused the IRES sequence from EMCV (13) upstream of a Cre recombinase gene equipped with a nuclear localisation signal (15), creating a generally applicable IRES-Cre cassette. We also made a fusion of EMCV IRES to the rtTA transactivator lacking nuclear localisation signal (6). The type 2 family of IRES sequences is very sensitive to changes in the sequence surrounding the second cistron initiation ATG (16,17) and the EMCV IRES derivative used here is mutated from the wild-type. Furthermore, neither the wild-type EMCV nor our cassettes have the optimal vertebrate sequence for translation initiation (12,18; Fig. 1A). Nevertheless, this IRES mutant has been shown to work in transgenic mice (13). To test our cassettes we made bicistronic vectors by cloning them downstream of the human neurotrophin-3 (NT-3) cDNA (19) in the expression plasmid pXM (20; Fig. 1B). To ascertain IRES derived effects, the Cre and rtTA genes were also inserted directly after NT-3, without the intervening IRES. The different expression vectors were electroporated into COS cells as previously described (19).

Total cellular protein from the Cre-series of electroporated COS cells was obtained by lysing 4×10^6 cells in 1 ml of lysis

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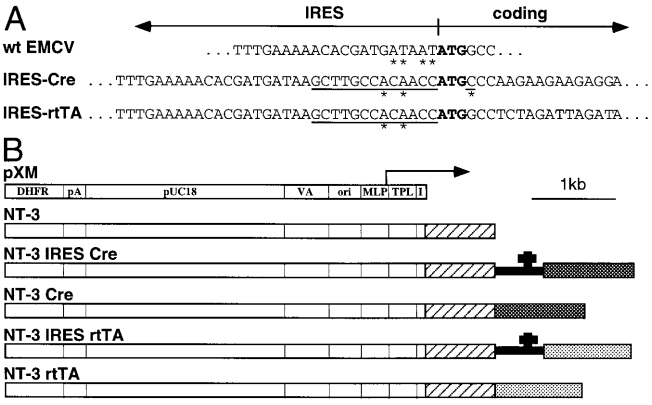

Figure 1. (**A**) Sequence comparison of wild-type EMCV IRES and the junctions between IRES and Cre or rtTA in our cassettes. The preferred initiation codon is shown in bold. Underlined sequences indicate changes from the wild-type EMCV. Nucleotides that do not conform to the optimal vertebrate translation initiation sequence are marked with asterisks. (**B**) pXM expression vector constructs used to verify the function of our IRES-Cre and IRES-rtTA cassettes in COS cells.

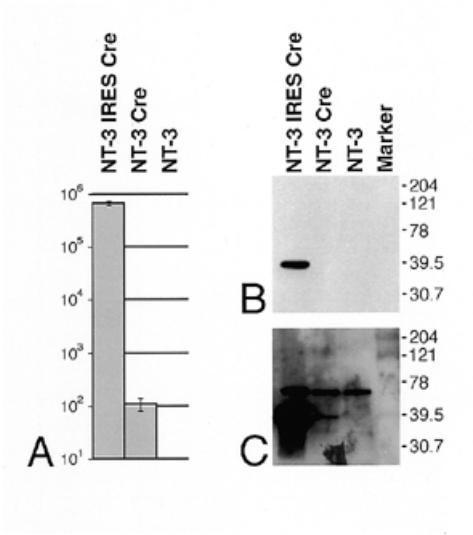

Figure 2. (**A**) Densitometric quantification of produced Cre protein. Mean ± SD of three independent experiments. (**B**) The expected 39 kDa Cre protein is seen on the blot and (**C**) on an overexposure of the same blot together with a 65 kDa non-specific band.

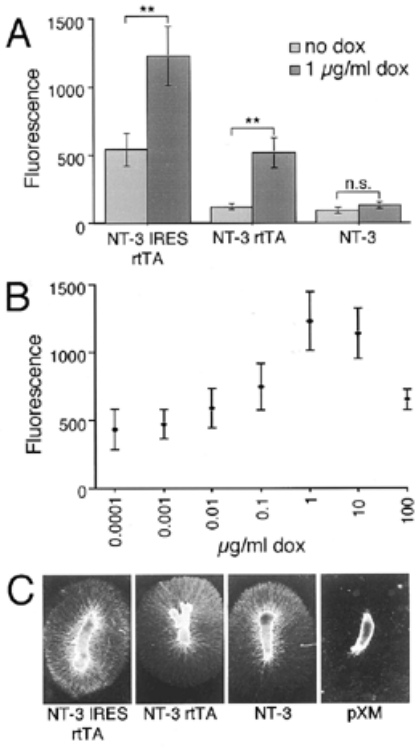

Figure 3. (**A**) β-galactosidase activity in COS cells co-electroporated with the reporter plasmid and NT-3 IRES rtTA, NT-3 rtTA or NT-3. The rtTA transcriptional activator was or was not induced with 1 μg/ml doxycycline (dox) as indicated. (**B**) A dose response curve for doxycycline induced β-galactosidase in cells electroporated with NT-3 IRES rtTA. Data represent six independent experiments. (**C**) Neurite extension from chicken E9 Remak's ganglion in a biological assay for NT-3 activity. pXM without insert served as a negative control.

buffer (1% SDS, 10 mM Tris pH 7.4, 1 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF), boiling for 10 min, passing the lysate repeatedly through a 21, 23 and 27 gauge needle and finally removing the debris by centrifugation at 4°C, 13 500 *g* for 10 min. Five micrograms of protein were immunoblotted using mouse anti-Cre antibody (21; MMS106, Babco), peroxidase conjugated anti-mouse IgG antibody (A-2304, Sigma) and ECLplus detection (Amersham). Comparing NT-3 IRES Cre and NT-3 Cre, we conclude that the EMCV IRES makes translation from the second cistron 10^3–10^4 times more efficient (Fig. 2A). As can be seen in Figure 2B, our IRES-Cre cassette works well in COS cells and the expected 39 kDa Cre protein is produced. Over-exposure (Fig. 2C) indicated weak Cre production from the NT-3 Cre vector. This small leakage from the second cistron in a bicistronic vector lacking an IRES sequence has been seen previously (22).

β-galactosidase produced from a tetracycline responsive lacZ reporter plasmid (pUHG 16-3, M.Gossen, unpublished) was assayed as a measure of the rtTA activity. 4×10^6 cells from the rtTA-series of electroporated COS-cells were lysed in 1 ml of dH_2O, whereafter debris was pelleted by centrifugation at 4°C, 13 500 *g* for 5 min. Supernatant containing 5 μg of protein was assayed immediately in 200 μl of 50 μM phosphate buffer pH 7.2, 1 mM $MgSO_4$, 0.2 mM $MnSO_4$, 2 mM EDTA, 0.05% azide and 0.1 mM methylumbelliferyl-β-galactoside. The accumulation of methylumbelliferone (excitation wavelength 365 nm, emission wavelength 450 nm) was measured in a microplate fluorimeter (Dynatech Microfluor) after 4 h of incubation at room temperature. Addition of doxycycline to the culture media induces β-galactosidase activity in COS cells co-electroporated with the reporter plasmid and a plasmid expressing rtTA, but not with the NT-3 control vector (Fig. 3A). The 2–3-fold induction in cells electroporated with NT-3 IRES rtTA is very low compared with reported inductions of several orders of magnitude with the rtTA system (6). We believe that the extremely strong pXM/COS cell expression system overloads the cells with rtTA, resulting in leaky transcription of the lacZ reporter and therefore high background. Moreover, since the reporter plasmid lacks a SV40

origin it will not replicate in COS cells, limiting the maximum production of β-galactosidase. With the NT-3 rtTA construct, lacking IRES, the amount of rtTA produced was sufficient to mediate induction while the non-induced background level is not significantly elevated from that of the NT-3 controls. From this we conclude that the IRES-rtTA system works, but may become leaky if too strongly expressed. A dose response curve for doxycycline with cells electroporated with NT-3 IRES rtTA (Fig. 3B) correlates to previous studies in HeLa cells (6) and shows optimal induction of the reporter at a concentration of 1 μg/ml and toxic effects at higher doses. Conditioned COS-cell media from the electroporations was tested in a neurite extension assay for biologically active NT-3 (23). In accordance with previous studies (16), no alterations in NT-3 levels could be detected when we introduced a second cistron, IRES or not, into the NT-3 expression vector (Fig. 3C; Cre-series not shown).

In this paper we have shown IRES-Cre and IRES-rtTA cassettes to work well in cultured cells. It remains to be shown that these cassettes work in mice when driven bicistronically by an endogenous tissue-specific promoter and we are currently pursuing this issue. The approach to obtain tissue-specific expression for conditional knockouts outlined here should also be applicable to other systems, such as the fusions of Cre or Flp to various steroid hormone receptor ligand binding domains.

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