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A reliable way of obtaining stable inducible clones

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ABSTRACT

Inducible gene expression systems provide a powerful tool for the analysis of gene product functions. The 'Tetracycline (Tc) expression system' has been widely and successfully used in many instances. However, this system remains somewhat tedious to use due to: (i) the establishment of a primary cell line constitutively and stably expressing the Tc-regulated transactivator and (ii) the obtention of a secondary line expressing the gene of interest in a Tc-dependent manner. In order to facilitate these two critical steps, we devised an efficient and molecular biology-free strategy allowing the successful selection of clones expressing any cDNA under tight regulation.

The 'Tetracycline (Tc) expression system' relies on the constitutive and stable expression of a Tc-regulated transactivator (tTa) (primary transfection), which will then drive the expression of the gene of interest inserted downstream of a tTa responsive promoter (secondary transfection) (1). This expression is an inverse function of the Tc concentration (Tc-off system) of the culture medium. One of the major problems encountered when using the Tc system is the establishment of a reliable primary cell line expressing tTa in a constitutive and stable manner. Indeed, it has often been reported that tTa expressing cells were unstable or difficult to obtain. It was also shown that the amount of tTa necessary for the system to work is actually low (10 000 molecules/cell). We thus designed a new strategy to obtain cell lines stably expressing tTa. To that end, we constructed a vector which codes for the puromycin resistance under the control of a tTa responsive promoter (tetO-CMV) (Fig. 1A). This vector was cotransfected in C2C12 and 3T3 cells in Tc-free medium with pUHD15-1 (encoding the tTa under CMV control). Three days after transfection, the cells were passaged (1–3) and puromycin (2.5 μg/ml) was added, still in the absence of Tc. After 2 weeks, puromycin resistant clones were picked and grown in duplicate 24 well plates. Both were grown in the presence of puromycin, but only one was kept in the presence of 1 μg/ml Tc. The clones growing in Tc-free medium but dying in the presence of Tc (i.e. ~20%) were selected as primary cells, since they match the criteria of primary tTa lines: stability of a constitutively expressed functional transactivator, insured by its capacity to drive the expression of the puro^r cassette only in the absence of Tc (Fig. 1A). Six of these clones were further characterized by transiently transfecting a tetO-CMV-luc reporter (pUHD13-3). All were found to be inducible in a Tc-dependent manner and to display low basal levels, even though the absolute induction factor was variable from one clone to the other (from ~10–100× in these transient assays). The other clones, representing ~80% of the puromycin resistant clones, were growing in the presence of Tc, reflecting integration events whose net result is puro^r expression in a tTa-independent manner.

Once a stable tTa line is available, the other caveat of the Tc inducible system is the obtention of a secondary line expressing the gene of interest under tight regulation. An elegant procedure has previously been presented by Kirchoff *et al*. (2). They used a vector containing a dicistronic sequence consisting of the gene of interest, an IRES and the secreted embryonic alkaline phosphatase (SEAP) cDNA, under the control of a tetO-CMV promoter. However, their method requires the induction of the clones being screened for their Tc-dependent SEAP expression, and thus eliminates the isolation of clones expressing cytotoxic products. We developed another approach in which the secondary lines were derived from primary tTa lines by cotransfection using a pGK-hygro construct and pTIS-X, a bicistronic vector containing X, the gene of interest, and the IRES-SEAP sequence, in Tc-containing medium (Fig. 1B). Hygromycin selection was applied 48 h post transfection and clones were picked after 2 weeks. After growth for 2 days in duplicate 96 well plates in phenol red-free medium, either in the absence (test plate) or in the presence (stock plate) of 1 μg/ml Tc, 50 μl of culture supernatant from each clone were transferred to a microtitration plate. In order to eliminate non-SEAP phosphatase activity (from the FCS used in the culture medium), plates were heated at 60°C for 10 min, and SEAP activity was measured as follows using a simple colorimetric assay (3). Fifty μl of $MgCl_2$ 2 mM, Diethanolamine 2 M and PNPP 10 mM were added to the culture supernatant and incubated at 37°C. After 1 min to 10 h, the clones displaying SEAP activity were visualized by their strong yellow coloration. More than 80% of these positive clones remained negative in the presence of Tc, indicating a Tc-dependent expression of the dicistronic sequence encompassing both the cDNA of interest and the SEAP coding sequence (Fig. 1C). After expansion from the stock plate, further analysis of these SEAP positive clones revealed that most of them (>80%) were indeed expressing the gene product of interest in a Tc-dependent manner (Fig. 2).

We were thus able to successfully express different gene products in different cell types without having to go through the tedious and time consuming western analysis of clones based solely upon their ability to express a drug resistance, which is not a reflection of their ability to respond to Tc. Furthermore, this

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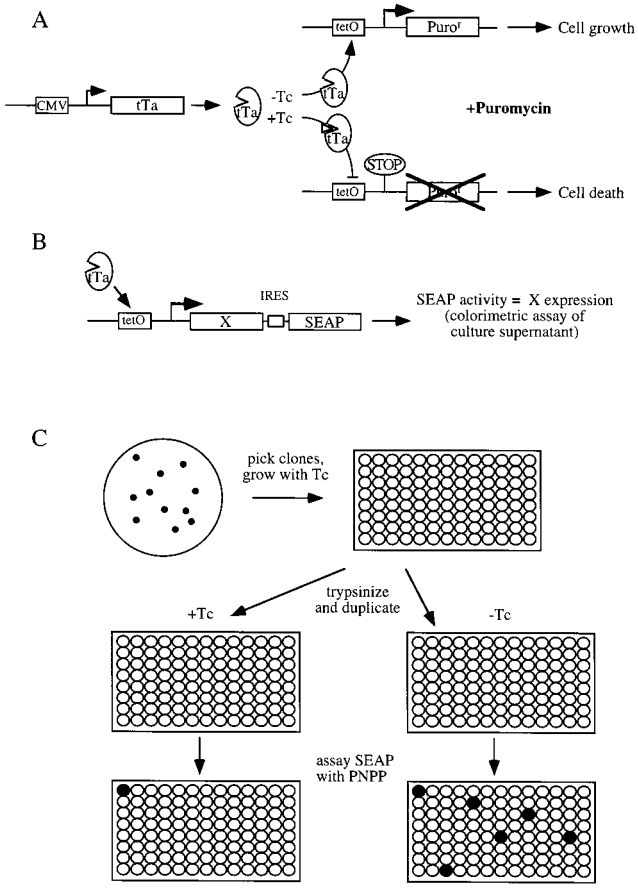

Figure 1. (**A**) Strategy for the primary cell-line selection. The tTa responsive promoter drives the expression of the puro^r cassette, allowing for cell growth in the absence of Tc and puromycin, while the cells die in the absence of Tc and the presence of puromycin. (**B**) Construction used for the selection of secondary cell lines. The gene of interest is expressed as a dicistronic transcript together with the coding sequence of the secreted alkaline phosphatase. (**C**) Selection of positive secondary clones. Clones were picked from the master plate after 15 days of selection using yellow tips, and transferred to a 96 well plate, were they were expanded for 2–3 days, in the presence of Tc. Using a multichannel pipette, the clones were then trypsinized and split in duplicate plates, in 10% FCS DMEM phenol-red free medium, with (stock) or without Tc (test). After 24–48 h, 50 µl from each well were assayed for SEAP activity as described in the text and the positive clones were identified, after 1 min to 10 h, by the yellow coloration. The positive clones found exclusively in the Tc plate were further expanded from the stockplate.

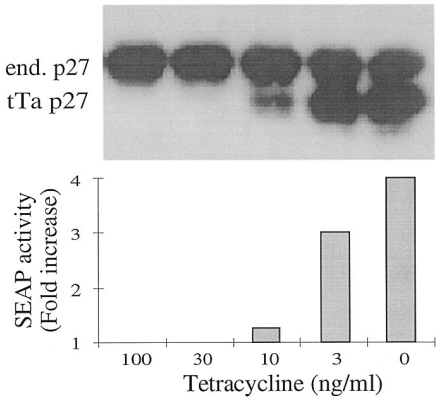

Figure 2. Example of Tc-dependent gene expression and its correlation with the SEAP colorimetric assay. The supernatants of a multiwell plate containing tTa cells stably transfected with pTIS-p27, treated with the indicated amount of Tc, were analyzed as described in the text for SEAP activity (bar graph). Each well was processed in parallel for western blot analysis using a p27 antibody. The endogenous p27 is detected in all conditions (end. p27), whereas the exogenous p27 (tTa p27), slightly smaller, is expressed in a Tc-dependent manner. One should notice the very tight regulation, since no exogenous p27 is detected in the presence of 100 ng/ml Tc.

stategy ensures that: (i) the primary cell line is stable and functional for tTa expression (otherwise the cells would be counterselected by puromycin in the presence of Tc) and (ii) the secondary line can be easily selected in duplicate wells, thus avoiding having to express potentially toxic gene products during the selection process. Even if the expressed products would result in cell death (which would be visible by microscopic observation), the concomitantly secreted alkaline phosphatase would still give a positive reading.

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