

A dicistronic construct allows easy detection of human CFTR expression from YAC DNA in human cells

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ABSTRACT

We have made a dicistronic construct where the picornaviral internal ribosome-entry site (IRES) driving the expression of the β -geo gene has been inserted into the 3' untranslated region of the human *CFTR* gene present in a YAC. When introduced into the human cell line Caco-2 expressing the *CFTR* gene, the expression of the dicistronic gene can be detected by lacZ staining and follows the accumulation of the endogenous *CFTR* mRNA upon differentiation of the cells. These data demonstrate that this IRES-based approach presents an alternative to mRNA *in situ* hybridisation and allows detection of expression in an autologous system.

Yeast artificial chromosomes (YACs) are powerful tools in the analysis of gene function and regulation as they are large enough to clone most mammalian genes intact with their associated long range transcriptional controlling elements. In addition the very large cloned DNA can be subtly modified using homologous recombination in the yeast host before being transferred intact into cells or transgenic mice (see review, 1). These techniques have been used to characterise genomic elements involved in the developmental regulation of the β -globin gene (2,3) and to study the hormonal regulation of the Apo (a) gene (4).

We have previously characterised a YAC (yCFTR) carrying the 200 kb human cystic fibrosis transmembrane regulator (*CFTR*) gene and have shown that it is well expressed in transgenic mice and complements the null CF phenotype (5). *CFTR* is expressed at very low levels and it was necessary to use ^{35}S *in situ* hybridisation to demonstrate the tissue specific expression of the YAC transgene. In this report, we are describing an alternative way to detect the expression of this *CFTR* gene, where an internal ribosome-entry site (IRES) driving expression of a β -geo gene (fusion between β -galactosidase and neomycin resistance gene) is used. IRES elements have previously been used to study gene expression, either in mice (6), in tissue culture (7) or to detect the expression of virally encoded transgenes (8). We have now introduced a picornaviral IRES- β geo cassette (6) into the 3'-end of the *CFTR* gene on the YAC.

The dicistronic YAC, yCFIRES, was constructed using the pop-in-pop-out procedure (9,10). A gene targeting vector was

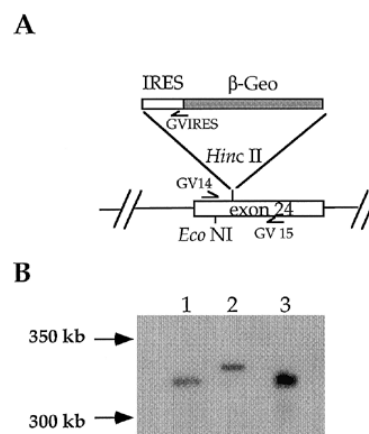


Figure 1. (A) Construction of the dicistronic YAC. The targeting vector pRS-IRES was constructed as follows. The PCR product GV14/GV15 (nt 4392–4415 and complementary to 5340–5360, respectively, in the *CFTR* cDNA, GenBank accession number M28668) was cloned into pCRII (Invitrogen). The *XhoI*–*NotI* fragment containing the IRES- β -geo cassette from pIRES- β Geo (6) was blunt ended with T4 DNA polymerase and cloned into the unique *HincII* site of pCRII-GV14/GV15. The insert was then excised with *SpeI* and *XhoI* and cloned into the same sites in the polylinker of pRS406 (Stratagene) to give the pop-in-pop-out targeting vector pRS-IRES. pRS-IRES was linearized (partial digestion with *EcoNI*) and used to transform yeast spheroplasts of a *ura*⁻ mutant of the original yCFTR selected by plating on 1 mg/ml 5FOA. The pop-out procedure (9,10) was then applied. (Primer GVIREs: 5'-ACGTCCCGTGGTTCGGG-3'). (B) High molecular weight yeast genomic DNA was separated on a 1% agarose gel and examined by Southern blot analysis with a *CFTR* cDNA probe. Lane 1, original yCFTR YAC; lane 2, pop in YAC clone; lane 3, pop out YAC clone.

generated, linearised within the segment of homology and transfected into yeast spheroplasts containing the 320 kb yCFTR. PCR analysis was performed to select the homologous integration of the targeting vector, using the primers GV14/GVIREs (Fig. 1A). Pulse-field gel analysis performed on one of the pop-in recombinant clones selected by PCR revealed that the YAC had increased in size (Fig. 1B, lane 2). The pop-out recombinants were screened by PCR and the selected clones containing the IRES- β geo element were positive with the primers GV14/GVIREs (Fig. 1A) and negative with the primers GV14/GV15 (Fig. 1A). Pulse-field gel analysis of one of these recombinants (yCFIRES) showed that the YAC had

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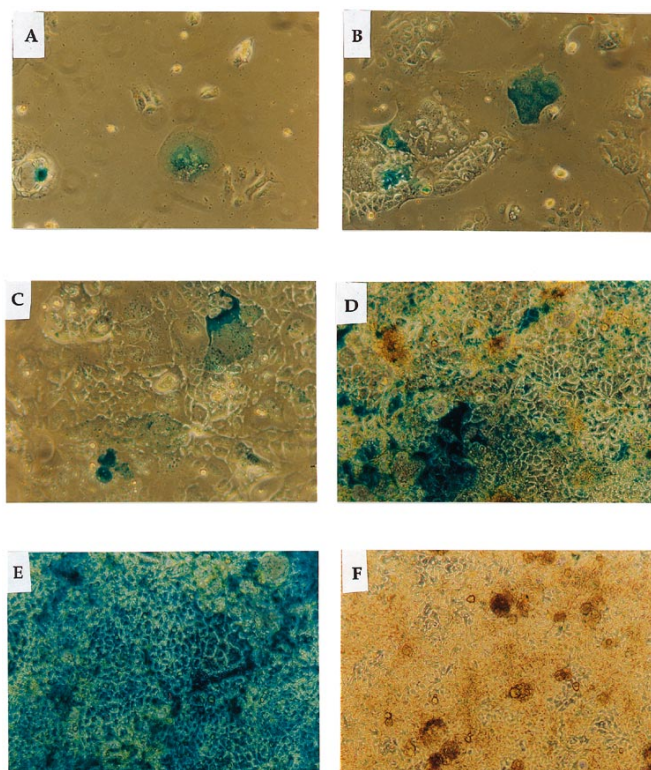


Figure 2. Differentiation-dependent expression of the dicistronic *CFTR* gene in Caco-2 cells. Transfer of the YAC into Caco-2 cells was carried out as described previously (12). Caco-2 cells stably transfected with the dicistronic YAC were seeded at 2×10^5 cells per cm^2 in Dulbecco's modified Eagle's medium containing 20% fetal calf serum and 400 $\mu\text{g}/\text{ml}$ of G418. An assay for β -galactosidase was performed on days 1 (A), 3 (B), 7 (C), 12 (D) or 20 (E) after seeding. No lacZ staining was detectable at these times on untransfected Caco-2 cells as shown for 20 days after seeding (F).

returned to an intermediate size, between the original yCFTR and the pop-in clone (Fig. 1B, lane 3) as expected, and no other rearrangements were detected. The percentages of correct homologous recombination were 50% for the pop-in and 61% for the pop-out steps.

yCFIRES was then introduced into the human colon adenocarcinoma cell line Caco-2, a cell line able to differentiate into an enterocyte-like cell and already reported to express a fully functional CFTR protein (11). Polyethylene-glycol mediated fusion (12) of yeast spheroplasts with these human cells generated, from a typical fusion experiment, between one and two G418-resistant colonies per 2×10^6 Caco-2 cells. Four independent colonies were then expanded and expression of the dicistronic yCFIRES was assayed by staining for lacZ activity. In these experiments, 2×10^5 cells per cm^2 were seeded and a β -galactosidase assay was performed 1, 3, 7, 12 and 20 days after seeding. The results presented in Figure 2 clearly show that the staining is barely detectable when the cells are sparse and actively dividing (Fig. 2A, day 1). The staining increases when the Caco-2 cells are confluent (Fig. 2B–D, days 3, 7 and 12, respectively) until the cells are fully differentiated (Fig. 2E, day 20).

In Caco-2 cells, the differentiation process has previously been shown to be accompanied by a 10-fold increase in mRNA level, while the content of the mature 170 kDa CFTR protein decreases (11). The results presented in Figure 2 indicate that the β -geo protein produced from the dicistronic gene follows the level of mRNA rather than the complex post translational regulation of the CFTR protein as expected.

As two weeks of exposure are usually necessary to detect the *CFTR* mRNA by ^{35}S *in situ* hybridisation (5), this approach provides a rapid and simple alternative for detection of mRNA expression. yCFTR is able to complement null CF mice (5) and the dicistronic yCFIRES will be used to characterise the as yet unidentified DNA elements involved in the regulation of expression of the *CFTR* gene, after deletion of candidate regulatory regions (13,14) by homologous recombination in yeast. This technique should be generally very useful for reporting the expression of a functional gene present in a YAC in an autologous system.

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