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Abstract

Reporter gene systems have been developed to allow investigators to visually identify transformed cells and to follow the transcriptional activity from promoter regions preceding the reporter genes. These reporter genes encode protein products which can be easily assayed and which can be strained for in the transformed cell.

Use of the Neurospora tyrosinase gene as a reporter gene in transformation experiments.

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Reporter gene systems have been developed to allow investigators to visually identify transformed cells and to follow the transcriptional activity from promoter regions preceding the reporter genes. These reporter genes encode protein products which can be easily assayed and which can be strained for in the transformed cell. The *E. coli* beta-galactosidase and beta-glucuronidase genes have been extensively used as reporter genes. The presence of these proteins can be determined by staining with X-gal and X-gus and the enzyme levels can be easily quantified by enzymatic assay. Although widely used, these two systems have some drawbacks for the study of gene expression in eukaryotic systems. Since the X-gal and X-gus substrates do not efficiently penetrate eukaryotic cells, the cells must be lysed prior to staining. Being *E. coli* genes, the codon usage is quite different from the codon usage within the eukaryotic cell. The use of the Neurospora tyrosinase gene as a reporter would overcome these difficulties.

We have developed a vector which allows for the usage of the Neurospora crassa tyrosinase gene as a reporter system in transformation experiments. The tyrosinase gene encodes a 75,000 MW protyrosinase peptide which is subsequently proteolytically cleaved to give an active tyrosinase molecule (Kupper et al. 1990 Pigment Cell Research 3:207-213). Tyrosinase catalyses the formation of melanin (an insoluble black pigment) from tyrosine or DOPA. The tyrosinase gene has the appropriate codon usage, translation start site, intron splice sites, and polyadenylation/termination site for Neurospora. Since tyrosine can be translocated into the cells, there is no need to lyse the cells in order to stain for tyrosinase activity. Furthermore, the levels of tyrosinase activity can be easily assayed spectrophotometrically. Under most growth conditions the endogenous tyrosinase gene is repressed and the level of background tyrosinase activity from the endogenous gene is very low.

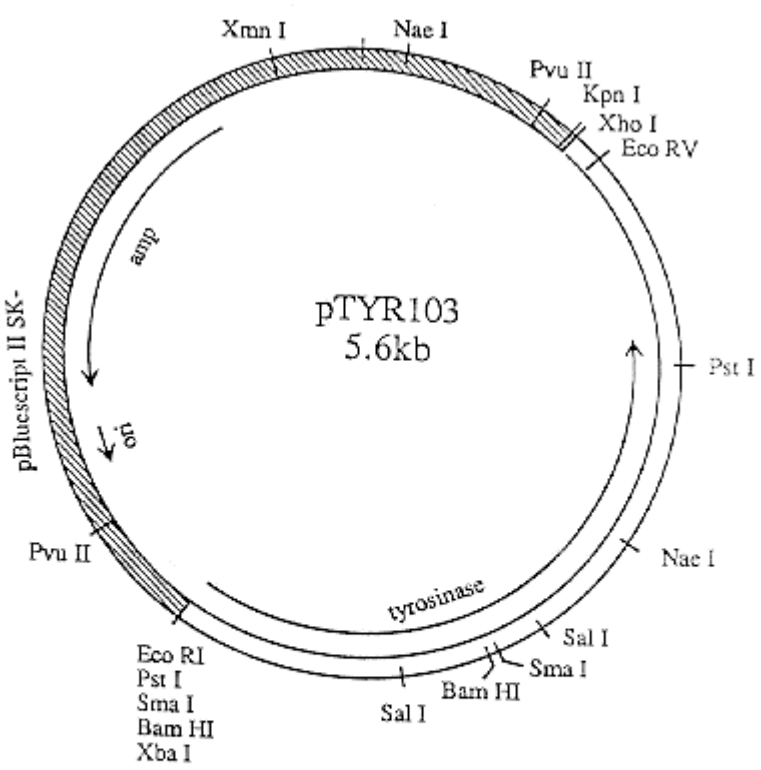
We isolated the tyrosinase gene by preparing three oligonucleotides encoding short regions of the published tyrosinase amino acid sequence (Lerch 1982 Proc. Natl. Acad. Sci. 35:3635-3639). These oligonucleotides were radioactively labeled and used to identify two cosmids in a cosmid library (Vollmer and Yanofsky 1986 Proc. Natl. Acad. Sci. 83:4869-4973) which contained the tyrosinase gene. The tyrosinase gene was subcloned from the cosmid into the pUC9 vector system and sequenced. The nucleotide sequence encoded the published amino acid sequence and closely matched the sequence of the tyrosinase gene which was published during the course of our research (Kupper et al. 1989 J. Biol. Chem. 264:17250-17258). We found that an *Eco*RI/*Xho*I fragment contained all of the coding sequences as well as 5' and 3' sequences (including the polyA addition site). This region was subcloned into the pBluescript vector to generate the plasmid pTYR1.

To prepare a reporter gene construct, we wanted to remove the 5' sequences up to a point near the start of translation site while retaining the coding and 3' sequences. Unwanted 5' sequences

were removed from the pTYR1 by *Bal*31 digestion. pTYR1 was linearized by digestion with the *Eco*RI restriction endonuclease and subjected to digestion with *Bal*31 exonuclease. Following the *Bal*31 digestion, *Eco*RI linkers were added to the digested ends. The DNA was then subjected to digestion with *Eco*RI and *Xho*I restriction endonucleases. The fragment containing the tyrosinase gene was purified by electrophoresis in an agarose gel and ligated into pBluescript vector which had been digested with *Eco*RI and *Xho*I. Sequencing of several plasmids allowed us to identify those which would be of value for use as reporter gene vectors. One of the plasmids, pTYR103, was chosen as an expression vector. pTYR103 contains 18 nucleotides of tyrosinase sequence upstream of the AUG start of translation codon. pTYR103 can be used in preparing constructs in which promoter regions are inserted upstream of the tyrosinase sequences. pTYR103 contains unique restriction sites for *Eco*RI and *Xba*I upstream of the tyrosinase gene. A map of pTYR103 is given as Figure 1. pTYR103 has been deposited in the Fungal Genetics Stock Center.

To test pTYR103 as a reporter system, we inserted the regulatory region from the Neurospora *grg-1* (*ccg-1*) gene upstream of the tyrosinase sequences. The resultant expression plasmid, pGRG-1/TYR103 was used to transform Neurospora cells. The cells used for the transformation were *aro-9;qa-2* mutants and the cells were cotransformed with the *qa-2*-containing pRAL-1 (Akins and Lambowitz 1986 Mol. Cell. Biol. 5:2272-2278) and the pGRG-1/TYR103 plasmids. After five rounds of conidial isolation, the transformants were spotted onto sorbose plates and allowed to grow for 2 to 3 days. One ml of a 10 mM tyrosine suspension was added to the plates. We found that 42 of 66 transformants from the cotransformation experiment stained black within a few hours while none of the transformants from a control transformation (transformation with pRAL-1 vector alone) stained. Figure 2 shows four transformants growing on sorbose medium and stained for tyrosinase activity. The upper left and lower right quadrants contain control transformants (pRAL-1 transformant). The upper right and lower left quadrants have pGRG-1/TYR103 cotransformants.

The protyrosinase encoded by the tyrosinase gene is an enzymatically inactive precursor molecule which must have the carboxyl terminus removed by proteolytic processing to become an active enzyme. Kupper et al. (1990 Pigment Cell Research 3:207-213) have recently shown that when the endogenous tyrosinase gene is induced in mycelia, the protyrosinase produced is inefficiently processed and most of the molecules remain in the inactive protyrosinase form. Upon disruption of the cell the protyrosinase is proteolytically processed to the active form. The inefficiency of intracellular proteolytic processing in mycelia was demonstrated by Western blot analysis and by the increase in tyrosinase activity seen as a function of incubation of the extract at 25øC prior to assaying for tyrosinase activity. We found the same phenomenon when looking at the protyrosinase produced from our expression system. However, we found that the levels of tyrosinase activity (processed protyrosinase) were adequate to allow for the convenient staining of transformed cells (Figure 2). The level of tyrosinase present in the transformants can be easily determined by an enzymatic assay. When quantitating the levels of tyrosinase activity in transformants, we suggest that the cellular extracts are dialyzed against 50 mM sodium phosphate buffer, pH 6, for at least two hours at room temperature to remove endogenous substrate and to allow for the proteolytic processing of the protyrosinase molecules. We have also found that we can follow the production of the tyrosinase mRNA in transformants by Northern blot.

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GGATCCCCCGGGCTGCAGGAATTCC GGCCATAGAGCAGCAACC  ATG  AGC  ACC
BamHI-SmaI--PstI--EcoRI-  ------------------  met  ser  thr
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Figure 1. A restriction endonuclease map of pTYR103. pBluescriptII SK- vector and tyrosinase sequences are shown in the striped and unstriped portions of the map respectively. The sites for various endonucleases are shown. pTYR103 has unique cloning sites for *Eco*RI and *Xba*I into which regulatory sequences can be inserted upstream of the tyrosinase gene. Below the map, the nucleotide sequence at the 5' end of the tyr gene in pTYR103 is shown. The *Bam*HI, *Sma*I, *Pst*I and *Eco*RI sites are from the pBluescript vector. The initiation of translation codon and subsequent codons are denoted.

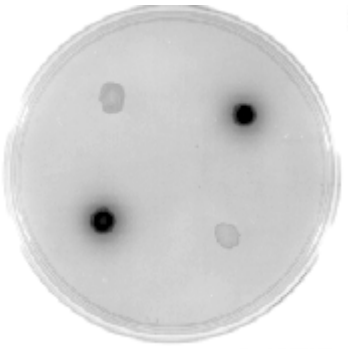

Figure 2. Staining of transformants containing pTYR103 plasmid sequences. pGRG- 1/TYR103 transformants (in upper right and lower left quadrants) and control transformants (transformed with pRAL1) were placed on a sorbose plate and allowed to grow for

2 days. The transformants were stained for tyrosinase activity by adding 1 ml of 10 mM tyrosine suspension to the plate. The plate was photographed 4 hours after adding the tyrosine.