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The Effects of a Keratin Promoter on the Expression of Mouse Tyrosinase in Various Cultured Cell Types via Retroviral Infection

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Summary—A retroviral vector was constructed containing a cDNA for mouse tyrosinase under control of a modified (with intron removed) chick feather keratin promoter. Cultured chick embryo keratinocytes infected with this vector showed bright fluorescent labeling with an antibody (T1) specific to mouse tyrosinase, but did not produce visible pigment. Cultured chick embryo fibroblasts infected with the vector also showed positive fluorescent labeling with the T1 antibody, but the labeling appeared less bright than that seen in infected keratinocytes. Cultured albino (c^a/c^a) melanocytes infected with the vector developed dark, discrete pigment granules. Mock infected keratinocytes, fibroblasts, and melanocytes had little or no background immunofluorescence when labeled with T1. Chick hepatocytes infected with the keratin vector could not be distinguished from mock infected hepatocytes cultures, even by T1 labeling. These results suggest that the modified keratin promoter imparts partial tissue specificity to genes under its control. The tyrosinase expression seen in keratinocytes, and the pigment production seen in albino melanocytes infected with the keratin vector may make it a vector useful for tagging gene transfer in albino chickens.

Key words: Transfection, Retrovirus, Tyrosinase, Mouse, Chicken, Keratin Promoter, Melanocyte, Keratinocyte, Hepatocyte

Introduction

Gene transfer by retroviral vectors is very effective in chick embryos and cultured cells because a single infection transfers the gene into many cells (Shuman, 1991, Brumbaugh, and Oetting., 1989). Hughes *et al.* (1986, 1987, 1990), developed a useful series of vectors based on the avian leukosis virus (ALV). One type vector, a replication

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competent virus, showed effective infection and highly expressed inserted transgenes (Crittenden *et al.*, 1989). This replication-competent ALV-based vector (RCAN) was used in this study to elucidate the expression of mouse tyrosinase in cultured chick embryo cells. For the insertion of genes into chickens or cells, a marker gene that is easily identifiable through inspection is potentially useful. Tyrosinase may provide such a marker by producing easily visible pigment in the skin and feathers of birds that receive the gene. A cDNA for mouse tyrosinase has been isolated, sequenced (Yamamoto *et al.*, 1987), and used to produce melanin in albino mouse melanocytes (Yamamoto *et al.*, 1989). This gene has since been used to study the expression patterns of several promoters in mice.

Albino chickens, which do not normally produce functional tyrosinase, have also been used, and the mouse tyrosinase gene has been shown to function in embryo cells from these chickens. RCAS (Hughes *et al.*, 1987) vectors have been constructed which contained the cDNA for mouse tyrosinase with constitutive, mouse, or quail tyrosinase promoters. Mouse tyrosinase was expressed with the constitutive promoter, and resulted in pigment production in cultured albino chick melanocytes (Whitaker *et al.*, 1989, Frew *et al.*, 1992, Akiyama *et al.*, 1994). The same construct was used to infect several chick embryo cell types, all of which expressed the gene and turned dark (Frew *et al.*, 1992). In non-melanocytes, the expression of tyrosinase resulted in morbidity and mortality, probably due to the production of toxic intermediates in the pigment production pathway (Frew *et al.*, 1992).

The constitutively promoted vector was also tested *in vivo*. Fertile autosomal albino chicken eggs were injected with the virus, and some of the down on newly hatched chicks showed an orange phenotype, compared to the light yellow of control chicks. The orange chicks were weak, and died within a few days of hatch, while chicks that did not have the orange down survived (Frew *et al.*, 1992). White Leghorn eggs were also injected, and chicks infected with the same virus had a highly variable phenotype; some of these chicks had darker legs or wings, and some had orange down on their head and neck (Frew *et al.*, 1992). The White Leghorn chicks with the darker phenotypes also failed to thrive and died shortly after hatching; some of the dead chicks were necropsied, and showed dark lesions on their livers indicating tyrosinase production by some of the hepatocytes (Frew *et al.*, 1992).

The above studies suggest that the toxicity associated with pigment production in non-melanocytes should be controlled by a promoter that would limit tyrosinase expression to cells in which it would not have adverse effects on the host (Frew *et al.*, 1992). Feather keratin promotors might provide such tissue specificity, since

the keratinocytes differentiate with a large burst of keratin production, die soon after, and become the feathers of the chicken. Toxicity of the pigment production intermediates should not be a problem, as the keratinocytes are programmed to die after they differentiate normally. Since the dead keratinocytes are then externalized by incorporation into feathers, any toxic substances in them should not affect the general health of the chicken. The feathers could then be easily inspected visually for pigmentation, and birds receiving the gene selected on that basis.

The promoters of feather keratin genes have a highly conserved structure that includes an intron. A cluster of 18 avian feather keratin (CFK) genes spanning 53 kb of DNA has been isolated, characterized and sequenced (Presland *et al.*, 1989). The 5′ non coding region, intron, and 3′ non-coding regions are highly conserved within the gene family (Gregg and Rogers, 1986).

Experiments with the feather keratin gene found a large increase in the transcription of the reporter gene when the intron was removed from the promoter region (Koltunow *et al.*, 1986). Since the gene expression studies using this CFK gene showed higher transcription levels using the CFK promoter without the intron, and since space for the promoter was very limited in the vector, the CFK promoter with the intron removed was chosen as the promoter in these studies. Cultured keratinocytes, hepatocytes, fibroblasts and melanocytes were infected with constructed virus and monitored for tyrosinase expression.

Materials and Methods

Vector Construction:

The CFK gene was a gift from Dr. George Rogers of the Department of Biochemistry, University of Adelaide, South Australia. The gene contained the coding region for the chicken down feather keratin gene B (Presland *et al.*, 1989) also called #9 (Molloy *et al.*, 1982), and also sequences 5′ and 3′ to that region (Dr. G. Rogers, personal communication) for a total of 2.9 kb (Fig. 1). The keratin promoter was isolated from the 5′ region of the CFK gene using the Pst1 fragment contained in the pGEM®-2 vector from Dr. Rogers (Fig. 2). The primer constructed for the 5′ end of the sequence (CFK-L) contained the Bsp 106 (Cla 1) recognition sequence (AT/CGAT) proceeded by GCGCGC as a clamp site (Fig. 2). The remaining 18 bases of the primer were the same as the published sequence for bases 37–54 so hybridization would occur with the complementary strand. The downstream primer sequence (7110) hybridized with 45 bases 5′ to the upstream splice site of the intron (Fig. 2). The effect was to amplify

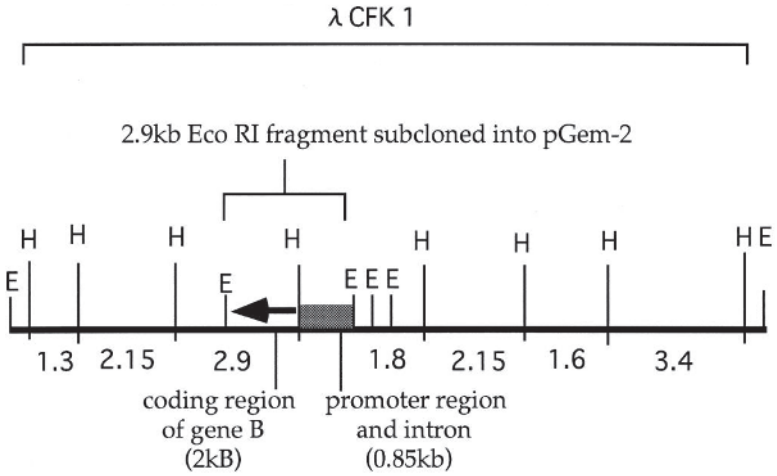

Figure 1. Map of the keratin genes surrounding the chicken feather keratin (CFK) gene B. The coding region for CFK gene B is contained in the segments between the Hind III and Eco RI sites indicated by the arrow. Approximately 400 bp of the CFK promoter region (shaded region) was amplified by a polymerase chain reaction (PCR) for use in the vector construction.

the sequence 5′ to the splice site, and also add a Sal I site and a clamp on the 3′ end of the amplified sequence. The predominant PCR product was approximately 400 base pairs. This product was cut with Bsp 106 (Stratagene, La Jolla, CA) and Sal I (Promega) to provide "sticky ends" for insertion into the pBluescript® II SK+ Phagemid vector (Stratagene). All SK vector constructs were cut at the Sca I site in the ampicillin resistance gene in addition to the sites required to insert the desired fragment. Therefore, all the plasmids were constructed from three fragments.

The three pieces were ligated together to produce the vector containing the amplified region of the CFK promoter: the Bsp 106 and Sal I cut PCR product containing the 5′ region of the CFK gene, the pBluescript® II SK vector cut with Sca I and Sal I, and the pBluescript® II SK vector cut with Sca I and Bsp 106. This construct was called the Bluescript-KP vector. The 3 piece ligation was required because the Sal I and Bsp 106 sites are separated on the pBluescript II vector by only 8 bases. Thus, the 2.3kb fragment containing the 5′ region of the CFK gene was isolated and used to make the final SK-KP construct. The procedure used to a construct KP-tyrosinase-J plasmid was similar to that used in construction of the Japanese quail tyrosinase promoter-mouse tyrosinase cDNA plasmid (Akiyama *et al.,* 1994). The final construct (KP tyrosinase-J) contained a modified promoter of the CFK gene (0.4 kb) as shown in Figure 3 spliced to the complete mouse tyrosinase cDNA (1.6 kb).

Figure 2. The 780 bp published sequence of the 5′ untranslated region of the chicken feather keratin (CFK) gene B, modified to 424 bp by intron exclusion for use as the promoter for the RCAN-KP vector. Also shown are the 5′ and 3′ PCR primer sequences, restriction sites used, and oligonucleotide linker sequence.

Viral production and titer determination:

Line 15B1 fertile chicken eggs were procured from the United States Department of Agriculture Avian Disease and Oncology Laboratory (East Lansing, MI). This line of chickens is known to grow the RCAS and RCAN vectors rapidly to high titer, and contains only two non-expressing endogenous Avian Leukosis Viruses (ALV) sequences. Thus, the normal 15B1 cells do not express the viral p27 antigen on cell surfaces,

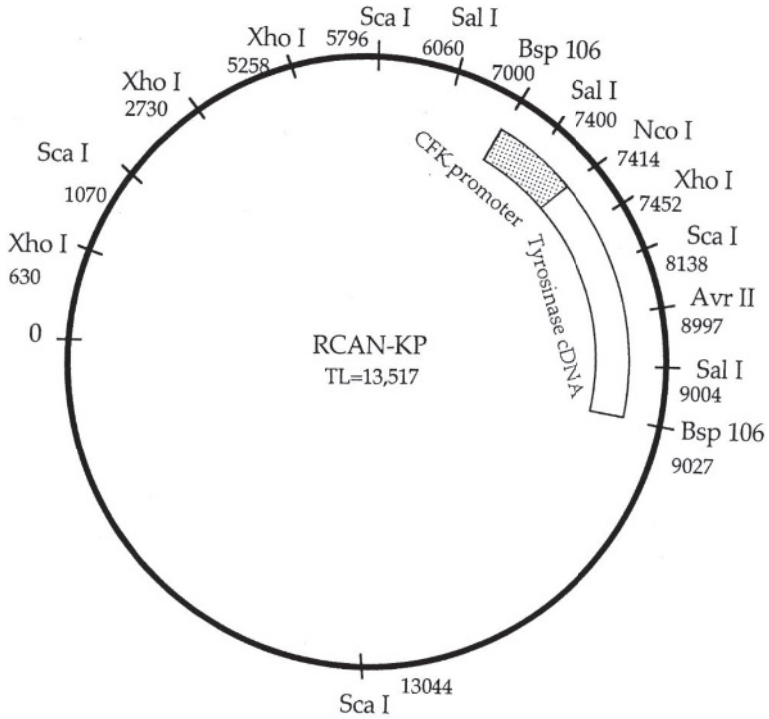

Figure 3. Completed proviral plasmid RCAN-KP with Bsp 106 insert containing the CFK promoter and mouse tyrosinase cDNA. Important restriction site locations are also noted. The Sal I sites at locations 6060 and 7400 were used to excise a 1.34 kb fragment to confirm the insert was in the correct orientation.

and the opportunity for recombination between the inserting retroviral sequences and endogenous retroviral sequences is reduced (Frew *et al.*, 1992). Fibroblasts were isolated from 12-day-old White Leghorn chick embryos of the 15B1 strain according to the method of Frew *et al.* (1992).

Viral stocks were produced by transfecting plasmid DNA into 15B1 Fibroblasts that were passed normally following transfection.

An enzyme-linked immunosorbent assay (ELISA) for the p27 antigen of the Avian Leukosis Virus (ALV) (Idexx, Portland, ME.) was performed on supernatants from various 15B1 fibroblast cultures. Supernatants from 15B1 fibroblasts transfected with the RCAN-KP vector construct showed a strong positive reaction for the p27 viral antigen 11 days after transfection. Supernatants from these cells were used to infect the other cultures in this study. Supernatants from mock transfected 15B1 cultures of the same age showed no reaction to the p27 viral antigen ELISA test.

Viral titers were in the range of 103–105 infectious particles/ml. The viral titer of

the RCAN-KP-Tyrosinase J was almost as strong as RCAS-Tyrs J (Whitaker *et al.*, 1991), RCANBPQJ, and RCANBPmodQJ (Akiyama *et al.*, 1994).

Cell culture:

Melanocytes: Autosomal albino (c^a/c^a) chicken stocks were maintained in Manter Hall at the University of Nebraska-Lincoln. These chickens do not make functional tyrosinase, and their melanocytes cannot form melanin because of the loss of six bases at a copper binding site of tyrosinase (Tobita-Teramoto *et al.*, 2000). Melanocytes were cultured from autosomal albino chicken eggs according to the method of Giss *et al.* (1982). They were infected and mock infected according to Whitaker *et al.* (1989), Akiyama *et al.* (1994).

Hepatocytes: Hepatocytes were cultured from 19-day-old albino (c^a/c^a) chick embryos using the technique of Bannister and O'Neill (1981) and Frew *et al.* (1992). The medium was a modified Krebs-Ringer's Bicarbonate Buffer (MKRBB) (4.8 mM NaCl, 120.2 mM KCl, 1.2 mM $MgSO_4$, 2.0 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 2.0 ml phenol red, 1 mM EGTA, 3.4 mM $CaCl_2$/liter ddH_2O, pH 7.2). Culture dishes were infected the day after culture by a complete medium change that contained 50% supernatants from RCAN-KP-transfected fibroblasts. Some hepatocyte cultures were mock infected by the same method using supernatants collected from mock-transfected fibroblasts on the same date. Thirteen days after infection, cover slips from both RCAN-KP-infected and mock infected cultures were fixed and stained with anti-mouse tyrosinase T1 antibody for immunofluorescence microscopy.

Keratinocytes: Line 15B1 keratinocytes were cultured from the dissociated 15B1 chick embryo trunks, previously described in Frew (1990) and were fed Keratinocyte Growth Medium (KGM^{TM}) (Clonetics, San Diego, CA) every second day. Keratinocyte cultures (15B1) were infected and mock infected by a complete medium change that contained 50% fibroblast supernatants and 50% fresh medium on the day after thawing and plating. To induce keratin production 1.8 mM $CaCl_2$ was added to the keratinocyte cultures (Frew, 1993). The end result was a mixture of differentiated keratinocytes and fibroblasts.

Fibroblasts: Fibroblast cultures were also tested by infection and mock infection with the RCAN-KP vector. They were cultured according to the methods described in the Viral Production and Titer Determination section.

This research has been carried out following the animal rules in the University of Nebraska, USA.

Immunofluorescence Microscopy and Cell Staining:

All types of cells were plated and grown on cover slips. The cultured cells were fixed with 3.5% paraformaldehyde in 0.1M phosphate-buffered saline (PBS), pH 7.5, for 20 min. at 20℃ . After rinsing with PBS, the fixed slips were dipped in −20℃ acetone for 5 min. for postfixation. Cells were rinsed gently and incubated with anti-p27 antibody (ALV-specific antibody; SPA-FAS Inc., Norwich, CT) (Smith *et al.*, 1979) diluted 1:300 or with anti-mouse tyrosinase T1 antibody (Yamamoto and Takeuchi, 1981) diluted 1:200 at 4℃ overnight. Tyrosinase T1 antibody was raised against a trypsin digested form of T3-membrane bound tyrosinase from mouse melanoma cells. This antibody does not cross-react with a chick tyrosinase (Yamamoto and Takeuchi, 1981). Both p27 and T1 antibodies were raised in rabbits. These antibodies were reacted with biotinylated anti-rabbit IgG (Amersham, Arlington Heights, IL), then reacted with diluted streptavidin-conjugated fluorescein isothiocyanate (Amersham) following a routine method (Akiyama *et al.*, 1994). Stained specimens were observed with an epifluorescence microscope (Nikon, Microphot Epi F1) and photographed using Kodak Ektachrome (ASA 200) or X-Pan (ASA 400) film.

To confirm differentiation in keratinocyte cultures, they were stained with rhodanile blue. Keratinocyte cultures were first fixed in 10% formalin, then in 100% methanol. They were stained with 0.05% rhodanile blue (Sigma, St. Louis, MO) in water for 20 minutes, and then rinsed with running water for 5 seconds. Rhodanile blue stains keratin red and other cytoplasmic components and nuclei blue (Smith and Bruton, 1977). This staining procedure therefore distinguishes between differentiated keratinocytes and other cell types, including undifferentiated keratinocytes and fibroblasts.

Results

RCAN-KP virus expression in 15B1: Cultures of 15B1 fibroblasts infected with supernatants from the transfected cells, as expected, showed a positive immunofluorescent labeling reaction with antibodies against the viral p27 antigen when labeled for immunofluorescent microscopy (Fig. 4). Mock infected fibroblasts showed only a low level of nonspecific labeling. Cultures of 15B1 keratinocytes infected with RCAN-KP supernatants, and mock infected 15B1 keratinocyte cultures showed similar results to the above fibroblasts when labeled with anti-p27 antibodies for immunofluorescent microscopy. Melanocytes and hepatocytes of the 15B1 genotype have also been shown to be positive for the anti-p27 antibodies when infected with any

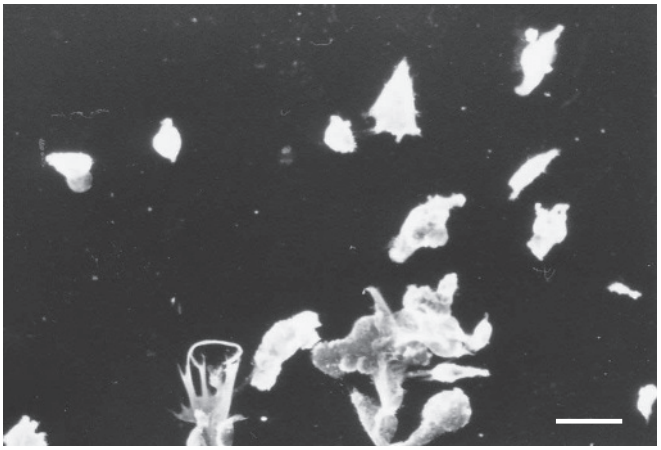

Figure 4. RCAN-KP-infected 15B1 fibroblasts fixed 11 days after infection and labeled with p27 antibody. These cells showed a strong labeling reaction for the viral p27 antigen. Bar = 50 µm.

of the RCAN family of viruses (Whitaker *et al.*, 1989, Frew *et al.*, 1992). These reactions confirm viral infection and the presence of the desired tyrosinase reporter gene and its accompanying promoter region.

Melanocytes: Autosomal albino (c^a/c^a) melanocytes infected with RCAN-KP virus produced pigment granules, which became discernible as early as day 7 after infection, and were quite definite in all infected cultures by day 12. Melanization continued to increase until the cells were fixed on the 20th day after infection (Figs. 5a and 6b). Infected c^a/c^a melanocytes labeled with T1 antibody showed a positive immunofluorescent reaction (Fig. 6a). Mock infected melanocytes failed to produce any pigment (Fig. 5b), and only background fluorescence was seen. Pigment granules in RCAN-KP infected melanocytes (Fig. 5a, arrows) were smaller than those produced by constitutive (RCAS) vectors (Whitaker *et al.*, 1989, Frew *et al.*, 1990). The number of the pigment granules seen in RCAN-KP infected melanocytes seemed to be as numerous as those in melanocytes infected with RCAN vectors driven by tyrosinase promoters (Akiyama *et al.*, 1994). The possible reasons for this unexpected result will be addressed in the Discussion.

Hepatocytes: No immunofluorescence reaction with T1 was found in RCAN-KP-infected hepatocytes (Fig. 7a and b, arrows). The mock infected cultures also showed no reaction at all. The morphology of the cells was similar between the two groups, both having numerous large vesicles, which are typical of liver cells, which spread out to cover large areas. The RCAN-KP infected cells did not round up and aggregate like those produced by constitutive (RCAS) vector infection (Frew *et al.*, 1990).

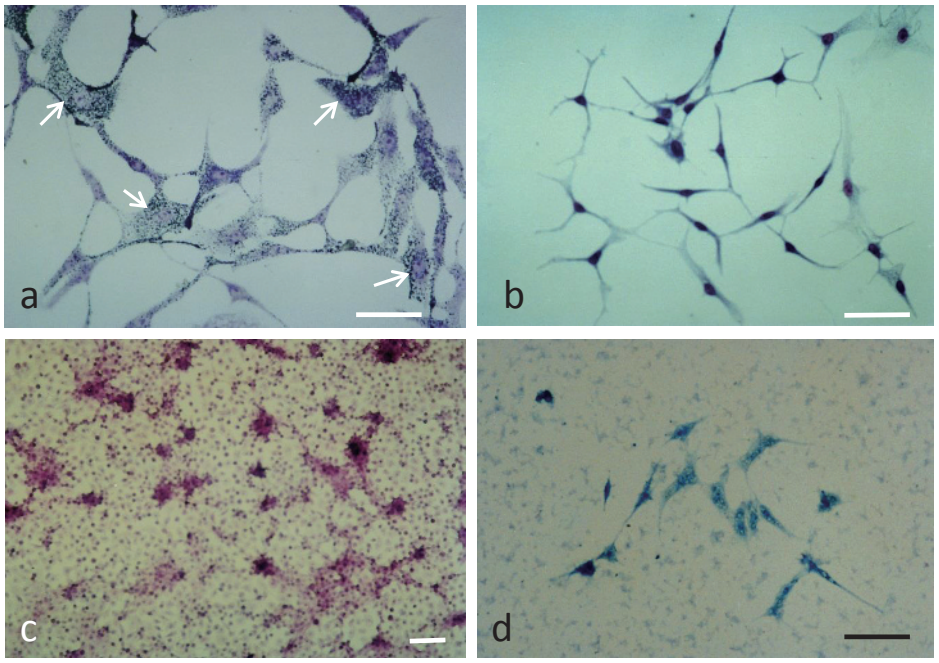

Figure 5. a: RCAN-KP-infected Giemsa-stained c^a/c^a melanocytes fixed 20 days after infection. Dark pigment granules in these cells can be seen as black spots (arrows). b: Mock-infected Giemsa-stained c^a/c^a melanocytes fixed 20 days after infection. No pigment granules can be seen. c: c^a/c^a keratinocytes stained with rhodanile blue. Only cross-linked keratin retains the red rhodamine B stain. d: c^a/c^a fibroblasts from the same dish (to assure equal staining procedure) as Fig. 5a, retaining only the nile blue stain. Bar = 50 µm.

Keratinocytes: Two days after the addition of 1.8 mM $CaCl_2$ to the keratinocytes' growth medium, keratin production was seen in the form of large aggregates of cells that appeared to the naked eye as white grains in the culture dishes. Under the light microscope, these keratin particles appeared as large mounds or ridges of cells. Rhodanile blue staining confirmed keratin production in these cultures as shown by the deep red-purple color in the clumped areas of Figure 5c. Keratinocytes infected with RCAN-KP showed a strong positive immunofluorescent labeling with for the T1 antibody to mouse tyrosinase when fixed and labeled on day 7 after infection and after stimulation with $CaCl_2$ (Fig. 6c, white arrows). However, these cultures failed to produce visible pigment (Fig. 6d, arrows).

The red staining, confirming keratin production coincident with the T1 immunofluorescence demonstrating strong tyrosinase production show that RCAN-KP-infected, differentiated keratinocytes produce more tyrosinase than do fibroblasts found in the same field (Fig. 6c, arrowheads). This shows an increase in transcription

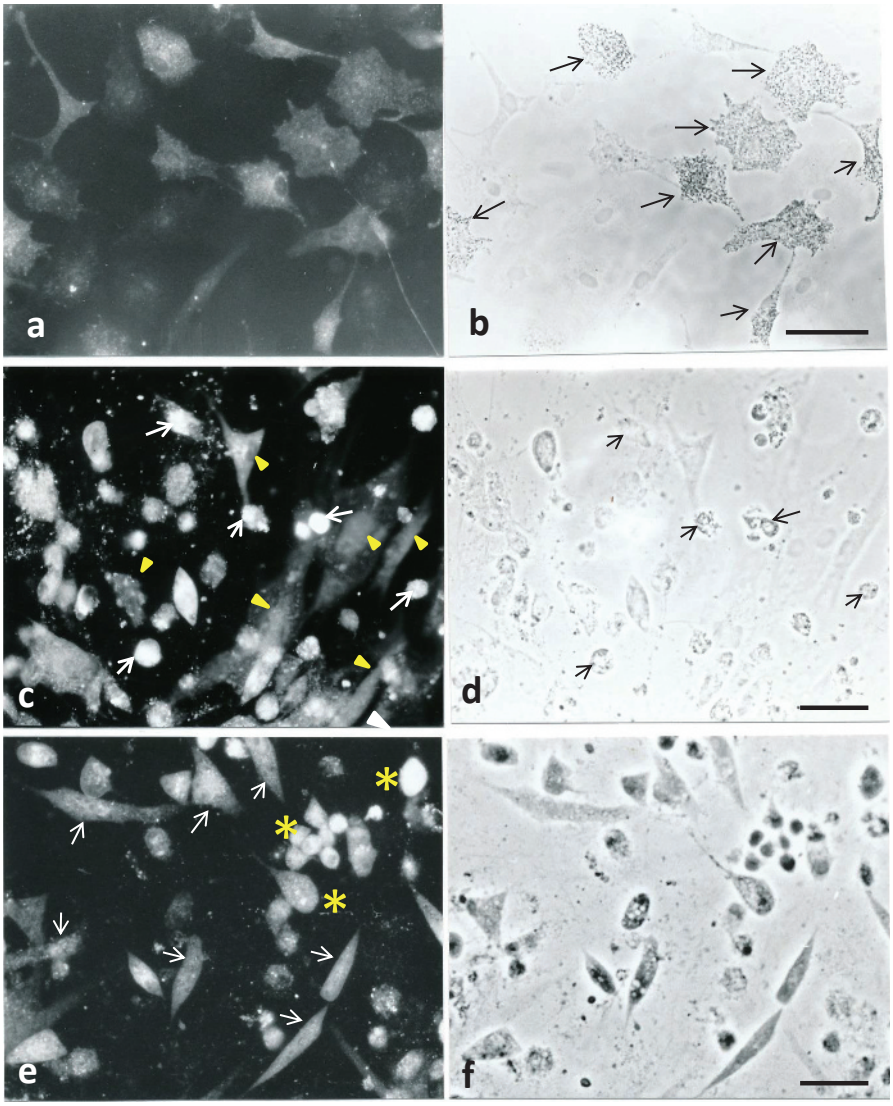

Figure 6. a: RCAN-KP-infected c^a/c^a melanocytes fixed 19 days after infection and labeled with T1 antibody, shown in a fluorescent micrograph. b: Phase contrast micrograph of the same field as a. Pigment granules can be seen as black spots (arrows). c: RCAN-KP-infected c^a/c^a keratinocytes fixed 7 days after infection and labeled with the T1 antibody. These cells showed a strong positive labeling reaction for mouse tyrosinase (arrows). Yellow arrowheads show fibroblasts. d: Phase contrast of the same field as c. e: RCAN-KP-infected 15B1 fibroblasts fixed 11 days after infection, labeled with T1 antibody (arrows). These cells showed a weaker labeling reaction than keratinocytes to mouse tyrosinase. Some of the keratinocytes in the same field (small round cells) are denoted by a yellow ∗. f: Phase contrast of the same field as e. Bar = 50 μm.

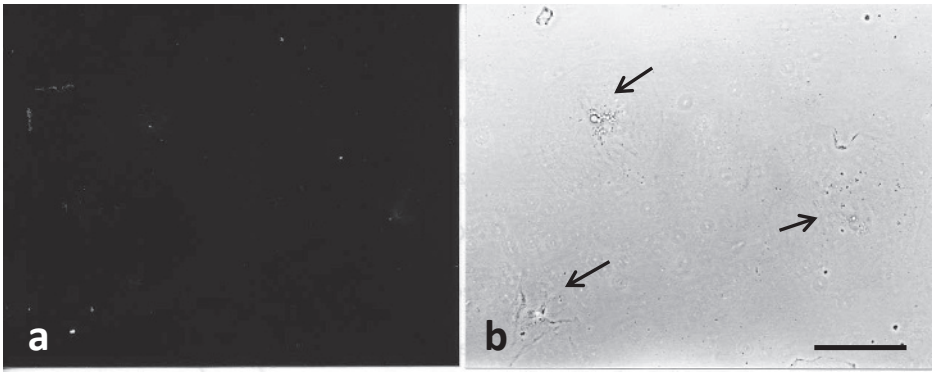

Figure 7. a: RCAN-KP-infected c^a/c^a hepatocytes fixed 13 days after infection and labeled with T1 antibody. Only dim background fluorescence from non-specific labeling can be seen. b: Phase contrast of the same field as a. Arrows show hepatocytes. Bar = 50 μm.

efficiency by the modified keratin promoter in differentiated keratinocytes above the level of transcription efficiency from the same promoter in fibroblasts.

Fibroblasts: Cultures of 15B1 fibroblasts do not produce high amounts of keratin as shown by the blue colonies in Figure 5d. When infected with the RCAN-KP vector, however, they showed a moderately immunofluorescent labeling reaction with the T1 antibody (Fig. 6e, white arrows). Mock infected fibroblasts showed only background fluorescence with the T1 antibody. In the RCAN-KP infected fibroblasts, no dark pigmentation was visible in any of the cultures, even to the naked eye, as had been the case with fibroblast cultures infected with the constitutive RCAS-Tyrosinase-J vector (Whitaker *et al.*, 1989), or through the microscope (Fig. 6f). Expression of tyrosinase was therefore quantitatively lower in fibroblasts infected with RCAN-KP than in those infected with the constitutive RCAS vectors. The expression of transgene analyzed by immunofluorescence is summarized in Table 1

Discussion

The presence of the viral construct is evidenced by detection of the viral p27 antigen in the case of keratinocytes and fibroblast as shown in Table 1. The infectability of hepatocytes and melanocytes in previous studies has been determined in a similar manner.

It was anticipated that non-keratin producing cells such as hepatocytes would show no pigment and no T1 immunofluorescence. This lack of expression shows the promoter used has some specificity, since a similar construct using a constitutive promoter did

Table 1. Visible and immunofluorescent staining comparisons of the responses of various cell types to infection with the RCAN-KP vector.

Cell Type	P27[a]	T1[b]	Visible pigmentation
Melanocytes	ND	++	++
Hepatocytes	ND	−−	−−
Keratinocytes	++	++	−−
Fibroblasts	++	+	−−

ND: not determined in this study. a: confirms presence of viral vector, reporter gene and accompanying promoter. b: confirms presence of reporter gene (tyrosinase) expression.

produce pigmentation and T1 immunofluorescence in hepatocytes (Frew *et al.*, 1990, Frew, 1993).

Additional evidence for specificity was the strong immunofluorescence seen in keratinocytes (Table 1). This result was expected since the promoter was originally for keratin, which is expressed at high levels (up to 50% of protein in the cell; Koltinow *et al.*, 1986). At such high levels of expression, the pigmentation was also expected to be produced in sufficient abundance to visually pigment keratinocytes infected with the RCAN-KP vector. No visible pigmentation was noted, however. Further investigation reveals at least 35 different keratin genes in the genome (Kemp *et al.*, 1975). The complex of the keratin gene cluster is likely to have quite finely tuned expression. Moderate production from each of them would produce a large amount of keratin. Since only one promoter coupled with tyrosinase was present in the infected cells, however, it is reasonable to expect only a moderate amount of tyrosinase gene expression. Alternatively, a few of the feather keratin genes could be responsible for most of the message, with the others being transcribed at a lower level. Gene B, from which the promoter was derived, could have been one of these less-transcribed genes.

Fibroblasts expressed enough mouse tyrosinase to produce T1 immunofluorescence, but not as much as keratinocytes (Table 1). Again, specificity is shown but not exclusivity. Expression with the constitutive promoter produced visually pigmented fibroblasts (Frew *et al.*, 1992) but expression with this promoter did not. Therefore, the modicum of expression must be explained. By their name and nature, fibroblasts contain fibers of some sort. The derivation of these cells from dissociated chick embryo trunks means that at least some precursor keratinoblasts are present. If fibroblasts are a general class of pseudo undifferentiated cells, then it is likely that some of the fibers produced are driven by the gene B promoter used in this study. This would explain the

levels of tyrosinase detected in this cell type.

Why should melanocytes express keratin promoted pigment? The quantity of pigment produced indicates that, in melanocytes, the chick feather keratin promoter produces levels of expression at least as high as the mouse or Japanese quail tyrosinase promoters (Akiyama *et al.*, 1994) but not as high as the constitutive promoter (Frew *et al.*, 1992). The bright T1 labeling confirms that the buildup of dark pigment granules was due to mouse tyrosinase, and not native chicken tyrosinase. Since pigment production is the normal function of melanocytes, the other genes needed for melanin production are active, and all the other factors are also present for pigment production. Consequently, pigment production is optimized and likely to be more efficient than in other cell types.

In addition to the cells being optimized for pigment production, the pigment produced in melanocytes is also concentrated in pigment granules (melanosomes). This concentration of the tyrosinase in melanosomes also results in uneven fluorescence when melanocytes are labeled with T1. Optimization of pigment production and concentration of the pigment in melanosomes make tyrosinase production and pigmentation in melanocytes difficult to compare with other cell types. Given the granular sequestering of tyrosinase products in melanocytes, it is possible that expression is in reality about the same as that seen in fibroblasts. Again we have a cell type that is replete with fibers of various types. Mayerson and Brumbaugh (1981) demonstrated that the presence of an array of different filament types in melanocytes. It is possible that one of these filament types could be transcribed from the gene B promoter used in this study, resulting in the activation of tyrosinase production in melanocytes infected with the RCAN-KP vector.

The viral long terminal repeat (LTR) used for the promoter in the RCAS-Tyrosinase-J may be a stronger promoter than the modified gene B promoter, or the modified gene B promoter may not operate at full efficiency in fibroblasts, or both. The possibility that the modified gene B promoter exhibits some tissue specificity may be supported by the results from mixed cultures of fibroblasts and keratinocytes, and by the results in hepatocyte cultures. The lower level of fluorescence seen in RCAN-KP-infected fibroblasts compared to RCAN-KP-infected keratinocytes labeled with T1 in mixed-culture micrographs (Figs. 6c and 6e) suggests that some differences in expression are occurring between different tissue types.

The results of this study, in principle, indicate the efficacy of using a keratin promoter to control the expression of tyrosinase in cultured chick embryo cells and possibly in embryos, *in vivo*. The levels of expression were not as high as expected or

needed to allow easy identification of the expression of the transgene. Expression in melanocytes as well as keratinocytes would not be a problem because the expression in both cell types is not likely to lead to toxicity. Expression in fibroblasts, however, if not precursors to keratinocytes, could pose a toxicity problem *in vivo.* Further experiments, searching for a promoter of a very highly expressed keratin gene, whose expression levels would lead to easy identification of transgene expression in culture and *in vivo* are needed.

Acknowledgements

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