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一、中文摘要

keratin 3 和 12(k3/12)是只表現在眼球表面的一對特異纖維蛋白質(intermediate filament)，而調節 k3 和 k12 之基因表現，例如 cis-DNA regulatory element，目前尚在研究當中。目前的研究當中尚未有定論在 k12 cis-DNA regulatory element 上游需多大的 DNA 序列才能有效的表現出其調節能力。而且 k12 的 promoter 無法有效轉殖在 primary conjunctival epithelial cell 中,雖然在 subculture 之細胞或是 SV40 T antigen transformed 之 conjunctival epithelial cell 中卻可以有效的轉殖，但是在這些細胞中無法有效的表現 k12 蛋白質。但是以 k12 promoter-β-galactosidase DNA construct 轉殖在這些細胞中發現 k12 之 5'-端上游 0.2 kb 的 cis-DNA 可以有效的表現出其 promoter 之活性。但是在 transgenic mouse 的實驗卻無法有效表現其活性。

為了解決這一個問題，我們設計以 in vivo particle-mediated gene transfer(''HeliosTN Gene Gun System'')技術來測試 k12 promoter-reporter construct 的活性。但是為了進一步鑑定更長的 promoter DNA 序列以便進行 transgenic mouse 或 in vivo transfected rabbits 的實驗。而經由此模式我們可以瞭解 k12 基因的調控，而能將此實驗結果運用於 k12-reporter construct 之 transgenic mouse 或 transfected rabbits 之實驗當中。進而製造眼角膜的 k12 promoter 和一些 growth factors 或 cytokine 的 chimera 應用於眼表層疾病的研究和治療。
本計畫在美國辛辛那提大學醫院眼科高惠陽博士回國擔任本計畫之共同主持人指導之下，在 k12 Gene Gun in vivo transfection assay 的實驗上已有初步的結果，我們發現在位於 k12 promoter 上游的 4 對 Pax-6 pair binding elements 對調控 k12 gene 在角膜上的表現有相當大的影響，當我們 delete 掉 k12 promoter 上的 Pax-6 elements 時 k12 在角膜上的表現就變弱了，同時在結膜和皮膚上的基因轉殖則沒有差別。所以我們認為 Pax elements 對 k12 基因在角膜上的表現相當重要。k12 一般只能表現在角膜表皮細胞中，而不能表現在結膜細胞和培養之角膜細胞中，所以它是具有角膜特異性的一種基因。而是否 Pax-6 在角膜細胞中伴演著控制其器官特異性分化之作用或是有其它 transcriptional factor 影響其表現是接下來我們所想瞭解的事情。

關鍵詞：角蛋白、基因槍、
專題計畫、報告格式、國科會

Abstract

The regulatory cis-DNA elements of the cornea-specific keratin 12 gene has been studied for a long time. Conventional ways of identifying this cell-specific promoter using cultured rabbit corneal epithelial cells derived from cornea explants is studied by many other authors and had following conclusions. First, for unknown reasons , these primary corneal epithelial cells could not be effectively transfected using liposomes. Second, the subcultured epithelial cells from cornea explants could be transfected but they de-differentiated and ceased to express keratin 12. Third, SV40 large T-antigen transformed rabbit corneal epithelial cell line is also used to test K12 promoter-β-gal DNA constructs, because they could be efficiently transfected by liposomes or electroporation. This established

corneal epithelial cell line maintains many epithelial cell characteristics, but they no longer express keratin 12. However, this cell line is still of value to examlne K12 promoter-β-gal DNA constructs for their functionality, because it is easy to maintain and still possesses many epithelial cell characteristics, e.g. stratification, expression of keratins, etc. From the studies of Dr. Kao, a 0.2 kb element in the 5'-flanking region of the keratin 12 gene is identified that could direct the expression of β-galactosidase by using this cell line. As the 5'-end flanking sequences increased in the promoter-reporter gene constructs, the levels of β-galactosidase expression decreased in the transfected rabbit corneal epithelial cells. These observations imply that 5’ end sequence of the Krt1.12 gene may contain sufficient and essential regulatory cis-DNA elements for corneal epithelial cell-specific expression of the reporter gene(s), e.g. CAT (chloroamphenicol acetyltransferase) and β-galactosidase. Thus, a selective group of the reporter DNA constructs is tested with the transgenic mouse technique. Unfortunately, none of the transgenic mouse lines expressed reasonable levels of the CAT activity in cornea or other tissues.

In order to circumvent these problems, we have tried the newly developed in vivo particle-mediated gene transfer techniques (Helios™ Gene Gun System") for a few trials to examlne the promoter activities of reporter gene constructs. The results of these "Gene Gun" experiments indicate that our reporter gene constructs (1.0 KZ, 2.5 KZ) contain regulatory cis-DNA elements for cornea epithelial cell expression of β-galactosidase. However, more experiments are needed to identify and characterize the DNA elements which are essential and sufficient for cornea epithelial cell-specific expression of reporter gene(s). Thus, the purpose of this study is to apply the "Gene Gun" system in the study of ocular surface epithelium. Then we can identify the exact structure of cis-regulatory DNA element of Krt1.12 gene. The identification of such DNA elements is a prerequisite for creating transgenic mouse models which may over-express cytokines, or carry domlnant negative mutations of receptors for future studies of corneal physiology, and development of a gene therapy strategy for treating corneal disease.

In the first year study, we found that the activity of k12 promoter increased to a constant level from 1.0 to 2.5 kb by *in vivo* Gene Gun delivery transfection. This result encourageed us to investigate the more detailed structure with k12 promoter. At first we focused on the Pax-6 pair boxes located within 2.5 KZ. There are four Pax-6 pair boxes located upstream from the transcriptional initiation site to 2.5 kb. We construct several deletion constructs, including 2.0 KZ / 0.2 KZ, 2.0 KZ /0.4 KZ, and 2.0 KZ /0.6 KZ deletion of Pax-6 elements, and we found that the promoter activities decreased when the number of Pax elements decreased. It suggests that the Pax pair boxes in the promoter of k12 did play important roles in the regulation of k12 expression.

Keywords: Research Project, Report Style, National Science Council

二、緣由與目的

The Importance of Using of Transgenic Mice in Studies of Gene Function

The transgenic technique of microinjection of cloned DNA into fertilized mouse eggs was first accomplished nearly simultaneously in the laboratories of Brinster, Costantini, Ruddle, Mintz, and Wagner (1,2). Since then numerous applications of transgenic animal technology have been reported. Altered phenotypes resulting from transgene expression demonstrated that introduced genes can exert profound effects on animal physiology. Transgenic mice have been important for the study of gene expression by hormonal and developmental control (1,3,4). Transgenic mice have also been invaluable for producing animal models of cancer and other diseases and testing the efficacy of gene therapy by the use of cell over expressing functional gene product, or dominant negative mutation of transgenes driven by cell type-specific promoters (5-8). In addition, cell-cell interactions and cell lineage relationships have been explored by cell-specific expression of toxin genes in transgenic mice (1,9,10). For example, recent studies suggest that attenuated and inducible toxins hold promise for future transgene ablation experiments (11,12).

The transgenic mouse technique has been successfully applied in several ocular tissues, i.e., lens, retina, except corneas. For example, lens-specific crystalline promoters have been used to express toxins, i.e., diphtheria toxin α and ricin, in several lines of transgenic mice. These lines resemble phenotypes of micro-ophthalmia, anophthalmia, etc (13,14). Similarly the use of opsin promoter-driven diphtheria toxin α has lead to the creation of mouse models for the studies of retinal degeneration (15). However, similar mouse models for the study of corneal physiology have not been obtained due to the lack of availability of functional cornea-specific promoters that can be used for the creation of such lines. To achieve the goal of creating transgenic mouse lines for the study of corneal cell biology, two prerequisites must be met: **1) To identify and characterize genes that are specifically expressed by corneal cells, i.e., epithelium, stroma and endothelium; 2) To identify the *cis*-regulatory elements of such cornea-specific genes.**

In Search of Cornea-Specific Genes

In order to create transgenic mouse lines over-expressing or carrying dominant negative mutations in cornea, it is imperative to identify and characterize the genes that are cornea-specific. The identification of the regulatory elements for such genes can then be used to prepare transgenic mice for the study of corneal physiology. To date, two house-keeping enzyme genes, transketolase and aldehyde dehydrogenase have been shown to be highly expressed by corneal epithelial cells (16-18). Keratin 12 and keratin 3 are also expressed by corneal epithelial cells (19-22). Keratocan is expressed by corneal keratocytes (23).

Cornea Epithelial Cell-Specific Keratin 12 and Keratin 3

Keratins are a group of water-insoluble proteins that form 10 nm intermediate filaments in almost all epithelial cells (24,25). About 30 different keratin molecules have been identified (24). These can be divided into acidic and basic neutral subfamilies according to their relative charge, immunoreactivity and sequence homologies to type I and II wool keratins (1,2,27-28). *In vivo*, a basic keratin is usually co-expressed or "paired" with a particular acidic keratin (24,26,27,29). In cell culture and in many hyperproliferative diseases, the expression of the keratin pair of K6 (56 kD) and K16 (48 kD) becomes dominant (30). The pair of a basic K5 (58 kD) and an acidic K14 (40 kD) keratin is expressed in basal cells of all stratified epithelia (8). The expression of basic/acidic keratin pairs of K1 (64-67 kD)/K10 (56.5 kD), K4 (59 kD/K13 (51 kD) and K3 (64 kD)/K12 (55 kD), is characteristic for the suprabasal cells of epidermal, esophageal, and corneal epithelium, respectively (31-34).

Expression of the K3/K12 pair has been found in human, bovine, guinea pig, rabbit, and chicken corneas, and is regarded as a marker for cornea-type epithelial differentiation (19,22,23,29,). Although the K3/K12 pair is considered to be the cornea-type differentiation marker, recent evidence indicates that K3 can be expressed in several tissues other than the corneal epithelium (19,30). It is of interest to note that the expression of keratin3 in mouse cornea has not yet been confirmed (31). In contrast, the expression of Keratin12 is restricted to the corneal epithelium (19,20,21,32-35). Dr. Kao (Cincinnati University) has recently cloned the mouse cornea-specific keratin 12 gene and examined its spatial and temporal expression during development (21,36). His results also indicate that keratin 12 is only expressed by corneal epithelial cells.

Sun and coworkers have identified the regulatory *cis*-DNA elements of keratin 3 in cultured keratinocytes derived from epidermal epithelial cells that do not express keratin 3 (37). However, the corneal specificity of the keratin 3 promoter has not been demonstrated in transgenic mice. Many investigators have attempted to identify the *cis*-regulatory elements of cornea-specific *Krt1.12* gene in recent few years. But the results from these unpublished studies reveal that the regulatory *cis*-DNA elements for cornea-specific keratin 12 expression is more complex than those of other reported keratin gene promoters, i.e., keratin 14, keratin 10 and keratin 1. These promoters have been used to create transgenic mice that over express cytokines or dominant negative mutations of the receptors for studies of skin biology (38-41).

Strategy for the Identification of Corneal-Specific Promoters and the Creation of Transgenic Mouse Lines.

The conventional way of identifying tissue-specific promoters is by transfecting cultured cells that express the gene of interest with promoter-reporter gene constructs, i.e., β-galactosidase, chloramphenical acetyl transferase (CAT), luciferase, green fluorescent protein, growth hormone, etc. The promoter-reporter gene constructs that direct cell type-specific expression are then used to create

transgenic mice by microinjection into fertilized mouse eggs. The reporter enzyme activity in various tissues is then measured to identify the tissue-specific regulatory *cis*-DNA elements. When there is no appropriate cell line available for the identification of promoters of tissue-specific genes, one of the few systems currently being used to analyze promoter and transgene activity *in vivo* is the transgenic mouse (1,2). This system has proven to be a valuable and powerful approach for studying transgene expression *in vivo*. It is, however, limited in its ability to provide a convenient and quantitative analysis of transgene activity. Variability of expression of reporter gene activity is observed among different lines of transgenic mice carrying the same promoter-gene construct (42). Previous studies have shown that transgene expression is influenced by both the integration site and the copy number of the transgene in the host chromosome (43). Techniques for generating transgenic mice are not yet routinely applicable to other mammalian systems and are also costly, laborious, and time-consuming. These limitations make transgenic mouse models impractical as a routine procedure for the identification of tissue-specific promoters. The newly developed particle-mediated gene transfer via the "Gene Gun" technique provides a convenient and easy routine method for the search for cornea specific promoters (44-48).

In vivo Transgene Expression via Particle - Mediated Gene Transfer: Gene Gun.

As mentioned above, the expression patterns of most mammalian transgene constructs are usually analyzed by using cell culture systems (49). However, the cultured cells do not always reflect the exact mechanism in which the gene is regulated *in vivo* (50). Transgenic mouse techniques suffer the setbacks of expression variation among transgenic mice, time consumption, and expense. With the recent progress in gene therapy research (51), there is a need for an efficient gene delivery and assay system to characterize promoter-therapeutic gene constructs *in vivo*, allowing examination of transgene expression levels and physiologic effects. It is also highly desirable that this system be applicable to many different somatic tissues and mammalian species pertinent to gene therapy experiments.

Recently, Bio-Rad (Hercules, CA) has introduced the "Helios™ Gene Gun System" for an *in vivo* expression of transgenes via delivery of DNA-coated gold particles into the somatic cells of living animals (46-48). This system provides many advantages that are superior to *in vitro* transfection of cultured cells and *in vivo* transgenic mouse models; e.g., simplicity, tissue-specificity. This technique has been successfully used in studies of cancer, infectious diseases, wound healing, *in vivo* and *in vitro* immune response in animals, and gene expression (44,48,49). According to the preliminary experiments from Dr. Kao, we introduce this project to evaluate the suitability of the Gene Gun System for *in vivo* expression of K12 promoter-β-gal constructs in rabbit corneas.

In the past decade, the Dr. Kao's laboratory has isolated and characterized genes that are expressed specifically by the

corneal epithelium, stroma and endothelium cells. He has isolated and characterized the cornea epithelial cell-specific mouse keratin 12 gene (20,21,32,34,35,51) and the corneal keratocyte-specific mouse keratocan gene. In this cooperated application, however, we will focus our discussion on keratin 12.

Identification and Characterization of the Cornea Epithelial Cell-Specific Keratin 12 Gene.

Expression of the K3/K12 pair is characteristic of corneal-type epithelial cell differentiation (20-22,25,32,34,35,51,48,49). However, the mechanism that regulates this cornea-specific keratin 12 expression remains unknown. To provide a better understanding of the cornea-specific expression, the K12 cDNA has been examined during development, corneal wound healing and in cultured corneal and conjunctival epithelial cells (20,21,32,34). The expression of keratin 12 is limited to corneal epithelial cells. This cornea-specific expression is exemplified by the observation in which the integrity and strength of corneal epithelial cells is compromised in homozygous keratin 12-deficient mice (51). These mice do not suffer any pathology in other epithelia, skin, or esophagus (51). The murine *Krt1.12* gene spans 6,567 base pairs of murine genomic DNA, and the mRNA encoding keratin 12 is distributed into eight exons. Chromosome mapping reveals that murine *Krt1.12* is located within the Krt1 complex of mouse chromosome11 (35). In addition to the production of authentic keratin 12 mRNA, the *Krt1.12* gene gives rise to several alternate poly(A)+ RNAs by the use of alternative splicing in intron 2; an alternative promoter in intron 1, and/or both. Sequence analysis indicates that the transcripts derived from alternative splicing and/or the alternative promoter do not have long open reading frames for keratin or keratin-like molecules. It is not known whether these alternate keratin 12 poly(A)+ RNAs have any biological function, e.g. the regulation of keratin 12 gene expression (35).

Identification of the Cornea Epithelial-Specific Keratin 12 Promoter Using Cultured Rabbit Corneal Epithelial Cells

The upstream about ~ 2600 bp of 5'-end DNA of *Krt1.12* gene has been cloned and sequenced(as shown in Figure 1). In this 5'-end sequence, four Pax-6 elements are found between –910 and –2,000 bp 5'-flanking the transcription initiation site of the *Krt1.12* gene. Pax-6 is a transcription factor containing both a homeodomain (HD) and a paired domain (PD) (52,53). It functions as an essential regulator of eye development in both Drosoplila and vertebrates (54-66). Several genes preferentially expressed in the eye such as lens crystallin (57,58) and rodopsin (59) have been shown to be regulated by Pax-6, and specific Pax-6 PD binding sites are found in their 5' regulatory sequence (59). Pax-6 is also highly expressed by corneal and conjunctival epithelia in adults, suggesting that Pax-6 may directly regulate gene expression in the cells of these tissues (56). Thus, it is very likely that keratin 12 expression may also be regulated at least in part by Pax-6.

Summary of the Identification of Keratin 12 Promoter

The use of K12 promoter-CAT constructs in transgenic mice failed to produce positive and exciting results. The exact reasons for this failure are not known. It is possible that K12 promoter-CAT gene constructs do not contain sufficient and essential elements for cornea-specific expression, or that the integration sites of transgenes in the mouse genome produce inhibitory effects on their expression. Although there are numerous successes in creating transgenic mice, the outcome of the microinjection of fertilized eggs is hard to predict. What can be done is to characterize the promoter-reporter gene constructs using an *in vitro* system, e.g. cultured cells, prior to creating transgenic mice. Unfortunately, there is no cell line that expresses keratin 12 that can be used to functionally characterize our DNA constructs. The newly developed Gene Gun technique provides us a unique opportunity to test the promoter reporter gene constructs for their cornea-specific expression *in vivo.* Dr. Kao's preliminary results using the "Gene Gun" demonstrate that the reporter gene constructs can be evaluated for their expression in cornea, conjunctival and epidermal epithelial cells.

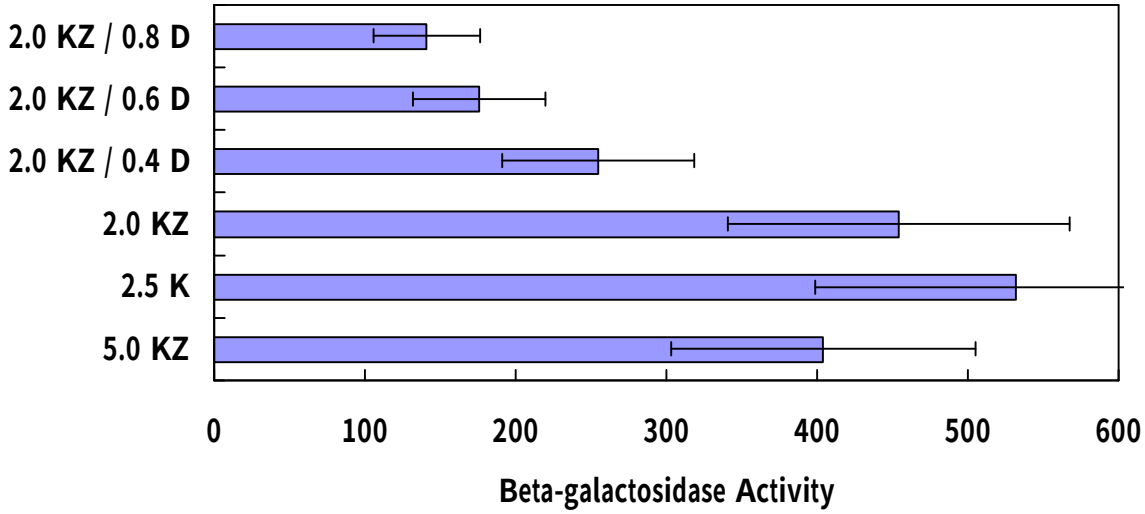
The results of *in vivo* transfection of K12 promoter β-gal constructs with the Gene Gun, and *in vitro* co-transfection in cultured corneal epithelial cell with Pax-6 cDNA and K12 promoter β-gal constructs indicate that the existing constructs may have the essential and sufficient elements i.e., Pax-6 elements, for cornea-specific expression. For example, *in vivo* transgene expression via Gene Gun revealed that the 2.5KZ construct produced the highest β-galactosidase activity in rabbit corneas, and did not express the enzyme in skin and conjunctiva. The co-transfection with Pax-6 cDNA did not significantly increase the β-galactosidase activity in cultured T-antigen transformed rabbit corneal epithelial cells. These observations are consistent with the notion that 2.5 kb 5'-end sequence of the *Krt1.12* gene contains cornea-specific enhancer elements, i.e., Pax-6, and silencer elements that suppress the keratin 12 expression in conjunctival, epidermal epithelial and transformed rabbit corneal epithelial cells. However, it is mysterious that transgenic mice carrying the 1K-CAT construct (containing one Pax-6 element) did not express the enzyme activity in cornea. However, it should noted that because only one mouse line was obtained, the observation may be inconclusive for the failure of identifying cornea epithelial cell-specific regulatory cis-DNA elements. It is also possible, however, that one single Pax-6 element may not be sufficient for high levels of reporter expression in transgenic mice. Nevertheless, the use of the Gene Gun technique will allow us to identify the regulatory cis-DNA elements that are sufficient and essential for cornea epithelial cell specific expression of the *Krt1.12* gene.

Thus, it is appropriate to prepare DNA constructs that contain multiple copies of Pax-6 elements, to increase the expression level of the transgenes. These constructs will

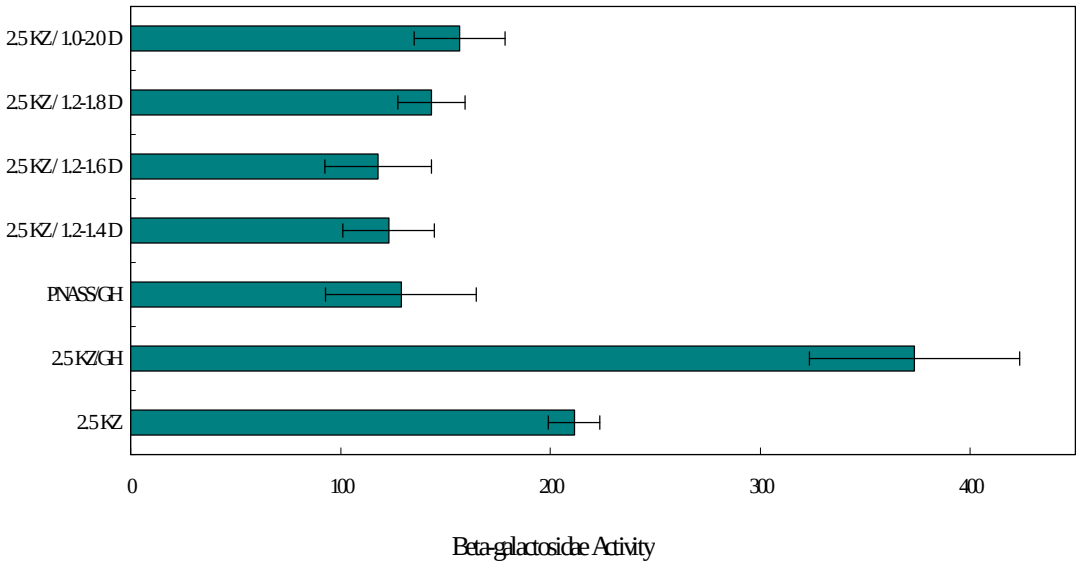
be examined by *in vitro* transfection of cultured rabbit corneal epithelial cells and *in vivo* with the Gene Gun.

結果與討論

Beta-galactosidase activity from the deletion constructs, deletion from 0.4 KZ to 1.0 KZ , decreases with the decrease of ratio of Pax-6 elements (Figures below).



We also made deletion constructs from 1.2 to 2.0 KZ, and replace the 3' poly A sequence end of original k12 constructs, which derived form SV40 3' end, with the growth hormone 3' poly A sequence. The results are shown in the figure below.



The deletion constructs, we made, are composed of Pax-6 sequences. So, we can know that Pax-6 is essential for the expression of k12 promoter. However, we do not know whether it does play a role in its expression in corneal epithelium or not. It is worthy of further studies to investigate the relationship between Pax-6 and other cis-DNA or trans-DNA elements. The future goal will be set on the exploration of the possible mechanism that regulates the cornea-specific gene expression. So far, we have a possible candidate expressing only in cornea epithelium. But we still don't understand its regulation and expression, we

will continue our studies to investigate in the following study. Our goals of the will be ：

1) Try to find out the relationship between Pax-6 and other cis-DNA elements and construct more specific Pax-6 deleted DNA constructs.
2) To study the role of 3' end poly A sequence on the cornea-specific expression of k12 promoter
3) If possible, we will follow the previous results and find out the most possible transcription factors interacting with Pax-6 and other cis-DNA elements.

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