Pakistan J. Zool., vol. 43(6), pp. 1183-1189, 2011.

Effect of First Intron on Ovalbumin Promoter Activity

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Abstract.- The present study was designed to investigate effect of first intron on ovalbumin promoter activity using immortalization of oviduct epithelial cells. The oviduct epithelial cells were cultured and then immortalized using pL-CTAgNeo virus. The pL-CTAgNeo virus maintained the structural integrity and growth of oviduct epithelial cells for 18 days whereas growth rate declined continuously with aging and cellular death after 7-10 days in controlled culture. The immortalized oviduct epithelial cells were transfected with pL-OV1345tPAGFP and pL-OV2964tPAGFP virus, respectively. The DNA and mRNA abundance of OV1345 virus was 46 and 14 times higher than OV2964 virus. The transcript effect of OV2964 virus was 3-fold higher than OV1345. The result showed that first intron in OV gene has a cis-regulation function for transcription.

Key words: Oviduct epithelial cells: immortalization: ovalbumin promoter activity: first intron.

INTRODUCTION

Transgenic biotechnology has resulted in excellent advancement in exploiting gene transfer techniques. Mostly animal genome has several introns (Bruno and Gunther 1989; Csuros *et al*., 2008). The ovalbumin gene includes several introns which are related to oviduct specific expression and drive reporter gene with high expression. The transcriptional regulation in ovalbumin gene is controlled by binding of sequence-specific DNA-binding proteins to promoters and enhancers (Blackwood and Kadonaga, 1998). Most eukaryotic genes are controlled by a complex array of cis-acting regulatory elements that modulate transcriptional activity. The expression of simian virus 40 (SV40) genes has been shown to transform cells and allow establishment of permanent cell lines (Gaudray *et al*., 1978; Asselin *et al*., 1985). The maintenance of the immortalized cell phenotype depends on the presence of the large T protein (Asselin *et al*., 1985). The SV40 genes have been introduced into a wide range of cells, and the induced phenotypes have been extensively studied (Gimbrone and Fareed, 1976; Rudland and Barraclough, 1990). The T antigen has been found to immortalize broad range of cells using recombinant genes with promoter that are either tissue-specific or ubiquitous (McKay *et al*., 1988; Paul *et al*., 1988; Hanahan, 1988). In this study, we cultured chicken oviduct magnum epithelial cells and then immortalized them using lentvirus (pL-CTAgNeo) containing SV40 large tumour antigen (TAG) and neomycin resistant gene (Neo). Immortalized cells were transfected with pL-OV1345tPAGFP and pL-OV2964tPAGFP viruses, respectively. Transcriptional efficiency of virus was observed to evaluate the influence of first intron on ovalbumin promoter activity.

MATERIALS AND METHODS

Construction of vectors

The SV40 large antigen (TAg) and neo were cloned from 293FT cells transfected with the pCMVSPORT6TAg.neo plasmid. The pL-eGFP plasmid was digested using *Xho1* and *Xba1* restriction enzymes and PCR product of TAg was sub-cloned in it. The constructed plasmid was named pL-CTAgBC. The pL-CTAgBC plasmid was digested using *Xba1* and *Kpn1* enzymes and PCR product of neomycin resistant gene was sub-cloned in it and new plasmid was called pL–CTAgNeo. The pL–CTAgNeo was digested with *Afl II* and *Xho*1 restriction enzymes. The agarose gel of

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0030-9923/2011/0006-01183 $ 8.00/0

digestion mix of pL-CTAgBC and pL–CTAgNeo was run to confirm plasmid size.

Ovalbumin promoter

The 16 months Isa brown laying hen, having 70% egg production was purchased from Qinglongshan Farm, Nanjing Jiangning District. The blood was collected from hen through wing vein and DNA was extracted using Invitrogen Kit. The two different sizes (1.345 and 2.964) of oviduct specific promoters were amplified. Both promoters only differ with first intron in 5' regulatory region. The primers

P1: 5'ACATTTACTGGGAAGCATATAGTATCATCAT3'
P2: 5'TGACTGCTAAAGGCACTCGAGCTTTCTA3',
and
P3: 5'CATGGTGAACC TCG AGTTGTCTAGAGCAAA3'

with *Nde1* and *Xho1* restriction sites were designed. The sense primer was same for both promoters and only differs in anti-sense. The PCR product of oviduct specific promoters was sub-cloned in pL-tPAGFP plasmid using *Nde1* and *Xho1* restriction enzymes. The constructed plasmids pL-OV1345tPAGFP and pL-OV2964tPAGFP were digested with *Afl II* and *BamH1* restriction enzymes and then visualized on agarose gel.

The pL-eGFP and pL-tPAGFP plasmids were obtained from key laboratory of Animal Genetics, Breeding and Reproduction, College of Animal Sciences and Technology, Nanjing Agricultural University, China.

Virus production

To prepare viral stock of pL–CTAgNeo, pL-OV1345tPAGFP and pL-OV2964tPAGFP vector, the 293FT cells were plated on 10cm dishes with 3.5µg of above mentioned plasmids, respectively, 5µg of gag/pol plasmid, 2.5µg of [illegible] plasmid and VSV-G plasmid by lipofectamine ™ 2000, respectively. The medium was changed after 6 h. Forty eight hour post transfection, culture medium was harvested and centrifuged at 3,000rpm for 15min at 4°C, filtered (0.45µm), followed by ultracentrifugation at 50,500xg for 120min at 4°C. The pellet was resuspended in phosphate buffered saline (PBS). Aliquots of each vector was stored at -80°C. For generation of lentivirus, 293FT cells were transfected with 9µg of ViraPower Packaging mix (Invitrogen) along with 5µg of each plasmid by lipofectamine. Twenty-four hours post transfection, the transfection mix was replaced with fresh culture medium without antibiotics. Virus-containing supernatant was harvested 72 h post transfection and cleared by centrifugation (3000rpm, 15min, 4°C) and filtered through a 0.45µm filter (Millipore). Thereafter, the virus was collected via ultracentrifugation in ultraclear centrifugation tubes for 2 h at 25,000rpm and 4°C. The pellet was allowed to dissolve overnight at 4°C in a volume of sterilized PBS that was 1% of the original medium volume. The next day, aliquots were prepared and stored at -80°C until use.

Culture of oviduct cells

The finely minced magnum tissue was suspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and collagenase at a concentration of 0.5mg/ml. The enzymatic disintegration of the oviduct tissues was performed into a 100ml glass bottle containing total volume of 20ml by incubating the vessel for 1hour in a shaking bed at 37°C. The tissue residue was removed by filtering the dissociation mixture with layers of gauze. In order to increase the purity of epithelial cells, the fibroblasts, blood and other cells were isolated from the epithelial cells. After isolation, the epithelial cells rapidly became weak compared with fibroblasts, blood and other types of cells. Epithelial cells were collected by centrifugation at 600rpm for 2min. The cells were washed twice using DMEM with 10% fetal bovine serum, penicillin (50µg/ml) and streptomycin (50µg/ml). After that the primary oviduct cells were grown at 37°C , 5%CO_2 in DMEM supplemented with 10mmol/L HEPES at pH 7.4, streptomycin, 8% chicken serum, 2% fetal calf serum, 7−10mol/L 17 β-estradiol (Sigma, USA), 6−10mol/L corticosterone (Sigma, USA), and 50µg/L insulin (Sigma, USA).

In vitro *transfection*

The 50µl pL-CTAgNeo virus was used for transfection of oviduct epithelial cells. The transfected cells were cultured for 18 days. 50µl of

pL-OV1345tPAGFP and pL-OV2964tPAGFP viruses were used for transfecting equal number of immortalised oviduct epithelial cells, respectively.

Efficiency of promoter

Forty eight hours post transfection of pL-OV1345tPAGFP and pL-OV2964tPAGFP viruses; equal number of oviduct epithelial cells was taken, DNA and RNA were isolated using Invitorgen Kit. The difference of integrated GFP efficiencies of pL-OV1345tPAGFP and pL-OV2964tPAGFP virus was quantified in immortalized oviduct epithelial cells. The mRNA was isolated using mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instruction. The quantity and quality of mRNA samples were analyzed by UV spectrophotometry. 1µl mRNA was subjected to semi-quantitative RT-PCR.

Semi-quantitative RT-PCR

GFP primers from pL-eGFP sequence:

Upstream
5' CTCGTGACCACCCTGACC TAC3'

and downstream
5'CACCTTGATGCCGTTCTT CTG3'

control gene - glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GenBank No. M11213) primers:

Upstream
5'AGGGCACTGTCAAGGCTG AGAACG3'

and downstream
5'GCTGAGATGATAACACGCTTAGCACCA3'

were designed. RT-PCR reaction system 12.5µL volume: DNA or cDNA sample 1µl, 0.025U TaKaRa Ex TaqTM R-PCR DNA polymerase (TaKaRa), 1.25µl 10×R-PCR buffer, 0.3mM dNTPs, SYBR Green 1, 3mM Mg^2 , 0.3µM primers. PCR conditions were : 94°C 10s; followed by 40 cycles at 94°C 3s, 62°C 15s and 72°C 20s, and last 72°C for 7min.

RESULTS

Vectors

The maps of various vectors *viz.* pL-CTAGBC, pL-OV1345t PAGFP and pL OV2964t PAGFP are shown Figure 1. The figure also shows the restriction products of three vectors. pL-CTAGBC (9363bp) after restriction with *Xba1* and *Kpn1*, produces three bands *viz.* 2075bp, 3632bp and 3656bp. pL–CTAgNeo (9591bp) after restriction with *Afl II* and *Xho1* produces 2075bp, 3860bp and 3656bp bands. pL-OV1345tPAGFP (10246bp) after restriction with *Afl II* and *BamH1* produces 1901bp, 2833bp and 3656bp bands. pL-OV2964tPAGFP (11870bp) after restriction digestion with *Afl II* and *BamH1* produce 1856bp, 1901bp, 3656bp and 4457bp bands.

Culture of oviduct cells

The oviduct epithelial cells were observed to grow well for first three days whereas an increase in the number of vacuoles with symptom of aging and slow growth was continuously observed in 7- 10 days culture. Cells appeared, gathering agglomerate and beat as the heart. Most of the adherent cells were seen polygonal; some fusiform and some in clusters. The 0, 2, 7, 9, 10 and 18 day image of oviduct epithelial cells are shown in Figure 2.

The oviduct epithelial cells transfected with pL-CTAgNeo expressed the proliferation performance and continuous growth till 18 days. Figure 2F reveals the image of immortalized oviduct cells at 18 days.

GFP DNA

The results of the quantitative view of pL-OV1345tPAGFP infection and its integration efficiency is significantly higher (about 46 times) than pL-OV2964tPAGFP virus. Figure 3 shows difference of abundance of DNA observed between pL-OV1345tPAGFP and pL-OV2964tPAGFP.

GFP mRNA

The measure of the mRNA expression level of pL-OV1345tPAGFP virus was also significantly higher (about 14 times) compared with pL-OV2964tPAGFP virus. Figure 4 shows the difference of abundance of mRNA expression level of pL-OV1345tPAGFP and pL-OV2964tPAGFP.

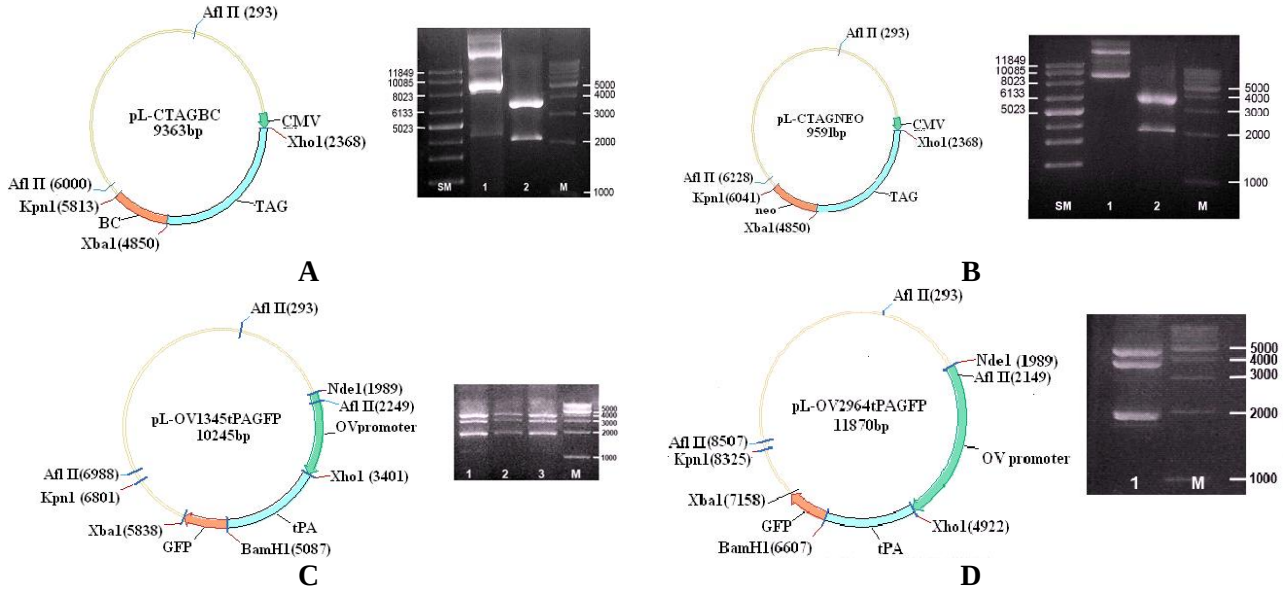

Fig. 1. **A,** Map of pL-CTAGBC vector, Lane 1, plasmid size 9363bp; Lane 2, plasmid digested to produce three bands 2075bp, 3632bp and 3656bp. **B,** map of pL-CTAgNeo Vector; lane 1, plasmid size 9591bp; lane 2, plasmid digestion into 2075bp, 3386bp and 3656bp, respectively; **C,** pL-OV1345tPAGFP Vector, size 11870 bp; plasmid digestion into 1856bp, 1901bp, 3656bp and 4457bp; **D,** pL-OV1345tPAGFP Vector, size 10246 bp; Lanes, 1, 2, 3, plasmid digestion into 1901bp, 2833bp and 3656bp. SM, supercoiled marker TaKaRa; M, Toyobo marker.

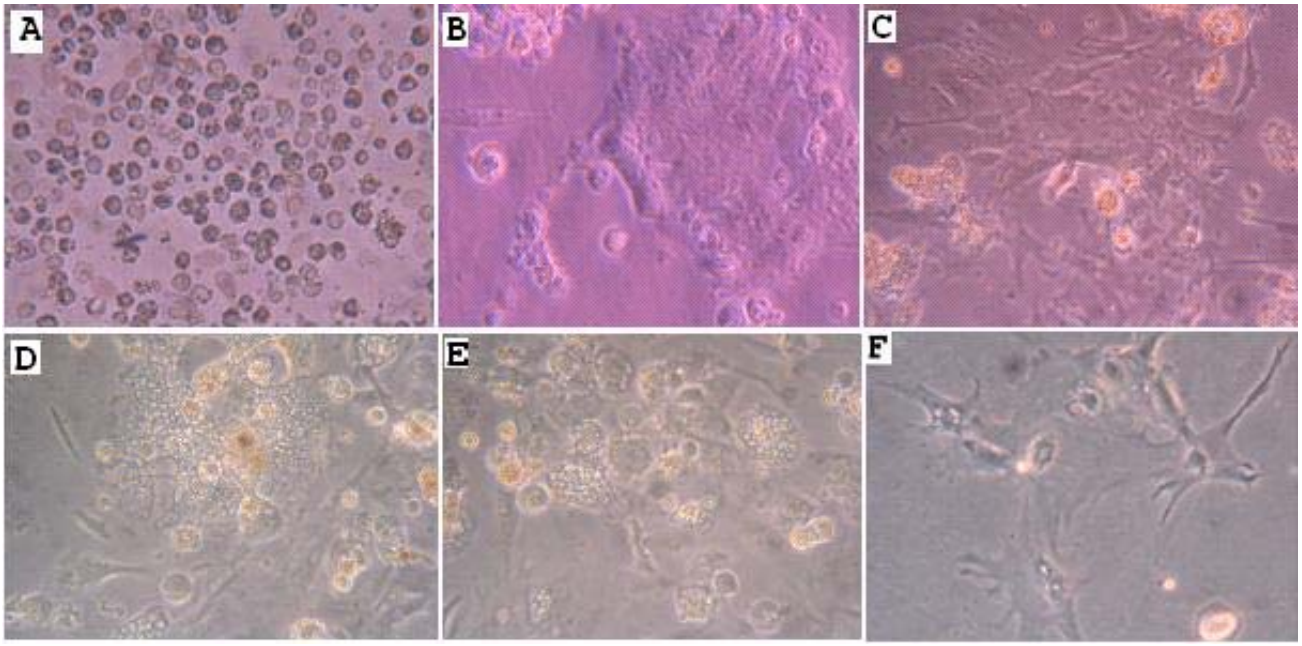

Fig. 2. Oviduct cell on day 0 (A), 2 (B), 7 (C), 9 (D), 10 (E) and 18 (F).

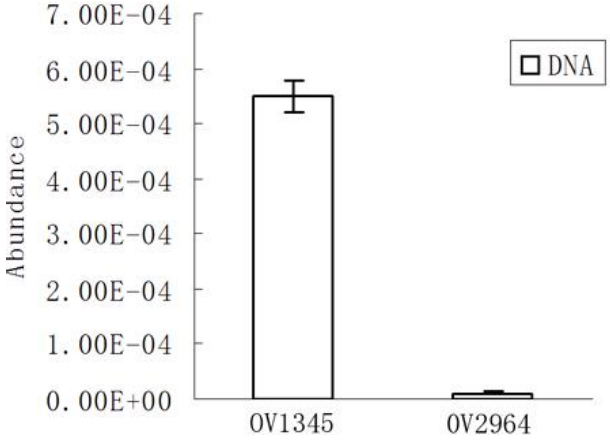

Fig. 3. The difference of abundance of DNA observed between pL-OV1345tPAGFP and pL-OV2964tPAGFP virus transfected with immortalized oviduct epithelial cells.

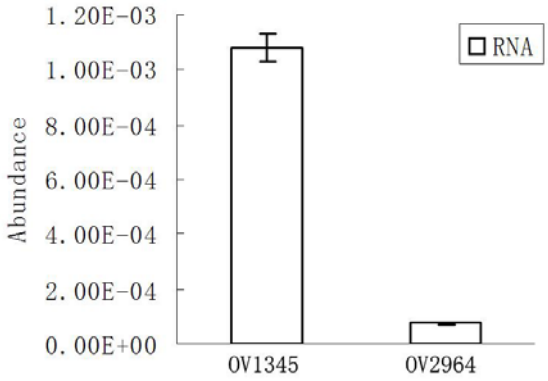

Fig. 4. The difference of abundance of mRNA expression level of pL-OV1345tPAGFPand pL-OV2964tPAGFP virus transfected with immortalized oviduct epithelial cells.

The transcript effect of virus with OV2964 was 3-fold higher than OV1345. Figure 5 shows difference of integration efficiencies of GFP of OV2964 and OV1345 promoter.

DISCUSSION

This study showed that oviduct epithelial cells agglomerated, showed beat like that of heart, most of the adherent cells appeared polygonal, some fusiform and sometime in clusters. The cell clusters

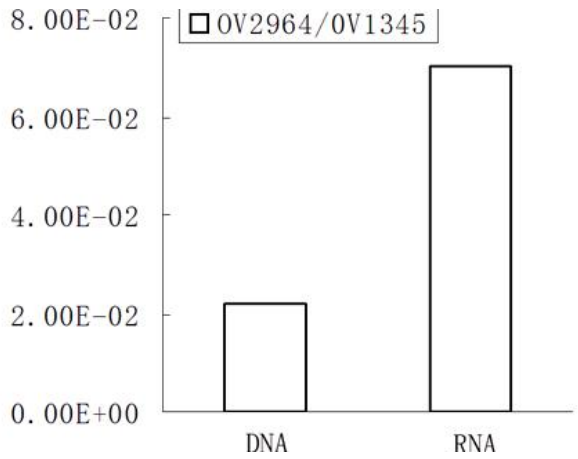

Fig. 5. Difference of integration efficiencies of GFP expression of pL-OV1345tPAGFP and pL-OV2964tPAGFP virus transfected with immortalized oviduct epithelial cells.

were attached to the bottom of the plastic dishes and organized into lumen-like structure. They also looked closely associated to maintain their structural integrity and it may be due to the intermediate filament cytoskeleton that holds epithelium together and is characteristic markers of epithelial identity. The close association of oviduct epithelial cells and their organization into lumen like structure are in consistent with results from other laboratories (Moll *et al*., 1986; Gao *et al*., 2005; Ouhibi *et al*., 1989). The oviduct epithelial cells grew well for first three days and growth rate declined thereafter. The cells were observed with increased vacuoles, aging and cellular death after 7-10 days. It is problematic to culture oviduct cells for longer periods, due to continuous decline in growth and rapid change of aging with cellular death. It is also not easy to transfect oviduct cell due to unavailability of cell line. The oviduct epithelial cells transfected with pL-CTAgNeo virus, maintained structural integrity and growth for 18 days. It revealed that using the SV40 large antigen and neo resistant gene, immortalized oviduct epithelial cells and oviduct epithelial cell line can be prepared. The results are in accordance with those of Okada *et al*. (2005), they immortalised cynomolgus monkey oviduct cells using pSV3-neoplasmid (American Type Culture Collection, Manassas VA, USA) that

contains both large and small T-antigens of the early region of SV40, and the bacterial neomycin phosphotransferase gene, which confers resistance to the antibiotic geneticin. Bertrand *et al*. (1991) also reported immortalization of human oviduct cell line by using a recombinant gene composed of the large T-encoding DNA of SV40 driven by the truncated vimentin promoter (HuVim 830-T/0). Introduction of this recombinant linear fragment into the nucleus of cells in primary cultures derived from various organs of different mammals was followed by integration and expression of the large T antigen, inducing cellular proliferation and resulting in permanent cell lines. It has also been gradually recognized that DNA transfection with chicken oviduct cells is problematic (Hiroshi *et al*., 1998). The expression of endogenous chicken ovalbumin gene is also strictly limited to the oviduct epithelial cells in the laying season. It is regulated by various factors including steroid hormones, cell-substratum, and cell-cell interactions (Muramatsu *et al*., 1995). The total volume of the mRNA expression of OV1345 virus was observed higher than 0V2964 virus, it is nearly 46-fold but the different of integration copy number and single-copy gene expression efficiency is 3 times than Ov1345. It showed that intron have most important role in the expression of OV promoter. However, it increases the vector size that affects virus infection efficiency due to the length of inserted genes, also cause difficulty in the virus production. It is not easy to evaluate effect of intron on ovalbumin promoter activity using the same virus titre and equal number of cells. It needs further investigation to explain the mechanism of intron effect. Gao *et al*. (2005) constructed an oviduct-specific expression vector (pOV), containing 3.0 kb of the 5′-flanking sequence of chicken ovalbumin gene and used various transfection procedures including electroporation, use of liposome and polyethyleneimine to transfect primary oviduct epithelial cells for finding more appropriate method of transfection. They also observed that transfection rate of oviduct epithelial cells mediated by polyethyleneimine produced slightly higher transfection compared the other two methods. At the same time, also showed the specificity of cells (organization) expressed by the exogenous gene under the drive of the constructed oviduct-specific expression vector. The efficiency of oviduct specific expression system is affected by various factors including steroid hormones, cell-substratum, and cell-cell interactions (Masami and Takami, 1990).

The oviduct cells grow well for first few days and number of vacuoles increased with aging and slow growth is seen in 7-10 days culture. The virus The oviduct epithelial cells pL-CTAgNeo transfected control aging and show continuous growth. It was also concluded that first intron has a cis function with major role in expression of promoter activity, and expression of oviduct specific expression system. The antibiotic could also affect physiological characteristic of the immortalized oviduct epithelial cells.

ACKNOWLEDGMENTS

This study was supported by high technology research and development program of China (863 Key program, Code No. 2007AA100504 and Scientific Research Foundation for Doctors, Jinling Institute of Technology, Code No. 403010004 and Natural Science Foundation of the Educational Commission of Jiangsu province, China, Code No. 09kjd230034.

REFERENCES

ASSELIN, C AND BASTIN, M., 1985. Sequences polyomavirus and simian virus 40 large T genes capable of immortalizing primary rat embryo fibroblasts. *J. Virol*., **56**: 958-968.

BERTRAND, S., PATRICK, V., CLAUDE, D. AND DENISE, P., 1991. Mammalian cell lines can be efficiently established *in vitro* upon expression of the SV40 large T antigen driven by a promoter sequence derived from the human vimentin gene. *Biol. Cell*, **73**: 7-14.

BLACKWOOD, E.M., AND KADONAGA J.T., 1998. Going the distance: A current view of enhance reaction. *Science*, **281**: 61–63.

BRUNO, L., AND GUNTHER, S., 1989. Cell-type specificity of regulatory elements identified by linker scanning mutagenesis in the promoter of the chicken lysozyme gene. *Nucl. Acids Res*., **17**: 8451-8463.

CHANG, S., 1989. *In vitro* transformation of human epithelial cells. *Biochim. biophys. Acta*, **823**:161-194.

CSUROS, M., ROGOZIN I.B. AND KOONIN, E.V., 2008. Extremely intron-rich genes in the alveolate ancestors inferred with a flexible maximum-likelihood approach.

Mol. Biol. Evol., **25**: 903-911.

GAO, B., HUAI-CHENG, S., CHENG-YI, S., ZHI-YUE, W., QIN, C. AND HONG-QIN, S., 2005. Transfection and expression of exogenous gene in laying hens oviduct *in vitro* and *in vivo*. *J. Zhejiang. Univ. Sci. B.,* **6**: 137–141.

GAUDRAY, P., RASSOULZADEGAN, M. AND CUZIN, F., 1978. Expression of simian virus 40 early genes in transformed rat cells is correlated with maintenance of the transformed phenotype. *Proc. natl Acad. Sci*., **75**: 4987- 4991.

GIMBRONE, M. AND FAREED, G., 1976. Transformation of cultured human vascular endothelium by SV40 DNA. *Cell*, **9**: 685-693.

HANAHAN, D., 1988. Dissecting multistep tumorigenesis in transgenic mice. *Annu. Rev. Genet*., **22**: 479-519.

HIROSHI, T., HISAKO, W., YASUSHIGE, O., HYI-MAN, P. AND TATSUO, M., 1998. Human alkaline phosphatase expression and secretion into chicken eggs *in vivo* gene electroporration in the oviduct of laying hens. *Biochem. biophys. Res. Commun*., **292**: 88-93.

MASAMI, Y. AND TAKAMI, O., 1990. Transfection of β-casein chi-meric gene and hormonal induction o its expression in primary murine mammary epithelial cells. *Proc. natl. Acad. Sci*., **87**: 3670-3674.

MCKAY, K., STRIKER, L., ELLIOT, S., PRINKERT, C., BRINSTER, R. AND STRIKER, G., 1988. Glomerular epithelial, mesangial and endothelial cell lines from transgenic mice. *Kidney Int*., **33**: 677-684.

MOLL, R., COWIN, P., KAPPRELL, H.P. AND FRANKE, W.W., 1986. Desmosomal proteins: new markers for identification and classification of tumors. *Lab Invest*., **54**: 4–25.

MURAMATSU, T., YOSHIMOTO, M., YAUSUSHIGE, O. and JUN-ICHI, O., 1997. Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos *in Ovo. Biochem. Biophys. Res. Commum*., **230**: 376-380.

OUHIBI, N., YVES, M., GÉRARD, B. AND BERNARD, N., 1989. Culture of epithelial cells derived from the oviduct of different species. *Human Reprod*., **4**: 229-235.

OKADA, H., HIROSEY, M.P., UDA, A., ITO, M. AND SANKAI, T., 2005. Characterization of an immortalized oviduct cell line from the cynomolgus monkey (*Macaca fascicularis*). *J. med. Primatol.*, **34**: 67–72.

PAUL, D., HYHNE, M., PINKERT, C., PIASECKI, A., UMMELMANN, E. AND BRINSTER, R., 1988. Immortalized differentiated hepatocyte lines derived from transgenic mice harboring SV40 T-antigen genes. *Exp. Cell Res*., **175**: 354-362.

RUDLAND, P. AND BARRACLOUGH, R., 1990. Differentiation of simian virus 40 transformed human mammary epithelial stem cell lines to myo epithelial.like cells is associated with increased expression of viral large T antigen. *J. Cell Physiol*., **142**: 657-665.

SACK, G., 1981. Human cell transformation by simian virus 40. *In Vitro*, **17**: 1-19.

(Received 1 October 2010, revised 27 January 2011)