ARTICULOS ORIGINALES COMPLETOS

EFFECT OF INTERVENING SEQUENCES IN THE TRANSIENT EXPRESSION OF TILAPIA GROWTH HORMONE IN MAMMALIAN AND FISH CELLS.

Oscar Hernández[1], Mary Claire Theron[2], Cloudine Puissant[2], Monique Bearzotti[3], Joe Attal[2], Pierre Yi LeBail[4], Edenaida Cabrera[1], Ricardo Lleonart[5], José de la Fuente[5], and Louis Marie Houdebine[2].

[1]*Center for Genetic Engineering and Biotechnology. P.O.Box 387 Camaguey 1, Cuba.* [2]*INRA. Jouy-en-Josas. Unité de Differenciation Cellulaire. 78352. France.* [3]*INRA. Jouy-en-Josas. Unité de Virus de Poissons. 78352. France.* [4]*INRA. Campus de Beaulieu. Lab. Phys. Poissons. 35042 Rennes, France.* [5]*Mammalian Cell Genetics Division. Center for Genetic Engineering and Biotechnology. P.O.Box 6162. Havana 6, Cuba*

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SUMMARY

The study of vertebrate regulatory sequences is essential for the understanding of gene regulation in these organisms. Several enhancer-promoter elements were studied in conjunction with two intervening sequences derived from the SV40 VP1 and rainbow trout growth hormone (tGH) genes. Transient expression of the reporter tilapia GH (tiGH) cDNA was assayed in carp, tilapia and hamster cells. Little or no effect on the tiGH expression was observed with the inclusion of the tGH intron in the fish cell lines. Only in carp cells, and in conjunction with the CMV enhancer-promoter regulatory sequences, a moderate stimulatory effect of the intron was observed. In hamster cells, the inclusion of the tGH intron led to a decrease in the tiGH expression. With the inclusion of the SV40 VP1 intron, a stimulatory effect was observed in hamster cells and a reduction in the fish cells. We concluded that, in transient expression experiments, the inclusion of a fish-derived tGH intron could increase the expression of a reporter tiGH gene depending on the fish cell line and the enhancer-promoter sequences employed.

RESUMEN

El estudio de secuencias reguladoras de vertebrados es escencial para el entendimiento de la regulación de los génes en estos organismos. Varios elementos promotores fueron estudiados combinados con intrones derivados de la proteina VP1 de SV40 y de el gen de la hormona de crecimiento de la trucha arcoiris (tGH). La expresión transiente del ADNc de la hormona de crecimiento de tilapia (tiGH) fue ensayada en células de carpa, tilapia y ovario de hamster chino. Poco o ningun efecto en la expresión de la tiGH se observó con la inclusión del intrón de la tGH en las líneas celulares de peces. Solamente en células de carpa y unido a la región promotora del CMV, un efecto estimulador moderado fue observado. En células de hamster la inclusión del intrón de la tGH provocó una disminución en la expresión de tiGH. El uso del intrón de SV40 causó una estimulación en células de hamster y una disminución de la expresión de la hormona en células de peces. Concluimos que en experimentos de expresión transiente, la inclusión del intrón proveniente de la tGH puede incrementar la expresión de la tiGH dependiendo de la línea celular así como de las secuencias promotoras empleadas.

INTRODUCTION

Transient *in vitro* expression systems have been extensively used for the study of regulatory sequences and in order to find efficient combinations for further use *in vivo* (Vergani *et al.*, 1991; Bearzotti *et al.*, 1992; Inoue *et al.*, 1990; Friedenreich and Schartl, 1990; Pasleau *et al.*, 1987). The study of the inclusion of intervening sequences and its effect on gene expression have also been addressed (Buchman and Berg, 1988; Huang and Gorman, 1990; Chapman *et al.*, 1991; Aronow *et al.*, 1989).

In transgenic animals, the substitution of cDNAs for the corresponding genomic fragments containing the intervening sequences have resulted, at least in some cases, in an increase in the protein expression *in vivo* (Palmiter *et al.*, 1991; Choi *et al.*, 1991; Wright *et al.*, 1991).

In fish, as well as in other organisms, results obtained by *in vitro* studies employing cell lines do not always reflect the activity of constructions *in vivo*. However, the use of appropriate cell lines, together with the possibility of employing other *in vivo* transient expression systems (Lleonart *et al.*, 1992; Etkin *et al.*, 1984; Chourrout *et al.*, 1986; Inoue *et al.*, 1989;

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La Habana, Cuba

Rokkones *et al.*, 1989; Chong and Vielkind, 1989; Stuart *et al.*, 1990), may lead to the establishment of gene regulation mechanisms and the construction of efficient constructs for *in vivo* gene expression studies. Studies on transgenic fish are still in their infancy. However, transgenic fish will be of considerable economic benefit for aquaculture and will enable scientists to make quantum leaps in their understanding of physiological and biochechemical mechanisms unique to fish, considering that fish respond much better than birds and mammals in terms of growth, when they receive the exogenous GH (Le Bail *et al.*, in press) and of the developmental biology of vertebrates in general (De la Fuente *et al.*, 1991; Powers, 1989).

We have studied several combinations of regulatory sequences in conjunction with two introns derived from the SV40 VP1 and tGH genes. In a transient expression system in fish and hamster cells employing the tiGH cDNA as a reporter gene, we found that the inclusion of a fish-derived tGH intron could increase the expression of a reporter tiGH gene depending on the fish cell line and the enhancer-promoter sequences employed. Finally, the best expressing constructs will have to be tested *in vivo* in a transient expression system (Lleonart *et al.*, 1992) and in transgenic fish to corroborate the results obtained here.

MATERIALS AND METHODS

Plasmid constructs

Transient transfections were made with seven plasmids containing the tiGH cDNA (Martínez *et al.*, 1992) fused to different regulatory elements (figure 1). Some of these elements included the SV40 origin of replication (SV40 ori), the human cytomegalovirus enhancer (CMVenh) and/or promoter region (CMVprom), the Roux sarcoma virus LTR promoter region (RSV), the thymidine kinase promoter (TKprom), the SV40 polyadenylation sequences, and intron sequences derived either from the SV40 VP1 protein (VP1) or from the rainbow trout tGH gene (INTT)(Agellon *et al.*, 1988). When the introns were absent, the corresponding constructions were designated as VP1. All constructs were inserted in a pBR322 derivative (figura 1). Details of the constructions are available upon request.

Cell lines and transient transfections

Three cell lines were used, EPC (epidermal carp cells derived from herpes virus-induced hyperplastic lesions) (Fijan *et al.*, 1983; Bearzotti *et al.*, 1992), TO2 (tilapia ovary cells) (Chen *et al.*, 1983) and CHO (chinese hamster ovary cells). The fish cells were cultured at 30°C (EPC) and at 37°C (TO2), respectively. The EAGLE medium modified by Stoker for BHK 21 cells, buffered by 20 mM TrisHCl pH 7.4, and with the addition of 10% tryptose phosphate broth, 10% fetal calf serum, penicillin (100 μ/ml) and streptomycin (100 μg/ml) was employed for fish cells culture. Before transfection, cells were washed three times with OPTI-MEM media (Gibco-BRL). For lipofectin transfection, 3 μg plasmid DNA were mixed in OPTI-MEM with 3 μl of lipofectin (Gibco-BRL), incubated for 15 min at room temperature and added to a 35 mm dish cell monolayer. Cells were incubated with the DNA-lipofectin mixture for 5 h, washed and incubated for 72 h in EAGLE medium. CHO cells were cultured in Ham F-12 medium (Gibco) supplemented with 10% fetal calf serum. Lipofectin transfection was done on 50% confluent cell monolayers. tiGH was assayed in the culture medium 72 h after transfection by using a RIA (Ricordel *et al.*, unpublished results). The 1% BSA blocker agent was not added to the CHO samples.

Northern blot analysis

Total RNA was purified from TO2 cells in the presence of guanidium isothiocyanate at acid pH to eliminate completely the plasmid from the aqueous phase and was extracted three times with phenol:chloroform (Puissant and Houdebine, 1990). Samples of 20 μg were run in 1% agarose denaturing gels and then transferred to Z-probe membranes (BioRad). Resulting imprints were hybridized to the tiGH cDNA probe (0.8 kb; Martinez *et al.*, 1992) labeled with ^{32}P-dCTP (Amersham) by the random primers technique. Hybridization solutions contained 500 mM sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA and 5 g/l dry milk.

Hybridization was at 65°C over night and the membranes were washed once for 15 min at 55°C in 40 mM sodium phosphate pH 7.2, 5% SDS, 1 mM EDTA and 2.5 g/l dry milk.

RESULTS AND DISCUSSION

The experiments described in this report were designed to study the effect of intervening sequences in the expression of tiGH cDNA in fish and hamster cells and to define appropriate enhancer-promoter combinations to design constructs for transgenic fish experiments.

We recovered the culture medium 72 h after transfection for protein analysis. The results are shown in (figure 1). Although several groups have reported on the positive effect of intervening sequences in gene expression (Chapman *et al.*, 1991; Choi *et al.*, 1991; Huang and Gorman, 1990; Palmiter *et al.*, 1991; Wright *et al.*, 1991), the inclusion of INTT in our experiments had little effect on tiGH expression in all fish cells used. However, with pS2CINTT-tiGH we had five times more expression than with pS2CVP-tiGH (without intron) in EPC cells. This finding desagrees with previous results where we used the CAT as a reporter gene and we found higher expression levels using the same construct without the INTT in EPC cells (data not shown), thus suggesting that there may be differences in the interaction between the intron and different coding sequences. We observed an inhibition of tiGH expression in CHO

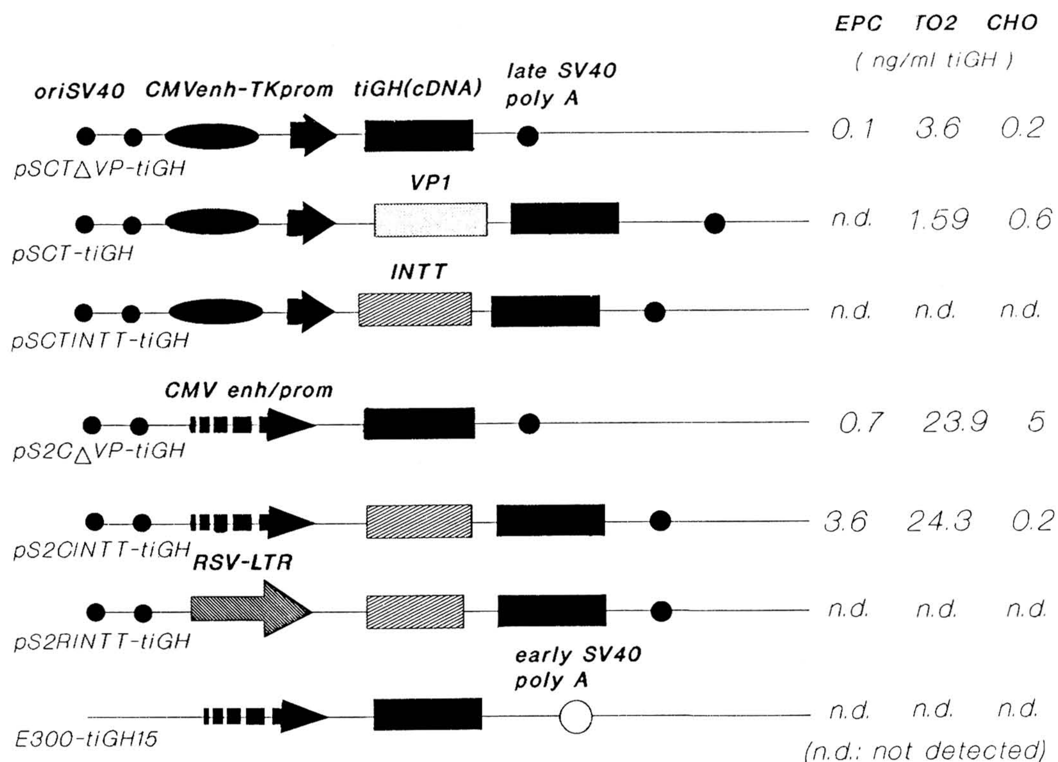

Fig. 1. Structure of the plasmids used for transfection in fish and mammalian cell lines. On the right panel, the results of the tiGH determination by RIA in culture medium for each transfected cell line are shown. Results expresed in ng/ml of tiGH are the means of duplicates from two independent dishes for each give construct.

cells when the INTT was included. This fact may reveal that the putative factors involved in mRNA processing and/or activation of transcription observed in some fish cell lines may not be fully represented in hamster cells (Inoue *et al.*, 1990).

The highest levels of expression were obtained in TO2 cells (figure 1). We observed in these experiments, at least, that the positive effect of INTT was promoter- dependent and it was only observed in carp cells (figure 1). The inclusion of VP1 intron maintained weak but detectable levels of tiGH in TO2 cells (1.6 ng/ml; figure 1). This suggested that the intron can be processed at least partially by this fish cell line. In CHO cells, the same intron (VP1) increased in three times the tiGH protein levels in the culture medium (figure 1), as would have been expected for a construct containing any other mammalian cDNA.

Although we did not have an internal control to normalize for intrinsic variations of the system (efficiency of transfection, cell doubling), the CMV enhancer-promoter present in S2C constructs (figure 1) led to the highest expression levels independently of the cell line. Northern-blot analysis was made in TO2 cells (figure 2). We found that the transcripts obtained from all the constructions had the expected size for the tiGH mRNA (0.8 kb), thus suggesting that the INTT and VP1 introns were well processed in these fish cell lines. The different transcript size observed for the constructs carrying the INTT intron was probably due to the presence of a 141 bp fragment from exons I-II of tGH (Agellon *et al.*, 1988).

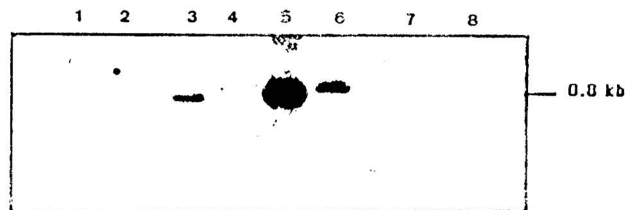

Fig. 2. Northern-blot analysis of RNA from transfected TO2 cells. Twenty g of total RNA from TO2 cells transfected with several constructs were run in a 1% agarose denaturing gel. The arrow indicates the RNA corresponding with tilapia growth hormone cDNA. Lane 1: E300-tiGH15, lane 2: pSCT-tiGH, lane 3: pSCTVP-tiGH, lane 4: pSCTINTT-tiGH, lane 5: pS2CVP-tiGH, lane 6: pS2CINTT-tiGH, lane 7: pS2RINTT-tiGH, lane 8: RNA from TO2 cells non transfected as negative control.

When we used the CAT reporter gene, the VP1 intron was not well processed in stable transfected EPC cells affecting the CAT expression in these cells (unpublished results). Similar results have been reported before and may indicate that mammalian introns might be not processed properly in some cases.

In conclusion, we have shown that in transient expression experiments, the inclusion of a fish-derived INTT intron can moderately increase the expression of tiGH in carp but not in tilapia cells and that this effect is dependent on the enhancer-promoter sequences employed. Experiments are in progress to test these constructs *in vivo* in transient expression systems and in transgenic tilapia.

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REFERENCES

AGELLON, L. B.; S. L. DAVIES; T. T. CHEN and D. A. POWERS (1988). Structure of a fish (rainbow trout) growth hormone gene and its evolutionary implications. *Proc. Natl. Acad. Sci. USA*. **85**:5136-5140.

ARONOW, B.; D. LATTIER; R. SILBIGER; M. DUSING; J. HUTTON; G. JONES; J. STOCK; J. Mc NEISH; S. POTTER; D. WIGINTON (1989). Evidence for a complex regulatory array in the first intron of the human adenosine deaminase gene. *Genes and Development* **3**:1384-1400.

BEARZOTTI, M.; E. PERROT; C. MICHARD-VANHEE; G. JOLIVET; J. ATTAL; M.C.THERON; C. PUISSANT; M. DREANO; J.J. KOPCHICK; R. POWELL; L.M. HOUDEBINE; D. CHOURROUT (1992). The expression of various gene constructs transfected into fish cells. *J. Biotechnology* **26**: 315- 325.

BUCHMAN, A. R. and P. BERG (1988). Comparison of intron-dependent and intron-independent gene expression. *Molec. Cell. Biol.* **8**:4395-4405.

CHAPMAN, B.S.; R.M.THAYER; K.A.VINCENT and N.L. HAIGWOOD (1991). Effect of intron A from human cytomegalovirus (Towne) immediate early gene on heterologous expression in mammalian cells. *Nucleic Acids Research* **19**:3979-398

CHEN, S.N.; S.C. CHI; Y. UENO and G.H. KOU (1983). A cell line derived from tilapia ovary. *Fish Pathology* **18**:13-18.

CHOI, T.; M. HUANG; C. GORMAN and R. JAENISCH (1991). A generic intron increase gene expression in transgenic mice. *Molec. Cell. Biol.* **11**:3070-3074.

CHONG, S.S.C. and J. R. VIELKIND (1989). Expression and fate of CAT reporter gene microinjected into fertilized medaka (*Oryzias latipes*) eggs in the form of plasmid DNA, recombinant phage particles and its DNA. *Theor. Appl. Genet.* **78**:369-380.

CHOURROUT, D.; R. GUYOMARD and L.M. HOUDEBINE (1986). High efficiency gene transfer in rainbow trout (*Salmo gairdneri* RICH.) by microinjection into egg cytoplasm. *Aquaculture* **51**:143-150.

DE LA FUENTE, J.; O.HERNANDEZ; L.GUILLEN; E.O.CASTRO; A.AGUILAR; L.HERRERA; C.ULIVER; A.PEREZ (1991) Transgenesis in fish. Applications in Biotechnology. *Biotecnología Aplicada* **8**:123-139.

ETKIN, L.D.; B. PEARMAN; M. ROBERTS and S. L. BEKTESH (1984). Replication, integration and expression of exogenous DNA injected into fertilized eggs of *Xenopus laevis*. *Differentiation*. **26**:194-202.

FIJAN, N; D. SULIMANOVIC; M. BEARZOTTI; D. MUZINIC; L. O. ZWILLENBERG;. C HILMONCZYK; J. F. VAUTHEROT and P.DEKINKELIN (1983). Some properties of the Epithelioma papulosum cyprini (EPC) cell line from carp Cyprinus carpio. *Ann. Virol. Inst. Pasteur* **134** (E): 207- 220.

FRIEDENRICH, H. and M.SCHARTL (1990). Transient expression directed by homologous and heterologous promoter and enhancer sequences in fish cells. *Nucleic Acids Research* **18**:3299-3305.

HUANG, M.T.F. and C. M. GORMAN (1990). Intervening sequences increase efficiency of RNA 3' processing and accumulation of cytoplasmic RNA. *Nucleic Acids Research* **18**:937-947.

INOUE, K.; K. OZATO; H. KONDOH; T. IWAMATSU; Y. WAKAMATSU; T. FUJITA; T. S.OKADA (1989). Stage-dependent expression of the chicken d- crystallin gene in transgenic fish embryos. *Cell Differenciation and Development*. **27**:57-68.

INOUE, K.; N. AKITA; S. YAMASHITA; T. SHIBA and T. FUJITA (1990). Constitutive and inducible expression of atransgene directed by heterologous promoters in a trout liver cell line. Bioch. Bioph. Res. Comm. **173**:1311-1316.

LeBAIL, P.Y.; PEREZ-SANCHEZ, J.; YAO, K.; and G. MAISSE (1992). Effect of GH treatment on salmonid growth: study of the variability of response. Comp. Biochem. Physiol. (in press)

LLEONART, R.; D. GARCIA DEL BARCO; O. HERNANDEZ; J. DE LA FUENTE (1992). Microinyection of DNA into tilapia (*Oreochromis aureus*) and common carp (*Cyprinus carpio*) one celled embryos: A novel system for *in vitro* transient gene expression studies. *Advances in Modern Biotechnology*. **1**:18.1

MARTINEZ, R.; D. GARCIA DEL BARCO; O. HERNANDEZ; R. LLEONART; F. HERRERA; E. CABRERA; J. DE LA FUENTE (1992). *Generation of transgenic tilapia with quimeric gene that direct the syntesis of mRNA in CHO cells.* Miami Short Reports. (Ed. by W. J. Whelan, F. Ahmad, H. Bialy, S. Black, M. Lou King, M. B. Rabin, L. P. Solomonson, L. I. K.Vasil) 2:68.

PALMITER, R.D.; E.P. SANDGREN; M.R. AVARBOCK; D.D. ALLEN; R.L. BRINSTER (1991). Heterologous introns can enhance expression of transgenes in mice. *Proc. Natl. Acad. Sci. USA* **88**:478-482.

PASLEAU, F.; F. LEUNG and J. KOPCHICK (1987). A comparison of bovine growth hormone expression directed by bGH genomic or intronless DNA in transiently transfected eukaryotic cells. *Gene* **57**:47-52.

POWERS, D.A. (1989) Fish as model systems. *Science* **246**:352-358.

PUISSANT, C. and L.H. HOUDEBINE (1990). An improvement of the single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Biotechniques* **8**:148-149.

ROKKONES, E.; P. ALESTROM; H. SKJERVOLD and K.M. GAUTVIK (1989). Microinjection and expression of a mouse metallothionein human growth hormone fusion gene in fertilized salmonid eggs. *J. Comp. Physiol.* B..**158**:751-758.

STUART, G. W; J. R.VIELKIND; J. V. McMURRAY; M. WESTERFIELD (1990). Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. *Development*. **109**:557-584.

VERGANI, G.; A. MAYERHOFER and A. BARTKE (1991). Acute effect of human growth hormone on liver cells *in vitro*: a comparison with livers of mice transgenic for human growth hormone. *Tissue and Cell* **23**:607-612.

WRIGHT, G.; A. CARVER; D. COTTOM; D. REEVES; A. SCOTT; P. SIMONS; I. WILMUT; I. GARNER and A. COLMAN (1991) High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. *Bio/Technology* **9**:830-834.