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# Regulation of Cell Growth and Alteration of Gene Expression in Human Hepatoma Cells by a Carp Maternal Gene

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**Pei-Yen Yeh and Fu-Hsie Yew (1993)** Regulation of cell growth and alteration of gene expression in human hepatoma cells by a carp maternal gene. *Bull. Inst. Zool., Academia Sinica* **32**(3): 204-213. A carp maternal cDNA library was used to transfect tumor-like tilapia ovary cells in culture, and a plasmid was subsequently rescued from a single cell clone. Transfection of the rescued plasmid into human hepatoma cells caused them to lose their tumorous characteristics; they were unable to pile up and failed to grow in soft agar. Moreover, we found that a co-culture of transformed and parental cells, both tilapia ovary and human hepatoma cells, could effectively suppress the tumorous phenotypes of the parental cells. The screening of several cellular proteins by western blot immunostaining showed that $\alpha$-fetoprotein and oncogenic proteins, *ras*, and *abl* were reduced in transformed cells; in addition, phosphorylated proteins with molecular weights corresponding to $pp60^{src}$ and EGF receptor were decreased. However, the tumor suppressor gene p53 product and a matrix protein collagen type IV were enhanced. Our results suggest that this maternal gene may be directly involved in cell growth and differentiation control.

**Key words:** Maternal gene, Growth regulation, Hepatoma cell.

*In vivo*, most somatic cells reside in a quiescent state. In the absence of growth factors, or as a result of their depletion, cells in culture are usually restricted to the G1 stage and are unable to divide. During early embryogenesis, however, blastomeres undergo rapid cell division, and in early embryos the cell cycle consists of only S phase and mitosis until reaching midblastula stage (Newport and Kirschner 1982). Previous studies on early embryogenesis have suggested that maternal factors which are present in eggs may overcome cell growth restrictions in somatic cells. Several growth factors and peptides that are homologous to oncoproteins have been identified in the maternal genes or proteins or mature eggs; these factors have been shown to affect embryonic development and blastoderm induction (for a detailed review, see Jessell and Melton 1992). In xenopus eggs, the maternal factor Vg1 which is homologous to transforming growth factor $\beta$ (TGF$\beta$) can cooperate with FGF to induce mesoderm formation (Weeks and Melton 1987, Kimelman and Kirschner 1987); however, in culture TGF$\beta$ has an inhibitory effect on myogenesis (Massague *et al.* 1986) as well as multiple effects on cell growth and differentiation in a cell type-dependent manner (Barnard *et al.* 1990, Rizzino 1988). The mechanisms and functions of these growth factors are still uncertain, and cell response

may be dependent on several additional factors, including variation in microenvironments (such as intercellular communication) and cell stage in the differentiation pathway.

When microinjected into inbred strain blastula embryos, mouse teratocarcinoma cells can develop totipotently to form a mosaic animal (Mintz and Illmensee 1975). This suggests that the tumorigenesis in teratocarcinoma cells is not due to irreversible changes in DNA, but more likely due to changes in extracellular environment; conversely, early embryo factors can redirect the regulation in growth and differentiation in teratocarcinoma cells. According to these findings, it is possible that those factors involved in the regulation of cell growth and differentiation may be found in maternal genes.

We attempted to find whether or not there is a maternal gene which is able to regulate the rate of cell growth, as well as to set the course which leads to differentiation. Our laboratory holds a spontaneously-transformed TO2 cell line, derived from tilapia ovary, which grows very fast (generation time 10 h) and piles up without affecting growth rate. Therefore, it appeared suitable to use TO2 cells as recipients for the transfection of a whole maternal cDNA library, from which we selected slow-growing clones and rescued maternal genes. The isolated gene was then used to transfect either human tumor cells or NIH3T3 cells so as to search for changes in tumor phenotype and gene expression.

In this report we describe a carp maternal gene which reversed the state of transformation of a human tumor cell and also changed the phenotype in other ways. Along with the detransformation, many changes in the expression of different cellular genes were also observed.

## MATERIALS AND METHODS

### Cells and Cell Cultures

TO2 cells derived from tilapia ovary were cultured in L-15 medium supplemented with 10% foetal calf serum (FCS) at 31°C (Chen *et al.* 1983); this is an established cell line which becomes spontaneously transformed in culture and with a generation time of 10h. Cells grow quickly at high densities even when pile up and form colonies in soft agar, but they cannot induce tumors in nude mice. NTU-BL, a cell line established from a hepatoma biopsy (Wen *et al.* 1990), was supplied by Dr. W. Wen (Institute of Biochemistry, College of Medicine, NTU); it was kept in Ham's F12 medium supplemented with 10% FCS, and incubated in a 5% $CO_2$, 37°C humidified incubator. NTU-BL cells can also be grown in serum-free media. At high densities, cells pile up to form domes, but have a reduced growth rate. These cells also grow in soft agar but cannot induces tumor in nude mice.

### Gene cloning, transfection, and plasmid rescue

Carp eggs were supplied by Dr. F. L. Huang (Dept. of Zoology, N.T.U.). Eggs were homogenized in proteinase K digestion buffer, followed by proteinase K digestion. RNA was extracted with phenol/chloroform and precipitated with ethanol. mRNA was selected via two runs of oligo-dT chromatography. The method of cDNA library construction followed the procedures of Okayama and Berg (1982). cDNA fragments were linked to pSV2 with two *Hind*III site ends. The entire cDNA library was used to transfect TO2 cells directly by calcium co-precipitation (Ausubel *et al.* 1987). Transfected cells were plated at low densities (about 200 cells per 10cm dish) to allow colonies to form from single cells. After 10 to 15 days, most cells formed large, piled-up colonies. A number of small (50 to 100

cells), non-expanding colonies formed; these were selected and subjected to further investigation; about twenty clones were selected from the original $10^6$ plated cells. Some of these selected clones did not grow well and were therefore discarded, but many of them showed regulated growth patterns; and from these, a TC19 clone was chosen for plasmid rescue due to its consistant behavior and easy handling.

Plasmid rescue from the genomic DNA of TC19 was done according to the methods described by Perucho *et al.* (1980). CsCl-purified plasmid was transferred along with pSV2neo (total DNA 17 $\mu$g, with plasmid-to-pSV2neo ratio 10:1) into $10^6$ NTU-BL cells by calcium precipitation. Transformed cells were selected with 500 $\mu$g/ml G418 for 20 days and maintained in 250 $\mu$g/ml G418; transforming frequency was less than $10^{-4}$. A single cell colony designated HC10 was selected for further analysis because of its constant morphology, dependable growth, and clear integration of the plasmid which was proven by Southern blot analysis (see Results section). In addition, a 10 cm dish of transformed cells (about 70 colonies) was pooled for use as a reference.

## Restriction map and Southern and Northern blot hybridization

The rescued plasmid (designated pSC19) was digested with various restriction endonucleases as indicated in figure legend and separated on a 0.8% agarose gel. Genomic DNA extraction and Southern blot hybridization was performed according to Ausubel *et al.* (1987). Afterwords, 10 $\mu$g DNA was digested with 50 U restriction endonuclease, separated in 0.8% agarose gel, and transferred to nylon filter (Hybond N, Amersham); the genomic DNA was then subjected to Southern blot hybridization. Digoxigenin-dUTP(DIG) random-labelled pSC19 was used as a probe, alkaline phosphatase-conjugated anti-DIG antibody was applied, and AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-1,2-deosetane) was used as substrate to develop DNA bands (all reagents except AMPPD purchased from Boehringer Mannheim; AMPPD was purchased from Tropix). Whole cell RNA was extracted by the guanidinium/phenol method described by (Chomczynski and Sacchi 1987). About 20 $\mu$g of RNA was separated on a 1% formaldehyde RNA denaturing gel. After transferring the RNA onto nitronyloncellulose filter, Northern blot hybridization was performed as previously described for Southern blot hybridization.

## Cell growth and characterization of transformed phenotypes

TO2 and transformant TC19 cells were seeded into 60mm dishes with 4 ml medium at initial cell density of $3.4\times10^4$; the plating efficiencies of the two cells were similar. Under co-culture conditions, cells were seeded at 1 to 1 ratio in equal total cell numbers. Cells were harvested every two days, and cell numbers were determined with a hemocytometer. Each data point represents the average of at least four independent experiments with a maximal deviation of 10%. Approximately $1\times10^5$ NTU-BL and HC10 cells were seeded in 60mm culture dishes with 4ml complete or serum-free medium per dish. Cells in some dishes were harvested every two days, and cell numbers were determined with a hemocytometer. Cells from each dish were further diluted into medium for soft agar growth assay; for these growth assays, $1.4\times10^4$ cells ($1\times10^5$ for pooled transformed cells) cells in 0.3% agar, F-12 (10% FCS) medium were poured onto a 0.6% agar layer (10% FCS, F-12 medium) and continuously cultured for 15-20 days. Colonies of more than 50 cells were counted. Another aliquat of cells was fixed in 3 parts absolute methanol to 1 part glacial

acetic acid, then briefly stained with Giemsa solution for microphotography.

### Western blot immunostain

NTU-BL and transformant cells were seeded at $1\times10^6$/60mm dish in serum-free medium and harvested 4, 6 and 8 days after plating; these cells were lysed directly with 2x SDS sample buffer. Whole-cell extract for $2\times10^5$ cells was subjected to SDS-PAGE electrophoresis. Proteins were separated with various gel concentrations according to antigen molecular weights, and subjected to a constant 8mA current for 12-16 hours. Western blot immunostaining was performed as described by Harlow and Lane (1988). The screened antibodies were listed as: EGF receptor (monoclone; sigma); *myc* (monoclone; ICI); *ras* and *src* (polyclone from sheep; ICI); *abl* and phosphotyrosine (polyclone from rabbit; Pharmingen); p53 (monoclone; Pharmingen); collagen type IV (rabbit polyclone; Collaborative Biomedical Products); and $\alpha$-fetoprotein (rabbit polyclone; Dako). All primary antibodies were applied in approximately 1 $\mu$g/ml concertration; alkaline phosphatase conjugated ABC (anti-biotin-conjugated) was used to develop the protein bands.

## RESULTS

We constructed a cDNA library from carp maternal mRNA with pSV2 as an expression vector. Two *Hind*III sites were created at the ends of the cDNA strands for ligation. The library was used to directly transfect a tilapia ovary cell line (TO2). Clone selection was based on morphology and growth rate (see Materials and Methods); since this was a functional assay rather than a conventional neomycin-resistance selection, and it is not a single gene transfection, the transformation frequency was difficult to estimate. Twenty clones were selected from the original $10^6$ seeded cells, so the frequency of occurrence of this phenotype was probably at least $2\times10^{-5}$.

The TC19 clone was chosen for investigation because of its consistancy in growth behavior. Fig. 1 shows the growth patterns of the TO2 cells and TC19 transfectant clone. In normal growth medium (L-15, 10% FCS), TO2 cells displayed log-phase proliferation throughout the culture period (up to $10^6$ per 60mm dish) while the transfectant TC19 cells reached a saturation point after four days ($3\times10^5$ per 60mm dish). A co-culture TO2/TC19 reached a saturation density similar to that for TC19 after six days; at this time, TO2 cells cultured alone piled up to form many domes, but the TC19/TO2 coculture cells did not (Fig. 2).

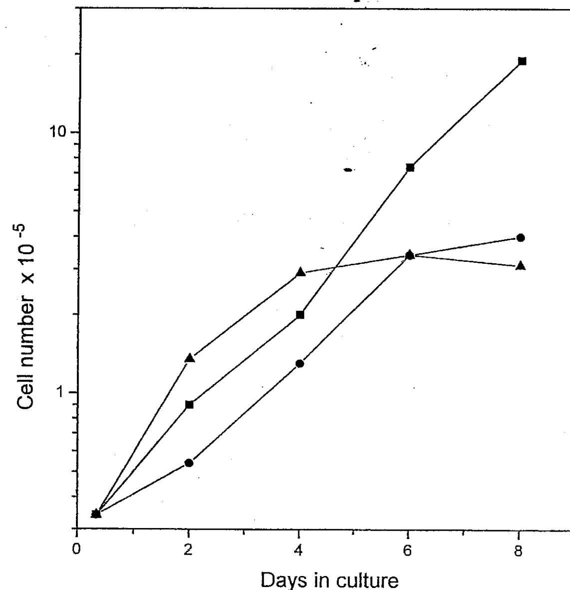


Fig. 1. Growth profiles of TO2 and TC19 cells. An initial number of $3.4\times10^4$ cells were grown in 4ml L-15 medium at 31°C and harvested every two days. Numbers of cells were determined with a hemocytometer. Each points is the average of at least four independent experiments. Deviation is within 10%.
■: TO2; ●:TC19; △: TO2/TC19 co-culture.

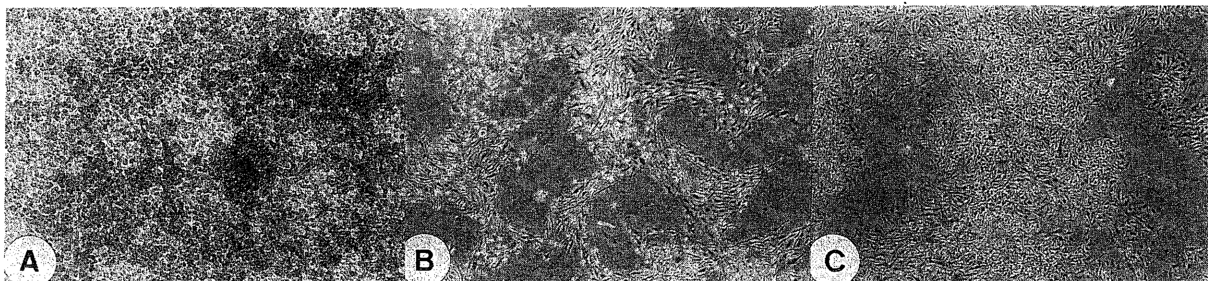


Fig. 2. TO2 and transformant TC19 cells. Cells were seeded a density of $3.4 \times 10^4$ per 60mm dish in L-15 medium and cultured for six days (Giemsa stained, 40x magnification)
a. TO2 cells; b. TC19 cells; c. cells in co-culture.

Plasmid pSC19 was rescued from TC19 cells. The maternal gene, a 3.3kb fragment released by *Hind*III digestion, was analyzed via restriction endonuclease digestion; the restriction map indicating single or double restriction sites is shown in Fig. 3.

Genomic Southern blot hybridization of HC10 cells is shown in Fig. 4a; the 3.3kb DNA fragment released from high molecular weight DNA by *Hind*III digestion and the ladder-like higher molecular weight bands suggest that pSC19 was integrated into the hepatoma genome in multiple copies without recombination. Results of the Northern blot hybridization revealed an approximately 3.2kb RNA transcript in HC10 cells (Fig. 4b).

The growth profiles of cells in 10% FCS culture medium showed no differences between hepatoma and transfectant HC10 cells (Fig. 5). However, after 10 days in culture, hepatoma cells piled up, but HC10

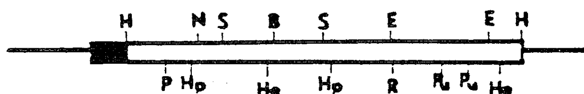


Fig. 3. Restriction map of 3.3kb maternal gene. Restriction sites are indicated as: H, *Hind*III; N, *Nco*I; S, *Sph*I; B, *BamH*I; E, *EcoR*I; P, *Pst*I; Hp, *Hap*I; He, *Hae*II; R, *Rsa*I and P, *Pvu*II. Solid box represents 323bp SV40 early gene promoter.

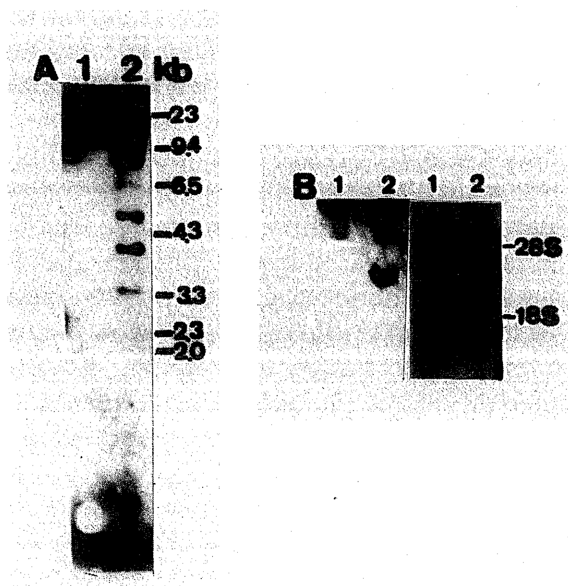


Fig. 4a. Southern blot hybridization of HC10 genomic DNA.
Lane 1: undigested DNA;
Lane 2: *Hind*III digested DNA.
The DNA fragment lengths were measured from *Hind*III digested λ-DNA.

Fig. 4b. Northern blot hybridization of HC10 whole-cell RNA.
Lane 1: Parental hepatoma NTU-BL RNA;
Lane 2: Transfectant HC10 RNA.
Both 28S and 18S RNA were used to show molecular weights and RNA quantities in the two cell-types.
Dig-random primed labelled pSC19 was used as probe.

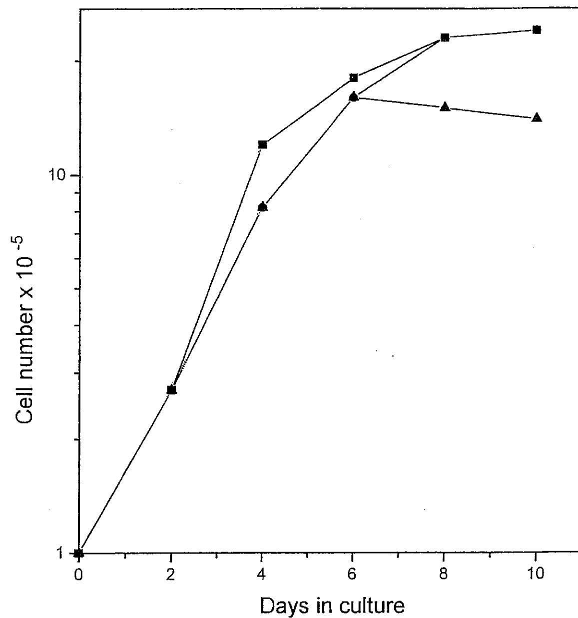


Fig. 5. Growth profiles of NTU-BL and HC10 cells. An initial number of $1 \times 10^5$ cells were plated in 4ml F-12 medium supplemented with 5% FCS and harvested every two days. Cell numbers were determined with a hemocytometer. Each point is the average of at least four independent experiments. Deviation is within 10%.
■: BL; △: HC10; ●: cells in co-culture.

Table 1. Colony formation in soft agar: To establish growth assays in soft agar, $1.4 \times 10^4$ cells ($1 \times 10^5$ for pooled transformed cells) in 0.3% agar (F-12 supplemented 10% FCS) medium were poured onto a 0.6% agar layer (F-12 medium, 10% FCS) and continuously cultured for 15-20 days. Colonies of more than 50 cells were counted.

| | NTU-BL alone | HC10 alone | pooled cells | HC10 & NTU-BL co-culture |
|---|---|---|---|---|
| 2 days | 502 ± 38 | 0 | 0 | 101 ± 20 |
| 4 days | 142 ± 18 | 0 | nd | 49 ± 4 |
| 6 days | 234 ± 56 | 0 | nd | 13 ± 2 |

nd: not determined

cells did not, even in a prolonged culture of up to 15 days. Co-cultures of the two cell types revealed the same behavior as HC10 cells cultured alone; they did not exhibit any piling up of cells (Fig. 5, 6). The results from an anchorage independent growth assay are shown in Table 1. We observed that HC10 and pooled transformed cells lost their ability to grow in soft agar. Though we observed a few colonies which had 8-16 cells in pooled transformed cells, prolonged culturing did not result in further growth.

The results of Western blot immunostains are presented in Fig. 7. We observed that *myc* protein, although present in very small amounts, was expressed in both NTU-BL and

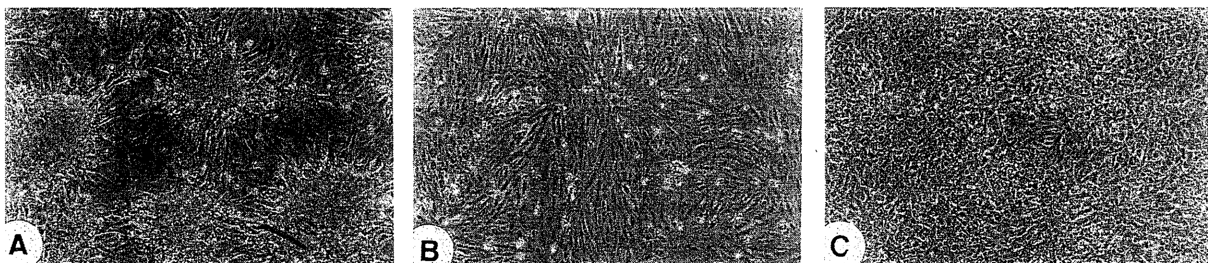


Fig. 6. NTU-BL and HC10 cells. Cells were seeded at a density of $10^5$ per 60mm dish in F-12 medium, with 5% FCS and were cultured for 10 days or 15 days. (Giemsa stain, 40x magnification).
a. NTU-BL cells; 10 days. b. HC10 cells; 15 days. c. co-cultured cells; 15 days.

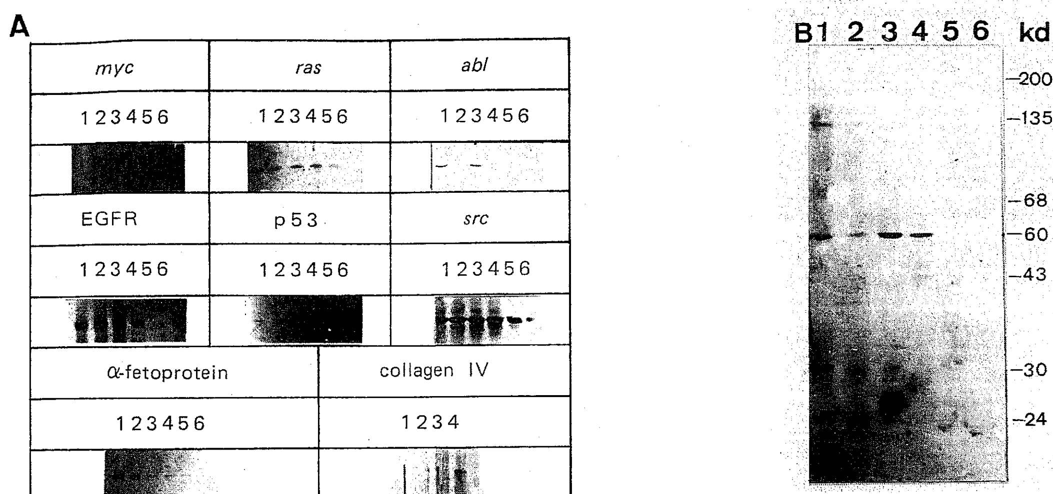


Fig. 7. Western blot immunostain of NTU-BL and HC10 cells. $1\times10^6$ cells were seeded in serum-free medium and harvested 4, 6 and 8 days after plating. $2\times10^5$ cells were lysed in SDS sample buffer and subjected to SDS-PAGE electrophoresis. Western blot immunostain was performed as described by Harlow and Lane (1988).

A. Lanes 1-3: NTU-BL on days 4, 6 and 8.
Lane 4-6: HC10 cells on corresponding days.
For collage type IV, cells were cultured under the previously described conditions for 10 days.
Lane 1: NTU-BL 10% FCS; Lane 2: NTU-BL serum-free; Lane 3: HC10 10% FCS; Lane 4: HC10 serum-free.

B. Immunostain of tyrosine phosphate. Bands with molecular weights corresponding to EGRF(135kd), *src*(60kd), and possibly *ras*(30 and 23kd) are indicated.

HC10 cells; *abl* protein was expressed in NTU-BL but not in HC10 cells. In NTU-BL cells, *ras* protein levels did not change during the culturing period, but they did decrease in HC10 cells as culture time was prolonged. Though total *src* protein amounts appear to remain constant in both cells, the phosphotyrosine stain revealed that protein bands with molecular weight corresponding to $pp60^{src}$ decreased in HC10 cells after prolonged culturing (Fig7B, 60kd). Also, the phosphorylated protein corresponding to the EGF receptor molecular weight (135kd) diminished in parental NTU-BL cells during culturing, and was completely absent from the transfectant HC10 cells. Two more phosphorylated proteins of approximately 30 and 24 kd was found to be present in the NTU-BL cells but not in the HC10 cells; this may represent phosphorylated *ras* protein. Tumor suppressor p53 protein was significantly enhanced in the HC10 transfectant cells. Collagen type IV was detected only in HC10 cells that were cultured for 10 days in serum-free medium , but not in the presence of 10% FCS; collagen type IV was completely undetectable in NTU-BL cells regardless of whether the cells were cultured with or without serum. The $\alpha$-fetoprotein level decreased in the NTU-BL cells as culture time was prolonged, but in HC10 cells the observed basal level did not change with time.

## DISCUSSION

The main functions of maternal genes are generally regarded as directing early embryo

cell growth and differentiation; further exploration of maternal mRNA roles may uncover those factors related to cell growth and differentiation control. The direct transfection of an entire cDNA library into cultured cells and subsequent search for altered phenotypes appeared to be a straight forward way to find the regulatory or determining elements. Although we cannot rule out the possibility that more than one maternal gene was integrated into the TC19 cells, following plasmid rescue we screened 10 *E. coli* colonies via alkaline mini-assays and always observed identical resultant patterns (data not shown). The possibility of a nonspecific integration being responsible for the observed changes in transformed cells seems remote, since the pooled transformed hepatoma cells showed a high frequency of appearing the same phenotype in soft agar growth assays. It also does not seem likely that specific changes in multiple gene activities could be due to nonspecific integration.

The TC19 cell growth profiles suggest that the gene is not a growth inhibitor, since the cells maintained a growth rate comparable to that of the parental cells during the first six days after plating. The results of co-culture experiments suggest that transformed cells regulate the growth of parental cells, either by producing diffusible factors or via cell-cell interaction. Kim *et al.* (1991) reported that the transfection of an *Rb* gene could induce the expression of TGF$\beta$, a growth inhibitor found in various cells. However, we do not believe that the 3.3kb maternal gene codes for a product such as *Rb*, since the transfection of an *Rb* gene under a strong promoter control arrests growth in the transformed cell (Su-Huang *et al.* 1988). We have yet to analyze whether or not TGF$\beta$ was present in the HC10-conditioned media; as TGF$\beta$ is secreted in a latent form with a higher molecular weight, and since its conversion to an active form requires further acid or protease treatment, its presence in HC10-conditioned medium is questionable. Finally, hepatocyte growth factor (HGF) has recently been shown to suppress tumor cell growth both *in vitro* and *in vivo* (see Furlong 1992 for a review, Shiota *et al.* 1992). Whether or not the HC10-conditioned medium contains HGF is presently under investigation.

Though many of their functions are still unknown, oncogenic proteins are likely associated with the control of cell growth. In addition, oncogene products have been shown to have major effects on differentiation for example, *myc* on haematopoiesis (Lachman *et al.* 1985, Lachman *et al.* 1986). However, it is very hard to distinguish those complicated interactions that constitute growth and/or differentiation. It remains possible that the blocking of cell differentiation in haematopoiesis by oncogenes is due to an overexpression of oncogene products which are known to stimulate cell growth and inhibit cell differentiation. In response to cellular gene expression and cell growth, several proteins such as *ras* and EGFR, were observed in different amounts in NTU-BL and HC10 cells. These results suggest that a major function of the maternal gene concerns growth control, and that it exerts this influence by regulating gene expression either directly or indirectly. On the other hand, $\alpha$-fetoprotein is strongly expressed in embryonic hepatocytes, hepatomas and during liver regeneration, but not in normal adult hepatocytes (Tamaoki and Fausto 1984). The reduced expression of $\alpha$-fetoprotein in HC10 cells may indicate that the maternal gene has an effect on cell differentiation; these together with other proteins create cellular microenvironments of differentiated cells.

The tumor suppressor gene p53 shows a high frequency of mutation or allele loss in human tumors (Donehower *et al.* 1992). In comparison with parental hepatomas, in HC10 cells there is either a strengthening

of p53 protein or an induction of p53 expression. We cannot rule out the possibility that mutated forms of p53 exist in HC10 cells, or if p53 is either overexpressed or at a normal level. From the observed suppression of transformed characteristics, we speculate that p53 in HC10 is under regulatory expression; further investigation should help to uncover those factors or pathways involved in p53 expression control.

We are currently trying to fractionate the HC10-conditioned medium in order to search for the factor or factors responsible for the inhibition of cell growth. Since parental NTU-BL cells cannot induce tumors in nude mice, we do not know whether or not the suppression of tumor phenotype by this maternal gene is complete. We are testing this by transfecting this gene into HeLa cells and looking for subsequent tumor induction in nude mice.

We do not know if our cDNA library of maternal genes used in the research was complete, nor the number of copies of each gene present in the maternal mRNA pool. Although the clones selected from the transfected TO2 cells were those showing regulatory growth, we cannot be certain that they were transformed by the same gene. Research on the gene sequencing and the purification of these growth regulating factors are in progress.

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# 一個鯉魚母系RNA基因可以調控肝癌細胞生長及基因表現

葉培彥　游復熙

體外受精的卵細胞中含有大量的母系RNA，一般相信這些RNA有主導胚胎細胞增生與分化的功能。我們從鯉魚卵中分離出母系RNA，並製成cDNA基因庫，再用它直接去轉型一吳郭魚卵巢細胞株TO2，TO2細胞株有類似癌細胞性質；可以堆積生長和無附著生長。因此我們可直接由生長性質挑出受母系基因轉型而生長受到抑制的單株群落。由其中之一TC19中，我們可將原來的母系基因釋出，並再次移殖入一人體肝癌細胞（NTU-BL）中。從眾多群落中我們選出一株HC10作深入研究。HC10不能堆積生長，也不能在洋菜液中無附著生長。由西方墨點方法檢查各種抗原，發現$\alpha$-fetoprotein和癌基因*ras*和*abl*的產物都減少許多；抑癌基因p53的產物和第四類collagen都有顯著增加。種種結果都顯示此一基因可能與胚胎細胞的增生與分化有密切關係。

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# Short Note

## Karyotype of the Emerald Green Tree Frog, *Rhacophorus smaragdinus*

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**June-Shiang Lai and Kuang-Yang Lue (1993)** Karyotype of the emerald green tree frog, *Rhacophorus smaragdinus*. *Bull. Inst. Zool., Academia Sinica* **32**(3): 214-216. Using bone marrow cell methanol-glacial acetic acid chromosome methodology, we karyotyped the emerald green tree frog *Rhacophorus smaragdinus*. Our results show it has 2n = 26 chromosomes, including five large and eight small pairs. Eight pairs are submetacentric in shape (Nos. 2, 3, 6, 7, 8, 11, 12, and 13); secondary constrictions were not observed. We compare the karyotype of *R. smaragdinus* with those of the two other *Rhacophorus* species endemic to Taiwan.

**Key words:** Karyotype, *Rhacophorus smaragdinus*, Taiwan.

*Rhacophorus smaragdinus* is rhacophorid frog endemic to Taiwan. It was first described by Lue and Mou (1983); tadpole morphology was described by Her *et al.* (1989). Concerning distribution, *R. smaragdinus* is restricted to the northern part of Taiwan (Lue and Lai 1990, Lue *et al.* 1991). Karyological studies of rhacophorid frogs in Taiwan have previously been performed by many researchers (Kuramoto 1989) with the exception of *R. smaragdinus*.

**Materials and Methods**—The three male and one female specimens used in this study were captured at Datong Shan (大桶山) near Taipei. For chromosome preparation we followed procedures described by Ota (1989). For terminology related to chromosomes we followed Levan *et al.* (1964).

**Results**—Based on photomicrographs, we found that the 2n chromosome number in *R. smaragdinus* is 26, including five large and eight small pairs (Fig. 1). Sex chromosome is not observed. Pair nos. 1, 4, 5, 9, and 10 are metacentric, while all others are submetacentric (Table 1). Secondary constrictions were not observed in this species.

Pair no. 1 is easily identified by its large size and metacentric form. Pair nos. 2 and 3 are easily defined by their nearly submetacentric shapes. However, pair no. 2 is significantly different from no. 3 in both size and shape (RL:$t = 3.818$, $df = 16$, $p < .05$; AR:$t = 3.35$, $df = 16$, $p < .05$). Pair nos. 4 and 5 are metacentric and also easily defined in terms of size and shape. Among the smaller chromosomes, nos. 9 and 10 were identified as metacentric; they are similar both in the relative length and arm ratio and are not significantly different from each other (RL:$t = 0.795$, $df = 16$, $.2 < p < .5$; AR:$t = -1.57$, $df = 16$, $.1 < p < .2$). Pair nos. 6 and 7 are different in size ($t = 2.014$, $df = 16$, $p < .05$). No. 7 and 8 are similar in shape ($t = .571$, $df = 16$, $p > .5$), as are nos. 11 and 12 ($t = .553$, $df = 16$, $p > .5$); however, pairs 11 and 12 differ in size ($t = 2.61$, $df = 16$, $p < .05$); Pair no. 13 is quite easy to identify due to its small size and submetacentric form.

Using data from both Table 1 and Kuramoto (1985), we drew an idiogram to compare the karyotypes of *R. smaragdinus*, *R. taipeianus*, and *R. moltrechti* (Fig. 2). The *t*-test results revealed that six pairs of chromosomes differed significantly in either relative length or arm ratio (or both) between *R. smaragdinus* and *R. taipeianus* ($p < .05$, pair nos. 3, 5, 6, 7, 8, and 11), as well as in nine pairs between *R. smaragdinus* and *R. moltrechti*. Among the three species, pair nos. 1 and 13 differed significantly in relative length (ANOVA, $df = 2,23$, $p < .05$), and pair nos. 1, 6, and 7 differed