tiation. Aflatoxin is also made by *con* strains on media lacking carbohydrate as a carbon source. The presence of the constitutive phenotype demonstrates that aflatoxin production need not be tied to cessation of growth. The process of induction of aflatoxin production must therefore be different than induction of sporulation, which is tied to cessation of growth in *Aspergillus*[17].

The differences found among these phenotypes cannot be accounted for by possible variations in the amount of inoculated conidia for each vial (ca 10^5), since sporulation (indicating idiophase) was always first observed at day 3. This indicates that the variation in the amount of inoculated conidia did not affect initiation of developmental differentiation. The *con* strains always produced aflatoxin within one day, well before idiophase induction, whereas the *reg* strains did not. The qualitative differences among strains of the three phenotypes on MMS and PMS media also cannot be accounted for by potential differences in initial inoculation levels since differences in production occurred throughout the time of growth, long after variation in the initial inoculum was obscured by the large amount of mycelium present.

Although some secondary metabolites are expressed constitutively[18] and others are only expressed in the idiophase, this is the first report which describes both the constitutive and idiophase-restricted phenotypes in different strains of a species that makes a particular secondary metabolite. The presence of these phenotypes provides an opportunity to study the genetic differences between constitutive and idiophase-restricted strains. Such studies may provide genetic models suited for molecular analysis that would explain how nutrition and growth phases regulate formation of aflatoxin and other secondary metabolites.

The presence of these three phenotypes in natural populations implies the presence of a wide variation in aflatoxin regulation. The mutation of a MMS-non-producer to aflatoxin production was effective here in identifying genes which potentially regulate aflatoxin production. Similar experimental designs, as well as parasexual analysis[14] of these mutants may further reveal the genetic nature of this variation.

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* Author for correspondence.

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A new cell line (XTY) from a tumor of *Xenopus laevis*

A. Fukui[a], A. Tashiro[a], H. Koyama[b], Y. Iwamura[c] and M. Asashima[a]

[a] *Department of Biology, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236 (Japan),* [b] *Department of Anatomy, School of Medicine, Yokohama City University, 3-9 Fukuura, Kanazawa-ku, Yokohama 236 (Japan), and* [c] *Department of Microbiology, Institute of Basic Medical Sciences, Tsukuba University, Tsukuba 305 (Japan)*
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Abstract. A new cell line (XTY) was derived from a tumor of a female *Xenopus laevis*. This cell line has been proliferating in standard amphibian culture medium for more than 4 years (470 generations) and has a doubling time of 75.5 h at 25 °C. The cells aggregate into large groups, and their stellate morphology and the expression of desmin demonstrated by immunocytochemistry suggest that their origin is not epithelial.
Key words. Xenopus laevis; tumor; cell line; XTY.

Several *Xenopus* cell lines have been established previously[1-4], but none originated from a *Xenopus* tumor. Among mammalian cell lines, many lines originating from tumor tissues have been found to be useful for studying the mechanism of transformation cell differentiation. Similarly, the *Xenopus* tumor cell line described here could provide a useful experimental system.

We have previously reported on the long-term cultivation of four cell types derived from a tumor of *Xenopus laevis*[5]. In the study reported here, we characterize one cell line that was established by long-term cultivation of one of these cell types.

Materials and methods

The primary culture was obtained in May 1983 from a subcutaneous tumor on the back of a female *Xenopus laevis*[5]. After 10 days, several cellular outgrowths from the tissue blocks were transferred to subcultures. All culture media contained Leibovitz's L-15 medium (50%) (Gibco, USA), fetal bovine serum (10%) (Hyclone, USA), distilled water (40%) and kanamycin sulfate (2 mg/ml) (Banyu, Tokyo, Japan)[4,6]. The cultures were kept at 25 °C and the culture medium was changed every 3 to 5 days. The cells were cultured in flasks (Falcon 3813, Primaria, 25 cm^2 style, Becton Dickinson, USA).

Histology. Large aggregates of XTY cells were fixed with 10% formaldehyde for 1 h. They were then dehydrated through an ethanol series and embedded in paraffin wax. Sections were cut 8-μm thick, and stained with hematoxylin and eosin.

Chromosomal analysis. The cultured cells were incubated in colchicine (0.25 μg/ml) for 8 h. They were then suspended in 0.25% trypsin and centrifuged. Resuspended cells were incubated in 0.075 M potassium chloride for 20 min, fixed with a 1:3 mixture of acetic acid and methanol, and stained with 2.5% Giemsa solution in phosphate buffered saline solution (1/10th standard concentration).

Ultrastructural analysis. Cultures were prefixed with 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1% tannic acid in 0.1 M cacodylate buffer, pH 7.3, for 1 h. After being rinsed in buffer, the specimens were postfixed with 2% osmium tetroxide in the same buffer for 1 h. The fixed cells were dehydrated through an ethanol series and embedded in Poly/Bed 812 resin (Polyscience, USA). Semithin sections at 0.5 μm were stained with 1% toluidine blue for light microscopy. Thin sections were doubly stained with uranyl nitrate and lead citrate, and were examined with a Hitachi H-600 transmission electron microscope.

Immunocytochemistry. XTY cells were seeded on a glass slide coated with 0.1% poly-L-lysine (Sigma, USA) solution. After 24 h of cultivation, cells were fixed in cold methanol (−20 °C) for 5 min, then washed with PBS (phosphate buffered saline) three times, incubated in PBS with 0.2% Triton-X 100 (Sigma, USA) for 5 min, and washed with PBS. Primary antibody incubation was done using rabbit anti-desmin (Medac, Germany, 1/100 diluted), rabbit anti-keratin (Medac, 1/50 diluted), or rabbit anti-neurofilaments (70 K subunit, Transformation Research, USA, 1/30 diluted). These polyclonal antibodies were diluted in PBS with 1% BSA (Sigma) for 1 h at room temperature. After they had been washed with PBS five times, the specimens were incubated with a secondary antibody (FITC-conjugated goat anti-rabbit IgG, Cappel, USA, 1/40 diluted) dissolved in PBS, for 30 min. The unbound secondary antibodies were washed away with PBS, and the specimens examined with an Olympus fluorescence microscope (model BHS-RFK) at 490 nm.

Immunoblot analysis. For immunoblots, XTY cells were homogenized in 50 mM Tris buffer (pH 7.4) and centrifuged at 12,000 rpm for 30 min. An equal volume of Laemmli's sample buffer (2× concentrated) was added to the supernatant and the mixture loaded onto a 10% SDS-polyacrylamide gel for electrophoresis (SDS-PAGE)[7]. Separated proteins were transferred electrophoretically to a nitrocellulose sheet (Schleicher & Schuell, Germany)[8]. The nitrocellulose sheet was soaked for 30 min in TBS (Tris buffered saline; 20 mM Tris-Cl pH 7.5 and 150 mM NaCl) which contained 1% skim milk. It was then incubated for 2 h at room temperature with each of the following rabbit polyclonal antibodies: anti-desmin (Medac, 1/1000 diluted), anti-keratin (Medac, 1/500 diluted), and anti-neurofilaments (Transformation Research, 1/300 diluted) in the same buffer. Then the nitrocellulose sheet was washed three times with TBS and incubated with horseradish peroxidase labeled goat anti-rabbit IgG (Bio-Rad, USA) for 2 h. Bound antibodies were detected by subsequent reaction with 0.05% DAB (diamino benzidine tetrahydrochloride, Dōjin, Japan) and 0.1% peroxide (Wako, Japan) in TBS. Whole proteins on the gels were visualized by silver staining[9].

Results

After 10 days of primary culture, fibroblastic cells grew out from the tissue blocks of *Xenopus* tumor. In some cases, the growth of these cells was followed by the formation of small groups of stellate cells and blood cell-like cells. After the cells had been subcultivated for about 3 years, four cell types could be distinguished in most culture flasks: blood cell-like cells, melanin-containing cells, fibroblastic cells, and stellate cells which we previously called neuroid cells[5]. The first three cell types stopped growing after 16 months; only the stellate cells continued to proliferate. After the cells had been subcultivated 20 times, an almost pure population of stellate cells, originating from 11 cells, was obtained. These cells proliferate with a doubling time of 75.5 h, and have been maintained for more than 1500 days (at least 470 mitotic generations). We call this cell line 'XTY' (*Xenopus* cell line derived from *T*umor, *Y*okohama).

The stellate cells vary in shape and size, with diameters ranging from 20 to 200 μm. They fall into two basic

morphological categories. One is a process-bearing type and the other is a large, flat, membranous type. The process-bearing type has a number of branching dendritic cell processes, some of which have surface enlargements emerging from a large cell body (fig. 1 A). The average diameter of the cell body is about 20 µm. The cells of the process-bearing type contain numerous granules and spherical structures. Both the cell body and the processes have membranous expansions. The flat membranous cell type, on the other hand, has no obvious processes. Instead, numerous thin fibers radiate from the perinuclear region and form a broad membrane (fig. 1 B). The two cell types may be interconvertible forms and represent different metabolic phases.

As the number of cells increases, the XTY cells tend to adhere to each other at the tips of their processes to form networks (fig. 1 C). Even when the cultures become confluent, contact, movement, or growth of XTY cells is not inhibited. When the cultures are nearly confluent, several groups of 10 or more overlapping cells begin to aggregate, forming piled-up structures (fig. 1 D). Later, these structures become cell aggregates. Sometimes these aggregates develop further into cellular balls, which often become detached from the substratum and float in the culture medium. The diameter of these cellular balls ranges from 100 to 2000 µm. The interior cells of an aggregate are not necrotic, and some show mitotic structures (fig. 2).

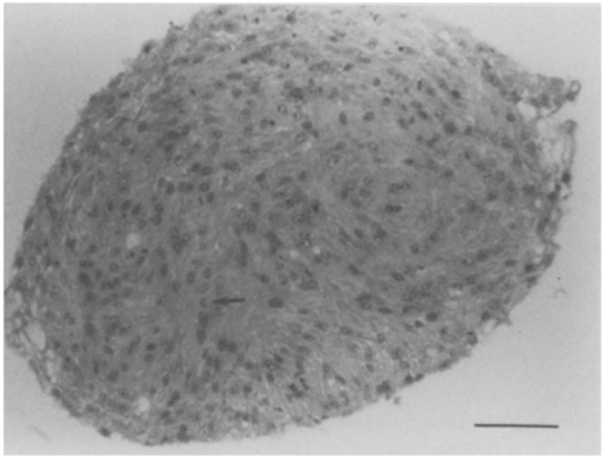

Figure 2. Aggregated XTY cells, stained in hematoxylin and eosin. Arrow points to a mitotic cell. Scale bar = 100 µm.

The number of metaphasic chromosomes of the XTY cells is 36 (fig. 3). As shown in figure 4, most cells contain 36 chromosomes, the normal diploid complement in *X. laevis*.

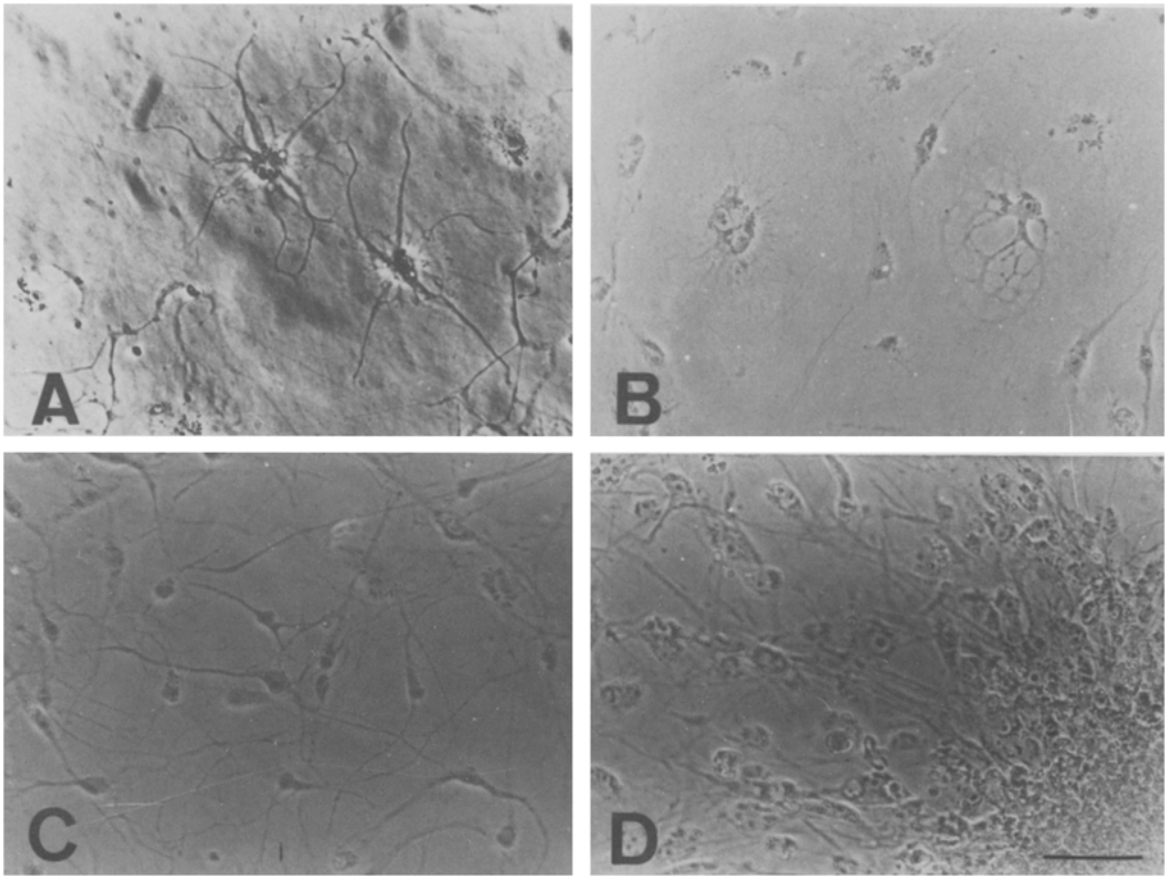

Figure 1. Phase-contrast photomicrographs of XTY cells. *A* Process-bearing type. *B* Flat membranous cell type. *C* XTY cells forming a network. *D* XTY cells forming an aggregate. Scale bar = 50 µm for all figures.

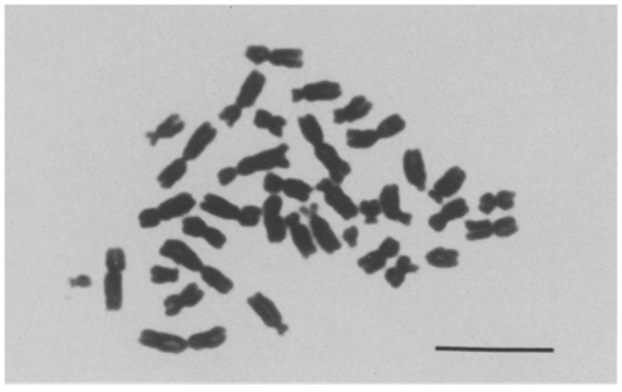

Figure 3. Metaphasic chromosomes of the XTY cells, stained in Giemsa. Scale bar = 10 µm.

The ultrastructural features of the XTY cells are shown in figure 5. The XTY cells have one or two prominent nucleoli, which consist of an electrodense core and a granular outer zone. The chromatin granules are not condensed into obvious clusters. The cells have no evident intercellular junctional or synapse-like structures. The cytoplasm is finely granular and contains rough endoplasmic reticulum (rER) and free ribosomes. Some tubular mitochondria lie scattered within the cytoplasm. The most prominent organelles are lysosome-like vacuolar structures. These contain multivesicular or multilamellar bodies. Many microtubules and filamentous structures run longitudinally within the cell processes.

According to immunocytochemical and biochemical examinations, XTY cells express desmin (fig. 6). Figure 6A shows the network structure of desmin in the cytoplasm as seen with indirect immunofluorescence. Antibodies to keratin and to neurofilaments did not reveal significant fiber structures. The results of an effort to identify desmin by an immunoblot analysis of proteins of XTY cells are presented in figure 6B. Lane 1 shows whole

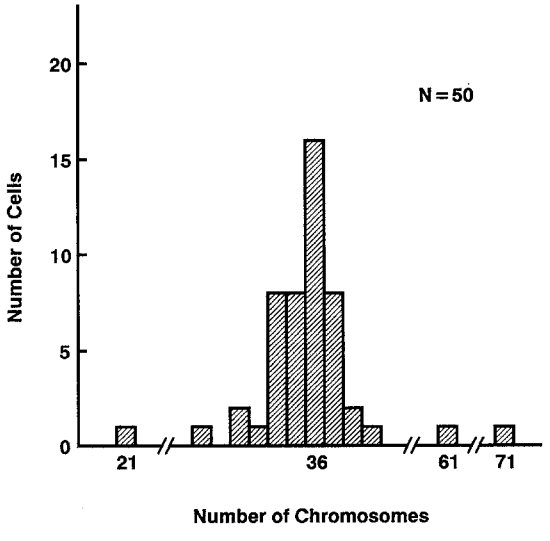

Figure 4. Histogram of chromosome numbers in XTY cells.

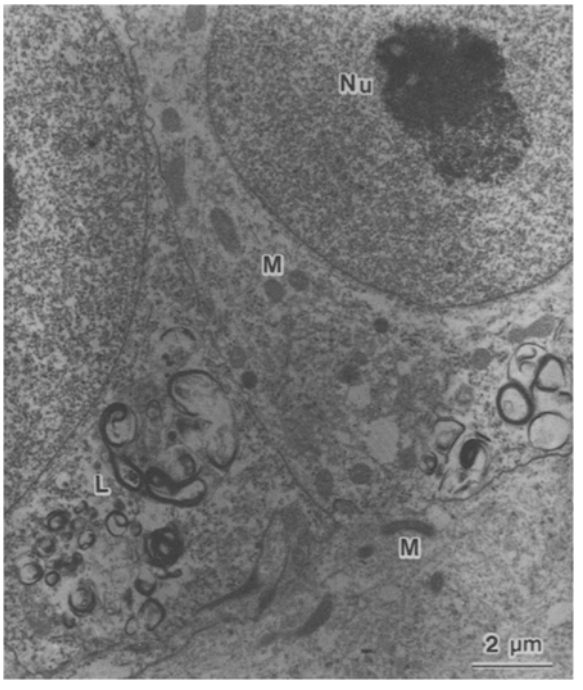

Figure 5. An electronmicrograph of XTY cells, stained with uranyl nitrate and lead citrate. Horizontal section (relative to substratum). L, lysosome-like structure; M, mitochondria; Nu, nucleolus.

proteins using silver stain, and lane 2 shows the result of Western blotting. In lane 2, the most intense band (arrow) was at 52 kD, which coincides with the molecular weight of desmin [10].

Discussion

The amphibians differ from mammals with regard to several properties. Among these are their tolerance of a wide range of temperatures [2, 4, 11], their well-developed ability to regenerate, and their development, which involves the metamorphosis of larvae to adults. Accordingly, amphibians have been used in experimental embryology, and they are more appropriate than mammals for experiments on cellular responses and differentiation at varying temperatures.

Here we report on several properties of the XTY cell line derived from a tumor of a female *Xenopus laevis*. These cells have no contact inhibition, form a focus, and aggregate around it. The cell line is heteroploid, but the mode of the chromosome number is diploid. This is a very important characteristic. Freed et al. previously described a haploid cell line [12, 13]. Only Pudney et al.[3] reported on a diploid cell line of *Xenopus laevis* which they called XTC. Recently, however, the XTC cells cultured in our laboratory were found not to be diploid. It is still necessary to compare our cell line XTY with the normal karyotype using a chromosome banding method.

In the XTY cells, several processes extend from the cell body and form a network-like structure. We were not able, however, to see neurofilaments using indirect immunocytochemical or electron microscopic techniques.

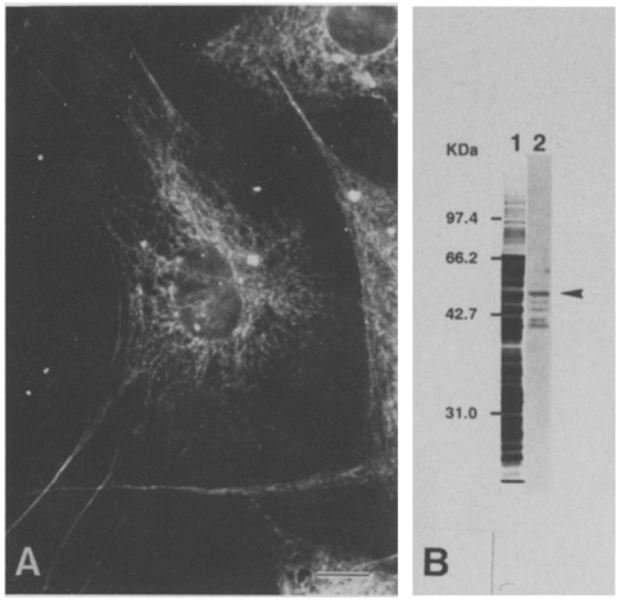

Figure 6. *A* Immunocytochemical demonstration of desmin. Scale bar = 10 µm. *B* Immunoblot analysis of proteins of XTY cells. Each lane loaded with 5 µg proteins. Lane 1: silver stain of polyacrylamide gel. Lane 2: immunoblot of anti-desmin antibody. Arrow points to 52 kD protein known to be desmin.

XTY cells also did not demonstrate any intercellular junctions and expressed desmin but not keratin. We therefore suggest that this cell line derives from interstitial rather than epithelial cells.

Immunoblot analysis shows desmin, which is a major protein of myogenic cells [10]. Minor bands seen in lane 2 of figure 6 might be degraded or nonspecific protein bound to contaminant antibodies contained in the primary antibody. Our results suggest that the XTY tumor cells are myogenic interstitial cells.

XTY cells form large aggregates which are different from the focus generally formed by transforming cells. The diameter of the aggregates is medium-sized (more than 2 mm). Some mitotic cells are present in the inner parts of aggregates; therefore, we consider that the cells within the aggregates do not undergo necrosis. This is also indicated by our finding that aggregates transferred to a new flask expand and proliferate. We believe that aggregated XTY cells represent a differentiated form of the XTY cells after confluency. When an aggregate (2 mm in diameter) was transplanted under the skin of the back of an adult *Xenopus laevis* tumor formation did not result.

In conclusion, the new cell line XTY has immortalized, and forms aggregates, but shows no malignancy. Morphology and ultrastructural features as well as the expression of desmin lead us to suggest that XTY cells are not epithelial, but interstitial. We plan to compare the XTY cell line with other *Xenopus* cell lines in order to determine its specific characteristics.

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Leaf-blade crimping in grasses: A new measure of growth

P. R. Espie, H. E. Connor[a] and I. J. McCracken

Forest Research Institute, P.O. Box 31-011, Christchurch (New Zealand), and [a] Centre for Resource Management, University of Canterbury, Christchurch (New Zealand)
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Abstract. In grasses, ligules compress and permanently crimp emerging leaf-blades (primary crimping). Ligule compression may also mark the abaxial surface of older leaves in some species (secondary crimping). Secondary crimping appears directly related to leaf daily growth rate. Leaf-blade crimping can be used for determination of relative tissue age and as a natural record of growth rate in grasses.
Key words. Grasses; crimping; leaf; leaf-blade; ligule; growth rate; *Chionochloa.*