GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

LAMPREY CELL CULTURES FOR IN VITRO PRODUCTION OF PHEROMONALLY ACTIVE BILE ACIDS

by:

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Research Completion Report

Lamprey cell cultures for in vitro production of pheromonally active bile acids

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Abstract

Petromyzonol sulfate (PS) is a bile acid produced by lamprey larvae that is able to stimulate the adult lamprey olfactory system and function as a migratory pheromone. Utilization of PS in the field may provide an environmentally safe, species-specific approach for controlling lamprey populations by influencing spawning behavior. However, utilization of this approach will require that a convenient and economical source of PS is available. The goal of this research is to develop methods for in vitro production of lamprey pheromonal bile acids from liver cell cultures. During the first year of this contract, long-term liver cell cultures from ammocoetes were derived in serum-free medium. From this work, it was determined that the liver cultures are able to synthesize and release PS along with a second bile acid, petromyzonol for several days before in vitro production decreased. The objective for the second year was to develop methods to stabilize PS production from the cultures. To address this problem we have utilized an approach that has been successfully employed to prolong bile acid production from mammalian liver cell cultures. This approach involves fusion of the bile acid producing cells to cells of an established hepatoma line. The resulting hybrid cell line exhibits the immortalized growth characteristics of the hepatoma cells while maintaining the ability to continuously synthesize the bile acids in culture. To pursue this approach, we have developed methods to fuse the lamprey liver cells to a rainbow trout hepatoma cell line (RTH-149) and select for stable hybrid clones. Using these methods, we have isolated several continuously growing lamprey-trout hybrid cell lines that exhibit the rapid growth characteristics of RTH cells. Currently, each cell line is being assayed for the production of PS. Once a PS producing line is isolated the cultures will be expanded and utilized for scaled-up production of the pheromone.

Introduction

Adult sea lamprey (*Petromyzon marinus*) migrate into tributary streams to lay their eggs before dying. After hatching from the eggs, the larvae burrow into the stream bed and exist for several years feeding on detritus and algae until physiological and environmental factors trigger the larvae to undergo metamorphosis and migrate into open water to begin the parasitic phase of their life cycle. During the larval stages, lamprey produce bile acids including petromyzonol sulfate (PS) that are released into the water and act as pheromones to attract spawning adult lamprey into the stream. The pheromonal bile acids may be used to alter sea lamprey spawning migration behavior and provide a species-specific strategy for controlling lamprey populations in the Great Lakes. We are working to develop lamprey cell cultures that will provide a continuous and convenient in vitro source of pheromonal bile acids.

Animal cell cultures are commonly used for in vitro production of bioactive compounds (Collodi and Barnes, 1992). The cultures can be induced to synthesize and release products into the medium providing a continuous source of the desired compound. In many cases, in vitro production of the compound is more convenient and economical than purification from whole tissue. In addition, since it is possible to maintain many cell types in the absence of serum or other undefined supplements, the desired compound can be obtained in medium containing very low levels of contaminating proteins, thereby simplifying the purification procedure (Cartwright, 1992). Another advantage of an in vitro production system is that several methods exist to increase yield of the desired compound. Scale-up of the cultures can be accomplished easily in a standard tissue culture facility by increasing the size or number of culture flasks or by genetically manipulating the cells to overproduce the desired compound.

With previous GLFC support we have established tissue culture methods and medium formulations for the derivation of cell cultures from adult and larval lamprey tissues (Ma and Collodi, 1996). Cultures were initiated from adult and larval liver, gill, muscle and gut and adult ovary, brain and kidney. The cultures from each tissue were maintained for a minimum of three weeks and most were maintained for several months and multiple passages.

The goal of the current research is to employ these previously developed methods to derive liver cultures from larvae that are able to synthesize and release pheromonal bile acids into the medium. The bile acid producing cell line will provide an in vitro system for continuous large-scale production of the pheromone for field studies.

Methods

Derivation of liver cell cultures from lamprey larvae

Lamprey larvae were maintained in a 10 gallon aquarium containing a sand substrate and fed a suspension of live yeast once a week. Before use each larva was transferred to a clean tank and starved for a period of 4 or 5 days. Larvae were sacrificed in 3-amino benzoic acid ethylester and the surface of the animal was sterilized in 95% ethanol followed by a 2 minute incubation in 0.5% bleach solution. Dissections were done aseptically in a laminar flow hood. Following dissection, each liver was placed in a petri dish containing culture medium supplemented with antibiotics (see below). The tissue was rinsed 4 or 5 times with fresh medium and then enzymatically dissociated by incubating 30 minutes in trypsin/EDTA solution (0.2% trypsin, 1 mM EDTA in PBS). Periodically, during trypsinization the tissue was disrupted by passing it through a pipette. The action of the trypsin was stopped by adding FBS to the tube. The dissociated cells were collected by centrifugation (500 x g, 5 min), resuspended in culture medium and transferred to a 35-mm culture dish that was treated with a commercial basement membrane preparation (matrigel, Collaborative Research). To initiate larger cultures, livers from 3 or 4 larvae were processed together and plated into a 25 cm^2 flask pretreated with matrigel.

Liver cells were grown in LDF basal nutrient medium containing sodium bicarbonate (0.15 mg/ml), HEPES buffer (15 mM; pH 7.0), selenium (10^{-8} M), penicillin (200 IU/ml), streptomycin sulfate (200 μg/ml) and ampicillin (25 μg/ml) (Ma and Collodi, 1996; Collodi et al., 1992). The LDF medium was supplemented with insulin (10 μg/ml), epidermal growth factor (20 ng/ml), trout serum (2%) and FBS (10%). Cultures were incubated at 18°C and medium was changed approximately once a week (Ma and Collodi, 1996).

Evaluation of liver cultures for PS production

Bile acid production from the liver cell cultures was determined by HPLC analysis of cell-conditioned medium in Dr. Peter Sorensen's laboratory at the University of Minnesota (Li et al., 1995). To obtain serum-free conditioned medium, liver cell cultures initiated as described above were transferred into serum-free LDF containing EGF and insulin and medium was collected every 72 hrs and stored frozen (-20° C). Cultures can be maintained for approximately 4 weeks in serum-free medium before they must be transferred back to serum for several days. Following this period in serum-containing medium the cells can be cultured again in serum-free conditions and additional medium can be collected. This cycle can be repeated several times and serum-free medium can be collected from the same culture for more than 6 months.

Medium collected from individual flasks was extracted with activated C_{18} solid phase extraction (SPE) cartridges and bile acids were eluted with methanol (Li et al., 1995). Bile acid analysis was conducted using a reverse-phase Nova-Pak C_{18} 4 μm column (4 mm x 10 cm) eluted with a step-wise gradient of ammonium dihydrogen phosphate (25 mM, pH 7.8) and acetonitrile. Eluted bile acids were passed through a second column (5 cm x 0.5 cm) containing 3α-hydroxysteroid dehydrogenase bound to

glutaraldehyde-treated aminopropyl glass beads. Buffer containing NAD (0.1 M Tris-HCl, pH 8.5, 2.7 EDTA, 1.63 mM DDT and 0.01 mM NAD) was introduced using a tee between the first and second columns at a constant rate of 1 ml/min. This enzyme oxidizes 3α-hydroxyl bile acids into 3α-keto bile acids and reduces NAD to NADH which is subsequently detected by a fluorescence detector with a narrow band excitation filter of 340 nm and a wide band emission filter with a range of 420-650 nm. Peak areas were calculated using a chromatography software program (712 System Controller). Bile acids were identified by comparing their retention times with those of standards.

Cell Fusions

Lamprey liver and RTH-149 cells were suspended together (3:1 ratio) in LDF medium containing 10% FBS (approximately 5 x 10^6 cells/ml) and polyethyleneglycol (PEG) (12.5% final concentration) was added. The cells were allowed to fuse for 10 to 12 minutes and then centrifuged out of the PEG. The cell pellet was rinsed twice with culture medium and the cells were resuspended in medium and placed in a flask. After allowing the cells to attach to the culture surface, FBS (10%) was added to the flask. The following day selection for hybrid colonies was initiated by adding ouabain (1.5 x 10^{-7} M) to the cultures. Lamprey cells used for the fusions were either obtained from previously initiated cultures or directly from whole livers. To isolate the cells from whole liver, the tissue was dissected and prepared for cell culture as described above. Potential hybrid colonies isolated by ouabain selection were expanded and eventually frozen in liquid nitrogen. Flow cytometric analysis was performed at the Purdue Cancer Center on each cell line derived from the isolated colonies to confirm that the line consisted of hybrid cells.

Results and Discussion

To determine if lamprey liver cells are able to synthesize pheromonal bile acids in vitro, cultures were initiated and conditioned medium was collected and assayed by HPLC for the presence of petromyzonol sulfate (PS), petromyzonol and allocholic acid. Methods were developed to maintain the cultures in serum-free medium in order to eliminate undefined components from the medium and facilitate the detection and purification of the bile acids. Livers were dissected from ammocoetes and cell cultures were initiated as described in the Methods. Results from approximately 100 samples of conditioned medium analyzed by HPLC demonstrated that the liver cells are able to produce PS in culture. The level of PS synthesis was highest during the first week of culture ($6.10 \pm .81$ μg/culture/72 hrs) and then decreased after 7 to 10 days ($1.22 \pm .29$ μg/culture/72 hrs). After two weeks in culture detectable amounts of PS continued to be produced by the liver cells ($0.57 \pm .35$ μg/culture/72 hrs) and production was maintained for up to 4 weeks ($0.20 \pm .07$ μg/culture/72 hrs). Some of the cultures also produced detectable levels of petromyzonol but allocholic acid was not detected in any of the cultures. Addition of the bile acid precursors, cholesterol and mevalonic acid did not have any effect on PS production. Also, addition of extracts prepared from lamprey liver and gall bladder in water, acetone and chloroform did not have any effect on PS synthesis by the cultures. Addition of the glucocorticoid hormone, dexamethasone, did enhance PS production in some of the cultures.

The pattern and amount of PS synthesized by the lamprey liver cells was similar to published levels of bile acid production from mammalian liver cell cultures. As we have observed with the lamprey cultures, bile acid production from the mammalian cells dropped after the first week in culture (Davis et al., 1983; Blumrich et al., 1994). However, in vitro production of bile acids from the mammalian cells has been stabilized by fusing the bile acid producing cells with cells from a continuously growing hepatoma cell line. The fused cells exhibit the growth characteristics of the hepatoma cell line (rapid, continuous growth in culture) and they are able to continue producing bile acids indefinitely in vitro (Petzinger et al., 1994).

To stabilize and prolong in vitro production of PS from lamprey liver cells, methods were developed to fuse the lamprey cells to cells of a continuously growing rainbow trout hepatoma line, RTH-149. RTH-149 is a well-characterized and widely used cell line that was derived from an aflatoxin induced trout hepatoma. Initial attempts to fuse the cells and isolate hybrid colonies using conventional methods were not successful so alternative techniques were developed for use with the lamprey cells. Fusion and hybrid selection methods are outlined in Figure 1. Cell fusions were performed by suspending RTH-149 and lamprey liver cells in medium in the presence of a low concentration of PEG. After fusing, the cells were cultured in medium containing FBS (10%) and ouabain. Ouabain is an ATPase inhibitor that is also used in mammalian selection systems. Lamprey liver cells and lamprey liver-RTH hybrid cells are resistant to ouabain whereas nonfused RTH and RTH-RTH hybrids are sensitive to the drug and die within 10 days. Nonfused lamprey liver cells are not able to proliferate in medium containing only FBS. Using this selection method, we have isolated 10 colonies of RTH-

lamprey liver cell hybrids in medium containing ouabain and FBS. Each selected colony was expanded into several flasks and a portion of the cells were frozen in liquid nitrogen. Cells derived from each colony were analyzed by flow cytometry to confirm that they were lamprey-RTH hybrids. Flow cytometry was used to measure DNA content in the cells and demonstrate that the amount of DNA contained in the hybrid cells was different than the amount found in each parent cell used in the fusion (Figure 2).

Morphology of the hybrid cells was unlike either parent cell type but the growth rate of the hybrid cells was similar to RTH-149 which proliferates much faster than the lamprey liver cells. Due to the higher growth rate and the ability to proliferate in medium supplemented only with FBS, the lamprey-RTH hybrids are easier and less expensive to maintain in culture than the lamprey liver cells. Therefore, once a hybrid colony that stably produces PS is identified it will be convenient and economical to scale up PS production. We are currently working with Dr. Peter Sorensen at the University of Minnesota to assay medium conditioned by the hybrid cells to determine if any of the lines isolated to date are able to synthesize PS.

Conclusions

The results from the first year of this project demonstrate that the lamprey liver cells, cultured in serum-free medium, are able to synthesize pheromonal bile acids and release them into the medium. In vitro synthesis of petromyzonol sulfate and petromyzonol was maintained for up to 10 days before production decreased. The objective for the second year of research was to develop methods to stabilize bile acid production from the cultures. The approach employed involves the fusion of bile acid producing cells to a continuously growing hepatoma cell line and isolation of hybrid clones able to stably synthesize bile acids. Methods were developed for the fusion of lamprey liver and rainbow trout hepatoma cells and continuously growing hybrid cell lines were selected. Isolated colonies were confirmed to consist of hybrid cells by flow cytometry. Hybrid colonies that exhibit rapid growth characteristics of the hepatoma cells are being screened for the production of petromyzonol sulfate. Once a PS producing line is isolated the cultures will be expanded and utilized for scaled-up production of the pheromone.

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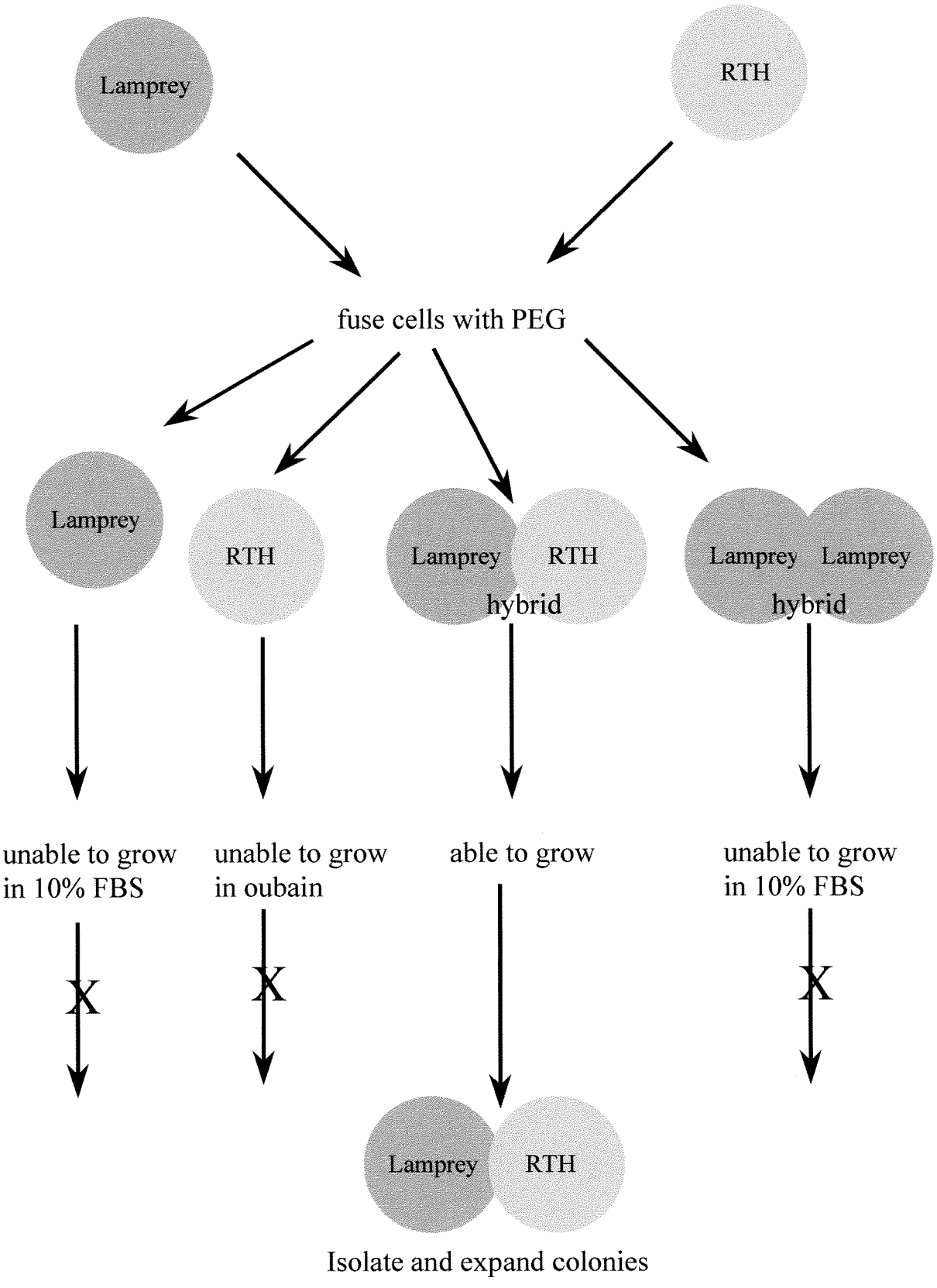
Figure 1. Outline of the method used for fusion of lamprey liver and rainbow trout hepatoma cells and selection of hybrid colonies.



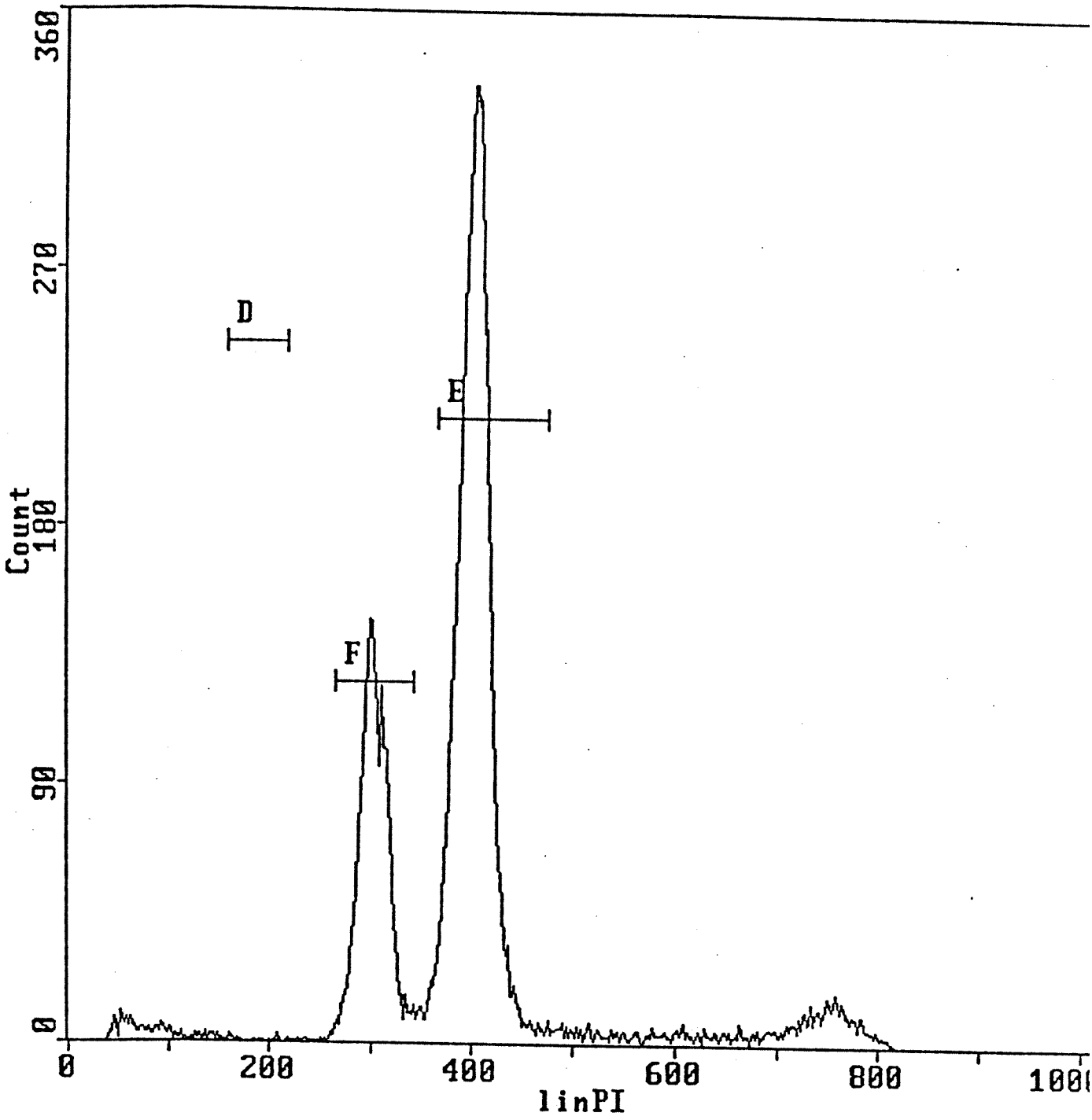


Figure. 2. Flow cytometric analysis of RTH-149 rainbow trout hepatoma cells (E) and RTH-lamprey liver hybrid cells (F). The figure shows the relative cell number (y axis) versus the relative amount of DNA/cell (x axis). The position of the peak for lamprey liver cells determined from a previous experiment is also shown (D).