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Title A CHROMOSOME ANALYSIS OF FIVE CELL LINES FROM FOUR SPECIES OF SALMONID FISHES

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Four cell lines derived from embryonic tissues of four species of salmonid fishes, and one from a hepatoma in rainbow trout have been established in this laboratory. The species represented are chinook, coho, and sockeye salmon; and steelhead and rainbow trout. All of these lines have been cultivated for approximately three years.

A chromosome analysis was performed on these cell lines and two primary cell cultures from embryonic tissues of chinook and coho salmon. Each analysis consisted of two parts, chromosome preparation and chromosome characterization.

Chromosome preparation involved exposing a growing culture to colchicine. The cells were harvested, flattened, expanded, fixed, and stained.

Chromosomes were characterized by selecting 100 metaphase

cells from stained preparations of each cell culture, and determining the number of chromosomes per cell and each chromosome's morphology.

At the time of analysis, the cell lines were about two years old, and the primary cultures were three to six weeks old. The modal numbers and ranges of chromosomes per cell for the five cell lines were as follows: chinook salmon line (TC-114), mode 71, range 18 to 190; coho salmon line (TC-119), mode 71, range 57 to 173; sockeye salmon line (SeE), mode 56, range 51 to 101; steelhead trout line (TC-137), mode 62, range 58 to 126; and rainbow trout line (TC-149), modes 54 and 60, range 18 to 144. Similar distributions for the two primary cell cultures were the following: chinook salmon culture, mode 68, range 35 to 172; and coho primary culture, mode 60, range 58 to 119.

These data indicate that the chinook and coho salmon, and the steelhead and rainbow trout hepatoma lines were heteroploid. Also, the sockeye salmon line, and coho and chinook salmon primary cultures had normal diploid chromosome constitutions.

All seven cell cultures showed ranges and/or distributions of chromosome numbers per cell that were greater than found in normal *in vivo* cells.

Chromosome morphology was determined by classifying each counted chromosome as either metacentric or telocentric. The

morphology of the chromosomes in each sample of 100 cells was represented by the average ratio of telocentrics to metacentrics per cell. The sockeye salmon and rainbow trout cell lines and the chinook primary cell culture had ratios similar to those reported for these species. The other cultures had ratios which differed slightly from those characteristic of the species.

A CHROMOSOME ANALYSIS OF FIVE CELL LINES FROM FOUR SPECIES OF SALMONID FISHES

by

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A CHROMOSOME ANALYSIS OF FIVE CELL LINES FROM FOUR SPECIES OF SALMONID FISHES

INTRODUCTION

This thesis describes some of the chromosomal properties of five cell lines derived from tissues of salmonid fishes. Four of these cultures were obtained from embryonic tissues of the following species: chinook salmon (Oncorhynchus tshawytscha (Walbaum)), coho salmon (O. kisutch (Walbaum)), sockeye salmon (O. nerka (Walbaum)), and steelhead trout (Salmo gairdneri Richardson). The fifth cell line was from hepatoma tissue obtained from an adult rainbow trout (S. gairdneri Richardson).

The chromosome analysis involved two main parts, chromosome preparation and chromosome characterization. The preparation of chromosomes consisted of treating a sample of cells in order to obtain a suitable number of cells in metaphase with distinct and dispersed chromosomes, then fixing and staining them. Chromosome characterization was accomplished by examining the stained preparations of a cell sample, randomly selecting 100 metaphase cells that satisfied certain criteria, and recording the properties of the chromosomes within these cells.

The chromosomal properties described were the number of chromosomes per cell and the general morphology of each chromosome counted. A chromosome's morphology was classified as either

metacentric or telocentric.

Chromosome analysis of these five cell lines was undertaken for several reasons: (a) an analysis is a basic means of indicating the genetic identity of a cell line, (b) in certain cases an analysis can be used to show the origin of a cell line, and (c) an analysis establishes the ploidy of a cell line.

The ploidy of the cell lines was of special interest in this investigation because it indirectly showed how the lines had genetically adapted to their in vitro environment. This adaption was important since, almost without exception, all known cell lines have changed from a normal diploid condition to a heteroploid or polyploid state. Most cultures that have remained diploid have not survived longer than about a year.

In addition to these cell lines, the chromosomes of two primary cell cultures were analyzed. One such culture was from coho salmon embryonic tissue, and the other was from chinook salmon embryonic tissue. Both of these primary cultures were started in the same way as the coho and chinook salmon cell lines.

The chromosomes of these primary cultures were analyzed to observe if the culture procedures used to start a new cell culture resulted in a population of diploid cells. Also, if these cultures showed a high percentage of diploid cells, these results would provide a means of checking the accuracy of the chromosome analysis procedures used in this investigation.

LITERATURE REVIEW

A chromosome analysis of cell lines from salmonid fishes requires two basic techniques, fish cell cultivation and chromosome analysis.

The first attempts at in vitro cultivation of fish tissues began around 1915. In 1921, Dederer was able to culture explants of Fundulus heteroclitus embryos in a crude medium consisting of sea water, Fundulus boullion, dextrose, and sodium bicarbonate. These cultures survived in this environment for periods up to ten days (1).

For the next 40 years little work was done concerning the in vitro cultivation of fish cells. During the 1950's interest was stimulated because of the advantages of fish tissue cultures as tools in virology. In 1957, Wolf and Dunbar cultivated tissues excised from salmonid fishes. Their work, the first attempted using salmonids, involved cultivation of explants of various types of tissues from three species of trout and the gold fish. The tissues were exposed to a variety of cultural environments, including different temperatures, types of media, and cell-attachment substrates. The trout tissues were kept alive for less than two months (27).

Later, Wolf adapted cell culture techniques for in vitro culture of mammalian cells to fish cells. The cultures were prepared by first dispersing tissue in a cold trypsin solution, then removing the

trypsin from the resulting cell suspension by centrifugation, washing the cells with saline, suspending the cells in growth medium, and finally dispensing the cell suspension into culture vessels (28).

Using this method, Wolf and Quimby were able to establish the first known cell line derived from salmonid fish tissues (29). Started in 1960, this cell line, obtained from rainbow trout gonad tissue, is still being cultivated (25).

Additional work was done with salmonid fish cell cultivation by Fryer, Yusha and Pilcher in 1963. Their efforts resulted in the establishment of four cell lines from three species of salmonid fishes. Three of these cultures were obtained from the embryonic tissues of coho salmon, chinook salmon, and steelhead trout. The fourth cell line was obtained from a hepatoma excised from an adult rainbow trout (6, 7). The techniques used to initiate these cell cultures are described in the Experimental Methods section of this thesis.

Another salmonid fish cell line was established in this laboratory by W. H. Wingfield (26). This line was derived from sockeye salmon embryonic tissues by the methods of Fryer, Yusha and Pilcher (7).

The chromosome analysis described in this thesis was performed on the five cell lines discussed above.

Such an analysis of cell lines involved preparation of chromosomes in a sample of cells, and an examination and characterization

of these prepared chromosomes.

The development of techniques used to prepare the chromosomes of in vitro cell systems for analysis began in 1952. At this time, Hsu published a report describing the numbers of chromosomes in human cells. His work was done with explants of human skin and spleen obtained from fetuses, and grown in plasma clots. The chromosomes were prepared by first fixing tissues in absolute methanol, then staining them with either May-Greenwald-Giemsa stain, or hematoxylin-eosin-azure stain (11).

In 1953, Hsu and Pomerat refined this procedure by exposing explants to a hypotonic solution before fixing them. This step caused the cells to swell, allowing the chromosomes to be dispersed over a larger area (13).

Another valuable step was added to the preparation of cellular material for chromosome analysis by Levan in 1956. In his analysis of chromosomes from several cell cultures obtained from human tumors, he increased the number of cells in metaphase by exposing the growing cells to colchicine for 24 hours. A 0.00005 percent solution of this chemical was added to growth medium to give a final concentration of 50×10^{-9} M colchicine. The presence of this compound prevented cells from dividing by arresting mitosis at the metaphase stage (15).

The use of colchicine was found to have other advantages. These

advantages were reported by Ford and Hamerton, and include the following: (a) it condenses chromosomes, making them more distinct and separate; and (b) it causes the chromatids of each chromosome to diverge, making chromosome morphology easier to distinguish (5).

Since the development of these essential steps required for chromosome preparation there have been many modifications. A general description of the most common of these techniques was presented by Rothfels and Siminovitch (21).

One of the most recent modifications was introduced by Pacha and Kingsbury in 1962. Their method differed from most techniques because cells were not expanded with a hypotonic solution. Instead, cells were flattened and expanded by placing them on the surface of an agar medium (17). This procedure is also described in detail under the Experimental Methods section of this report.

In addition to preparation, this chromosome analysis also involved chromosome characterization. This step consisted of microscopically examining a stained preparation of cells, selecting those in metaphase that satisfied certain criteria, and describing the number and morphology of the chromosomes within these cells.

Some generally accepted criteria used to govern the selection of metaphase cells were listed by Hungerford, and are as follows: (a) a cell must be fixed and stained so as to make the cell and its

chromosomes clearly visible; (b) a cell's chromosomes must be distinct from each other, and in the same focal plane; (c) a cell's cytoplasm must be uniform and its interior and perimeter unbroken, (d) a cell should have no other metaphase cells adjacent to it (14).

The properties of chromosomes usually described in a chromosome analysis are the number per cell and the morphology of each (9). The methods used to determine chromosome number and morphology vary with the purpose of the analysis.

A chromosome analysis can serve three purposes. It can provide a means of identification, indicate the origin, and establish the ploidy of a cell line.

A chromosome analysis is a fundamental means of defining the genetic identity of a cell line (20). This can be done by determining both the chromosome number and morphology of a cell line. Such an identification is much more reliable if the cells under examination contain marker chromosomes (2).

The origin of a cell line can be indicated by comparing the morphology and number of its chromosomes with those in normal in vivo cells obtained from similar tissues. This comparison is most conclusive when the chromosomal nature of the original tissue is distinctive, for example, if there is a high percentage of one type of chromosome morphology (2).

The ploidy of a cell line is found by counting the number of

chromosomes per cell in a sample of metaphase cells. The modal number of chromosomes per cell is the number from which the ploidy is determined (9).

Most established mammalian cell lines are heteroploid or polyploid. Normally, a primary cell culture is diploid; then, during a period of about a year, or approximately 50 transplantations, the diploid state changes to aneuploid or polyploid. If this change does not occur the culture degenerates and dies (10).

Cold-blooded animal cell cultures have been reported to show chromosome behavior similar to mammalian cell cultures. They appear to be initially diploid, then if the cultures develop into cell lines, they also change ploidy (8).

A chromosome analysis of nine primary cultures from nine species of fish belonging to the family Centrarchidae indicated these cells to be diploid. These results were confirmed with parallel chromosome analyses of in vivo gonad tissues from the same species (19).

The work reported in this thesis includes an attempt to determine whether two primary cultures from two species of salmonids were diploid. The experimental design used was similar to that used by Peterson and Fogh in their comparison of the FL line of human amnion cells with a primary culture obtained from similar tissues (18).

The aneuploid or polyploid nature of cold-blooded animal cell

lines in documented in the literature. Three such lines are described in Table I.

TABLE I. A DESCRIPTION OF THREE COLD-BLOODED ANIMAL CELL LINES WHICH ARE REPORTED TO BE ANEUPLOID.

Name of Cell Line[1]	Source of Cells	Year Started	Normal Diploid Number	Some Chromosome Characteristics
RTG-2 (29, 25)	Gonads of Rainbow Trout	1960	60	Mode: 59 Range: 40 to 100 58% of cells counted had 59 ± 2 chromosomes
FHM (8)	Tissues of Northern Fathead Minnow	1962	50	Mode: 51 Range: 38 to 89 76% of cells counted had 51 ± 2 chromosomes
BT (30)	Tongue of Bullfrog	1961	26	Mode: 42 Range: 24 to 79 70% of cells counted had 44 ± 3 chromosomes

[1] References concerning cell lines are listed with the name of each line.

EXPERIMENTAL MATERIALS AND SUPPLIES

Materials for Initiation and Care of Cell Cultures

This investigation required materials used for routine maintenance of fish cell lines and initiation of certain primary cell cultures.

Cell Cultures

The five cell lines used in this research are described in Table II. The two primary cultures were obtained from embryonic tissues of coho and chinook salmon.

TABLE II. A DESCRIPTION OF THE FIVE CELL LINES FROM SALMONID FISHES USED IN THIS CHROMOSOME ANALYSIS.

Designation of Line	Species of Origin	Source of Cells	Date Started	Age in Months by 5/66	Number of Transplants
TC-114	Chinook Salmon	Whole Embryos	Jan., 1963	40	40
TC-119	Coho Salmon	"	Feb., 1963	39	46
TC-137	Steelhead Trout	"	June, 1963	35	35
TC-149	Rainbow Trout	Hepatoma Tumor	Aug., 1963	33	29
SeE	Sockeye Salmon	Whole Embryos	Oct., 1963	30	32

Growth Medium

One hundred ml of complete growth medium contained the following ingredients: 76.6 ml of Minimum Essential Medium (MEM) (Eagle), less glutamine (3); 2.0 ml of a penicillin and streptomycin solution, 25,000 units per ml and 25,000 micrograms per ml, respectively, dissolved in MEM; 1.0 ml of glutamine (100X) (Microbiological Associates); 0.4 ml of mycostatin (Squibb), 25,000 units per ml, dissolved in MEM; and 20.0 ml of agamma calf serum (Hyland Laboratories). The MEM was prepared in 400 ml quantities which could be stored for two weeks at 10° C. Each 400 ml volume contained the following components: 375.0 ml of Earle's Balanced Salt Solution (BSS) (4), 8.0 ml of MEM Essential Amino Acids (50X), 4.0 ml of MEM Non-Essential Amino Acids (100X), 4.0 ml of MEM Vitamins (100X), 4.0 ml of sodium pyruvate (100X), and 5.0 ml of 11.0 percent sodium bicarbonate.

Earle's BSS was made up in this laboratory as a 10X solution and kept at -20° C until needed. To prepare 1X BSS, the 10X solution was diluted with double distilled water, dispensed into Pyrex bottles (375 ml per bottle), and autoclaved for 20 minutes. The 1X BSS was refrigerated at 10° C. Except for the 11.0 percent sodium bicarbonate solution, all the other MEM components were obtained commercially from Microbiological Associates Incorporated. A more

detailed description of the above information is given by Fryer (6).

Glassware

Pyrex Erlenmeyer flasks were used to hold growth medium for fluid changes and serial transplants. Cell cultures were cultivated in Pyrex 150 ml milk dilution bottles. Centrifugation was performed in 50 ml round-bottom centrifuge tubes (Kimax).

Glassware was routinely washed with Micro-Solv (Microbiological Associates). Culture containers were soaked in 25 percent sulfuric acid for 12 hours prior to washing. After washing, each piece of glassware was rinsed ten times in tap water and five times in deionized distilled water.

Rubber Materials

Rubber stoppers were used to seal culture bottles. The stoppers were No. 2, white latex (West Company). White latex rubber policemen attached to glass rods were used to scrape cells off glass surfaces.

New rubber materials were specially treated before being used for culture work. The treatment involved boiling them for 15 minutes in 0.5N sodium hydroxide, rinsing in tap water, boiling in 4.0 percent v-v hydrochloric acid, rinsing in tap water, then rinsing thoroughly in deionized distilled water.

Trypsin Solution

A 0.2 percent trypsin solution was used to remove cell monolayers from glass surfaces and to disperse embryonic tissue. This solution was prepared by adding two grams of powdered trypsin (1:300, Nutritional Biochemicals Corporation) to 100 ml of 10X GKN (16) in a one liter volumetric flask. The flask was then filled two-thirds full with double distilled water, and 6 ml of 0.2 percent phenol red was added. The pH of this mixture was adjusted to approximately 7.4 by adding small quantities of crystalline sodium bicarbonate.

The volumetric flask was placed on a rotary shaker until the mixture was dissolved. The solution was then diluted to one liter with double distilled water and sterilized through a Seitz filter.

Seitz filter pads were prepared by first rinsing them with a one percent acetic acid solution, then with a one percent sodium bicarbonate solution, and finally with double distilled water until the filtrate was a neutral pH. The filter apparatus was sterilized in an autoclave for 20 minutes.

Sterility Testing Medium

Sterility checks were made on growth medium and cell suspensions. The sterility testing medium was thioglycollate broth (Difco) dispensed 10 ml per culture tube. Sterility was determined by

pipetting one ml of the solution in question into the broth and incubating it at 25° C for two to three days.

Enzyme Dispersion Apparatus

A Teflon-covered magnetic stirring bar, 1.5 inches long, was used to suspend minced embryo tissues in a 0.2 percent trypsin solution. The bar was turned with a magnetic stirring device (Laboratory Mag Mix) placed underneath a 250 ml Erlenmeyer flask containing the trypsin, tissues and magnetic bar.

Instruments

Stainless steel curved-tipped forceps, stainless steel scissors, and scalpels with No. 10 blades and No. 3 handles (Bard-Parker) were used to prepare embryos for enzyme dispersion. Instruments were sterilized in an autoclave for 20 minutes.

Cell Counting Materials

Cell counts were performed by diluting one ml of cell suspension with two ml of 0.01 percent crystal violet in 0.1 M citric acid, then counting the stained cells in a hemocytometer. A more detailed explanation of this technique is presented elsewhere (22).

Incubator

Cell cultures were cultivated at 23° C in a walk-in incubator. The incubator was equiped with a thermostat which regulated an electric heater and a cooling unit.

Materials for Chromosome Analysis

The materials required for chromosome analysis by this procedure can be separated into two groups, materials for chromosome preparation and those for chromosome characterization.

Materials for Chromosome Preparation

The chromosome preparation techniques used in this analysis were a modification of a method developed by Pacha and Kingsbury (17). The materials needed are listed below in the approximate order in which they were used.

Colchicine Solution. A colchicine solution was used to accumulate cells in metaphase. The solution was 0.001 percent colchicine (Nutritional Biochemicals Incorporated) in sterile double distilled water (12).

Agar Medium. An agar medium was used to expand and flatten cells. It contained 1.5 percent Difco agar and 0.5 percent sucrose

in distilled water. This mixture was melted and dispensed into screw-cap tubes, 20 to 25 ml per tube. The tubes were sterilized in an autoclave for 15 minutes, cooled, and refrigerated.

When the agar medium was needed, tubes were placed in a steamer until the agar was melted. Tubes were cooled to approximately 50° C and emptied into sterile 15 x 100 mm petri dishes, one tube per dish. After the agar solidified, the dishes were inverted and incubated at 37° C for about 12 hours. When dried, they were either cooled to 20° C and used, or refrigerated.

Glassware. Petri dishes, 15 x 100 mm, were used for agar medium. Cells were prepared on 25 x 75 mm microscope slides (precleaned, selected, Van Waters and Rogers), and covered with 22 x 40 mm coverslips (No. 1, Van Waters and Rogers).

Bouin's Fixative. Bouin fluid contained the following reagent-grade chemicals per 105 ml: 75 ml of picric acid (1.4 grams per 100 ml distilled water), 25 ml of 40 percent formaldehyde, and 5 ml of glacial acetic acid (16).

Acid Hydrolysis. Hydrolysis was performed in a 0.1 N hydrochloric acid solution. Six hundred ml of this solution was placed in a one liter beaker and held in a water-bath at 60° C.

Reagents for May-Greenwald-Giemsa Method. Fixed and

hydrolyzed cells were stained by the May-Greenwald-Giemsa method (16). The following reagents were required: May-Greenwald stain (Allied Chemical), 0.25 percent in absolute methanol; Giemsa stain (Allied Chemical), 1 gram in a 132 ml solution of one part glycerol to one part absolute methanol; and four solutions of acetone and/or xylene. These four solutions included: undiluted acetone, undiluted xylene, a mixture of two parts acetone to one part xylene, and a mixture of one part acetone to two parts xylene.

Staining Rack. The staining procedure was performed in a "Lab-Tek Staining Set." The set consisted of a stainless steel rack, six staining dishes, and a slide holder.

Mounting Medium. Mounting medium (Technicon) was used to secure coverslips to microscope slides.

Materials for Chromosome Characterization

The characterization of prepared chromosomes in this analysis was simplified by the use of a binocular microscope with a camera attachment. This equipment and additional materials needed to characterize chromosomes are described below.

Microscope. Stained preparations were examined with a Leitz Ortholux microscope. Direct light intensity was regulated with a

Leitz transformer and lamp. The microscope contained a camera attachment equiped with an ocular and low-power eyepiece.

Photographic Materials. Photographs were made with a Leica 35 mm camera. The film used was either Kodak panatomic X or Adox KB 14.

Film was developed in a Koda-Craft Roll-Film Tank using Kodak D-76 Developer, SB-5 stop bath solution, and Kodak Fixer.

Projection Equipment. Developed film was placed in a film-strip projector, and the images of the negatives were projected on 8 x 11.5-inch sheets of paper. Paper was clipped to a vertical piece of fiber board adjacent to the microscope. The distance between the projector and the fiber board was about four feet.

EXPERIMENTAL METHODS

Methods of Cell Culture Initiation and Care

Cell lines were serially transplanted approximately once a month, and their growth medium was changed at intervals between five and ten days. Primary cell cultures were initiated and their medium changed at five-day-intervals; for the purposes of this investigation they were not transplanted.

Care of Cell Lines

Cell lines were serially transplanted by first removing the growth medium from culture bottles and treating the cell monolayers with an equal volume of 0.2 percent trypsin solution for 15 to 30 minutes. Any cells still attached to the glass were removed with a rubber policeman. Next, the cell suspensions were transferred into 50 ml centrifuge tubes and centrifuged at 1000 rpm for ten minutes.

After centrifugation, the trypsin was discarded and the cells were resuspended in 5 ml of growth medium. This suspension was triturated 10 times with a 10 ml pipet, and pipetted into enough growth medium to give a total volume two to three times greater than the amount of growth medium originally removed from the culture bottles. This total volume depended upon the amount of cell growth in these bottles. The suspension was dispensed (8 ml per bottle) into

sterile 150 ml milk dilution bottles. The bottles were sealed with rubber stoppers and the stopper and neck of each bottle were enclosed in a piece of sterile aluminum foil. Culture containers were then placed in the 23° C incubator.

Growth medium changes on stock cultures of cell lines were made ten days after serial transplantations, and at ten-day intervals thereafter until they were subcultured again. Cell lines and primary cultures that were being used for chromosome analysis received fluid changes at five-day intervals, and 24 hours prior to the preparation of chromosomes for analysis.

Methods of Primary Cell Culture Initiation

The methods used to initiate the primary cultures which developed into the five cell lines analyzed in this investigation have been previously described (6, 7). Because two additional primary cultures were used in this research, these methods will be briefly mentioned.

The primary cultures were obtained from embryonic tissues of coho and chinook salmon. Whole embryos were removed from eggs and minced. These tissues were suspended in a 0.2 percent trypsin solution at a ratio of approximately one gram of tissue to 30 ml of trypsin. Continuous mixing of the tissues (at 20° C) was performed by a magnetic stirring apparatus (previously mentioned).

Tissues were dispersed in this manner for 30 minutes, then the flask was removed from the stirring device and the fragments were allowed to settle. The supernatant was poured off and discarded because the initial enzyme treatment usually yielded large amounts of cytotoxic materials. The remaining fragments were re-suspended in an equal volume of fresh trypsin and mixed for 40 additional minutes. Dispersion was again discontinued and the supernatant poured off; however, this fluid was saved since it contained large numbers of suspended cells. More fresh trypsin solution was added to the tissues and the above process was continued until enough cells were dispersed, or until the fragments were completely dissociated.

The suspension of cells in trypsin was transferred into centrifuge tubes, centrifuged at 1000 rpm for ten minutes, and the trypsin solution discarded. Cells were diluted in enough growth medium to give a concentration of 1.5 to 2.0 x 10^6 cells per ml and were then dispensed into culture bottles. The culture containers were incubated at 23° C.

Methods of Chromosome Analysis

Of the two steps in chromosome analysis, preparation was more complicated than chromosome characterization. However, the latter was more time consuming. In this investigation, each preparation of a cell culture took about three hours of working time, while each

characterization took approximately 20 hours.

Chromosome Preparation

The technique used to prepare chromosomes involved the following sequence of steps: (a) pretreatment with colchicine, (b) preparation of cell suspension, (c) cell expansion, (d) cell fixation, (e) cell hydrolysis, (f) staining by the May-Greenwald-Giemsa method, and (g) coverslip attachment. Steps (c), (d), and (e) were developed by Pacha and Kingsbury (17) and were used with only slight modifications. Step (a) was described by Hsu and Klatt (12). The remaining steps, (b), (f) and (g), are standard techniques in cytology and cell culture.

Pretreatment with Colchicine. Cell cultures had to achieve an adequate rate of cell division before colchicine pretreatment was effective. A culture was considered to be dividing at a sufficient rate when its prepared cells contained enough metaphase cells to make chromosome characterization efficient. Cell lines had enough metaphase cells two to three weeks after a transplantation, and primary cultures contained enough such cells after three to six weeks.

Twenty-four hours prior to pretreatment with colchicine, the growth medium was changed on the cultures. The treatment was started by adding 0.2 ml of a 0.001 percent colchicine solution to the eight ml of growth medium. The cells were exposed to colchicine

for 16 to 24 hours, depending upon the apparent division rate of the cell culture. The pretreatment was terminated by removing the growth medium.

Preparation of Cell Suspension. Eight ml of 0.2 percent trypsin solution was added to the culture bottle. It was allowed to disperse the cell monolayers for two hours at 20° C. Any cells still attached to the bottle were scraped off with a rubber policeman, and the suspension was allowed to stand for another 30 minutes. Next, the cells were transferred into a centrifuge tube and centrifuged at 1000 rpm for 10 minutes. The trypsin was discarded and the pellet of cells suspended in one ml of a solution composed of ten percent agamma calf serum in MEM. This suspension was triturated 20 times with a one ml pipet.

A 0.25 ml aliquot of the suspension was counted with a hemocytometer. Depending upon the cell concentration, the remaining 0.75 ml of suspension was diluted with enough ten percent serum solution to give a final concentration of 3 to 4 x 10^6 cells per ml.

Cell Expansion. An agar medium containing 1.5 percent agar and 0.5 percent sucrose was used to expand and flatten cells. One-hundredth ml drops of the above cell suspension were placed at 0.75 inch intervals over the surface of the agar medium; about 15 drops per petri dish. The drops were absorbed in 15 to 20 minutes,

leaving opaque, circular layers of cells about 0.25 inch in diameter on the surface of the medium.

Next, a scalpel was used to cut around the edge of each cell layer. The resulting pieces of agar medium were removed from the petri dish with a spatula and carefully placed cells-side-down on glass microscope slides. Usually, six pieces of medium could be put on one slide.

Cell Fixation. Fixation was accomplished by placing slides in a petri dish and immersing the pieces of agar medium with Bouin's fixative. The fixation time was between 45 to 60 minutes. After fixation, the slides were lifted from the fixative and the pieces of medium were removed. This latter step required care because the layer of cells which had attached to the slide could easily be scraped off. The slides were then rinsed in distilled water.

Cell Hydrolysis. Cells were hydrolyzed to remove background RNA. The slides with fixed cell preparations were placed in a slide holder. Then, the holder and slides were placed in 0.1 N hydrochloric acid at 60° C for 15 to 20 minutes. Next, the slides were rinsed three times in two changes of distilled water, and excess water removed.

Staining by The May-Greenwald-Giemsa Method. The

May-Greenwald-Giemsa method was used to stain all cell preparations (16). The slide holder and slides were first placed in May-Greenwald stain for ten minutes, then in Giemsa stain for 20 minutes. The slides were dehydrated by quickly rinsing them in two changes of acetone, three rinses in a solution of two parts acetone to one part xylene, three rinses in a solution of one part acetone to two parts xylene, and a final treatment in pure xylene for ten minutes. Then, the holder and slides were removed from the xylene, and the slides were taken from the holder and air-dried for 30 minutes to two hours.

Coverslip Attachment. A clean glass coverslip was placed over each slide and held in place with mounting medium (Technicon). Excess mounting medium was removed by applying pressure to the coverslip.

The slides could be immediately examined with a microscope, using the high-dry objective. After two days, the mounting medium was dry enough to allow slides to be observed with oil immersion.

Chromosome Characterization

Chromosome characterization involved microscopically examining stained cell preparations of the seven cell cultures analyzed in this investigation. From each of these preparations, 100 cells in

metaphase were randomly chosen, and the number and morphology of the chromosomes within each cell were recorded.

Selection of Metaphase Cells. Slides containing cell preparations were first scanned using the low-power objective. When a metaphase cell was observed which appeared suitable for chromosome characterization, it was examined more closely with the oil immersion objective.

The final selection of a cell was governed by the following criteria: (a) the cell had to be fixed and stained so as to make the cell and its chromosomes clearly visible; (b) the cell's chromosomes had to be distinct from each other, and in the same focal plane; (c) the interior and perimeter of the cytoplasm had to be uniform and unbroken; (d) a cell could have no other metaphase cells adjacent to it (14).

If a cell fulfilled the above criteria it was photographed. Also, the location of the cell on the slide was determined using a mechanical stage micrometer so that the cell could be relocated when chromosome characterization was performed.

Determination of Chromosome Number and Morphology. The technique used to determine the number of chromosomes per cell was designed to accommodate the facilities available in this laboratory. Two images of a given cell were used as the chromosomes were

counted. The first image was relocated on a slide and viewed with the oil immersion objective. The second was projected on a sheet of paper beside the microscope. This latter image was provided by the negative of the photograph of the cell projected by a film-strip projector. The image on the sheet of paper was used to mark off the chromosomes as they were counted. The same cell viewed through the microscope was used as a check on the projected image of the negative.

When a chromosome was counted, it was marked as either a metacentric or telocentric chromosome. The metacentrics were marked with an "X" and the telocentrics with an "O". After all the chromosomes in a cell were marked, the total number of each type of chromosome was determined and these two numbers were added to give the number of chromosomes per cell.

Classification of chromosomes as metacentric or telocentric was based on the relative location of the centromere on each chromosome. The location of the centromere on a metacentric was somewhere in the central portion of the chromosome, showing four distinct chromatid arms. A telocentric had the centromere at the end of the chromosome, with two relatively long chromatid arms on one side and no arms, or very short ones, on the other side. In certain cases the difference between metacentric and telocentric chromosomes was very slight. This made classification somewhat arbitrary, and lessened the accuracy of morphology determinations.

EXPERIMENTAL RESULTS

A chromosome analysis was performed on five salmonid fish cell lines currently maintained in this laboratory in order to establish their ploidy and to provide one means of identifying them. In addition, two primary cell cultures initiated from embryos of salmonid fishes were analyzed to find their ploidy. For each analysis of a cell culture two kinds of information are presented: the history of the culture and its treatment at the time of chromosome analysis, and the chromosomal properties determined in the analysis.

History of Cell Cultures at the Time of Analysis

All cell lines were about two years old and had undergone between 20 and 27 transplantations when their chromosomes were prepared for analysis. Lines were allowed to grow for about two weeks after a transplantation before they were exposed to colchicine. After 16 to 28 hours of colchicine pretreatment, the cells were harvested and their chromosomes prepared. A more detailed description of the above information is given in Table III.

The two primary cell cultures were three to six weeks old at the time of analysis, and were exposed to colchicine for 24 hours. These experimental conditions are described in Table IV.

TABLE III. THE AGE, NUMBER OF TRANSPLANTATIONS, AND PRETREATMENT OF CELL LINES AT THE TIME OF CHROMOSOME ANALYSIS.

Cell Line Species and Designation[1]	Chinook Salmon TC-114	Coho Salmon TC-119	Steelhead Trout TC-137	Rainbow Trout TC-149	Sockeye Salmon SeE
Hours of Exposure to Colchicine	20	16	24	24	28
Period of Culture Growth Prior to Analysis (Weeks)	2	1.5	2	2	3
Number of Transplantations at the Time of Analysis	22	27	20	20	26
Age of Cell Line at the Time of Analysis (Months)	24	25	23	23	22

[1]The chinook, coho and sockeye salmon, and steelhead trout cell lines were obtained from embryonic tissues. The rainbow trout line was from hepatoma tissue of an adult rainbow trout.

TABLE IV. THE AGE AND HOURS OF EXPOSURE TO COLCHICINE FOR PRIMARY CULTURES AT THE TIME OF CHROMOSOME ANALYSIS.

Primary Cell Culture and Source[1]	Chinook Salmon, Whole Embryos	Coho Salmon, Whole Embryos
Hours of Exposure to Colchicine	24	24
Weeks of Culture Growth at the Time of Analysis	5-6	3-4

[1] These cultures were obtained from tissues of whole embryos by enzyme dispersion and cultivation in growth medium at 23° C.

Chromosomal Characteristics of Cell Cultures

Each chromosome characterization of a cell culture involved counting the number of chromosomes per cell in a sample of 100 metaphase cells, and classifying each chromosome, as it was counted, as either metacentric or telocentric. The 100 cells were chosen in a random manner dictated by the previously mentioned criteria.

The chromosomal properties of the chinook (TC-114), coho (TC-119), and sockeye (SeE) salmon cell lines are described in Table V. Similar properties of the steelhead (TC-137), and rainbow (TC-149) trout cell lines are found in Table VI. Table VII lists these characteristics for the chinook and coho salmon primary cultures.

These results were compared with chromosomal properties of normal *in vivo* cells of the same species reported by Simon, and

TABLE V. CHROMOSOME DISTRIBUTIONS AND MORPHOLOGY IN CHINOOK (TC-114), COHO (TC-119), AND SOCKEYE (SeE) SALMON CELL LINES.

TC-114

Number of Cells Containing Indicated Chromosome Numbers:

	3	4	3	5	4	3	8	9	12	4	11	5	4	5	13	6	1
Number of Chromosomes per Cell:	18	61-63	65	66	67	68	69	70	71	72	73	74	75	76	78-113	129-141	190

TC-119

Number of Cells Containing Indicated Chromosome Numbers:

	2	6	10	6	9	4	1	1	1	3	6	11	21	6	2	2	2	1	5	1
Number of Chromosomes per Cell:	57	58	59	60	61	62	63	65	67	68	69	70	71	72	73	74	107	112	134-142	173

SeE

Number of Cells Containing Indicated Chromosome Numbers:

	1	3	9	16	53	6	4	3	2	2	1
Number of Chromosomes per Cell:	51	53	54	55	56	57	58	59	60	96	101

Cell Line	Modal Number	Normal Diploid Number[1]	Range of Chromosome Numbers	Ratio of Telocentric to Metacentric Chromosomes Normal[1]	Cell Lines	Ploidy
TC-114	71	68	18-190	0.89	0.97	Heteroploid
TC-119	71	60	57-173	0.15	0.28	Heteroploid
SeE	56	56	51-101	0.22	0.23	Diploid

[1] As determined on fertilized ova of chinook, coho, and sockeye salmon by Simon (23).

TABLE VI. CHROMOSOME DISTRIBUTIONS AND MORPHOLOGY IN STEELHEAD TROUT (TC-137) AND RAINBOW TROUT (TC-149) CELL LINES.

TC-137

	Number of Cells Containing Indicated Chromosome Numbers:																
	5	2	5	10	25	14	4	7	8	4	2	3	4	2	1	3	1
Number of Chromosomes per Cell:	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	126

TC-149

	Number of Cells Containing Indicated Chromosome Numbers:																
	5	1	12	7	11	8	8	9	12	6	4	6	1	1	2	5	2
Number of Chromosomes per Cell:	18-38	53	54	55	56	57	58	59	60	61	62	63	64	65	67	109-121	130-144

Cell Line	Modal Number(s)	Normal Diploid Number[1]	Range of Chromosome Numbers	Ratio of Telocentric To Metacentric Chromosomes Normal[1]	Ratio of Telocentric To Metacentric Chromosomes Cell Lines	Ploidy
TC-137	62	60	58-126	0.36	0.24	Heteroploid
TC-149	54, 60	60	18-144	0.36	0.35	Heteroploid

[1] As determined on fertilized ova of rainbow trout by Simon and Dollar (24).

TABLE VII. CHROMOSOME DISTRIBUTIONS AND MORPHOLOGY IN CHINOOK AND COHO SALMON PRIMARY CELL CULTURES.

Chinook Primary Cell Culture

	Number of Cells Containing Indicated Chromosome Numbers:												
	1	1	3	7	64	6	3	2	4	1	1	6	1
Number of Chromosomes per Cell:	35	64	66	67	68	69	70	72	73-89	125	126	132-140	172

Coho Primary Cell Culture

	Number of Cells Containing Indicated Chromosome Numbers:							
	5	7	70	10	4	1	1	2
Number of Chromosomes per Cell:	58	59	60	61	62	63	117	119

Primary Cell Culture	Modal Number	Normal Diploid Number[1]	Range of Chromosome Numbers	Ratio of Telocentric to Metacentric Chromosomes Normal[1]	Cell Cultures	Ploidy
Chinook	68	68	35-172	0.89	0.86	Diploid
Coho	60	60	58-119	0.15	0.26	Diploid

[1] As determined on fertilized ova of chinook and coho salmon by Simon (23).

Simon and Dollar (23, 24).

The modal number of chromosomes was found to be different in four of the cell lines from that characteristic of the species. The sockeye salmon line and the two primary cell cultures had modal numbers of chromosomes which were the same as the normal diploid numbers of these species. All seven cell cultures showed a range and distribution of chromosome numbers per cell that were greater than found in normal in vivo cells.

The ratio of telocentric chromosomes to metacentric chromosomes in each of the seven cell cultures was found by dividing the total number of telocentrics by the total number of metacentrics in each sample of 100 metaphase cells. The differences between these ratios and those reported for normal cells were never greater than 0.13. The sockeye salmon line, rainbow trout line, and chinook salmon primary culture had ratios which agreed with the ones found in normal in vivo cells from the same species.

The above data show that four of these salmonid cell lines are heteroploid, and one cell line has a diploid chromosome constitution. That all the lines derived from embryonic tissue were originally diploid, was indicated by the results of the primary culture analyses. Both primary cultures were definitely diploid in number.

Five photographs of metaphase cells from each of the cell lines

are presented in Figures 1 through 5. These illustrate the general appearance of the chromosomes in the lines, and are examples of the metaphase cells which were used to determine their chromosomal characteristics.

Figure 1. Metaphase cell from the coho salmon cell line (TC-119) containing 59 chromosomes. Oil immersion, magnification approximately 2,600 X.

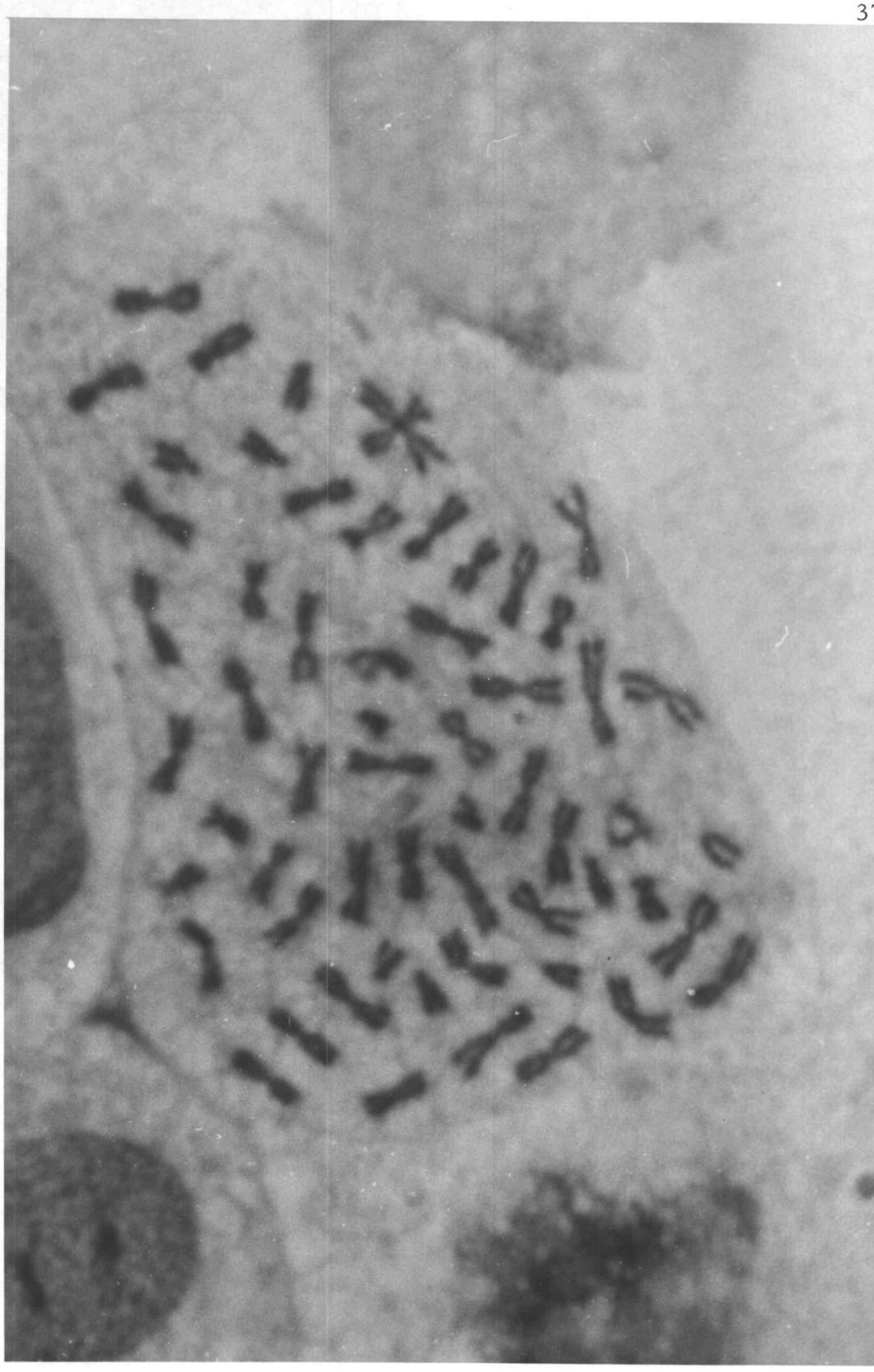

Figure 1

Figure 2. Metaphase cell from the steelhead trout cell line (TC-137) containing 62 chromosomes. Oil immersion, magnification approximately 1,500 X.

Figure 3. Metaphase cell from the sockeye salmon cell line (SeE) containing 56 chromosomes. Oil immersion, magnification approximately 1,500 X.

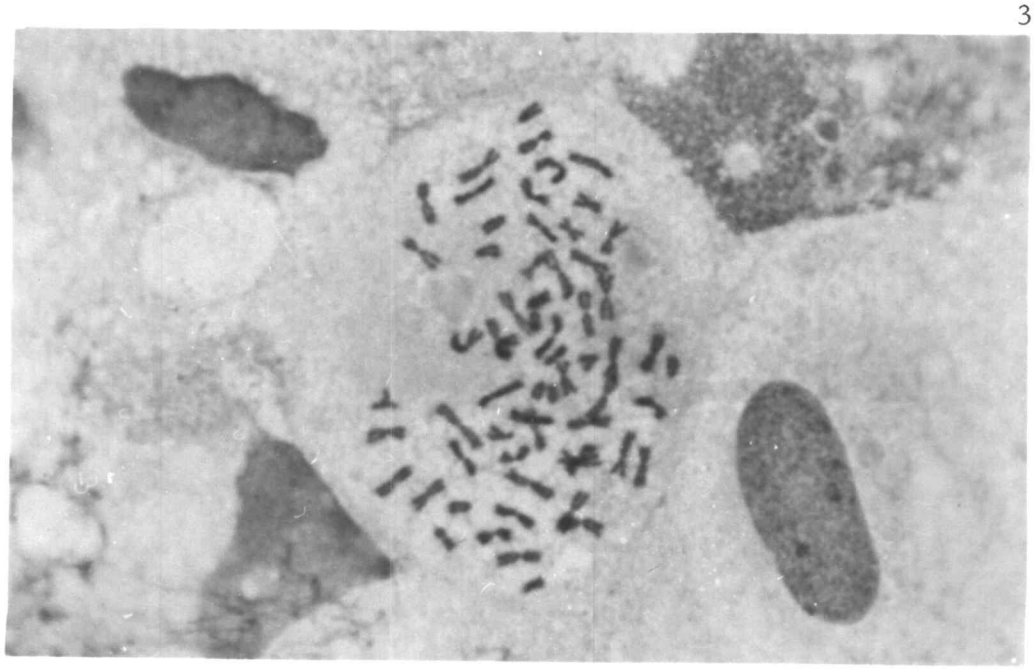

Figure 2

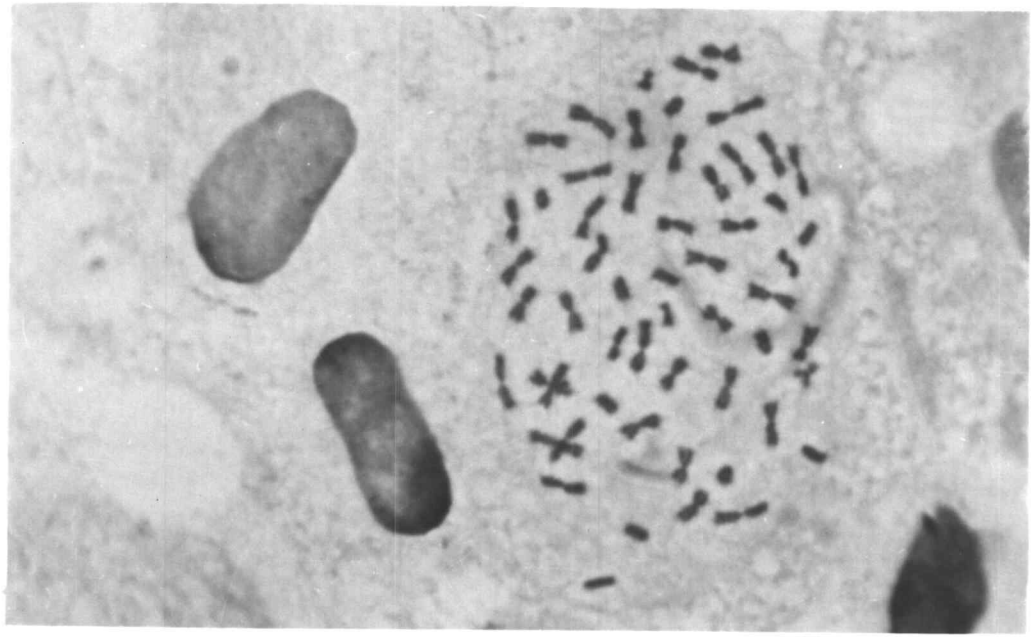

Figure 3

Figure 4. Metaphase cell from the rainbow trout hepatoma cell line (TC-149) containing 54 chromosomes. Oil immersion, magnification approximately 1,500 X.

Figure 5. Metaphase cell from the chinook salmon cell line (TC-114) containing 62 chromosomes. Oil immersion, magnification approximately 1,200 X.

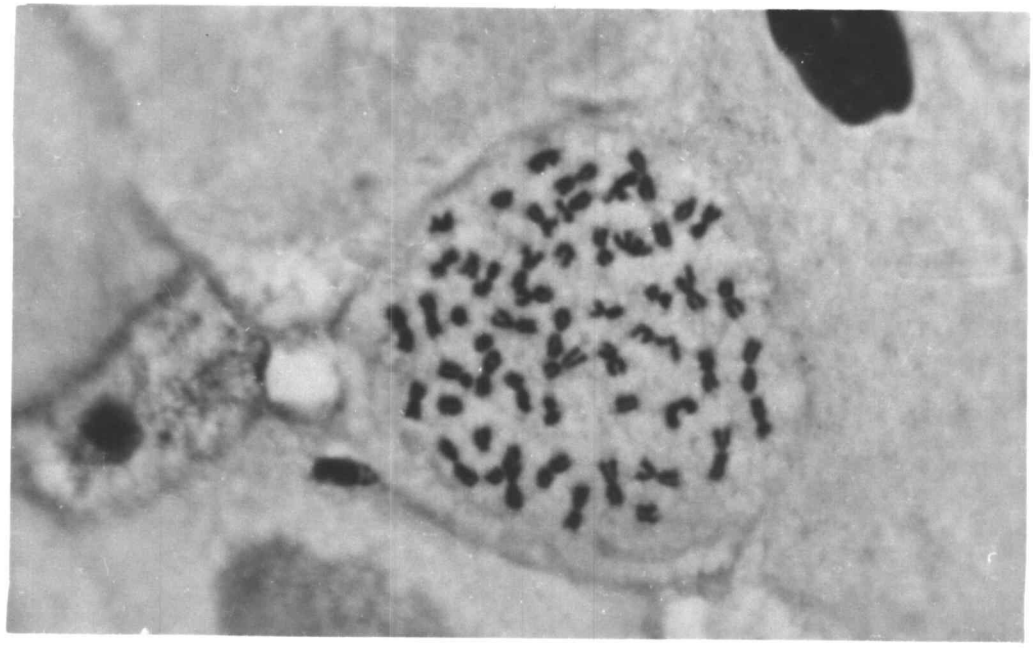

Figure 4

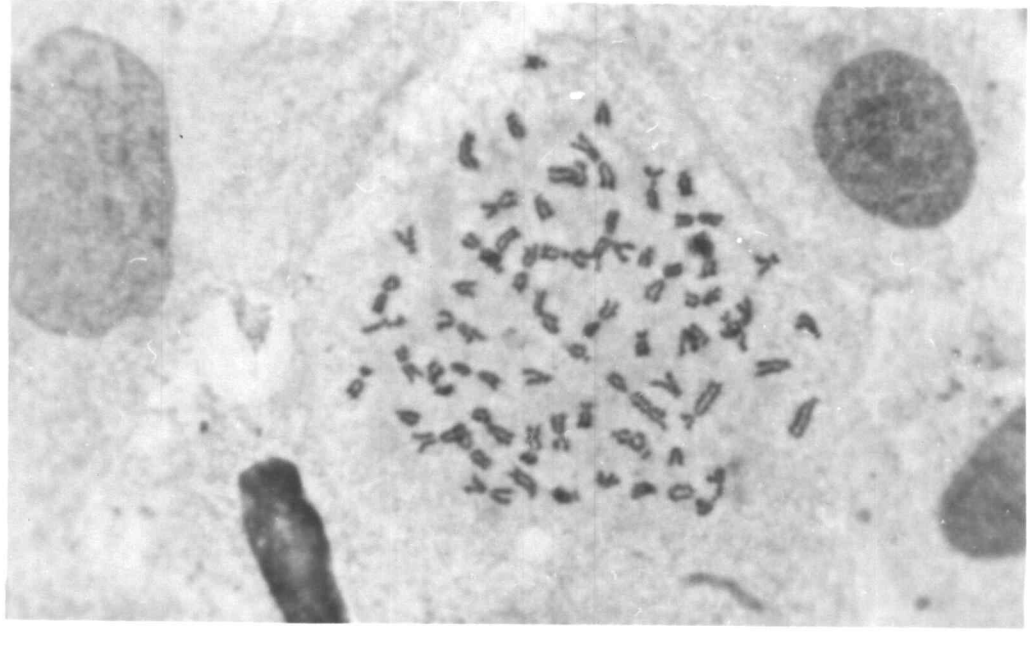

Figure 5

DISCUSSION

Most established cell lines (those capable of indefinite in vitro cultivation) have been found to have chromosome distributions other than diploid (9). Four of the cell lines analyzed in this investigation also demonstrated deviations from diploidy. In addition, the four lines are around three years old and have undergone approximately 40 transplantations. These three factors strongly indicate that the chinook and coho salmon, and steehead and rainbow trout cell lines are established cell lines.

The degree of deviation of the modal chromosome numbers in these four lines from the normal diploid numbers reported for these species corresponds with the apparent relative growth rates of these lines. These relative rates of growth were estimated from the intervals at which the lines could be subcultured. Cell lines are generally subcultured when they have formed monolayers with sufficient cells to easily undergo a two to three-fold expansion. The coho salmon line showed a modal number ten chromosomes higher than the diploid number, and it is the fastest proliferating of the cell lines. The chromosome distributions of the chinook salmon and rainbow trout lines had modes which were four chromosomes away from the normal diploid number, and these lines grow at a slower rate. The steelhead trout cell line has the slowest growth rate, and

it had a modal shift of only two chromosomes.

That the chinook and coho salmon primary cell cultures were diploid is not surprising. However, the fact that the sockeye salmon cell line was found to be diploid is of interest. This line was cultivated for two years in the same growth medium and under similar laboratory conditions as the four heteroploid lines; yet it retained the normal diploid modal number. It is possible that this line was quasi-diploid, i.e., the line may have had the diploid mode, but possibly did not contain the normal set of chromosomes. Presently, the sockeye line is showing signs of degeneration similar to those described by Hayflick and Moorhead in their work with diploid human cell strains (10). This would suggest that the line is diploid, and has not fully adapted to in vitro cultivation.

The presence of at least one tetraploid cell in most of the cell cultures analyzed is not unusual. Simon reported that in all five of the salmonid species he analyzed 50 percent of the embryos contained one or more polyploid cells (23).

Three of the cultures, the chinook and coho salmon cell lines and coho salmon primary culture, had ratios of telocentric to metacentric chromosomes higher than similar ratios found in normal in vivo cells of these species. Such deviations mean increases in the numbers of telocentrics per cell. These increases could be real or due to errors made in classifying the chromosomes during analysis.

This latter possibility is partially supported by the idiograms of in vivo chinook and coho salmon cells analyzed by Simon. Each species had a certain number of submetacentric chromosomes per cell (23). It is possible that some of these were classified as telocentrics in this investigation.

The ranges and distributions of chromosome numbers in all these cell cultures and the heteroploid condition of the four cell lines show that many cells have either lost or gained chromosomes. Four generally accepted mechanisms may account for these changes. One is the result of multipolar spindles which cause a cell to divide into more than two daughter cells, leaving some cells with fewer chromosomes. Another is known as nondisjunction; in this case daughter chromatids do not split apart at anaphase, but both go to one pole, causing a gain of one chromosome in one daughter cell and a loss of one in the other. Chromatid loss is a third mechanism and involves the loss of a chromatid to the cytoplasm during cell division. The fourth mechanism is the cause of polyploid cells. Known as endomitosis, it occurs when the chromosomes duplicate and divide, but the cell does not divide (9).

Although each primary cell culture had approximately 70 percent of its cells with the normal diploid chromosome number, other chromosome analyses of primary cell cultures have not shown such wide distributions of chromosomes per cell (18, 19). Several

explanations could account for these differences. Possibly the chinook and coho cultures were genetically less stable in the in vitro conditions used to initiate them. Or, because they were three to six weeks old at the time of chromosome analysis, rather than the three to 20 days reported by the above authors, there was more time for cell division abnormalities to occur.

Gravell and Malsberger reported a wide distribution of chromosomes per cell in their analysis of a fathead minnow primary cell culture. This culture also had more than 70 percent of its cells with the same chromosome number. They suggested that this variation was due to the age of the culture (two months) at the time of analysis (8).

The modal number of chromosomes per cell in a heteroploid cell line is also known as the stem-line number. It is commonly believed that cells with the stem-line number of chromosomes have a selective advantage over other cells in a culture. Thus, through cell division abnormalities a cell line can adapt to in vitro environments by natural selection of cells with sets and numbers of chromosomes best suited for maximum proliferation (9).

SUMMARY AND CONCLUSIONS

1. A chromosome analysis was performed on five cell lines and two primary cell cultures derived from salmonid fishes in order to establish their ploidy or demonstrate their genetic identity. Each analysis involved first chromosome preparation and finally characterization.
2. Chromosomes were prepared by modifications of methods developed by Hsu and Klatt (12), and Pacha and Kingsbury (17).
3. At the time of chromosome analysis the cell lines were about two years old with 20 to 27 transplantations, and the primary cultures were between three and six weeks old.
4. Chromosome characterization was accomplished by selecting 100 metaphase cells from stained preparations of each cell culture, counting the number of chromosomes per cell, and classifying them as either metacentric or telocentric.
5. The modal numbers and ranges of chromosomes per cell for the five cell lines were as follows: chinook salmon line (TC-114), mode 71, range 18 to 190; coho salmon line (TC-119), mode 71, range 57 to 173; sockeye salmon line (SeE), mode 56, range 51 to 101; steelhead trout line (TC-137), mode 62, range 58 to 126; rainbow trout line (TC-149), modes 54 and 60, range 18 to 144. Similar distributions for the two primary cell

cultures were the following: chinook culture, mode 68, range 35 to 172; coho salmon culture, mode 60, range 58 to 119.

6. The chinook and coho salmon and the steelhead and rainbow trout cell lines were found to be heteroploid. These properties, plus their present ages and numbers of transplantations indicate that these lines are capable of indefinite in vitro cultivation.

7. The sockeye salmon cell line and the coho and chinook salmon primary cultures showed diploid chromosome constitutions. The sockeye line is now demonstrating signs of deterioration; this is another indication that it is diploid and has not fully adapted to in vitro cultivation.

8. All seven cell cultures had ranges and distributions of chromosome numbers per cell that were greater than those found in normal in vivo cells.

9. The chromosome morphology of each cell culture was represented by the sum of all the telocentric chromosomes divided by the sum of all the metacentric chromosomes. These ratios in the rainbow trout and sockeye salmon cell lines, and the chinook salmon primary culture were similar to those characteristic for these species. The remaining cell cultures had ratios which deviated from normal ratios by 0.08 to 0.13.

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