Eurythermic and stenothermic growth of cultured fish cells and their thermosensitivity

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Summary

RBCF-1 cells derived from the caudal fin of the goldfish (*Carassius auratus*) have been established at 37°C, and could grow at a wide range of temperatures (20–37°C). To examine the mechanism(s) of this eurythermic property, we isolated clones from RBCF-1 cells with different optimal growth temperatures. R37 clone grew continuously at 37°C but could not grow at 27°C. R27 clone grew continuously at 27°C but could not grow at 37°C. Thus R37 and R27 cells may be referred to as stenothermic. The R3727 clone, which was derived from R37 cells selected at 27°C, and the cell hybrids of R37 and R27 cells, could proliferate for a long time and form colonies at 27–37°C, like progenitor RBCF-1 cells, so they may be referred to as eurythermic. The shape of colonies of R3727 cells changed with incubation temperature; they resembled those of R37 cells at a higher temperature (37°C), while at a lower temperature, (27°C), they were like those of R27 cells.

The lethal effects of heat treatment were compared among RBCF-1 clones. R27 cells were most sensitive, and R37 cells were most resistant. The heat treatment could induce the four major heat-shock proteins (hsp30, hsp42, hsp70 and hsp90). Marked induction of hsp70 and hsp30 was observed at 33°C for R27, at 37°C for R3727 and at 40°C for R37. With R3727 cells, induction of hsps and thermosensitivity were highly dependent on the incubation temperature before heat treatment.

Key words: cultured fish cells, heat-shock proteins, eurythermy, stenothermy, thermosensitivity.

Introduction

Changes in water temperature affect the distribution and the survival of poikilothermic animals such as fish. Cells of fish are supposed to survive and continue growing at a wide range of temperatures. These 'eurythermic' characteristics of cell growth are retained even when cells are transferred to *in vitro* culture, using media and growth conditions similar to those used for mammalian cell culture (Wolf & Quimby, 1969). In general 'the optimal growth temperature', i.e. the temperature at which growth of cultured cells is most rapid, is a little higher than the preferred temperature of the intact animal. For example, rainbow trout (*Salmo gairdnerii*) as individuals live in water at temperatures from 8 to 12°C, but their growth is most rapid at about 15°C, although physiological processes are suboptimal at 15°C (Wolf & Ahne, 1982). Cultured cells from rainbow trout proliferate most rapidly at 20°C (Wolf & Ahne, 1982). For cultured mammalian cells, the range of permissive growth temperatures is 36–39°C, with the optimum at 37°C. Within this range of temperatures, mammalian cells have a fairly constant growth rate and metabolic rate, each falling off outside this range (Sisken *et al.* 1965). This range of growth temperatures of cultured mammalian cells is quite narrow (stenothermic) compared with that of most cultured fish cells (eurythermic). Shima *et al.* (1980) established a cell line designated RBCF-1 from the caudal fin of the goldfish (*Carassius auratus*). The primary culture was started at 37°C, a temperature higher than that for other goldfish cell lines, and cells obtained thereafter could grow within the wide temperature range 20–37°C. In this study we obtained three clones (R37, R27 and R3727) from RBCF-1. R37 and R27 clones could grow only within a narrow range of temperatures, their optimal growth temperatures being 37°C and 27°C, respectively. A cell line designated R3727 was derived from a colony from a R37 cell formed at 27°C. This cell line continued to grow at 37°C as well as at 27°C. The fused cells of R37 and R27 cells also retained eurythermic growth characteristic of the original RBCF-1 cells. We also compared the thermosensitivity and induction of heat-shock proteins (hsps) for these cell clones.

Materials and methods

Cell lines

Cultured goldfish cell clones R37, R27 and R3727 derived from

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RBCF-1 (Shima *et al.* 1980) were grown attached to plastic dishes in a HEPES (10 mM)-buffered medium L-15, supplemented with 15% fetal bovine serum (FBS). R37 cells and R27 cells were maintained at 37°C and 27°C, respectively. R3727 cells were derived from the R37 cell line selected at 27°C, and cultured at 33°C. R37H7 and R27H4 clones were obtained by transfection by pSV2-Hyg, which confers the gene of resistance to hygromycin to R37 and R27 cells. R37N1 and R27N2 clones were obtained by transfection by pSV2-Neo, which confers the gene of resistance to neomycin (G418) to R37 and R27. The details of transfection using RBCF-1 cells will be reported elsewhere (Hayasaka *et al.* unpublished). The selection markers were introduced into the cells to facilitate isolation of R37/R27 hybrid cells.

Population doubling time and plating efficiency

Each 60 mm dish was seeded with 5×10^5 cells in 5 ml of the growth medium. The medium was renewed every three days. The number of cells per dish was determined at various time points, and at least three dishes were used for each point. To determine the plating efficiency, 100–1000 cells were inoculated in a 60 mm dish and incubated without a medium change, followed by fixation and staining of colonies formed. Numbers of colonies with 32 or more cells were counted. Colonies took 7–10 days to become visible when cells were incubated at 37 or 33°C, and 14–18 days at 27°C. At least three dishes were used for each cell clone.

Electrofusion

Electrofusion was done using an Electro Cell Fuser (Model ECF-2001: Riko Kagaku). The fusion experiments were carried out at room temperature. The hygromycin-resistant clone (R37H7 or R27H49) and G418-resistant clone (R37N1 or R27N2) were harvested, and equal numbers of cells were mixed and pelleted by centrifugation. Then 10^6 (in total) cells were suspended in 200 μl of 0·35 M-sucrose under an alternating electric field of 0·16 kV cm^{-1} and 1 MHz. The cells were allowed to align and form pearl chains for 10 s. Cell fusion was initiated by a pulse, the intensity of which was 2·8 kV cm^{-1}, with a duration of 100 μs. Then the cells were inoculated into selection medium (300 μg of G418 and 300 μg of hygromycin B per ml of growth medium). The medium was changed once a week, and after three weeks the surviving colonies were isolated.

Heat treatment of cells

To examine the synthesis of hsps, the cells, grown to near confluence (10^6 cells in a 25 cm^2 plastic flask), were subjected to higher temperatures by partially immersing the flask in a temperature-regulated waterbath. The volume of medium in the flask was only 5 ml to make temperature equilibration rapid. To measure the thermosensitivity in terms of survival, 100–1000 cells were inoculated into a 25 cm^2 plastic flask and the same heat treatments were done at about 12 h after inoculation. The medium was changed immediately after the heat treatments, and the cells were incubated for colony formation.

The cells were pre-incubated in methionine-free DMEM for 2 h before temperature shift. The cells were labeled for one hour with Tran ^{35}S-label™ (ICN, an *Escherichia coli* hydrolysate labeling reagent containing L-[^{35}S]methionine; final activity of ^{35}S was 10 μCi ml^{-1}) at various temperatures. The incorporation was terminated by removing the labeling medium and washing the cells with phosphate buffered saline (PBS). The cells were then harvested, suspended in 1 ml of PBS and transferred to a microcentrifuge tube for centrifugation at 3000 revs min^{-1} for 3 min in the cold. In order to prepare cell extracts, the cell pellet was suspended in Laemmli sample buffer (62·5 mM-Tris, 2% SDS and 5% β-mercaptoethanol; Laemmli, 1970) to a concentration of 10^4 cells μl^{-1}, and solubilized by immersing in boiling water for 2 min.

Table 1. *Plating efficiencies of RBCF-1 cell clones at different temperatures*

Clone	37°C	33°C	27°C
R37	0·56 ± 0·02	0·56 ± 0·03	0·01 ± 0·005
R27	0	0·13 ± 0·03	0·31 ± 0·07
R3727	0·40 ± 0·02	0·56 ± 0·02	0·12 ± 0·01

Electrophoresis and autoradiography

The 5 μl cell extract, which is equivalent to 5×10^6 cells, was analyzed on 10% polyacrylamide–SDS slab gels with a 4·5% stacking gel using the discontinuous buffer system of Laemmli (1970). Slab gels were run at a constant current of 10 mA and stained with silver (2D-Silver Stain Kit 'DPC'; Daiichi Pure Chemical). After staining, the gels were dried and autoradiographed using Kodak X-Omat R5 film for 14 days in a −80°C refrigerator.

Results

Table 1 summarizes the plating efficiencies of three cell clones derived from RBCF-1. R27 cells formed colonies at 27°C but not at 37°C, whereas R37 cells formed colonies at 37°C, and also a few colonies at 27°C. Both cell clones could form colonies at 33°C. A clone designated as R3727 was isolated from a single colony of R37 formed at 27°C. This clone could form colonies at both 27°C and 37°C. Plating efficiency at 27°C was rather lower than that at 37°C. R37 cells formed colonies with a characteristic pattern of cell arrangement at 37°C. The cells were spindle-shaped, and cells aligned with each other in one direction forming a strip of cells, which is the typical shape of colonies of RBCF-1 during early passages (Shima *et al.* 1980). R27 cells were smaller, and formed colonies which showed a more dispersed pattern of cellular arrangement. R3727 cells formed colonies whose shapes were like those of R37 cells at 37°C, and like those of R27 cells at 27°C. Fig. 1 shows the growth curves for these clones at various temperatures. It was found that R37 cells continued to grow at 37°C, but at 27°C, they grew during the first few days and then the number of cells decreased. R27 cells grew at 27°C but they soon died at 37°C. R3727 cells continued to grow at both temperatures at similar rates, although in the case of RBCF-1 cells, the population doubling time was largely temperature dependent (Shima *et al.* 1980).

Table 2 shows the fusion efficiency of the experiments. R37H7 and R27H4 were clones containing the hygromycin resistant gene, while R37N1 and R27N2 were clones containing the G418 resistant gene. The introduction of the exogenous genes did not cause any change of optimal growth temperature (data not shown). Hybrid clones were most frequently obtained at 33°C. At 37°C, very few hybrid clones were found. Table 3 shows the plating efficiencies of eight hybrid clones isolated at 33°C. All hybrid clones retained resistance to both selection

markers for at least several months (data not shown). Three clones (clones 6, 7 and 8) formed colonies at 27 °C, but only a few colonies were formed at 37 °C, and one clone (clone 3) formed colonies at 37 °C but only a few colonies were formed at 27 °C. The other four clones (clones 1, 2, 4 and 5) could form colonies at both temperatures. So the phenotypes for lower (27 °C) and higher (37 °C) growth temperatures seemed to complement each other. The sensitivities of R37, R3727 and R27 to the lethal effects of heat treatment (from 27 °C to 42 °C) were examined (Fig. 2) using colony formation assay. R37 cells were the most resistant to heat treatment, and R27 cells were the most sensitive. However, in the case of R3727 cells, thermosensitivity was highly dependent on the incubation temperature before heat treatment. When the R3727 cells were incubated at 27 °C for 12 h, the sensitivity was similar to that of R27 cells incubated at 27 °C, and when the cells were incubated at 37 °C for 12 h the sensitivity was similar to that of R37 cells at 37 °C (Fig. 3).

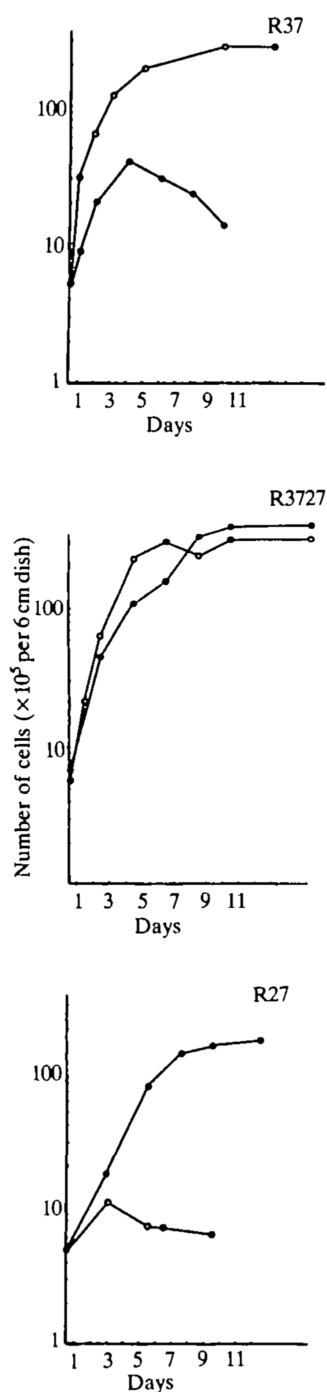


Fig. 1. Growth curves of three clones of RBCF-1. The cells were cultured at 27 °C, ●, and 37 °C, ○.

Table 2. *Frequency of hybrid clones of RBCF-1 cells (no. of colonies per 10^6 cells)*

Hybrid	Selection temperature 37 °C	33 °C	27 °C
R37H7 × R27N2	0	114·9 ± 9·6	69·9 ± 5·4
R37N1 × R27H4	15·6 ± 5·4	170·1 ± 12·9	39·9 ± 15·3
R37H7 × R27N2	0	156·9 ± 18·6	48·0 ± 5·23

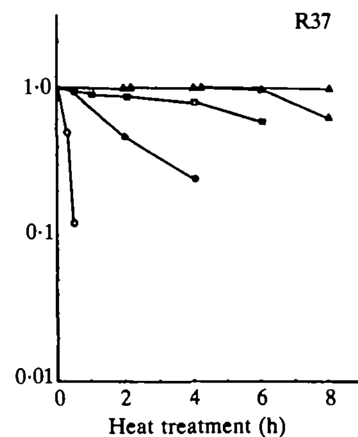
Table 3. *Plating efficiencies of RBCF-1 hybrid cell clones (R37H7 × R27N2) at different temperatures*

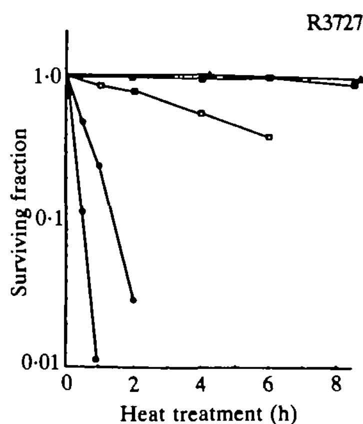
Clone	37 °C	33 °C	27 °C
R37H7	0·66 ± 0·03	0·65 ± 0·04	0·003 ± 0·003
R27N2	0	0·058 ± 0·006	0·18 ± 0·03
Hybrid clone			
1	0·14 ± 0·008	0·71 ± 0·01	0·53 ± 0·02
2	0·24 ± 0·03	0·45 ± 0·04	0·25 ± 0·01
3	0·63 ± 0·02	0·66 ± 0·03	0·005 ± 0·002
4	0·48 ± 0·01	0·60 ± 0·03	0·43 ± 0·03
5	0	0·17 ± 0·01	0·15 ± 0·01
6	0·05 ± 0·003	0·18 ± 0·01	0·30 ± 0·02
7	0·04 ± 0·01	0·23 ± 0·003	0·11 ± 0·01
8	0·03 ± 0·006	0·42 ± 0·02	0·30 ± 0·02

Fig. 4 shows the induction of heat-shock proteins at various temperatures. Just after starting radio-labeling, the cells were transferred to various temperatures and were kept for 1 h. Four major proteins with molecular weights of 30K (K = $10^3 M_r$) (hsp 30), 42K (hsp 42), 70K (hsp 70) and 90K (hsp 90) were induced by heat treatment in all cell clones. Under non-stressed conditions, a low level of synthesis of hsp90, hsp70, and hsp42 was observed in all cell clones, but synthesis of hsp30 could not be detected. A marked induction of hsps (especially hsp70 and hsp30) was observed in the 33 °C treatment of R27 cells, at 37 °C for R3727 cells and at 40 °C treatment for R37 cells. Fig. 5 shows the proteins produced by R3727 cells after 12 h incubation at 37 °C and 27 °C. No marked synthesis of hsps was observed at 37 °C, and there was no significant difference in autoradiographic profiles among the three samples.

Discussion

The eurythermic characteristics of fish cells are retained even in cultured cell lines *in vitro*; they can grow continuously in a wide range of temperatures, as they can in the intact animal. In general, the optimal incubation temperature for cultivation of cells *in vitro* is slightly





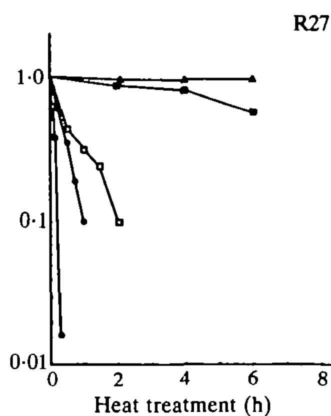


Fig. 2. The sensitivities of three clones of RBCF-1 to heat treatments. R27, R3727 and R37 cells were cultured at 27°C, 33°C, and 37°C, respectively. The cells were transferred to the various temperatures and then cultured for colony formation. The cells were treated at the following temperatures for various times: 27°C, ▲; 33°C, △; 37°C, ■; 40°C, □; 42°C, ●, and 45°C, ○.

above that preferred by the intact animal (Wolf & Ahne, 1982). Cultured cells of warm-water fish seldom grow at 5–10°C; their optimal growth temperature is generally in the range 22–25°C, and their upper limit for growth is about 30°C. Cultured cells of some tropical fish can be adapted to grow at 37°C (Wolf & Quimby, 1969).

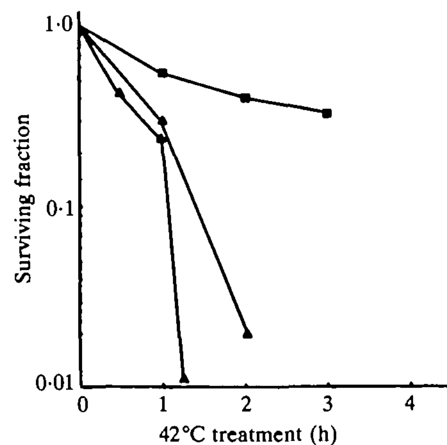


Fig. 3. The effect of incubation temperature on the sensitivity of R3727 cells to the 42°C heat treatment. The cells were incubated at 27°C, ▲; 33°C, △ and 37°C, ■, for 12 h, and then transferred to 42°C for various times.

Metabolic studies in the goldfish (*Carassius auratus*), in which the rate of oxygen consumption and levels of physical activity were measured, have shown a maximum in the range of 25–30°C, with a gradual decline at lower temperatures and a sharp decline at higher temperatures (Kanungo & Prosser, 1959*a*,*b*). Also in the goldfish, the upper lethal temperature was found to be 39°C, and 37°C was the sublethal temperature (Garcia & Johnson, 1972). Rio *et al.* (1973) reported that the optimal growth temperature of cultured goldfish cells was 20°C, and the upper temperature limit for growth and viability was 24°C. However, RBCF-1 cells, which were initially cultured at 37°C, showed a wide range of growth temperatures. This cell line continued to grow at 20–37°C, and at the optimal temperature (37°C) the cells grew very rapidly. Population doubling time at 37°C was about 12 h; at 20°C the population doubling time became longer, about 58 h (Shima *et al.* 1980). No mammalian cell line can grow over such a wide range of temperatures. Therefore this goldfish cell line provides a very useful system for studying the evolutionary steps in vertebrates, from 'poikilothermy' to 'homeothermy'. To elucidate the factors that determine the optimal growth temperature, we isolated the cell clones R37 and R27, which have a narrow temperature range for growth. R37 cells could not continue to grow at 27°C, but, with very low efficiency, some cells could form colonies at both 37°C and 27°C, like R3727 cells. On the other hand, R27 cells never formed colonies at 27°C, and they died out soon after being transferred to 37°C. 33°C was the common permissive temperature for the growth of all cell clones examined. Recently we checked the growth of primary cultured goldfish cells at 27°C and 37°C, and found that they could grow at a wide range of temperatures (like R3727 cells) for a long time, irrespective of the temperature used for primary culture (Sato *et al.* unpublished). So it appears that goldfish cells can originally grow at a wide range of temperatures, and long subcultivation at fixed temperatures may result in the selection of stenothermic clones. Cell fusion experiments suggested that the R27 cells failed to grow at a higher temperature (37°C),

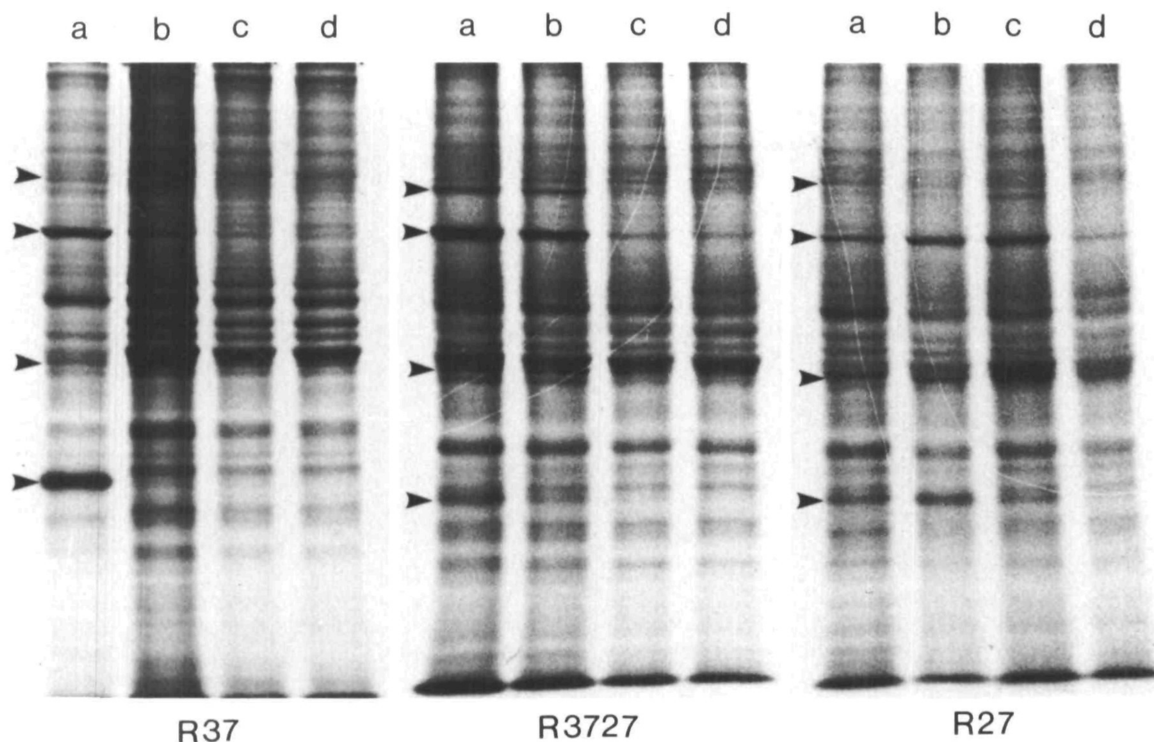


Fig. 4. The induction of heat-shock proteins in RBCF-1 clones. Before the experiments, R27, R3727, and R37 cells were cultured at 27°C, 33°C and 37°C, respectively. After 2 h incubation in methionine-free DMEM, the cells were labelled with ^{35}S for 1 h at various temperatures: a, 40°C; b, 37°C; c, 33°C and d, 27°C. Then cells were washed and harvested. 10^6 cells were suspended in 100 μl of lysis buffer. 5 μl of sample was analyzed on 10% polyacrylamide–SDS slab gels. The arrowheads indicate the four major heat shock proteins, hsp90, hsp70, hsp42 and hsp30.

and R37 cells failed to grow at a lower temperature (27°C), but they were able to complement each other's narrow range of temperature for growth, and the cells become eurythermic (Table 2 and Table 3).

These results prompted us to examine thermosensitivity and induction of hsps in various cell clones. Heat-shock proteins are found in many eukaryotic and prokaryotic cells; their synthesis is induced if the cell is exposed to high temperatures or other forms of stress. The induction of hsps in many organisms so far reported is equally rapid, but the maximum induction temperature varies in correlation with the normal range of environmental exposure. The maximum synthesis of hsps in salmon and trout is observed at about 28°C, in slime molds at 30°C, in sea urchins at 30–32°C, in *Drosophila* at 36–37°C and in chicken fibroblasts at 45°C (Kelley & Schlesinger, 1978; Kothary & Candido, 1981; Lindquist, 1980, 1986). These temperatures for maximum induction of hsps may correlate not with the difference of hsps genes but rather with the optimal growth temperature of cells, since hsp genes introduced into other cells with different optimal growth temperatures could be activated by the heat treatment that could induce the hsps of the recipient cells (Bienz, 1984; Corces *et al.* 1981). Koban *et al.* (1987) reported that in the case of hepatocytes prepared from channel catfish, *Ictalurus punctatus*, acclimatized to various temperatures, the temperature for induction of hsp synthesis is relatively independent of acclimatizion temperature, and that the heat-shock response may operate near a particular set-point temperature, which may be determined by genetic rather than environmental control.

Many reports have been presented in support of the hypothesis that the synthesis of hsps is a crucial element in the acquisition of thermotolerance. The temporal association between the development of thermotolerance and the appearance and disappearance of hsps were known in some mammalian cell lines (Hahn & Li, 1982). Mammalian cell lines selected for increased survival at high temperature produced hsps constitutively (Li & Werb, 1982). Cultured rainbow trout cells synthesized the major heat-shock proteins when the incubation temperature was shifted from 22°C to 28°C, and developed tolerance to 32°C (Mosser & Bols, 1989). However, some results suggest that the synthesis of hsps may not be a requirement for the development of thermotolerance (Aujume & Firko, 1988; Widelitz *et al.* 1984). The question of whether hsps affect cell growth in eurythermic animals like goldfish should contribute to the understanding of the mechanisms of adaptation to the thermal environment.

Fig. 3 shows that R27 cells were the most sensitive to the lethal effects of heat treatment, while R37 cells were the most resistant. But this difference may be attributed to the difference in the incubation temperature, since in R3727 cells the incubation temperatures before the heat treatments affected the thermosensitivity of cells (Fig. 3). The temperature that induced marked synthesis

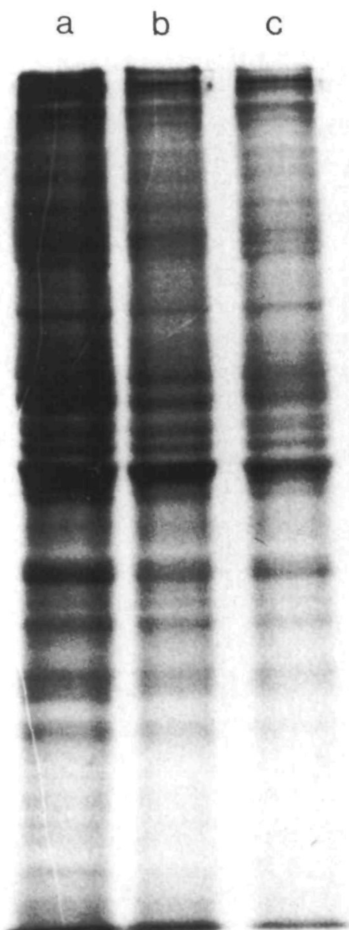


Fig. 5. The protein synthesis of R3727 cells incubated at various temperatures for 12 h. The cells were incubated at a, 37°C; b, 33°C and c, 27°C, and then the cells were harvested and transferred into the lysis buffer (10^4 cells μl^{-1}).

of hsps was 33°C for R27, 37°C for R3727, and 40°C for R37. The temperatures that induced some hsps (hsp70 and hsp30) may correlate with the incubation temperature before heat treatment, which could modify the thermosensitivity of cells. In R3727 cells, the hsps were induced when the cells were transferred from 33°C to 37°C. But once R3727 cells were transferred to and kept at 37°C for 12 h, marked synthesis of hsps was no longer observed, as in the case of R3727 cells incubated at 33°C or 27°C. If the hsps cause thermotolerance in R3727 cells, it would be expected that the hsps would accumulate in cells cultured at 37°C for 12 h. We are now trying to determine the amounts of hsps in cells at various temperatures by Western blotting analysis.

Goldfish cells can be cultured easily and grow rapidly. Various clones which grow at different temperature ranges could be obtained as reported here. We believe that they are very useful tools for investigating the evolutionary change from poikilothermy to homeothermy, and also the mechanisms of eurythermic and stenothermic properties in vertebrates from a molecular aspect. Their wide range of growth temperatures also helps us to examine the biological effect of temperature on vertebrate cells, such as induction and regulation of hsps.

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