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Culture Shock

Synthesis of Heat-shock-like Proteins in Fresh Primary Cell Cultures

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The isolation of *Xenopus* liver, lung and testis cells by collagenase digestion of the tissue, followed by Percoll density gradient centrifugation, was characterized by the preferential synthesis of two proteins whose size and charge were similar to 70 and 85 kD heat-shock proteins. The synthesis of these two heat-shock-like proteins, relative to that of total protein, declined gradually in the first 3–4 days after the cells were plated out for primary culture. In fresh primary cultures of liver parenchymal cells albumin mRNA concentration declined rapidly and plateaued at 3–4 days of culture. Freshly isolated male *Xenopus* hepatocytes were refractory to induction by estrogen of vitellogenin gene transcription but they reacquired hormonal response during the first 3 days of culture. Both of these differentiated phenotypic functions of the *Xenopus* hepatocytes were quantitatively associated with the decline in synthesis of hsp-like proteins in freshly prepared primary cell cultures. Freshly isolated or heat-shocked hepatocytes exhibited a rounded shape with little intercellular contacts, whereas during the recovery period of 3 days they acquired a flattened shape with a high degree of intercellular and cell–substratum interaction. These results present the first evidence for the preferential synthesis of heat-shock-like proteins by procedures for establishing primary cell cultures. They emphasize the necessity of monitoring normal and heat-shock protein synthesis and cell morphology before using primary cell cultures for studying normal regulatory and developmental processes.

A variety of cellular stresses, especially thermal, but also changes in pH, nutrient deprivation or exposure to toxic chemicals, induce the synthesis of a typical set of proteins, best defined as heat-shock proteins (hsp's). Their induction is characteristically accompanied by an abrupt cessation of much of the cell's normal RNA and protein synthesis and a reorganization of cytoskeletal elements (see refs [1, 2] for reviews). While studying the de novo activation of vitellogenin genes by estrogen in primary cultures of *Xenopus* hepatocytes [3, 4], we noticed that freshly isolated hepatocytes were refractory to hormonal stimulation and that the acquisition of full hormonal responsiveness took 3 days in culture. As heat-shock itself led to a transient paralysis of hormonal responsiveness [5], we investigated the possibility that hsp's may be formed during preparation of cells for primary culture and that these newly synthesized proteins may be associated with the early refractory period. Here we report that hsp-like proteins are synthesized in *Xenopus* hepatocytes during the setting up of primary cultures. The preferential synthesis of these proteins was correlated with a rapid disappearance of albumin mRNA from the cells during the first 4 days in culture. Simultaneously, the response of the hepatocytes to estrogen, as determined by the de novo accumulation of vitellogenin gene transcripts, is progressively ac-

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quired as the synthesis of these stress or culture-shock proteins declines during the first 3 days of culture. The onset and decline of the newly-synthesized hsp-like proteins, the loss of albumin mRNA and the acquisition of hormonal response are also paralleled by a significant alteration in cellular morphology, intercellular aggregation and attachment to substratum.

MATERIAL AND METHODS

Cell Culture

The preparation of purified parenchymal cells from *Xenopus laevis* by collagenase disruption of the liver followed by Percoll density gradient centrifugation was as described earlier [4]. The hepatocytes were cultured in 33 mm-diameter plastic Petri dishes in Wolf & Quimby's medium at a density of 2.3×10^5 cells/cm^2.

Hybridization and Quantification of Vitellogenin and Albumin Messenger RNA

Total RNA was extracted from cultured hepatocytes by the LiCl/urea procedure as previously described [4]. Poly(A)-enriched RNA to be used as mRNA standard for disc hybridization assays was prepared from the livers of hormonally untreated male *Xenopus* (for albumin mRNA) or from female *Xenopus* treated with estrogen in vivo (for vitellogenin mRNA), as described by us earlier [3].

Disc hybridization assays for *Xenopus* vitellogenin or albumin mRNA were performed with aminothiophenol paper as previously described [3], except that the hybridization probe used for vitellogenin mRNA estimation was an equal mixture of plasmids pXlvc 10, pXlvc 18, pXlvc 19, pXlvc 23 containing vitellogenin cDNA inserts corresponding to the four expressed *Xenopus* vitellogenin genes, obtained originally from Professor W. Wahli, University of Lausanne, Switzerland. For albumin mRNA determination the plasmid pXlalb 7420 containing cDNA insert corresponding to the 74 kD *Xenopus* albumin gene was constructed in our laboratory. The plasmid DNA was linearized and labelled with ^{32}P by nick-translation with [α-^{32}P]dCTP and [α-^{32}P]TTP (2000–3000 Ci/mmole) to a specific activity of $5–50\times10^7$ cpm/μg. Standard curves were constructed for each disc hybridization assay with purified vitellogenin or albumin mRNA standards, diluted appropriately with yeast tRNA. Hybridization and washing conditions were as previously described [3]. The values were expressed as parts of albumin or vitellogenin mRNA per million parts of total RNA (ppm). All work with recombinant DNA was carried out under the safety requirements and guidelines recommended by GMAG (Genetic Manipulation Advisory Group, UK).

Radioactive Labelling of Proteins Synthesized in Culture

In order to determine the pattern of proteins synthesized by the cultured hepatocytes and the appearance of hsp-like proteins, the culture medium was replaced with fresh medium containing 30 μCi of [^{35}S]methionine (1150–1300 Ci/mmole) for the 12 h of incubation at the time and temperature indicated. At the end of the labelling period the culture medium was removed, the cells rinsed in phosphate-buffered saline (PBS) and homogenized in 0.1% SDS. Incorporation of [^{35}S]methionine into hot trichloroacetic acid (TCA)-insoluble material was determined on duplicate 10 μl samples of the cell homogenate and equal amounts of acid-insoluble radioactivity or protein were analysed by SDS-10% PAGE on slab gels [6]. Protein concentration was determined by the method of Bradford [7]. Gels were fluorographed according to Bonner & Laskey [8], using Fuji RX X-ray film at −70°C. The density of the film image was measured using a Joyce-Loebl microdensitometer in order to determine the relative distribution of radioactivity in the different protein bands.

Morphological Examination

The effect of culture stress and heat-shock on the morphology of primary cultures of *Xenopus* hepatocytes was monitored at different stages of culture and at different temperatures by scanning electron microscopy (SEM). The cells were fixed on the culture dishes for 1 h in 1% glutaraldehyde in

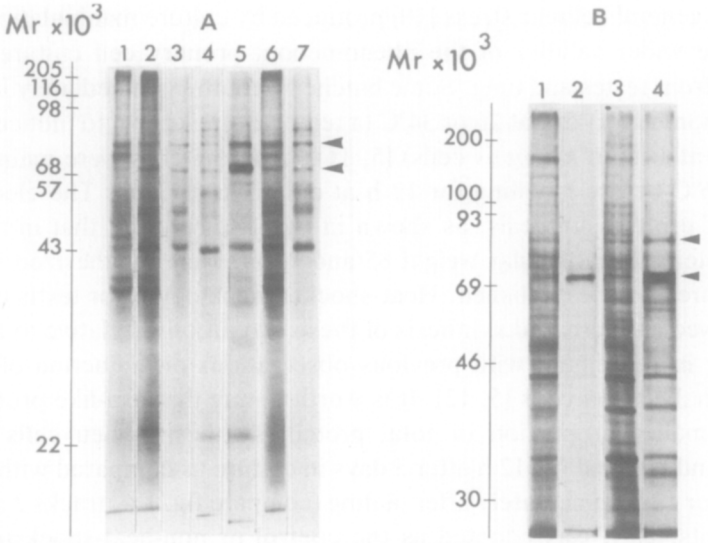

Fig. 1. Synthesis of heat-shock-like proteins in primary cultures of cells derived from *Xenopus* liver, testes and lung. The different tissues were processed at 26°C in the same way as for obtaining primary cultures of hepatocytes and plated at a density of 2×10^5 cells/cm^2 in 33-mm cultures dishes. Immediately after plating, some batches of cells were incubated for 12 h with 30 μCi [^{35}S]methionine per dish in 2 ml Wolf & Quimby medium, either at the normal temperature of 26°C or the heat-shock temperature of 34°C. Other batches of cells were maintained at 26°C for 3 days before labelling with [^{35}S]methionine. At the end of the 12-h labelling period, the cells were homogenized in 0.1% SDS, total TCA-precipitable radioactivity determined and pattern of proteins synthesized analysed by 10% SDS-PAGE, followed by fluorography [5, 24]. The figure illustrates typical fluorograms of proteins synthesized by different cells. *1*, Hepatocytes labelled at 26°C, 12 h after plating; *2*, hepatocytes labelled at 26°C, 72–84 h after plating; *3*, testes cells labelled at 26°C, 0–12 h after plating; *4*, lung cells labelled at 26°C, 0–12 h after plating; *5*, hepatocytes labelled at 34°C, 0–12 h after plating; *6*, hepatocytes labelled at 34°C, 72–84 h after plating; *7*, testes cells labelled at 34°C, 0–12 h after plating. Equal amounts of proteins were loaded on each track. Arrows indicate position of the two major heat-shock proteins of M_r=70 and 85 kD. (*B*) Synthesis of heat-shock-like proteins in primary cultures of *Xenopus* hepatocytes at 20°C. All procedures as in (*A*) for *Xenopus* hepatocytes, except that all cells were isolated and maintained at 20°C. *1*, Hepatocytes labelled at 20°C, 0–12 h after plating; *2*, hepatocytes labelled at 34°C, 0–12 h after plating; *3*, hepatocytes labelled at 20°C, 48–60 h after plating; *4*, hepatocytes labelled at 34°C, 48–60 h after plating. *Arrows* indicate position of the two major heat-shock proteins of M_r 70 and 85 kD.

Barth X medium, pH 7.4 and washed in Barth X containing penicillin and streptomycin, before dehydration in increasing concentrations of ethanol and critical point-drying [9]. Specimens were then gold-coated, visualized and photographed in a Jeol JSM-35 CF SEM.

RESULTS AND DISCUSSION

Synthesis of Heat-shock-like Proteins at the Early Stages of Primary Culture

The nature of proteins synthesized in freshly prepared and heat-shocked hepatocyte cultures was analysed by gel electrophoresis with particular emphasis on heat-shock proteins, since their presence in the freshly prepared cells would

indicate a general cellular stress [10] produced by culture manipulations. In order to test the wider validity of the phenomenon, primary cell cultures were also prepared from testes and lung. Some batches were then immediately labelled with [^{35}S]methionine for 12 h at 26 or 34°C (a temperature known to induce heat-shock protein synthesis in *Xenopus* cells) [5, 11]. Other batches were maintained for 3 days at 26°C before labelling for 12 h at either 26 or 34°C. The electrophoretic pattern of protein synthesis, as shown in fig. 1*A*, revealed that in all instances major proteins of molecular weight 85 and 70 kD were synthesized immediately after cultures were established. Heat-shocked hepatocytes or testis cell preparations showed an increased synthesis of these two proteins relative to that at 26°C, this being in agreement with previous observations on induction of heat-shock proteins in *Xenopus* cells [5, 12]. It is worth noting that hsp-like proteins constituted a smaller proportion of total protein synthesis when cells were heat-shocked and labelled for 12 h after 3 days in culture as compared with those heat-shocked for 12 h immediately after plating (compare fig. 1*A*, tracks *1* and *6*). In all experiments, 26°C was adopted as the control or non-heat-shock temperature, since earlier work has shown that there is no preferential synthesis of hsp's at this temperature in primary cultures of *Xenopus* hepatocytes, cell lines or oocytes [5, 11, 12]. Nevertheless, in order to eliminate the possibility that 26°C might constitute a mild thermal shock, we carried out all procedures, from isolation of hepatocytes to their maintenance and labelling with [^{35}S]methionine, at 20°C. As shown in fig. 1*B*, there was no difference in the labelling of the 70 and 85 kD hsp-like proteins, of hepatocytes at 20 and 26°C, thus confirming that a mild thermal shock is not contributing to the results obtained at 26°C. Cells from other tissues also behaved similarly. Two-dimensional gel analysis confirmed that the 70 kD protein is identical in the case of freshly prepared and heat-shocked hepatocytes (fig. 2) and that this 70 kD protein resembles that of a similar size and charge previously identified in heat-shocked *Xenopus* oocytes [11]. Similar results were obtained with heart and lung cells at the onset of the culture period (results not shown). By monitoring the synthesis of hsp-like proteins in hepatocyte cultures maintained for 3 days, a decline in synthesis of the 85 kD protein was quite noticeable, which coincided with the enhanced competence of these cells to respond to estrogen, as described below.

Albumin mRNA and Inducibility of Vitellogenin mRNA in Freshly Prepared Primary Cultures

Many cellular, metabolic and developmental activities are known to be disrupted coincidental with the appearance of newly-synthesized hsp's produced by thermal shock and a variety of stresses [1, 2]. Since our laboratory is interested in the expression of albumin and estrogen-induced vitellogenin genes in cultured *Xenopus* hepatocytes [3, 4, 5, 13], it was important for us to establish what would be the consequence on this function of the formation of large amounts of hsp-like proteins and their subsequent decline in the first days of freshly prepared cultures

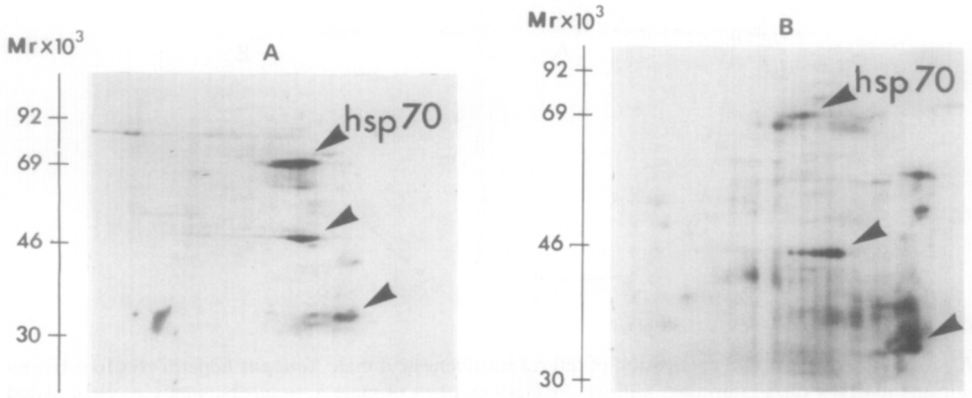

Fig. 2. Synthesis of hsp-like proteins in fresh primary cultures of hepatocytes maintained at the normal (26°C) and heat-shock (34°C) temperatures, as revealed by two-dimensional gel electrophoretic analysis of labelled proteins. Male *Xenopus* hepatocytes were labelled with 30 μCi [^{35}S]methionine per dish for 12 h immediately following plating and maintained at either 26°C or 34°C as described in fig. 1. TCA-precipitable ^{35}S-labelled proteins were resolved by isoelectric focussing between pH 3.5 and pH 10, equilibration in 10% glycerol–5% β-mercaptoethanol–2.3% SDS–62.5 mM Tris, pH 6.8 before electrophoresis on 10% SDS–polyacrylamide slab gels in the second dimension [24] and fluorography [24]. (*A*) Hepatocytes labelled with [^{35}S]methionine for 12 h at 34°C; (*B*) hepatocytes labelled for 12 h at 26°C. The position of hsp 70 is marked, while the arrows denote positions of other identical proteins in both samples. Equal amounts of proteins were loaded on the gels. Note that fewer proteins are labelled in heat-shocked cells.

of these cells. For this purpose, primary cultures of adult male *Xenopus* hepatocytes were prepared and maintained in serum-free Wolf & Quimby culture medium at 26°C [4]. In some experiments, RNA was extracted from the cells and the albumin mRNA concentration measured by hybridization to cloned *Xenopus* albumin cDNA. In other experiments, batches of cells were exposed to 10^{-6} M estradiol for successive periods of 8 h immediately after they were plated out at the end of which the quantity of vitellogenin mRNA induced during this period was determined by hybridization to ^{32}P-nick translated cloned *Xenopus* vitellogenin cDNA containing plasmids [3]. As shown in fig. 3*A*, albumin mRNA, which is the most abundant messenger in liver parenchymal cells, started disappearing rapidly as soon as the cells were plated out. The half-life of the messenger was calculated to be ~8 h and the decay plateaued out at 3 days of culture. This result is reminiscent of the finding of Clayton & Darnell [14] of the rapid loss of the bulk of liver mRNA, including albumin mRNA, in primary cultures of rat hepatocytes.

Accompanying the disappearance of the albumin mRNA in an almost reciprocal fashion was the loss of responsiveness to estrogen of the freshly prepared cultures. We know from previous studies that while vitellogenin gene transcription is readily induced by the hormone in the male *Xenopus* liver in vivo, there is a refractory period of hormonal response in the first 3 days of setting up primary hepatocyte cultures [3, 4]. It is therefore significant, as shown in fig. 3*B*, that the

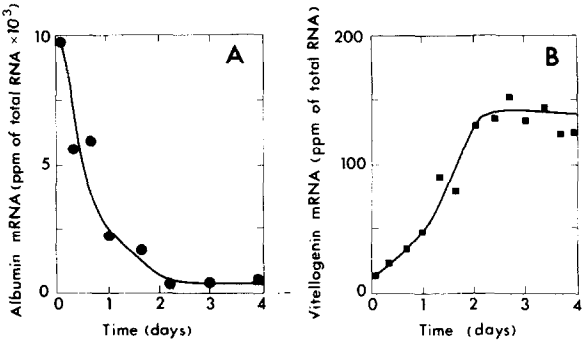

Fig. 3. Refractoriness and acquisition of full responsiveness of male *Xenopus* hepatocytes to estrogen during the first 4 days in primary culture. Primary cultures of male *Xenopus* hepatocytes were plated on uncoated plastic dishes at a density of 2×10^5 cells per 33-mm dish in 2 ml serum-free Wolf & Quimby medium, and maintained in culture at 26°C. (*A*) Albumin mRNA concentration in total cellular RNA was quantitated by disc filter hybridization to ^{32}P-nick-translated plasmid containing *Xenopus* albumin cDNA corresponding to 74 kD albumin [26]. (*B*) Immediately after plating out (time 0) and over the next 4 days, the culture medium of different batches of cells was supplemented with 10^{-6} M estradiol-17β. Eight hours after exposure to the hormone, RNA was extracted from the cells and 10 μg aliquots were found to aminothiophenol paper discs in triplicate [9]. Vitellogenin mRNA was quantitated by disc filter hybridization to ^{32}P-nick-translated plasmids containing *Xenopus* vitellogenin cDNA inserts.

amount of vitellogenin mRNA induced immediately after culture is very low but during the following 4 days the capacity of the cells to accumulate vitellogenin mRNA increased tenfold. We also know from our earlier studies [3, 4] that the metabolism of estradiol remains unchanged during this period, a single dose of 10^{-6} M estradiol being adequate to maintain vitellogenin gene transcription for at

Table 1. *The relationship between hsp synthesis and vitellogenic response of Xenopus hepatocyte cultures to estrogen during and upon recovery from heat shock*

Heat shock period (hours at 34°C)	Recovery period (hours at 26°C)	Hsp synthesized (% total protein)	Response to E_2 (ppm Vg mRNA)
–	–	<2	140
12	–	19	0
12	12	13	10
12	24	7	40
12	36	3	100

Male *Xenopus* hepatocytes were cultured for 5 days at 26°C. Batches of cells were then heat-shocked for 12 h at 34°C, before being returned to 26°C for varying periods of time. Cells were labelled with [^{35}S]methionine for the last 12 h of each of the incubation periods to determine the extent of synthesis of proteins appearing as hsp 70 and 85 (determined by scanning fluorograms as shown in fig. 1). The response to estrogen (E_2) was quantitated as described in fig. 3 as the amount of vitellogenin (Vg) mRNA accumulating during an 8 h period following the addition of 10^{-6} M estradiol immediately after the heat shock or recovery periods.

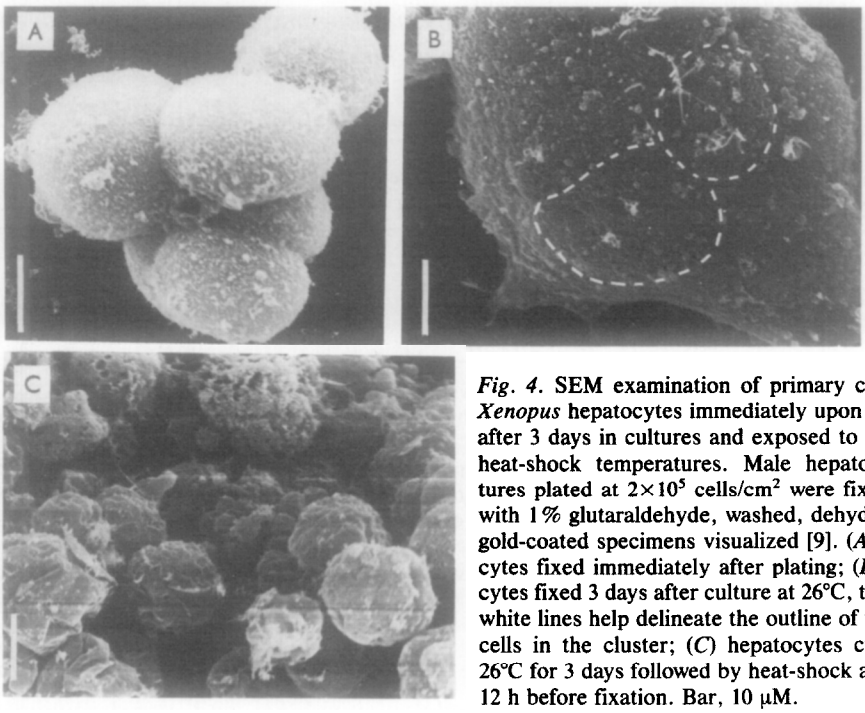

Fig. 4. SEM examination of primary cultures of *Xenopus* hepatocytes immediately upon plating or after 3 days in cultures and exposed to normal or heat-shock temperatures. Male hepatocyte cultures plated at 2×10^5 cells/cm^2 were fixed in situ with 1% glutaraldehyde, washed, dehydrated and gold-coated specimens visualized [9]. (*A*) Hepatocytes fixed immediately after plating; (*B*) hepatocytes fixed 3 days after culture at 26°C, the broken white lines help delineate the outline of two of the cells in the cluster; (*C*) hepatocytes cultured at 26°C for 3 days followed by heat-shock at 34°C for 12 h before fixation. Bar, 10 μM.

least 12 h. Re-exposure of cells to estradiol 24 h after the first addition also did not enhance the accumulation of vitellogenin mRNA (results not shown), so that the 'memory effect' [3, 13] does not appear to operate during this early period in culture. In order to verify that functionally the hsp-like proteins produced upon culture shock have a similar effect as that produced by heat shock, we subjected some batches of cells to thermal shock. From other studies in our laboratory [5], we know that short periods of heat shock cause a transient paralysis of activation by estrogen of vitellogenin genes in primary cultures of *Xenopus* hepatocytes. It is therefore significant, as shown in table 1, that the loss of responsiveness upon culture shock in terms of vitellogenin mRNA accumulation is comparable to that described after heat shock. The continuing synthesis of small amounts of hsp's during the recovery from heat shock is known not to interfere with hormone responsiveness [5].

Morphological Studies

Simultaneously to the above biochemical experiments, scanning electron microscopic examination of the cells was carried out over a period of the first few days after setting up the cultures. It showed that, when initially plated out, the cells are dispersed, rounded, exhibit little cell–cell interaction and have no obvious attachment to the surface of the culture dish (fig. 4*A*). During the

Table 2. *Association between hsp synthesis, vitellogenic response to estrogen and morphology of Xenopus hepatocytes, as a function of time and temperature of culture*

Culture conditions (days/temperature °C)	Hsp synthesized (% total protein)	Response to E_2 (ppm Vg mRNA)	Cell morphology (roundedness/ aggregation)
0/26	9.1	20	+++/−
1/26	4.0	50	++/+
3/26	2.5	140	−/+++
3/26→34	13.0	0	+++/−

Male *Xenopus* hepatocytes, freshly prepared (0 days in culture) or cultured for 1 or 3 days at 26°C were labelled with [^{35}S]methionine for the last 12 h in order to measure the extent of synthesis of proteins appearing as hsp 70 and 85 (determined by scanning fluorograms as shown in fig. 1). In the batch of cells indicated at the bottom of table 2, hsp's were induced by transferring the cells after 3 days in culture at 26–34°C for the 12-h labelling period at the end. The response to estrogen (E_2) was quantitated as described in fig. 3 as the amount of vitellogenin (Vg) mRNA accumulating during an 8-h period following the addition of 10^{-6} M estradiol at 0, 1 or 3 days in culture. Cell morphology was semi-quantitatively expressed as the proportion of cells seen by scanning electron microscopy (see fig. 4) on a relative scale of virtually all cells (+++) being rounded and disaggregated or being flattened and aggregated (−).

following 3 days the cells reform multicellular complexes and reveal obvious attachments to the substratum such that it becomes difficult to discern individual cells (fig. 4*B*). The similarity between the rapid loss of albumin mRNA or the refractory period of estradiol responsiveness after culture preparation and that after heat shock of the *Xenopus* hepatocyte cultures (table 1, fig. 3) led us to examine cellular morphology of primary cultures first maintained at 26°C for 3 days before heat shock at 34°C. Following transfer to 34°C for 12 h the cells become rounded again and lose their intracellular contacts (fig. 4*C*). Maintenance of these heat-shocked cells at 26°C for several days led to re-acquisition of the morphology shown in fig. 4*B*, which paralleled the recovery from heat shock of the transcription of vitellogenin genes induced by estrogen. The latter phenomenon has been described by us elsewhere [5].

GENERAL COMMENTS

Primary cultures of cells isolated from embryonic or adult tissues are increasingly used to study expression or maintenance of differentiated functions [15]. A problem often encountered is the refractory period seen following the isolation of the cells when they fail to respond to various stimuli [4, 5, 16, 17]. The observation that the majority of liver specific mRNAs disappear rapidly during the early stages of primary culture of rat hepatocytes is particularly relevant [14]. Although various explanations have been proposed for this failure to maintain or express differentiated function in fresh culture, such as loss of hormones or growth

factors [18], nutrient deprivation [19], loss of cell–cell contact [20], or the build-up of toxic waste products [21], no definitive biochemical mechanism is yet available. Our studies show for the first time that preparation of primary cultures leads to the preferential synthesis of hsp-like stress proteins and which may be responsible for the rapid breakdown of the most abundant liver mRNA, i.e. albumin mRNA, and the refractoriness of the cells to elicit a normal response to the hormone. They may also be relevant to the earlier findings of a sharp drop in albumin synthesis and secretion by freshly prepared organ cultures of *Xenopus* liver [22]. These studies also show that cell–cell interactions may be critical for the retention of the normal differentiated function of these cells. The possible association between the synthesis of hsp-like proteins and changes in cell morphology at the onset of culture and the temporary loss of hormonal response of *Xenopus* hepatocytes are summarized in table 2.

Although our experiments have demonstrated the preferential synthesis of hsp-like proteins after isolation of cells and during the early stages of their culture, we also considered the possibility that to some extent these proteins were normal cellular products in the whole animals. Attempts were therefore made to label these proteins in *Xenopus* liver in vivo, but the unavoidable stress applied to the animal during this procedure, the complex dynamics of amino acid uptake and the heterogeneous cell population in the organ vitiated the measurement of a possible basal level of hsp synthesis in vivo.

What exactly is responsible for the appearance of newly-synthesized stress or culture-shock proteins upon setting up cells in primary culture is not known but, since in our studies the *Xenopus* hepatocytes recovered full responsiveness to estradiol as determined by the accumulation of in vivo levels of vitellogenin mRNA [3], it is unlikely that nutritional and hormonal deprivation or toxic waste product build-up are the causal agents. It is more likely that damage to intercellular and intracellular architecture caused by disaggregation or some other manipulation of cells is responsible and that cell–cell contact and cell–substratum interactions may be the important factors underlying the loss of mRNA and refractoriness to hormonal stimuli, followed by the subsequent gradual recovery of response to external regulatory signals [14, 23]. Assuming that it is heat-shock proteins that are actually responsible for the initial loss of phenotypic functions, we do not know how these ubiquitously encountered stress proteins bring about their effects. Whatever the causal mechanisms, our findings demonstrate that monitoring the synthesis of hsp-like stress proteins induced by culture shock would allow the determination of optimum conditions of isolation and culture, so that primary cell cultures can be more effectively used as models for exploring regulatory and developmental processes.

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