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Alterations Of Gene Expression Induced By Temperature And Thyroid Hormone In Amphibian Cell Cultures

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ALTERATIONS OF GENE EXPRESSION INDUCED BY TEMPERATURE AND THYROID HORMONE IN AMPHIBIAN CELL CULTURES

by

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Submitted in partial fulfilment
of the requirements for the degree of
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ABSTRACT

The epidermis of the American bullfrog, Rana catesbeiana, consists of a simple 2-3 cell layer epithelial covering for most of its larval life. During this time, larvae are exposed to a variety of exogenous and endogenous influences which may alter the expression of epidermal cell genes. In order to study exogenous influences and to circumvent the possible influences of tissue-tissue interactions on the gene expression of epidermal cells, a method was developed for culturing epidermal cells from larval R. catesbeiana. This study examined the effect(s) of brief fluctuations in temperature and the influence of thyroid hormone (T_3 (triiodothyronine); a known inducer of precocious differentiation in situ) on protein synthesis in primary epidermal cell cultures from R. catesbeiana larvae.

Primary epidermal cell cultures from wholly larval (tail fin) and adult (hind limb) organs were incubated briefly at temperatures between 22 and 36.5°C. The types of proteins synthesized by epidermal cell cultures from either tissue were indistinguishable at these temperatures. However, at temperatures exceeding 22°C (32, 34 and 36.5°C, specifically) epidermal cell cultures depressed their synthesis of 'control' proteins and synthesized a novel heat shock protein (HSP) of 65 kd (pI 6.7-6.8); synthesis of an additional HSP (M_r=25 kd) was

detected only at 36.5°C. The synthesis of these HSPs is transient as control protein synthesis is virtually restored 4 hr after returning the cultures to the control (22°C) temperature. The results indicate that these cells respond to fluctuations in temperature by the depressed synthesis of proteins normally made at 22°C and the new and/or enhanced non-coordinate synthesis of HSPs.

The effect of T_3 on the water-insoluble proteins synthesized by epidermal cell cultures was examined. Hind limb epidermal cell cultures maintained in the presence of T_3 (3×10^{-10} moles/mL) for 36 hours were found to synthesize water-insoluble proteins which corresponded closely in M_r and pI to keratins typical of stratification and/or keratinization; synthesis of these proteins did not occur at 36 hr when culture medium lacked T_3. Many of the water-insoluble proteins extracted from hind limb epidermal cell cultures are precipitable with rabbit anti-human cytokeratin antibodies. The results suggest that T_3 promotes a precocious induction of certain water-insoluble proteins in hind limb epidermal cell cultures and that these proteins have immunochemical properties similar to mammalian keratins.

The presence of T_3 in the culture medium did not induce changes in the water-insoluble proteins synthesized by primary tail fin epidermal cell cultures after 36 hr or 5 days. These results suggest that longer exposure to, or greater concentration of T_3 - either of which are

required *in situ* by organs that regress at metamorphic climax - may be required to elicit a differentiative (in this case, degenerative) effect on the type of water-insoluble proteins synthesized by tail fin epidermal cells or that T_3 may not induce any changes in the water-insoluble proteins synthesized by tissues destined for degeneration.

Treatment of larval *R. catesbeiana* *in situ* with T_3 (3×10^{-10} moles/ gram body weight) demonstrates that the water-insoluble proteins synthesized by hind limb epidermal cells from T_3-treated and control larvae are the same but different from those induced by T_3 in cultured epidermal cells. The discrepency may be due to (1) an initial dedifferentiation making cultured cells more responsive to T_3 or (2) the effects of T_3 may be more evident in the absence of any tissue-tissue interactions.

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NOMENCLATURE

°C	degrees Celsius
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
F.C.S.	fetal calf serum
FITC	fluorescein isothiocyanate
HSP	heat shock protein
IEF	isolectric focusing
IEF-SDS-PAGE	isoelectric focusing in the first dimension followed by polyacrylamide gel electrophoresis in the presence of SDS in the second dimension
IgG	immunoglobulin G
kd	kilodalton
KSBA	keratin protein extraction buffer
M_r	relative molecular mass
MEM	minimal essential medium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
PPO	2,5-diphenyloxazole
RIPA	radioimmune precipitation buffer
RNA	ribonucleic acid
SBA	protein extraction buffer
SDS	sodium dodecyl sulfate
T_3	3,3',5-triiodothyronine
T_4	thyroxine
TRIS	Tris(hydroxymethyl) aminomethane

XKEC Xenopus laevis kidney epithelial cells

PART 1

INTRODUCTION

1.1 General Introduction

In anurans, metamorphosis is a complex postembryonic process during which organs required for adult life become functional while larval organs regress (reviewed by Frieden and Just, 1970, Dodd and Dodd, 1976; see also Metamorphosis: A Problem in Developmental Biology, Second Edition, 1981). The process of metamorphosis is initiated by endogenous elevation of thyroid hormone. Exogenous thyroid hormone introduced to the larvae by injection, immersion or hormone-containing implants will precipitate precocious metamorphosis. In larvae of the American bullfrog, Rana catesbeiana, approximately one year is necessary to complete natural metamorphosis while only 10-14 days are required to complete the process if metamorphosis is induced.

The natural habitat of R. catesbeiana is widespread in North America, extending from Canada to the southern United States. During most of the larval period the epidermis, which exists as a relatively simple, two-cell layered epithelium, is subjected to the rigors of the external environment, not the least of which is

temperature. Epidermis which will eventually be deemed useless at metamorphic climax (tail) must survive stresses such as temperature with the same effectiveness as epidermis which will proliferate (hind limb).

Once this natural hurdle provided by the external environment is conquered, larval R. catesbeiana must respond appropriately to changes in its internal environment caused by elevation of endogenous thyroid hormone levels. The animal's tissues respond in a truly remarkable and precice way to programmed cell death and cell proliferation signaled by thyroid hormone. For example, tissue destruction proceeds from the distal end of the tail and ceases at its base while the neighboring body epidermis responds by acquiring differentiation specific epidermal changes (stratification and keratinization) that equip R. catesbeiana for a terrestrial life (reviewed by Fox, 1975).

While there are inherent drawbacks in the use of primary epidermal cell cultures from Rana catesbeiana, rare opportunities are also provided to the researcher. Cell cultures from amphibians are notoriously fastidious (Rafferty, 1976). Those from R. catesbeiana are no exception. Furthermore, obtaining and maintaining antiseptic conditions are difficult because of the propensity of parasites within these animals (Rafferty, 1976; Freed and Mezger-Freed, 1970).

Epidermal cell cultures from larvae can be exposed to

the extremes of temperature which are a natural part of its habitat. By examining the total proteins synthesized in vitro, subsequent to brief incubations at sub- and supraoptimal temperatures, one can determine if the epidermal cells have altered their gene expression relative to cultures maintained at the 'control' temperature. The recent interest in the acquisition of thermotolerance makes such questions pertinent. Thermotolerance is particularly relevent to an organism that can neither remove itself from its environment when it proves stressful nor regulate its body temperature.

Larval epidermal cultures also permit the manipulation of the extracellular growth environment. With the removal of tissue-tissue interactions, the response of epidermal cells specifically to exogenous thyroid hormone can be determined. Also, by culturing epidermal cells from animals in different stages of development, in the presence of thyroid hormone, the ability of the cells to respond to thyroid hormone can be assessed. The epidermal-specific keratins are a family of highly conserved, water-insoluble proteins which are differentially expressed during development. Their extraction and analysis provides a method of detecting the response and relative maturity that thyroid hormone induces in epidermal cells in vitro.

The introductory chapter will briefly review the current literature which discusses induction of new gene

expression by environmental stress. The regulation of the stress-responsive genes and the acquisition of thermotolerance by exposure to environmental stress will also be considered. The response of larval epidermis to natural and thyroid hormone induced metamorphosis will be discussed in the second aspect of the introduction. Finally, the structural properties of keratins and their differential expression during development and in epidermal cell cultures will be reviewed.

1.2 The Expression of Novel Proteins in Response to Heat and Other Stresses

1.2.1 The Induction of the Heat Shock Response

In response to a brief incubation at an elevated temperature, cells from an organism respond by the new and/or enhanced synthesis of a particular group of proteins, the heat shock proteins (reviewed by Ashburner and Bonner, 1979). The expression of the complete complement of heat shock proteins (HSPs) is not, however, necessarily coordinate (Lewis et al., 1975; Mirault et al., 1978; Lindquist et al., 1982; Ketola-Pirie and Atkinson, 1983; Atkinson and Dean, 1985; Dean and Atkinson, 1985). Schlesinger and colleagues (1982) have shown that HSPs from many organisms are not only similar in molecular mass (M_r) and isoelectric point (pI) but are also immunologically related. Antibodies directed

against HSP70 and HSP89 from heat shocked chicken fibroblasts displayed distinct cross-reactivity with proteins of similar M_r from yeast, slime molds, corn seedling roots, worms, frogs, Drosophila, rodents and humans (Schlesinger et al., 1982).

Concomitant with the enhanced synthesis of this select group of proteins (HSPs) is a generalized depression in the synthesis of 'control' proteins (Ashburner and Bonner, 1979; Atkinson, 1981a; Findly and Pedersen, 1981; Baszczynski et al., 1982; Ketola-Pirie and Atkinson, 1983). Furthermore, following temperature elevation, cells from an organ kept in tissue culture have been shown to respond by synthesizing more HSPs than are observed when the organ is maintained in situ (Hightower and White, 1981; Landry et al., 1982; Kim et al., 1983). It is generally agreed that this response to heat is ubiquitous throughout the plant and animal kingdoms. A similar response to heat stress has been demonstrated in prokaryotes (Travers and Mace, 1982; Yamamori et al., 1982; Neidhardt et al., 1982).

Stress provided by sources other than heat can cause changes in protein synthesis. Research in this, and other laboratories, has demonstrated that this response can be elicited in some animal systems by metal ions (Levinson et al., 1979; 1980; Kothary and Candido, 1982; Atkinson et al., 1983; Somerville, 1984; Atkinson and Dean, 1985; Schlesinger, 1985), sodium arsenite

(Schlesinger et al., 1982; Atkinson et al., 1983; Somerville, 1984; Atkinson and Dean, 1985; Heikkila et al., 1985b), amino acid analogs (Kelley and Schlesinger, 1978; Li and Laszlo, 1985), amino acid deprivation (Levinson et al., 1979), anoxia (Valezquez and Lindquist, 1984), ethanol (Li et al., 1982; Rodenhiser et al., 1986), mechanical injury (White, 1980; Currie and White, 1981; White and Currie, 1983; Hightower and White, 1981; Hammond et al., 1982; Heikkila et al., 1985b) and cold (Fink and Zeuthen, 1980; Ketola-Pirie and Atkinson, 1983). Although some environmental insults result in the complete battery of HSPs being synthesized, others fail to activate specific proteins or activate 'stress-specific' proteins.

1.2.2 The Regulation of the Synthesis of Heat Shock Proteins

The regulation of the expression of heat shock proteins is believed to exist at three levels. The first involves the shift in transcription from genes coding for control proteins to those which specify heat shock proteins (reviewed by Ashburner and Bonner, 1979; see also Bonner, 1982; 1985; Lindquist et al., 1982 and Lindquist and DiDomenico, 1985). Regulation also occurs at the levels of RNA processing (Mayrand and Pedersen, 1983; Lindquist and DiDomenico, 1985) and translation of RNAs (Storti et al., 1980; Beinz, 1982; 1984; Beinz and Gurdon, 1982; Kruger and Beneke, 1981; 1982; Ballinger and

Pardue, 1985).

In Drosophila tissue culture cells (Schneider-line 2), transcription of the 70 and 26 kd HSPs is constitutive, albeit at a low level, in non-heat shocked cells (Findly and Pedersen, 1981). Subsequent to temperature elevation, Findly and Pedersen (1981) observed that in Drosophila tissue culture cells the induction of transcription of genes coding for HSPs was asserted quickly and the HSP mRNAs became 'superprevalent' within the nucleus (Findly and Pedersen, 1981).

Heat shock gene regulation also occurs at the level of processing transcribed RNA into functional messages (Lindquist and DiDomenico, 1985). Mayrand and Pedersen (1983) have demonstrated this phenomenon in the Drosophila K_c0 and HeLa cell lines. Under heat shock conditions, the assembly of 'control' heterogeneous nuclear RNA (hnRNA) into heterogeneous ribonucleoprotein (hnRNP) is abortive. They suggested that heat shock favors the processing and/or transport of mRNAs which require little or no modification to be functional and that heat shock mRNAs are among this group. Indeed, it has been verified that all but one of the heat shock proteins of Drosophila do not contain intervening sequences, making splicing an unnecessary modification of transcribed RNA (Holmgren et al., 1979). The HSP83 is the notable exception (Lindquist and DiDomenico, 1985). In Drosophila, this HSP (83kd) is constitutively synthesized, has the lowest

temperature of induction, is not induced to the same degree as the other HSPs at high temperatures (Lindquist and DiDomenico, 1985) and is the last to be repressed after return to control temperatures (Lindquist et al., 1982).

Translational control of protein synthesis has also been observed following temperature elevation. From the pool of newly transcribed HSP-mRNAs and pre-existing mRNAs, translational preference for the mRNAs coding for heat shock genes is displayed. The mRNAs present prior to temperature elevation are not degraded in most systems but remain within the cytoplasm in an non-translatable form (Mirault et al., 1978; Storti et al., 1980; Ballinger and Pardue, 1985). Upon return to the 'control' temperature, the availability of pre-existing 'control' mRNAs allows for the quick resumption of normal protein synthesis (Ballinger and Pardue, 1985). In Drosophila, translation of mRNA in a homologous cell-free translation system derived from heat shocked cells displays translational preference for heat shock mRNAs (Kruger and Beneke, 1982). However, translation in a heterologous system (i.e. the cell-free rabbit reticulocyte lysate system) does not distinguish between heat shock and control mRNAs and translates both with equal efficiency (Storti et al., 1980; Kruger and Beneke, 1982; Ballinger and Pardue, 1985).

Lindquist and her collaborators have extended the

results of the transcriptional shift to heat shock genes and their translation using transcriptional and translational blocks (Lindquist et al., 1982; reviewed by Lindquist and DiDomenico, 1985). They have stated that HSP synthesis is autoregulated in Drosophila, requiring the accumulation of HSPs to inhibit further transcription of HSP genes (Lindquist et al., 1982; Lindquist and DiDomenico, 1985). Upon return to a control environment, HSP70, at the very least, must have attained a certain concentration within the cell to restore normal protein synthesis. However, by repressing the synthesis of HSPs with cycloheximide, they found that translation of pre-existing mRNAs was nonetheless, inhibited. By blocking transcription of HS genes with actinomycin D, thereby repressing HSP-mRNA accumulation and ultimately HSP synthesis, they still observed a translational inhibition of 'control' mRNAs (results are reviewed in Lindquist and DiDomenico, 1985).

Quail red blood cells (RBC's) incubated in the presence of transcriptionally inhibiting concentrations of actinomycin D reveal a slightly different picture (Atkinson and Dean, 1985) than is observed in Drosophila (Lindquist et al.,1982; Lindquist and DiDomenico, 1985). RBC's incubated at a heat shock temperature (45°C) in the presence of 2 µg of actinomycin D/mL of medium did not synthesize HSPs. However, they did synthesize proteins which were indistinguishable from RBC's incubated at

control temperature (37° C) in the presence (2 μg/mL) or absence of actinomycin D (Atkinson and Dean, 1985). The repression of translation of pre-existing mRNA species at heat shock temperatures in the presence of Actinomycin D observed in Drosophila was not apparent in quail RBC's. The observations from quail RBC's, unlike Drosophila, clearly demonstrate that there is a transcriptional requirement for detectable HSP synthesis (Atkinson and Dean, 1985).

Beinz has shown that in Xenopus oocytes, control of HSP synthesis is solely at the level of translation (Beinz, 1982; Beinz and Gurdon, 1982). The HSP70, which was observed following heat treatment, represented an already transcribed mRNA whose translation was repressed at the 'control' temperature. That this mRNA is stored within the cytoplasm in an untranslatable form is verified by transcriptional inhibition. In spite of enucleation and α-amanitin treatment prior to heat shocking the oocytes, the HSP70 was synthesized after temperature elevation. Furthermore, when RNA was isolated from control and heat shocked oocytes and translated in vitro, identical proteins were synthesized. Subsequent studies with cDNA clones confirmed the presence of HSP70 mRNA in non-heat shocked oocytes (Beinz, 1984). Quantitation of HSP70 mRNA reveals no significant increase in its transcripts after heat shock (Beinz, 1984).

1.2.3 Acquisition of the Heat Shock Response during Development

As discussed above, oocytes from Xenopus have the capacity to respond to heat stress in the absence of RNA transcription (Beinz, 1982; Beinz and Gurdon, 1982). However, results from embryonic Xenopus have demonstrated that this ability is transient; during a specific period of embryogenesis, heat shock treatment caused a generalized depression of protein synthesis and ultimately proved lethal to the embryos (Beinz, 1984; Heikkila et al., 1985a; 1985b; Nickells and Browder, 1985). The resumption of heat shock protein synthesis (HSP87, HSP76, HSP70, HSP68, HSP57 and HSP42 (or HSP43)) has been noted in fine-cell blastulae and in all older embryos (Heikkila et al., 1985a; 1985b). In medium-cell blastulae, the response to heat was not consistent and, when observed, fewer HSPs (87, 70 and 68 kd) were synthesized (Heikkila et al., 1985a; 1985b; Nickells and Browder, 1985). More recently, Nickells and Browder (1985) have shown that the animal and vegital poles of the embryo displayed differential synthesis of HSPs. The synthesis of a 35 kd HSP was unique to the cells of the vegital pole which also synthesized greater amounts of the HSP57 and HSP43.

A period of heat sensitivity has also been demonstrated in embryonic sea urchins. From fertilization through to early blastula stages, heat shock caused an overall depression of protein synthesis with no

accompanying HSP synthesis (Giudice, 1985; Heikkila et al., 1985b). The pre-blastula embryos, incapable of recovering from this stress, died (Giudice, 1985; Heikkila et al., 1985b). When 16-cell blastomere embryos were dissociated they acquired the heat shock response at the same time as undissociated controls. These results indicated that, in the sea urchin embryo, the ability to respond to a heat stress is not a function of cell-cell interaction. Rather, the response to heat corresponded to a particular length of time following fertilization (Giudice, 1985). The ability to respond to heat in intact or dissociated embryos occurred at the same time that the embryo synthesizes new mRNAs necessary for development to proceed (Giudice, 1985).

A similar stage-dependent response to heat treatment has been observed in mouse and rabbit embryos (Heikkila et al., 1985b). Embryo stages preceeding blastocyst development did not synthesize detectable HSPs. In vitro translation of mRNAs from heat shocked rabbit blastocysts has demonstrated that there is increased labeling of the HSP70 reflecting an accumulation of HSP70 mRNA. The presence of HSP70 mRNA in heat shocked embryos and its absence from controls has been verified by Northern hybridization (Heikkila et al., 1985b). Similar response to sodium arsenite and mechanical injury have also been elicited from rabbit blastocysts (Heikkila et al., 1985b).

In Drosophila, heat induction of HSP70 and HSP68

synthesis could not be detected prior to the blastoderm stage of embryonic development (Graziosi et al., 1980). However, the HSP84 was observed in unfertilized eggs (as an unlabeled protein) and at all stages in control and heat treated embryos. These results have been verified and extended by Zimmerman, Petri and Meselson (1983). They found that during normal ovarian development in Drosophila, in the absence of any environmental insult, mRNAs coding for the HSP83, HSP28 and HSP26 accumulated. These mRNAs remained abundant in embryos until the blastoderm stage of development. Furthermore, deliberate heat stress of the embryos failed to induce heat shock RNA synthesis. HSP70-mRNA was not detectable at any stage during ovarian development or heat shock of pre-blastoderm embryos.

1.2.4 The Functional Significance of Heat Shock Protein Synthesis

From the results presented it appears that the synthesis of HSPs is correlated with the development of thermotolerance possibly by some form of homeostatic regulation at the cellular level (Heikkila et al., 1985b). Following initial exposure to a heat stress, vertebrates and invertebrates demonstrate an improved tolerance to subsequent heat exposures and to temperatures which would otherwise prove lethal (Dean and Atkinson, 1983; 1985; Velazquez and Lindquist, 1984; Atkinson and Dean, 1985;

Heikkila et al., 1985a; 1985b; Li and Laszlo, 1985; Nickells and Browder, 1985); the acquisition of thermotolerance extends to the improved tolerance of other stresses (Velazquez and Lindquist, 1984). It is the synthesis of particular HSPs, notably the HSP70 and/or HSP68 which have been most strongly implicated in the acquisition of thermotolerance by an organism. This is inferred by the absence of their synthesis and the subsequent lethality of elevated temperature incubation in early embryos from many organisms (as discussed previously).

To demonstrate further the role of the HSP70 in the acquisition of thermotolerance, Velazquez and Lindquist (1984) used monoclonal antibodies directed against the HSP70 of Drosophila. They felt that by determining the intracellular location of the HSP70 with immunofluorescence, a functional correlation between HSP70 synthesis and thermotolerance might be possible. Their results showed that in the presence of an induced stress (heat or anoxia), most of the HSP70 is concentrated within the nucleus and continues to concentrate there for the duration of the stress. Upon return to a control environment, the HSP70 is transported to the cytoplasm at a rate which varies with the length or severity of the stress. From these, and other results (reviewed by Lindquist and DiDomenico, 1985), they suggest that HSP70 is involved in the post-transcriptional regulation of its

own synthesis and possibly the synthesis of other HSPs. The mechanism of regulation they propose is by direct interaction with the 5' leader sequences of its own mRNA. The leader sequences of Drosophila HSP mRNAs are long by comparison to other mRNAs and contain an abundance of adenine. Coupled with the evidence that HSP70 is an RNA-binding protein and its synthesis is autoregulated, they propose a direct functional relationship between HSP70 synthesis and its own regulation.

The possible importance of the low M_r HSPs cannot be ignored in studies of thermotolerance. In a mutant form of Dictyostelium, in which the low M_r HSPs are absent, thermotolerance fails to develop (Loomis and Wheeler, 1982).

Based on the results from his laboratory, Tanguay and colleagues are less certain of the functional significance of the HSPs (reviewed by Tanguay, 1985). They have found that during biochemical fractionation of Drosophila cells, both the HSP68-HSP70 and HSP22-HSP28 groups are isolated in the nuclear fraction, are highly resistant to nuclease treatment and are not extracted at high salt concentrations. This supports their involvement as components of the nucleoskeleton or cytoskeleton. In subsequent immunofluorescence studies using polyclonal antibodies directed against the HSP23 and HSP68-70, the presence of these proteins was localized to the cytoplasm during the recovery period.

The HSP83, which is constitutively synthesized, was shown by cell fractionation and immunofluorescent studies to be exclusively located in the cytoplasm at control and heat shock temperatures. The more peripheral location of HSP83 during heat shock temperatures, lends support to the proposition that it is, in some way, involved with the plasma membrane (Tanguay, 1985). While the results from biochemical fractionation of Drosophila cells are supported by the immunofluorescent localization of HSPs within these cells, more direct evidence of HSP synthesis and thermotolerance remains to be elucidated.

1.3 Spontaneous and Thyroid Hormone-Induced Development and Regression of Anuran Epidermis

1.3.1 The Epidermis of Anuran Larvae and Adults

The anuran epidermis consists of 2-3 epithelial cell layers for approximately the first third of larval life (Leeson and Threadgold, 1961; Frieden and Just, 1970; Wright, 1973; Fox, 1975; Whitear, 1975). As larval development proceeds and the animal approaches metamorphic climax, the number of cell layers increases and characteristics typical of epidermis become apparent. As cells migrate to the external surface, they and their nuclei flatten (Fox, 1975). The cells of the outer epidermal layer which are joined laterally by tight junctions, display numerous mucous granules along their

external surface (Fox, 1975). Keratinization is first noted at stage XII in Rana pipiens (Wright, 1973) and by stage XVI, developing skin glands are observed (Derby, 1968; Kollros and Kaltenbach, 1952).

Within the cytoplasm of the larval epidermal cells a granular endoplasmic reticulum, which may be well-developed, ribosomes and polysomes and an extensive Golgi apparatus with many associated round smooth-surfaced vesicles are observed (Fox, 1975).

A variety of other cells are scattered among the keratinocyte population of the larval epidermis. These include epidermally derived Flask cells (Whitear, 1975; Fox, 1975). Flask cells are larger than the 'typical' epidermal cells and extend through more than a single epidermal cell layer (Whitear. 1975). Also present are Merkel cells (Nafstad and Baker, 1973), melanophores, larval Leydig cells, granular cells and epidermal neuromasts (Fox, 1975).

Keratinocytes are the major cell type of the 5-9 cell layers of the adult anuran epidermis (Parakkal and Alexander, 1972). Desmosomes are prominent between adjoining cells (Parakkal and Alexander, 1972; Whitear, 1974; Fox, 1975). As epidermal keratinocytes move towards the external surface they flatten, develop a dense thickened membrane and become keratinized as the stratum corneum (Parakkal and Alexander, 1972; Lavker, 1974; Whitear, 1974; Fox, 1975). The surface stratum corneum

is covered by a thin layer of mucus contributed by Flask cells, Leydig cells and epidermal and dermal glands (Parakkal and Alexander, 1972; Whitear, 1974). This layer of mucus effectively binds water and aids in respiration through the skin (Parakkal and Alexander, 1972). Some intracellular organelles persist in the cells of the stratum corneum but prior to sloughing are eliminated by autolysis (Fox, 1975; 1981). Tonofilaments remain apparent but eventually exist as a homogeneous mass within the keratinocytes (Fox, 1975).

The stratum granulosum underlies the stratum corneum. Deeper in the interior are the strata mucosum, spinosum and germinitivum, respectively (Fox, 1975). The cells of these layers progressively flatten and become more squamous in appearance as they approach the external surface (Parakkal and Alexander, 1972; Fox, 1975). Immature inner epidermal cells are cuboidal, spherical or columnar (Fox, 1975; 1981). Along the inner margin of the basal cell layer, abundant hemidesmosomes are observed (Fox, 1981). Together with associated tonofilaments, the hemidesmosomes form the figures of Eberth (Fox, 1981). The basal layer of cells is anchored to a well-defined basal lamina by hemidesmosomes. The adepidermal membrane which underlies the basal lamina and dermis (Salpeter and Singer, 1959) contains poorly oriented collagen fibres (Parakkal and Alexander, 1972).

Scattered among the epidermal keratinocytes are Flask

cells which constitute approximately 10% of the epidermal cell population (Whitear, 1975). These cells are believed to aid in epidermal sloughing (Whitear, 1975). Merkel cells also persist in the adult epidermis, making up approximately .3% of the cell population (Nafstad and Baker, 1973). Desmosomal junctions have been observed in both of these cell types (Fox, 1975). Melanophores, histiocytes and granular leukocytes are also detected but do not exhibit desmosomes (Frieden and Just, 1970; Fox, 1975).

1.3.2 Thyroid Hormone-Induced Metamorphosis

Administration of exogenous thyroid hormone (T_3 or T_4) to responsive anuran larvae results in the precocious differentiation of adult organs required for terrestrial life and the degeneration of larval aquatic organs (Gudernatsch, 1912; Kaltenbach, 1959; 1968; Kollros, 1961; Derby, 1968; Etkin, 1968; Frieden and Just, 1970; Atkinson and Little, 1972; Just and Atkinson, 1972; Wright, 1973; Atkinson, 1981b; Wright et al., 1981). It is generally agreed that the regression of larval aquatic organs is initiated by higher levels of T_3 or T_4 than are required for the development of adult organs (Kaltenbach, 1959; 1968; Etkin, 1968; Frieden and Just, 1970; Fox, 1981).

The ability of amphibia to respond to thyroid hormone (T_3 or T_4) involves the acquisition of tissue competence. This response may also be dependent on the level of

circulating thyroid hormone or require the presence of other hormones (Kaltenbach, 1959; 1968; Etkin and Gona, 1967; Derby, 1968; Etkin, 1968; Frieden and Just, 1970; Atkinson and Little, 1972; Just and Atkinson, 1972; Atkinson and Just, 1975; Wright et al., 1979; 1981; Atkinson, 1981b). Some tissues are rendered responsive to thyroid hormone by exposure to a sustained level while other tissues require a gradual increase in thyroid hormone (Kollros, 1961; Derby, 1968; Etkin, 1968; Frieden and Just, 1970; Atkinson, 1981b). Other factors which have been sited as important to the induction of precocious metamorphosis are the maintenance temperature of treated animals (Ashley et al., 1968; Derby, 1968), T_3 versus T_4 as the hormone of choice (Ashley et al., 1968; Frieden and Just, 1970; Atkinson, 1981b), the route of thyroid hormone administration (immersion, intraperitoneal injection or T_4-containing cholesterol implants) (Kaltenbach, 1959; 1968; Ashley et al., 1968; Etkin, 1968; Frieden and Just, 1970; Atkinson, 1981b) and the minimum dose of T_3 or T_4 required to initiate a metamorphic response (Kaltenbach, 1959; Ashley et al., 1968; Derby; 1968; Etkin, 1968; Frieden and Just, 1970; Atkinson, 1981b).

The many biochemical and anatomical (gross and microscopic) changes resulting from spontaneous or thyroid hormone-induced metamorphosis have been extensively reviewed. Therefore, they will not be dealt with here.

Interested readers are directed to the excellent reviews of Weber, 1969; Frieden and Just, 1970; Dodd and Dodd, 1976 and the more recent ones of Atkinson (1981b), Broyles (1981), Fox (1981), Frieden (1981), Kollros (1981), Smith-Gill and Carver (1981), and White and Nicholls (1981) found in Metamorphosis: A Problem in Developmental Biology, Second Edition (1981).

1.3.2(a) Thyroid Hormone-Induced Epidermal Development

Attempts to determine whether the events surrounding normal maturation of the epidermis of anurans at climax are duplicated during induction of metamorphosis by administration of exogenous thyroid hormone, have been examined. The morphological changes which accompany the maturation of epidermis from larvae to adulthood have been useful markers for assessing the effects of thyroid hormone.

Kollros and Kaltenbach (1952) were able to stimulate precocious epidermal differentiation by implanting thyroxine-cholesterol pellets under the dorsal skin of stage VIII (Taylor and Kollros, 1946) R. pipiens. Glandular development in the immediate area of the pellet was initiated by the third day following implantation. In addition, the epidermis stratified, cornified, molted and showed changes in pigmentation. These changes are characteristic of epidermis at metamorphic climax. As distance from the thyroxine-cholesterol pellet increased,

the effects of thyroid hormone decreased. Implantation of control pellets (cholesterol only) did not produce differentiative changes.

Wright (1973) demonstrated that the morphological hallmarks of epidermal differentiation are precociously induced in hind limb epidermis of R. pipiens following submersion in T_4. Autoradiographic analysis of epidermal tissue sections from stage X animals (Taylor and Kollros, 1946) demonstrated a DNA synthetic pattern typical of more mature epidermis, i.e. restriction of cell division to the basal layer. The decrease in DNA synthesis in the outer layer of epidermal cells was detected as soon as 6 hours after treatment with T_4. Within 24 hours of T_4 treatment, the 3 cell layers which constitute the epidermis displayed individual patterns of DNA synthesis. Controls showed a uniform pattern of DNA labeling.

Similar results have been observed by Dhanarajan (Dhanarajan, 1979; Dhanarajan and Atkinson, 1981). Following a single injection of T_3 to stage VI-VII R. catesbeiana larvae, differentiation-specific changes were apparent within 48 hr. Acommpanying an overall increase in limb length was increased stratification of the epidermis which became more pronounced with time. Eight days after T_3 injection, epidermal glands are well-developed.

In premetamorphic X. laevis, T_3 and T_4 have been shown to be effective inducers of precocious

differentiation of dorsal and lateral epidermis *in vivo* and *in vitro* (Vanable and Mortensen, 1966; Reeves, 1977). Organ cultures of dorsal skin treated with T_4 and subsequently examined for glandular development, displayed a limited period of positive response (Vanable and Mortensen, 1966). This period of T_4-responsiveness was found in mid-larval stages. Cultures initiated from young larvae or animals in whom the forelimbs were about to, or had just emerged, revealed that T_4 was inhibitory to glandular development. Examination of polysomes from larval epidermis *in vivo* and epidermal cell cultures, has shown that premature synthesis of an adult-specific keratin (Reeves, 1975) is initiated following T_3 treatment (Reeves, 1977).

1.3.2(b) Regression of the Anuran Tail

The regression of the anuran tail is an ordered process involving the sequential appearance of specific biochemical changes. These biochemical changes are ultimately observed as the programmed death of the cells that make up the tail (Weber, 1969; Frieden and Just, 1970; Fox, 1975; 1981; Kerr et al., 1974; Merrifield, 1979; Atkinson, 1981b). When metamorphosis is precociously induced by exogenous thyriod hormone, temperature, age and species of the tadpole, dose of thyroid hormone and its route of administration are all important factors in assessing tissue response

(Kaltenbach, 1959; 1968; Weber, 1962; Derby, 1968; Etkin, 1968; Frieden and Just, 1970; Ashley et al., 1975; Dodd and Dodd, 1976; Atkinson, 1981b). The suseptibility of the various tissues which make up the tail to different levels of thyroid hormone and the resulting effects of tissue-tissue interactions are also important considerations in evaluating this organ's regression (Kaltenbach, 1959; 1968; Salzmann and Weber, 1963; Etkin, 1968; Kim et al., 1977; Atkinson, 1981b; Niki et al., 1982).

During metamorphic climax, the epidermal cells which keratinize and are sloughed from the tail are not replaced by the germinative cell population in anurans (Wright, 1973; Fox, 1975). The tail epidermis does not differentiate and displays no peak in mitotic labeling during larval development (Wright, 1973). This is in contrast to the observations from hind limb epidermis. The morphological events surrounding the regression of the tail are similar, however, whether metamorphosis occurs spontaneously or is induced (Fox, 1975).

Kaltenbach (1959) demonstrated that when thyroxine-cholesterol implants contained 40% thyroxine, well demarcated areas of localized absorption could be induced in dorsal and ventral tail fins of R. pipiens. When thyroxine-cholesterol implants contained 20% thyroxine general metamorphic events were accelerated. However, the dorsal or ventral tail fins into which the

implants were placed displayed resorption in only 10% of the animals thus treated. Implants containing 60% thyroxine resulted in precocious metamorphosis and resorption of the tail fin anterior and posterior to the implant.

Quantitative studies on the response of isolated tail discs from Rana pipiens have shown that lower levels of thyroxine elicit a response than in the intact animal (Derby, 1968). Although regression of tail discs in vitro was successful at low levels of T_4, a longer period of time was required for resorption. Excessive doses of T_4 did not expedite the resorptive process (Derby, 1968). However, reduction of the latent period preceeding the resorptive response has been demonstrated. Kim and colleagues (1977) exposed isolated tail discs from premetamorphic Rana pipiens to thyroxine. Tail discs, previously unexposed to T_4, which were docked between T_4-treated discs displayed regression within 24 hours. Untreated discs incubated in thyroxine containing medium had a minimum 4 day latent period before a detectable resorptive response was apparent.

The recent work of Niki and colleagues (1982) has demonstrated the importance of dermal-epidermal interactions for the successful degeneration of the tail. They found that when epidermis was stripped from blocks of tail tissue taken from stage X R. catesbeiana larvae, the tails did not regress in the presence of T_3. Tissue

blocks in which the epidermis was left adherent or those to which it was applied after stripping followed by incubation in T_3-containing medium showed typical tail regression.

Biochemically, there is a shift in the enzyme population from those concerned with energy metabolism to those involved in breakdown of the tissues of the tail during metamorphic climax (Weber, 1969; Frieden and Just, 1970; Dodd and Dodd, 1976; Atkinson, 1981). The use of various species of Rana and Xenopus laevis have enlightened researchers as to the quantitative enzymatic changes that occur and permitted their localization to particular tissues. Although autolytic destruction mediated by lysosomal acid hydrolases is a likely method of tissue destruction (Eeckhout, 1969; Frieden, 1968; Weber, 1969; 1978; Frieden and Just, 1970; Dodd and Dodd, 1976; Atkinson, 1981b) prior to macrophage or 'phagosome' engulfment, some believe that it cannot account for the entire process (Weber, 1969; Kaltenbach et al., 1979; Merrifield, 1979; Atkinson, 1981b). Indeed, Merrifield (1979) has shown that the T_3-induced decrease in actin and tropomyosin was partially, at the very least, the result of translational inhibition.

Kaltenbach and colleagues (1979) found that the enzyme distribution among the various tissues of the tail was, in general, the same whether metamorphosis was spontaneous or induced. In their histochemical

localization study, lysosomal and non-lysosomal enzymes displayed corresponding increases and decreases under both metamorphic states. They found that the staining for TPPase, 5'Nase, Mg^{2+} - and Ca^{2+} - activated ATPase becomes more intense in epidermis and connective tissue. This corresponds to an increase in lysosomal, Golgi apparatus and phagocyte activity. The activity of acid phosphatases and cathepsin-like esterases has been localized to the dermis in tail rudiments of spontaneously metamorphosing X. laevis (Weber, 1963) Although the type and distribution of enzymatic change is the same in spontaneous and thyroid hormone-induced metamorphosis, the quantitative changes in enzyme activity are seldom of the same magnitude when metamorphosis is artificially stimulated (Eeckhout, 1969; Frieden, 1968; Weber, 1969; Dodd and Dodd, 1976; Atkinson, 1981b).

1.4 The Expression of the Keratin Family of Proteins During Epidermal Development

1.4.1 Molecular Structure of Type I and Type II Keratins

Intermediate filaments (7-11 nm) along with microfilaments (4-6 nm) and microtubules (23-25 nm) make up the cytoskeletal network of vetebrate cells (reviewed by Lazarides 1980). The identification of intermediate-type filaments in Drosophila suggests that a similar cytoskeletal network may be present in insects (Walter and Biessman, 1984). Since only one subclass of intermediate filament is usually expressed by a particular

cell type, identification of the intermediate filament and/or its subunits is an indication of the cells' tissue of origin. For example, keratins (and cytokeratins) are synthesized by epidermis and a variety of epithelia, desmin by muscle cells, vimentin by fibroblasts and other cells of mesenchymal origin, glial fibrillary acidic protein (GFAP) by astroglia and the neurofilament proteins (NF-L, NF-M and NF-H) by neuronal cells (reviewed by Lazarides, 1980, Osborn et al., 1981, Franke et al., 1981e and Osborn and Weber, 1982). (The term 'cytokeratins' was first implemented by Franke and colleagues (Franke et al., 1978) to distinguish keratins extracted from living cells from the keratins found in terminally differentiated stratum corneum. This term is now in common useage.).

The ability of different subclasses of intermediate filaments from rodent, bovine and invertebrate sources to form copolymers, albeit of variable stability (Steinert et al., 1981), and the production of a monoclonal antibody which cross-reacts with all subclasses of intermediate filaments in vertebrates and invertebrates (Pruss et al., 1981), suggested that common structural properties existed (Roop et al., 1984). More recently, sequence analysis of rodent and human intermediate filament genes has indicated that all intermediate filaments likely arose from a common ancestral gene (Krieg et al., 1985).

Indeed, intermediate filaments from all organisms examined to date have a central rod domain of 311-314

amino acids which consists of 4 distinct α-helical subdomains, 1A, 1B, 2A and 2B (Hanukoglu and Fuchs, 1982; 1983; Steinert et al., 1985b). The amino acid sequence of the rod domain is made up of quasi-repeat heptad that facilitates an α-helical coiled-coil conformation (Hanukoglu and Fuchs, 1983; Steinert et al., 1983; 1985a; 1985b). Along the rod domain, acidic and basic amino acid residues are distributed in a distinct 28 residue periodicity (reviewed by Steinert et al., 1985b). Within the α-helical regions of the rod domain, the sequence of residues 8-20 of segment 1A and the last 30 residues of 2B are highly conserved. These two α-helical regions are the amino and carboxy terminal segments of the rod domain (Steinert et al., 1985a; 1985b).

The 4 α-helical regions are separated by 3 non-α-helical linker regions, L1, L12 and L2. The linker L12 which joins segment 1 and 2 has the potential to form a β-sheet. The keratin family of intermediate filaments which constitutes 2 of the 3 intermediate filament 'Types' (based on the designation of Crewther et al., 1978 from sheep wool α-keratins) has remarkably similar rod domains (Steinert et al., 1985b). A great deal of sequence homology is observed between the rod domains of desmin, vimentin and GFAP (Type III intermediate filaments) (Steinert et al., 1985a).

Analysis of sequence data from mouse and humans has shown that distinct heterogeneity between Type I and Type

II keratins is contributed by the 2 end domains which are bilaterally symmetrical with respect to the rod domain (Hanukoglu and Fuchs, 1982; 1983; Kim et al., 1983; Steinert et al., 1983; 1985a). On the amino- and carboxy-terminal sides of the rod domain of Type II keratins are two highly conserved globular subdomains, H1 and H2 (Steinert et al., 1985a). The mass differences in the keratin Types I and II are due to 2 subdomains of variable size and sequence. The variable subdomains, V1 and V2, which are rich in glycine and/or serine residues may be up to 130 amino acids long in large keratins (Steinert et al., 1985a; 1985b). In keratins of low molecular mass the size of V1 and/or V2 is reduced, and in some, it is non-existent (Steinert et al., 1985b).

The amino- (N) and carboxy-terminal (C) subdomains of Type I and Type II keratins are usually of basic pH and heterogeneous in size and amino acid sequence (Roop et al., 1984). In mouse, by comparing amino acid sequence data, Roop and colleagues (1984) have found that each keratin bears a unique C subdomain. Preparation of synthetic peptides corresponding to the C subdomains of 4 mouse keratins, demonstrated that when used as antigens, keratin specific antibodies were generated.

The keratins are obligate heteropolymers (Steinert et al., 1981; Steven et al., 1983). That is, for filament assembly the presence of different subunit proteins is required. cDNA analysis has shown that subunits within

assembled filaments must represent Type I (acidic) and Type II (neutral-basic) keratins (Fuchs et al., 1981; 1983; 1984; Fuchs, 1983; Sun et al., 1983a; 1983b; 1984; Steinert et al., 1985b). Members of each Type are coordinately expressed in a tissue- and differentiation-specific manner (reviewed by Fuchs et al., 1984). With the possible exception of only 2 of the many keratins observed in epidermis and a variety of epithelium, different mRNAs encode each keratin (Fuchs and Green, 1979; Fuchs, 1983; Fuchs et al., 1983; 1984; Hanukoglu and Fuchs, 1983; Kim et al., 1983).

Sauk et al., (1984) have demonstrated that the assembly of keratin filaments in vitro is facilitated by the presence of the non-α-helical N- and C-terminal domains. Removal of the end domains which are chymotrypsin sensitive (Steinert et al., 1983) and subsequent renaturation of the remaining α-helical fragments demonstrated an increase in the lag period for filament assembly. In addition, the filaments that did form were anomalous. The ordered structure characteristic of intermediate filaments was absent (Sauk et al., 1984).

1.4.2 The Morphology of the Developing Mammalian Epidermis

All mammals are surrounded by an epidermis that withstands ravages from the internal and external environment for the life of the organism. In developing

mammalian embryos, the epidermis is initially observed as a simple epithelium, a single cell layer in thickness (Banks-Schlegel, 1982; Moll et al., 1982b; Holbrook, 1983; Dale et al., 1985). As development proceeds, the layers of epithelial cells increase to 2, and eventually become stratified into 5-9 layers prior to birth (Jackson et al.,1981; Banks-Schlegel et al., 1982; Moll et al., 1982b; Schweizer and Winter, 1982; Holbrook, 1983; Dale et al., 1985). Formation of specialized epidermal structures is coincident with epidermal development. For example, when the epidermis of human fetuses consists of only 3 cell layers (including the protective periderm at 13 weeks) hair germs are histologically detectable (Moll et al., 1982b; Dale et al., 1985).

With the increase in epidermal cell layers, morphological properties associated with a differentiated epidermis can be distinguished in the superficial cells (Banks-Schlegel, 1982; Schweizer and Winter, 1982; Dale et al., 1985). For example, at day 16 of fetal mouse development, the epidermis consists of a basal cell population and an obvious stratum spinosum (Schweizer and Winter, 1982). Prior to this stage, the epidermis could only be distinguished as basal cells, and superficial cells of intermediate development (Schweizer and Winter, 1982).

The advent of this morphological distinction between the cell layers also heralds the restriction of

proliferative activity to the basal cell population. In mouse and humans, it has been shown that *in vivo* and *in vitro* the basal cells are smaller than the suprabasal cells which are committed to terminal differentiation (Sun and Green, 1976; Green, 1977; Watt and Green, 1981; Hennings et al., 1983a; 1983b; Skerrow and Skerrow, 1983; Schweizer et al., 1984a; Dale et al., 1985). By the seventeenth day of fetal mouse development, the superficial cells resemble the flattened cells characteristic of the granular layer (Schweizer and Winter, 1982). While still *in utero*, the outermost cells of mammalian epidermis keratinize, die and form the cornified layer (Banks-Schlegel, 1982; Schweizer and Winter, 1982; Holbrook, 1983; Dale et al., 1985). This represents the terminally differentiated state of the epidermal keratinocyte.

As stated above, once epidermal cells can be morphologically distinguished, cell division becomes restricted to the basal cell population. Watt (1984) has shown that *in vitro*, the commitment of particular epidermal cells to terminal differentiation is selective, rather than random and cannot be assessed by typical morphological criteria such as size. In cell cultures of human keratinocytes, the cells which are committed to terminal differentiation are less adherent to the substrate. In addition, they are positive (by immunofluorescence) for involucrin, the protein precursor

of the cornified envelope of differentiated keratinocytes. When stratification of monolayer cultures is induced, only the involucrin-positive cells become upwardly mobile and form desmosomes and tight junctions with their neighboring (now) superficial cells. If protein synthesis is inhibited at the time of stratification, the cells are still involucrin-positive, indicating their commitment to differentiation prior to stratification.

By immunoblot analysis, it has been shown that approximately 10% of the basal cells of mouse epidermis express a differentiation specific (60 kd) keratin (Schweizer et al., 1984a). Immunofluorescent staining of basal keratinocytes confirmed the small percentage of cells positive for the presence of the 60 kd keratin. This demonstrates that as seemingly undifferentiated cells of the basal population, the developmental fate of these cells has, nonetheless, been determined.

1.4.3 Expression of The Keratin Family of Proteins in Developing and Mature Epidermis

The development and maturation of the epidermis necessitates the development of special qualities of durability which make the whole (epidermis) greater than the sum of its parts (individual keratinocytes). Accompanying the morphological changes of epidermis during embryonic development, are changes in the synthesis of the keratins, a family of water-insoluble proteins. The

keratin subunits have M_rs of 40-70 kd and assemble into the 7-11 nm tonofilament network of developing and mature epidermis. A well-developed tonofilament network and the highly insoluble cornified envelope impart strength and durability to the epidermis.

Nineteen mammalian cytokeratins (excluding their isoelectric variants) identified in a variety of normal and pathological conditions have been catalogued (Moll et. al., 1982a); a more recent compilation suggests that the keratin group of intermediate filaments consists of upwards of 30 different proteins (Steinert et al., 1985b). Although the keratins extracted from epidermis present a seemingly complex picture, individual cells within the tissue express only 2-5 keratins at a given time (Fuchs et al., 1984).

In mammals, the emergence of specific patterns of epidermal keratin expression both <u>in utero</u> and after birth have recently come to the fore (Fuchs and Green, 1978; Banks-Schlegel, 1982; Schweizer and Winter, 1982; Tseng et al., 1982; Woodcock-Mitchell et al., 1982; Roop et al., 1983; Skerrow and Skerrow, 1983; Sun et al., 1983a; 1983b; Dale et al., 1985). Dale et al. (1985) have recently conducted a monoclonal and keratin-specific antibody study of epidermal keratin expression during human embryonic and fetal development. From their results, they concluded that there are no embryonic-specific keratins. However, the expression of keratins which are associated with

stratification (50 and 58 kd (reviewed by Sun et al., 1983a; 1983b)) and keratinization (67 and 56.5 kd (reviewed by Sun et al., 1983a; 1983b)) precede the morphological manifestation of these properties. Coincident with the detection of the 67 and 56.5 kd keratins is the disappearance of the 40 and 52 kd keratins observed early in development. The loss of low M_r keratins when the synthesis of high M_r keratins begins has been observed in fetal rabbit epidermis (Banks-Schlegel, 1982) but was not apparent in results from fetal mouse (Schweizer and Winter, 1982).

Dale and colleagues concluded that the expression of specific keratins by epidermal cells may be what commits the tissue to stratification and keratinization. The same cannot be said for filaggrin, the precusor protein of the amorphous matrix which is associated with keratohyaline granules. Detection of filaggrin is simultaneous with follicular and subsequently, interfollicular keratinization (Dale et al., 1985).

The pattern of epidermal keratin synthesis and the mitotic restriction to the basal layer established during human fetal development persist throughout the life of the animal (Woodcock-Mitchell et al., 1982; Skerrow and Skerrow, 1983; Sun et al., 1983a; 1983b; 1984). In a non-pathological condition, basal cells continue to synthesize 46, 50, 56 and 58 kd keratins while suprabasal cells persist in the synthesis of 65-67 and 56 kd keratins.

as well as some low M_r keratins (Fuchs and Green, 1980; Woodcock-Mitchell et al., 1982; Skerrow and Skerrow, 1983; Sun et al., 1983a; 1983b; 1984). Deviation from the normal state of keratin synthesis has been correlated with hyperproliferative diseases (Skerrow and Skerrow, 1983; Weiss et al., 1984) and malignancies (Moll et al., 1982c; reviewed by Fuchs et al., 1984). Indeed, alterations in the keratin-specific expression by epithelial tissues is proving to be diagnostically valuable (Moll et al., 1982c; Fuchs et al., 1984).

Mammalian epidermal cell cultures often express keratins that are different from what is observed in intact epidermis (Fuchs and Green, 1978; Banks-Schlegel, 1982; Roop et al., 1983). Fuchs and Green (1978) first showed that keratin-enriched fractions from human foot callus had M_rs of 65, 63, 55, 46, 45 and 41 kd. However, keratinocyte cultures from human foreskin synthesized keratins with M_rs of 58, 56, 50, 48, 46 and 40 kd (Fuchs and Green, 1978; Sun et al., 1984) although morphologically, some of the cultured cells appeared to be terminally differentiated (Green, 1978). Although more recent results have demonstrated the presence of the high M_r keratins in human epidermal cell cultures, the authors attribute the discrepency to different sources of medium supplements (Banks-Schlegel and Harris, 1983). Comparison of the results from human epidermal cell cultures with epidermal cell cultures from rabbit

(Banks-Schlegel, 1982) and mouse (Roop et al., 1983) verified that the absence of high M_r keratins from human keratinocyte cultures was not an unique finding (Fuchs and Green, 1978).

1.4.4 Effect of Growth Environment on the Differentiation of Cultured Cells that Synthesize Keratins

A valuable advancement in the area of epidermal research has been the development of successful tissue culture techniques. *In vitro* examination of the cells derived from epithelium has revealed that many of the morphological properties of intact epidermal or epithelial tissue are retained by its cells in culture. In appropriately supplemented tissue culture medium, increasing cell size with progressive differentiation, desmosome formation, stratification, formation of the cross-linked envelope and shedding of the surface cells into the medium have been repeatedly documented (Sun and Green, 1976; Green, 1977; 1980; Rice and Green, 1979; Banks-Schlegel and Green, 1980; 1981; Green et al., 1981a; 1981b; Watt and Green, 1981; Hennings et al., 1983a; 1983b). The maintenance of these properties *in vitro* has permitted the researcher the convenience of manipulation afforded by tissue culture without the influence of factors other than those provided in the culture medium. As will be discussed below, specific properties of the epidermis are markedly effected by medium supplements.

1.4.4(a) The Effect of Vitamin A Supplemented Medium on Epidermal Differentiation

The presence of vitamin A or other retinoids in growth medium has been shown to alter the differentiation of epithelium *in vitro* (Reeves and Laskey, 1975; Fuchs, 1981; Eichner et al., 1984). Human epidermal keratinocytes grown in the absence of vitamin A were more adherent to neighboring cells and to culture dishes. In addition, a decrease in cell motility was observed (Fuchs, 1981). In vitamin A-free medium, morphological properties typical of a keratinizing, terminal differentiating epithelium are observed; nuclear destruction, an abundance of keratohyaline granules, birefringence and stratum corneum formation are apparent (Reeves and Laskey, 1975; Fuchs, 1981; Eichner et al., 1984). When cultures are maintained in vitamin A-free medium, the release of terminally differentiated squames to be sloughed into the medium is inhibited in cell cultures from humans (Fuchs, 1981) but not in epidermal cell cultures from *Xenopus laevis* (Reeves and Laskey, 1975).

The absence of vitamin A from growth medium has been shown to alter the keratins synthesized by epidermal cell cultures (Fuchs, 1981; Eichner et al., 1984). Human epidermal cell cultures grown and maintained in vitamin A-free medium synthesized a 67 kd water-insoluble protein (Fuchs, 1981; Eichner et al., 1984). With increasing

prominence of the 67 kd protein, two lower M_r water-insoluble proteins of 40 and 52 kd became less intense (Fuchs, 1981; Eichner et al., 1984). Immunoprecipitation (Fuchs and Green, 1981) and immunoblot (Eichner et al., 1984) verified that the 67 kd protein was a keratin. The presence of adult-specific keratins in Xenopus laevis epidermal cell cultures has also been reported (Reeves and Laskey, 1975)

The translation of poly(A+) mRNA from epidermal keratinocytes grown in the presence or absence of vitamin A showed that mRNA coding for the 67 kd was extracted only when cells were grown in vitamin A free medium (Fuchs, 1981). The detection of the 67 kd keratin is particularly interesting since it is not usually detected in cultured human keratinocytes (Fuchs and Green, 1978). Using monoclonal antibodies, Eichner et al. (1984) demonstrated that the increase in the 67 kd keratin (Type II, AE2 and AE3 positive) is coordinated with an increase in th 56.5 kd keratin (Type I, AE1 and AE2 positive). The relationship between the detection and subsequent increase in the 67 and 56.5 kd keratins and keratinization is discussed above.

1.4.4(b) The Effect of Calf Serum Supplemented Medium on Epidermal Differentiation

In mouse mammary epithelium, the presence of calf serum has little effect on the cytokeratins synthesized in

culture (Asch and Asch, 1985). However, the absence of serum from medium inhibits the growth of fibroblasts which often contaminate epithelial cell preparations. The preparation of serum-free medium eliminates the detection of the intermediate filament characteristic of fibroblasts, vimentin (Asch et al., 1981; Asch and Asch, 1985).

Keratinocyte cultures from newborn human foreskin maintained in serum-free medium develop detergent-insoluble filaments and a cornified envelope and are sloughed into the medium (Green, 1977). However, digestion of cellular nuclei, which is also a characteristic of terminal differentiation, is not detected (Green, 1977). Unfortunately, Green (1977) did not do accompanying keratin protein analysis of the epidermal cells maintained in serum-free medium.

1.4.4(c) The Effect of Medium Calcium Levels on Epidermal Cells in Culture

The differentiation of epidermal cell cultures from human and mouse are markedly affected by low calcium levels in the medium ($<.1$ mM) (Boyce and Ham, 1983; reviewed by Hennings et al., 1983a and 1983b). In low calcium concentrations epidermal cell cultures will grow for limited periods as a monolayer, displaying a polygonal morphology typical of basal cells. Desmosome formation, keratohyaline synthesis, cornified envelope formation and

normal keratin filament assembly are inhibited. Tonofilament bundles that are observed are poorly developed, with most displaying a perinuclear arrangement. Keratin synthesis remains unaffected by low calcium levels (reviewed by Hennings et al., 1983a).

1.4.4(d) The Effect of Cell Density on Keratin Gene Expression

In non-pathological conditions one intermediate filament subclass is usually expressed by a particular cell type. Although epithelial cells in culture may co-express cytokeratins and vimentin (Franke et al., 1979; Osborn et al., 1980; Franke et al., 1981b; 1981d; Masuda et al., 1985), the most notorious examples of this phenomenon are from the long established PtK_1, PtK_2 and HeLa cell lines (Osborn et al., 1980; Franke et al., 1981d). Recent evidence from Lane et al. (1983) has shown that in situ early mouse embryos (8.5-13.5 days old) express both cytokeratins and vimentin in parietal endoderm cells. She and her collaborators suggested that the co-expression of the 2 intemediate filament subclasses may be due to the absence of cell contacts. Indeed, with increasing cell density, a suppression of vimentin expression is observed (Lane et al., 1983).

The effect of cell density on cytokeratin synthesis in bovine mammary gland epithelium (BMGE) cultured in medium supplemented with only fetal calf serum or in

medium containing insulin, hydrocortisone and prolactin in addition to fetal calf serum, has been elaborated by Ben Ze'ev (1985). BMGE maintained as a low density monolayer culture in medium supplemented with fetal calf serum, synthesized large amounts of vimentin as well as cytokeratins typical of this cell line. In addition to an apparent decrease in vimentin synthesis, BMGE cultured at high density also synthesized an acidic 45 kd cytokeratin not detectable at low densities. When the cell cultures were maintained in medium which was hormonally supplemented, vimentin synthesis was not observed at low or high density plating. Detection of the acidic 45 kd protein was still apparent only in high density cultures. Use of monoclonal antibodies verified the novel acidic 45 kd protein as a cytokeratin (Ben Ze'ev, 1985). The synthesis of vimentin by cytokeratin synthesizing epithelial cells is, however, not a universally observed phenomenon in sparsely seeded cultures (Rheinwald et al., 1984).

1.4.4(d) The Effect of Common Medium Supplements and 3T3 Feeder Layer on Keratinocyte and Epithelial Cell Growth

It is common practice for a feeder cell layer of lethally irradiated 3T3 mouse fibroblasts to be used to adhere cultured keratinocytes to the surface of the culture dish (reviwed by Green, 1980). In its absence,

Rheinwald and colleagues (1984) have found that vimentin is synthesized along with the keratins specific the particular cell type. Upon return to a 3T3 feeder layer, the synthesis of vimentin is supressed and keratins are again the only intermediate filament type observed (Rheinwald et al., 1984).

Epidermal growth factor (EGF), hydrocortisone, insulin and cholera toxin are frequently used as supplements to promote keratinocyte proliferation in vitro (reviewed by Green, 1980). EGF, a murine derived growth factor, has been shown to markedly enhance the proliferative capacity of keratinocytes (Green, 1977; reviewed by Green 1980 and Rheinwald et al., 1984). A decrease in the number of terminally differentiating cells is concomitant. Once cultures are confluent, the tendency to cell division is reversed, and a stratum corneum of terminally differentiated squames is formed at the surface of the epithelium (Green, 1980).

Growth of human epidermal keratinocytes in the absence of EGF and hydrocortisone promotes morphological differentiation (Eichner et al., 1984). Immunoblot analysis demonstrated that keratins specific for keratinization (67 and 56.5 kd) were synthesized, albeit at low levels, in vitro (Eichner et al., 1984). With the exception of the studies of Fuchs (1981) and Eichner et al. (1984), the assessment of the differentiative capacity of various medium supplements is based largely on the

morphological properties of the cells (as discussed above). The influence of medium supplements on keratin synthesis has largely been ignored (Rheinwald et al., 1984).

1.5 Summary and Thesis Objectives

1.5.1 Summary

Alterations in gene expression occur naturally during the developmental process. Activation of a particular gene or 'family' of genes may be transient. The proteins synthesized as a result of their induction are required for only limited periods. Although some genes or gene families may be expressed throughout development, more 'mature' gene products may be induced as development proceeds. In addition, while the expression of some genes is ubiquitous, other genes may be tissue-specific. Transient and ubiquitous (heat shock) and continuous and tissue-specific (keratin) gene expression were examined.

The transient and ubiquitous expression of genes which environmental stress or combination of stresses induce is required while the stress is present. The synthesis of the proteins for which the stress-responsive genes code is paramount, halting normal protein synthesis in most systems until the stress is removed. Upon return to the control environment, the restoration of normal

protein synthesis is gradual, but eventually the stress (heat shock) proteins are not detectable.

The synthesis of keratins is, unlike heat shock proteins, continuous and tissue-specific. The expression of keratin genes, as detected by sensitive antibody studies, occurs early in vertebrate epidermal development (Banks-Schlegel, 1982; Schweizer and Winter, 1982; Dale et al., 1985). As development proceeds, keratins continue to be expressed but are replaced by differentiation-specific keratins which reflect the increasing maturity of the epidermal cells. Once the 'maximum' maturity in keratin expression is attained by epidermal cells, they are terminally differentiated and die.

Primary epidermal cell cultures from larval Rana catesbeiana provide an excellent system for examining the transient, ubiquitous and continuous, tissue and differention-specific expression of heat shock and keratin genes, respectively. The former can be elicited by brief incubations of epidermal cell cultures at supraoptimal temperatures. The latter can be induced in situ or in organ culture by exogenous thyroid hormone (Kollros and Kaltenbach, 1952; Vanable and Mortensen, 1966). Accelerated maturation of primary epidermal cell cultures from larvae was attempted by addition of thyroid hormone to the growth medium.

1.5.2 Thesis Objectives

The research described herein addressed the following questions:

1) Does the long-established Xenopus laevis kidney epithelial cell line respond to brief incubations at sub- or supraoptimal temperatures by synthesizing new and/or enhanced proteins? If so, are these proteins the result of newly transcribed mRNAs or are they present in a non-translatable form at the 'control' temperature (22° C)?

2) In Rana catesbeiana, do primary epidermal cell cultures from organs which are wholly larval (tail fin) or adult (hind limb) respond to brief incubations at elevated or depressed temperatures by synthesizing new and/or enhanced proteins? If so, are the same proteins synthesized by each tissue?

3) During the first 36 hours of cell culture, are primary epidermal cell cultures from different stages of larval Rana catesbeiana responsive to thyroid hormone, as demonstrated by the selective synthesis of particular water-insoluble proteins? If the cells are responsive to thyroid hormone, do cell cultures from different stages of larvae respond by synthesizing the same water-insoluble proteins?

4) When epidermal cell cultures from larval R. catesbeiana are maintained for 5 days, are the water-insoluble proteins synthesized in the presence or absence of thyroid

hormone different than those observed during the first 36 hours of cell culture? Do epidermal cell cultures from different larval stages synthesize the same water-insoluble proteins during the final 36 hours of the 5 day culture period?

5) Are the water-insoluble proteins synthesized by R. catesbeiana epidermal cells in culture keratins? If so, are the same keratins expressed by epidermal cell cultures when the source of the epidermis is from larvae of different stages of morphological differerentiation?

6) Does the epidermis of thyroid hormone-treated larvae synthesize the same water-insoluble proteins in situ as epidermal cell cultures during the first 36 hours following exposure to exogenous thyroid hormone?

7) Do animals at different stages of metamorphic climax synthesize the same proteins? Are these proteins the same as those observed in epidermal cell cultures?

PART 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals

Tadpoles of the American bullfrog Rana catesbeiana were obtained from either Howe Brothers Minnow Farms, Box 111, Atlanta, Texas or Sea-Mac Marketing, a division of Haltech Scientific Supply, Mount Albert, Ontario. They were maintained at room temperature in previously aged and aerated water on a diet of canned spinach and a 12 hr light/dark cycle.

2.1.2 Chemicals

Unless otherwise stated, all reagents required for electrophoresis were purchased from Fisher Scientific Company, Toronto, Ontario, Bio-Rad Laboratories, Richmond, California or J. T. Baker Chemical Co., Phillipsburg N.J. Low molecular weight calibration kits were a product of Pharmacia Fine Chemicals, a division of Pharmacia Inc., Piscataway, N.J.

Sodium deoxycholate, dithiothreitol, phenylmethyl sulfonylfluoride, ethylenediamine tetraacetic acid, 2-mercaptoethanol, N,N'-methylene-bis-acrylamide, 3,3',5-triiodothyronine sodium salt and gentamycin sulfate

were purchased from Sigma Chemical Company, St. Louis, Missouri.

Cell culture supplies, unless specified to the contrary, were products of Grand Island Biological Company (GIBCO), Grand Island, New York. Fetal calf serum was obtained from either GIBCO or Animal Health Laboratories, Markham, Ontario. Culture dishes were from GIBCO, Falcon Lab Ware, a division of Becton Dickinson Canada, Mississauga, Ontario or Corning Glass Works, Science Products Division, Corning, New York.

2.2 Methods

2.2.1 Culturing Methods

The cell culture methods for the perpetuation of the established kidney epithelial cell line from Xenopus laevis and the primary epidermal cell cultures from Rana catesbeiana tail fin and hind limb involve different procedures. For this reason, each method of cell culture will be described in detail.

2.2.1(a) Culturing an Established Line of Kidney Epithelial Cells from Xenopus laevis

Established adult kidney epithelial cell cultures of the African clawed-toe frog Xenopus laevis (obtained from Dr. J. Gall, Yale University) were maintained according to the method of Rafferty (1976). Xenopus laevis kidney epithelial cells (XKEC) were cultured in modified mammalian

medium (Medium 199) in Corning T-75 plastic tissue culture flasks. The mammalian medium was diluted by 6% (v/v) with sterile double-distilled water and supplemented with 1.5% (w/v) lactoalbumin hydrolysate, 15% fetal calf serum, 75 µg/mL gentamycin sulfate and 1.5 µg/mL fungizone. The cells were kept actively growing by using an incubation temperature of 22°C in a darkened tissue culture chamber.

Growth of cell cultures was assessed visually by phase contrast microscopy. When epithelial cell sheets approached confluence, exhausted medium was replaced with fresh medium twelve hours prior to the establishing of daughter cultures. Cells were displaced from the culture flask, transferred to a 15 mL conical bottom, sterile centifuge tube and pelleted for 10 minutes on a table top centrifuge. Cell pellets were suspended in 5 mL of fresh medium and 10% of this suspension was distributed to T-75 culture flasks containing 20 mL of fresh medium and maintained at 22°C in a darkened tissue culture chamber.

2.2.1(b) Primary Cultures of Rana catesbeiana Hind Limb and Tail Fin Epidermal Tissue

The method used for culturing amphibian hind limb and tail fin epidermal cells, and lung tissue was according to that described previously by Ketola-Pirie and Atkinson (1983). Tadpoles of the American bullfrog Rana catesbeiana were immobilized on ice and staged according to the method of Taylor and Kollros (1946). Those tadpoles

which were found to be within larval stages XII-XV, based on previously defined surface anatomical features (Taylor and Kollros, 1946) had their notocords severed. The tail fins and hind limbs were excised from these animals and the epidermal tissues of these organs stripped from underlying muscle. The isolated epidermal tissues were passed through four or five consecutive changes of Hank's balanced salt solution (without phenol red indicator) diluted 33% with sterile double-distilled water containing 75 µg/mL gentamycin and 1.5 µg/mL fungizone. The tissues were minced into 2.0 mm^2 pieces and 12-15 were plated per gelatinized (Bacteriological gelatin, BBL Microbiological Media, a division of Becton Dickinson, Cockeysville, MD) 15 mm petri dish (Hauschka, 1972). The tissues were maintained at 22° C with a mammalian medium (Medium 199) which was diluted 66% with sterile double-distilled water. The medium was supplemented with 75 µg/mL gentamycin, 1.5 µg/mL fungizone and 5% fetal calf serum (final concentration). On the first day of cell culture, each explant was sustained with a single drop of the modified medium administered with a Pasteur pipet. A few additional drops of medium were added to the periphery of the culture dishes to maintain the humidity. Every day thereafter, for four consecutive days, the old medium was replaced with 1 mL of fresh medium.

2.2.1(c) Determination of Protein Synthetic Activity in Amphibian Cultures Exposed to and Recovering from Temperature Shifts

The procedures used for the incubation of amphibian cell cultures at various temperatures for brief periods was similar to that used by Atkinson (1981a). Prior to a thermal shift, the modified medium was removed from XKEC and from primary epidermal cell cultures grown at 22° C and replaced with Minimal Essential Medium (MEM) lacking leucine (GIBCO) or methionine (Flow Labs). All cell cultures from Rana catesbeiana were exposed to temperatures below the optimum growing temperature for 2 hours, above 22° C for 1 hour or at the optimal temperature (22° C) for 1 hour. XKEC cultures were incubated similarly at all temperatures for two hours. At designated times after the switch to leucine-free medium, the cultures from Rana catesbeiana were labeled with 3.3 μCi/mL of L-[U-^{14}C]leucine (New England Nuclear (NEN), Boston, MA) and allowed to incorporate the [^{14}C]leucine for 2 hours at 22° C. Similarly treated cultures from Xenopus laevis were switched to methionine-free medium and labeled with 60 μCi/mL of [^{35}S]methionine (1196.3 Ci/mMol L-[^{35}S]methionine (NEN) or to leucine-free medium and labeled with 3.3 μCi/mL of L-[U-^{14}C]leucine (NEN) for 2 hours at 22° C. At the end of the labeling period, the cells were lysed in a solution

containing 9 M urea, 1 mM PMSF and 5% 2-mercaptoethanol (Atkinson 1981a) by a shearing force provided by a tight-fitting teflon pestle attached to a Sorvall tissue homogenizer (set at 4). Insoluble material was pelleted and the supernatants used immediately or stored at -20°C.

Following a 1 hour heat stress in leucine-free medium at 34°C, 5-day cell cultures from Rana catesbeiana tail fin epidermis were allowed to recover at 22°C for 0, .5, 1, 2, 3 and 4 hours. At the end of these specified times, 3.3 μCi/mL of L-[U-^{14}C]leucine was added to each culture dish and incorporation of the isotope allowed to proceed at 22°C for 2 hours. Cell lysates were prepared by the method described above.

2.2.1(d) Photographing of Primary Epidermal Cell Cultures

To assess morphologically the effect of temperature on hindlimb, tail fin and lung epithelial cell cultures, representative groups of cells were photographed after various periods in culture. A Nikon inverted phase contrast microscope (model MD, Nippon Kogaku Inc., Garden City, NY) with attached Yashika 35 mm SLR camera containing 2415 technical film (Kodak Canada Inc., Toronto, Canada) was used to photograph the cells. A green filter was used to obtain suitable contrast.

2.2.2 Isolation of RNA from Xenopus laevis Kidney Epithelial Cells and its Translation in vitro

The extraction of total cellular RNA followed the sodium perchlorate extraction of Lazardi and Engleberg (1979) and Wilcockson (1975), as modified by Sachs et al. (1980). In an effort to eliminate RNase activity introduced by handling, disposable gloves were worn, all glassware for RNA extraction was autoclaved and previously unopened chemicals used.

To obtain suitable amounts of RNA, 16-20 T-75 tissue culture flasks (containing 1 X 10^6 cells/flask) were used for each temperature studied. The flasks were placed at 22, 35 or 41.5°C for 2 hours. At the end of the 2 hour period, the medium was decanted from cultures incubated at 22 or 35°C and the cells adhering to the flasks were washed with a cold saline solution and harvested (by scraping with a rubber policeman) in cold saline. The method was slightly altered for cultures incubated at 41.5°C, since visual assessment revealed that a significant proportion of these cells had lifted from the culture flasks. In this case, the medium was decanted but not discarded and the cells which remained adherent were scraped from the culture flasks and kept separate from the cell population suspended in the medium.

In all instances, the cells which were pelleted for 10 minutes at 2500 x g were suspended in 3 mL of the first RNA isolation buffer containing 50 mM TRIS-HCl (pH 7.5), 5%

SDS, 200 mM NaCl, 15 mM EDTA and 0.05% proteinase K (Boehringer Mannheim) in a 15 mL Kimax or Corex centrifuge tube and incubated at 35°C for 15 minutes followed by a 5 minute incubation at 55°C. Since non-nucleic acid material is contained within the pellets, they were discarded. To the supernatants, 2 mL of 3.5 M $NaClO_4$ was added and mixed by vortexing. The suspension was incubated at 55°C until it became clear. A total of 20 mL of $NaClO_4$-saturated 80% ethanol solution was then added, mixed by vortexing and incubated at 4°C for 1 hour. The nucleic acids were pelleted at 4°C by centrifuging at 3,000 x g for 10 minutes and the pellets briefly air-dried. The pellets were suspended in 5 mLs of a 25 mM TRIS-HCl (pH 7.5), 5% SDS, 7.5 mM EDTA solution followed by the addition of a 20 mL volume of the $NaClO_4$-saturated 80% ethanol solution. This mixture was incubated at 4°C for 1 hour and the nucleic acids pelleted by centrifuging for 20 minutes at 4°C. To air-dried pellets 7.5 mL of a 25 mM TRIS-HCl (pH 7.5), 0.2% SDS, 1 mM EDTA solution was added. Precipitation of nucleic acids was accomplished by adding 12.5 mL of 3 M $NaCH_3COO$ (pH 6.0) and 4.5 mL of isopropanol to this solution and incubating overnight at -20°C. The nucleic acids were pelleted by centifuging at 3000 x g for 20 minutes at -10°C. The pellets were suspended in a 10% solution of 3 M $NaCH_3COO$ stock solution diluted in 95% ethanol. Precipitation of nucleic acids was allowed to proceed overnight at -20°C. Nucleic acids were pelleted

by centrifuging at -10°C for 20 minutes and the pellet dried overnight by lyophilization.

RNA-containing pellets were solubilized in 50 µL of sterile double-distilled water and stored at -70°C. RNA content was determined and volumes equivalent to 2.5 µg of RNA translated *in vitro* for 60 minutes at 36.5°C in a rabbit reticulocyte lysate system (Pelham and Jackson, 1976) using [^{35}S]methionine as the labeled amino acid (NEN). Translation was terminated by placing the reaction tubes on ice. Samples of the translation products were used immediately or stored at -70°C.

2.2.3 Use of Thyroid Hormone to Examine its Effects on the Synthesis of Water-Insoluble Proteins in Rana catesbeiana Epidermal Cell Cultures

Thyroid hormone in the form of 3,3'5-triiodothyronine sodium salt (T_3) (stock solution of 1 x 10^{-10} moles/mL) was used to supplement the growth environments of primary epidermal cell cultures of *R. catesbeiana*. Explants and cells in culture were supplied with T_3 by its addition to growth medium or its direct application to epidermal explants.

2.2.3(a) Extraction of Water-Insoluble Proteins (Keratins) from Rana catesbeiana Epidermal Cell Cultures

Extraction of water-insoluble proteins from *Rana catesbeiana* cultured epidermal explants and cells followed

the methods described for epidermal tissues from other animal systems (Sun and Green, 1978b; Fuchs, 1981; Wu and Rheinwald, 1981). Cells and explants were scraped from 60 X 15 mm petri dishes and transferred to 15 mL conical bottom centrifuge tubes and each dish rinsed with 1-1.5 mL of 40 mM TRIS-HCl (pH 7.4). The cells and explants were spun at 2500 X g for 10 minutes in a table top centrifuge, the supernatants discarded and the pellets resuspended in 1-1.5 mL of cold (4°C) 40 mM TRIS-HCl (pH 7.4). The suspended explants and cells from tail fins were homogenized with a tight fitting teflon pestle attached to a Sorvall tissue homogenizer at a setting of 4 for 2-4 minutes and cells and explants from hind limb epidermis were homogenized by 100 strokes of a cold Dounce homogenizer. These homogenates were then sonicated with a Kontes micro-ultrasonic cell disruptor (Kontes Scientific, Vineland, NJ) for 4 X 15 seconds.

The final, sonicated homogenates were centrifuged for 20 minutes at 8000 x g in an SS-34 rotor in an RC2-B high speed centrifuge set at 4°C. The supernatants from the 8000 x g centrifugates were discarded and the pellets were resuspended in 2 mL of a 20 mM TRIS-HCl (pH 7.4) buffer containing 1% Triton X-100. The suspended pellets were sonicated (4 x 15 seconds) and incubated at room temperature for 10 minutes. After incubation, the suspensions were centifuged for 10 minutes at 8000 x g and the supernatants discarded. The 8000 x g pellets were

suspended in a keratin solubilizing buffer (KSBA) containing 20 mM TRIS-HCl (pH 7.4), 2% SDS, 1 mM EDTA, 10 mM dithiothreitol and 1 mM PMSF, sonicated for 4 x 15 seconds, and boiled for 3 minutes. The solubilized samples of the water-insoluble proteins were clarified by centrifuging for 5-7 minutes at 8000 x g and either used immediately or stored at -20°C.

2.2.3(b) 36 Hour Epidermal Cell Cultures for the Extraction of Water-Insoluble Proteins (Keratins)

R.catesbeiana tadpoles (stages IX-XI) were immobilized on ice, their notocords severed and their hind limbs and tail fins excised. The epidermal tissues were stripped from underlying muscle and prepared for cell culture as described in section 2.2.1(b). Cell cultures of hind limb epidermis (stages IX-XI) were divided among four different preparations of media (i) MEM-methionine (ii) MEM-methionine + 5% fetal calf serum (iii) MEM + 3 µL T_3/mL medium (3 X 10^{-10} moles T_3/mL medium) and (iv) MEM-methionine + 5 µL T_3 applied directly to each explant (T_3 concentration = 5 X 10^{-10} moles/explant) and maintained in a darkened tissue culture chamber for 36 hours. Primary cell cultures from tail fin (stages IX-XI) and hindlimb (stages XII-XV and XVI-XIX) epidermis were maintained using medium preparations (ii) and (iii) described above. All of the MEM-methionine used in their

preparation was diluted 66% with sterile double-distilled water and supplemented with 2 mM glutamine (1 mL of 200 mM glutamine stock solution/100 mL medium), 75 µg/mL gentamycin sulfate and 1.5 µg/mL fungizone. At the time of plating 60 µCi/mL of [^{35}S]methionine was added to each culture dish. The following day, 1 mL of appropriate medium and 60 µCi/mL of [^{35}S]methionine were added to each dish. After 36 hr in culture, the cells were lysed and water-insoluble fractions were prepared as described in section 2.2.3 (a).

2.2.3(c) Five Day Epidermal Cell Cultures for the Extraction of Water-Insolube Proteins (Keratins)

Tadpoles of the American bullfrog Rana catesbeiana were immobilized on ice, staged and their notocords severed. The tail fins and hind limbs were excised from animals of stages IX-XI and only hindlimbs were removed from animals of stages XII-XV and XVI-XIX. The epidermal tissues were prepared for cell culture as described in section 2.2.1(a). Separate epidermal cell cultures from hind limb of animals in stages IX-XI were maintained in a darkened tissue culture chamber at 22°C for 3.5 days in each of the following medium preparations: (i) modified mammalian Medium 199 (ii) Medium 199 + 5% F.C.S. (iii) Medium 199 + 3 µL T_3/mL medium (T_3 concentration = 3 X 10^{-10} moles/mL medium or (iv) Medium 199 + 5 µL T_3/explant.

Cell cultures from hindlimb of animals in stages XII-XV and in stages XVI-XIX, and tail fin epidermis were maintained in medium preparations (ii) and (iii) described above. All medium preparations were supplemented with 2 mM glutamine/mL medium, 75 µg/mL gentamycin sulfate and 1.5 µg/mL fungizone. On the first day of cell culture a single drop of one of the four above medium preparations was applied to directly to each explant. To maintain the humidity within each culture dish a few drops of the appropriate medium was added peripherally. Subsequently, one milliliter of fresh medium was given each day for 2 days. After 3 days of incubation diluted medium was removed from all culture dishes and replaced with MEM-methionine (Flow Laboratories) which did not have added serum or T_3 or contained 5% F.C.S., 3 µL T_3/mL or 5 µL T_3 applied directly to each explant. At the time of plating 60 µCi/mL of [^{35}S]methionine was added to each culture dish. The following day, 1 mL of fresh medium and 60 µCi of [^{35}S]methionine was added to each culture dish. Eighteen hours later the cells were lysed and water-insoluble fractions prepared as outlined in section 2.2.3(a).

2.2.3(d) Immunofluorescence of 36 Hour Epidermal Cell Cultures from Hindlimb (Stages IX-XI)

Primary epidermal cell cultures from tadpoles' hind limb (stages IX-XI) were maintained for 36 hours on collagen coated 22 x 40 mm glass coverslips in 60 mm plastic petri dishes. Each culture was covered with diluted modified mammalian medium (Medium 199) supplemented with either fetal calf serum or thyroid hormone (3×10^{-10} moles/mL) as detailed in section 2.2.3(b). At the end of 36 hours, coverslips were removed and washed by dipping them ten times in phosphate buffered saline (PBS) (pH 7.6). The cells adhering to the coverslips were fixed by immersing the coverslips into a 3% formaldehyde solution for 20 minutes at room temperature. The fixed cells, adhering to the coverslips, were rinsed in PBS (pH 7.6), immersed in acetone cooled to -20° C and maintained at -20° C for 20 minutes. The cells adhering to the coverslips were washed in PBS (pH 7.6) and either used directly or stored at 5° C in PBS.

Two 50 µL drops of commercially purchased rabbit antiserum to purified human cytokeratins (DAKO Corporation, Santa Barbara, CA) diluted 1:400 in PBS (pH 7.6) were placed at the bottom of each petri dish and the coverslips placed cell side down. The petri dishes were covered and incubated at 37° C for one hour. PBS (pH 7.6) was used to float the coverslips off the petri dishes and to wash out

excess primary antibody. The secondary antibody, FITC conjugated goat anti-rabbit IgG (GIBCO), was diluted 1:10 with PBS and two 50 µL drops of it were placed in plastic tissue culture dishes. The coverslips were placed cell side down and again incubated at 37° C. Petri dishes were flooded with PBS to lift coverslips from the surface. Excess secondary antibody was removed by repeated washings in PBS. The cells were washed sequentially in Dulbecco's PBS (pH 7.4) and Sorenson's buffer. The coverslips were mounted on slides with glycerol-PBS (pH 9.0) and sealed to the slides with melted dental wax.

To determine if the epidermal cells exhibited positive immunofluorescence with the rabbit antiserum to human cytokeratins a Leitz epifluorescent attachment on a Leitz photomicroscope II was used. Tri-X film (Kodak Canada, Toronto, Ontario) was used to photograph representative cells.

2.2.3(e) Immunoprecipitation of Keratins from Five Day Hindlimb Epidermal Cell Cultures

Premetamorphic Rana catesbeiana were divided into three stage groups (i) IX-XI (ii) XII-XV and (iii) XVI-XIX (Taylor and Kollros, 1946) and the hind limbs excised from immobilized animals. The epidermis was stripped from underlying muscle and primary cultures were prepared and maintained in 5% F.C.S. supplemented medium as outlined in section 2.2.3(c). On the fifth day, samples were prepared

by enriching for the keratin protein fraction as described in section 2.2.3(a) but solubilized in 200 µL of radioimmune precipitation buffer, RIPA (.05 M TRIS-HCl (pH 7.2), 1% triton X-100, 1% sodium deoxycholate, .1% SDS, .15 M NaCl and 1 mM PMSF (Atkinson et al., 1983)). Samples were then sonicated with a Kontes micro-ultrasonic tissue disruptor (4 x 15 seconds), boiled for 3 minutes and clarififed by centrifugation for 5 minutes in a Beckman microfuge. Supernatants containing the RIPA soluble proteins were removed and stored at -20° C. The insoluble pellets were suspended in 200 µL of SBA, sonicated (4 x 15 seconds) and boiled for 3 minutes. Clarification was performed by centrifuging samples for 5 minutes in a Beckman microfuge (Figure 1).

Alternatively, to determine whether all of the water-insoluble proteins which could potentially be precipitated by rabbit anti-human cytokeratin antibodies were being solubilized in RIPA and SBA, companion samples were solubilized in a final solution of 9 M urea and 1 mM PMSF and sonicated (4 x 15 seconds) (Figure 1).

Volumes equivalent to 5 x 10^5 cpm of incorporated [^{35}S]methionine were aliquoted and those samples solubilized in SBA or 9 M urea diluted four-fold with RIPA. All samples were mixed with commercially purchased rabbit antiserum to purified human cytokeratins (DAKO Corporation, Santa Barbara, CA) in amounts equivalent to 8.4 µg (dilution of 1:100) or 50 µg of antibody. The mixtures

Figure 1

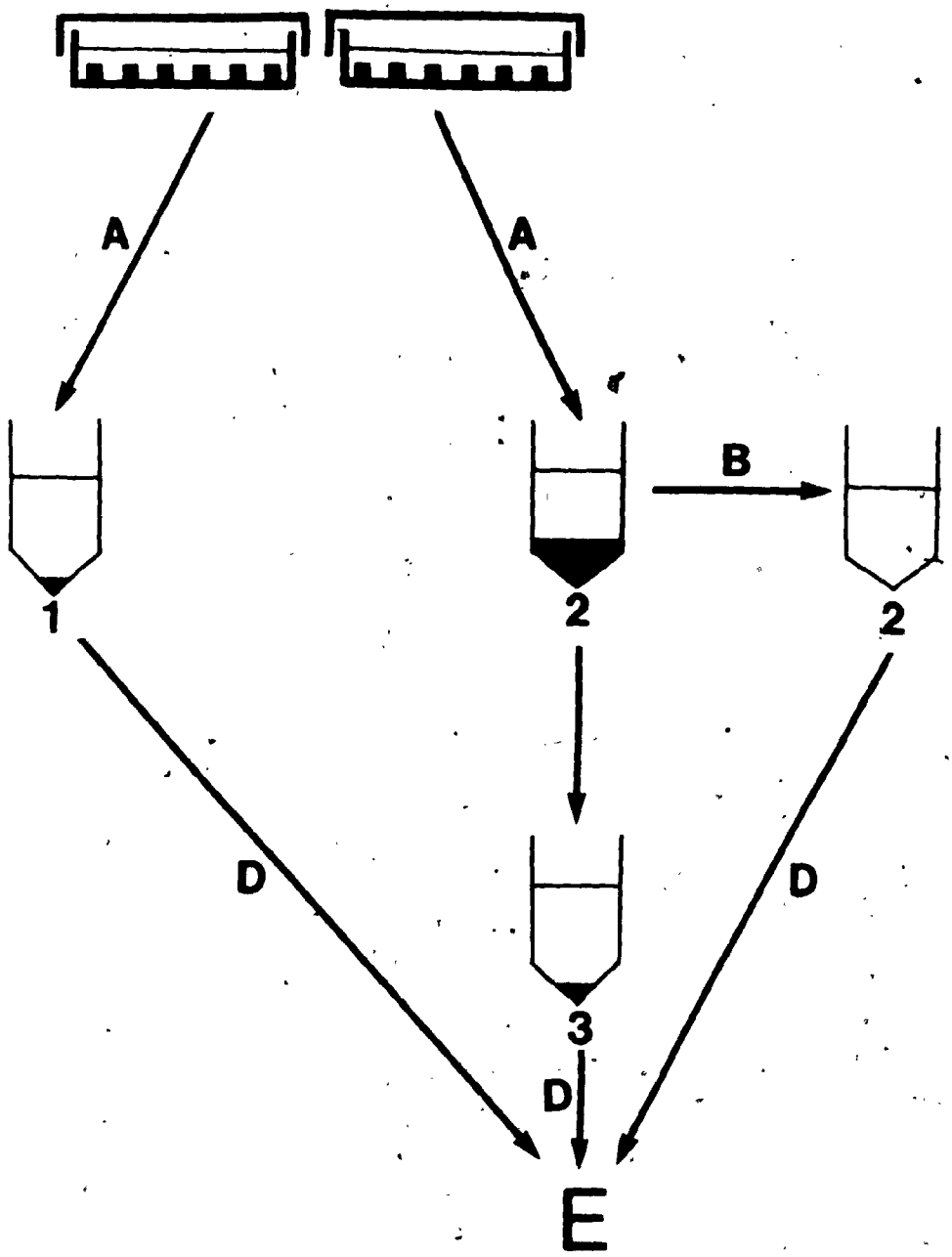
A Fractions enriched for water-insoluble proteins were solubilized in 9 M urea (fraction 1) or radioimmune precipitation buffer, RIPA, containing 1% Triton X-100, 1% sodium deoxycholate, .1% SDS, 0.15 M NaCl, 0.05 M TRIS-HCl (pH 7.2) and 1 mM PMSF (fraction 2).

B Since a large amount of the water-insoluble fraction was insoluble in RIPA, the supernatant containing those water-insoluble proteins which were soluble in RIPA was removed and stored at -20°C (fraction 2).

C To the large RIPA-insoluble pelllet 200 µL of buffer containing 2% SDS, 60 mM TRIS-HCl (pH 6.8), 10% glycerol, 1 mM PMSF was added. The mixture was vortexed, sonicated, boiled and insoluble material which remained insoluble pelleted by microfuging (fraction 3).

D Supernatant volumes equivalent to 5×10^5 cpm from each fraction (1, 2 and 3) were immunoprecipitated with rabbit anti-human cytokeratin antibodies.

E The individual immunoprecipitates from fractions 1, 2 and 3 were washed and solubilized in a 50 µL final volume of buffer containing 2% SDS, 60 mM TRIS-HCl (pH 6.8), 10% glycerol, 1 mM PMSF and 5% 2-mercaptoethanol. Acid-precipitable cpm were determined from each fraction and the water-insoluble proteins in each fraction were separated by SDS-PAGE.


A
A
B
1
2
2
D
D
3
D
E

were incubated for 20 minutes at room temperature and followed by the addition of 100 µL of formalin-fixed Staphylococcus aureus Cowan I strain (Calbiochem-Behring, a division of American Hoescht Corp., La Jolla, CA; standardized to bind 2+/-1 mg IgG/mL) (Atkinson et al., 1983). Samples were microfuged and the pellets washed thoroughly 3 times with RIPA. The precipitates were solubilized in 50 µL of 60 mM TRIS-HCl (pH 6.8), 2% SDS, 1 mM PMSF, 5% 2-mercaptoethanol buffer (SBA) boiled for 3 minutes and then microfuged for 5 minutes (Atkinson et al.,1983). Samples were either used immediately or stored at -20°C.

2.2.4 Methods for Studying the Synthesis of Proteins in Epidermal Cells from Hind Limb in situ

Spontaneously metamorphosing and premetamorphic R. catesbeiana were labeled with a radioactive amino acid in situ. The length of time maintained subsequent to labeling differed. In addition, different methods were used to extract proteins from hind limb epidermis. Therefore, the procedures involved in both instances will be considered in detail.

2.2.4(a) Extraction of Water-Insoluble Proteins (Keratins) from Premetamorphic Rana catesbeiana Labeled in situ.

Premetamorphic Rana catesbeiana tadpoles were

immobilized on ice and divided into three groups based on their developmental stage: (i) IX-XI (ii) XII-XV and (iii) XVI-XIX (Taylor and Kollros, 1946). During this study all animals were starved and maintained at 22°C. In order to minimize the effects of fluctuating endogenous levels of T_3, these experiments were conducted in the late autumn and early winter.

Precocious metamorphosis was induced by a single injection of 3×10^{-10} moles/gram body weight of triiodothyronine (T_3). Tadpoles designated as controls were injected with an equal volume of .01 N NaOH (i.e. 3 µL of NaOH/gram body weight). Animals were injected concurrently with 60 µCi/mL of [^{35}S]methionine and T_3 or NaOH. A second injection of 60 µCi/mL of [^{35}S]methionine was administered 18 hours after the first injection. At the time of sacrifice, animals were immobilized on ice, staged and their notochords severed. Hind limbs were excised and the epidermis stripped from underlying muscle. The water-insoluble proteins were extracted from epidermal tissues as described in section 2.2.3(a) and these samples used immediately or stored at -20° C.

2.2.4(b) Extraction of Total Proteins from Hind Limb Epidermis of Spontaneously Metamorphosing *Rana catesbeaiana*

Spontaneously metamorphosing *Rana catesbeiana* were briefly immobilized on ice, staged (Taylor and Kollros,

1946) and injected through the proximal tail musculature with 3.3 μCi/mL of L-[U-^{14}C]leucine or 60 μCi/mL of [^{35}S]methionine. Consciousness was restored by placing animals in a small volume of previously aged and aerated tap water. The animals were maintained at 20°C for 4 hours after which time they were sacrificed by cervical dislocation or severing the notochord. Hindlimbs were removed and the epidermis stripped from underlying muscle. Any muscle tissue which remained adherent was scraped from the epidermis with a scalpel. Epidermal tissues were minced into 2 mm^2 pieces and solubilized with a Sorvall tissue solubilizer at a setting of 4 in 9 M urea and 1 mM PMSF and 15% 2-mercaptoethanol. Extractions were clarified by centrifuging at 5000-7000 x g in a Sorvall RC2-B high speed centrifuge at 4°C. Samples were used immediately or stored at -20°C.

2.2.5 SDS Polyacrylamide Gel Electrophoresis and Fluorography

To analyze polypeptides, electrophoretic separation on polyacrylamide gels was used. One dimensional separation of proteins on the basis of molecular mass (M_r) was effected in the presence of SDS using a slightly modified method of Laemmli (1970). Prior to electrophoresis, samples solubilized in 9 M urea were adjusted to contain 2% SDS, 60 mM TRIS-HCl (pH 6.8), 10% glycerol, 1 mM PMSF and 5% 2-mercaptoethanol. When buffer containing 2% SDS was

used to solubilize proteins, samples were boiled for 3 minutes before electrophoretic separation. Heat shock experiments on primary cultured cells from the epidermis of premetamorphic American bullfrogs and the established X. laevis kidney epithelial cell line utilized 3-15% gradient polyacrylamide separating gels containing 0.1% SDS. Water-insoluble proteins were separated on 7.5-20% linear gradient gels containing 0.1% SDS. In all instances, separating gels were overlaid with a 3% stacking gel and a 25 mM TRIS-HCl (pH 8.3) running buffer added to upper and lower baths of electrophoretic tanks. Electrophoresis was conducted at 5 mA/gel until bromphenol blue, the tracking dye added to the upper running buffer bath, reached the stacking gel/separating gel interface at which time the milliamperage was increased to 15 mA/gel. Electrophoresis was considered complete when the tracking dye reached the bottom of the separating gel when 3-15% gradients were used. Separating gels which contained 7.5-20% polyacrylamide were electrophoresed for an additional 2 hours subsequent to the tracking dye reaching the bottom of the slab gel.

Two-dimensional analysis involving the separation of proteins on the basis of their isoelectric point in the first dimension and their M_r in the second dimension, followed the method of O'Farrell (1975). Isoelectric focusing was performed on 10 cm x 0.3 cm tube gels containing 8 M urea, 4% acrylamide, 1% Nonidet P-40 (NP-40)

(BDH Chemicals Ltd., Poole, England) and 2% ampholines. To establish the pH range, ampholines in the pH range of 3.5-10 (LKB Instruments Inc., Rockville, Maryland) were used to separate total proteins from primary epidermal cell cultures from R. catesbeiana and X. laevis kidney epithelial cell cultures synthesized during heat-stress. The in vitro translation products of total cellular RNA from heat-stressed X. laevis kidney epithelial cells (XKEC) were separated in the first dimension using pH 5-8 ampholines (Pharmacia Fine Chemicals, a division of Pharmacia Inc., Piscataway, N.J.). This ampholine range was also used to separate the proteins from XKEC incubated at 22 and 36.5°C for 1 hour and 34°C for 2 hours prior to the addition of [^{35}S]methionine. For all two-dimensional separations of water-insoluble proteins synthesized by primary epidermal cell cultures from R.catesbaina, a mixture of .3 mL of pH 5-7 (LKB) and .2 mL of pH 3.5-10 ampholines was used. Before the addition of lysates, the tube gels were prefocused at 200 V for 15 minutes, 300 V for 30 minutes and 400 V for 1 hour in an upper cathode buffer of .05 N NaOH and lower anode bath of .05 N H_3PO_4. Cathode and anode buffers were replaced with fresh ones at the end of the prefocusing period.

Ampholines, equivalent to 2%, were added to samples solubilized in 9 M urea. Prior to the addition of ampholines (2%) and NP-40 (1%), samples solubilized in buffer containing 2% SDS were saturated with urea.

Isoelectric focusing was performed for 12-18 hours at 400 V. One hour before the end of electrofocusing the voltage was increased to 800. Tube gels were equilibrated in 2 changes of buffer containing 2% SDS, 60 mM TRIS-HCl (pH 6.8), 10% glycerol, 1 mM PMSF and 5% 2-mercaptoethanol over a 20 minute period prior to overlaying slab gels for electrophoresis in the second dimension. The isoelectric focusing gels were secured to slab gels with 1% Noble agar dissolved in 1% SDS. Electrophoresis in the second dimension was carried out in accord with the conditions described above for one-dimensional separation.

To characterize the isoelectic point of electrofocused proteins, the pH gradients of the electrically focused standard gels were determined by directly measuring the pH in intact gels with a pH probe (Bio-Rad Gel Pro-pHiler, Bio-Rad laboratories, Richmond, CA) or by slicing companion gels and determining the pH of the water extract (Saleem and Atkinson, 1976).

At the end of the separation of proteins on slab gels, proteins were stained with Coomassie G-250 or R-250 (Bio-Rad Laboratories, Richmond, California), destained and photographed. Gels were then prepared for fluorography according to the method of Bonner and Laskey (1974) and Laskey and Mills (1975). Briefly, gels were taken through 3 consecutive changes of dimethyl sulfoxide (DMSO) of 1/2 hour each and then placed in a solution of 2,5-diphenyloxazole (PPO)/DMSO (22% w/v) for 3 hours. At

the end of this period, gels were rinsed overnight in cold running water and subsequently dried under vacuum onto Whatman 3MM filter paper on a heated gel dryer (Bio-Rad Model 120). X-ray film (RP X-Omat, Kodak Canada, Toronto, Ontario), pre-flashed to an optical density of 0.15, was placed flashed side down on dried gels containing incorporated radioactive amino acid and exposure of the film carried out at -70°C in the dark. Exposed X-ray films were developed as outlined by Kodak Canada for the development of X-rays.

2.2.6 Quantitation of Protein, RNA and Incorporated Radioactive Amino Acid Content in Cell Lysates and Water-Insoluble Fractions

2.2.6(a) Quantitation of Protein

Protein determinations were performed according to the method of Lowry et al. (1951) and the turbidometric assay method of Comings and Tack (1972) using bovine serum albumin (BSA) as the standard. Standards and samples were read on a Gilford spectrophotometer and from the optical density curve drawn from known quantities of the BSA standard, the protein content of lysates was determined by interpolation.

2.2.6(b) Determination of RNA Content for in vitro Translation

To determine the amount of RNA to be added to the

rabbit reticulocyte *in vitro* translation system it was first necessary to calculate the amount of total cellular RNA extracted from *X. laevis* kidney epithelial cells. To this end, 2.5 and 5 µL aliquots of RNA in solution were diluted with 1 mL of double-distilled water and optical density readings were performed at A_{260} and A_{280}. From these values, RNA content was calculated using the following formula : optical density (A_{260}) x dilution factor x 50 µg RNA/mL/optical density unit (Kidder, 1972).

2.2.6(c) Preparation of Samples for the Determination of Incorporated [^{14}C]leucine or [^{35}S]methionine

The amount of radioactivity incorporated into proteins from total cell lysates and water-insoluble fractions was determined by acid-precipitation of proteins. To a 10 µL aliquot of sample, 1 mL of 10% trichloro acetic acid (TCA) was added and precipitation allowed to proceed on ice. The acid-precipitable proteins were pelleted by centrifugation at 5000g for 20 minutes, the supernatants discarded and the pellets washed with ether. Samples were vacuum-dried overnight and the pellets solubilized in 100 µL of buffer containing 60 mM TRIS-HCl (pH 6.8), 2% SDS and 5% 2-mercaptoethanol. A 50 µL aliquot of this suspension was added to 10 mL of Triton X-100/toluene/Omnifluor (NEN) cocktail (Turner, 1968).

The amount of [^{35}S]methionine incorporated into proteins synthesized from total cellular RNA extracted from

X. laevis kidney epithelial cells in the rabbit reticulocyte in vitro translation system and the water-insoluble proteins precipitated with rabbit anti-human cytokeratin antibodies was determined in a slightly different manner. A one microliter volume of the sample was spotted onto 1 square centimeter pieces of Whatman 3MM filter paper and boiled for 20 minutes in a 10% TCA solution containing 'cold' methionine. Boiling was stopped and the filter papers precipitated in the TCA solution by the addition of ice. The filter papers were taken through 2 changes each of distilled water, 95% ethanol and acetone, respectively and allowed to air-dry. Once dried the filter papers were placed in liquid scintillation vials (NEN) to which 1.5 mL of NCS tissue solubilizer (Amersham Corp., Arlington Heights, Illinois) was added. The vials were incubated for 30 minutes at 55°C and allowed to cool before adding 10 mL of tolene/Omnifluor (NEN) cocktail and 17 µL of glacial acetic acid. Radioactivity was determined on a Beckman LS-255 scintillation counter.

PART 3

RESULTS

3.1 Established Xenopus laevis Kidney Epithelial Cell Cultures

Although Rafferty (1976) had shown that the established X. laevis kidney epithelial cell line (XKEC) could both survive and reproduce across a wide temperature range, little had been done to elucidate quantitative (Rafferty, 1976) or qualitative changes in the proteins synthesized at various temperatures. Based on earlier results (Rafferty, 1976) for X. laevis kidney epithelial cells, 22°C was considered and used as the optimum growing temperature in this study. To assess any changes in gene expression (as reflected by the proteins synthesized) which may result from incubation at these temperatures, the proteins from these cells were separated by gel electrophoresis and the newly synthesized proteins observed by fluorography.

3.1.1 Heat Shock Induction of the Established Adult Xenopus laevis Kidney Cell Line

The incubation of Xenopus laevis kidney epithelial cells for 2 hr at elevated temperatures results in the new

and/or enhanced synthesis of a number of polypeptides. These polypeptides are not easily resolved by one-dimensional fluorography (Figure 2). However, the detection of these proteins is facilitated by separation in two dimensions. Proteins with M_rs of 95.5 kd (pI 6.6), 80 kd (pI 6.6), 75 kd (pI 7.0), 68 kd (pI 6.2), 59 kd (pI 5.7), 43 kd (a polypeptide which resolves into 3 separate isoelectric variants with pIs of 5.6, 6.0 and 6.7) and 38 kd (pI 6.6) are readily observed (Figure 3). The 75 kd protein is first noted following incubation at 32°C and continues to be synthesized at 34 and 36.5°C. At 34°C, the 80, 68 and 59 kd are enhanced and the 3 isoelectric variants of the 43 kd protein and 38 kd protein are first detected. The synthesis of the 43 kd and 38 kd proteins is increased at 36.5°C. Interestingly, a protein with an M_r of 57 kd (pI of 5.6), undetected at lower temperatures, is a major protein product at 34°C and is apparently synthesized to a lesser degree at 36.5°C. The 95.5 kd protein (pI 6.6) appears to represent an enhanced synthetic product; it is detectable at the lower temperatures examined, but is particularly prevalent at 34 and 36.5°C (Figure 3, panels D and E). Although the depressed synthesis of some proteins is apparent at higher temperatures, it is not remarkable (Figure 3, area within the circles).

A one hour incubation at 36.5°C is sufficient to

Figure 2.

Fluorogram of one dimensional SDS-PAGE (3-15% gradient gel) separation of the proteins synthesized by XKEC following a 2 hr incubation at control (22°C), depressed (<22°C) and elevated (>22°C) temperatures. Unless specified to the contrary, [^{35}S]methionine was the labeling probe. Cell cultures were incubated at 5 (lane 1), 10 (lane 2), 15 (lane 3), 22 (lane 4, [^{14}C]leucine), 30 (lane 6), 32 (lane 7), 34 (lane 8, [^{14}C]leucine), 34 (lane 9) and 36.5°C (lane 10). The positions of the molecular mass markers are indicated on the left and the new and/or enhanced proteins determined by two-dimensional separation on the right. Approximately 20,000 cpm of acid-precipitable proteins were applied to each lane.

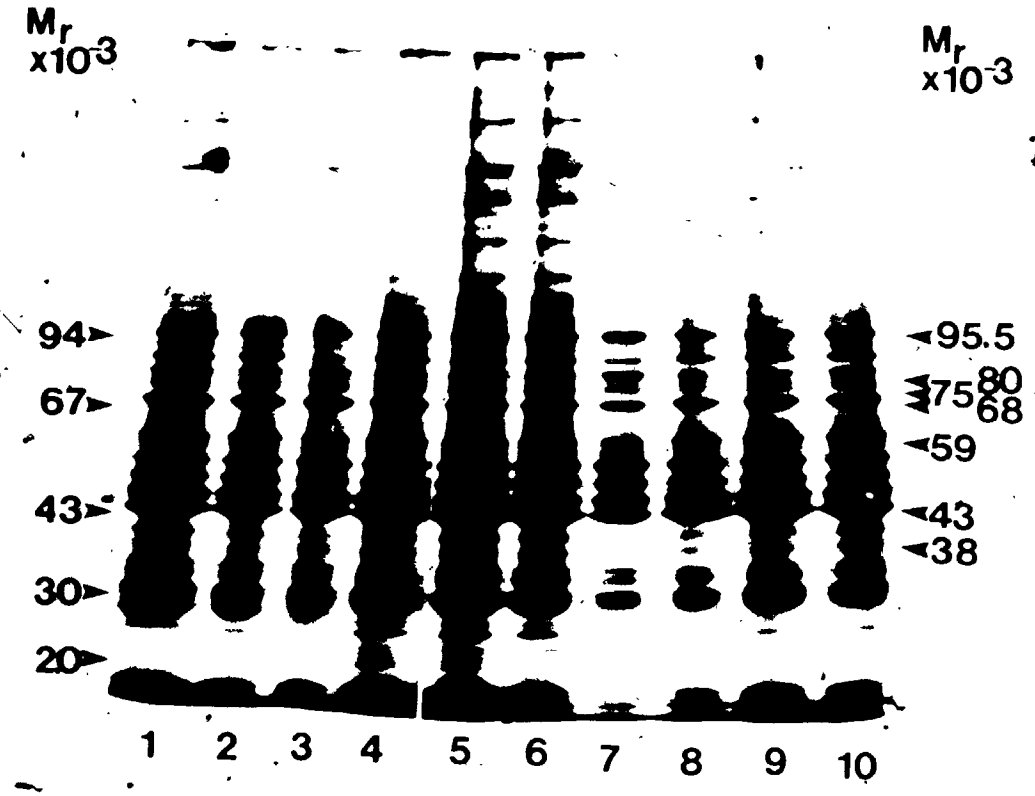

Mr x10-3
Mr x10-3
94
67
43
30
20
95.5
80
75
68
59
43
38
1 2 3 4 5 6 7 8 9 10

Figure 3.

Fluorographic analysis of two-dimensional polyacrylamide (3.5-10 ampholines in the first dimension and 3-15% gradient in the second) gel electrophoretic separation (IEF-SDS-PAGE) of the polypeptides synthesized in the presence of [^{35}S]methionine by XKEC following a 2 hr incubation at 22 (control, A), 30 (B), 32 (C), 34 (D) and 36.5°C (E). New and/or enhanced synthesis of proteins with M_rs of 95.5, 80, 75, 68, 59, 57, 43 and 38 kd are indicated by arrowheads. The 75 kd protein is first noted at 32°C (arrowhead in panel C) while the remainder of the new and/or enhanced polypeptides are observed at 34 and 36.5°C (arrowheads in panels D and E, respectively). The synthesis of some proteins is depressed when XKEC are incubated at higher temperatures (see area within the circles in all panels). Molecular masses of the standards and the new and/or enhanced proteins are indicated on the left and right, respectively. The pH range is indicated along the bottom of each gel. Approximately 100,000 cpm of acid-precipitable lysate was applied to each electrofocusing gel.

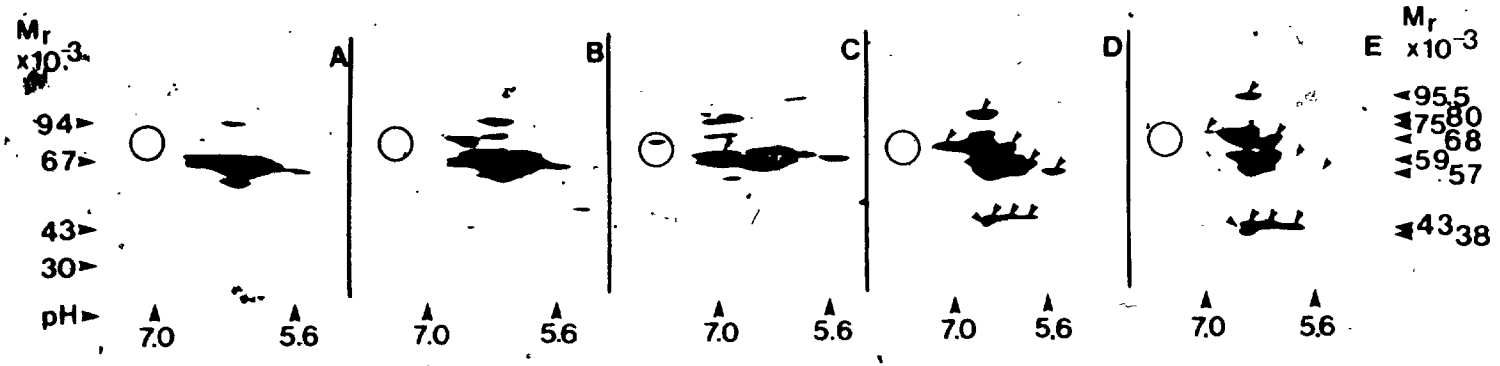
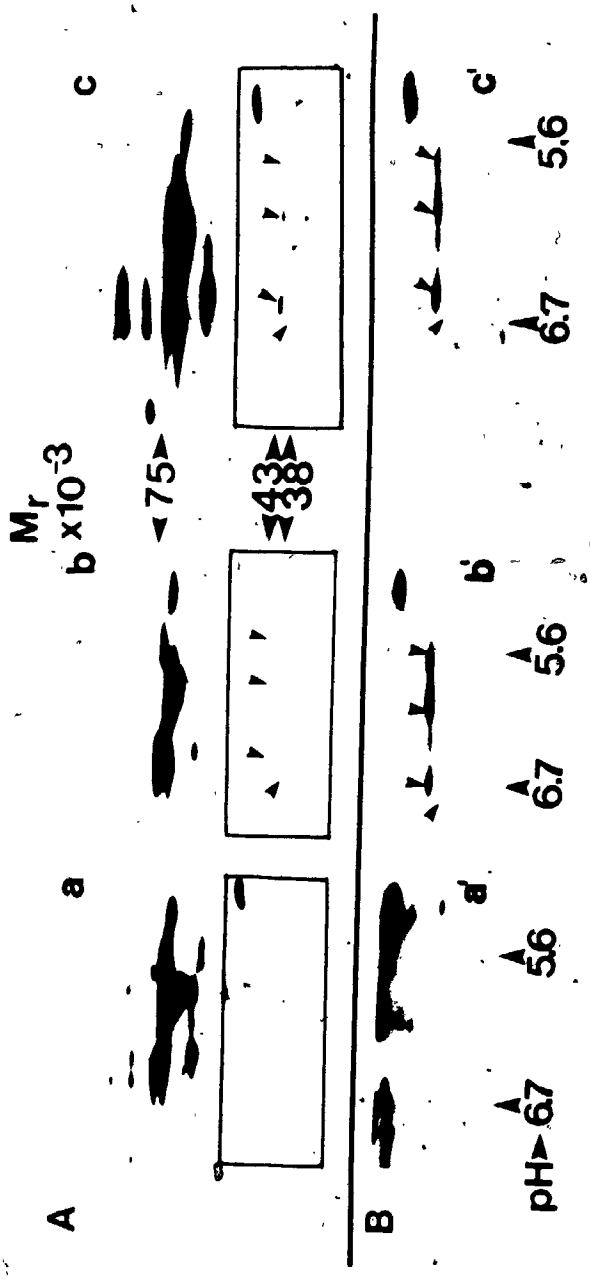

Mr x10-3
94
67
43
30
pH
A
B
C
D
E
7.0
5.6
Mr x10-3
95.5
80
75
68
59
57
43
38

Figure 4.

Fluorographic analysis of two-dimensional (3-15% gradient) polyacrylamide gel electrophoretic (IEF-SDS-PAGE) separation of XKEC incubated at control (22°C) and heat shock (36.5°C) temperatures (Part A, panels a and b, respectively) for 1 hr and at 34°C for 2 hr (Part A, panel c). The apparent new synthesis of the 3 distinct isoelectric variants of the 43 kd and the 38 kd proteins are indicated by arrowheads in panels b and c (A). The enclosed areas in Part A are repeated after longer exposure of the fluorogram in Part B. The panels shown in B demonstrate more profoundly the absence of these polypeptides at 22°C (a') and their presence at 36.5°C (b') and 34°C (c'). Approximately 50,000 cpm of acid-precipitable lysate was applied to each electrofocusing gel. An expanded 5-8 pH range was used to separate proteins in the first dimension. Molecular masses are indicated between panels b and c in Part A and the pH range along the lower panels a', b' and c' in Part B.


M_r x10^{-3}
75
43
38
a
b
c
a'
b'
c'
A
B
pH
6.7
5.6
6.7
5.6
6.7
5.6

induce the synthesis of the 43 and 38 kd polypeptides (Figure 4, part A, panels a and b). These proteins correspond to the 3 isoelectric variants of the 43 kd protein and the 38 kd protein noted following a 2 hr incubation at 34°C (Figure 4, Part A, panels b and c and Part B, panels b' and c'). With the exception of these low M_r proteins, the new and/or enhanced synthesis of other proteins (as discussed above) are not detected subsequent to a 1 hour incubation.

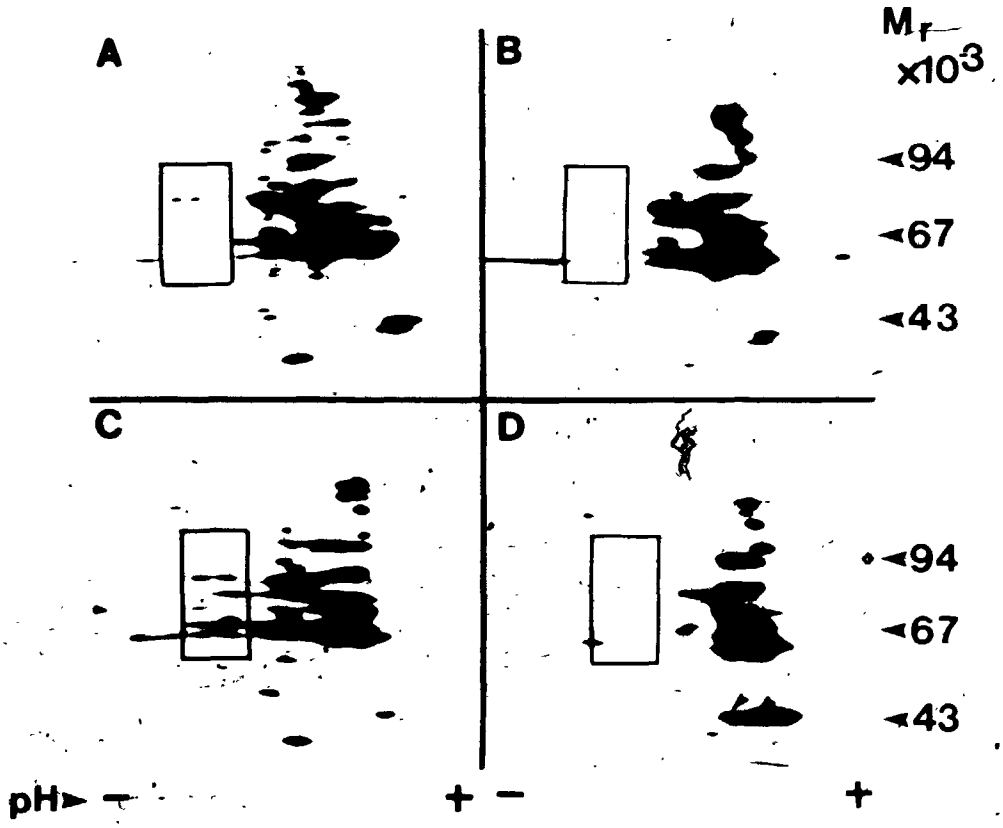
Exposure of X. laevis kidney epithelial cells (XKEC) to sub-optimal temperatures (10 and 15°C) does not result in the new and/or enhanced synthesis of any particular polypeptides, including any of those which are noted at heat shock temperatures. When cells are incubated at 10 or 15°C no deviation from the normal (22°C) pattern of protein synthesis is apparent by fluorography (Figure 5). In addition, the depressed synthesis of some polypeptides, synthesized by cells incubated at the control tempererature (22°C) is detectable but not remarkable at depressed temperatures in this system (see area within the rectangles in Figure 5).

3.1.2 In vitro Translation of Total Cellular mRNA Isolated from Control and Heat Shocked Xenopus laevis Kidney Epithelial Cells

The isolation and in vitro translation of total

Figure 5.

Fluorographic analysis of the polypeptides separated by IEF-SDS-PAGE (3-10 ampholines in the first dimension and 7-17.5% gradient gel in the second dimension) of the polypeptides synthesized by XKEC incubated at 10 (panel A), 15 (panel B) and 22°C (panel C). Panel D is a 2 hr incubation at 34°C included to demonstrate that the new and/or enhanced synthesis of the 43 and 38 kd proteins (arrowheads) are detectable with these particular conditions of separation. The areas within the rectangles represent the depressed synthesis of some proteins observed following incubation at 10°C (A), 15°C (B) and 34°C (D) relative to the control temperature of 22°C (C). The position of co-electrophoresed molecular mass standards are indicated to the right.


A
B
C
D
M_r
$\times 10^3$
94
67
43
94
67
43
pH
−
+
−
+

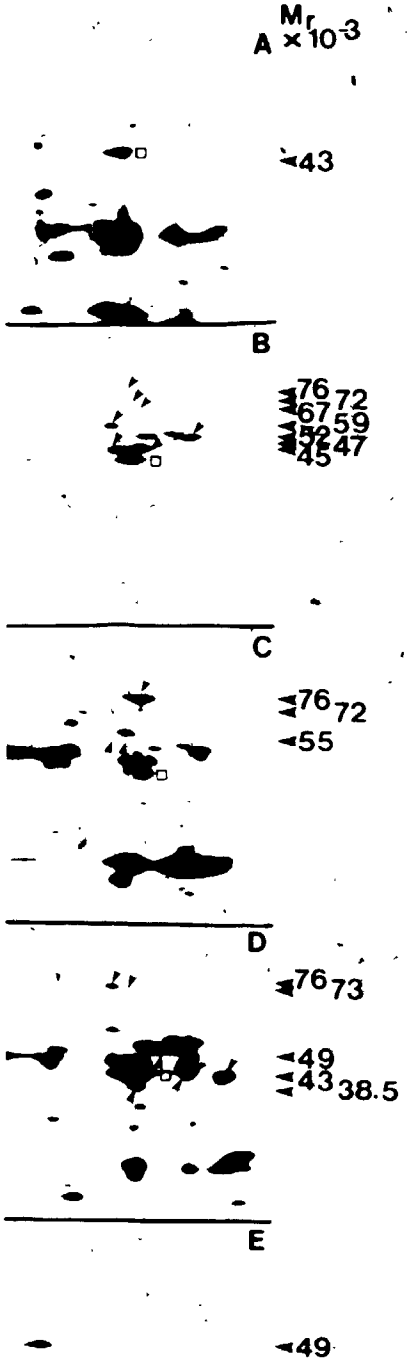
cellular RNA from Xenopus laevis kidney epithelial cells (XKEC) incubated at 22 and 34°C reveals that heat shock markedly alters the mRNA population available for translation. From the results shown in Figure 6, it is noted that while many of the same mRNA species are translated in control and heat-shocked cells, the translation of the control RNAs is depressed in cells incubated at 34°C. Proteins with M_rs of 76 kd (pI 5.9), 72 kd (pI 5.9), 68 kd (pI 5.7), 59 kd (pI 5.9), 52 kd (pI 5.3), 47 kd (pI 5.7) and 45 (pI 5.9) are novel and/or enhanced translational products of cells incubated at 34°C (Figure 6, panel B).

A 2 hr incubation at 41.5°C caused a significant proportion of the Xenopus laevis cells to lift from the flask. The cells which lifted were kept separate from those which remained adherent. Total cellular RNA was extracted from both groups (hereafter referred to as LXKEC and AXKEC for lifted and adherent cells, respectively) and translated in vitro.

The proteins synthesized in vitro from the AXKEC RNA population closely paralleled the products observed when RNA from cells incubated at 34°C for 2 hr is translated. With the exception of a relatively minor 55 kd protein which resolves into 2 isoelectric variants with pIs of 5.9 and 6.0 (Figure 6, panel C, arrowheads), the differences which exist between cells incubated at 34°C and 41.5°C

Figure 6.

Two-dimensional separation (IEF-SDS-PAGE) of the *in vitro* translation products from total cellular RNA isolated from control (22°C, panel A), heat-shocked at 34°C (panel B), adherent XKEC following incubation at 41.5°C (panel C) and lifted XKEC following incubation at 41.5°C (panel D). The molecular masses of the new and/or enhanced proteins subsequent to heat-shock are indicated on the right and the particular proteins by arrowheads within the panels. An open square in panels A, B, C and D is used to the mark the position of a 43 kd referance protein. Panel E represents the protein(s) synthesized from the RNA which is endogenous to the translation kit. Ampholines expanded through the 5-8 pH range were used in the first dimension and a 7-17.5% polyacrylamide gel in the second. Approximately 150,000 cpm of acid-precipitable protein were applied to each electrofocusing gel.


A
Mr ×10-3
43
B
76
72
67
59
52
47
45
C
76
72
55
D
76
73
49
43
38.5
E
49
pH - 5.9 5.2

(AXKEC) are in the intensity of some proteins. Particularly apparent is the intensity of the 76 and 72 kd proteins (both with pIs of 5.9) in AXKEC.

The translation products from LXKEC RNA exhibit a number of variations. In addition to the 76 kd (pI 5.9) which is less intense in the LXKEC, a 73 kd protein (pI 5.75) is clearly resolved. Although the 73 kd protein may be present in the AXKEC cells, the synthesis of the 76 kd polypepetide is so intense as to inhibit clear detection of the 73 kd protein. Also, 2 isoelectric variants of a 49 kd (pIs 5.45 and 5.3), a 38.5 kd (pI 5.7) and an acidic 43 kd (pI 4.85) protein are translated at obviously discernable levels (Figure 6, panel D). The molecular masses of the translation products with M_rs of 76, 68, 59, 43 and 38 kd correspond to proteins observed in heat-shocked XKEC <u>in vivo</u>.

3.2 Primary Epidermal Cell Cultures from Rana catesbeiana Tadpole Hind Limb and Tail Fin

Movement of epidermal cells out from tail fin or hind limb explants is observed within 6 hours after plating. Within 24 hours of plating, the epidermal cells form either a continuous migrating layer, or completely seal the wounded epidermal explant. This is in agreement with reepithelialization studies of isolated Rana tail fin discs (Derby, 1968; 1978) and embryonic rabbit epidermal explants (Banks-Schlegel, 1982). After 5 days of cell culture the epidermal cells begin to lift from the plate, undergo cell death, or are overgrown by fibroblasts.

The effects of increasing or decreasing temperature have an obvious morphological impact on the epidermal cell cultures from hind limb or tail fin (Figures 7 and 8, respectively). As the increment from the optimum growing temperature of 22°C is increased in either positive or negative direction, the area around the cells becomes refractile, the previously continuous epidermal cell sheets are interrupted by numerous discontinuities and the cells themselves have an altered attenuated morphology. The greater the temperature shift, the more profound is the morphological impact.

3.2.1 Thermal Shift Induction of New Polypeptide Synthesis in Primary Epidermal Cell Cultures from R.catesbeiana Tadpoles

Figure 7.

The effect of temperature elevation and depression on 5 day epidermal cell cultures from hind limb of R. catesbeiana (stages XII-XV). Subsequent to a 2 hr incubation at 5 or 15°C and a 1 hr incubation at 22, 30 or 36.5°C, representative areas of epidermal cell sheets were photographed by phase contrast microscopy. (x 85)

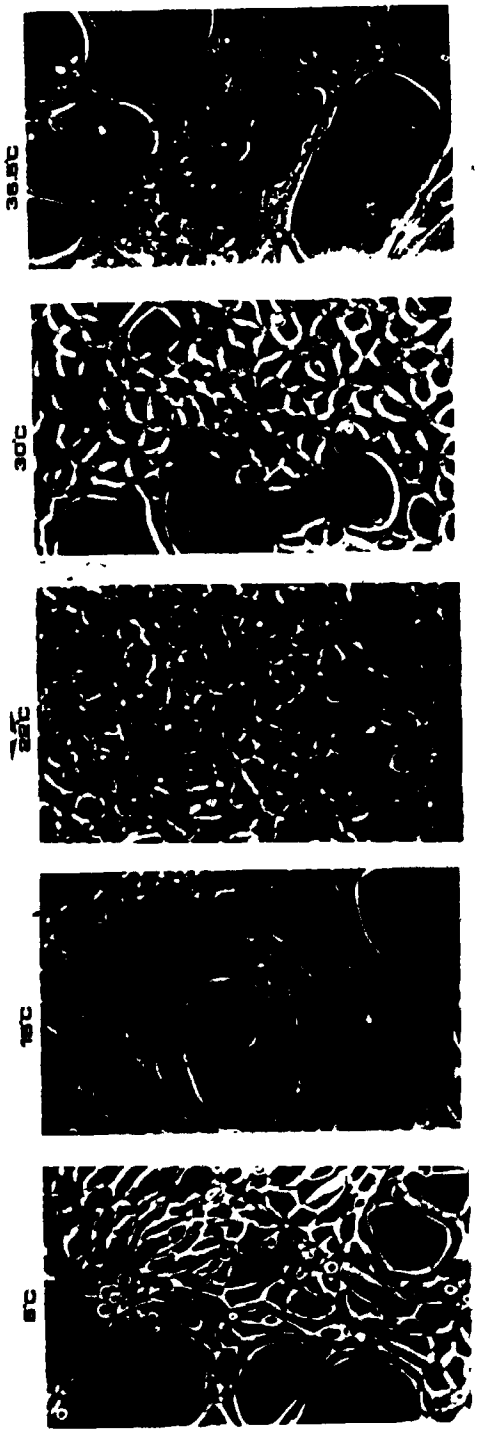

36.5°C
30°C
22°C
15°C
8°C

Figure 8.

The effect of temperature elevation on 5 day epidermal cell cultures from tail fin of R. catesbeiana (stages XII-XV) following a 1 hr incubation at control (22°C) and 32 and 36.5°C. Representative areas of the epidermal cell sheets were photographed by phase contrast microscopy (x 85).

36.5°C
32°C
22°C

Incubation of tail fin or hind limb epidermal cell cultures at temperatures between 10°C and 30°C did not result in detectable qualitative changes in the proteins synthesized. However, cells incubated at 5°C for 2 hours or at 32°C, 34°C or 36.5°C for 1 hour results in the new and/or enhanced synthesis of a protein with an M_r of 65 kd (Figure 9). The synthesis of this polypeptide becomes more pronounced as the temperature is increased (Figure 9); at 36.5°C it is the major polypeptide synthesized. The shift of these cultures to 36.5°C also resulted in the depressed synthesis of the proteins normally made at 22°C and the induction of a protein with M_r of 25 kd which is not detectable at other experimental temperatures (Figure 9). Although the 65 kd polypeptide is detectable at 5°C, neither the 25 kd protein nor the generalized depression of the proteins synthesized at the control temperature is apparent.

Two-dimensional analysis of hind limb and tail fin cultures indicates that the apparently novel 65 kd protein has a pI of 6.7-6.8 (Figures 10 and 11, respectively). However, as the temperature is increased, its synthesis becomes so pronounced that the pI extends across a range of 6.5-7.0. The pI of the 25 kd protein was determined to be 6.5. This method of protein separation also demonstrated the absence of the 25 kd protein at all temperatures but 36.5°C (Figures 10 and 11).

Figure 9.

Fluorogram of one-dimensional SDS-polyacrylamide (3-15% gradient) gel electrophoretic separation of the polypeptides synthesized by 5-day primary epidermal cell cultures from R. catesbeiana (stages XII-XV). Lanes 1 and 9 illustrate the proteins synthesized by hind limb epidermal cells following incubation at the temperature extremes of 5 and 36.5°C, respectively. The pattern of polypeptides synthesized by tail fin epidermal cells is demonstrated following incubation at 5 (lane 2), 10 (lane 3), 15 (lane 4), 22 (lane 5), 30 (lane 6), 32 (lane 7), 36.5°C (lane 8). The molecular masses of new and/or enhanced polypeptides are indicated by arrowheads on the left and right. Approximately ½10,000 cpm of acid-precipitable lysate were applied to each lane.

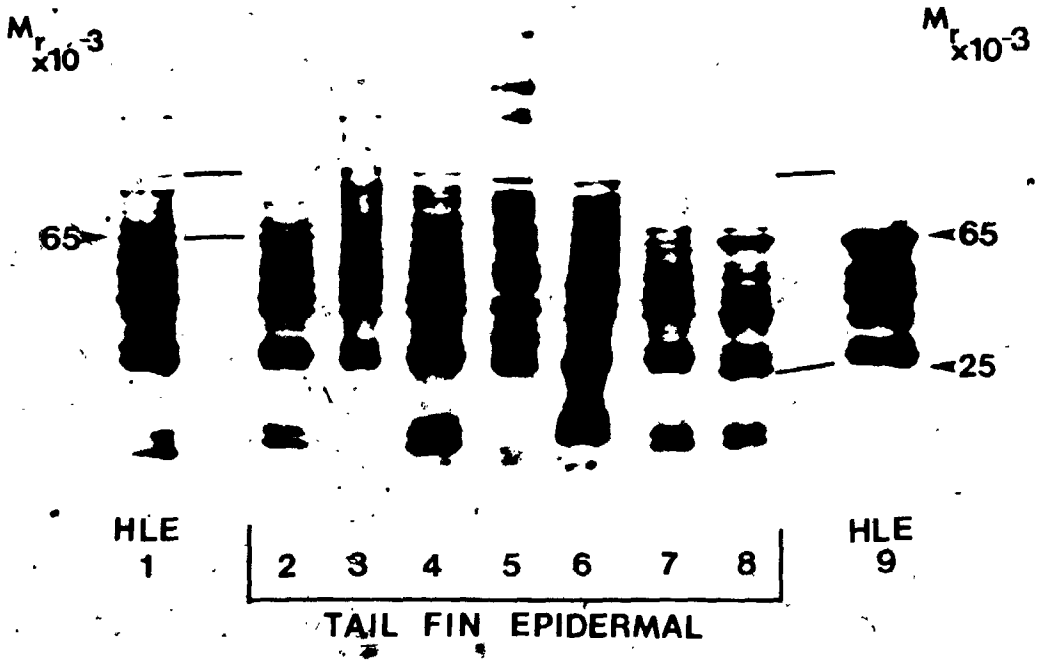

M_r x10^{-3}
M_r x10^{-3}
65
65
25
HLE 1
2 3 4 5 6 7 8
HLE 9
TAIL FIN EPIDERMAL

Figure 10.

Fluorographic analysis of two-dimensional polyacrylamide gel electrophoretic (IEF-SDS-PAGE) separation of the proteins synthesized by 5-day primary cultures from hind limb of R. catesbeiana (stages XII-XV) following incubation at (from left to right) 5, 10, 15, 22, 32, 34 and 36.5°C. Arrowheads indicate the temperature dependent appearance of a 65 kd protein with pI 6.7-6.9 at 5, 32, 34 and 36.5°C. A 25 kd protein with pI 6.7-6.9 at 36.5°C is also indicated by an arrowhead. Ampholines in the pH range of 3.5-10 were used in the first dimension and a 3-15% gradient gel to separate proteins in the second dimension. To each first dimensional electrofocusing gel approximately 50,000 cpm of acid-precipitable lysate was applied. The positions of coelectrophoresed M_r markers are indicated to the left.

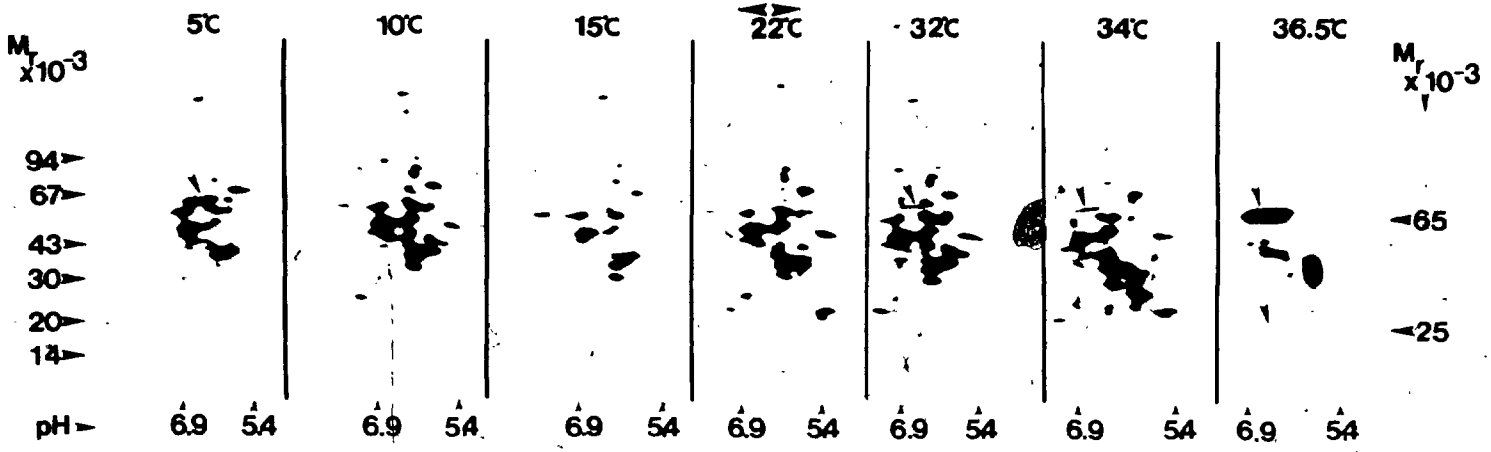
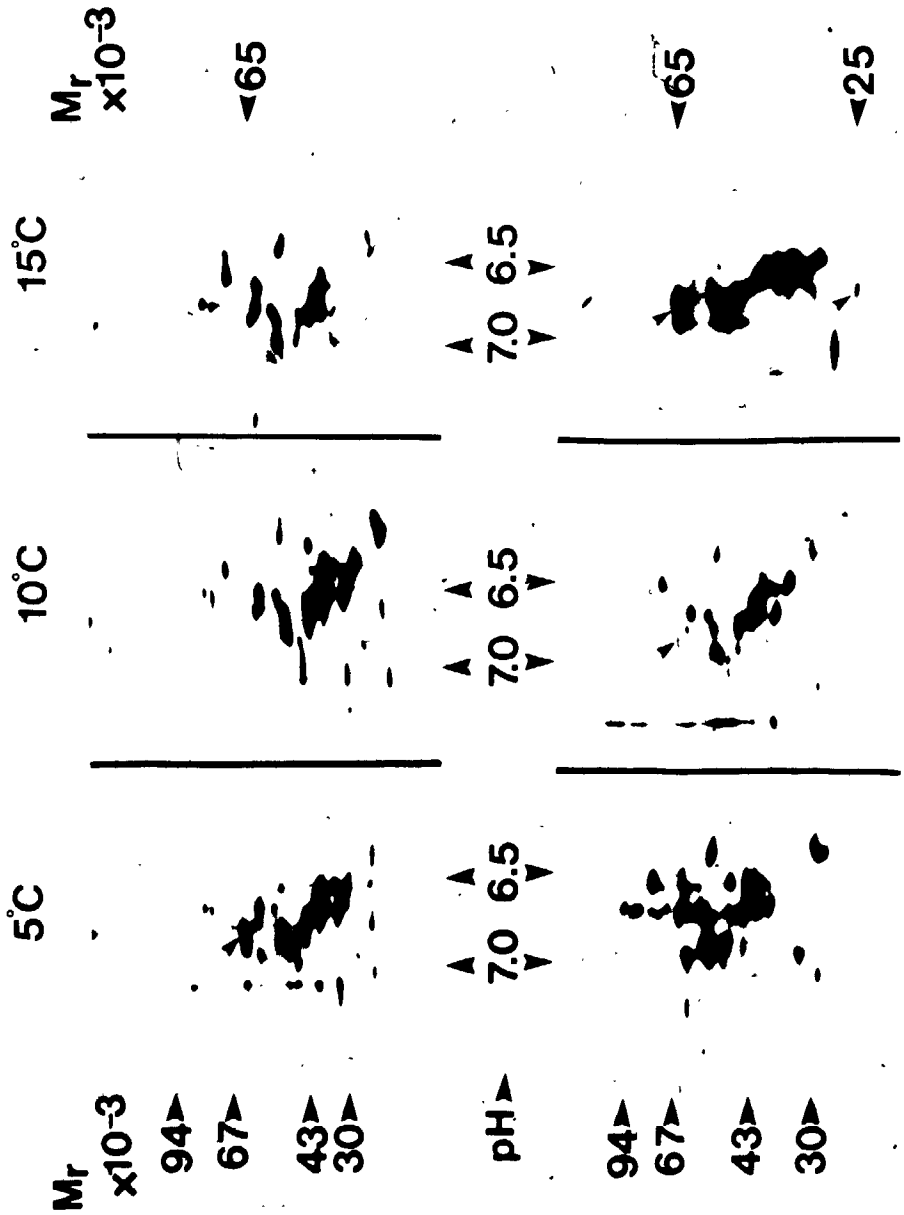

5°C
10°C
15°C
22°C
32°C
34°C
36.5°C
M_r x10^{-3}
94
67
43
30
20
14
65
25
pH
6.9
5.4

Figure 11.

Fluorographic analysis of IEF-SDS-PAGE separation of the proteins synthesized by 5-day cell cultures from the tail fin epidermis of R. catesbeiana (stages XII-XV) subsequent to incubation at 5, 10 and 15°C for 2 hr and 22, 32 and 36.5°C for 1 hr. The presence of the 65 kd protein at 5, 32 and 36.5°C is indicated by arrowheads. An arrowhead also indicates the 25 kd protein detected only at 36.5°C. Ampholines in the pH range of 3.5-10 were used to electrofocus gels in the first dimension. A 3-15% gradient gel was used in the second dimension. Approximately 50,000 cpm of acid-precipitable protein was applied to each electrofocusing gel. The positions of coelectrophoresed M_r marker proteins are indicated to the left.


Mr x10-3
<65
<65
<25
15°C
10°C
5°C
6.5
7.0
6.5
7.0
6.5
7.0
36.5°C
32°C
22°C
Mr x10-3
94
67
43
30
pH
94
67
43
30

3.2.2 Recovery of Tail Fin Epidermal Cell Cultures from Heat Shock (34°C)

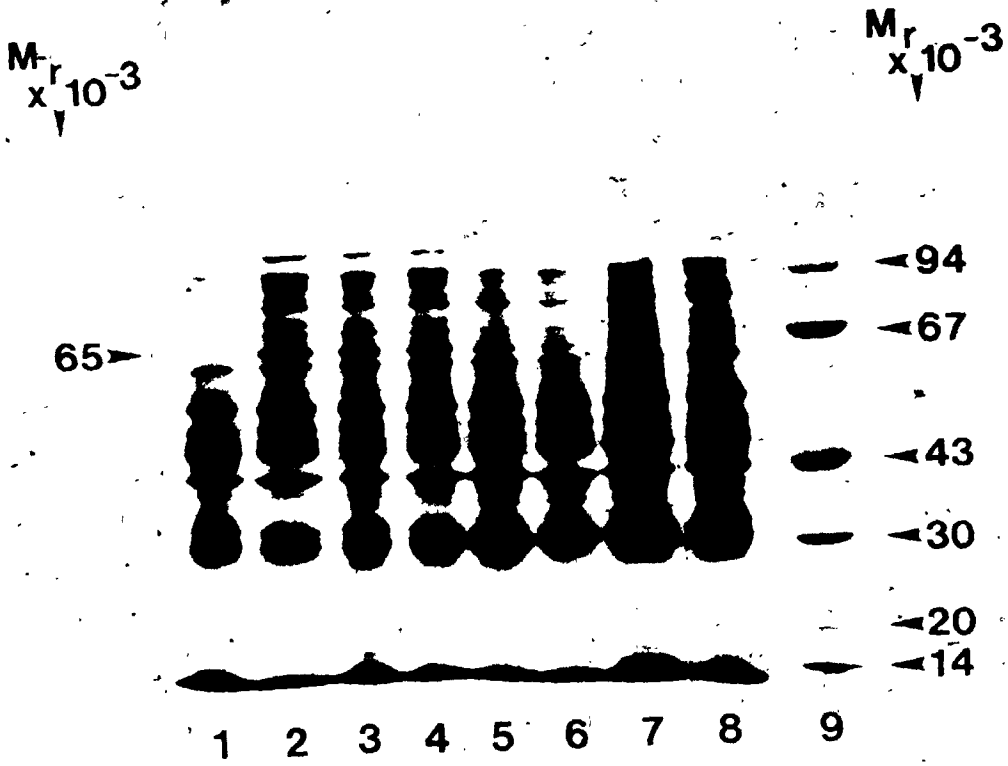
Since epidermal cell cultures from hind limb and tail fin respond to temperature stress by synthesizing the same novel proteins (Figures 10 and 11), only tail fin cultures were used to determine if a control pattern of protein synthesis is restored in cells returned to 22°C following a heat shock. The cells, incubated at 34°C for one hour, still synthesize detectable quantities of the 65 kd protein after a .5, 1, 2 or 3 hr recovery period before a 2 hr labeling time with [^{14}C]leucine. After a 4 hr recovery, the proteins synthesized resemble those of the control (Figure 12).

3.3 Water-Insoluble Proteins Synthesized by Epidermal Cell Cultures from R.catesbeiana Tadpoles

Since epidermal cell cultures from tail fin and hind limb respond to sub- or supra-optimal temperatures by synthesizing new/and or enhanced proteins was demonstrated, I was curious to determine if epidermal cells in culture would respond to differences in their nutrient environment by synthesizing new and/or enhanced proteins. Thyroid hormone (3,3',5-triiodothyronine; T_3), a well-documented inducing agent of precocious differentiation in anuran larval tissues, provides an excellent supplement to examine this phenomenon in vitro. Because the keratins (a family of water-insoluble proteins) change in response to the

Figure 12.

Fluorogram of one-dimensional SDS-PAGE (3-15% gradient gel) separation of the proteins synthesized by 5-day primary epidermal cell cultures from R. catesbeiana tail fin during recovery from a 1 hr heat shock at 34°C. Cell cultures were allowed to recover for 0 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 3 (lane 6) and 4 hr (lane 7). Proteins synthesized by cultures maintained at the control temperature were coelectrophoresed with the samples prepared for the recovery series and are shown in lanes 1 and 8. Molecular mass marker proteins are shown on the right. Approximately 10,000 cpm of acid-precipitable cpm were applied to each lane. The position of the 65 kd HSP is indicated to the left.


M_r x 10^{-3}
M_r x 10^{-3}
65
94
67
43
30
20
14
1 2 3 4 5 6 7 8 9

differentiative state of epidermal tissue, they provide an ideal marker for assessing whether epidermal cells in culture from larval anurans could mount a response to thyroid hormone. The water-insoluble proteins synthesized in medium supplemented with fetal calf serum would be considered as those synthesized in a control environnent.

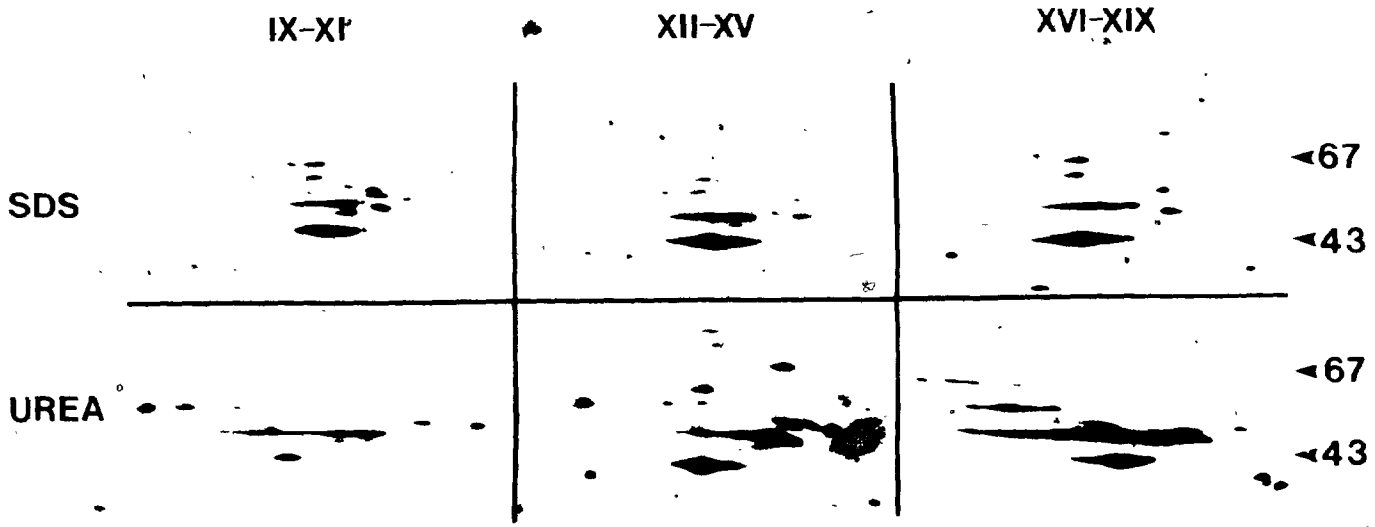
Variable solubility has been demonstrated among some of the proteins extracted in water-insoluble fractions. In addition, a particular denaturing agent is sometimes found to give superior results by 1- and 2-D SDS-PAGE. For these reasons, hind limb epidermal cell cultures from stages IX-XI, XII-XV and XVI-XIX maintained in the presence of 5% fetal calf serum (F.C.S.) and labeled with [^{35}S]methionine for the last 36 hours of cell culture were extracted in KSBA (a keratin protein extraction buffer containing 2% SDS) or 9 M urea (Figure 13). Since there was no significant difference in the solubility properties of the water-insoluble proteins in either solution, the SDS-containing KSBA was used for final preparation of these fractions (Figure 13) in subsequent studies.

3.3.1(a) Water-insoluble Proteins Synthesized by Thirty-Six Hour Epidermal Cell Cultures from Tadpole Tail Fin (Stages IX-XI)

The maintenance of epidermal cell cultures from stages IX-XI tail fin for 36 hr in medium containing either 5% F.C.S. or 3 µL T_3/mL medium results in the synthesis of 2

Figure 13.

Two-dimensional IEF-SDS-PAGE separation of the keratin-enriched fractions from 5 day hind limb epidermal cell cultures solubilized in KSBA or 9 M urea to determine whether certain water-insoluble proteins were preferentially solubilized in one of the two commonly employed denaturing buffers. Volumes equivalent to 75,000 cpm of acid-precipitable lysate were added to each elecrofocusing gel. Ampholines in the pH range of 5-7 (.3 mL) and 3.5-10 (.2 mL) were used to separate proteins in the first dimension and a 7.5-20% polyacrylamide gradient gel in the second dimension.


IX-XI
XII-XV
XVI-XIX
SDS
UREA
67
43
67
43

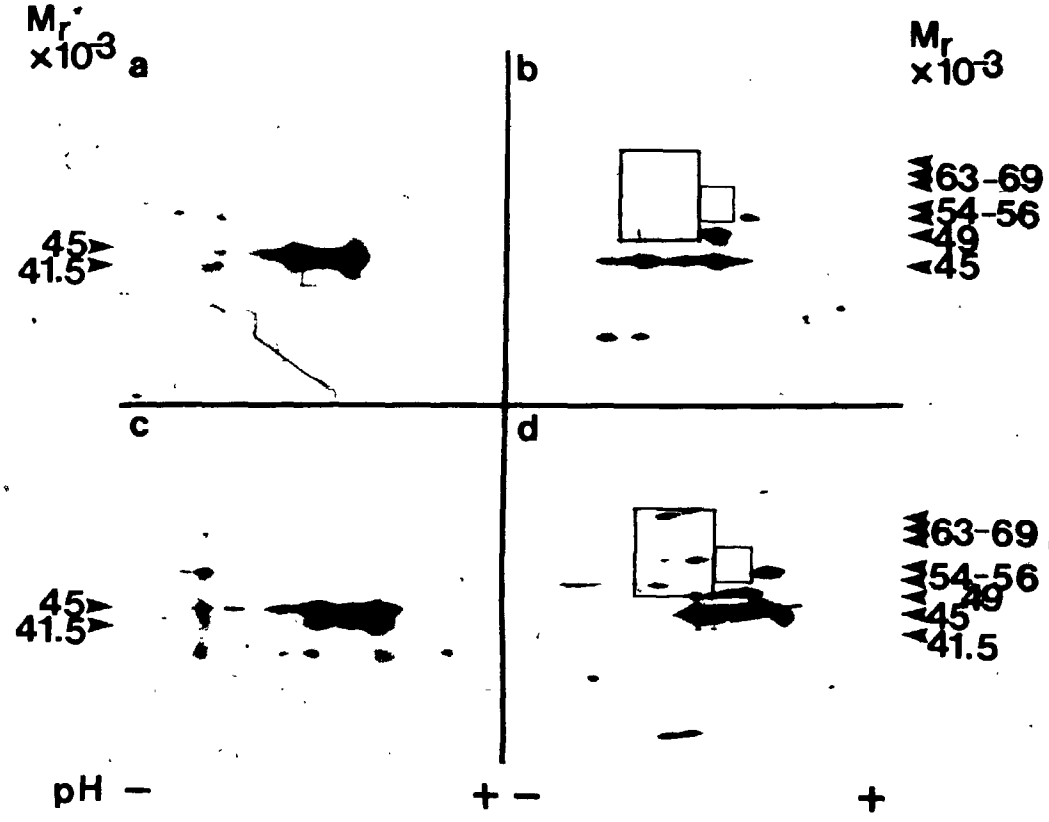
major proteins with M_rs of 41.5 and 45 kd (Figure 14, panels a and c). Although the 41.5 kd polypeptide is expressed as at least 3 isoelectric variants in cells grown in either medium, its synthesis is markedly enhanced in cells maintained in a T_3 supplemented environment. The 45 kd protein is expressed as 2 isoelectric variants in the presence of 5% F.C.S. and 3 in T_3 containing medium.

3.3.1(b) Water-Insoluble Proteins Synthesized by Five Day Epidermal Cell Cultures from Tadpole Tail Fin (Stages IX-XI)

Two-dimensional analysis of 5 day cell cultures from R. catesbeiana tail fin epidermis (stages IX-XI) demonstrates that numerous proteins are present within the 40-70 kd range in the water-insoluble fractions. Multiple isoelectric variants of the 41.5 kd protein are present in both medium preparations. Significant synthesis of proteins with M_rs of 49 and 55 kd also occurs during the labeling period. A 56 kd protein is present as at least 2 isoelectric variants in T_3 supplemented medium. A more acidic protein of 56 kd is noted in the presence of F.C.S. (Figure 14, panels b and d). Also detected in both medium conditions, but apparently enhanced in medium containing F.C.S., are proteins with M_rs of 49 and 54 kd. Although basic proteins with M_rs of 63-69 kd are synthesized by cells maintained in medium containing either F.C.S. or T_3, their presence is enhanced considerably when T_3 is the

Figure 14.

Two-dimensional polacrylamide (7.5-20% gradient) gel IEF-SDS-PAGE separation of the water-insoluble proteins synthesized by 36 hr and 5-day epidermal cell cultures from tail fin (stages IX-XI).
Panel a: 36 hr cell cultures maintained in MEM supplemented with 5% F.C.S.
Panel b: 5-day cell cultures maintained in MEM supplemented with 5% F.C.S.
Panel c: 36 hr cell cultures maintained in MEM supplemented with 3 μL T_3/mL medium.
Panel d: 5-day cell cultures maintained in MEM supplemented with 3 μL T_3/mL medium.
Areas within the large squares in panels b and d define the basic, high M_r water-insoluble proteins whose synthesis is enhanced in the presence of T_3. The small square outlines the position of an acidic 56 kd water-insoluble protein synthesized by epidermal cells from tail fin maintained in the presence of 5% F.C.S. for 5 days. A pH gradient expanded through the 5-7 range (.3 mL plus .2 mL 3.5-10 ampholines) was used in the first dimension. Approximately 100,000 cpm of acid-precipitable lysate was applied to each electrofocusing gel. M_rs of the water-insoluble proteins within 40-70 kd are indicated on the left for 36-hour cell cultures and on the right for epidermal cells maintained for 5 days.


Mr ×10-3
a
b
Mr ×10-3
63-69
54-56
49
45
45
41.5
c
d
63-69
54-56
49
45
41.5
45
41.5
pH −
+ −
+

supplement.

3.3.1(c) Water-Insoluble Proteins Synthesized by Thirty-six Hour Cell Cultures from Tadpole Hind Limb Epidermis (Stages IX-XI)

Epidermal cells from tadpole hind limb (stages IX-XI) were maintained in culture for 36 hours in one of 4 medium conditions (unsupplemented, + 5% F.C.S., 3 µL T_3/mL medium or 5 µL T_3 applied directly to each explant) to which [^{35}S]methionine was added. When the water-insoluble proteins are separated on the basis of their molecular mass, major proteins with M_rs of 42, 43, 45, 51 and 53 kd are detected (Figure 15, lanes 1-4). In the presence of T_3, the synthesis of 45, 53, 58 and 63 kd water-insoluble proteins are particularly enhanced (Figure 15, lanes 3 and 4, positions indicatedby solid triangles). Although not in the molecular mass range assigned to the keratins, the synthesis of a 73 kd protein is enhanced in the presence of both T_3 concentrations (Figure 15, lanes 3 and 4, position indicated by a solid triangle).

Two-dimensional analysis of the water-insoluble proteins synthesized in the variously supplemented medium during the first 36 hours of cell culture emphasizes the variation among the 40-70 kd proteins. In addition to the major proteins cited above, a minor protein with an M_r of 49 kd is synthesized by cells maintained in any of the four medium conditions (Figure 16, panels a, c, e and g).

Figure 15.

One-dimensional fluorographic analysis of the water-insoluble proteins synthesized by hind limb epidermal cells (IX-XI) cultured for 36 hr at 22°C and labeled with [^{35}S]methionine for the culture period. Lane 1 represents the water-insoluble proteins synthesized by cells maintained in MEM-serum, lane 2 MEM supplemented with 5% F.C.S., lane 3 MEM containing 3 µL T_3/mL medium and lane 4 MEM-serum and 5 µL T_3 applied directly to each explant. The water-insoluble proteins with M_rs of 51, 53, 58, 63 and 73 kd synthesized during the first 36 hr of cell culture are indicatd by solid triangles between lanes 3 and 4. Their synthesis is particularly enhanced in the presence of T_3.

Lanes 5-8 are the water-insoluble proteins synthesized by 5-day cell cultures from hind limb labeled with [^{35}S]methionine for the last 36 hr of the culture period. Lane 5 is the water-insoluble proteins synthesized in unsupplemented MEM, lane 6 in the presence of 5% F.C.S., lane 7 in MEM supplemented with 3 µL T_3/mL medium and lane 8 when 5 µL T_3 is applied directly to each explant but the MEM is otherwise unsupplemented. The molecular masses of these proteins are indicated on the right and co-electrophoresed M_r standards on the left. The proteins are separated on a 7.5-20% gradient gel. Approximately 20,000 cpm of acid precipitable lysate was applied to each lane.

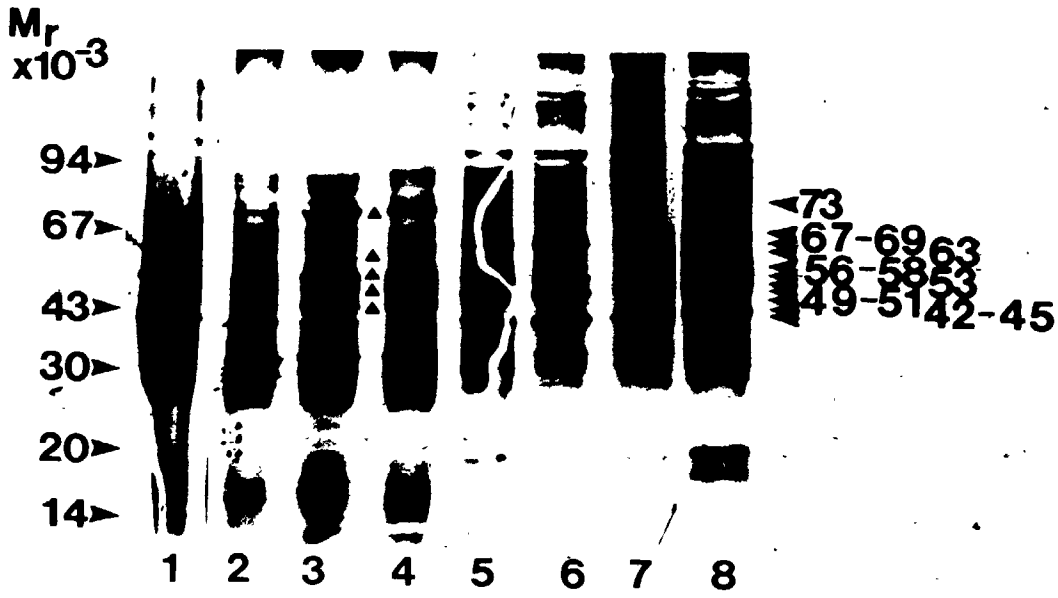

Mr
x10-3
94
67
43
30
20
14
1 2 3 4 5 6 7 8
73
67-69
63
56-58
53
49-51
42-45

Figure 16.

Fluorographic analysis of the two-dimensional separation of the water-insolube proteins synthesized by 36 hr and 5 day epidermal cell cultures from hind limb (stages IX-XI) maintained at 22° C.

Panel a: 36 hr cell cultures maintained in unsupplemented MEM

Panel b: 5-day cell cultures maintained in unsupplemented MEM

Panel c: 36 hr cell cultures maintained in MEM + 5% F.C.S.

Panel d: 5-day cell cultures maintained in MEM + 5% F.C.S.

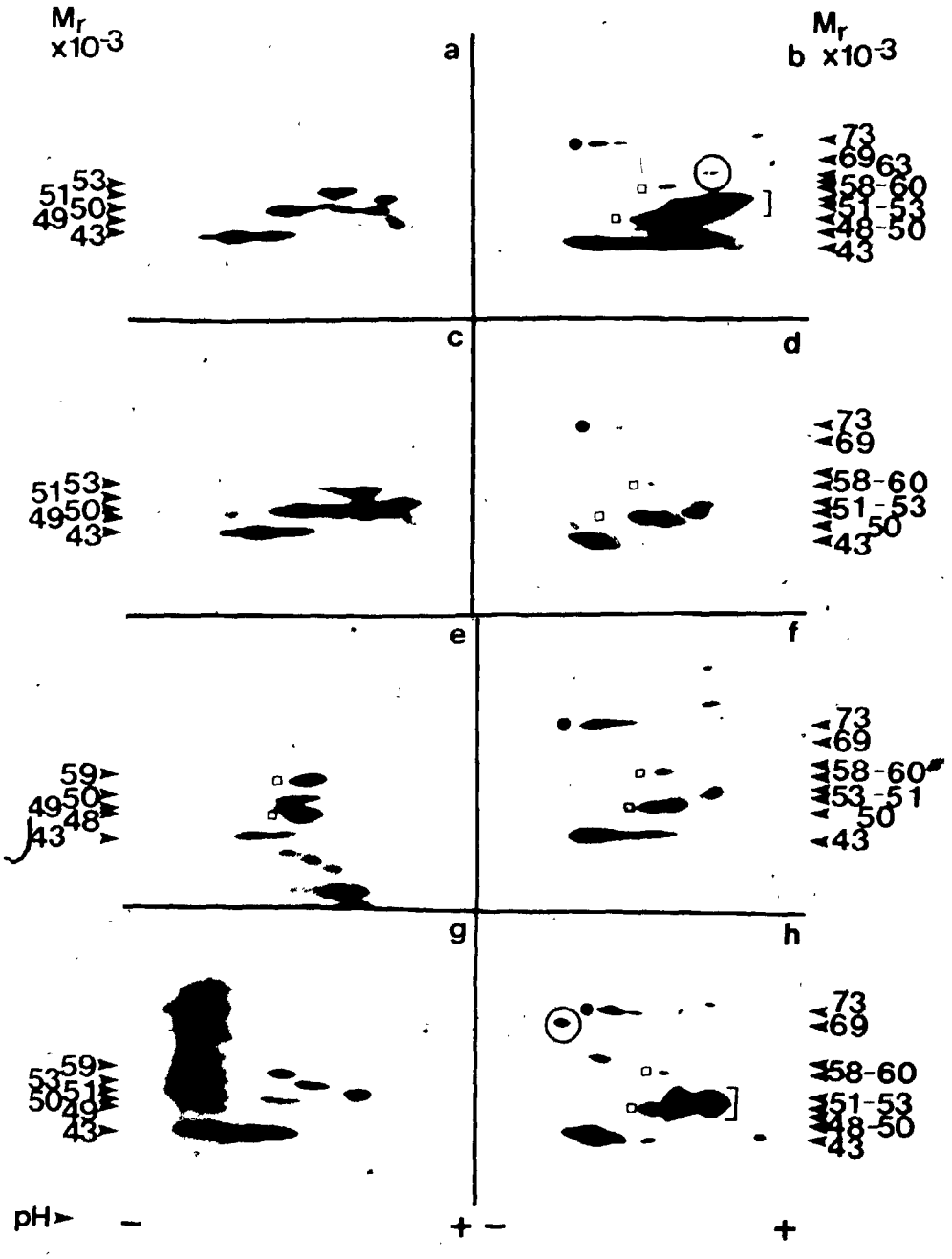
Panel e: 36 hr cell cultures maintained in MEM + 3 μL T_3/mL medium

Panel f: 5-day cell cultures maintained in MEM + 3 μL T_3/mL medium

Panel g: 36 hr cell cultures maintained in unsupplemented MEM but 5 μL T_3 applied directly to each epidermal explant

Panel h: 5-day cell cultures maintained in unsupplemented MEM but 5 μL T_3 applied directly to each epidermal explant.

Open squares are used to mark the position of the water-insoluble proteins with M_rs of 48 and 59 kd resolved when 36 hr epidermal cell cultures are maintained in MEM supplemented with 3 μL T_3/mL medium (panel e). In panels b, d, f and h, the open squares mark the water-insoluble proteins believed to correspond to the 48 and 59 kd proteins indicated in panel e. The open circle in panel b surrounds an acidic, water-insoluble 63 kd protein observed only when cells are maintained in unsupplemented MEM. In panel h, the open circle outlines a basic, water-insoluble protein with an M_r of 69 kd whose synthesis is enhanced when 5 μL of T_3 is applied directly to each explant. The square bracket outlines the 51/53 kd dumbell-shaped doublet in panels b and h. The solid circle indicates the position of the 73 kd water-insoluble protein in panel b, d, f and h. The electrofocusing gels contained a mixture of .3 mL of pH 5-7 and .2 mL of pH 3-10 ampholines. A 7.5-20% gradient gel was used to separate the proteins in the second dimension. Molecular masses of the proteins of interest are indicated on the left for cell cultures maintained for 36 hours and on the right for 5-day cell cultures. Approximately 150,000 cpm of acid-precipitable lysate was applied to each electrofocusing gel.


Mr x10-3
a
Mr
b x10-3
53
51
50
49
43
73
69
63
58-60
51-53
48-50
43
c
d
53
51
50
49
43
73
69
58-60
51-53
50
43
e
f
59
50
49
48
43
73
69
58-60
53-51
50
43
g
h
59
53
51
50
49
43
73
69
58-60
51-53
48-50
43
pH
–
+
–
+

A water-insoluble protein with M_r of 48 kd is synthesized by cells grown in all media except the medium to which 5 µL T_3 was applied directly to each explant (Figure 16, panels a, c, e and g). The presence of T_3 at either concentration markedly enhanced the synthesis of a 59 kd protein which also is detected at low levels in the presence of serum or unsupplemented medium. The greatest similarities in the water-insoluble proteins synthesized are observed among cells maintained in unsupplemented, 5% F.C.S. and 5 µL T_3 applied directly to each explant medium preparations (Figure 16, panels a, c and g).

While either T_3 concentration is effective in stimulating the synthesis of the 59 kd, the acidic 51 and 53 kd proteins observed in the other medium preparations are not detected in medium supplemented with 3 µL T_3/mL medium (Figure 16, panel e). The water-insoluble proteins synthesized by cells maintained in medium supplemented with 3µL T_3/mL medium appear quite different from those observed in the other growth conditions during the first 36 hr of cell culture. They are however, very similar to the water-insoluble proteins synthesized by 5 day cell cultures from hind limb (stages IX-XI) (Figure 16, compare panels e to b, d, f and h. Reference proteins are indicated by open squares).

3.3.1(d) Water-Insoluble Proteins Synthesized by Five Day Cell Cultures from Tadpole Hind Limb Epidermis

(Stages IX-XI)

The water-insoluble proteins synthesized by 5 day hind limb epidermal keratinocyte cultures (stages IX-XI) during the last 36 hours of cell culture were visualized by fluorography. One-dimensional separation revealed that the major water-insoluble proteins had M_rs of 69, 67, 58, 56, 53, 49 and 43 kd are observed within the 40-70 kd range. A 45 kd protein is faintly detected in all medium conditions with the exception of 5% F.C.S. When 5 µL T_3 is applied directly to each explant a 63 kd water-insoluble protein is enhanced.

From two-dimensional fluorograms the differences that exist between the proteins synthesized by 5 day cell cultures in the four medium conditions do not seem so dramatic as that observed in cultures maintained for only 36 hr. This may, in part, be due to the greater variety of water-insoluble proteins synthesized within the 40-70 kd range in 5 day cultures (Figure 16, panels b, d, f and h).

The 63 kd protein which was detectable in one-dimensional fluorograms is most intense when cells grown in the absence of serum are separated in two dimensions (Figure 16, panel b, protein within the circle). When 5 µL T_3 is applied directly to each explant, the 69 kd protein is more prominent than in other growth conditions (Figure 16, panel h, protein within the circle). Although present in all growth conditions, the 51 and 53 kd doublet separates into two distinct isoelectric variants when 5 µL

T_3 is applied to individual explants (Figure 16, panel h). It is likely that the 53/51 kd doublet is present as the 2 isoelectric variants in MEM-serum since these proteins extend across a pI range which corresponds to that observed with 5 µL T_3 (Figure 16, panel b). In the presence of 5% F.C.S. or 3 µL T_3/mL medium the doublet clearly resolves into the single most acidic isoelectric variant.

Cells maintained in MEM-serum or in MEM and 5 µL T_3/explant also apparently synthesize greater amounts of 50 and 48 kd proteins. They can, in these growth conditions, be resolved into 2 isoelectric variants. The enhanced synthesis of the 73 kd protein in 36 hour cell cultures (Figure 15, lanes 3 and 4) maintained in a T_3 supplemented environment but not detected on two-dimensional fluorograms, is resolved in all four growth conditions from 5 day cultures (Figure 16, panels b, d, f and h).

It is interesting to note that the pattern of water-insoluble proteins synthesized during the 36 hour and 5 day cell culture periods are not the same for tail fin and hind limb epidermis from animals of the same stage (IX-XI). In these two sources of epidermis for cell culture, the differences observed in the water-insoluble proteins synthesized display a reverse dependence on the length of time in cell culture. Unlike the similarity between calf serum and T_3 supplemented medium noted in 36 hour tail fin cell cultures, epidermal cell cultures from

hind limb exhibit clearly detectable differences in the proteins synthesized within the first 36 hours of cell culture. However, after 5 days in cell culture the most notable differences are noted in the water-insoluble proteins extracted from tail fin. The water-insoluble proteins synthesized by 5 day cell cultures from hind limb maintained in either calf serum or 3 µL T_3/mL medium (Figure 16, panels d and f) appear identical by IEF-SDS-PAGE separation.

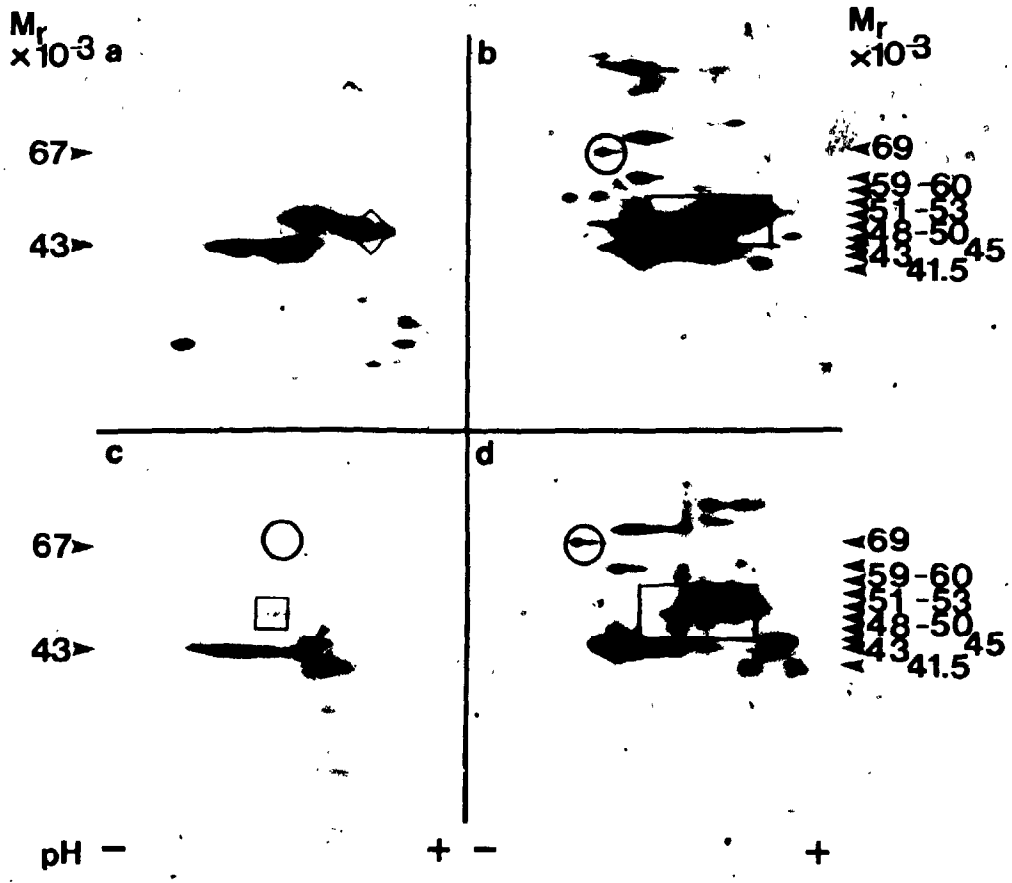
3.3.2(a) Water-Insoluble Proteins Synthesized by Thirty-six Hour Cell Cultures from Tadpole Hind Limb Epidermis (Stages XII-XV)

Hind limb epidermal cells from stages XII-XV maintained in culture for 36 hours synthesize water-insoluble proteins with M_rs of 41.5, 43 and 53 kd in the presence of 5% F.C.S. or 3 µL T_3/mL medium (Figures 17, panels a and c).

When medium is supplemented with 3 µL T_3/mL medium, the synthesis of a water-insoluble protein with M_r of 69 kd is observed (Figure 17 panels c, area within the circle) and the relative synthesis of a 45 kd protein is increased (Figure 17, panel c, small arrowhead). The 53 kd protein which is a major synthetic product in calf serum supplemented medium, is surrounded by a square in panel c (T_3 supplemented medium) as its presence is not so easily detected. In the presence of F.C.S., a 48 kd

Figure 17.

Fluorograms of IEF-SDS-PAGE separation of the water-insoluble proteins extracted from 36 hr and 5-day cell cultures from hind limb epidermis (stages XII-XV) maintained at 22°C.
Panel a: 36 hr cell cultures maintained in MEM + 5% F.C.S.
Panel b: 5-day cell cultures maintained in MEM + 5% F.C.S.
Panel c: 36 hr cell cultures maintained in MEM supplemented with 3 µL T_3/mL medium.
Panel d: 5-day cell cultures maintained in MEM supplemented with 3 µL T_3/mL medium.
The presence of a water-insoluble protein with M_r of 48 kd, detected in 36 hour cell cultures maintained in the presence of F.C.S., is outlined by a diamond in panel a. In the presence of T_3, a 69 and 45 kd water-insoluble proteins are synthesized (panel c, circle and arrowhead, respectively). The position of the 53 kd protein is outlined by a square in panel c. The rectangles in panels b and d outline the water-insoluble proteins of M_r 48-50 (whose synthesis is enhanced in the presence of F.C.S. (panel b)) and 51-53 kd (which are more intensely synthesized in the presence of T_3 and whose position is marked by open triangles in panel d)). A circle outlines the 69 kd water-insoluble protein in panels b and d. The molecular masses of co-electrophoresed M_r standards are indicated on the left and to the right are the M_rs of the water-insoluble proteins synthesized by 36 hr and 5 day cell cultures. Approximately 200,000 cpm of acid-precipitable lysate were applied to each electrofocusing gel. The pH gradient and separating gel were as outlined for the previous figures of water insoluble protein separation.


Mr ×10-3 a
67
43
b
Mr ×10-3
69
59-60
51-53
48-50
43
45
41.5
c
67
43
d
69
59-60
51-53
48-50
43
45
41.5
pH
−
+
−
+

water-insoluble protein is observed (Figure 17, panel a, region within diamond).

3.3.2(b) Water-Insoluble Proteins Synthesized by Five Day Cell Cultures from Tadpole Hind Limb Epidermis (Stages XII-XV)

The maintenance of epidermal cell cultures from hind limb (stages XII-XV) for 5 days results in the synthesis, during the last 36 hours, of a greater number of water-insoluble proteins and of ones which have greater molecular masses. Also, as was apparent in epidermal cultures from stages IX-XI, the resolution of the proteins synthesized is markedly improved.

When water-insoluble proteins are separated by 2-D gel electrophoresis the differences detected in the proteins synthesized in the presence or absence of T_3 are largely quantitative rather than qualitative. In general, when cells are maintained in T_3 supplemented medium, there is a decrease in the synthesis of the lower molecular weight proteins. For example, the relative synthesis of 48 and 50 kd proteins is depressed relative to cells maintained in F.C.S. (Figure 17, panels b and d, areas within the rectangles) and more isoelectric variants of higher M_r basic proteins are resolved within the 40-70 kd range.

In the presence of T_3, the synthesis of the basic, isoelectric variants of the 69 and 60 kd proteins and an acidic 45 kd protein are stimulated. When medium is

supplemented with 5% F.C.S., the synthesis of 50 kd, 57 kd, present as 2 basic isoelectric variants, and an acidic 49 kd protein are enhanced. The basic and acidic components of the 51/53 kd dumbell shaped doublet are clearly resolved in T_3 containing medium (Figure 17, area within the rectangle, positions indicated by open triangles). The synthesis of the basic variants of this doublet is depressed in the presence of F.C.S.

3.3.3(a) Water-Insoluble Proteins Synthesized by Thirty-six Hour Cell Cultures from Tadpole Hind Limb Epidermis (Stages XVI-XIX)

Cultures of 36 hour hind limb epidermal cells from animals in stages XVI-XIX synthesized water-insoluble proteins with M_rs of 49, 51, 53, 55 and 63-69. In the presence of T_3, proteins with an M_r of 53-55 and 73 kd are enhanced (Figure 18, panel c). In addition to the water-insoluble proteins cited, cells maintained in F.C.S. supplemented medium also synthesized 46 and 43 kd proteins. Low levels of a 60 kd protein are also noted (Figure 18, panel a).

As was previously apparent, the water-insoluble proteins from cells maintained in T_3 supplemented medium are similar to those obtained in 5 day cell cultures. In this instance, the similarity is most remarkable when compared to calf serum supplemented cell cultures (Figure 18, panels b and c. The position of proteins in common

Figure 18.

Two-dimensional IEF-SDS-PAGE separation of the water-insoluble proteins extracted from 36 hr and 5-day hind limb epidermal cell cultures maintained at 22°C (stages XVI-XIX).

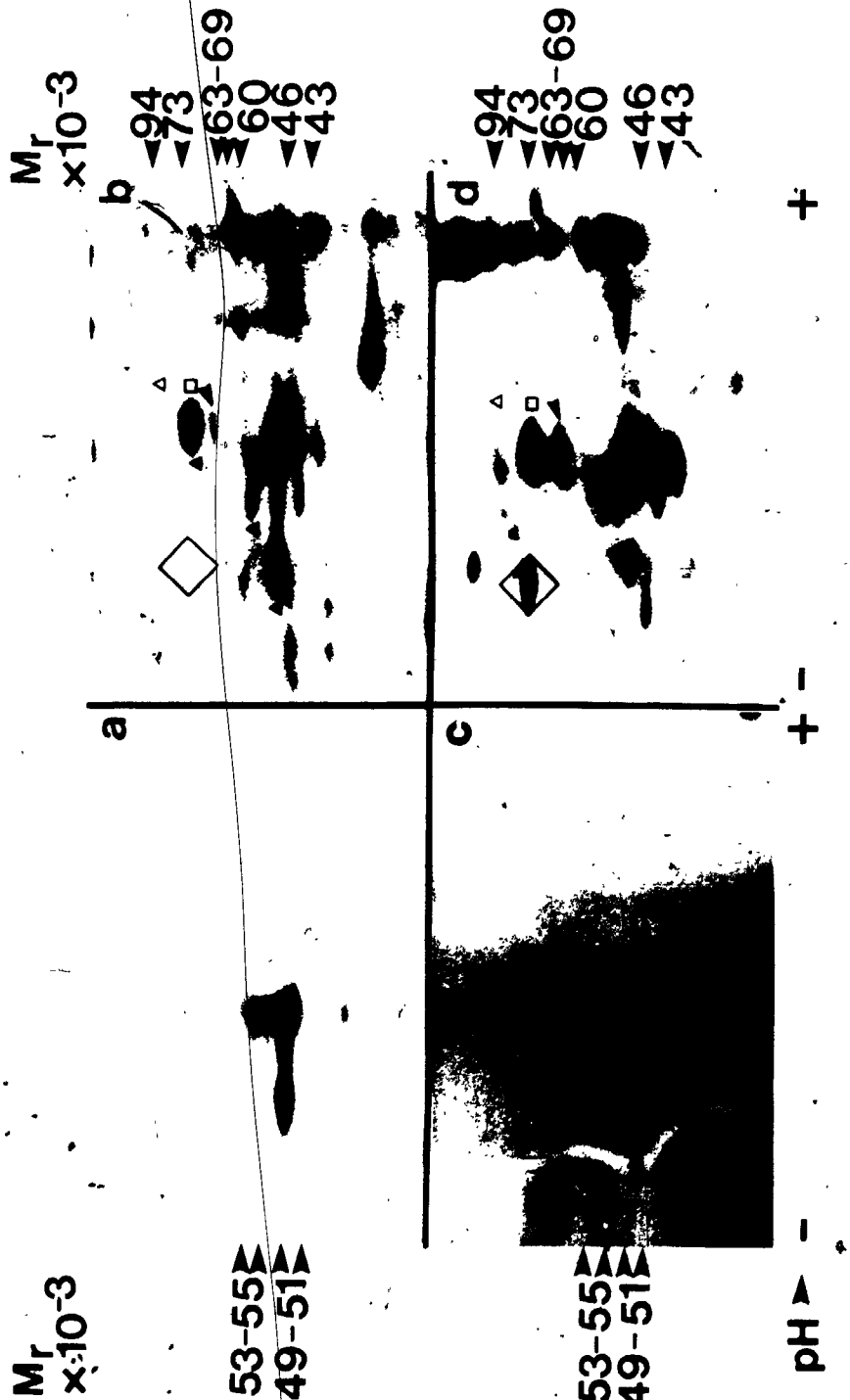
Panel a: 36 hr cell cultures maintained in MEM + 5 % F.C.S.

Panel b: 5-day cell cultures maintained in MEM + 5% F.C.S.

Panel c: 36 hr cell cultures maintained in MEM + 3 μL T_3/mL medium.

Panel d: 5-day cell cultures maintained in 3 μL T_3/mL medium.

The positions of the 73, 53-55, and 49-51 kd proteins detected in 36 hr cell cultures maintained in T_3 (panel c) which correspond to water-insoluble proteins synthesized by 5 day cultures in the presence of F.C.S. (panel b) are indicated by solid triangles. Within the region outlined by the diamond is a basic, water-insoluble protein with an M_r of 73 noted only when hind limb epidermal cells (stages XVI-XIX) are maintained for 5 days in the presence of 3 μL T_3/mL medium (panels b and d). The enhanced synthesis of the basic 73 kd protein in the presence of T_3 is marked by an open square (panels b and d). Only in 5 day cell cultures maintained in the presence of T_3 is the 94 kd water-insoluble protein resolved (panels b and d, position indicated by open triangles). Ampholines in the pH ranges of 5-7 (.3 mL) and 3.5-10 (.2 mL) were used in isoelectric focusing gels. A 7.5-20% polyacrylamide gradient was used to separate water-insoluble proteins in the second dimension. Approximately 200,000 cpm of acid-precipitable lysate were applied to each electrofocusing gel.


M_r ×10^{-3}
94
73
63-69
60
46
43
b
d
a
c
+
-
+
-
53-55
49-51
pH

are indicated by enclosed triangles).

3.3.3(b) Water-Insoluble Proteins Synthesized by Five Day Cell Cultures from Tadpole Hind Limb Epidermis (Stages XVI-XIX)

During the last 36 hr of cell culture, cells maintained in the presence or absence of T_3 synthesize water-insoluble proteins with M_rs of 63-69, 53-55, 49-51, 46 and 43 kd. The presence of T_3 stimulates the synthesis of 63-69 kd proteins (Figure 18, panels b and d, arrowheads). The synthesis of an acidic 73 kd protein is also enhanced ((Figure 19, panels b and d, position indicated by open squares). Although a basic 73 kd protein (Figure 19, panels b and d, protein is outlined by a diamond) is not detected in the presence of F.C.S., this growth environment stimulates the synthesis of acidic 60 kd proteins.

3.4 Immunological Studies on Thirty-six Hour and Five-Day Epidermal Cell Cultures from Hind Limb

Although water-insoluble proteins within the 40-70 kd range are noted in 36 hour and 5 day cell cultures, the mere presence of these proteins and the changes noted in the proteins synthesized in various media conditions does not verify that these proteins are, in fact, keratins. Since keratinization as reflected by birefringence in hindlimb epidermis from stages XII-XV has been demonstrated

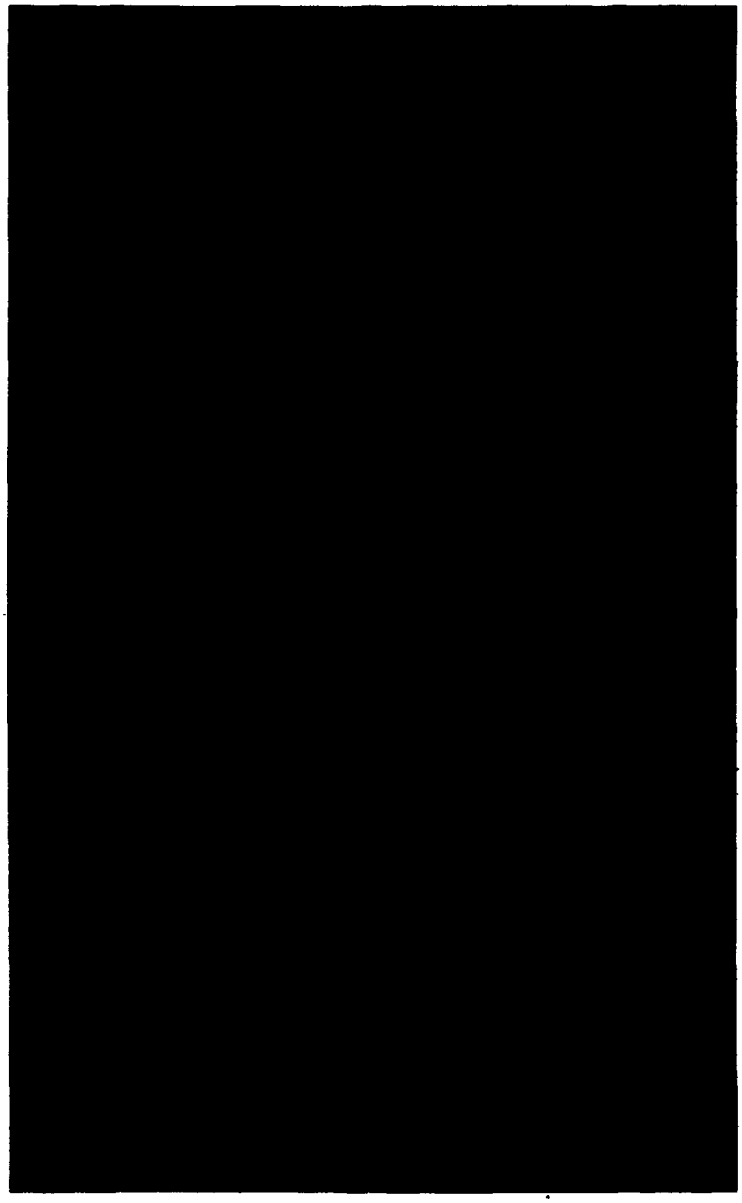
(Wright, 1973), it was felt that if positive immunofluorescence for antibodies against human cytokeratins could be demonstrated in 36 hour cell cultures from hind limb from stage IX-XI tadpoles, their presence was assumed in epidermal cell cultures from more mature larvae. Since the water-insoluble proteins within 40-70 kd are few in 36 hour cell cultures, immunofluorescence was chosen to demonstrate the presence of keratin-like proteins. Since the number of water-insoluble proteins is markedly increased in cell cultures from hind limb epidermis maintained for 5 days, immunoprecipitation was used as a method for a more precise identification of which epidermal proteins were keratin-like proteins.

3.4.1 Immunofluorescence of Thirty-six Hour Epidermal Cell Cultures from Tadpole Hind Limb (Stages IX-XI)

The ability of epidermal explants to elaborate sheets of epidermal keratinocytes is not facilitated on glass coverslips, a necessity for immunofluorescence. The tendency of the explants is to seal the wounded area. The coating of glass coverslips with 2% collagen does not significantly alter this condition. The explants are easily maintained on plastic coverslips but the scattering of light which these cause make them unsuitable for immunfluorescence. Although growth was not prolific, representative cells noted on collagen coated glass were observed and photographed.

Figure 19.

Indirect immunfluorescence of 36 hr cell cultures from hind limb epidermis maintained in 5% F.C.S. (panel A) or 3 μL T_3/mL medium (panel B). Note the felt-like appearance of the tonofilament network and the intense staining of the perinuclear regions. The intensely staining small cells typical of the basal cell population (Schweizer et al., 1984a) are indicated by arrowheads in panel B.



Epidermal cells sustained in the presence of F.C.S. or T_3 for 36 hr exhibited positive immunofluorescence (Figure 19, A and B). The tonofilament network is not well-developed but shows a rather felt-like appearance when cells are maintained in F.C.S. or T_3 supplemented medium. This staining pattern has been observed in epidermal cells from middle layers of epidermis in mammals (Schweizer et al., 1984a). Although nuclear regions are negatively fluorescent in cells from both groups, the perinuclear region stains intensely. Among the group of epidermal cells grown in the presence of T_3, some cells show intense immunofluorescence (Figure 19, panel B, arrowheads) and appear to be smaller than the surrounding cells. This pattern of immunofluorescence has been identified as the characteristic staining pattern of the basal cell population of epidermis in newborn mouse (Schweizer et al., 1984a). The marked appearance of these intensely staining 'basal-like' cells in the presence of T_3 suggests that T_3 does indeed stimulate the growth of these cells. While this method demonstrates that keratin-like proteins are, in fact, present in 36 hour cell cultures from stages IX-XI hind limb epidermis, the cells from a particular growth environment cannot be distinguished on this basis,

3.4.2 Immunoprecipitation of the Keratins from Hind Limb Epidermal Cells Cultured for Five Days

Primary cultures from hindlimb were maintained in the

presence of 5% F.C.S. for five days. Although all molecular weight classes of water-insoluble proteins within 40-70 kd are synthesized in this growth condition, not all isoelectric variants are represented.

Six or seven keratin proteins within the 40-70 kd M_r range were precipitated from each stage group, with those solublized in 9 M urea (fracton 1) consistently giving the clearest results (Figure 20). From fractions 2 and 3 together, all of the keratins immunoprecipitated from urea solubilized samples are resolved. However, in neither of these buffers alone are all of the keratins immunoprecipitated. Samples solublized in RIPA (fraction 2) apparently preferentially exclude the highest M_r components which are easily resolved in fractions solubilized in buffer containing 2% SDS (fraction 3).

From keratin-enriched fraction 1 (as elaborated in Figure 1) (stages IX-XI) proteins with M_rs of 60, 52, 49, 46 and 40 kd were immunoprecipitated (Figure 20, lane A and Figure 21, Part A, lanes 1, 2 and 3). The 65 and 60 kd proteins are barely detectable.

Proteins with M_rs of 67, 64, 57, 52, 49 and 46 kd were immunoprecipitated from hind limb epidermal cells from stages XII-XV (Figure 20, lane B and Figure 21, Part B, lanes 1, 2 and 3). Immunoprecipitation of the 67, 56 and 46 kd proteins was most prominent in keratin-enriched fractions 1 and 3 (Figure 21, Part B, lanes 1 and 3, respectively).

Figure 20.

One-dimensional separation of the keratins immunoprecipitated from 5 day epidermal cells cultured at 22°C in MEM + 5% F.C.S. Keratin-enriched fractions were solubilized in 9 M urea and volumes equivalent to 5 x 10^5 cpm immunoprecipitated with rabbit anti-human cytokeratin antibodies. The keratins immunoprecipitated from stages IX-XI are shown in lane A, from stages XII-XV in lane B and from stages XVI-XIX in lane C. Molecular masses of immunoprecipitated proteins are indicated to the left and right.

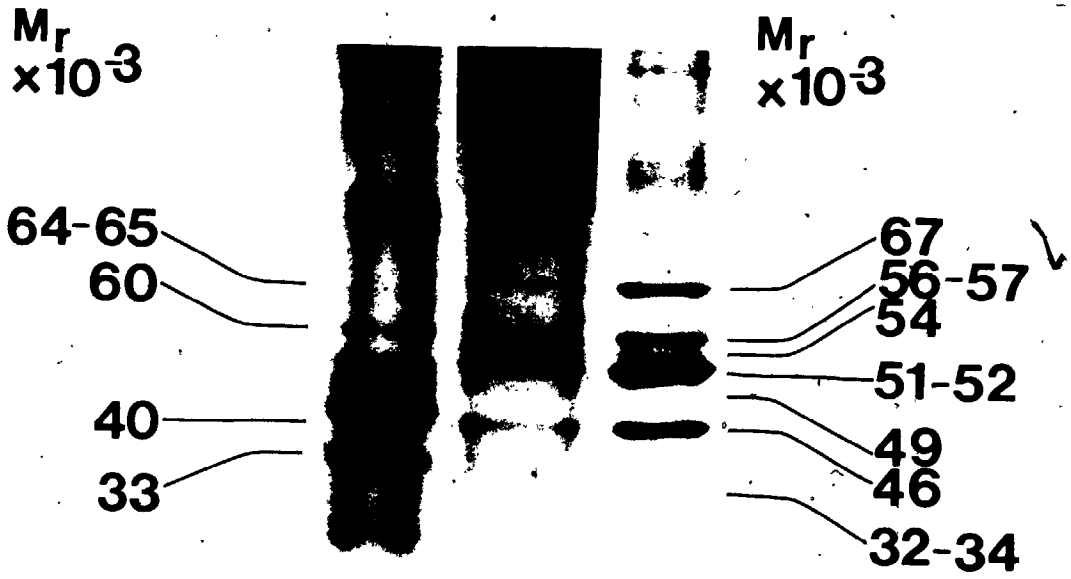

Mr
×10-3
64-65
60
40
33
Mr
×10-3
67
56-57
54
51-52
49
46
32-34
A
B
C

Figure 21

One-dimensional separation of the water-insoluble proteins immunoprecipitated from 5 day hind limb epidermal cell cultures maintained at 22°C in the presence of 5% F.C.S. with rabbit anti-human cytokeratin antibodies.

A: Stages IX-XI

Lane 1: proteins immunoprecipitated from keratin-enriched fractions solubilized in 9 M urea.

Lane 2: proteins immunoprecipitated from keratin-enriched fractions solubilized in RIPA buffer.

Lane 3: proteins immunoprecipitated from fraction 3 which was solubilized in SBA.

The presence of the 48 kd protein is restricted to immunoprecipitates from fractions solubilized in 9 M urea.

B: Stages XII-XV

Lane 1: proteins immunoprecipitated from water-insoluble fractions solubilized in 9 M urea.

Lane 2: proteins immunoprecipitated from water-insoluble fractions solubilized in RIPA buffer.

Lane 3: proteins immunoprecipitated from fraction 3 which was solubilized in SBA.

Note the prominence of the 57 and 46 kd proteins in lane 2 (RIPA buffer solubilized fractions) and of the 67 kd protein in lane 1 (samples solubilized in 9 M urea).

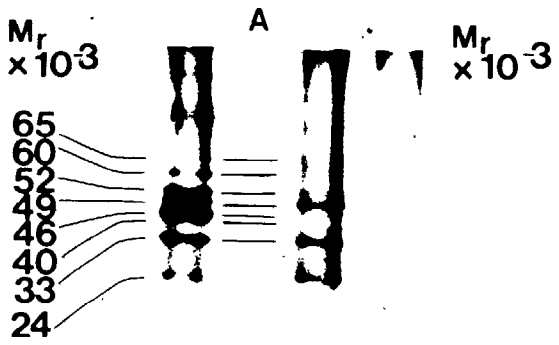
C: Stages XVI-XIX

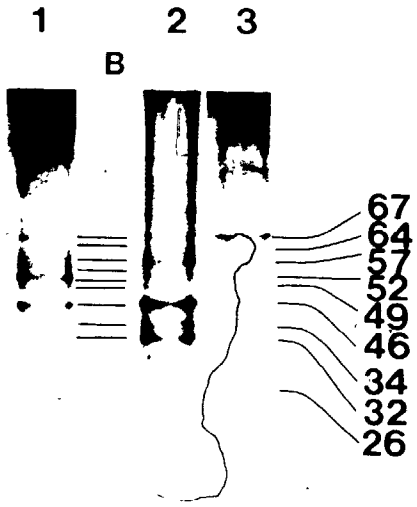
Lane 1: proteins immunoprecipitated from keratin-enriched fractions solubilized in 9 M urea.

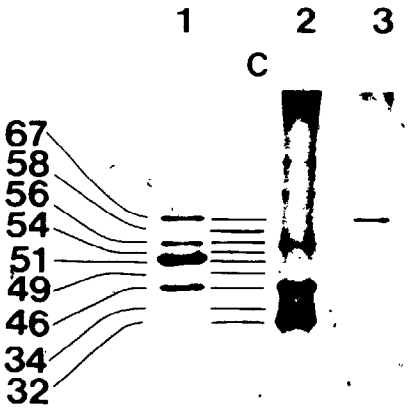
Lane 2: proteins immunoprecipitated from keratin-enriched fractions solubilized in RIPA buffer.

Lane 3: proteins immunoprecipitated from fraction 3 which was solubilized in SBA.

Note the prominence of the 20-30 kd proteins immunoprecipitated from RIPA buffer solubilized samples of all stages. Molecular masses of the immunoprecipitated proteins within 40-70 kd are indicated to the right and to the left. Approximately 50,000 cpm of acid-precipitable lysate was used for immunoprecipitation.


A
M_r $\times 10^{-3}$
65
60
52
49
46
40
33
24
M_r $\times 10^{-3}$


1 2 3
B
67
64
57
52
49
46
34
32
26


1 2 3
C
67
58
56
54
51
49
46
34
32

1 2 3

Proteins with M_rs of 67, 56, 54, 51, 49 and 46 kd were precipitated from hind limb epidermal cells, stages XVI-XIX (Figure 20, lane C and Figure 21, Part C, lanes 1, 2 and 3). A 58 kd protein is noted only in precipitates from samples solubilized in RIPA (fraction 2) (Figure 21, Part C, lane 2).

Additional contaminating proteins with M_rs in the 20-40 kd range were immunoprecipitated from all stage groups. These immunoprecipitated proteins were most evident in fractions extracted in RIPA buffer (Figure 21, Parts A, B and C, lane 2 in all Parts.) These proteins were not subtle contaminants but among the major proteins observed.

3.5. Water-Insoluble Proteins Synthesized in situ by Hind Limb Epidermis in T_3 or NaOH Injected Animals

Water-insoluble proteins were extracted from hind limb epidermis from whole animals. The purpose was two-fold. First, curiousity arose as to whether the proteins extracted from tissue culture cells would be synthesized when tissue/tissue interactions are maintained in whole animals. Also, are the effects of T_3 in epidermal keratin-like proteins from whole animals manifested as early as 36 hr following injection or is a latent period required before differences are detected between control (NaOH) and T_3 injected animals?

A single injection of T_3 or NaOH was given 36 hr before sacrificing. This was followed immediately by an injection of 60 μCi/mL of [^{35}S]methionine; a subsequent

injection of [^{35}S]methionine was given 18 hr later. All induced metamorphosis experiments were performed during the winter months to minimize the effects of endogenous thyroid hormone fluctuations which would occur during spontaneous metamorphosis.

3.5.1. Protein Synthesis in Hind Limb Epidermis During the First Thirty-six Hours Following Injection

At the time of sacrifice, tadpoles were staged (Taylor and Kollros, 1946) to determine if any effects of T_3 had been manifested. Unlike hind limbs from controls, those from T_3-injected animals showed increased vascularization as has been noted previously (Dhanarajan, 1979; Dhanarajan and Atkinson, 1981).

Water-insoluble proteins common to T_3 or NaOH injected animals within all stage groups had M_rs of 64, 60, 53, 49, 48 and 43 kd (Figure 22). In animals from the 3 stage groups injected with T_3, a 56.5 kd protein is resolved that is not observed in any of the stage groups from the control animals (Figure 22, lanes 1, 2 and 3, open arrowheads). A protein with M_r of 67-69 kd is enhanced in hind limb epidermis from T_3-injected animals and in sham injected stages XVI-XIX (Figure 22, lanes 1, 2, 3 and 6, indicated by small squares). With the exception of the 56.5 kd protein, T_3 and sham-injected hind limb epidermis from stages IX-XI and XVI-XIX appear to be the same (Figure 22, lanes 1 and 4, and 3 and 6). Among the two groups of water-insoluble proteins extracted from stages XII-XV,

those with M_rs of 67-69 and 56.5 kd are synthesized subsequent to T_3 injection only (Figure 22, lanes 2 and 5).

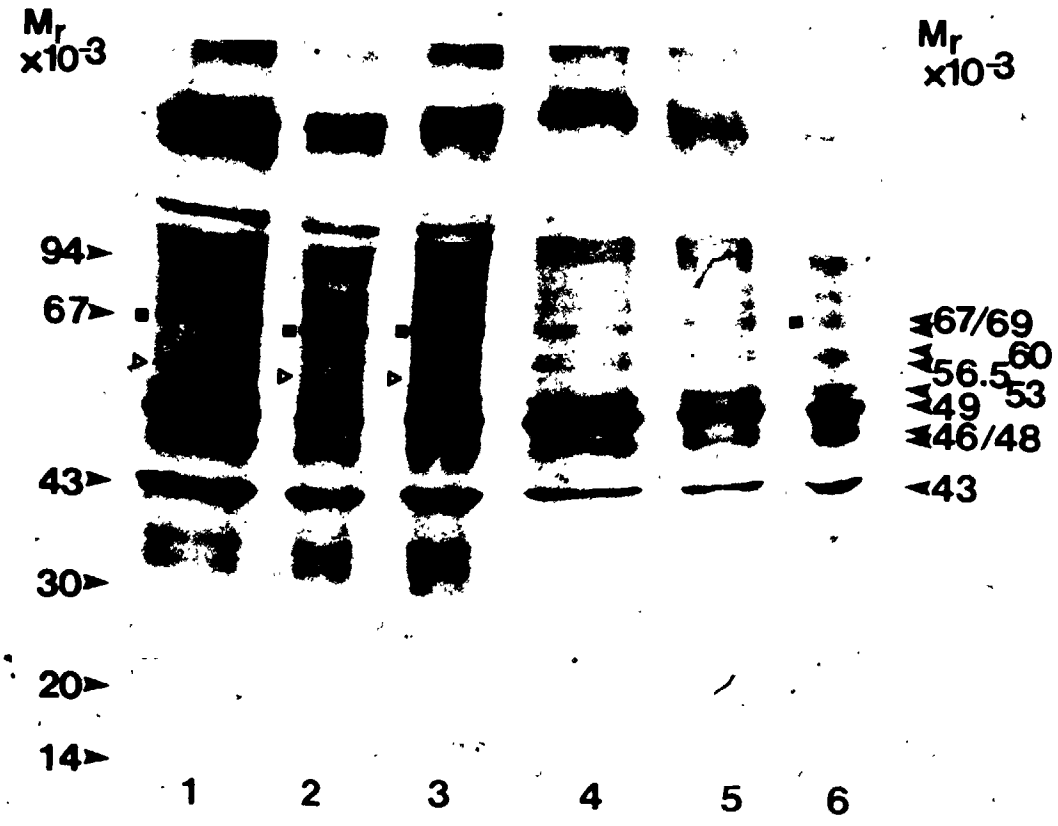
The specific activity (cpm/µg protein) of the water-insoluble proteins was not sufficiently high to allow separation by two dimensional electrophoresis.

3.6 Protein Synthesis in Hind Limb Epidermis from Spontaneously Metamorphosing Tadpoles

In an effort to determine whether the proteins synthesized by hind limb epidermis from animals in which metamorphosis is induced and epidermal cell cultures maintained in a variously supplemented medium bears any similarity to those extracted from epidermis of spontaneously metamorphosing animals, R. catesbeiana at various stages of metamorphic climax were injected with [^{14}C]leucine or [^{35}S]methionine. Both isotopes were used to determine if preferential synthesis of some proteins was exhibited with a particular isotope. Four hours subsequent to their injection with the radioactive probe, the animals were sacrificed. Since the epidermis is rapidly maturing at this time, actually reaching its synthetic peak at stage XXI (Wright, 1973; Atkinson, 1981b), total proteins were extracted. In addition, preliminary studies showed that the proteins synthesized during the labeling period were, for the most part, in the M_r range of the keratins. Since previous studies demonstrated no preferential solubility, 9 M urea was

Figure 22.

Fluorographic analysis of the water-insoluble proteins synthesized *in situ* by hind limb epidermis during a 36 hr period following injection with T_3 or NaOH. Animals were injected with 60 μCi/mL of [^{35}S]methionine 36 and 18 hr prior to sacrifice. Lanes 1-3 are water-insoluble proteins synthesized following T_3 injection from stages IX-XI (lane 1), XII-XV (lane 2) and XVI-XIX (lane 3). Lanes 4-6 are the water-insoluble proteins synthesized subsequent to NaOH injection from stages IX-XI (lane 4), XII-XV (lane 5) and XVI-XIX (lane 6). The apparently novel synthesis of the 56.5 kd and the enhanced synthesis of 67-69 kd water-insoluble proteins detected subsequent to T_3 injection (lanes 1-3) are indicated by open arrowheads and filled squares, respectively. The positions of co-electrophoresed M_r standards are indicated on the left and the proteins synthesized within 40-70 kd on the right. A 7.5-20% polyacrylamide gel was used to separate the proteins.


Mr
×10-3
94
67
43
30
20
14
1
2
3
4
5
6
Mr
×10-3
67/69
60
56.5
53
49
46/48
43

used.

3.6.1 Fluorographic Analysis of the Proteins Synthesized by Epidermis from Animals in Metamorphic Climax

The major proteins synthesized *in situ* by hind limb epidermis from tadpoles in metamorphic climax had M_rs of 65, 62, 57-58, 54, 50, 45-47 and 43 kd. Among the various stages of metamorphic climax, no marked differences can be detected in actual protein products by one-dimensional separation (Figure 23). IEF-SDS-PAGE separation of the proteins synthesized during a 4 hr labeling period with [^{14}C]leucine revealed that during stage XXI the intensity of the basic proteins is apparently markedly increased with the highest M_r, basic proteins of 62 and 58 kd extending across a wide basic pH range (Figure 24, panels a, c and e) as is the more acidic 47 kd protein. In addition, the 43 kd protein which was so prominent in keratin-enriched fractions from tissue culture cells (stages IX-XI and XII-XV) and premetamorphic *in situ* studies is barely resolved due to the density of the the 45-47 kd proteins in all instances.

When [^{35}S]methionine is used as the labeled amino acid, the number of isoelectric variants between the basic 57-58 kd proteins increases from 2 at stage XXI to a minimum of 3 at stages XXII and XXIII (Figure 24, panels b, d and f). With [^{35}S]methionine as with

Figure 23.

One-dimensional fluorographic analysis of total proteins within 40-70 kd synthesized by R. catesbeiana in various stages of spontaneous metamorphic climax. The proteins were synthesized during a 4 hr labeling period with [^{14}C]leucine. Stage XX (lane 1), stage XXI (lane 2), stage XXII (lane 3), stage XXIII (lane 4), stage XXIV (lane 5) and stage XXV (lane 6) are represented. The M_rs of coelectrophoresed standards are indicated on the left and of 40-70 kd proteins synthesized on the right. A 3-15% polacrylamide gradient gel was used to separate the proteins. Approximately 10,000 cpm of acid-precipitable proteins were applied to each lane.

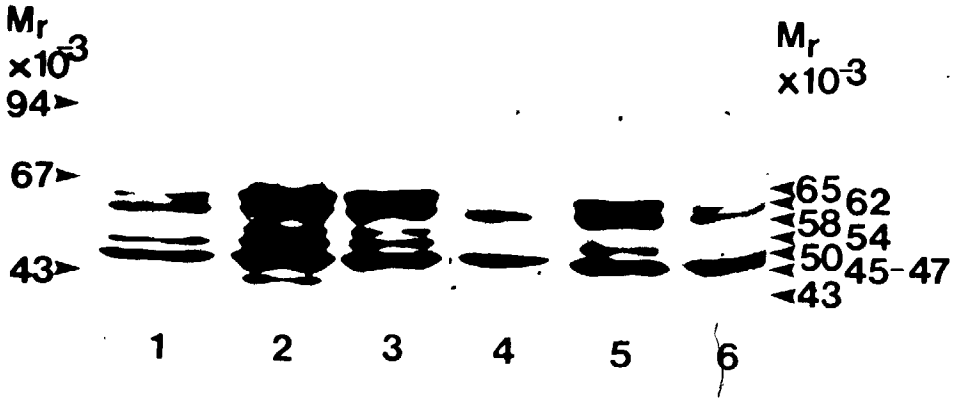
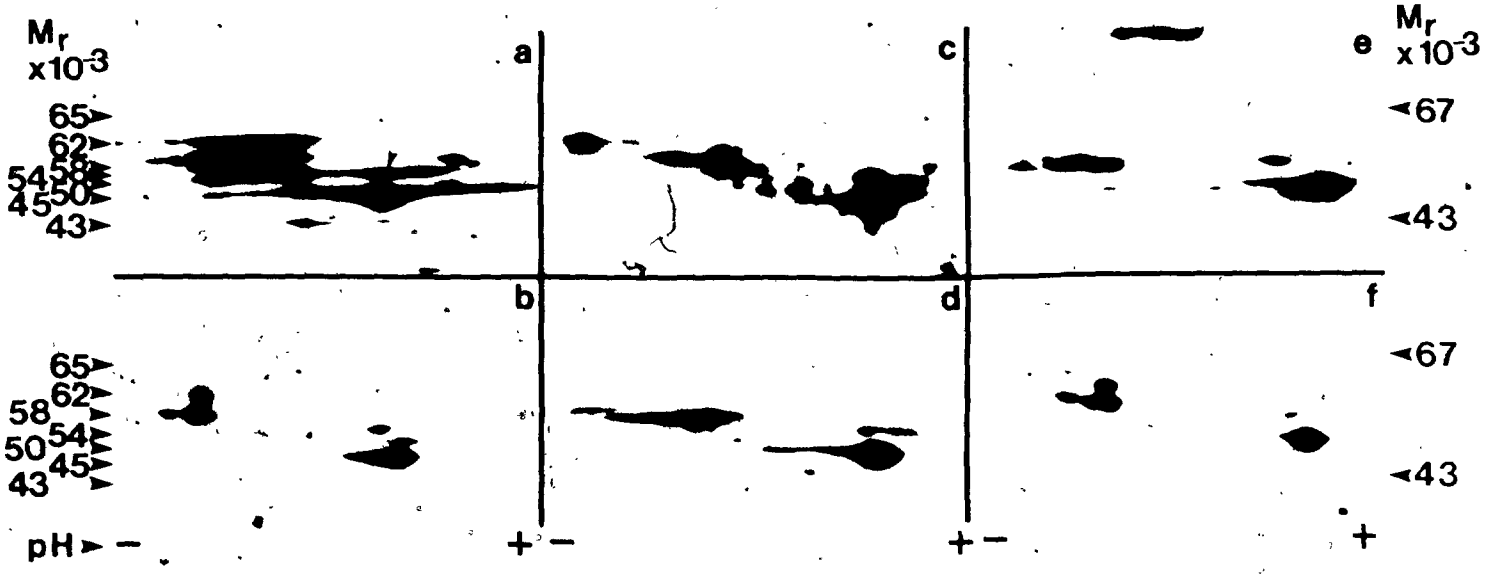

Mr
x10-3
94
67
43
Mr
x10-3
65
62
58
54
50
45-47
43
1
2
3
4
5
6

Figure 24.

Two-dimensional IEF-SDS-PAGE separation of the total proteins synthesized by hind limb epidermis from selected stages of spontaneously metamorphosing R. catesbeiana in a 4 hr labeling period during which they were injected with [^{14}C]leucine (panels a, c and e) or [^{35}S]methionine (panels b, d and f). Although the basic high molecular mass proteins are more intense when animals are labeled with [^{14}C]leucine, they are more clearly resolved into their isoelectric variants when [^{35}S]methionine is the labeled amino acid. The increase in the synthesis of the 50 kd protein(s) by animals staged as XXI and labeled with [^{14}C]leucine (panels a arrowhead) relative to animals from stages XXII (panels c) and stages XXIII (panels e) are readily apparent when the proteins are separated in two-dimensions. Molecular masses of the proteins within 40-70 kd are indicated on the left and co-electrophoresed standards on the right. The pH gradient used for electrofocusing gels was a mixture of pH 5-7 (.3 mL) and pH 3.5-10 (.2 mL). A 7.5-20% polyacrylamide gradient gel was used to separate proteins in the second dimension. Approximately 30,000 cpm of acid-precipitable lysate were applied to each electrofocusing gel.


Mr x10-3
65
62
54
50
45
43
a
b
c
d
e
f
Mr x10-3
67
43
65
62
58
54
50
45
43
67
43
pH
−
+
−
+
−
+

[^{14}C]leucine, the major discrepencies during these 3 stages of metamorphic climax are largely in intensity of specific proteins and the apparent decreased synthesis of high M_r, basic proteins as climax proceeds.

PART 4

DISCUSSION

4.1 Qualitative Assessment of the Proteins Synthesized by Xenopus laevis Kidney Epithelial Cells Incubated at Different Temperatures

The nutrient and temperature parameters for sustained cell growth of the Xenopus laevis kidney epithelial cell line (XKEC) have been described (Rafferty, 1976). However, little quantitative or qualitative data accompanied these descriptions (Rafferty, 1976). In this study, the amount of [^{14}C]leucine or [^{35}S]methionine incorporated into proteins synthesized by XKEC subsequent to a 2 hr incubation at temperatures between 5 and 36.5°C was quantitated. The results demonstrated that the maximum incorporation of either radioactive amino acid occurred at 34°C. This temperature is slightly less than the prolonged incubation of XKEC at 37°C from which Rafferty (1976) obtained inconsistent results on their survival. The ability of Xenopus cells to tolerate elevated temperatures was usually accompanied by chromosomal transformation (Rafferty, 1976).

To determine whether XKEC respond qualitatively to changes in temperature, the total proteins synthesized

were examined. Since 26°C is regarded as the temperature above which diploid cell cultures from amphibians undergo a declining growth, 22°C was used as the 'control' temperature. IEF-SDS-PAGE separation of the proteins synthesized by XKEC following a brief incubation at temperatures between 5 and 36.5°C demonstrated that the increase in radioactive amino acid incorporation at 34°C is accompanied by marked changes in the new and/or enhanced proteins synthesized. Although the initial change in protein synthesis (detection of a 75 kd protein) is first noted at 32°C, new and/or enhanced protein synthesis at 34°C is striking. The HSPs detected at 34°C and also at 36.5°C which have M_rs of 95.5 kd (pI 6.6), 80 kd (pI 6.6), 75 kd (pI 7.0), 68 kd (pI 6.2), 59 kd (pI 5.7), 43 kd (pI 5.6, 6.0 and 6.7) and 38 kd (pI 6.6) are very similar to those observed by Heikkila et al. (1985a; 1985b) and Nickells and Browder (1985) for embryonic Xenopus.

Results from Xenopus oocytes, and more recently, XKEC, showed that major HSPs with approximate M_rs of 70 and 30 kd were synthesized subsequent to incubation at elevated temperatures (Beinz, 1982; 1984; Beinz and Gurdon, 1982). A protein of 38.5 kd was the only product approaching 30 kd observed in this study. This discrepancy may be due to either the extended period of incubation used (4 hr) or the genetic differences which have likely arisen among the XKEC line maintained in

various places (Beinz, 1982; 1984; Beinz and Gurdon, 1982).

Since the XKEC line provides an available source of RNA, large numbers of cell cultures were incubated at the control and uppermost 'heat shock' temperature used to examine *in vivo* protein synthesis. The major cell-free translational products from the RNA obtained from cells incubated at 34° C had M_rs of 76, 72, 68, 59, 52, 47 and 45 kd. These M_rs are similar to those observed *in vivo*. Although the mRNA extracted from cells incubated at the control temperature (22° C) results in translational products with M_rs of 45 and 47 kd, the synthesis of these proteins is markedly enhanced at 34 °C. These results indicate that the mRNA extracted from heat-shocked XKEC is either newly transcribed or is present in a form which is not translatable prior to incubation at an elevated temperature.

In addition, cells were incubated at 41.5° C for two hours. At this temperature, many cells lift from the surface of the culture flask. The cells which remained adherent (AXKEC) were kept separate from those which lifted (LXKEC) and the total cellular RNA extracted and translated *in vitro*. The cells which lifted did not synthesize a 76 kd protein to the same degree noted in AXKEC; not resolved in AXKEC are a 73 kd, an acidic 49 kd, 2 isoelectric variants of a 43 kd and 38.5 kd proteins. The differences in RNA/protein product observed in the two

cell populations created by incubating XKEC at 41.5°C may be the result of their means of propogation. Since these cultures were not clonally derived prior to heat shock, there is no way of knowing that genetic integrity existed among the cell population. Further, the maintenance of these cells for two decades confers upon them the qualities of a transformed cell line, thus making the occurrence of spontaneous mutations likely.

In recent years, a great deal of interest in the relationship between HSP synthesis and subsequent thermotolerance has been generated (McAlister and Finkelstein, 1980; Burdon, 1982; Li and Werb, 1982; Dean and Atkinson, 1983; Velazquez and Lindquist, 1984; Dean and Atkinson, 1985; Giudice, 1985; Heikilla et al., 1985a; 1985b; Li and Lazlo, 1985; Nickells and Browder, 1985). In particular, the presence of the HSP70 has been the focal point of discussions on thermotolerance; its synthesis has been repeatedly associated with the ability of an organism to withstand environmental insults (reviewed by Heikkila et al., 1985b and Li and Laszlo, 1985). The results obtained by others leads to the speculation that among the differences in the RNA population of AXKEC there is an RNA species which conveys upon this adherent cell population some protein which better allows it to cope with this stress. Unlike the selective translation of HSP mRNAs observed in the homologous protein synthetic machinery from heat-shocked

Drosophila cells (Kruger and Beneke, 1981; 1982), heterologous systems do not display translational preference of particular mRNAs (Storti et al., 1980; Lindquist, 1981; Lindquist and DiDomenico, 1985; Ballinger and Pardue, 1985). Therefore, the presence of an mRNA isolated from heat-shocked XKEC should be translated if, in fact, it has been transcribed.

In a recent study Velazquez and Lindquist (1984) demonstrated that tolerance to physiological stresses such as heat shock and anoxia was related to the presence of the HSP70 in Drosophila larvae. In light of this development, the HSP76 in AXKEC could be considered highly suspect in conveying thermotolerance to AXKEC whose ability to remain attached to the culture flask is, at least, less affected by incubation at an elevated temperature. The synthesis of this protein is so enhanced in AXKEC that if the HSP73 observed in LXKEC is synthesized, it cannot be resolved.

These data clearly show that XKEC respond to brief elevations in their temperature environment by qualitatively changing the proteins synthesized. The response is most apparent at 34 and 36.5°C as demonstrated by the many new and/or enhanced proteins detected. These qualitative changes are not observed following incubation at depressed (5, 10 and 15°C) temperatures. Cell-free translation of RNA from XKEC reveals that the RNA from heat-shocked XKEC is either newly transcribed or in an

untranslatable form prior to incubation at 34°C.

4.2 Qualitative Assessment of the Proteins Synthesized by Primary Epidermal Cell Cultures of Rana catesbeiana Incubated at Different Temperatures

The parameters established for the successful cell culture of Xenopus, and a variety of mammalian epidermal and epithelial cell cultures proved invaluable for the cell culture of larval epidermis from R. catesbeiana. In particular, cell cultures from epidermis have, in the past decade, become the focus of much attention since epidermis provides the researcher with a wonderfully labile system (Reeves, 1975) by virtue of the various stages of maturity present among its cell layers (Rheinwald and Green, 1975b; Sun and Green, 1976; 1978b; Green, 1977; Tseng et al., 1982; Woodcock-Mitchell et al., 1982; Skerrow and Skerrow, 1983; Breitkreutz et al., 1984; Schweizer et al., 1984a; Dale et al., 1985). The constant turnover of the epidermal cell population and the effective protective barrier it provides against temperature, chemical and physical assaults (Green et al., 1981a) which are a natural part of the environment, make this an excellent system for in vivo and in vitro study.

The definition of particular requirements for the propogation of cultured keratinocytes and epithelia has been elaborated. Successful perpetuation of human keratinocytes and a variety of rat epithelial cells has

been accomplished using a substrate of lethally irradiated 3T3 mouse fibroblasts (Rheinwald and Green, 1975a; 1975b; Sun and Green, 1976; Green 1977; Green et al., 1981a; Phillips and Rice, 1983). Others have shown that collagen coated culture dishes provide an equally suitable substrate (Karasek and Charlton, 1971; Mancianti and Karasek, 1983). Although dermal substrates would provide an adherent for epidermal tissue pieces, some heterotypic dermal/epidermal interactions have been shown to alter the pattern of keratin synthesis in mouse (Schweizer et al., 1984b). Also, homotypically recombining tail fin epidermis with the underlying tail dermis in R. catesbeiana has a profound effect upon the response of this tissue to thyroid hormone (Niki et al., 1982). Since the focus of this study was to determine alterations in gene expression in developing epidermis, purity of epidermal explants without enzyme intervention to achieve this end and minimal stimulation by the binding substrate were desired. For these reasons, a 2% collagen coating commonly used in tissue culture (Hauschka, 1972) was chosen to adhere epidermal explants to culture dishes.

Attempts to clonally perpetuate keratinocyte colonies from larval R. catesbeiana epidermis by dispersing the cells with a trypsin solution were not successful. The use of epidermal explants was, therefore, required to obtain keratinocyte cultures. By this method, epidermal cells could be observed moving from tail fin or hind limb

epidermal explants within 6 hours after plating (Ketola-Pirie and Atkinson, 1983). These phenomena have been noted in other systems. In rabbit embryos, Banks-Schlegel (1982) noted that prior to stratification of the epidermis, trypsin dispersion for clonal perpetuation of keratinocyte colonies was ineffective. When explants of epidermis were used, she too, observed the relative quickness of movement of keratinocytes from the tissue pieces.

Once successful at maintaining the primary epidermal cells from this amphibian (larval R. catesbeiana), I was curious to determine if these cells would respond to temperatures in the same way as XKEC, an established cell line. Also, the age-dependent ability of animals to elicit a response to a temperature stress has recently been the focus of much attention (Heikkila et al., 1985a; 1985b; Nickells and Browder, 1985). Since the primary epidermal cells from R. catesbeiana were obtained from larval animals, the question of ability to respond to temperature seemed particularly relevant. With minimal manipulation, larval anuran epidermis can be subjected to the temperature stresses which are a natural part of its habitat.

When cell cultures from larval tail fin and hind limb epidermis were incubated at elevated temperatures, the cells responded by dramatically altering their gene expression. A one hour incubation at a temperature of

32°C or greater is sufficient to elicit a depression of the synthesis of the proteins normally made at the control temperature (22°C). A 65 kd protein, pI 6.7-6.8, previously not detected by fluorography is observed at 32°C. With increasing temperature, both the synthesis of the 65 kd protein and depression of the usual pattern of protein synthesis become more pronounced. Only at 36.5°C is a 25 kd protein with pI 6.7-6.8 observed. As has been noted in other systems (Lewis et al., 1975; Mirault et al., 1978; Atkinson, 1981a; Lindquist et al., 1982), the synthesis of these HSPs is both temperature dependent and non-coordinate.

The incubation of primary epidermal cells from tail fin and hind limb at suboptimal growing temperatures for 2 hours resulted in no detectable alteration of the protein synthetic pattern except at 5°C. The 65 kd protein, pI 6.7-6.8, was observed at this temperature. The depressed synthesis of other proteins was not apparent in 'cold shock' conditions.

Since cells from the primary cultures examined have different developmental fates and represent constituents from tissues which are wholly larval (tail fin) or adult (hind limb) (reviewed by Atkinson, 1981b), it is concluded that the ability of cells from this organism to respond to cold or heat shock by rapid changes in gene expression is neither tissue specific nor dependent on the differentiative state of these cells.

Observations made by phase contrast microscopy show that morphologically, the primary epidermal cells responded in a similar manner to supra- or suboptimal temperatures. Unlike the resistance to morphological perturbations noted in XKEC, the morphology of the epidermal cells from larval Rana were highly susceptible to temperature changes. The typical polygonal shape of epidermal cells was markedly altered. Many cells became increasingly refractile and elongated in appearance at 5, 30 and 36.5°C.

These morphological features were similar to those observed when epidermal cells are maintained in low or calcium free medium (Hennings et al., 1983a; 1983b; Steinert and Cantieri, 1983). Although the epidermal cells would grow and divide for limited periods at low calcium concentrations, desmosome formation, keratin synthesis, stratification and ultimately, terminal differentiation were severely affected (Watt and Green, 1982; Hennings et al., 1983a; Steinert and Cantieri, 1983; Breitkreutz et al., 1984). The cells are also characterized by distinct intercellular spaces which increase the refractility of the epidermal cell sheet and a perinuclear arrangement of tonofilament bundles which could account for an altered cellular morphology (Hennings et al., 1983a). The observations of epidermal cells from other organisms maintained in medium containing low calcium are particularly interesting since the cell

cultures in this study were switched to a calcium free environment during temperature stress and the 2 hr labeling period.

Incubation of primary epidermal cell cultures from R. catesbeiana clearly demonstrated that these cells, like XKEC, respond to temperature elevations by synthesizing new and/or enhanced proteins. Although the actual number of new and/or enhanced proteins synthesized in response to temperture elevation by primary epidermal cell cultures from Rana was noticeably fewer than in XKEC, the response was more profound. First, with increasing temperature the synthesis of new and/or enhanced proteins became increasingly intense. Also, the reduction in 'control' protein synthesis was more apparent in primary cultures from Rana. Completely undetected in XKEC, but readily apparent in primary epidermal cell cultures from R. catesbeiana, is the synthesis of a novel protein(s) of 65 kd in response to incubation at 5°C.

4.3 Qualitative Assessment of the Water-Insoluble Proteins Synthesized by Primary Epidermal Cell Cultures from Larval R.catesbeiana Tail Fin and Hind Limb

Keratins are the water-insoluble proteins synthesized by epidermal cells in situ and in vitro. The keratin family of proteins provide an ideal marker for the

assessment of epidermal differentiation. Since primary cultures from tail fin and hind limb incubated at elevated or depressed temperatures could not be distinguished from each other on the basis of the proteins synthesized or morphological features of the epidermal sheets arising from their explants, the question as to whether they would respond differently to T_3 medium supplements arose. In situ, the gross and microscopic morphological response of the epidermis to endogenous or induced T_3 elevation is quite different in these organs. However, when removed from the animal, the influences of the surrounding tissue are eliminated. In this instance, would the epidermal cells from hind limb and tail fin synthesize the same water-insoluble proteins? This seemed particularly relevant since, in a recent study, Niki et al. (1982) demonstrated the necessity of the epidermis for degeneration of the tail in T_3-treated R. catesbeiana larvae.

It has been shown that the presence of many growth medium supplements affect the progression of keratin synthesis or morphological differentiation in cultures of human keratinocytes (Green, 1978; Fuchs and Green, 1978; 1980; Rheinwald et al., 1984), a variety of rat epithelia (Masuda et al.,1985; Phillips and Rice, 1983) and mouse and bovine mammary epithelium (Asch and Asch, 1985; Ben Ze'ev, 1985). In this study, four different growth conditions were used to maintain hind limb epidermal cell

cultures from premetamorphic animals (stages IX-XI). This was necessary to determine whether changes observed in water-insoluble protein synthesis were due to the presence of T_3 in medium or were contributed by some other factor(s). Since the epidermal tissues in this study were not enzymatically cleared of contaminating underlying tissue, it was necessary to determine if fibroblasts were contributing to the profile of water-insoluble proteins extracted. The growth of fibroblasts (which synthesize the intermediate filament protein vimentin) proliferates in the presence of F.C.S. and is suppressed in its absence (Asch et al., 1981; Rheinwald et al., 1984; Asch and Asch, 1985). By IEF-SDS-PAGE vimentin can easily be detected by a characteristic 'step' separation pattern (Franke et al., 1980b; 1980d; Ochs et al., 1983; Asch and Asch, 1985). When epidermal cells from hind limb (stages IX-XI) were maintained in culture for 36 hours or 5 days in unsupplemented MEM or MEM + 5% F.C.S. evidence of active fibroblast contamination based on the criteria described above could not be detected.

When epidermal cells from hind limb (stages IX-XI) were maintained in MEM + 3 µL T_3/mL medium for 36 hours, the water-insoluble proteins synthesized resembled those extracted from 5 day hind limb epidermal cell cultures (stages IX-XI). Although fewer water-insoluble proteins within 40-70 kd are noted after only 36 hr, the pattern of synthesis was similar to that observed after 5 days,

particularly in the presence of F.C.S. Interestingly, the water-insoluble proteins synthesized by 36 hr cell cultures when 5 μL T_3 was applied directly to the explants more closely paralleled the protein products observed from cells grown in MEM-serum and MEM + 5% F.C.S. The direct application of T_3 to explants may reflect a less efficient means of exposing epidermal explants to thyroid hormone as opposed to its addition directly to the growth medium.

In the presence of 3 μL T_3/mL of medium and 5 μL T_3/explant, the synthesis of a 59 kd water-insoluble protein was detected. A 59 kd protein (cytokeratin 4 in the human catalog of cytokeratins, Moll et al., 1982a) has been observed in stratified non-keratinizing epithelium from mammals (Moll et al., 1982a; Tseng et al., 1982; Woodcock-Mitchell et al., 1982; Sun et al., 1983a; 1983b; Eichner et al., 1984; Dale et al., 1985). Also, the absence of low M_r water-insoluble proteins (51 and 53 kd) in extractions from hind limb epidermal cells maintained in MEM + 3 μL T_3/mL medium indicates the stratification of epidermis (Dale et al., 1985). Indeed, it has recently been shown that the synthesis of keratins typical of a stratification and the depressed synthesis of keratins associated with a simple epithelium precede morphological stratification of the epidermis (Dale et al., 1985).

A positive immunofluorescence test for epidermal keratins was observed in 36 hr epidermal cell cultures from hind limb (stages IX-XI) maintained in the presence

or absence of T_3. Although the well-developed tonofilament network characteristic of epidermis and a variety of epithelia from mammalian sources (Sun and Green, 1978a; Sun et al.,.1979; 1983a; 1983b; Franke et al., 1979; 1981a; 1981b; 1981c; 1981d; 1981e; Breikreutz et al., 1981; 1984; Debus et al., 1982; Osborn, 1983) was not observed, the pattern is typical of basal and intermediate layers of epidermis from newborn mouse (Schweizer et al., 1984a). The more diffuse staining and beginings of tonofilanent formation are typical of intermediate epidermal layers (Schweizer et al., 1984a). The lack of a well-defined tonofilament network likely reflects the inadequate quantities of Type II keratins required for tonofilament formation (Fuchs, 1983; Fuchs et al., 1983; 1984; Steinert et al., 1985b). The intensely fluorescent cells noted in epidermal cells cultured in 3 µL T_3/mL medium, are characteristic of the small cells of the basal layers (Schweizer et al., 1984a). This demonstrates that T_3 is effective in sustaining the germinative cells required for maintenance of the epidermal cell population. These findings are in accord with the epidermal development of R. catesbeiana from stages IX-XI. Morphologically, the epidermis is simple and 2 cell layers thick. Birefringence, characteristic of keratinization, is not observed (Matoltsy, 1964; Wright, 1973; Fox, 1975; 1981; Atkinson, 1981b).

When hind limb epidermal cells from stages XII-XV and

XVI-XIX were cultured for 36 hours, the water-insoluble proteins synthesized bear a greater similarity to those observed from 5 day cell cultures maintained in F.C.S. than to their 36 hour counterparts. The presence of a 69 kd water-insoluble protein extracted from stages XII-XV epidermal cells cultured in T_3 supplemented medium is observed in keratinizing epidermis (Fuchs and Green, 1980; Moll et al.,1982a; Sun et al., 1983a; 1983b; 1984; Dale et al., 1985). This is in agreement with the developmental progression of the epidermis observed in Rana from this stage group. At stage XII, keratinization of the epidermis covering the hind limb is first detected (Wright, 1973).

Synthesis of water-insoluble proteins (M_r=63 kd) which correspond to keratinization of the epidermis were noted in 36 hr hind limb epidermal cells (stages XVI-XIX) maintained in the presence of F.C.S. In T_3-supplemented medium, an additional water-insoluble protein of 73 kd was detected. While a protein with this M_r was observed by one-dimensional separation of extractions from 36 hr hind limb epidermal cell cultures (stages IX-XI), it could not be detected by IEF-SDS-PAGE. Although the M_r does not correspond to previously defined epidermal keratins (Lazarides, 1980; Steinert et al., 1985b), it is in agreement with the results of Franke and colleagues (1981d) for desmoplakin, a major protein of desmosomes. In addition, the synthesis of desmoplakin is coordinated

with the increased number of cellular contacts that occur as epidermis stratifies (Dale et al., 1985).

Epidermal cells from stages IX-XI and XII-XV hind limb maintained in culture for 5 days synthesized water-insoluble proteins during the final 36 hr that are characteristic of a maturing epidermis as defined by their increasing M_r (59-69 kd) and basic pI (Fuchs and Green, 1980; 1981; Banks-Schlegel, 1982; Moll et al., 1982a; Tseng et al., 1982; Woodcock-Mitchell et al., 1982; Skerrow and Skerrow, 1983; Sun et al., 1983a; 1983b; 1984; Eichner et al., 1984; Dale et al.,1985; Steinert et al., 1985b). The epidermal cells from these two groups synthesize water-insoluble proteins which are, for all intents and purposes, identical; the most striking variation between the proteins of these two stage groups is quantitative rather than qualitative.

Hind limb epidermal cells (stages XVI-XIX) maintained in T_3 supplemented medium synthesize enhanced levels of 63-69 kd proteins associated with terminal differentiation of the epidermis (as discussed above). The synthesis of water-insoluble proteins of lower M_r appear quantitatively and qualitatively similar in the presence or absence of T_3. Thyroid hormone did stimulate the synthesis of a water-insoluble protein of 94 kd in 5 day epidermal cell cultures from this stage group. However, the significance of this protein is not apparent. In this (stage XVI-XIX), and all 5 day hind limb epidermal cell

cultures (stages IX-XI and XII-XV), the 73 kd water-insoluble protein (discussed above) was among the major proteins observed. The detection of this protein corresponds to the morphological maturation of epidermal cell cultures observed by others after 5 days (Reeves and Laskey, 1975; Green, 1977).

The water-insoluble proteins extracted from tail fin epidermal cell cultures do not display differences in response to their sustaining medium during the first 36 hours of cell culture. After five days in cell culture, quantitative differences in the water-insoluble proteins synthesized in the presence or absence of T_3 are evident. In the presence of T_3, epidermal cells from tail fin synthesized enhanced quantities of basic, higher M_r water-insoluble proteins. The epidermal cell cultures from tail fin respond to T_3 in a manner similar to that observed in the intact animal. Organs required for larval life have a higher threshold to T_3 and a relatively protracted period prior to eliciting a response to T_3 (reviewed by Freiden and Just, 1970 and Atkinson, 1981b) compared to organs required for adult life.

During the first 36 hours of cell culture, hind limb epidermal cells respond to T_3 medium supplements by synthesizing water-insoluble proteins characteristic of differentiation. After 5 days in cell culture, the response of hind limb epidermal cells is both temporal and environmental. These cell cultures synthesized higher M_r

proteins of basic pI in response to the time in culture. However, the synthesis of these water-insoluble proteins is somewhat enhanced in T_3-supplemented medium. Primary epidermal cell cultures from tail fin did not respond to T_3 after 36 hr in cell culture. T_3-induced differences in epidermal cell cultures from tail fin were subtle quantitative differences detected after 5 days.

4.4 Immunoprecipitation of Keratins from Five Day Hind Limb Epidermal Cell Cultures

It was necessary to determine whether the water-insoluble proteins synthesized by primary hind limb epidermal cell cultures from the 3 larval stages were, indeed, keratins. If, in fact, they proved to be keratins, were they differentially expressed based on the maturity of the larvae from which the epidermal cell cultures were derived?

The keratins immunoprecipitated from 5 day cell cultures revealed that the high M_r proteins characteristic of a maturing stratified squamous epidermis are most prominent in 9 M urea solubilized fractions from stages XII-XV and XVI-XIX. The detection of 67 and 51 kd keratins as major proteins in immunoprecipitates from stages XVI-XIX agrees with previous observations from keratinizing mammalian epidermis (Roop et al., 1983; Steinert et al., 1985b). The keratins immunoprecipitated from hind limb epidermal cell cultures (stages IX-XI) were

of the type noted in immature epithelium or non-keratinizing stratified squamous epithelium. The 60 kd protein (cytokeratin 4 from Moll et al., 1982a) was detected but the 65 kd protein was barely resolved in stages IX-XI. Interestingly, a 60 kd water-insoluble protein which was a major synthetic product among the high M_r proteins on IEF-SDS-PAGE separation of stages XII-XV and XVI-XIX was not detected in their immunoprecipitates. Also, the number of proteins precipitated within the 40-70 kd range is less than observed on two-dimensional fluorograms. To date, a single polyclonal antiserum or monoclonal antibody which has the capacity to recognize all keratin subunits has not yet been made (Franke et al., 1981a; Tseng et al., 1982; Asch and Asch, 1985). It may be that the 60 kd protein although present, is in a configuration which is particularly stable or masks antigenic determinants.

Furthermore, Franke et al. (1983) have demonstrated that keratins vary in their solubility properties in different urea concenntrations. In this study, the concentration of urea was decreased to allow reaction with antibodies. The 60 kd protein, as well as other water-insoluble proteins which correspond in M and pI to previously catalogued keratins (Moll et al., 1982a), may be particularly suseptible to spontaneous reformation in vitro as has been observed for some members of the keratin family of proteins (Steinert et al., 1978; Franke et al.,

1983; Steinert and Cantieri, 1983).

The variable solubility of different keratin subunits was also demonstrated in this study. The almost complete exclusion of high M_r keratins in immunoprecipitates from RIPA solubilized fractions illustrates this buffer's inability to (1) alter the configuration of high M_r keratins to allow reaction of epitopes with affinity purified polyclonal antibodies or (2) maintain high M keratins in solution thereby resulting in their exclusion prior to the addition of the antibody.

Proteins within 20-40 kd were detected in immunoprecipitates from 5 day hind limb epidermal cell cultures (all stages). Since the α-helical subunits of mammalian keratin filaments have been shown to have an M_r of 24-26 kd. this would account for the proteins which are detected within this M_r range (Steinert and Idler, 1975; Fuchs and Green, 1978; Steinert et al., 1980; Weber and Geisler 1982; Steinert and Cantieri, 1983). Unidentified proteins of M_r 20 kd in mouse mammary epithelium (Asch and Asch, 1985), 20 kd (+/- 6 kd) in X. laevis epidermis (Reeves, 1975) and 37 kd in human esophageal epithelium (Banks-Schlegel and Harris, 1983) have also been identified by immunoprecipitation or immunoblots. Others have suggested that co-precipitation of proteins with an approximate M_r of 37 kd respresents filaggrin, a major protein of the amorphous matrix in epidermal cells.

Although some contaminating proteins

immunoprecipitate with rabbit anti-human cytokeratin antibodies, the results clearly show that in hind limb epidermal cell cultures from stages associated with keratinization of the epidermis in situ (Matoltsy, 1964; Wright, 1973), high M_r, differentiation specific keratins are immunoprecipitated. This is particularly apparent in immunoprecipitates from stages XVI-XIX in which the presence of the 67 kd is enhanced. Since an equal number of cpm were used in all fractions reacted with anti-human cytokeratin antibodies, this finding is relevant.

4.5 Proteins Synthesized by Hind Limb Epidermis in situ by Spontaneously Metamorphosing and Preclimactic Rana catesbeiana

The effect of endogenous and induced T_3 elevation on epidermal cell protein synthesis in situ was examined. From animals in the process of spontaneous metamorphosis, total hind limb epidermal cell proteins were examined. Water-insoluble proteins were extracted from hind limb epidermis of larval animals injected with T_3 or NaOH.

When separated by 1-D or 2-D gel electrophoresis, hind limb epidermis from spontaneously metamorphosing animals synthesized the same proteins with some quantitative and slight qualitative variations. The resolution of all high M_r proteins detected by 1-D analysis was not observed when the proteins are separated in 2 dimensions. Although certain isoelectric variants

of some basic, high M_r proteins were not resolved in all conditions, the proteins synthesized were, nevertheless, characteristic of terminally differentiating epidermis. In addition, the separation of the total proteins from the hind limb epidermis of spontaneously metamorphosing animals are similar to those noted in keratin-enriched fractions from 5 day hind limb epidermal cell cultures (stages XVI-XIX) solubilized in 9 M urea. This demonstrates that: (1) the pattern of synthesis of keratins in cell cultures from stages XVI-XIX are similar to that observed during spontaneous metamorphosis and (2) the major proteins synthesized by hind limb epidermis during spontaneous metamorphosis appear to be of the keratin-type.

With the exception of a 56.5 kd protein, the water-insoluble proteins synthesized <u>in situ</u> during the 36 hr following injection with NaOH or T_3 appeared to be identical on the basis of their M_r regardless of the treatment or the stage of the larvae. A 56.5 kd protein has been associated with hyperproliferation in mammalian epidermis (Weiss et al., 1984). It is possible that T_3 stimulation of hind limb epidermis to differentiate could also provoke hyperproliferation. However, without 2-D separation, the 56.5 kd protein cannot be clearly distinguished from the acidic 56 kd protein observed in epidermal cell cultures from tail fin.

From water-insoluble protein extracts from larval

Rana catesbeiana labeled in situ the synthesis of 63-69 kd was detected. This is particularly interesting since these proteins were not observed to any great extent in 36 hour cell cultures from the same staged animals. A dedifferentiation of the epidermal tissue accompanying wounding and cell culture may be occurring in vitro. Such occurrances have been noted in mammalian epidermis and epidermal cell cultures (Fuchs and Green, 1978; 1980).

IEF-SDS-PAGE separation of the water-insoluble proteins synthesized by larval epidermal cells from R. catesbeiana provides a useful method for examination of changes in protein synthesis. However, in addition to keratin-like proteins, contaminating proteins were also extracted. The coextraction of water-insoluble proteins which may or may not correspond to the 40-70 kd range assigned to the keratin family of proteins has been noted in other systems (Reeves, 1975; Banks-Schlegel, 1982; Lane, 1982; Asch and Asch, 1985). For example, a water-insoluble protein with an M_r of 43 kd was extracted from in situ and in vitro labeling of all 36 hr and 5 day hind limb stage groups. This protein which likely represents actin, has been recorded by others (Sun and Green, 1977; Fuchs and Green, 1978; Green, 1980; Gray, 1981; Green et al., 1981b; Banks-Schlegel, 1982; Lane, 1982; Ochs et al., 1983; Breitkreutz et al., 1984; Asch and Asch, 1985; Ben Ze'ev, 1985) as a contaminant in the preparation of keratin-enriched fractions. The presence

of such contaminants exemplifies the need for immunological assessment of water-insoluble protein synthesis.

PART 5

CONCLUSIONS

The following conclusions can be made based on the results presented:

(1) Incubation of the established Xenopus laevis kidney epithelial cell line at 34 and 36.5°C results in the synthesis of many new and/or enhanced proteins. These proteins represent RNAs which are either newly transcribed or transcribed at markedly increased levels following temperature elevation. When RNA from cells incubated at 41.5°C is translated in vitro, the synthesis of a 76 kd protein is markedly enhanced in the cell population which remains adherent to the culture flask. This protein may confer thermotolerance to the adherent cell population.

(2) In Rana catesbeiana, the response of primary epidermal cell cultures from organs which are wholly larval (tail fin) or adult (hind limb) to a temperature stress is identical. A novel 65 kd protein is observed at 5°C and 32-36.5°C. Only at 36.5°C is a 25 kd protein detected. Therefore, in this system the synthesis of stress-induced proteins is noncoordinate in epidermal cell cultures from

either source.

(3) During the first 36 hours of cell culture, T_3 induces the precocious synthesis of water-insoluble proteins associated with epidermal differentiaton in primary epidermal cell cultures from hind limb (stages IX-XI, XII-XV and XVI-XIX). T_3 does not induce the same water-insoluble proteins in cell cultures from all stages. In hind limb epidermal cell cultures from stages IX-XI, the water-insoluble proteins induced by T_3 have a similar M_r and pI to those associated with epidermal stratification. T_3-supplemented medium induces the synthesis of water-insoluble proteins which correspond to keratinization of the epidermal cells in cultures from stages XII-XV and XVI-XIX.

(4) The water-insoluble proteins synthesized by primary hind limb epidermal cells after 5 days in culture are indicative of epidermal differentiation. However, these are not T_3-induced. Rather, the cells seem to reflect epidermal maturation which results from the length of time the cells are maintained in culture.

(5) Primary epidermal cell cultures from tail fin are not responsive to T_3 after 36 hr or 5 days. Although the enhanced synthesis of some water-insoluble proteins was observed in the presence of T_3, the induction of specific

proteins associated with epidermal maturation only in T_3-supplemented medium is not apparent. The absence of a T_3-induced response can be attributed to insuffucient T_3 levels or possibly inadequate exposure time. Alternatively, T_3 may not induce distinct changes associated with epidermal maturation in tissues whose ultimate fate (at metamorphic climax) is regression.

(6) Many of the water-insoluble proteins synthesized by primary epidermal cell cultures from R. catesbeiana are keratins. The keratins precipitated from hind limb epidermal cell cultures (stages XVI-XIX) indicate a more mature state of epidermal differentiation than is observed in the keratins immunoprecipitated from stages IX-XI and XII-XV.

(7) In situ, the water-insoluble proteins synthesized by hind limb epidermal cells from larvae (stages IX-XI, XII-XV and XVI-XIX) during the 36 hr following treatment with T_3 or NaOH are similar to each other but different from those observed in cell culture. Water-insoluble proteins with M_rs indicating epidermal keratinization are observed in situ. The conflicting results between the water-insoluble proteins synthesized in situ and in cell culture may be due to an inital dedifferentiation of the hind limb epidermal cells maintained in culture. Alternatively, in the absence of tissue-tissue

interactions, cultured hind limb epidermal cells may be more susceptible to the effects of T_3.

(8) The proteins extracted from the epidermis of Rana catesbeiana in various stages of metamorphic climax are similar to each other. The M_rs and pIs are similar to the water-insoluble proteins observed from 5 day hind limb epidermal cell cultures.

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