Meeting Report

HORMONAL REGULATION OF SPECIFIC GENE EXPRESSION

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1. Introduction

A FEBS Advanced Course (No. 53) was held September 19–22, 1978 at The Middlesex Hospital Medical School on the 'Hormonal Regulation of Specific Gene Expression'. The course coincided with the celebrations to mark the 50th Anniversary of the Foundation of the Courtauld Institute of Biochemistry. A Symposium was held on the first day at which the speakers surveyed various aspects of the subject for the comparative nonexpert. The subsequent course lasted two and a half days and consisted of talks by invited speakers and shorter contributions from participants. As with the last course on a related but different subject [FEBS Letters (1976) 72, 215–226] we, as organizers, were asked to produce a survey of the papers presented and of the discussion and in particular to provide a bibliography that would serve as an entrée to the field. Rather than try to provide a blow by blow account we have tried to pinpoint the present status of the various aspects of the subject. All the major contributors have had an opportunity to comment on a draft report and we hope that there is a reasonable concensus concerning the views expressed.

The plan of the course was to consider first the progress that has been made in developing cell cultures that have either been shown to respond to hormones or have the potential of doing so. There followed several sessions in which current work on various hormones was considered in turn. Particular emphasis was placed on the use of in vitro systems, either cell cultures or explants. In the last session the contribution of current developments in recombinant DNA research to endocrinology was reviewed.

There are two broad approaches to an understanding of gene expression in eukaryotes. Either one can utilize systems in which a few well-defined proteins are produced in response to specific stimuli, or one can study systems in which the specific genes of interest are reiterated to such a degree that they may be isolated by physical means alone. The present course was almost entirely confined to examples of the first of the two approaches.

2. Isolation and maintenance of hormonally responsive cell cultures

The prospect of culturing animal cells under carefully defined conditions has for long been an attractive proposition to those who are interested in delineating the precise mode of action of the various hormones. A major problem has been the dependency of such cell cultures on the presence of serum in the medium. It has been proposed that the role of serum in cell culture is to provide cells with complexes of hormones [1]. G. H. Sato (San Diego) has made a study of the growth requirements of various types of cells in the absence of added serum. He has shown that usually a mixture of several hormones is required and that the precise mix differs as between the different cell types [2,3]. The following cells have been studied, GH_3 pituitary tumor, HeLa, melanoma, Swiss and Balb 3T3, MDCK dog kidney, rat and mouse neuroblastomas, human mammary tumor, rat pheochromocytomas, rabbit aorta and smooth muscle, normal rat ovarian and C6 glioma. The defined media can be used for primary culture. The following generalizations emerge as regard media.

All cells need insulin and transferrin. Among other substances required for GH_3 cells are triiodothyronine and thyrotrophin releasing hormone, parathyroid hormone, fibroblast growth factor and somatomedin C. The production and characterization of the various somatomedins is clearly a growth area in this field. In addition to defining the growth factor *per se* it is, in some cases, also necessary to adsorb purified serum proteins to the plastic surface to allow proper cell attachment. It is likely that well-characterized substances such as fibronectin and polylysine will suffice for this purpose. Conditioning factors may be necessary for some cells. It is probable that these will turn out to be new polypeptide growth factors.

The overall impression from Sato is that steady progress is being made and the complexity of the systems is not as formidable as originally perceived. There are clearly already cell lines available whose hormonal requirements are completely defined and similar information concerning all other cell lines should be available in the near future.

J. F. Tait (Department of Physics, the Middlesex Hospital Medical School) described the use of purified and unpurified dispersed cells to investigate the mode of action of trophic hormones. The adrenal cortex contains at least three types of steroid producing cells, those originating from the zona glomerulosa (ZG), fasciculata (ZF) and reticularis (ZR). These may exclusively produce certain steroids, e.g., aldosterone by the zona glomerulosa. However, they all produce at least one steroid, corticosterone, in common but its mode of stimulation differs for the three types:

(a) The zona fasciculata production is stimulated by ACTH and by very few other factors. Tissue culture, dispersed and in situ ZF cells show similar characteristics.

(b) The zona glomerulosa production is stimulated by small changes in K^+ concentration, angiotensin II, ACTH and for rat adrenals in vitro, serotonin [4–6]. The maximum stimulatory ratio (stimulated/basal production) for ZG is lower than for ZF cells. Dispersed and in situ cells but not, according to published results [7], tissue cultured ZG cells show similar characteristics.

(c) Production by zona reticularis cells, as far as is known, is stimulated by the same factors as ZF cells but the stimulatory ratio for ACTH is lower. Also the ZR cells quantitatively have a different pattern of steroid output compared with ZF cells [8,9].

The characteristics of these cells make them potentially powerful tools for the investigation of the mode of action of trophic hormones, including effects on gene expression, because by comparison between cell types, the causality of certain correlations can be tested. However, for this and other purposes, homogeneous cells must be used, otherwise even a 5% contamination can lead to ambiguous results [10]. Also purification of the cell preparation will tend to eliminate damaged cells. The employment of superfusion techniques would be an advantage so that in situ conditions can be more closely reproduced [11]. The effects of product inhibition or utilization of precursors from contaminating cells may be reduced. Also in superfusion experiments, the cells can act as their own control. This may be important as the variation in significant intermediate factors, such as cyclic AMP may be small.

As regards the specific methods studied:

(i) Unit gravity sedimentation has been employed to obtain the three types of adrenal cells in a reasonably pure form. Their characteristics before and after such procedures were discussed [10]. Recently a simpler but effective method of purification using gel filtration has been applied [12].

(ii) For superfusion of large or heavy cells, embedding techniques have been used [13]. Elutriation techniques have also been investigated [14] but the problem of continuous loss of homogeneous cells has not been overcome. The most successful technique has been to incorporate the cells into gel columns [15]. These, after modifications, can also be used for wash out experiments, useful in investigating effects on inorganic ionic fluxes [16].

U. Gehring (Heidelberg) described his recent work on the cell genetic analysis of steroid hormone action. Certain murine lymphoma cells are killed upon exposure to high physiological or pharmacological doses of glucocorticoids. This unique type of cellular response has been used for selecting a series of glucocorticoid-resistant clones. Three major phenotypes of resistance have been obtained with defects in steroid binding to the specific receptor or in the

nuclear interaction of the receptor–steroid complex. Cell hybridization studies suggest that these defects reside in the receptor molecule itself rather than in any modulators associated with the receptor system. These phenotypes of resistance belong to the same complementation group and thus both the steroid binding site and the nuclear interaction site of the receptor molecule appear to be contained within the same molecular unit, probably within the same polypeptide chain.

Hybrids between wild-type and resistant lymphoma cells are glucocorticoid sensitive. Resistance, however, arises quite frequently in these hybrids with a rate about 100-fold higher than in the parental wild-type cells. In the majority of individual hybrid cells, steroid resistance is acquired concomitantly with the loss of one chromosome.

The cytotoxic glucocorticoid effect in lymphoma cells is related to the extent of receptor saturation. A critical amount of receptor has to be complexed with steroid for the response to occur. A series of cell genetic experiments show that the cytotoxic actions of glucocorticoids and cyclic AMP, although similar in extent and kinetics, occur by independent mechanisms. Nevertheless, pretreatment of wild-type lymphoma cells with dibutyryl cyclic AMP makes the cells more susceptible to the cytotoxic action of glucocorticoids [17].

B. A. Cooke (Royal Free Hospital School of Medicine, London) and co-workers (Erasmus University, Rotterdam) have purified Leydig cells from rat testes and have studied the mechanism of action of lutropin on these cells during in vitro incubation. It was shown that protein synthesis is involved in stimulation by lutropin of testosterone production and that the protein(s) involved has a half-life of <13 min. Further studies demonstrated that lutropin induces synthesis of a protein with mol. wt 21 000 – this was detected by labelling the Leydig cell proteins with [^{35}S]methionine followed by separation by polyacrylamide gel electrophoresis and autoradiography of the dried gel. Evidence was obtained which indicated that the induction of this protein is mediated by cyclic AMP and involves synthesis of mRNA. Its half-life is >30 min and therefore is probably not the proposed regulator protein involved in control of steroidogenesis. Incubation of Leydig cells from immature rat testes showed that a protein with mol. wt 21 000 was also induced by lutropin in these cells. In addition lutropin also induced proteins of mol. wt 11 000, 21 000, 27 000 and 29 000. At high concentrations of lutropin there was a decrease in the synthesis of a protein with mol. wt 13 000. Induction of the 21 000 mol. wt protein was not detectable in lutropin-sensitive neoplastic Leydig cells but induction of two proteins with the same molecular weight as detected in Leydig cells from immature rats was demonstrated (mol. wt 27 000 and 29 000). None of the proteins detected have a short half-life and further work now indicates that the synthesis of proposed short half-life protein(s) involved in steroidogenesis may not be under the control of lutropin, i.e., it is synthesized independently of lutropin and plays a permissive role [18].

J. Taylor-Papadimitriou (ICRF, London) described the growth requirements of human epithelial cells in culture. While epithelial cells can be cultured from non-malignant human mammary tissues and secretions, conditions for the routine culture of malignant epithelial cells from primary breast carcinomas have not been found. A study has been made of the factors affecting the growth of the non-malignant mammary epithelial cells which can be obtained in homogeneous culture from milk, and, by spillage, from benign tumours. To date, maximum growth of epithelial cells has been obtained in the presence of fibroblast feeders in medium RPMI containing insulin and hydrocortisone, foetal calf serum and epidermal growth factor (EGF). Short term effects of oestradiol and progesterone on DNA synthesis can be demonstrated in the absence of serum but require feeders. The growth stimulatory effects of serum, EGF and the steroid hormones are all inhibited by interferon.

The growth requirements of epithelial cells from milk (which morphologically appear to be of two types) and from fibroadenomas (morphologically one type) are similar although the serum requirement is more exacting for fibroadenoma cells. On the other hand, EGF, feeders, hydrocortisone and other hormones, tested with a range of serum concentrations, have not improved the growth of human mammary carcinoma cells, which appear to differ considerably in their growth requirements from normal and benign tumour epithelial cells [19–21].

The properties of a promising cell line, Rama 25, derived from a mammary tumor induced in a rat by dimethylbenzanthracene was described by P. S. Rudland (ICRF, London). During rapid proliferation, Rama 25 cells appear as a single undifferentiated epithelial type; at high cell densities, however, small numbers of two other cell types are formed, which respectively resemble secretory and myoepithelial cells of the mammary gland, as judged by light and electron microscopy and immunofluorescent staining for casein (milk proteins). These additional cell types cannot be explained as contaminating cell populations since the cell line has been cloned several times; furthermore, the proportion of either can be increased by dimethylsulphoxide under different conditions. Specific epithelial features are seen by histological and ultrastructural examination of tumors formed by Rama 25 cells in immunodeficient mice. A line of the myoepithelial-like cells, Rama 29, isolated from a Rama 25 culture by cloning, has also been obtained. They propose that the undifferentiated cell type is a form of mammary stem cell which can differentiate in culture [22]. With reference to the stimulation of casein synthesis there is a long period of at least 8 h, after the addition of dimethyl sulphoxide, before casein synthesis and secretion are induced [23].

J.-P. Kraehenbuhl (Lausanne) has used dispersed mammary epithelial cells from rabbits. Dispersed cells were prepared from virgin, pregnant and lactating rabbits by dissociating mammary glands by enzyme digestion, calcium chelation and mechanical disruption. Epithelial cells were separated from interstitial cells by isopycnic centrifugation on dense albumin solutions [24].

^{125}I-labelled prolactin and ^{125}I-labelled human growth hormone, iodinated by the lactoperoxidase method, were used as tracers to characterize binding of lactogenic hormones to dispersed cells. Association and dissociation rates were temperature dependent and indicated internalization of radiolabelled ligand. Internalization was further confirmed by EM autoradiography. High affinity (10^{-10} M) binding sites were detected at all stages of differentiation. However, the concentration of binding sites varied through pregnancy and lactation in an inverse relation to blood concentration of progesterone.

Finally mammary cells from midpregnant rabbits were cultured on collagen gels as described by the group of Pitelka [25]. The cells responded to lactogenic hormones by increasing their rate of casein secretion, as measured by a radioimmunoassay, and by higher protein synthesis, as determined by incorporation of [^{3}H]leucine in trichloroacetic acid-insoluble material. The response was dose dependent over the incubation period. However the K_d of the biological response (5×10^{-8} M) was 1.5 orders of magnitude higher than the K_1 of binding (10^{-10} M).

3. The effect of thyroid, glucocorticoids and androgens

P. A. Andreasen (Leeds) described progress with the purification of glucocorticoid receptors. Because of the low concentration of the receptors in the tissues and their lability it has not so far proved possible to purify a receptor to homogeneity. Two methods were described:

(a) Chromatography on negatively charged matrices;
(b) Chromatography on Blue dextran–Sepharose.

Since in method (a) the primary cytosolic form does not bind whereas the heat-activated form does, the purification involves two consecutive chromatographies with the receptor in its unactivated and activated form, respectively. In (b) the glucocorticoid–receptor complex binds and can be eluted with polyanions like heparin; theophylline alters the salt-elution pattern. The receptor is being characterized by the use of aqueous dextran–polyethylene glycol two-phase partitioning [26].

The use of cultured rat pituitary cells was described by H. M. Goodman (San Francisco). These cells are being used to clone the genes for the hormones synthesized with a view to studying the regulation of the synthesis of these hormones by other hormones. The GC cells synthesize growth hormone (GH) and the G_1H_4 cells synthesize both GH and prolactin. These hormones and placental lactogen all have similar primary structures.

Rat GH is synthesized as a precursor with a signal peptide of 26 amino acids at the N-terminus. This is converted to GH by EDTA-stripped microsomal membranes. The synthesis of GH is regulated by

dexamethasone. The mRNA for GH has been purified and a cDNA probe prepared. From the use of this probe it is apparent that the induction of GH synthesis is at the level of transcription. The effect of triiodothyronine and dexamethasone is synergistic and results in a 45-fold stimulation in the synthesis of GH. The induction depends on the conditions of culture of the rat pituitary tumour cells. The medium must be changed every three days to get a positive effect, otherwise the hormones cause a decrease in the synthesis of GH. Insulin may also have a positive effect but the stimulation is not comparable with that caused by triiodothyronine and dexamethasone ([68,69]; also see [27]).

The effect of estradiol on the synthesis of prolactin in the pituitary of the male rat during primary and secondary stimulation was described by G. Vassart (Free University of Brussels). Prolactin mRNA was isolated from the pituitaries of rats chronically treated with estradiol which had been thyroidectomized to reduce the content of GH mRNA. The isolated prolactin mRNA was 81% pure by cell-free translation. cDNA was prepared and shown not to cross hybridize with GH mRNA. A comparison was made of prolactin synthesis in pituitary fragments and of prolactin mRNA levels measured both by cell-free translation and by molecular hybridization to prolactin cDNA. The results show that primary exposure of male rats to estradiol results in a considerable accumulation of prolactin mRNA before any stimulation of prolactin synthesis. During secondary stimulation prolactin mRNA and synthesis increase faster in an almost parallel way [28,29].

The control of gene expression by androgens was considered by three speakers (M. G. Parker, Cambridge; S. J. Higgins, Leeds; W. Heyns, Leuven). Parker explained that androgens stimulate the synthesis and secretion of four rat prostate proteins which together account for 50% of the total protein synthesis in the gland [30]. Cell-free translation studies and molecular hybridisation utilizing cDNA probes indicate that protein synthesis is regulated mainly via alterations in mRNA concentration. Firstly, translation of prostatic mRNA in several cell-free systems results in the synthesis of four androgen-dependent polypeptides but their migration on polyacrylamide gels containing SDS differed slightly from the authentic secretory polypeptides [31]. Processing of these translation products was obtained by adding microsomal membranes derived from dog pancreas or rat ventral prostate to the cell-free system which resulted in polypeptides comigrating with the four polypeptides synthesized in tissue minces. Secondly, cDNA complementary to the most abundant prostatic poly(A)-containing RNA was fractionated and used as a probe to test the effect of androgens on these species of RNA [31]. Testosterone markedly stimulated their cellular concentration and further studies, using the technique of hybrid arrested translation [32] indicated that the class of abundant poly(A)-containing RNA was mRNA coding for four polypeptides secreted as part of the prostatic fluid by the rat ventral prostate.

Heyns described work on the characteristic prostatic binding protein, which binds several steroids with a low affinity. This protein is contained in and secreted by the rat ventral prostate and is composed of two subunits linked together by non-covalent forces; after reduction of disulphide bridges each subunit dissociates further into two components. One of these is present in both subunits [33,34]. It is likely that some of the polypeptides described by Parker correspond to these components. The binding protein, measured by radial immunodiffusion constitutes about 45% of the total cytosolic protein in adult male rats and drops to 4.8% three weeks after castration. Treatment with androgens restores the initial value, but cyproterone acetate counteracts their effect [35]. Similar results are found for the incorporation of [^{3}H]leucine by prostatic slices into immunoprecipitated protein and for the mRNA activity of prostatic RNA measured in a wheat germ extract [34].

Higgins described the effect of testosterone which controls the synthesis of two major secretory marker proteins of the rat seminal vesicle largely through effects on specific mRNAs. However testosterone does not appear to act in a differential fashion in that the proportion of total protein synthesis devoted to these major proteins and the concentrations of their mRNAs within the mRNA population change but little during androgen manipulation [36,37]. In this respect the response of the seminal vesicle is very different from that of a closely related androgen target, the ventral prostate.

4. The effect of oestrogens

In the preceding symposium P. Chambon (Strasbourg) spoke of the organisation of the eukaryotic genes and of current ideas concerning the way in which particular genes may be selected for transcription. The rapid progress in the development of the nucleosome-concept of chromatin structure and of the application of electron microscopy to the detailed analysis of genes has brought the subject to an exciting stage and Chambon was able to show how the peculiar properties of hen oviduct chromatin have been exploited. (For an example of the use of micrococcal nuclease to prepare transcriptionally active and inactive chromatins from hen oviduct see [38].)

G. S. McKnight (Seattle) opened the discussion on the synthesis of egg white proteins. They have recently developed a chick oviduct tissue culture which responds to estrogen in vitro. The induction of ovalbumin and conalbumin mRNAs in culture is quantitatively similar to that observed in vivo and occurs with the same kinetics. There is a 3 h lag before ovalbumin mRNA begins to accumulate and protein synthesis is required during this time. The induction of conalbumin mRNA occurs with a <1 h lag but is also completely blocked by inhibitors of protein synthesis.

The culture system is being used to measure the rates of mRNA transcription for both ovalbumin and conalbumin during the early phase of induction. Preliminary results indicate that the rate of both ovalbumin and conalbumin mRNA synthesis is under hormonal control and that there is a 2 h lag before the rate of transcription on the ovalbumin gene increases. Similar results have been obtained in vivo by measuring the rates of ovalbumin and conalbumin mRNA synthesis in nuclei isolated at various times after administering hormones.

Other hormones are also active in the oviduct culture system. Dexamethasone as well as other glucocorticoids induces egg white mRNAs in vitro and have recently been found to be active in vivo. Progesterone also causes induction in vitro as well as in vivo; the combination of estrogen and progesterone causes a transient inhibition of conalbumin mRNA accumulation when given during an estrogen induction. Insulin is required in the culture medium for mRNA induction with either estrogen or progesterone [39].

A. E. Sippel (Berlin) reported on work on estrogen regulation of egg white protein mRNA synthesis in the chick oviduct. His results showed that the rapid accumulation of all four major egg white protein mRNAs during secondary hormone induction is due to an increase in their transcriptional rates in the nucleus as well as to an increase in their stability in the cytoplasm [40]. The group in Berlin is currently concentrating their efforts on the gene for lysozyme. They have compared the sequence organization of cloned mRNA sequences with that of the gene in the genome. They have found that the structural sequences are interrupted in cellular DNA by at least four intervening sequences so that the total length of the gene in the genome is 5-times the size of the mature mRNA [41].

Bert O'Malley (Houston) described the work of their laboratory on the control of gene expression in the chick oviduct. The steroid hormones (estrogen and progesterone) that lead to induction of synthesis of a number of egg white proteins in the oviducts of chicks or hens, do so by first inducing an increase in the net intracellular levels of their respective mRNAs. The increase in mRNA appears to be due primarily to increased gene transcription since the rate of transcription in nuclei and chromatin, as determined by radiolabelled nucleoside triphosphate incorporation into specific mRNA, is enhanced by steroid hormones.

The ovalbumin structural gene is split into eight segments by seven large intervening sequences (introns, inserts) of unknown origin, giving an overall length of the gene of >8000 base pairs of DNA coding for a $mRNA_{ov}$ of only 1859 bases [42]. These intervening sequences are unique (1 copy/haploid genome) and are transcribed fully (but only in chick oviduct cells). Recently, they have identified a series of probably high molecular weight precursors (>40 S) to mature $mRNA_{ov}$ (18 S). Their data are consistent with the hypothesis that the gene is transcribed initially into a high molecular weight precursor molecule and polyadenylated. Subsequently, the transcripts complementary to intervening sequences are successively excised and the structural sequences are rejoined until all intervening sequence RNA have been removed and the mature cytoplasmic $mRNA_{ov}$ has been formed. A series of mRNA intermediates

in this process have been identified and isolated [43].

The expression of this natural gene is regulated by steroid hormones in conjunction with their specific intracellular receptors. Upon entry into the cell, the steroid binds with high affinity and specificity to a cytoplasmic receptor. Following an 'activation' reaction, the hormone–receptor complex is translocated to the nuclear compartment and binds to chromatin 'acceptor sites'. This sequence of events precedes, and presumably initiates, the enhanced transcription of specific genes which is characteristic of steroid hormone action in target cells [44].

J. R. Tata (NIMR, Mill Hill) in his review in the symposium on 'The Cell Biology of Hormonal Regulation of Gene Expression' drew attention to the advantages of studying the induction of the synthesis of vitellogenin following the administration of estrogen to male chickens or toads. This protein is synthesized in the liver and is then transported in the serum to the ovary where it is split into the two proteins phosvitin and lipovitellin.

D. J. Shapiro (Urbana) described their work on the mechanism by which estrogen induces cell differentiation and transcription of the gene coding for vitellogenin in *Xenopus laevis*. Vitellogenin mRNA is undetectable (<0.5 molecules/cell) in livers of unstimulated male *Xenopus* and is induced to ~ 50 000 molecules/cell by administration of estradiol-17β. Induction proceeds without cytodifferentiation and DNA synthesis. They have recently developed an improved system for in vitro induction of vitellogenin by mRNA by physiological doses of estradiol-17β. These long-term organ cultures (which last several weeks) exhibit induction kinetics comparable to those observed in vivo.

Analysis of the rate of vitellogenin mRNA accumulation by hybridization to vitellogenin cDNA has led them to the interesting observation that restimulation of cells previously induced to synthesize vitellogenin mRNA, but inactive in vitellogenin mRNA synthesis at the time of restimulation results in a far more rapid and efficient activation of the vitellogenin gene than occurs on initial stimulation. In order to examine the possibility that estrogen stimulation results in the permanent conversion of the vitellogenin gene into an open DNase I-sensitive conformation, they have developed techniques for separation of hepatocytes from other liver cells and are examining the susceptibility of vitellogenin nucleosomes to DNase digestion.

The major focus of their efforts in this system concerns the synthesis, structure and processing of the vitellogenin mRNA precursor in isolated nuclei which complete, but as yet do not initiate, RNA chains, and the isolation and analysis of cloned vitellogenin DNA fragments [45,46].

L. Träger (Frankfurt) reminded the participants that steroids are not only effective in higher organisms but also show several biological activities in lower organisms. Both *Pseudomonas testostone* and *Streptomyces hydrogenans* are examples of prokaryotes which respond to steroids in the culture medium with an increased synthesis of certain enzymes. In the case of *Streptomyces hydrogenans* the synthesis of four different enzymes can be enhanced by various steroids. He is concerned with the synthesis of two of these enzymes, namely 20β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase, the synthesis of which increases within 30 min of the addition of steroid. The synthetic glucocorticoid, pregnadiendiolone, has high activity for the induction of 20β-hydroxysteroid dehydrogenase but shows only low activity with respect to 17β-hydroxysteroid dehydrogenase. Testosterone or estradiol on the other hand show activities in the opposite order. In the case of 20β-hydroxysteroid dehydrogenase they could show by immunological methods that the increase of the enzyme activity is due to an increase in protein synthesis.

The simplest hypothesis for the differential activity of the steroids would involve high-affinity receptor proteins, which would function in a similar manner to those that regulate bacterial operons. In fact, they were able to isolate a high molecular weight protein from the microorganism which shows a high affinity–low capacity binding to progesterone. But surprisingly, this protein shows only a very low affinity for those corticosteroids which serve as strong inducers in the case of 20β-hydroxysteroid hydrogenase; moreover, this protein exhibits no affinity for DNA isolated from the same microorganism. It seems likely that this protein is involved in the transport of steroids into the cell. The same conclusion has been

drawn by Watanabe for the high-affinity testosterone-binding protein from *Pseudomonas testosteroni*. So far therefore, there is no evidence for or against the existence of steroid-binding regulatory elements in *Streptomyces hydrogenans*. In *Streptomyces hydrogenans* they studied the total RNA and nucleotides after addition of steroids. All steroids tested so far have caused a rapid and pronounced drop in RNA content and RNA synthesis. Despite the initial decline of total RNA, whereby 20%, sometimes up to 50% of the cellular RNA is degraded within 20 min, the production of mRNA for 20β-hydroxy-steroid dehydrogenase is enhanced as early as 10 min after steroid addition. Because of the hydrolysis of an essential part of the so-called stable RNA of the cells, free nucleotides are secreted into the culture medium. Furthermore, steroids decrease the nucleoside triphosphate pool for purines very rapidly. Because the pool of GTP decreases more markedly than that of ATP, the ratio of ATP to GTP increases temporarily. Moreover, most of the steroids tested impair cell growth. Whether the reduction of ATP and GTP pools could be part of the cellular signals for enzyme induction, as has been shown for other microorganisms, has still to be proven.

Therefore steroids behave simultaneously as stimulators of anabolic processes (induction of several enzyme syntheses) as well as inhibitors of several metabolic processes (destabilization of RNA and diminished nucleoside triphosphate regeneration) in the same cell [47,48].

5. The action of peptides

R. K. Craig (Courtauld Institute London) explained in his contribution to the symposium why the mammary gland is an excellent model eukaryote system in which to study, not only the fundamental mechanisms required for the control of protein synthesis but also, the cellular mechanisms involved in the post-translational modification and vesicularization of secretory proteins. The growth and differentiation of the mammary gland is controlled by an intricate combination of both steroid and peptide hormones while the proteins synthesized and secreted by the fully differentiated gland are limited to a few well-defined species [49]. He also described how studies on the guinea pig and other lactating animals may be used to explore the potential of human mammary tumours to synthesize the milk protein, α-lactalbumin. It is hoped that these studies will be of value in indicating whether patients will benefit from subsequent hormone therapy [50].

J. M. Rosen (Houston) and L. M. Houdebine (Jouy-en-Josas) presented more detailed accounts of their respective studies on the control of milk protein synthesis. Rosen explained that the binding of prolactin to a membrane receptor had been demonstrated to be a necessary prerequisite but may not be sufficient, for the induction of casein synthesis [51]. Recent studies had indicated that certain peptide hormones, including prolactin, insulin and epidermal growth factor may enter the cell [52]. Thus, regulation of specific gene expression by prolactin may be mediated through a surface membrane site via an indirect mechanism or via the direct interaction of the internalized peptide hormone, or a fragment thereof, with the genetic constituents. Alternatively the internalized, processed peptide hormone receptor–complex may act as a 'second messenger' for gene activation.

The ability to quantitate the level of a specific mRNA following the addition of a peptide hormone may permit the identification of potential mediators of peptide hormone action. Accordingly, mammary gland organ culture, which is performed in a serum-free, chemically-defined medium, has been utilized to study the mechanism by which prolactin regulates specific gene expression. The high level of endogenous casein mRNA, quantified with a specific cDNA hybridisation probe, in the mid-pregnant gland rapidly decreased during culture in the presence of insulin and hydrocortisone (IF) alone; after 48 h only 4% of the original level was observed and by 72 h, the casein mRNA level decreased to near steady-state levels. Prolactin addition after the initial 48 h period resulted in the rapid accumulation of casein mRNA. Within 1 h after prolactin addition to a culture of 13 day pregnant mammary tissue a 1.3-fold induction relative to the IF controls was observed, increasing to 2.1-fold by 4 h and 6.5-fold by 24 h.

Using these data transcription rates of 3.1 and 10.9 molecules.min^{-1}.$cell^{-1}$ were obtained in the absence and presence of prolactin, respectively. This

3.5-fold increase in the rate of casein mRNA transcription was not sufficient, however, to account for the experimental accumulation of casein mRNA (7-fold above control in 24 h). Thus, these results imply that prolactin in addition to increasing the rate of casein mRNA synthesis may also decrease mRNA breakdown. Using a method developed by Kafatos [53], the observed accumulation of casein mRNA was best approximated when a half-life of 8.5 h was assumed in the absence of prolactin, and a value of >50 h, was assumed in the presence of prolactin. Thus, a combined effect of prolactin on both the rate of transcription and degradation of casein mRNA could best account for the observed mRNA accumulation seen in organ culture.

A more direct method which measures the rate of transcription and turnover of pulse-labelled casein mRNA sequences using a cDNA-cellulose affinity column has also been employed [54]. A purified 15 S rat casein mRNA fraction was used to enzymatically synthesize cDNA covalently linked to cellulose [55]. The specificity of the cDNA–cellulose affinity resin was demonstrated and it was then employed under conditions of DNA excess to detect casein-specific sequences present in RNA extracted from mammary explants pulse-labelled for 30 min with [^{3}H]uridine at various times after prolactin addition. In the presence of insulin and hydrocortisone ~0.09% of the pulse-labelled RNA was selectively retained. Within 30 min after the addition of prolactin casein-specific transcripts increased to 0.38%. This level of casein mRNA transcription was maintained for the next 24 h in the presence of prolactin. Thus, prolactin rapidly increased the rate of synthesis of casein mRNA ~4.2-fold in these experiments in excellent agreement with the previous results.

Two steroid hormones, hydrocortisone and progesterone, were also able to modulate the prolactin induced accumulation of casein mRNA. Hydrocortisone alone did not increase the accumulation of casein mRNA, but this steroid potentiated the action of prolactin. Simultaneous progesterone addition was able to inhibit in a dose-dependent fashion the induction of casein mRNA.

Current studies are directed towards the elucidation of the organization of the three casein genes and their primary transcription products using cloned DNA probes for each of the milk protein mRNAs. In addition, pulse-chase studies are being performed in order to study the kinetics of mRNA processing and turnover. Thus, mammary gland organ culture may provide an excellent model system for studying peptide hormone action and perhaps even mammalian mRNA metabolism in general [56,57].

Houdebine reminded the course that the initiation of lactation is preceded by a development of the mammary gland which includes a cellular multiplication leading to the appearance of epithelial cells, the organization of the tissue ducts and acini and the hypertrophy of the epithelial cells. The action of the various hormones in the rabbit was being studied after their injection into the animals and after their addition to the culture medium of mammary explants. The synthesis of casein was estimated by immunoprecipitation after incubating mammary fragments with ^{14}C-labelled amino acids at the end of the hormonal treatments. The concentration of casein mRNA in total and polyribosomal RNA was determined:

(a) By their translation in a cell-free system followed by an immunoprecipitation of the synthesized casein;
(b) By their hybridization to a cDNA probe synthesized by using a casein mRNA fraction partially purified by immunoprecipitation of the casein synthesizing polyribosomes.

During pregnancy in the rabbit, the synthesis of casein is progressively increased and the amount of casein mRNA is coincident with the rate of casein synthesis. In the pseudo-pregnant rabbit, when the mammary gland is partially developed but has no secretory activity, prolactin induces an increase of casein synthesis and a parallel accumulation of casein mRNA. Glucocorticoids although quite inactive alone, provoke a marked amplification whereas progesterone reduces the action of prolactin with respect to these two parameters. Hence, the action of these stimulations on casein synthesis is mediated in a large part by an accumulation of casein mRNA which is roughly proportional to the rate of casein synthesis, at this stage of mammary gland development. Thyroxine stimulates the action of prolactin on casein synthesis without any parallel accumulation of casein mRNA.

In the virgin mature rabbit, that is before the

appearance of epithelial cells, prolactin induces also some accumulation of casein mRNA, but this activation of casein genes is not accompanied by a significant casein synthesis. Hence, the mammary cell is sensitive to prolactin at the early stage of mammary gland development and the availability of casein mRNA is not the only factor limiting the synthesis of casein.

The onset of lactation is accompanied by an hypertrophy of the mammary cell which is then endowed with the cellular machinery (ribosomes, translational factors, endoplasmic reticulum, Golgi apparatus . . .) required for the mass synthesis of the secretory products. This transformation is ensured essentially by prolactin which favours the binding of casein synthesizing polyribosomes to the membrane of the endoplasmic reticulum. Glucocorticoids seem not to be a major factor to support these effects and progesterone exerts a potent inhibitory effect on the hypertrophy of the mammary cell.

The effect of prolactin and glucocorticoids on casein synthesis is mediated partly by a stabilization of casein mRNA [58,59,60].

P. Briand (Copenhagen) spoke of their work on the hormonal influence on mammary tumours in GR mice. In the GR strain of mice, van Nie has shown that mammary tumors can be induced by treating females with progesterone and oestrone. The tumors can be transplanted to castrated animals provided the animals are treated with progesterone plus oestrone. If hormone treatment is discontinued, the tumors regress. The cell proliferation kinetics have been studied in vivo by injecting thymidine intraperitoneally into animals which were killed at regular intervals and a labelled mitosis curve worked out. The results showed that the hormone treatment was required to maintain the cells in the proliferative state. No effect of hormones on cell loss could be demonstrated.

The treatment with progesterone plus oestrone can be substituted by treatment with prolactin intraperitoneally three times daily and approximately the same growth rate is obtained. By serial transplantation, hormone-independent tumors will arise. Oestrogen, progesterone, and prolactin receptors have been demonstrated in the hormone-dependent, but not in the hormone-independent tumors. Hormone-dependent tumor cells have been explanted in monolayer cultures. In these primary cultures, thymidine incorporation can be stimulated by the addition of oestradiol, progesterone, prolactin, epidermal growth factor to a synthetic medium containing 5% fetal bovine serum and insulin [61–63].

H. O. Voorma (Utrecht) described work on the regulation of tyrosine aminotransferase (TAT) in hepatoma cell lines. Induction of TAT has been carried out in synchronized HTC and H35 cell cultures. HTC cells were synchronized with the aid of colcemid and mitotic shake off. H35 cells were synchronized by serum depletion, which arrests the cells in G_0 or early G_1. TAT was induced with dibutyryl cAMP (DBC) in HTC cells in different phases of the cell cycle. No induction was observed in early G_1, G_2 and mitosis, whereas in late G_1 and S phase the cells were sensitive to DBC and TAT induction occurred.

Dexamethasone (DEX) was used in H35 cells for induction of the enzyme. Again they observed that induction of TAT predominantly occurs in late G_1 and S phase. Although the pattern of induction and the response to DBC and DEX look similar at the 5 h point of induction, gross differences are seen in the kinetic analysis of both types of induction. DBC-dependent induction takes off immediately, whereas DEX-dependent induction shows a 1.5 h lag time. Even in the presence of actinomycin D a smaller but significant induction of TAT by DBC is found. These data indicate that time is needed between the synthesis of mRNA and its translation [64].

G. Vassart (Brussels) described a congenital defect in the synthesis of thyroglobulin (Tg). Tg is a large glycoprotein (19 S; mol. wt 660 000) actively synthesized by the thyroid cell. It is composed of two 330 000 mol. wt subunits synthesized on the rough endoplasmic reticulum from translation of a 33 S mRNA. Following its secretion in the follicular lumen of the thyroid, Tg becomes iodinated on tyrosyl residues. Some iodotyrosyl residues condense into iodothyronines constituting the thyroid hormones T_3, T_4. These, to become active, must be released from the Tg molecules by proteolysis at the level of lysosomes. Tg is thus the obligatory precursor of normal thyroid hormones.

Some pathological situations in man, as well as in animals, are characterized by the congenital absence of Tg associated with a goiter and profound

hypothyroidism. They have investigated such a disorder occurring in goats. Tg cDNA was transcribed from normal 33 S mRNA and used to determine whether Tg mRNA sequences could be found in RNA prepared from the thyroid of goiter tissue. The results may be summarized as follows:

(1) Tg mRNA sequences are definitely present in the goiter tissue but at a concentration 10–30-times lower than normal.
(2) No Tg synthesizing polyribosomes can be found in the goiter tissue.
(3) The subcellular distribution of RNA having a base sequence characteristic of Tg mRNA is abnormal in the goiter. Being subnormal in amount in the nucleus they are virtually absent from the membrane-bound polyribosome fraction where Tg synthesis normally takes place.
(4) Tg mRNA sequences from the goiter cannot be translated into immunoprecipitable Tg when injected into the oocyte of *Xenopus laevis*. At a similar concentration, normal Tg mRNA sequences are readily translated by the oocytes.

The results suggest that this congenital defect may be due to abnormal processing and/or transport of the Tg mRNA precursor, or to the fast turnover of an abnormal Tg mRNA in the cytoplasm [65,66].

6. Application of recombinant genetics to the investigation of gene expression

Recent developments in recombinant DNA research have made it possible to obtain DNA which is completely homogeneous with respect to the coding sequences of a particular protein. A common approach is to isolate the mRNA fraction from cells which are synthesizing the appropriate protein, prepare a cDNA and to clone this by introducing the cDNA into a plasmid which is used to transform a disabled strain of *E. coli*. Such cDNA may be used in various ways to further research aimed at the elucidation of the regulation of specific gene expression and several of the reports already referred to have mentioned cDNA. These have indicated its use as a probe for quantifying the amount of a particular mRNA in a cell, or among the various cell organelles, but it can also be used to monitor the way in which a particular piece of DNA in the genome is selected for transcription and how the mRNA produced as a primary transcript is processed.

In this last session the speakers not only gave examples of the way in which recombinant DNA research was being used at present, but the participants also heard from those who were using the methodology on systems that are not hormonally controlled. As an example of this there was a discussion of current ideas concerning the organisation in the genome of globin genes.

H. M. Goodman (San Francisco) opened the discussion on this topic by describing their work on the structure, expression in bacteria and regulation in cultured cells of the growth hormone gene. The growth hormone (GH) gene is expressed by cultured rat pituitary (GC, GH_3) cells and is related to the genes for prolactin and chorionic somatomammotropin (HCS, placental lactogen). In the cells, thyroid (T_3) and glucocorticoid hormones each induce GH mRNA and the two hormones are synergistic. Further, insulin can prevent the ability of glucocorticoids to induce GH mRNA, an effect that can be overcome by T_3. Glucocorticoids and T_3 appear to affect $<0.5\%$ of the expressed genes, yet, within 30 min the 30 000 steroid receptors increase chromatin binding sites for RNA polymerase from about 10^5 to 1.5×10^5/cell. The cellular milieu can be further manipulated such that glucocorticoids de-induce GH production and chromatin capacity for polymerase binding. A working hypothesis is that the steroid rapidly modifies chromatin structure in a gross way that nonetheless affects expression of only a few genes. The direction of the modification (stimulatory or inhibitory), determined by other hormones and the cellular milieu, dictates whether glucocorticoids will positively or negatively regulate the expression of a specific gene.

cDNA to human (HGH) and rat GH mRNA and to HCS mRNA has been cloned in bacteria and sequenced. The cDNA to human mRNA was purified to >99% homogeneity prior to cloning. This was achieved by: isolation of a fragment of sequences coding for amino acids 24–191 of the protein and part of the 3′ non-coding region, internal cleavage of the fragment and gel purification of the pieces; and reconstruction of the original fragment. The three cDNAs have a striking nucleic acid sequence homology, suggesting that the three genes have a common evolutionary origin.

Cloned cDNA to RGH mRNA from plasmid pMB9 was ligated to the *Pst*I endonuclease restriction site in the ampicillin resistance gene of plasmid pBR322. A new protein of the size expected of the fusion between the β-lactamase (that confers ampicillin resistance) and GH was produced by the new plasmid; in addition, material from these clones, but not from other *E. coli* clones without or with pBR322, produced material that reacted to antisera to GH. Thus, these bacteria appear to produce a protein containing amino acids in a sequence occurring in growth hormone [67–75].

P. Little (St Mary's Hospital, London) described the analysis of normal and abnormal haemoglobin genes. They have used techniques, originally devised to study the rabbit β-globin gene [76], to produce a 'map' of restriction enzyme sites round the linked human β- and γ-globin genes [77], and the linked γ-globin genes. This has enabled them to define precisely the intergene distances and structures of the various human β-like globin genes. They have then used the derived maps to examine the globin DNA from patients suffering from the inherited disease β-thalassaemia. This disease is characterised by a complete, or total absence of, β-globin proteins and, in some cases, may represent eukaryotic control mutations. The possibility of using similar experimental techniques as a diagnostic method was also briefly discussed.

R. M. Kay (ICRF, Mill Hill) described work on the analysis of clones containing *Xenopus* globin sequences, emphasising in particular the advantages of phase separation for rapid plasmid DNA isolation [78] and HART analysis as a method of identifying inserted coding sequences [79].

P. Chambon (Strasbourg) then provided a detailed analysis of the organisation of the ovalbumin structural gene [80–83]. In addition he also presented confirmatory evidence, that like the Houston group, they also had obtained evidence for the presence of a high molecular weight precursor of $mRNA_{ov}$. The egg white protein story was then again taken up by O'Malley (Houston), who after presenting evidence that the ovomucoid gene contained inserts and was also transcribed as a high molecular weight transcript, proceeded to describe DNA sequences data obtained from cloned genomic 'intron' regions of the ovalbumin gene. This was of particular interest, as it showed that considerable sequence diversity occurred in the intron region within the same species, thus explaining the minor disparity between restriction data obtained in Strasbourg and that obtained in Houston.

In an attempt to gain some insight into the process by which intervening sequence RNA is converted from precursor molecules to mature messenger RNA_{ov}, a collaborative effort between Brownlee's laboratory (MRC, Cambridge) and O'Malley's laboratory has resulted in the determination of the DNA sequence in the junctions between structural and intervening sequences in the ovalbumin gene [84].

G. U. Ryffel (Bern) spoke of the analysis of clones containing DNA coding for vitellogenin of *Xenopus*. All available data suggest that estrogen activates transcriptional and/or post-transcriptional events in the nucleus [85].

As a first approach for understanding these events they have cloned vitellogenin cDNA in PMB9 [86]. Clones containing vitellogenin cDNA of $1–3.7 \times 10^3$ bases were mapped on the vitellogenin mRNA by R-loop analysis in the electron microscope and by hybridisation to specific RNA fragments [87]. All clones containing a terminal part of the mRNA proved to contain the 3′-end of the vitellogenin RNA. Clones with an internal segment or the mRNA were orientated in the electron microscope by hybridisation of a cDNA to the RNA in the R-loop.

Heteroduplex analysis in the electron microscope revealed that the cloned DNAs consists of two main groups, (A + B), which do not cross hybridize under the spreading conditions used. Each main group is divided into two subgroups which form paired heteroduplexes with characteristic substitution loops. Hybridisation experiments between the main groups revealed sequence relatedness between A and B group sequences with a T_m some 20°C lower than in homoduplexes. On the other hand hybrids between clones of subgroups in A or B showed a 5 1°C lower melting temperature than homoduplexes. The presence of four different classes of vitellogenin cDNA clones was further confirmed by restriction analysis.

By hybridization of specific restriction fragments of one subgroup to the vitellogenin mRNA used for R-loop analysis in the electron microscope, they demonstrated that R-loops contain perfectly matched

RNA exclusively. Using RNA of a single animal in R-loop formation with all subgroups provides convincing evidence that a single frog synthesizes all four different RNA sequences they have found in their vitellogenin cDNA collection [88]. All these data lead to the conclusion that the vitellogenin genes in *Xenopus* constitute a small family of related sequences which are under estrogen control.

7. Concluding remarks

As we indicated in the Introduction the availability of cell cultures that are susceptible to the action of hormones under 'physiological conditions' will obviously be a great boon to the development of this field. Sato, who has already made remarkable contributions in this field, seems very hopeful of virtual complete success in this regard but some of the audience were mesmerised not only by the complexity of the serum-free defined media but also by the prodigous effort involved in this kind of research. It is apparent though that even without embarking on such painstaking research that some cells have comparatively modest requirements and so even today there are a number of excellent systems. Perhaps the explant culture described by Shapiro for the study of the effect of estrogen on liver cells deserves especial mention for it may encourage others to try their luck. It seems that if cell cultures are not available organ explants often will suffice.

Two very significant developments emerged from the results so far obtained from the work on the site of action of hormones. Firstly it seems probable that even peptide hormones such as prolactin may act in the nucleus (Kraehenbuhl). The dogma concerning hormone action that we teach our students, that peptide hormones confer their activity by binding to receptors on the cell surface whereas steroid hormones bind to specific receptor proteins in the cytoplasm en route to the nucleus, may well turn out to be an oversimplification. Secondly it seems from the work of McKnight on egg white proteins that a single hormone acting on a single cell may have a quite different temporal effect with respect to different proteins. This surely introduces a fascinating extension of the degree of refinement of the site of action of a hormone. The development of recombinant DNA technology has clearly already provided powerful probes whereby such phenomena can be explored.

The course amply demonstrated the need for a truly multidiscipline approach to the subject and indeed the need to draw together from time to time people with widely different expertise. Thus the excitement for those exploring the regulation of specific gene expression is that they are working at a time when major advances are being made in our understanding of chromatin structure, eukaryotic cells in culture and recombinant DNA technology.

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