Gene Expression

Regulation at the RNA & Protein Levels

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Gene Expression: Regulation at the RNA and Protein Levels

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Preface

In 1988, Australia celebrated the bicentennial anniversary of the first European settlement of those far off shores. The number of would-be biochemists in the party is not on record, but with the passing years, the processes of evolution (analysed and enunciated by an opportunistic visiting scientist) have given rise to a vibrant, contemporary biochemical community. In tribute, the Biochemical Society resolved to devote its annual symposium for 1988 to a topic that, while retaining its perennial widespread appeal, would manifest on this occasion a particularly strong representation from Australia. The notion met with rapid approval 'down under' and joint sponsorship was generously offered by the Australian Biochemical Society. The title of this volume reflects the subject matter that was ultimately selected, but reveals nothing of the manner in which it was conceived. This must have been the first ever event run by the Biochemical Society where the initial planning was carried out by emissaries from both Societies in a rickety firetower high above a forest in the German Democratic Republic!

The symposium itself took place at the 627th meeting of the Biochemical Society, held in Nottingham from 20 to 22 July. Five speakers each represented Australia and the U.K. with other contributions given by Japanese, European and American invitees. Thanks are due to all of our guests for their willingness to participate (involving travel in some cases of vast distances), for the stimulating presentations that they gave and for the timely delivery of the manuscripts that comprise this volume.

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Cardiff, Adelaide and Nottingham

Abbreviations

ALA	2-Alalyl-2-isopropyl acetamide
ALV-S	5-Aminolaevulinate synthase
BPDI	Bovine pancreatic trypsin inhibitor
BPV.	Bovine papilloma virus
CAT	Chloramphenicol acetyl transferase
cMGF	Chicken myelomonocytic growth factor
CSF	Colony stimulating factor
CTF	CCAAT transcription factor
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDF	Eosinophil differentiation factor
endoH	Endoglycosidase H
env	Envelope
ER	Endoplasmic reticulum
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSH	Reduced glutathione
GSSH	Oxidized glutathione
HA	Haemagglutinin
HGF	Haemopoietic growth factor
HRM	Haemopoiesis response modifier
hsp	Heat-shock protein
ICM	Inner cell mass
IGF	Insulin-like growth factor
IL-3	Interleukin 3
IL-5	Interleukin 5
LIF	Leucocyte inducing factor
LPS	Lipopolysaccharide
LTR	Long terminal repeat
3MA	3-Methyl adenosine
M-CSF	Macrophage colony stimulating factor
MEL	Murine erythroleukaemia cell
MHC	Major histocompatibility complex
MSV	Murine sarcoma virus
PDI	Protein disulphide-isomerase
PE	Parietal endoderm
PHA	Phytohaemagglutinin
pIg	Polymeric immunoglobulin
pIgR	Polymeric immunoglobulin receptor
RA	Retinoic acid
RSV	Rous sarcoma virus
SC	Secretory component
SDS/PAGE	SDS/polyacrylamide-gel electrophoresis
snRNP	Small nuclear ribonucleoprotein particle
SV40	Simian virus 40
THBP	Thyroid hormone binding protein
VE	Visceral endoderm
VSV	Vesicular stomatitis virus

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Gene Expression and Differentiation in F9 Mouse Embryonal Carcinoma Cells

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Synopsis

The mouse embryonal carcinoma cell line F9 differentiates *in vitro* in a manner analogous to the formation of extraembryonic (parietal or visceral) endoderm from inner cell mass cells in early embryogenesis. After retinoic acid addition to cells in monolayer culture, differentiation to parietal endoderm proceeds over several days. Early changes in gene expression are seen before differentiation becomes irreversible, and may be mediated post-transcriptionally. Midway through differentiation, transcription of a group of endogenous and exogenous (viral) genes rises. Increased activity of the DNA-binding transcription factor AP-1 has been implicated in this rise in transcription, but it has not been determined whether this is the only factor involved. In the third phase of differentiation, a group of proteins characteristic of parietal endoderm appears. The F9 cell system may be significant in being among the first in which altered transcription factor activity responsible for changing gene expression during differentiation is understood.

F9 Cell Differentiation as a Model for Events in Early Embryogenesis

During the earliest phase of growth and cell division in the mouse embryo, cells maintain identical and completely undifferentiated characteristics, at least until the 8-cell morula is formed. Subsequently these cells divide to produce the trophectoderm and inner cell mass (ICM) lineages, the latter giving rise to all embryonic and most extraembryonic tissues. At around 4 days of development, a subset of ICM cells differentiates to form the primitive endoderm. Some of these cells begin to migrate on to the trophoblast where they lay down a thick layer of extracellular matrix material and assume the characteristics of parietal endoderm (PE) cells [1].

Endoderm cells remaining associated with the egg cylinder form the visceral endoderm (VE), a continuous epithelial layer supported by a thin basement membrane. As embryogenesis proceeds, the VE cells form the visceral yolk sac which expands to surround the developing embryo. A characteristic of cells of the visceral yolk sac is production of large amounts of α-fetoprotein [1].

The mouse embryonal carcinoma cell line F9 is one of many embryonal carcinoma cell lines established in culture from early embryos. F9 cells in their

undifferentiated state have characteristics similar to those of the ICM, and show a very low rate of spontaneous differentiation *in vitro* (reviewed in [1]). Addition of retinoic acid (RA) to F9 cells in monolayer culture, however, induces morphological and biochemical changes over a period of several days. Product cells have many of the characteristics of parietal endoderm [2]. This process of differentiation can be enhanced by addition of exogenous cyclic AMP, to which the cells become sensitive soon after differentiation begins [3]. However, F9 cells appear not to differentiate as extensively as equivalent cells in the embryo, since synthesis of extracellular matrix proteins occurs at a much lower level in differentiated F9 cells than in PE cells from the early embryo [1].

F9 cells can be induced to form VE if they are cultured as floating aggregates before addition of RA. α-Fetoprotein-producing cells form as a layer on the outside of the aggregates after 4 or more days of RA treatment [1].

As discussed by Hogan *et al.* [1], F9 cells differentiating in culture may be of use in examining some of the critical questions of cell–cell and cell–matrix interactions involved in controlling extraembryonic endoderm formation in the embryo. They have already proven to be extremely valuable in analysing changing patterns of gene expression, and in particular, in understanding how these changes are controlled at different stages of differentiation.

Changes in Gene Expression During F9 Cell Differentiation *in vitro*

Fig. 1 summarizes the current state of knowledge on changing patterns of gene expression during RA-induced differentiation of F9 cells. For convenience, both PE and VE cells are depicted as deriving from a common primitive endoderm intermediate. This does not strictly describe the process of differentiation *in vitro*, since the two cell types are produced under different culture conditions. However, marker studies suggest that they both derive from a common intermediate cell type, both *in vitro* and *in vivo* [1].

Consideration of the data summarized in Fig. 1 suggests that the differentiation process and associated changes in gene expression can be divided into three phases. The characteristics of each of these phases are discussed below, although only the middle phase will be considered in any detail: the most extensively studied alterations in gene expression occur during this phase.

Phase I—the induction phase

Events critical for subsequent stages of the differentiation process occur within the first few hours of exposure of cells to RA. Initially, the effects of RA remain reversible by addition of sodium butyrate [4], but after about 8 h, the cells become determined and differentiation proceeds, even in the absence of further exposure to RA. It is believed that RA acts at the level of the nucleus with attachment to DNA via a specific receptor [5,6]. Determining the sites where this activated receptor protein is bound early in RA-induced F9 cell differentiation should be instructive in identifying the early critical responses that control subsequent steps of cell differentiation. Some genes the RNA levels of which alter early after RA addition have been identified (see Fig. 1). These

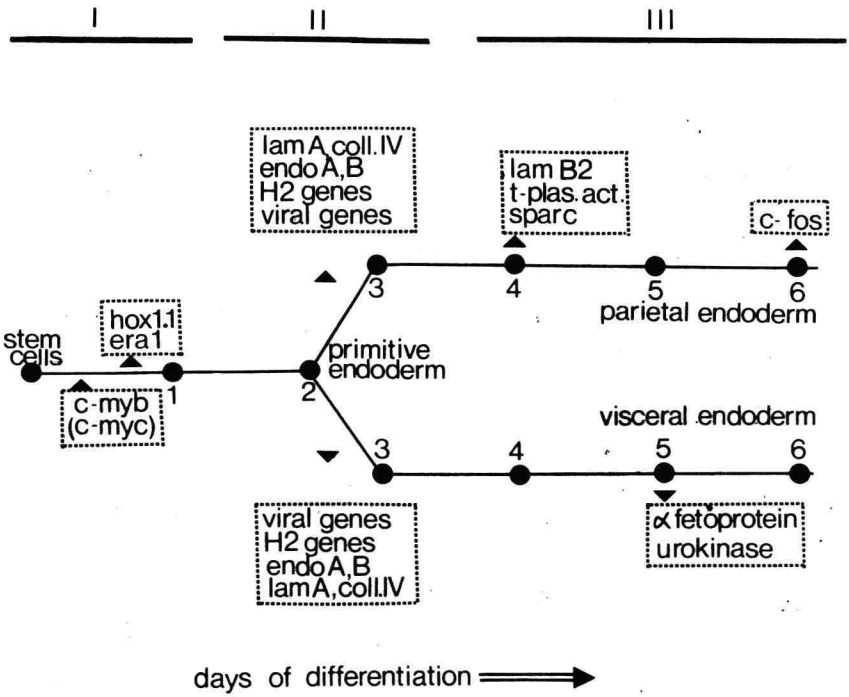

Fig. 1. *Changes in gene expression during differentiation of F9 cells in vitro*

The diagram shows alternative pathways for differentiation of F9 stem cells to parietal endoderm (monolayer culture) or visceral endoderm (clumped cell culture) via a putative primitive endoderm intermediate (reviewed in [1]). The timing and extent of changes in the levels of gene products (RNA and/or protein) are summarized from published information: c-*myc* and c-*myb* [9,10,35]; c-*fos* [10]; laminin chains and collagen type IV [12,15,36,37]; *endo A* and *B* [12,38,39]; *hox 1.1* [7]; *era 1* [8]; MHC Class I *H2* genes [23,37,40]; tissue plasminogen activator and urokinase [15,16,41]; α-fetoprotein [12] and viral genes [41]. Time points show days after RA addition, with the three phases of differentiation proposed in the text outlined at the top.

include a homoeobox-containing gene [7], the *Era-1* gene, of as yet unknown function [8], and several oncogenes [9,10]. These last alterations may play a role in the changes in cell properties (loss of tumorigenicity, decrease in cell growth rate, etc.) that accompany differentiation, although no direct role for any of the proteins has yet been identified. It appears that some, at least, of the changes reported early in differentiation may be controlled by altering the stability of mRNAs rather than by a direct effect on transcription [7,9].

Phase II—the primitive endoderm phase?

After 2–3 days of RA treatment, F9 cells change in shape and a new pattern of gene expression becomes apparent. Increased transcription is seen from a wide range of endogenous genes including those encoding components of the extracellular matrix, cytoskeletal proteins (*endo A* and *B*) and genes of the major histocompatibility complex (Fig. 1). At the same time, transcription of a group

of exogenous genes, from viruses such as SV40, polyoma, cytomegalovirus and a range of retroviruses, increases from its previous low level (reviewed in [11]). These changes appear to be common to differentiation pathways leading to both PE and VE, and occur before those changes that are confined to one pathway or the other (Fig. 1). Since many of the proteins are expressed to some extent in primitive endoderm in the embryo [1], the changes in this phase *in vitro* would be consistent with the appearance of a primitive endoderm-like intermediate cell type. Regulation of genes activated during Phase II has been studied in some detail and is discussed more fully below.

Phase III—appearance of fully differentiated cells

Subsequent to the 'common' transcriptional changes of Phase II, altered gene expression characteristic of either PE or VE cells is seen. For VE cells, the most obvious change is increased α-fetoprotein expression [12], while for PE cells, expression of the SPARC and laminin B2 matrix proteins and of tissue plasminogen activator occurs [13–16; M. J. Sleigh, unpublished work]. Little information is available on how the activation of these genes is controlled. A key question will be whether the regulation system for Phase III genes shares and/or is dependent on prior changes responsible for the activation of Phase II genes, or whether the two events occur independently.

Regulation of viral genes in Phase II of F9 cell differentiation

By far the most extensively studied event in F9 cell differentiation is the activation of the 'common' set of genes during Phase II. Initial work in this area was based on the assumption that viral gene expression would provide a model for understanding the regulation of endogenous genes [11]. From our current state of knowledge, it seems that this assumption may be only partly valid [17].

Early studies on viral gene regulation in F9 cells, and in the related EC cell line PCC4, demonstrated that regulation was at the level of transcription and was mediated through transcriptional enhancer sequences (reviewed in [11]). These enhancer sequences show low, or zero activity in undifferentiated cells, but normal activity after cell differentiation. Some evidence for negative regulation of viral enhancer sequences in F9 cells has been obtained. Gorman *et al.* [18] showed that the enhancer from the murine sarcoma virus (MSV) reduced the level of transcription from associated gene promoters in F9 cells — increased transcription was seen when the MSV enhancer was removed or when cells also contained DNA sequences from the long terminal repeat of Rous sarcoma virus (RSV). In the latter case, it appeared that the RSV sequences were able to sequester a DNA-binding factor normally acting to block transcription through the viral enhancer. Competition studies using sequences from the polyoma virus enhancer to remove suppression from the SV40 early gene promoter/enhancer have provided additional evidence for suppression of virual enhancer sequences in F9 cells [19]. However, the activity reported for SV40 enhancer sequences in F9 cells in different laboratories varies from zero

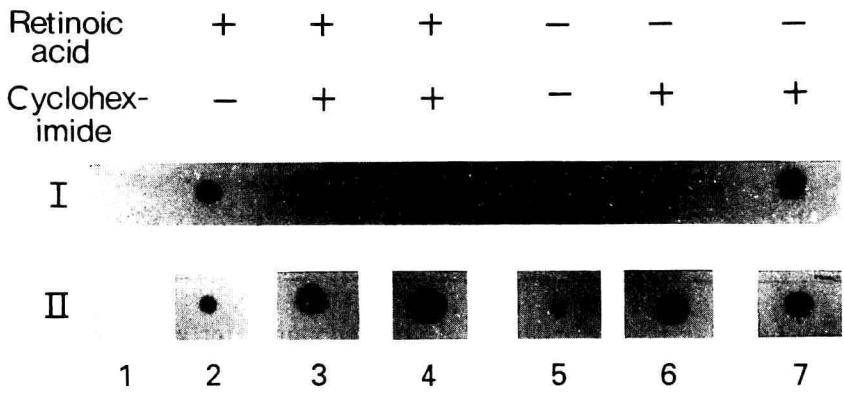

Fig. 2. *Nuclear run-on analysis of pSV_2CAT transcription in FCN1 cells*

Nuclei were isolated from FCN1 cells [17] after treatment with RA (5×10^{-7} M) for 5 days (lanes 2–4) and cycloheximide (10 μg/ml) for 3 or 16 h (lanes 3 and 6, or 4 and 7, respectively). Nuclei from untreated F9 cells containing no pSV_2 CAT gene were used as a control (far left lane). Initiated transcripts in the isolated nuclei were extended in the presence of [α^{32}P]UTP. RNA was purified by precipitation with trichloroacetic acid and equal numbers of counts from each nuclear sample were hybridized to filters containing CAT gene DNA (0.2 μg) [42,43]. Experiments I and II were carried out using independent nuclear preparations.

or negative [18] to approximately 2-fold (compared with 50–100-fold in differentiated cells; [20]) or fully active [21]. These differences are presumably dependent on differences in the cells or their method of culture. This means that it is very difficult to compare results from one laboratory to another and whether viral enhancer sequences have negative or merely reduced activity in F9 cells is not universally established.

In attempting to identify how viral enhancer sequences are activated during F9 cell differentiation, we carried out some studies with the protein synthesis inhibitor cycloheximide. The original rationale for the experiment was that if a transcriptional repressor was present in undifferentiated cells, then inhibiting protein synthesis could lower the concentration of the repressor and so increase transcription from viral gene promoters. If the repressor was absent from differentiated cells, then the same increase in transcription should not be seen in these cells.

Cloned F9-derived cell lines containing copies of the SV40 promoter/enhancer driving the bacterial chloramphenicol acetyl transferase gene (CAT; [22]) were established and the effect of cycloheximide on CAT gene transcription determined by dot-blot analysis of cellular RNA and nuclear run-on analysis [17]. Fig. 2 shows such an analysis for the cell line FCN1, which contains approximately 50 integrated copies of pSV_2CAT and an RA-induced increase in CAT enzyme activity (due to increased expression of the integrated genes) of approximately 12-fold. Retinoic acid treatment induced a corresponding modest increase in CAT gene transcription rate, but cycloheximide treatment (sufficient to block protein synthesis by 80–90%) increased transcription much more substantially. Surprisingly, this was seen in both differentiated and undifferentiated cells, suggesting that whatever the effect of cycloheximide

Table 1. *Order of gene activation in EC cell lines in response to different inducers*

Data summarized in the Table show that when EC cell differentiation is induced by cooling or by RA treatment, the order of activation of the genes from SV40, polyoma virus (Py), for the cytoskeletal protein endo A and for the MHC Class I (*H2*) gene is variable [24,41,47].

Cell line	Inducer	Order of gene activation
PCC4	Cooling	Py, endo A, SV40, *H2*
	Retinoic acid	
F9	Cooling	endo A, *H2*, (not Py), SV40
	Retinoic acid	Py, SV40, H2, endo A

(removal of a repressor, activation of a transcription factor, etc.) it was able to exert this effect both before and after differentiation.

Are endogenous genes activated in Phase II controlled in the same way as viral genes?

In the original hypothesis, it was proposed that viral gene regulation in differentiating F9 cells would provide a model for control of co-activated endogenous genes. On this basis, cycloheximide treatment would be expected to activate transcription from endogenous genes through the same pathway as that affecting SV40 transcription.

Reports in the literature describe evidence for negative regulation of the *H-2L*^{d} gene, a member of the major histocompatibility complex (MHC) Class I gene family and activation of transcription of the gene in F9 cells by cycloheximide treatment [23]. The *endo A* gene, coding for a cytoskeletal protein, has been reported to show increased transcription in the F9-related cell line PCC4 following cycloheximide treatment [24], and activation of polyoma virus expression by cycloheximide has also been described [25]. In F9 cells, no evidence for cycloheximide-induced activation of *endo A* gene transcription could be obtained [17] and evidence for the *H-2L*^{d} gene was questionable under circumstances where strong activation of SV40 expression was seen. These results demonstrate that viral and endogenous genes showing transcriptional activation during F9 cell differentiation are not always susceptible to identical mechanisms of gene regulation.

Models for Phase II gene activation

Table 1 summarizes information on the order (or extent) of gene activation during Phase II in F9 and PCC4 cells where differentiation is triggered by different inducing agents. If increased expression of this set of four genes can only result from altered activity of a single transcription factor, then the order of gene activation should be similar whenever increased expression is observed. Since this is not the case, then either gene activation involves altered activity of two or more factors (one of which could be a represssor) or it can be achieved by two or more alternative pathways, each involving altered activity of a single transcription factor.

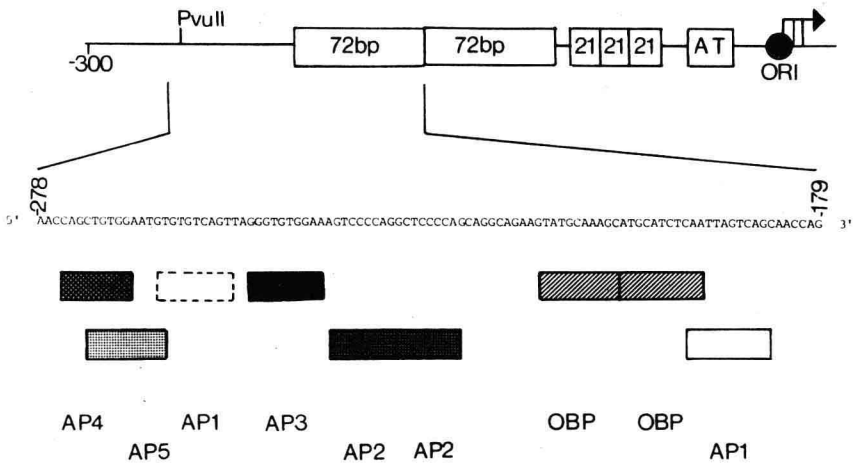

Fig. 3. *Protein binding sites in the enhancer region of SV40*

The Figure shows the promoter/enhancer region for the SV40 early transcription unit, including the Sp1-binding 21 bp GC-rich repeats, a TATA box-like sequence and start points for transcription (arrow) located near the origin of replication. The expanded region shows one of the 72-bp repeats and upstream sequences, with locations of the binding sites for transcription factors AP1-5 and the octamer binding protein (OBP). This information is taken from [27], but a second AP1 binding site (around -260) not included in this reference, but located previously [44], is also shown.

There is considerable indirect evidence to support the feasibility of the second of these hypotheses. Studies from a number of laboratories have shown that the SV40 enhancer region contains multiple, sometimes overlapping, binding sites for at least six different proteins, which are presumed to act as transcription factors (Fig. 3; reviewed in [26,27]). Mutational studies of the enhancer, to identify regions important for enhancement in different cell lines [21], as well as studies on DNA binding factors [28], have suggested that there is considerable variation among cell types in the subset of factors binding to DNA and involved in transcriptional enhancement. For example, in HeLa cells, the factors AP4, AP5, AP3 and the octamer binding protein appear to be most significant, and in differentiated F9 cells, AP4, AP5, AP1 and the octamer binding protein contribute most to activity.

Presumably, the enhancer would increase in activity in these cells if active AP1 or AP2 (for HeLa) or AP2 or AP3 (for differentiated F9s) appeared. Possibly, different inducing agents acting on F9 or PCC4 cells can change the activities of different DNA binding factors. The order of gene activation in response to the inducers would then depend on the relative abilities of different factors to stimulate the activity of each gene (influenced by availability, location and strength of relevant binding sites).

Many mutants of polyoma virus able to replicate in EC cells have been isolated. A common feature of these mutants is the presence of alterations in the enhancer region (Fig. 4; reviewed in [29]). Interestingly, mutants isolated in F9 cells have changes different from those in PCC4 mutants. This is consistent with the results of Table 1, which suggests that the initial spectrum of transcription