Chapter 4
Phosphorylation Dynamics in Mammalian Cells

D.A. Gilbert[1] and K.D. Hammond[2]

Abstract Living cells are auto-dynamic because of control systems operating in their periodic mode. They comprise diverse regulatory networks and are thus multi-oscillators covering a wide range of characteristics. Phosphorylation reactions are involved in virtually all aspects of cell function. Here, we outline a range of our studies on ATP and protein phosphorylation in order to highlight certain features of ultradian dynamics not widely recognised nor appreciated. Our work in this field alone supports the multi-oscillator concept of the living cell and confirms its complexities not least with regard to the significance of temporal oganisation of dynamic processes. The findings support the view that the regulation of cell function, properties and behaviour is achieved through modulation of the dynamic characteristics and are consistent with our concepts of differentiation and cancer.

Key words Oscillations, cell dynamics, ATP, phosphorylation, differentiation

The living cell is most surely dynamic,
Its behaviour so very erratic.
Why is it so true
That all but a few
Insist on treating it static?

4.1 To Begin …

The nature of life determines all that happens in biology yet it is all too rarely taken into account when attempting to understand biological phenomena (Gilbert and Lloyd, 2000). The prime attribute of life is auto-dynamic behaviour and that

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is also the way we decide its existence. Of utmost significance to this feature are control systems, which, paradoxically, can favour both constancy of composition and also give rise to auto-dynamic behaviour (e.g. Gilbert, 1968). Cellular properties, function and behaviour thus depend on the quantitative aspects of cellular control systems and therefore organisation of processes in time, not least their coordination (Gilbert, 1968, 1969, 1974b, 1984). But the principles of dimensional analysis (e.g. Segel, 1980) show quite clearly that we can only explain quantitative and temporal features in terms of factors which, explicitly or implicitly, have mass and time embodied in them. This implies a total dependency of cellular processes on reaction rates, not gene structure. Here we merely focus attention on the existence of oscillatory behaviour in a number of cellular systems associated with phosphorylation. But we emphasise that the existence and characteristics of periodicities are governed entirely by the *sets* of reaction rates concerned. Thus, although we do not specifically mention the latter, we do acknowledge and imply their total significance.

Since predicting the cellular involvement of phosphorylation processes in theoretical studies on the nature of the cell cycle and cancer (Gilbert, 1974a; cf. also MacKinnon and Gilbert, 1992), we have been making experimental observations on the dynamics of such reactions. *In vitro* studies on mitosis by others have confirmed that several phosphorylated components are indeed so involved (e.g. Kirschner, 1992) but we have focused on reactions not directly concerned with mitosis and outline some studies here, in order to stress the complexity of cellular processes in time. We start with some of the 'simpler' issues.

Conventional approaches to cell biochemistry have indicated the importance of coenzymes and cofactors to function, properties and behaviour. This implies a dependence not only on the levels of such compounds but also on the way they change with time. In turn, those aspects reflect the synthesis and degradation (utilisation) and inter-conversion. There is still a detrimental focus of attention on the roles of genes but apart from other aspects, explanations for dynamic problems cannot come just from the identification of participating compounds nor is it adequate to interpret everything in terms of individual constituents when life is dependent on systems of components. Coenzymes and cofactors are dominant in cell function and behaviour for at least two reasons; firstly, they are involved in a wide range of reactions (which gives them the ability to coordinate processes) and secondly, those properties give rise to looped dependencies that provide control action. Without the cyclic sets of reactions, they can only act as regulators. Some problems associated with such studies have been considered by Gilbert and Ferreira (2000).

4.2 ATP and Related Aspects

We begin by considering one special phosphorylated compound of this kind. ATP is a component which is necessary for many of the reactions of interest and we briefly mention some of our findings because of this widespread significance. As mentioned

above, by implication we are also concerned with the reactions involved in its synthesis, degradation and inter-conversion with ADP; one can expect it to be involved in several distinct control systems. We therefore briefly widen our remit slightly to include some comments on glycolysis and mitochondrial function even though those topics alone deserve a whole chapter and, no doubt, will receive attention elsewhere in this volume. The above considerations also provide reasons to expect oscillatory changes in the level of this component, ATP, and so is the case. Indeed, a number of authors have reported periodic variations so we focus on the complexity aspect not, we believe, hitherto considered by others although suggested by the complicated waveforms.

4.2.1 ATP

Figure 4.1A and B show Enright periodogram analyses of ATP oscillations observed in L cells and in murine erythroleukaemic (MEL) cells, respectively. In agreement with Gilbert and Lloyd (2000), these diagrams alone add further weight to the view

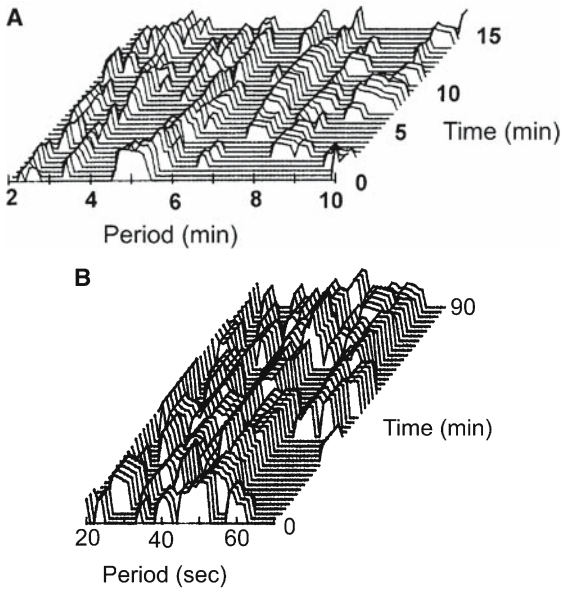

Fig. 4.1 ATP is involved in many cellular reactions and it is reasonable to expect many of them to form parts of distinct control systems that can oscillate in a relatively independent manner. That being so, the ATP level in a cell would reflect the summation of several rhythms. As can be seen in these Enright periodograms, time series analyses of the fluctuations in two mouse cell lines, L cells (Fig. 4.1A) and MEL cells (Fig. 4.1B), support that argument. Comparable results have also been obtained using a hamster kidney cell line

that cells are multi-oscillators. Each diagram indicates the existence in the data of several rhythms of different periods (frequencies) contributing to the total variation in the level of ATP in the indicated cells – and presumably all cells (we have observed similar results with HaK cells). The distinct bands of different periods strongly suggest the involvement of diverse metabolic processes although, because of the nature of the rhythmic processes, one cannot entirely rule out contributions from harmonics or morphological dynamics (Visser et al., 1990). However, there is an additional factor which cannot be dismissed, namely that we are dealing with summation of changes in sub-populations of cells which exhibit distinct periods of oscillation, as detected in studies on morphological periodicities (Visser et al., 1990). Not observed in these particular examples are drifts in the periods of some rhythms (e.g. Gilbert et al., 2000), seemingly due to gradual depletion of nutrients which also confirm theoretical arguments that the dynamic state is not rigidly fixed in characteristics.

Although some are not directly concerned with phosphorylation processes, it is pertinent to mention briefly oscillations associated with glycolysis and mitochondrial action which are responsible for ATP production.

4.2.2 Glycolysis

Rhythmic changes have been observed in the activities and isozyme patterns of a number of the enzymes and hexokinase and lactic dehydrogenase (LDH) in particular (see Gilbert and Lloyd, 2000). We note reports that the latter enzyme and certain other glycolytic enzymes are phosphorylated at tyrosine-specific sites (Cooper et al., 1983; Cooper et al., 1984).

The term ‘glycolytic oscillator’ usually refers to periodic changes generated by phosphofructokinase (PFK) and it could be assumed that other glycolytic enzyme periodicities we have reported are all due to the PFK oscillator. However, they cannot all be the result of that periodicity because variable and distinct timing differences exist between the various rhythms. It should be noted that much early discussion surrounded the possibility of more than one glycolytic oscillator. Studies on LDH indicate that independent periodic variations occur in the levels of individual isozymes (Ferreira et al., 1996b) and there is some evidence for the involvement of regulators in the way of differences between electrophoretic and extract pyruvate activity measurements. A controversial observation in our work on LDH was that both the activity and the amount of active isozyme oscillated in cell- and particle-free preparations (Ferreira et al., 1996a), as do the glycolytic substrates (see Edmunds, 1988). Such preparations can thus not be assumed to have constant composition, nor can the primary oscillations observed be attributed to interaction between cytoplasm and mitochondrion or to plasma membrane reactions. The behaviour, however, may relate to covalent modification of the enzyme protein by phosphorylation. In other studies, we have drawn attention to theoretical considerations on the efficiency of oscillating glycolysis, particularly in relation to the phenomenon of ageing (Gilbert, 1995).

4.2.3 Mitochondrial Action

Our studies on oscillations in the inter-conversion of NAD/NADH (the system redox state), and the effect thereon of insulin, have been described (e.g. Visser et al., 1990; Gilbert and Lloyd, 2000). This multi-frequency rhythm is usually attributed primarily to the PFK rhythm. By way of a change, here we include evidence for oscillatory changes in the redox state of FAD/FADH that can tentatively be associated with mitochondrial reactions and, hence, ATP synthesis (Fig. 4.2). The latter power spectrum analysis diagram again implicates the existence of a number of periods and also shows that the FAD oscillation is not rapidly stopped by suddenly dropping the temperature from 37°C down to 4°C; indeed the power is enhanced by the treatment (it should be pointed out that the data has been enhanced to emphasise the difference between the unaffected and cold shocked sections of the data, see Visser et al., 1990). Similar results have been noted with light scattering morphological studies – see for example Visser et al. (1990). It may be asked why such treatment should stimulate the rhythms. Elsewhere (Gilbert and Visser, 1993), we have shown that insulin stimulates cell surface vibrations and it was suggested that the non-specific actions of insulin on metabolism are due to effective stirring of the surrounding diffusion layer. And the FAD kind of effect discussed here could thus reflect a general attempt of cells to counteract a drop in metabolic rate by increasing the uptake of nutrients.

4.2.4 Phosphoamino Acid Phosphatases

Before the advent of suitable phosphorylated protein substrates, we developed a spectrophotometric assay for determining the hydrolysis of phosphotyrosine (Ferreira et al., 1996c). Figure 4.3, shows oscillations in this activity in MEL cells

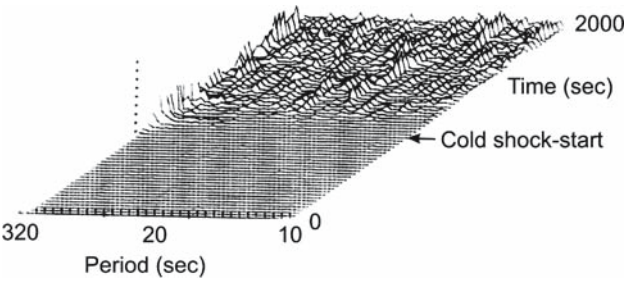

Fig. 4.2 We have used a difference fluorescence method (exciting the cells with light of one frequency and detecting the emitted light at another) to follow changes in the redox state of FAD in cells and here we show the stimulating effect of a sudden cold shock (from 37°C down to 4°C) on the oxidation state in a suspension culture of MEL cells using power spectrum analysis of the data. Although it appears that a range of oscillations occur only after the change in temperature, this is because we enhance the effect by only plotting values which are greater than an arbitrarily set threshold (Visser et al., 1990). Note the non-linear period scale of this method of analysis

and the effect thereon of insulin while the accompanying diagram, Fig. 4.4, also indicates that the hormone affects the rhythm and makes it evident that this activity is inhibited by lowering the temperature.

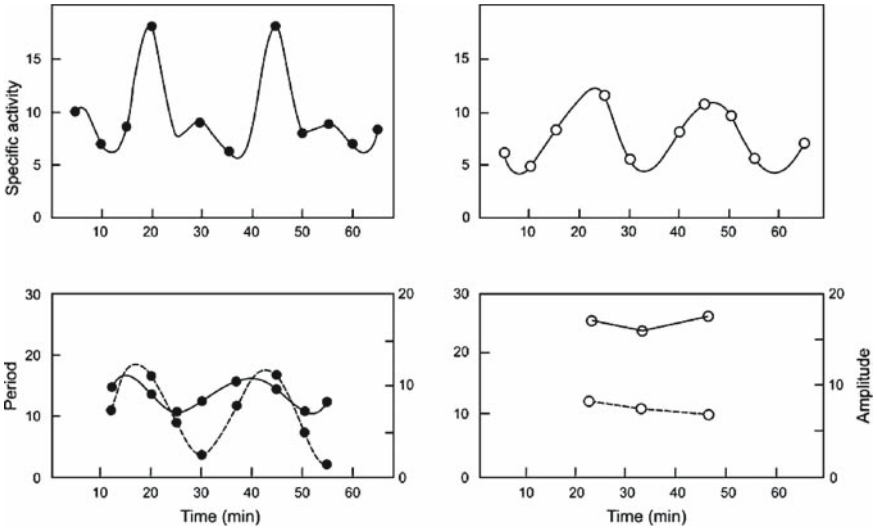

Fig. 4.3 The phosphotyrosine phosphatase (PTPase) activity in MEL cells has been found to oscillate and, as seen here, insulin affects this rhythm as it does for several cellular oscillations, consistent with the widespread effects of the hormone on cellular processes. It is not clear though if the latter acts directly on the PTPase periodicity or through another system which modulates the rhythm. Control cells are shown on the left and insulin-treated cells on the right. Period (———). Amplitude (.....)

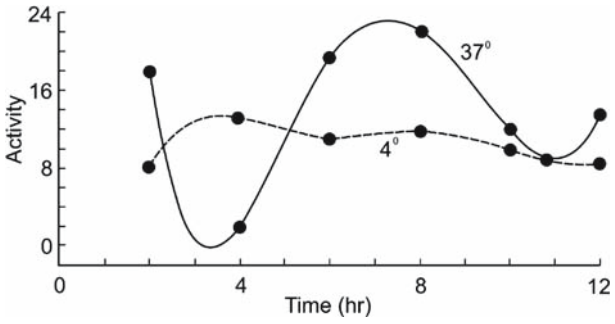

Fig. 4.4 Here it can be seen that the phosphotyrosine phosphatase oscillation in MEL cells is inhibited at low temperature. This diagram suggests an oscillation period of the order of hours, whereas the last figure seems to indicate a period of minutes. Both can be wrong but the former is certainly way off the mark. This is because of the problem of aliasing and hence a dependence on the sampling interval

4.2.5 Temporal Coordination

A widely ignored but major feature of great importance is the temporal co-ordination of cellular processes in time. The temporal relationship between two rhythms we thus study by using phase plane plots wherein the corresponding pairs of data values of interest, determined in the same cell extract, are plotted against each other (Gilbert, 1969). In these diagrams, time does not appear explicitly but as a movement of the point in the plane of the graph. In steady state situations the various data points fall on defined lines which are positive in slope where the two rhythms concerned are in-phase and negative in slope where they are out-of-phase, assuming that their periods are the same. Where an intermediate time relationship exists, the result is a closed curve under these conditions. More complex outcomes are obtained if the periods differ. In Fig. 4.5A and B, we give examples showing different phasings between the LDH and hexokinase isozyme rhythms in different cells. For LDH we have used the rates toward butyrate (B) as one substrate and pyruvate (P) for the alternate substrate to estimate isozyme pattern; on the other hand, for our

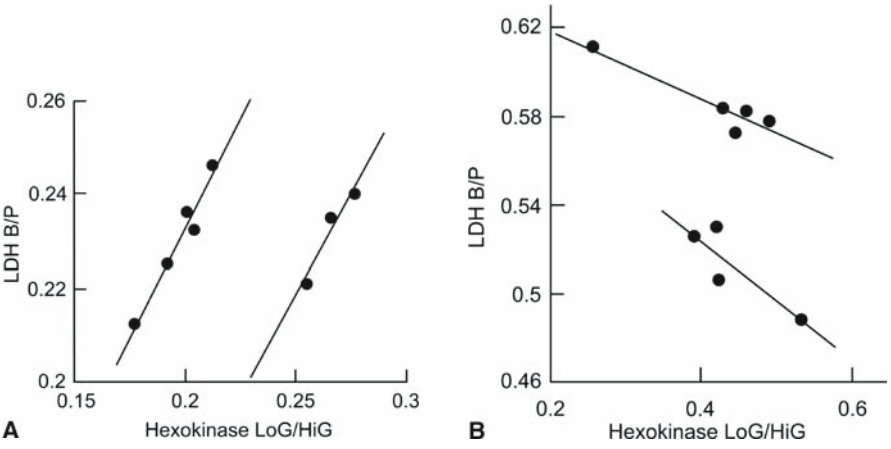

Fig. 4.5 Especially when the frequencies of two rhythms are the same, it is possible to study the phase relationship between them by plotting corresponding values, determined in the same extracts, against one another. This approach has been used in these two phase plane plot diagrams to observe the relative timings of periodic variations in the effective isozyme patterns for lactic dehydrogenase (B/P) and hexokinase (LoG/HiG) in HaK (Fig. 4.5A) and HeLa (Fig. 4.5B) cell lines. It can be seen that in both these cases, the data values fall on one or other of two straight lines, but in one the lines are of positive slope while of negative slope in the second. Our interpretation of this result is that (a) the two rhythms were in phase (positive slope) in one cell line but out of phase (negative slope) in the other and (b) metabolic switching was occurring in both cases giving rise to the pairs of lines. The different slopes thus support the view that cells can exhibit different patterns of temporal organisation and that the two periodicities are not due to the same rhythmic process. The highly linear nature of the lines indicates that both cells were essentially in a steady state (cf. Gilbert, 1974b, 1984)

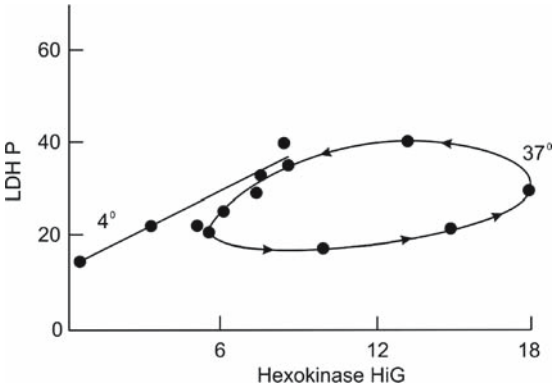

Fig. 4.6 With these rat hepatoma cells, the phase plane plot of the activities of LDH and hexokinase initially yielded a closed cyclic curve indicating that the two rhythms were in an intermediate phase relationship when the cells were at 37°C. However, when the temperature was rapidly decreased to 4°C, there was a rapid fall in the mean level of the hexokinase activity and the two rhythms then oscillated in phase, as shown by the linear plot

hexokinase studies we used a low glucose level (LoG) or high value (HiG). Also evident in both these diagrams are shifts in the relationships caused apparently by metabolic switching. The same approach has been used to study the effects of agents on timing relationships and Fig. 4.6 shows that a shift from 37°C to 4°C caused a rapid drop in the mean activity of hexokinase and a change in the phasing of the oscillation relative to the LDH rhythm.

All our results clearly support the suggestion that, under defined conditions, cells have characteristic patterns of temporal organisation (Gilbert, 1968, 1984).

4.3 Protein Phosphorylation Dynamics

There is overwhelming evidence that the reversible phosphorylation of proteins influences virtually all aspects of cellular function, from interactions at the cell membrane, through signal networks to transcriptional control in the nucleus but little attention has been paid to the temporal aspects of these processes, except with regard to mitosis. In the following sections, we consider the more general aspects but stress the cyclic behaviour relating to protein phosphorylation, highlighting the fact that cellular control of differentiation may be achieved through modulation of the rhythms (see Gilbert, 1984).

In the studies we describe, we have used the murine erythroleukaemic, MEL, and human acute promyelocytic leukaemic, HL60, cell lines. These cell lines, proliferating in culture, can be induced to differentiate and lose their malignant characteristics by a variety of agents; they thus provide valuable models for studying

the molecular and cellular dynamics involved in differentiation, as well as the pathogenesis of cancer and its reversal (Rovera et al., 1979; Breitman et al., 1980; Reuben et al., 1980). The findings we discuss are consistent with our concept (Gilbert, 1984) that differentiation and cancer involve changes in the set of control systems dynamics, that is in the patterns of temporal organisation, by modification of the frequencies, amplitudes, means and phasings of cellular rhythms.

4.3.1 Phosphorylation Potential

The highly dynamic nature of protein phosphorylation processes, even greater than cell cycle studies seem to indicate (Norbury and Nurse, 1992), was illustrated in a study of changes in the phosphorylation potentials of certain specific proteins (Ferreira et al., 1994a). When MEL cell preparations were incubated with ^{32}P-ATP, and then subjected to SDS-PAGE, autoradiographic analysis showed a very intense band, a major component of the cells, designated protein X, corresponding to a molecular mass of 81 kDa; a fainter band, corresponding to a mass of 63 kDa and designated protein Y, was also seen. Remarkably, variations in phosphorylation of as much as 100-fold were found within an interval of 10 min. The identities of the X and Y proteins are not known and we are unable to define the nature of the underlying reactions, although we note a report describing the stimulatory effect of growth factors on the *in vivo* phosphorylation (possibly autophosphorylation) of an 80 kDa protein in 3T3 cells (Rozengurt et al., 1983).

Time plots revealed the irregular periodic nature of the changes for both protein X and protein Y despite the random selection processes involved; both the periods and amplitudes seemed to be rhythmically modulated, but apparently by different processes. Periodogram analysis of the data (Fig. 4.7) confirmed the complexity of the phosphorylation dynamics of the two proteins. Although there was some similarity between the X and Y protein oscillations, there was a lack of any distinct phase relationship between them; the ratio of intensities fluctuated in a pseudoperiodic manner with variations up to 50-fold. There may be several explanations for the complexity of the rhythms even if one assumes that the X and Y bands represent single proteins. Variations may be the result of changes in activities of kinases and phosphatases and the extents and multiplicity of phosphorylation (reflecting the involvement of several distinct kinases affecting different residues). As kinases are generally very specific, it seems probable that those catalysing the reactions for protein X and protein Y are different, irrespective of whether only one enzyme is involved in each case. If both activities vary with time in a periodic way, the observed complexities can be understood, especially if the frequencies are distinct. More than two oscillations seem to be involved for both proteins and this could account for the lack of a particular relationship in the phase plots, as might competition effects. However, the question arises as to how two, possibly more, kinase activities can be regulated independently in periodic manner. Changes in protein concentration may be another factor; oscillations are known to occur in protein synthesis

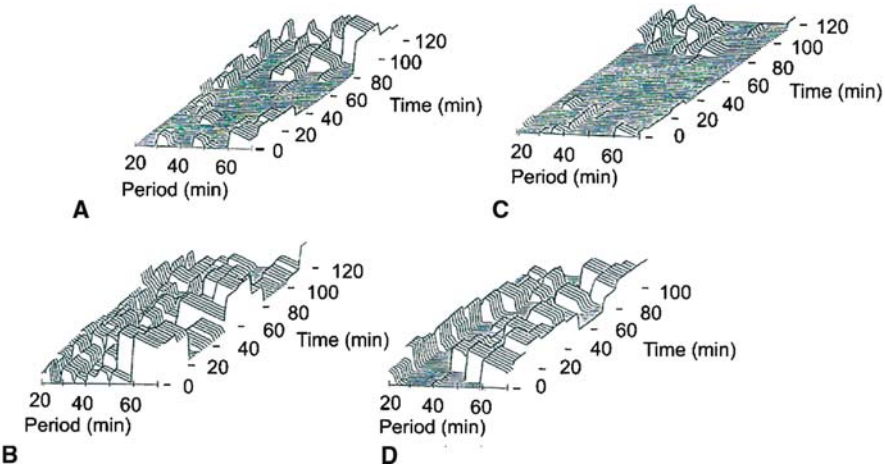

Fig. 4.7 These Enright periodograms show the phosphorylation dynamics of two proteins, protein X (**A** and **B**) and protein Y (**C** and **D**), in MEL cells. **A** and **C**: untreated control cells. **B** and **D**: cells treated with insulin. For the rhythms whose periods fall within the range shown, the magnitudes (the relative variance) tend to vary with time, but are more marked after addition of the hormone (Adapted from Hammond et al., 1998)

(although not dependent on ATP) and in the rate of incorporation of amino acids into protein (Brodsky et al., 1992).

Insulin was found to enhance the protein X and Y oscillations in a complex manner, in that it may affect the mean level of particular rhythms contributing to the overall behaviour as well as the dynamic characteristics of that contribution. The action of the hormone seemed to be general, but not all oscillations were affected similarly. Insulin stimulates rhythmic variations in cell morphology (Visser et al., 1990) and its effects on phosphorylation are in fact comparable. As with morphological changes, the apparent frequency dependent response to the hormone may be due to rapid, discrete change in the periods of certain oscillations rather than simple increases and decreases in their amplitudes. The complexity of the response to insulin is consistent with the multiplicity of its effects on cellular processes.

4.3.2 Protein Kinases and Phosphoprotein Phosphatases

Clearly, phosphorylation processes are highly dynamic, even under resting conditions, and it has become increasingly evident that the cyclic interconversion of the regulatory enzymes, the protein kinases and phosphoprotein phosphatases, represents the dynamic process in which the steady state equilibrium between active and inactive forms varies depending on the parameters determined by the metabolic state of the system. The emergent array of phosphoprotein phosphatases has shown that nature

has created a diversity of structure and function far exceeding that of the protein kinases (Shi et al., 1998; Virshup, 2000; Janssens and Goris, 2001; Cohen, 2002; Sim and Ludowyke, 2002).

A possible role for serine/threonine phosphatases in regulating granulocytic differentiation of HL60 cells was suggested by Morita et al. (1992) and Tawara et al. (1993). Our studies of the serine/threonine phosphatases, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), and of protein tyrosine phosphatase 1B (PTP1B) enzyme protein expression during proliferation and differentiation of MEL and HL60 cells, which involved SDS-polyacrylamide gel electrophoretic analysis of cell extracts followed by immunoblotting using specific antibodies, showed marked temporal variations in the intensities of immunoreactive bands. The magnitude of the fluctuations for PP1 was as much as seven- to eightfold and for PP2A and PTP1B up to 10–12-fold (Hammond et al., 1998; Bhoola and Hammond, 2000; Bodalina et al., 2005).

The molecular mass of 38 kDa we observed for PP1 in HL60 cells corresponded closely to that given by Mumby and Walter (1993) for the catalytic subunit of the protein. Differentiation of HL60 cells along the granulocytic and monocytic pathways, induced by all-trans retinoic acid (ATRA) and phorbol myristate acetate (PMA), respectively, was associated with variations in the patterns for the PP1 band. With PMA, there was also a marked change in the mean expression of PP1. Representative results showing the effect of ATRA are given in Fig. 4.8. In MEL cells, we found a single immunospecific protein of mass 33 kDa. Temporal variations in the expression of this protein were seen and there were differences between the patterns in proliferating and hexamethylene bisacetamide (HMBA)-induced differentiating cells. In some cases, there were changes in amplitude and phasing, while in others there was a dampening or an overall increase in protein expression in HMBA-treated cells as compared with controls. An example is shown in Fig. 4.9. The difference plot effectively removes mean variations and provides further illustration of the dynamic nature of the system. Instantaneous period and amplitude both varied with

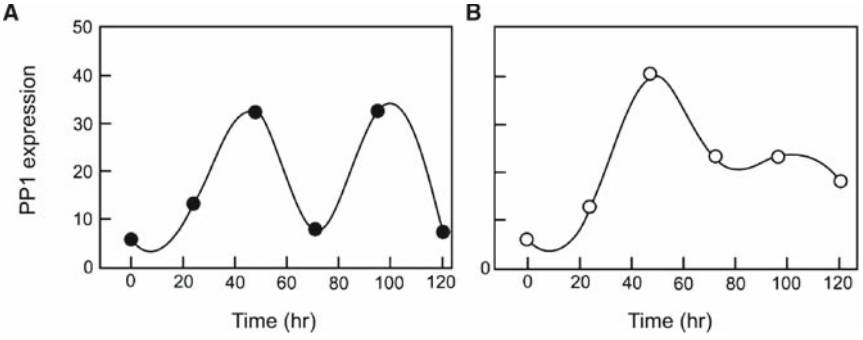

Fig. 4.8 The expression of PP1 in HL60 cells shows a rhythmic pattern of behaviour (**A**) that is modulated during ATRA-induced differentiation along the granulocytic pathway (**B**). The aliasing problem (see caption to Fig. 4.4) is obviously also relevant here, again because of the long sampling interval

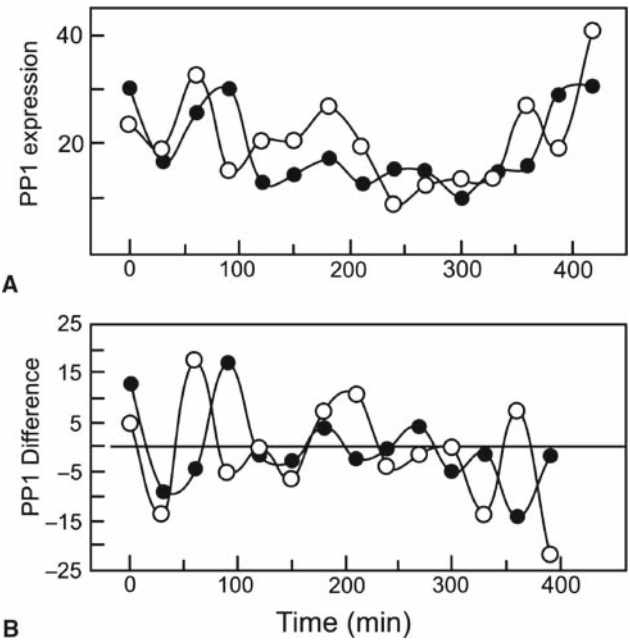

Fig. 4.9 Variations in the expression of PP1 can also be seen in untreated, proliferating (•) and HMBA-treated, differentiating (◦) MEL cells (**A**). The difference curves (**B**) provide further illustration of the dynamic nature of the system

time making it difficult to estimate mean period and amplitude; variations noted between the untreated and the HMBA-treated cells indicated that changes were taking place during cell differentiation.

In HL60 cells, with an antibody specific for the catalytic subunit of PP2A, we detected three bands of mass 34, 37 and 46 kDa. The 34 and 37 kDa bands were predominant and may represent different phosphorylation states (Mumby and Walter, 1993); the 46 kDa band was a minor form. All three of the immunoreactive proteins we observed showed temporal behaviour and the patterns were modified during induced differentiation along the granulocytic and monocytic pathways. A dampening effect on the expression of the 34 kDa PP2A catalytic subunit, of possible regulatory significance, was seen on treatment with either ATRA or PMA. During ATRA induced differentiation, our results showed different trends in the expression of the 34 and 37 kDa subunits suggesting that their expression may be modulated independently. With PMA, the trends became similar, and in phase, for the different subunits. Preparations from MEL cells, when probed for PP2A, showed two bands of masses 32 and 36 kDa; the 36 kDa band probably represented the catalytic subunit and the 32 kDa band a cleaved species of the catalytic subunit (see Tung et al., 1984, 1985). Time dependent variations in expression of the 32 and

36 kDa subunits were observed, using 15, 30 and 60 min and 12 h sampling times and HMBA modified the dynamics. Figure 4.10 shows ratios for the two subunits and a phase plane plot. Clearly, the two proteins were oscillating and there was some evidence that in certain instances they were doing so in phase with one another. This suggests a common underlying process, but on the other hand, there were differences that implied a certain degree of independence. As with PP1, the period and amplitude curves varied with time; mean periods covered a wide range. Observations again demonstrated that sampling frequency is an important factor in determining period and the true values may be much lower because of the problem of aliasing (Hammond et al., 1998; Gilbert and Ferreira, 2000; Calvert-Evers and Hammond, 2002).

We have also observed differentiation-associated changes in oscillatory behaviour for expression of PTP1B in HL60 cells. Our findings for expression of PTP1B, as for PP1 and PP2A, have indicated that the dynamic control mechanisms operating during induced differentiation along the granulocytic and monocytic pathways are different. The catalytic domain of PTP1B was detected as a 46 kDa band, which was usually the major form; in addition, a 42 kDa form was seen. During differentiation along the granulocytic pathway, induced by ATRA, there was an overall increase in

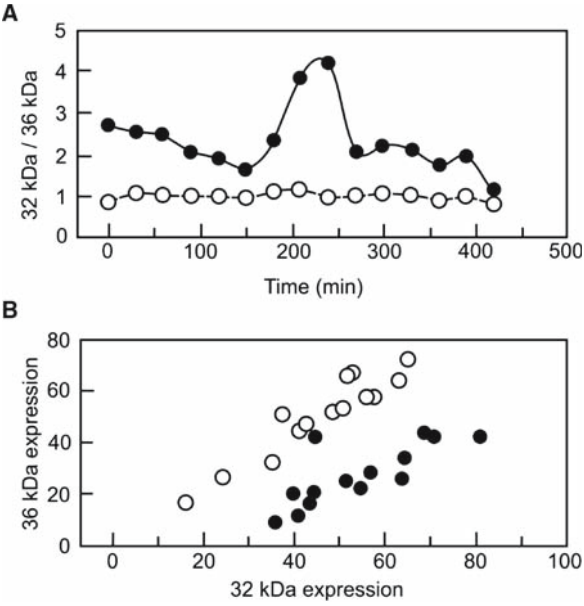

Fig. 4.10 The relationships between the 32 and 36 kDa PP2A components showed some variability; the two proteins were oscillating and in certain instances they were in phase with one another. In this experiment, their expression in untreated, proliferating (•) and HMBA-treated, differentiating (◦) MEL cells is plotted as ratios (**A**) and as a phase plane plot (**B**)

PTP1B expression and a dampening effect on the oscillation. For PMA-induced monocytic differentiation, there was an increase in the magnitude of the oscillation (Fig. 4.11). Further, the effect seen with PMA was remarkable with a complete change in distribution of the two forms in a time dependent manner and predominance of the 42 kDa subunit after 48 h. This change could have been the result of dephosphorylation, or alternatively, calpain catalysed cleavage of the protein may have caused a shift in the mobility (Frangioni et al., 1993). Studies have indicated that HeLa cells undergo phosphorylation of PTP1B during mitosis when the slower moving band is seen; in synchronous cells the faster band predominated (Schievella et al., 1993). In a study of the effects of different lysing buffers on PTP1B expression, we showed that when fluoride and phosphate were included, the protein was detected exclusively as the higher molecular mass form; in buffers from which these substances were omitted only the lower band was seen (Calvert-Evers and Hammond, 2000a).

Studies of the dynamics relating to protein concentrations and the activities of protein tyrosine phosphatase (PTP) and protein tyrosine kinase (PTK) have provided yet more evidence of oscillatory rhythms (Calvert-Evers and Hammond, 2000b, 2002, 2003). These involved examination of time-dependent variations during proliferation and differentiation, linear correlations and phasing relationships on a time basis and variations in the characteristics of the rhythms. Quantitative assessment of activity of PTPase and PTK in proliferating and ATRA-induced differentiating HL60 cells was by non-radioactive photometric enzyme linked immunoassay.

Temporal organisation and time-dependent variations were observed for the enzyme activities of PTP and PTK and the amount of total protein extracted from both proliferating and differentiating cells. ATRA treatment significantly altered the patterns of the oscillating waveforms. For PTP enzyme activity, the effect of ATRA gave rise to four distinct patterns of oscillatory behaviour – dampening, partial phase shift, phase shift and aperiodic random effect. The patterns of behaviour seen

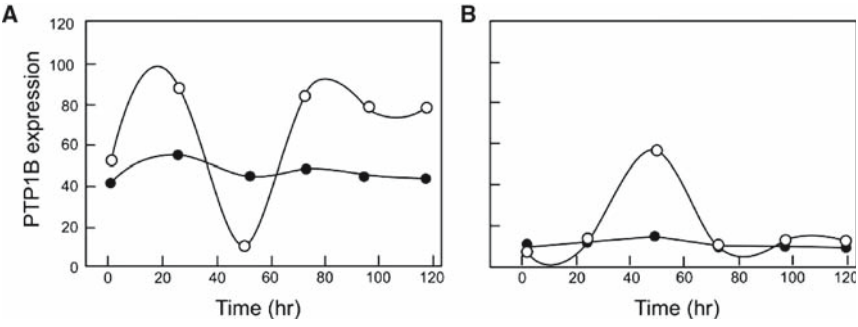

Fig. 4.11 Time dependent changes in the expression of the 42 (**A**) and 46 kDa (**B**) forms of PTP1B can be seen in HL60 cells. The patterns are enhanced during PMA-induced differentiation along the monocytic pathway. Untreated control cells (•); PMA-treated cells (◦)

for PTK activity on ATRA treatment included suppression or dampening, slight suppression with partial phase shift, increase in activity and a random irregular effect. Examples are shown in Figs. 4.12 and 4.13. No obvious relationship could be distinguished between the PTP or PTK enzyme activities and the protein concentration for either proliferating or differentiating cells; the poor degree of correlation suggested relative independence of these sets of oscillators. If temporal changes in PTP and PTK activities were caused simply by fluctuations in the amount of protein extracted from the cells, the timing relationship would not be apparent, as the enzyme activities should vary in unison with the protein changes. If the activities of the two enzymes were interdependent, phase plane plots should not have shown such marked deviation from linearity. As no particular relationships could be detected for these periodicities, it is possible that the frequencies of the rhythms are different, or that multiple interacting rhythms are involved. This is

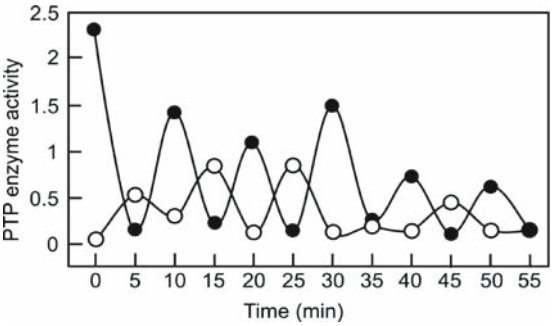

Fig. 4.12 Different types of behaviour are seen for PTP activity during ATRA-induced differentiation of HL60 cells along the granulocytic pathway. This figure shows an experiment in which there was a phase shift. Untreated control cells (•); ATRA-treated cells (◦). We note that the patterns seen for PTP specific activity were essentially the same (see Calvert-Evers and Hammond, 2000b)

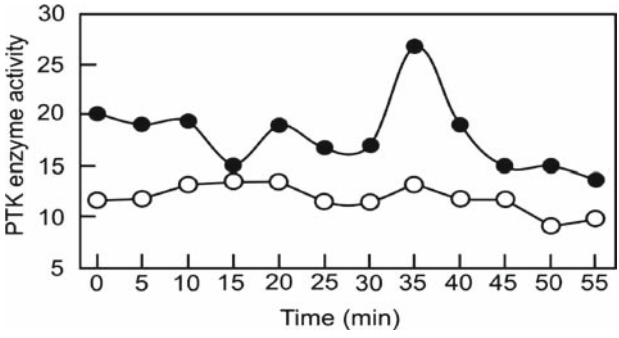

Fig. 4.13 Fluctuations in the activity of PTK in HL60 cells can be seen here. Differentiation along the granulocytic pathway induced by ATRA resulted in a dampening of the rhythms in this experiment. Untreated control cells (•); ATRA-treated cells (◦)

consistent with our findings for other studies (Gilbert and Tsilimigras, 1981; Ferreira et al., 1994b). Analysis of the data showed that the period and amplitude of PTP and PTK enzyme activities in HL60 cells varied with time and that there were distinct changes in these characteristics during differentiation along the granulocytic pathway. The periods covered a wide range of values (5–26 min) and were of a similar order of magnitude in proliferating and differentiating cells, and for both enzymes. Differences in the amplitude values for PTP in untreated and induced cell were obvious. A very noticeable decrease was observed where there was dampening or suppression of activity during differentiation. This observation provides for an extra dimension of metabolic control through differential modulation of rhythmic characteristics such as amplitude.

The wide variety of modulatory effects following treatment of cells with differentiating agents reflects their dynamic nature and the complex time-dependent interactions between the individual regulatory processes within the cell, between the metabolic control systems of different cells and with the environment.

4.3.3 Protein Kinase C

Protein kinase C (PKC) plays a critical role in regulating a multitude of signal transducing networks including certain mitogenic pathways controlling cell proliferation and differentiation (Nishizuka, 1992; Dekker et al., 1995; Toker, 1998; Buchner, 2000). Early work from our laboratory suggested a role for PKC in the differentiation of MEL cells (Sprott et al., 1991a) and results reported by other groups indicated the involvement of specific isoforms (Melloni et al., 1989; Melloni et al., 1990; Leng et al., 1993; Patrone et al., 1994; Pessino et al., 1994; Rosson and O'Brien, 1995; Mallia et al., 2000). Subsequently, we introduced the dimension of time to a study of the expression of the PKC isoforms α, ε and ζ, representing the classical, novel and a typical groups, respectively (Hammond et al., 2000a). We confirmed the presence of the α, ε and ζ forms of PKC in MEL cells and studied dynamic aspects of their expression. Expression of the isozyme proteins was determined by SDS-polyacrylamide gel electrophoresis followed by western immunoblotting using specific antibodies. Cyclic behaviour was seen for all three isoforms and on induction of MEL cell differentiation, using hexamethylene bisacetamide (HMBA), over time periods of several days, or of several hours, changes were apparent. We also presented the first reports of cyclic changes in the expression of mRNA. Expression of mRNA for PKCα and PKCε was measured following RNA extraction, reverse transcription, polymerase chain reaction and electrophoretic analysis on agarose gels. There were distinct differences between the patterns in proliferating and differentiating cells for both PKCα and PKCε mRNA; the effects differed somewhat in different experiments, reflecting the complexity of the system, but in general there was a change in amplitude and phasing rather than frequency (Fig. 4.14). In these studies, for both mRNA and protein expression, we gave comparisons of

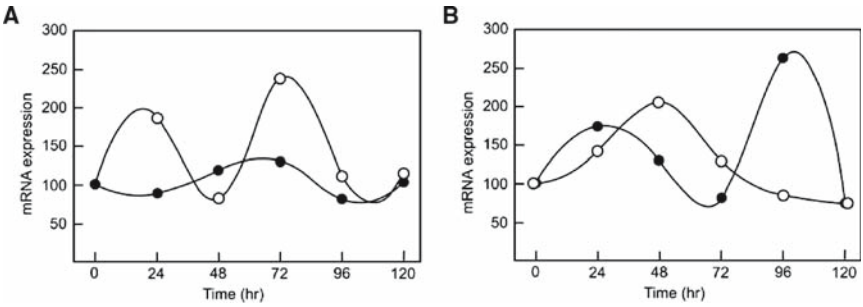

Fig. 4.14 Temporal variations in the expression of the mRNA for PKCα (**A**) and PKCε (**B**) seen in MEL cells and the effect of HMBA-induced differentiation are shown here. Untreated control cells (•); HMBA-treated cells (◦)

results for mean values with those corresponding to individual experiments. These were given particular mention as they demonstrated clearly the importance of examining the findings in a time-dependent manner for each of the data sets, in order to provide complete information without masking the dynamics. We suggested, based on our time studies, that modulation of the dynamics of the signals delivered by the different PKC isoforms is one of the molecular mechanisms involved in MEL cell differentiation. The rhythms of the different isoforms may vary in concert with each other as well as with those of transducing cascades, such as RAS/RAF (Kolch et al., 1993; Young et al., 1996; Van Dijk et al., 1997; Toker, 1998), in order to produce a coordinated response to extracellular effectors.

4.3.4 The RAS Signal Transducing Network

Another aspect of our work has involved studies of the *RAS* gene and its protein product in relation to induced differentiation of MEL cells. The *RAS* genes encode proteins that form part of a complex array of interacting networks regulated by kinases and phosphatases (Downward, 1990; Campbell et al., 1998; Chen et al., 1998; Malumbres and Pellicer, 1998). Several investigations suggested a role for the RAS protein in erythroid differentiation (Downward, 1990; Scheele et al., 1994; Ge et al., 1998; Matsumura et al., 1998); we showed changes in the activities of protein kinase A and protein kinase C, both of which interact with the RAS pathway and modulate its activity, during differentiation of MEL cells (Sprott et al., 1987, 1991a, b). We also saw temporal changes in expression of PKC isoforms in the study described above.

In our studies of *RAS,* we demonstrated cyclic changes in expression of H-*RAS* and N-*RAS* mRNA, as measured by northern blot analysis, and the protein product, as determined by SDS-PAGE and western immunoblotting (see Fig. 4.15)

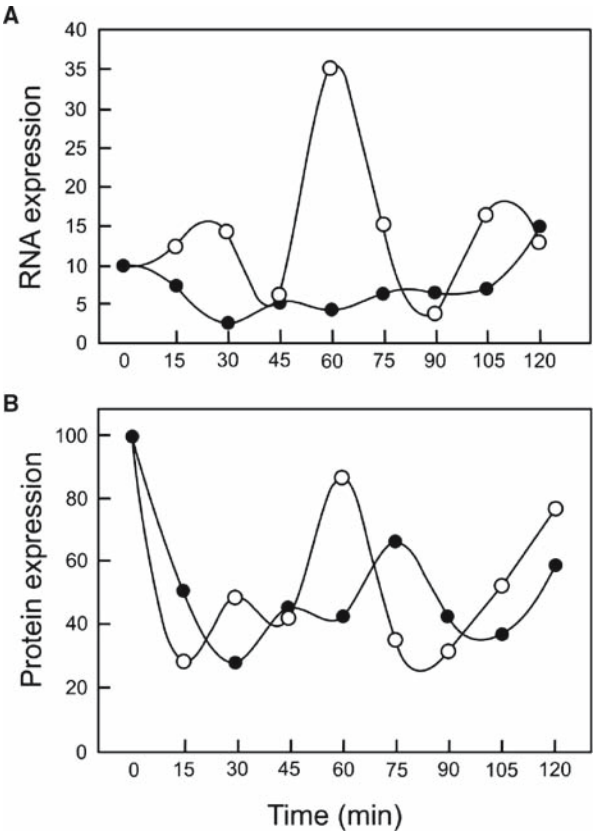

Fig. 4.15 The expression of the mRNA specific to H-*RAS* (**A**) and the expression of the RAS protein (**B**) in MEL cells show cyclic behaviour which is modified during induced differentiation. Untreated control cells (•); HMBA-treated cells (○)

(Hammond et al., 2000b). Once again, we showed that a range of results may be possible because of periodic behaviour and demonstrated that statistical comparisons based on single time point analyses provided an incomplete picture, which is not necessarily a reflection of the true situation and may be misleading. When mean values were assessed, increases in the expression of mRNA and protein were apparent after treatment with HMBA for 96 or 100 h; these changes may be associated with activation of the RAS pathway leading to terminal differentiation, or they may be coincidental depending on the phasing of the rhythms. Changes in frequency and phasing of the patterns of expression on treatment with HMBA were seen throughout the time courses studied, suggesting involvement of RAS in MEL cell differentiation from an early stage. It was clear that that regulation of a critical signal transducing protein, RAS, like that of the other kinds of proteins we have studied, is not just a matter of a simple increase or decrease in concentration, it is more complex involving modulation of the pattern of temporal control.

4.3.5 The P53 Protein

The P53 protein is a nuclear phosphoprotein, which, like other proteins, is constantly adjusting to changes in the cell; it is a tumour suppressor protein that responds to DNA damage, abnormal cell proliferation and hypoxia by arresting cell proliferation or inducing cell death (Hollstein et al., 1991; Lane, 1992; Greenblatt et al., 1994; Vousden, 2002). The p53 system is of particular interest to us for examining our concepts of temporal organisation not least because the protein has been reported to oscillate. There are feedback loops in which P53 controls the expression of its own regulator, MDM2, and a simple mathematical model has been presented, which suggests that oscillations in P53 and MDM2 proteins can occur in response to a stress signal (Bar-Or et al., 2000; Monk, 2003; Lahav et al., 2004; Tyson, 2004; Harris and Levine, 2005). Oscillations of both proteins occur on exposure of various cell types to ionising radiation. These may be associated with repair of DNA, preventing continuous, excessive P53 activation. Other studies have shown that DNA damage elicits quantal pulses of P53 (Lahav et al., 2004; Tyson, 2004). Monk (2003) considers time delays resulting from transcription, transcription splicing and processing and protein synthesis, which in principle can result in oscillatory mRNA and protein expression; it can be shown mathematically that the observed oscillatory expression of proteins, including P53, which are components of short feedback inhibition loops appear to be driven by transcriptional delays.

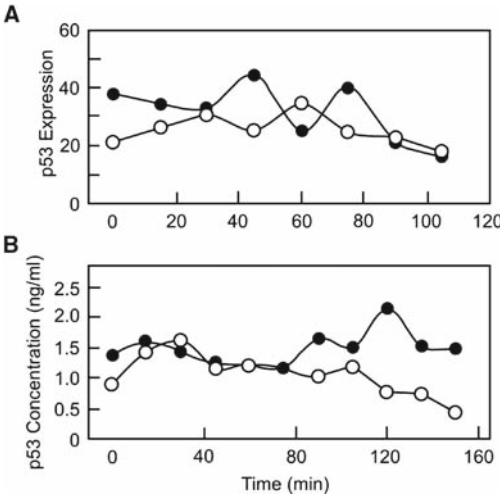

Fig. 4.16 Changes in the expression of the P53 protein in proliferating and differentiating MEL cells, measured by western blotting (**A**) or by ELISA (**B**). Untreated control cells (•); HMBA-treated cells (◦)

In our studies, aimed at exploring the contribution of P53 to the auto-dynamic nature of MEL cells, the protein was analysed both by western immunoblotting and by a more sensitive ELISA procedure; with both techniques, oscillatory behaviour, consistent with that seen in MCF7 cell populations (Lahav et al., 2004), was apparent (Bodalina et al., 2007). Our results were consistent with the view that HMBA influences P53 dynamics; (see Fig. 4.16) the period and amplitude curves differed in proliferating and differentiating cells. The period of the P53 rhythm was similar to that seen for PP1 and PP2A (30 min or less) in MEL cells (Bodalina et al., 2005). For PTK and PTPase in HL60 cells, the estimated period was lower and of the order of 10 min (Calvert-Evers and Hammond, 2002).

As in all our other investigations, the variations were complex as would be expected in the cellular environment where there are numerous interactions influencing protein expression. The models and experiments of Bar-Or et al. (2000) and Monk (2003), which revealed quantal pulses and harmonic oscillatory patterns, involved relatively uncomplicated situations in an ideal environment. Consistent behaviour is possible only in simple systems, that is in experiments where only a few isolated components are reacted together or in studies involving individual cells.

4.3.6 The Protein Phosphorylation Process

Support for the existence of numerous cellular periodicities associated with reversible protein phosphorylation is now considerable and includes rhythmic variations in expression of key phosphorylated and dephosphorylated proteins and enzyme protein expression and activity. The evidence indicates that phosphorylation of proteins is a dynamic process varying constantly according to the needs of the cell. A drastic change may result in an altered state of the cell, as in cancer. The expression of critical proteins may be affected as a result of disturbances to the balance between the activities of kinases and phosphatases. These dynamic changes and complex interactions appear to be reflected in the oscillatory patterns of cellular components, which may be used as a means of monitoring transformation and possibly modulating the process.

4.4 To Conclude …

All cellular reactions must comply with simple basic laws and principles but we think that here alone we have shown that the consequences can be extremely complex. Following from that truism is the realisation that our ability to analyse and interpret actual data is incredibly challenged.

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