A mathematical model of the regulation of the G₁ phase of Rb^{+/+} and Rb^{-/-} mouse embryonic fibroblasts and an osteosarcoma cell line

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Abstract. A mathematical model integrating the roles of cyclin D, cdk4, cyclin E, cdk2, E2F and RB in control of the G₁ phase of the cell cycle is described. Experimental results described with murine embryo fibroblasts (MEFs), either Rb^{+/+} or Rb^{-/-}, and with the RB-deficient osteosarcoma cell line, Saos-2, served as the basis for the formulation of this mathematical model. A model employing the known interactions of these six proteins does not reproduce the experimental observations described in the MEFs. The appropriate modelling of G₁ requires the inclusion of a sensing mechanism which adjusts the activity of cyclin E/cdk2 in response to both RB concentration and growth factors. Incorporation of this sensing mechanism into the model allows it to reproduce most of the experimental results observed in Saos-2 cells, Rb^{-/-} MEFs, and Rb^{+/+} MEFs. The model also makes specific predictions which have not been tested experimentally.

A large body of work has focused on understanding the regulation of the G₁/S transition in eukaryotes. Study of cell cycle progression has led to the elucidation of a variety of proteins which are required for appropriate entry into S phase. These proteins include the G₁ cyclins, D-type cyclins, cyclin E, and cyclin A, and their associated kinases, cdk4 and cdk2, which together form active complexes required for entry into S phase (Hunter & Pines 1995). The activity of cdk/cyclins can be inhibited by cdk inhibitors (cdis) such as p16ink4, p21cip1, p27kip1, and others (Hunter & Pines 1995). The retinoblastoma tumour suppressor protein, RB, is the principal known G₁ substrate for active cdk/cyclin complexes (Wang, Knudsen & Welch 1994, Weinberg 1995). RB interacts with the E2F-family of transcription factors and suppresses E2F-mediated transcription. E2F binding sites are observed in a number of genes involved in DNA synthesis and cell cycle progression, and E2F can promote the expression of these genes and entry into S phase (Nevins 1992, Johnson *et al.* 1994, DeGregori, Kawalik & Nevins 1995).

In this manuscript we have focused on processes involving D-type cyclins, cdk4, cyclin E, cdk2, E2F and RB. A pathway for G₁ progression has been described in which D-type cyclin expression is dependent on the presence of growth factors (Sherr 1994). The accumulated

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cyclin D interacts with cdk4 which phosphorylates RB leading to the release and subsequent activation of E2F (Sherr 1994, Weinberg 1995). Active E2F then drives the expression of cyclin E (DeGregori & Nevins 1995, Geng *et al.* 1996). Cyclin E associates with cdk2 and this complex can maintain RB phosphorylation, and in combination with free E2F promote entry into S-phase (DeGregori *et al.* 1995, Duronia & O'Farrel 1995).

Mathematical modelling of this pathway failed to account for some specific observations. For instance, it predicted that, in the absence of RB, MEFs should become growth factor independent, while it has been observed that *Rb*^{-/-} MEFs require growth factor to enter S phase (Herrera *et al.* 1996). Our model incorporates into the experimentally defined pathway a sensing mechanism to enhance cyclin E activity for increasing concentrations of RB and growth factor. We hypothesize that this sensing mechanism is lost in transformed cell lines such as Saos-2.

THE MODEL

This model focuses on the regulation of progression through the G₁ phase of the mammalian cell cycle involving cdk4/cyclin D, cdk2/cyclin E, E2F, and RB. Maturation promoting factor (MPF) is included in the model to allow for completion of the remainder of the cell cycle, i.e. S, G₂ and M phases. Most of the interactions presented in this model are supported by experimental evidence. The following are the known and assumed reactions built into this model. It is known that growth factors stimulate the production of cyclin D (at rate a_D) with half saturation concentration (h) during early G₁ (Sherr 1994), and we assume the cell is cultured in rich media and therefore contains a constant pool of growth factors (GF). Furthermore, the total cdk2, cdk4, E2F (E_T) and RB (R_T) concentrations are assumed constant throughout the cell cycle (Chen *et al.* 1989, Matsushime *et al.* 1992, Pagano *et al.* 1992). Free E2F controls the production of cyclin E (at rate a_E) (DeGregori *et al.* 1995, Ohtani *et al.* 1995, Geng *et al.* 1996). We assume that cdk4 and cdk2 are not limiting, and cyclin D/cdk4 (denoted by D) and cyclin E/cdk2 (E) complexes are formed as soon as the relevant cyclins are produced. Therefore, the formation rate of cyclin/cdk complexes are proportional to the production rate of cyclins. Hypophosphorylated free RB (denoted by R) sequesters E2F forming RB/E2F (R_s) at rate p_s . Hence, the formation of RB/E2F is proportional to the concentrations of free E2F ($E_T - R_s$) and hypophosphorylated RB. The cyclin D/cdk4 kinase phosphorylates RB (with parameters p_D , q_D) with higher affinity than cyclin E/cdk2 (p_E , q_E), due to the interaction of D-type cyclins with RB (Kato *et al.* 1993). In the process of phosphorylation of RB the RB/E2F complexes are disrupted and E2F is released (Nevins 1992, Weinberg 1995). Free E2F in concert with cyclin E/cdk2 will initiate S phase. We assume that a phosphatase activated by MPF, denoted by M , dephosphorylates RB (with parameters p_M , q_M) because RB is dephosphorylated at the end of mitosis (Ludlow *et al.* 1993). We model the phosphorylation and dephosphorylation of RB using Michaelis-Menten type kinetics, taking into consideration that the concentration of the enzyme, cdk/cyclin, is not constant throughout the cell cycle. For simplicity, we refer to hypophosphorylated and hyperphosphorylated forms of RB as unphosphorylated and phosphorylated RB, respectively. We assume that the activity of cyclin D/cdk4 decreases as a result of the degradation of cyclin D (at rate d_D) triggered indirectly by the activity of cyclin E/cdk2. It is known that cdk/cyclin complexes can promote cyclin degradation in G₁ (Lanker, Valdidieso & Wittenberg 1996). Cyclin E/cdk2 activity declines as cells progress through S phase. Since we do not include S and G₂ phase activities in this model, we introduce this feedback by assuming that MPF initiates the degradation of cyclin E (at rate d_E) which is necessary for the control of cyclin

E/cdk2 activity. Furthermore, it is known that MPF (cyclin B/cdc2) activates a cyclin-specific protease that degrades cyclin B (at rate d_M), and we assume it activates a protease that degrades cyclin E as well (Holloway *et al.* 1993, Murray 1995). Hence, the activity of both the RB phosphatase and the cyclin E protease are proportional to the MPF concentration. We assume cascade reactions initiated by transcription factor E2F (at rate f) and cyclin E/cdk2 (at rate a_M) help the cell cycle proceed, if undisturbed, through the S, G_2 and M phases. Hence, the concentration of E2F and cyclin E/cdk2 are proportional to the formation reactions of MPF. We also introduce the autocatalytic production of MPF (at rate g), that is initiated by cyclin E/cdk2 (Hoffmann *et al.* 1993, Obeyesekere, Tucker & Zimmerman 1994). Therefore, the term depicting the autocatalytic reaction is coupled with the concentration of cyclin E/cdk2 (i.e. M^E). These assumptions allow us to avoid modelling phases other than G_1 . Finally, we include the mechanism of sensing: cyclin E/cdk2 activity is enhanced by the total growth factor (with parameters α_1 , β_1) as well as total RB concentrations (with parameters α_2 , β_2). Figure 1 summarizes the biochemical interactions in our model.

In terms of the concentrations of the variables in our model, we interpret the beginning of the cell cycle as the time when the cyclin D/cdk4 (D) complex is increasing along with a high concentration of unphosphorylated RB (sum of R and R_s). We consider G_1 completion, and hence S initiation, to occur when the cyclin E/cdk2 (E) concentration is above a threshold, close to its peak, free E2F (difference of E_T and R_s) reaches maximum level and unphosphorylated RB drops below a threshold level. The subsequent S, G_2 and M phases are lumped together. Passage through these phases are lumped together. Passage through these phases is marked by an increasing unphosphorylated RB concentration, a decreasing cyclin E/cdk2 concentration, and a peak in MPF (M) concentration. Therefore, if the MPF concentration increases above a threshold (for example, one half of the concentration of MPF seen in a normal cell cycle profile), we consider that the cell has completed G_1 and, hence, has committed to progression through the rest of the cell cycle (see Figure 2). Finally, we consider that when the cell exits from M phase, the concentration of cyclin E/cdk2 is very low, unphosphorylated RB is very high, and MPF is decreasing rapidly.

Mathematically, our model describes the concentrations of five compounds, D (active cyclin D/cdk4 complex), E (active cyclin E/cdk2 complex), R (free unphosphorylated RB), R_s (E2F sequestered with RB) and M (maturation promoting factor, MPF), by the following first-order differential equations:

$$\dot{D} = a_D \left(\frac{GF}{h + GF} \right) - d_D ED \quad (1)$$

$$\dot{E} = a_E \left(1.0 + \frac{\alpha_1 GF}{\beta_1 + GF} \right) \left(1.0 + \frac{\alpha_2 R_T}{\beta_2 + R_T} \right) (E_T - R_s) - d_E ME \quad (2)$$

$$\dot{R} = \frac{p_M (R_T - R_s - R) M}{q_M + (R_T - R_s - R) + M} - p_s (E_T - R_s) R \quad (3)$$

$$\dot{R}_s = p_s (E_T - R_s) R - \frac{p_D R_s D}{q_D + R_s + D} - \frac{p_E R_s E}{q_E + R_s + E} \quad (4)$$

$$\dot{M} = a_M E + f (E_T - R_s) + g M^E - d_M M \quad (5)$$

Figure 1. Flow chart illustrating the protein interactions described by the model.

Figure 2. *continued*

Figure 2. Simulation of the cell cycle: (a) normal cell, (b) transformed cell and (c) Rb-null cell. Parameter values are given in Table 1. The initial values for a are $D=0.5$, $E=0.3$, $R=0.2$, $R_s=0.8$, $M=0.7$. The initial values for b and c are the same except $R=0.0$ and $R_s=0.0$. —, cycD/cdk4; ····, cycE/cdk2; ----, unpho-RB; —.—.—, free E2F; and — — — —, MPF.

In the above, the notation $\dot{Y}$ denotes the time derivative, dY/dt and the parameters are defined as before.

Quantitative information on reaction rates are not available in the literature. We converged on a set of parameter values by performing repeated simulations until we obtained cyclic concentration profiles that agree qualitatively with results cited in the literature.

SIMULATION RESULTS

This mathematical model was numerically solved using a mixed fifth and sixth-order Runge–Kutta–Verner integration algorithm. First, we reproduce the cyclic behaviour (cell proliferation) of a normal cell (e.g. a normal MEF cell); the simulation result is given by Figure 2a. The corresponding parameter values for obtaining this result are given in the first column of Table 1. These parameter values are stated in arbitrary units, c and t , for concentration and time, respectively. We may interpret the behaviour of the variables of these results relating to cell cycle as follows: Initially, the cyclin D/cdk4 complex is formed (activated) at a rate directly proportional to the concentration of growth factors. Almost all of E2F will be sequestered by unphosphorylated RB during the very early part of G_1 phase where a high concentration of unphosphorylated RB is seen. The increasing concentration of cyclin D/cdk4 will result in elevated levels of phosphorylated RB and, therefore, higher levels of free E2F (active). With the appearance of free E2F, the level of cyclin E/cdk2 will rise in the latter

part of the G_1 phase causing a drop of the cyclin D/cdk4 levels due to the degradation of cyclin D initiated by cyclin E/cdk2. The high concentrations of cyclin E/cdk2 and E2F will cause the MPF concentration to increase. Since MPF production is autocatalytic, its production rate increases with time, causing a rapid peak in MPF concentration. The high MPF concentration stimulates both a protease that degrades the cyclin E of the cyclin E/cdk2 complex and a phosphatase that dephosphorylates RB. These reactions result in lower cyclin E/cdk2 and higher unphosphorylated RB concentrations with lower free E2F levels. Since there is less cyclin E/cdk2 and E2F to stimulate MPF production, MPF degradation dominates and its concentration falls. The cell is now in the same state as it was at the beginning of the cycle and hence begins a new cell cycle. These results reproduce experimental descriptions of the concentration fluctuations during cell cycle progression. The cyclin D/cdk4, cyclin E/cdk2, RB, free E2F and MPF concentrations cycle periodically and remain positive for all time. Furthermore, RB is progressively phosphorylated with maximal phosphorylation concomitant with the high cyclin E/cdk2 complex and E2F concentrations.

We can reproduce the transformed cell cycle, which lacks RB (e.g. the cell cycle of Saos-2), using the same model with a minimal change in the parameters. To represent the absence of RB in this cell we set $R_T=0$. As mentioned previously, to represent the assumption that this transformed cell has lost the proposed sensing mechanism for changes in R_T and GF , we employ $\alpha_1=0$ and $\alpha_2=0$, respectively. We were able to obtain cyclic cyclin D/cdk4 and MPF profiles similar to profiles of Figure 2a for the transformed cell which contains the three parameter changes mentioned above, by changing only a_E from 0.038 to 0.0405 (+0.0025;

Table 1. The parameter values for the mathematical model to obtain a normal, Rb-/- and a transformed cell cycle. The arbitrary units (not SI units) for concentration and time are c and t , respectively. These parameter values were selected to obtain stable cell cycles for normal cells. There are no experimental values for these parameters in the literature

Parameter	Normal cell value	Rb-/- cell value	Transformed cell value
a_D	$0.1 \text{ } ct^{-1}$	$0.1 \text{ } ct^{-1}$	$0.1 \text{ } ct^{-1}$
a_E	$0.038 \text{ } ct^{-1}$	$0.038 \text{ } ct^{-1}$	$0.0405 \text{ } ct^{-1}$
a_M	$0.02 \text{ } t^{-1}$	$0.02 \text{ } t^{-1}$	$0.02 \text{ } t^{-1}$
h	$0.01 \text{ } c$	$0.01 \text{ } c$	$0.01 \text{ } c$
q_D	$0.6 \text{ } c$	$0.6 \text{ } c$	$0.6 \text{ } c$
q_E	$0.6 \text{ } c$	$0.6 \text{ } c$	$0.6 \text{ } c$
q_M	$0.8 \text{ } c$	$0.8 \text{ } c$	$0.8 \text{ } c$
f	$0.05 \text{ } t^{-1}$	$0.05 \text{ } t^{-1}$	$0.05 \text{ } t^{-1}$
g	$0.132 \text{ } c^{-2} \text{ } t^{-1}$	$0.132 \text{ } c^{-2} \text{ } t^{-1}$	$0.132 \text{ } c^{-2} \text{ } t^{-1}$
p_s	$0.15 \text{ } c^{-1} \text{ } t^{-1}$	$0.15 \text{ } c^{-1} \text{ } t^{-1}$	$0.15 \text{ } c^{-1} \text{ } t^{-1}$
p_D	$0.12 \text{ } t^{-1}$	$0.12 \text{ } t^{-1}$	$0.12 \text{ } t^{-1}$
p_E	$0.024 \text{ } t^{-1}$	$0.024 \text{ } t^{-1}$	$0.024 \text{ } t^{-1}$
p_M	$0.12 \text{ } t^{-1}$	$0.12 \text{ } t^{-1}$	$0.12 \text{ } t^{-1}$
d_D	$0.1 \text{ } c^{-1} \text{ } t^{-1}$	$0.1 \text{ } c^{-1} \text{ } t^{-1}$	$0.1 \text{ } c^{-1} \text{ } t^{-1}$
d_E	$0.05 \text{ } c^{-1} \text{ } t^{-1}$	$0.05 \text{ } c^{-1} \text{ } t^{-1}$	$0.05 \text{ } c^{-1} \text{ } t^{-1}$
d_M	$0.26 \text{ } t^{-1}$	$0.26 \text{ } t^{-1}$	$0.26 \text{ } t^{-1}$
α_1	0.2	0.2	0.0
α_2	11.0	11.0	0.0
β_1	2.0 c	2.0 c	2.0 c
β_2	44.0 c	44.0 c	44.0 c
R_T	2.0 c	0.0 c	0.0 c
E_T	1.5 c	1.5 c	1.5 c
GF	1.0 c	1.0 c	1.0 c

note that the total effective rate constant for cyclin E/cdk2 activity in normal MEF is 0.0599). This cell cycle simulation result is given by Figure 2b. Notice that the free E2F concentration is constant and R and R_s do not exist in this graph due to R_T being set equal to zero.

Based on these two sets of parameters, which reproduce normal and transformed cell cycles, we can simulate further experimental results.

The first experiment that we considered was obtaining Rb-null cells (i.e. Rb-null MEF). For that, we employed the same set of parameters as for normal cells (Table 1) with only R_T set equal to zero. As shown by Figure 2c, the cell cycle was undisturbed and the cell-cycle time was still the same as in Figure 2a. This agrees with experimental data by Herrera *et al.* (1996).

Next, we mimicked removing growth factors from the Rb-positive normal cell, the Rb-null cell, and the transformed cell. We accomplished this by setting $GF=0$ at time, $t=200$. As seen in Figures 3, the cells in both forms, Rb-positive (Figure 3a) and Rb-null (Figure 3b), arrest, i.e. no oscillations when growth factors are removed. However, the transformed cell continued to progress through the cell cycle in the absence of growth factors (Figure 3c). Following this, we simulated re-introduction of growth factors (back to $GF=1$) to the arrested cells, both normal and Rb-null. We utilized different values of R_T (total RB=1.0, 1.5, 2, 2.5, 3) for normal cells to look at potential effects of RB. In each experiment we changed GF from 1 to 0 at $t=200$ and later at $t=1800$ we reset $GF=1$ to mimic the re-introduction of growth factors to arrested cells. For comparison, the MPF values of these cases, including the Rb-null cells ($R_T=0$), are given in Figure 4. The behaviour of the other variables are similar to that of MPF (not shown). We assume that a cell is proliferating if its MPF levels rise above 3.0 in this study. Based on this, a normal cell arrested due to growth factor removal will resume cycling for low R_T values (up to 2), but will not resume cycling for higher R_T values. Furthermore, the normal cells with $R_T=1$ resumed cycling faster than the cells with higher R_T . Interestingly, however, the Rb-null cell ($R_T=0$) took longer to re-enter the cell cycle. In the following paragraphs, we state further experimental results obtained by computer simulations of our mathematical model (the parameter values given by Table 1) for the three specific cases as stated before: the normal cell, the Rb-null cell, and the transformed cell.

GF injection: We increased only the parameter GF , for all three cases, to simulate the effects due to the elevation of growth factor levels within a cell. The normal or the Rb-null cases did not show changes in the cell cycle time when the growth factors were doubled in value. However, the maximum values of MPF and cyclin D/cdk4 were higher when cells were injected with GF . The transformed cells did not show any changes in the cell cycle time or the levels of MPF or cyclin D/cdk4 in response to rising growth factor values. Results for these three cases, normal, Rb-null and the transformed cell, are shown in Figures 5a, b and c, respectively; only the profiles of cyclin D/cdk4 and MPF are shown, for clarity.

RB injection: This study was performed by increasing the R_T value on all three cases at times $t=200, 210, 220, \dots, 300$ to simulate injection of RB protein within G_1 or after. Raising R_T values for the transformed cells, where $R_T=0$, will arrest cell cycle progression in early G_1 irrespective of the time of injection (Figures 6a & b). Furthermore, these arrested transformed cells did not re-enter the cell cycle when growth factors were increased (GF was increased from 1 to 10) or cyclin D production rate, a_D , was increased (up to 0.5). However, for increased values of cyclin E production rate ($a_E=0.0505$) or total E2F ($E_T=1.9$) the arrested transformed cell re-entered the cell cycle (Figures 6c & d, respectively). The results were quite different for the injection of RB into the Rb-null cells. These cells did not arrest at all when injected with $R_T=1$ at any given time. However, increasing R_T values beyond

Figure 3. continued

Figure 3. Growth factor dependence: arrest of (a) normal, (b) Rb-null and not (c) transformed cell due to the removal of growth factors. Parameter values are from Table 1 except, $GF=0$ from $t=200$ for key see legend to Figure 2.

$R_T=1$ gave quite interesting results when performed on normal cells and Rb-null cells. These cells exhibited phase-specific behaviour for injection of RB at these greater values. In summary, both normal and Rb-null cells will arrest for some RB values if injected during G_1 phase, but not at non- G_1 times. Cell arrest is seen if R_T is changed from the original value 2 to a new value 3 for normal (Figure 7a) and from 0 to 2 for Rb-nulls (Figure 7b) during G_1 phase, and not at other times for these R_T values; RB injection at $t=240$ has no effect on cell cycle progression (Figures 7c & d, respectively). If RB is injected at values less than $R_T=5$ in normal cells (less than 6 in Rb-nulls) at non- G_1 times, the cell still cycled but for $R_T=5$ (6 for Rb-nulls) and greater, the cells arrested.

Inhibition of cyclin D: To simulate the effects of an inhibitor on cyclin D/cdk4 kinase activity, we reduced a_D to half of its original value. The inhibitor affects only the normal cell by arresting its cyclic behaviour. The cell behaviours for cyclin D/cdk4 inhibition on all the three cell types, normal, Rb-null and transformed cells, are given by Figures 8a, b & c, respectively.

Inhibition of cyclin E: Reduction of the activity rate of cyclin E/cdk2, a_E , to a half, arrests all three cell types irrespective of the time inhibition. Furthermore, in all three arrested cells the final level of cyclin E/cdk2 was slightly higher than that of cyclin D/cdk4. These three results are shown in Figures 9a, b & c.

Reduction of E2F: To simulate the effects due to a reduction of the transcription factor E2F we reduced its total concentration, i.e. decreased E_T . All three cell types show arrest within the cycle or in the next immediate cell cycle, depending on the time of reduction,

when E_T was reduced to 0.75. We only present the results of this experiment on the normal cell in Figure 10, as all three cases are similar in behaviour.

Based on these simulation results, normal cells can become arrested in two configurations based on concentration values of unphosphorylated RB. In the first state of arrest, the unphosphorylated RB level in the arrested cell was almost half of its total amount (close to 1); this was seen when the arrest occurred due to reduction of E2F concentration or inhibition of cyclin E/cdk2 activity. In the second state of arrest, the unphosphorylated RB level was higher compared to the first; this arrest was obtained when the activity of cyclin D/cdk4, was inhibited to half its value or after the removal of growth factors.

DISCUSSION

The mathematical model presented here describes the action of cyclin D/cdk4, cyclin E/cdk2, RB, E2F, and MPF on progression through the G_1 phase of the mammalian cell cycle. In order to accurately reflect experimental derived behaviour, the model includes a sensing mechanism for a normal cell wherein the activity of cyclin E is enhanced based on the total amount of RB and growth factor. This sensing mechanism is present in both Rb $+/+$ and Rb $-/-$ MEFs, which reflect a normal cell, but are absent in transformed Rb $-/-$ cell lines, such as the Saos-2 osteosarcoma.

Figure 4. Cell cycle entry from quiescence: MPF profiles of normal cells with different values of R_T , the total amount of RB, after re-injection of growth factors (GF is changed from 0 to 1) at $t=1800$. Initially, all these cells were in an arrested state due to growth factor removal from 1 to 0 at time $t=2000$. —, $RT=0.0$; ····, $RT=1.0$; ----, $RT=1.5$; -.-.-.-, $RT=2.0$; — — — —, $RT=2.5$; and -.-.-.-, $RT=3.0$.

Figure 5. continued

Figure 5. Changes in growth factors. Cell behaviour of an (a) normal, (b) Rb-null and (c) transformed cell due to the increased levels of growth factors. Parameter values are from Table 1 except, $GF=2$ from $t=200$. For key see legend to Figure 2.

Simulated experiments using this model reproduced many results obtained experimentally. It predicted the growth factor requirement for both Rb $+/+$ and Rb $-/-$ MEFs, which was recently reported (Herrera *et al.* 1996). The modelled cells enter the cell cycle in response to a threshold concentration of growth factor, and increasing growth factor above the threshold does not significantly effect cell cycle progression, which is consistent with a large body of evidence. The model predicts that all cell types simulated require the presence of cdk2/cyclin E for cell cycle progression. This result is in agreement with a number of findings that cyclin E is essential for entry into S phase, and that cdk2 inhibition can arrest the growth of Rb-deficient cells (Ohtsubo *et al.* 1995). The model also predicts that addition of RB to Rb-deficient tumour cells will lead to an arrest which is specific for the G_1 phase of the cell cycle. This has been reported in detail for Saos-2 cells (Goodrich *et al.* 1991, Templeton *et al.* 1991, Qin *et al.* 1992). Furthermore, our model shows that the RB-mediated growth arrest can be overcome by cyclin E or E2F overexpression (Hinds *et al.* 1992, Qin *et al.* 1995). Also, the model predicts that Rb-deficient cell lines either MEFs or Saos-2 do not require cyclin D/cdk4 activity for cell cycle progression. This is consistent with the finding that Rb-deficient cells are resistant to growth arrest mediated by the cdk4/cdk6 specific inhibitor p16ink4 (Lukas *et al.* 1995, Koh *et al.* 1995).

Despite these agreements there were several areas where the model failed to confirm known cell cycle phenomenon: (1) Rb-null MEFs are known to enter S phase from quiescence 2 h earlier than Rb $+/+$ (Herrera *et al.* 1996). However, in our simulations Rb-null cells re-enter the cell cycles more slowly than those cells with intermediate levels of

Figure 6. continued

Figure 6. Cell arrest (RB introduction) and rescue of transformed cells: transformed cells arrest when the total RB level (R_T) is elevated from 0 to 1 at time $t=200$ (a), and at time $t=240$ (b). These cells are released from arrest by increasing cyclin E/cdk2 activity (c), or by elevating the transcription factor, E2F (d). For key see legend to Figure 2.

Figure 7. continued

Figure 7. Cell arrest (RB introduction) only in G_1 times for normal cells. The total RB level is increased within the G_1 phase in a normal cell (a), and in a Rb-null cell (b), and after G_1 for normal (c) and Rb-null (d) cells. For key see legend to Figure 2.

Figure 8. continued

Figure 8. Cyclin D inhibition. Behaviour of a (a) normal, (b) Rb-null and (c) transformed cell when cyclin D/cdk4 is inhibited; activity rate of cyclin D/cdk4, a_D is reduced to half of its original value at time, $t=200$. For key see legend to Figure 2.

RB ($R_T=2.0$ the cells enter S phase very slowly, i.e. not until $t=6000$ (data not shown). Therefore, low levels of RB allow for more rapid entry into S phase, while at RB values of 0.0 there is a delayed entry. The Rb $-/-$ MEFs contain Rb-related proteins, p107 and p130, which may provide an Rb-like activity which is comparable to very low levels of RB. This Rb-like activity could allow for the accelerated entry into S phase (Cobrinik *et al.* 1996); (2) In our model cyclin D overexpression fails to reduce an RB mediated block in tumour cells. It has been published that cyclin D can reverse the cell cycle block of Saos-2 cells, but it is much less efficient than cyclin E (Hinds *et al.* 1992, Ewin *et al.* 1993); (3) Our model indicates that Rb-deficient tumour cell lines would be arrested via RB overexpression. This is not necessarily the case, as a number of Rb-deficient tumour lines are resistant to RB overexpression (Zacksenhaus *et al.* 1993, Wang *et al.* 1994). The tumour cells modelled in this paper reflect the behaviour of the Saos-2 osteosarcoma cell line. The genetic differences between tumour cells which allow for differential responses to tumour suppressors are unclear. In our model a tumour cell which is overexpressing E2F or cyclin E will become resistant to RB over-expression, suggesting that changes such as these could contribute to resistance to RB.

The model also raises a few interesting questions which have not been formally tested. First, the model includes a sensing mechanism in which growth factor and RB lead to the stimulation of cyclin E activity in the cell, and this mechanism is lost in transformed cells. This indicates that in 'normal' cells RB has both a positive and a negative role in the regulation of entry into S phase. The negative role is the documented inhibition of E2F

Figure 9. continued

Figure 9. Cyclin E inhibition. Behaviour of a (a) normal, (b) Rb-null and (c) transformed cell when cyclin E/cdk2 is inhibited; activity rate of cyclin E/cdk2, a_E is reduced to half of its original value at time, $t=200$. For key see legend to Figure 2.

activity (reviewed in Weinberg 1995). The positive role is a stimulation of cyclin E activity through an unknown mechanism. In transformed cells, which lack the sensing mechanism, introduced RB can only fulfil its negative growth regulatory function, which would explain why these cells arrest in G_1 at relatively low levels of RB. To date, no experimental data suggests a positive role for RB in progression through G_1 . However, since the negative and positive actions of RB are afterall coupled, it may be difficult to observe positive contributions of RB towards progression to S phase. Second, the model suggests that introduction of RB into 'normal' cells at non- G_1 times will have no effect on cell cycle progression even in the following G_1 -phase. This finding suggests that there exists a de-sensitization mechanism for RB: if the cell is in S phase when a large excess of RB is accumulated, the cell will become resistant to this RB in the following G_1 . Third, that cell cycle arrest in G_1 can do so in two discrete manners which can be differentiated based on the phosphorylation status of RB. G_1 arrest with phosphorylated RB occurs by inhibition of cyclin E or E2F, while arrest with unphosphorylated RB is caused by cyclin D inhibition or removal of growth factor. There is a very strong correlation between RB phosphorylation and cell cycle progression (reviewed in Wang *et al.* 1994). However, recent findings have shown that cells can be arrested in G_1 with phosphorylated RB (Chen, Knudsen & Wang 1996). Whether cells arrested by either inhibition of E2F or cyclin E contain phosphorylated RB remains to be determined. Furthermore, this model suggests that there exist two configurations of cell behaviour for cells with non-zero RB levels, a proliferative (cyclic) as well as a non-proliferative stage, with no changes in the parameters (results not shown). This

Figure 10. E2F inhibition. Cell arrest due to the reduction of E2F (a) at $t=200$ and (b) at $t=240$. For key see legend to Figure 2.

interesting mathematical result, known as two basins of attraction, as well as its biological explanation will be discussed by the same authors in a later contribution.

The model presented in this manuscript represents a more simplified version of the G_1 phase of the cell cycle than is encountered in reality, but more complex than a previously proposed model for the G_1 phase (Obeyesekere, Herbert & Zimmerman 1995). However, it is an important study since this model can replicate a significant number of experiments which have been carried out in the laboratory. Furthermore, the model makes specific predictions which have not been previously reported in the literature. Since laboratory experiments are quite time consuming in comparison with computer simulation of models, we believe that this type of study can help to serve as a bridge between biologists and mathematicians.

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REFERENCES

- CHEN PL, SCULLY P, SHEW JY, WANG JY, LEE WH. (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58**, 1193.
- CHEN Y, KNUDSEN ES, WANG JY. (1996) Cells arrested in G_1 by the v-Abl tyrosine kinase do not express cyclin A despite the hyperphosphorylation of RB. *J. Biol. Chem.* **271**, 19637.
- COBRINIK D, LEE MH, HANNON G *et al.* (1996) Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev.* **10**, 1633.
- DEGREGORI J, KOWALIK T, NEVINS JR. (1995) Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G_1 /S-regulatory genes. *Mol. Cell. Biol.* **15**, 4215.
- DURONIO RJ, O'FARRELL PH. (1995) Developmental control of the G_1 to S transition in *Drosophila*: cyclin E is a limiting downstream target of E2F. *Genes Dev.* **9**, 1456.
- EWIN ME, SLUSS KH, SHERR CJ, MATSUSHIME H, KATO J, LIVINGSTON DM. (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**, 487.
- GENG Y, EATON EN, PICN M *et al.* (1996) Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene* **12**, 1173.
- GOODRICH Y, WANG NP, QIAN YW, LEE EY, LEE WH. (1991) The retinoblastoma gene product regulates progression through the G_1 phase of the cell cycle. *Cell* **67**, 293.
- HERRERA RE, SAH VP, WILLIAMS BO, MKEL TP, WEINBERG RA, JACKS T. (1996) Altered cell cycle kinetics, gene expression, and G_1 restriction point regulation in Rb-deficient fibroblasts. *Mol. Cell. Biol.* **16**, 2402.
- HINDS PW, MITTNACHT S, DULIC V, ARNOLD A, REED SI, WEINBERG RA. (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70**, 993.
- HOFFMANN I, CLARKE PR, MARCOTE MJ, KARSENTI E, DRAETTA G. (1993) Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis. *EMBO J.* **12**, 53.
- HOLLOWAY SL, GLOTZER M, KING RW, MURRAY AW. (1993) Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell* **73**, 1393.
- HUNTER T, PINES J. (1995) Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. *Cell* **79**, 573.
- KATO J, MATSUSHIME H, HIEBERT SW, EWEN ME, SHERR CJ. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.* **7**, 331.
- KOH J, ENDERS GH, DYNLACHT BD, HARLOW E. (1995) Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* **375**, 506.
- LANKER S, VALDIVIESO MH, WITTENBERG C. (1996) Rapid degradation of the G_1 cyclin Cln2 induced by CDK-dependent phosphorylation. *Science* **271**, 1597.

- LUDLOW JW, GLENDENING CL, LIVINGSTON DM, DeCARPRIO JA. (1993) Specific enzymatic dephosphorylation of the retinoblastoma protein. *Mol. Cell. Biol.* **13**, 367.
- LUKAS J, PARRY D, AAGAARD L *et al.* (1995) Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* **375**, 503.
- MATSUSHIME H, EWEN ME, STROM DK *et al.* (1992) Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. *Cell* **71**, 323.
- MURRAY A. (1995) Cyclin ubiquitination: the destructive end of mitosis. *Cell* **81**, 149.
- NEVINS JR. (1992) E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**, 424.
- OBEYESEKERE MN, TUCKER SL, ZIMMERMAN SO. (1994) A model for regulation of the cell cycle incorporating cyclin A, cyclin B and their complexes. *Cell Prolif.* **27**, 105.
- OBEYESEKERE MN, HURBERT JR, ZIMMERMAN SO. (1995) A model of the G₁ phase of the cell cycle incorporating cyclin E/cdk2 complex and retinoblastoma protein. *Oncogene* **11**, 1199.
- OHTANI K, DeGREGORI J, NEVINS JR. (1995) Regulation of the cyclin E gene by transcription factor E2F1. *Proc. Natl. Acad. Sci. USA* **92**, 12146.
- OHTSUBO M, THEODORAS AM, SCHUMACHER J, ROBERTS JM, PAGANO M. (1995) Human cyclin E, a nuclear protein essential for the G₁-to-S phase transition. *Mol. Cell. Biol.* **15**, 2612.
- PAGANO M, PEPPERKOK R, VERDE F, ANSORGE W, DRAETTA G. (1992) Cyclin A is required at two points in the human cell cycle. *EMBO J.* **11**, 961.
- QIN XQ, CHITTENDEN T, LIVINGSTON DM, KAEIN WG JR. (1992) Identification of a growth suppression domain within the retinoblastoma gene product. *Genes Dev.* **6**, 953.
- QIN XQ, LIVINGSTON DM, EWEN M, SELLERS WR, ARANY Z, KAEIN WG JR. (1995) The transcription factor E2F-1 is a downstream target of RB action. *Mol. Cell. Biol.* **15**, 742.
- SHERR CJ. (1994) G₁ phase progression: cycling on cue. *Cell* **79**, 551.
- TEMPLETON DJ, PARK SH, LANIER L, WEINBERG RA. (1991) Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. *Proc. Natl. Acad. Sci. USA* **88**, 3033.
- WANG JY, KNUDSEN ES, WELCH PJ. (1994) The retinoblastoma tumor suppressor protein. *Adv. Canc. Res.* **64**, 25.
- WEINBERG RA. (1995) The retinoblastoma protein and cell cycle control. *Cell* **81**, 323.
- ZACKSENHAUS E, BREMNER R, JIANG Z *et al.* (1993) Unraveling the function of the retinoblastoma gene. *Adv. Canc. Res.* **61**, 115.