Gene Kinetics of the Cell Proliferation in Vivo *

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Abstract

A theory of the metabolism and gene expression of living cells is presented. The transcription and protein synthesis from the individual operons are calculated and combined with exponential decays of the enzymes, the metabolic pathways, and their transcripts, yielding the overall competition by different genes for the metabolic focus. Computations based on the theory simulate the cell cycle as a series of peaks of transcript followed by peaks representing the enzymes coded for while the G_0 phase appears as a regulated constant rate of enzymatic catalysis and gene expression prior to a trigger operon for commitment to division, which, when activated, may take the cell into the cell cycle. The computer simulations, which include contact inhibition - quiescence, commitment to division, cell cycle phase lengths, sequential cell cycles, as well as gene amplification, are in good agreement with bench-top experiments and suggest explicit molecular vehicles for the observed kinetics.

I INTRODUCTION

In the previous papers in this series [1, 2], empirical evidence was collected indicating that the cell proliferation is correlated with increased metabolic bulk fluxes and a model was proposed according to which the genetic program of the eucharyotic cell is predisposed to growth and proliferation whereas in the G_0 phase, the metabolic flux is instead conveyed into differentiated functions. Such a two-channel model of the cell proliferation is amply supported by experimental data [1, 2, 3, 4]. The fluxes are primarily regulated at the plasma membrane where all the nutrients first enter the cell, while the intracellular metabolic branching is governed by the second messenger cyclic AMP and other compounds having a differentiation-inducing effect. The abrogation of specialized functions and concomitant activation of the pathways characteristic of proliferation may be regulated from the plasma membrane, either when its coupled symport and antiport fluxes are released from inhibitory control at receptor sites or when its fluxes are unrestrained by inhibitory regulation following membrane perturbations, as is commonly observed in malignant tumor cells [2].

In the present paper, a mathematical formulation of the gene expression in the cell cycle is developed, which may explain these and other findings. The formulation is based on the measurable quantities amounts of enzyme, amounts of metabolites in an inducing pathway of the next arbitrary gene to be transcribed (which may also specifically be a trigger gene for the commitment to division and cell proliferation), amounts of transcript, and the rate of progression through the cell cycle or the phase lengths. The metabolic regulation and the rate of biological transport above or below steady state are expressed in the compressed form of an effective decomposition of the inducer being metabolized by the inducing pathway. The theory is first developed in detail and then adapted for computer simulations, followed by simulations of cycling cells, the commitment to division, and contact inhibition (quiescence). The results of the computations are evaluated throughout with reference to published gross cell biological as well as molecular data. Likely molecular effectors of the general mechanisms are identified.

II MATERIALS AND METHODS

Computations were performed on a NEC PC 8201A computer using n_{82}-BASIC programming language and required approximately 10 - 15 kbytes. Several versions of the program were written and gradually improved over a period of more than 5 years with identical or similar results (cf. [5]) The software allowed visual inspection in real time of the graphs representing cellular contents of mRNA and enzyme-pathway due to the activity of five different operons expressed sequentially in a running cell cycle and yielded numerical values of amounts of mRNA, enzyme-pathway, and cell cycle phase lengths. Further details from cerven@scienceandresearchdevelopmentinstitute.com

III THEORY

Cellular metabolism may be ascribed to a set of time-dependent probabilities, P, that a nutrient or a metabolic intermediate interacts with a target such as an enzyme or a gene-regulatory site,

$$P_{n,e,t} = \frac{n_e}{\Sigma E}\ exp(-A^* t) \tag{1}$$

where n_e is the number of copies of some particular enzyme, "e", or an operon, a promoter region, or any indirect vehicle of gene regulation, $E = \Sigma n_n$ is the number of copies of all the species of receptors and enzymes which may serve as recipients of the substrate, and A^* is a factor regulating the time (t) dependency of the metabolite's decay.

Consider then the special case that a single enzyme molecule or a regulatory site compete for a substrate metabolite with all the n_e enzyme molecules converting it and that mainly the latter determine the rate of decomposition of the substrate. This is described by a probability P_e,

$$P_{e,t} = \frac{1}{n_e}\ exp(-A_t t) \tag{2}$$

where, similarly to the kinetics of coagulation on "n" nuclei, cf. [6], the time-dependent breakdown of the substrate in an interval of time, Δt, depends exponentially on the amount of converting enzyme, n_e,

$$P_{e,\Delta t} = \frac{1}{n_e}\ exp(-A\ n_e) \tag{3}$$

The factor A accommodates enzyme regulation and additional substrate requirements and may be resolved into

$$A = C\ \frac{f(r)\ f(s)\ Z}{M} \tag{4}$$

wherein regulatory stimulation, $f(r)$, co-substrate levels on a scale starting below saturating ones, $f(s)$, and other stimulatory effects on the enzyme, Z, are growing functions which increase the probability of the substrate's inactivation by the competing enzymes. The factor M accounts for levels of substrate-inducer above or below steady state: Excess substrate, $M > 0$, implies that the probability of substrate inactivation declines less rapidly than during standard conditions, $M = 1$, whereas levels of substrate below steady state, $M < 0$, are equivalent of a more efficient inactivation of the substrate. C is a proportionality constant. The factor A thus expresses the effective decomposition of the substrate. Steady state levels of inducing metabolite, m_e, are further defined by

$$m_e = M_0\ n_e \tag{5}$$

that is, a proportionality by the factor M_0 between the amount of enzyme and the amount of its metabolite, similarly for an array of metabolites, $m_{e1},\ m_{e2},\ \ldots$, sequentially formed in an enzymatic pathway.

The transcription of two genes, OP_e and OP_f, competing for the same inducer, m_e, or for a transcription-activating factor or a transcription-activating complex that may be activated by m_e is now considered. The amounts of transcript (mRNA) synthesized from the two genes, R_e and R_f, are assumed to be proportional by the factors K_e and K_f to the amount of inducer preceding the enzymatic conversion, m_e,

$$R_e = K_e\ m_e\ , \quad R_f = K_f\ m_e\ , \quad \ldots . \tag{6}$$

and the probabilities of transcription from the two genes are also proportional to m_e,

$$P_{Re} = K_{Re} m_e\ , \quad P_{Rf} = K_{Rf} m_e\ , \quad \ldots \tag{7}$$

Furthermore, the amounts of gene-regulatory sites of the two competing operons are taken small compared to the amounts of converting enzymes, approaching a single site on the DNA molecule,

$$1 \leq\approx n_{op} \ll n_e \tag{8}$$

such that the probabilities of induction of the two genes by plain binding with equal affinity are equal,

$$P_{Re} \approx P_{Rf} \quad . \tag{9}$$

The probability of transcription from the single gene in a time interval, Δt, is

$$P_{Rf,\Delta t} = exp(-A\ n_e) = \frac{K_f\ m_e}{R_f}\ exp(-A\ n_e) \quad , \tag{10}$$

where the latter part accounts for the proportionality of Eq. 6 and Eq. 7 taken per molecule of mRNA. Provided the emerging mRNA is translated and the formed pre-enzyme activated, Eq. 10 defines the presence of the enzyme molecule. Furthermore, a non-vanishing probability that a product emerges from the enzymatic conversion of the single substrate molecule also defines the presence of the enzyme. The latter is complementary to the probability that the substrate remains available for binding to an enzymatic or regulatory site, Eq. 3. Taken per metabolite per enzyme, that complementary probability is

$$P_{\,CPe,\Delta t} = \frac{1}{m_e}\frac{1}{n_e}(1 -\ exp(-A\ n_e)) \quad . \tag{11}$$

Given the specified prerequisites, Eq. 10 and Eq. 11 are proportional,

$$\frac{1}{m_e\ n_e}(1 - exp(-A\ n_e)) \propto \frac{K_f\ m_e}{R_f}\ exp(-A\ n_e) \quad : \tag{12}$$

The growing probability of void events in the time interval (inducers lost, left side) is proportional (cf. Eq. 7) to the vanishing probability of transcription of the gene (right side). Eq. 12 is subsequently rearranged with the help of Eq. 5 and a proportionality constant $K \propto K_f$ introduced to get the amount of transcript synthesized at steady state, $R_{f,\Delta t}$. A quotient of a probability measure of the transcription event divided by a probability measure that the inducer is converted is thus obtained, each enzymatic conversion providing an opportunity for induction - gene activation as well:

$$R_{f,\Delta t} = \frac{K\ {M_0}^2\ {n_e}^3\ exp(-A\ n_e)}{(1 - exp(-A\ n_e))} \tag{13}$$

When further applying Eq. 13 to the suppression of genes by the formation of co-suppressors, the void event is equivalent of the formation of a co-suppressor and equivalently as applied to transcription-inhibiting factors and complexes. The types of metabolic events which are proportional, satisfying the equation with the specified prerequisites therefore include the following:

1. An inducer or a transcription-activating factor (complex) which is inactivated or broken down by the activity its own metabolic pathway activates the transcription of this same pathway or enzyme ($R_f = R_e$).

2. An inducer or transcription-activating factor (complex) which is inactivated or broken down by the activity of its own metabolic pathway activates the transcription of another pathway or enzyme.

3. The absence of a co-repressor or transcription-inhibiting factor (complex) which is generated from a gene-activating form by the activity of its own metabolic pathway activates the transcription of this same pathway or enzyme.

4. The absence of a co-repressor or transcription-inhibiting factor (complex) which is generated from a gene-activating form by the activity of its own metabolic pathway activates the transcription of another pathway or enzyme.

All these cases share the feature that the transcription-inhibiting principle emerges after the enzymatic conversion. The reverse situation that the enzymatic conversion results in a transcription-activating principle which requires some decreasing probability of an ensuing gene repression invites a separate consideration that is avoided here. By inference, Eq. 13 also applies to the case that

5. An enzymatic activity ascribed to the compound being inactivated by its own pathway stimulates the transcription of a gene.

Multiplying Eq. 13 with the factor $exp(A\ n_e)$ yields the form

$$R_{f,\Delta t} = K\frac{{M_0}^2\ {n_e}^3}{exp(A\ n_e) - 1} \quad , \tag{14}$$

which is recognized from Bose statistics.

Eq. 14 gives a measure of the number of transcripts synthesized at steady state, $M = 1$, as a function of the amount of enzyme, the amount of inducers or transcription-activating factors prior to the enzymatic conversion catalyzed by the enzyme "e" (Eq. 5) (equivalently, transcription-activating complexes), enzyme regulation, co-substrate levels, and small fluctuations of the level of transcription-activating principle out of steady state. The equation also applies to co-suppressors or transcription-inhibiting factors (complexes) formed from gene-activating principles by the action of the enzyme. The cell cycle may be regarded as divided into consecutive sub-segments of its phases characterized by their respective gene products [1, 7, 8] whereby the commitment to cell division is an all-or-none event [9] which is well accounted for by probabilistic models [10, 11]. The moving metabolic focus corresponds to the interpretation of Eq. 12 and Eq. 14 as describing the activation of consecutive genes characterized by transcripts $R_b,\ R_c, \ldots\ R_f, \ldots$ induced or activated by enzymes (pathways, inducers, transcription-activating factors or complexes, absence of co-suppressors, absence of transcription-inhibiting factors or complexes) $a,\ b, \ldots,\ e, \ldots$,

$$R_{f,\Delta t} = K_e\frac{{M^2}_{0,e}\ {n_e}^3}{exp(A_e\ n_e) - 1} \tag{15}$$

where $K_e,\ M_{0,e},\ n_e$, and A_e are constants and variables characteristic of the enzyme-pathway "e" and the subscript indicates the (alphabetic or numerical) order in which the genes are activated. The synthesis of each enzyme-pathway per interval of time, Δt, is proportional to the anabolic capacity, α, a function of the amounts and chemical activities of ribosomes, tRNAs, amino acids, etc., and proportional to the fraction of the pathway's own transcripts relative to all the various types of transcript competing for the ribosomal sites,

$$(n_e)_{\Delta t} = \alpha_{\Delta t}\Big(\frac{R_e}{\Sigma R}\Big)_{\Delta t} \tag{16}$$

whereby the transcripts in the numerator and the denominator are to be understood as sums of contributions from the preceding and actual intervals of time while also correcting for the transcripts' time-dependent, often rapid, breakdown or inactivation. The total amount of enzyme-pathway present is likewise a sum of contributions from consecutive intervals of time,

$$n_{e,t} = n_{e,\Delta t(n)} + \sum_{t=0}^{n-1} (n_e)_{\Delta t(t)} \quad , \tag{17}$$

with corrections for ongoing breakdown and regulatory inactivation of the enzyme. The decomposition of the transcripts and the enzymes-pathways ascribed to thermal breakdown and the action of nucleotidases, restriction enzymes, proteolytic enzymes, regulatory inactivation, etc., is described by respectively

$$R_{e,\Delta t(n)} = D_{R(n)}\ R_{e,\Delta t(n-1)} \tag{18}$$

and

$$n_{e,\Delta t(n)} = D_{e(n)}\ n_{e,\Delta t(n-1)} \tag{19}$$

where the factors $D_{R(n)} < 0$ and $D_{e(n)} < 0$ may vary depending on the sub-segment of time, $\Delta t(n)$, and the expression of hydrolytic and regulatory enzymes.

IV IMPLEMENTATION OF THEORY

When applying Eq. 1 - 19 to the cell proliferation, the genes are assumed to be regulated conventionally by metabolites acting as inducers, co-suppressors, or parts of regulatory transcription -activating or transcription-inhibiting complexes in consecutive intervals of time of the cell cycle phases. The gene-regulatory factors are assumed to have access to the genome, which may or may not require a permeability increase or transport across the nuclear pores.

Five consecutively transcribed genes ($OP_1 - OP_5$) were arranged in the computer program according to Eq. 15 - 19, which required approximately 12 kbytes including graphic display. Arbitrary numerical values of all the input variables were evaluated until consecutive activation of the genes characteristic of the phases of the cell cycle was observed, as is demanded by experimental data [8, 9, 12]. The contribution ${M_0}^2$ in Eq. 15, being less than $exp(M^{-1})$, M_0 was treated as a constant, neglecting that it may be a growing function of M. Since all the equation variables are monotonic functions of their respective measured biochemical quantity the effects of the latter could be unequivocally assessed by altering the numerical values of the variables according to their definitions. As a first approximation, all the equation variables were treated as independent. The sequence of computation of the biochemical reactions corresponds to the sub-cellular location of the metabolic events. For example, the order "protein synthesis - protein breakdown -unspecific mRNA breakdown - mRNA synthesis" corresponds to sequential biochemical reactions in the ribosomes (endoplasmic reticulum), the cytosol, and the nucleus. An example of graphs of gene expression obtained by this method is shown in Fig. 1. The cell cycle appears as a series of peaks of transcript followed by peaks representing the enzymes coded for and the gene expression can be studied as a function of the equation variables whereby the peak intervals indicate the rapidity of cell cycling and the phase lengths.

In order to simulate consecutive cell cycles based on Eq. 15 - 19, the assumptions were made that the peak transcripts are broken down on completion of mitosis, or else, that they have a short half-life of less than 1 % of the duration of the cell cycle, and that primarily the gene products and their associated pathways remaining in the cytosol in the post-mitosis phase, cf. [13] determine what will happen during the next cell cycle. The latter were therefore preserved upon completion of the cell cycle at the time of peak expression of OP_5, followed by entry into the next G_1 phase. Using this method, the progressive

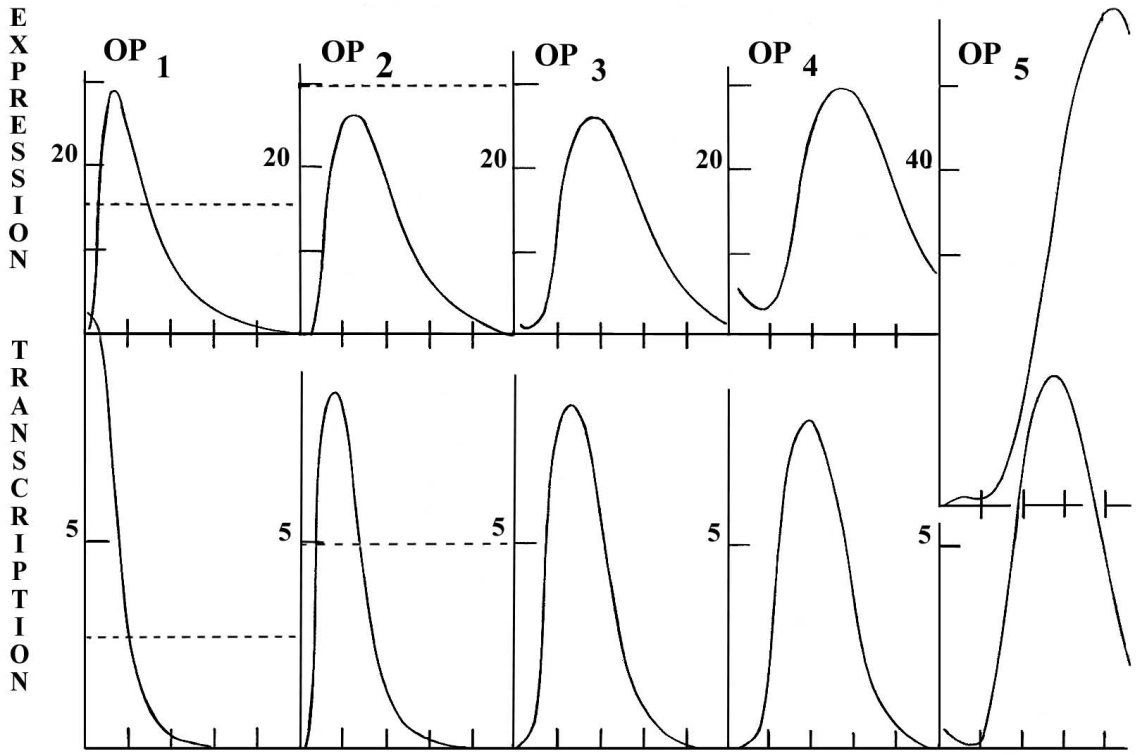

Fig. 1: The transcription and expression of five genes, $OP_1 - OP_5$, obeying the kinetics described by Eq. 15 - Eq. 19. The total amounts of transcript coding for a pathway are shown in the lower half of the drawing and the total amounts of corresponding enzyme in the upper half, each as a function of time starting at $t = 0$ in each of the ten sections of the drawing. A proliferating cell is represented by solid lines and a quiescent one by dashed lines. The graphs were computed based on a constant and uniform rate of decomposition of both transcript and enzymes from all operons by general hydrolytic pathways (Eq. 18 and Eq. 19), but these standard conditions were not mandatory for obtaining similar graphs. The graphs were reproduced in consecutive cell cycles - G_0 phases and reversibly appeared when switching from one of these modes of gene expression to another. The third operon, OP_3, plays the role of a trigger gene for commitment to division, which is activated by the preceding, second pathway represented by the enzyme e_2 and the transcript R_2., The first two operons played the dual roles of representing the G_1 as well as the G_0 phase. In the quiescent cell, the first gene, OP_1, was induced by the enzyme e_2 when the latter pathway became over-expressed above a numerical threshold value.

cell cycles were evident as five consecutive peaks of transcript with slightly lagging enzyme peaks, characterized by constant phase and amplitude, and phase locking within a few cell cycles (Fig. 1). The S phase was regarded as an interruption of the G_1 and G_2 phases and neglected. Like many of the cyclic gene activations characteristic of proliferating cells [7, 12, 14, 15, 16] DNA replication itself may be regarded as a cyclin-cdk -induced event [17]. Any observed peak of expression might then be chosen to represent the S phase. Accordingly, the peak intervals generated by the present gene kinetics may tentatively be identified with the cell cycle phases such that $OP_1 - OP_2$ corresponds to early G_{1a}, $OP_2 - OP_3$ to early G_{1b}, $OP_3 - OP_4$ to late G_1, and $OP_4 - OP_5$ to $G_2 - M$.

Besides studying the gene kinetics the proliferating cell it is also of interest to simulate commitment to division and the cessation of proliferation (contact inhibition - quiescence). When down-regulating the inducing metabolite of OP_3 by increasing the numerical value of A_2 (cf. Eq. 3, Eq. 4), prominent over-expression of the OP_2 gene was observed since the increasingly silent $OP_3 - OP_5$ genes did not efficiently compete for the anabolic pathways available. This feature was employed for defining contact inhibition -quiescence in the following way: The presence of G_0 genes was established by introducing a threshold on the e_2 enzyme product above which induction of OP_1 by e_2 and its substrate became effective. Thereby, the OP_1 and OP_2 genes were assigned the dual roles of representing the G_0 as well as the G_1 phases while the OP_3 gene was assigned the key role of regulating commitment to division. The induction of additional genes (above the threshold) in the presence of excess inducing pathway may be understood as the presence of transcription -activating factors shared by many genes, which are made available, activated or induced as a result of an obstructed induction, diminished expression, and/or activity of the gene product of the trigger gene for commitment following metabolic regulation of its inducing pathway(s). Genes playing key roles in the regulation of cell proliferation which are known to be involved in such schemes include Rb - p53 [19] - [22], cyclin D_1- E2F [23] - [27] and MAPK-Raf-Ras [28] - [33]. Using the method of computer simulation described, contact-inhibited cells appeared as a constant expression of differentiated functions ($OP_1 - OP_2$) spanning unlimited time and commitment-quiescence appeared as reversible switches between the constant expression and the five peaks characteristic of the cell cycle.

V MOLECULAR MECHANISMS OF COMMITMENT AND QUIESCENCE

A threshold for the commitment to division was imposed on the third operon, OP_3, by increasing the numerical value of the effective decomposition of its inducer/gene-activating principle, A_2, sufficiently to prevent its transcription and expression. This made the subsequent operons, $OP_4 - OP_5$, silent also, i.e. their transcription and expression were not detectable in comparison with the operons transcribed before the trigger event linked to OP_3. Such a setting of the equation variables corresponds to the well-established all-or-none character of the commitment to division and the ensuing gene expressions in un-transformed (non-neoplastic) cells. The magnitude of alteration of the numerical value of A_2 required for observing this characteristic feature was approximately 2.5 times its basal value associated with cell proliferation. The A_2 threshold further depended on the amount of constitutive

mRNAs competing with the transcripts from the five operons during translation (cf. Eq. 16), or competing for rate-limiting factors needed for mRNA synthesis.

Increasing numerical values of A_2 may be interpreted as stimulatory enzyme regulation on the enzymes converting the inducing metabolite, alternatively more supply of rate-limiting co-substrates for these enzymes. Such factors lead to down-regulation of the concentration of inducing metabolite, and are kinetically equivalent of levels of inducer below steady state, $M_2 < 1.0$ (cf. Eq. 4). The various possible contributions to the composite factor A in its role of expressing the effective concentration of inducing metabolite are summarized in Table I.

A. Rate Limitation by Enzyme Regulation	
Regulatory stimulation (inhibition) of the pathway	Effect on [i] , A_i
1	$\uparrow$ ($\downarrow$) $\downarrow$ ($\uparrow$)
2	$\uparrow$ ($\downarrow$) $\downarrow$ ($\uparrow$)
3	$\downarrow$ ($\uparrow$) $\uparrow$ ($\downarrow$)
4	$\downarrow$ ($\uparrow$) $\uparrow$ ($\downarrow$)
5	$\downarrow$ ($\uparrow$) $\uparrow$ ($\downarrow$)
B. Rate Limitation by Co-substrate Availability	
More (less) co-substrates for the pathway	Effect on [i] , A_i
1	$\uparrow$ ($\downarrow$) $\downarrow$ ($\uparrow$)
2	$\uparrow$ ($\downarrow$) $\downarrow$ ($\uparrow$)
3	$\downarrow$ ($\uparrow$) $\uparrow$ ($\downarrow$)
4	$\downarrow$ ($\uparrow$) $\uparrow$ ($\downarrow$)
5	$\downarrow$ ($\uparrow$) $\uparrow$ ($\downarrow$)

Table I. Anticipated direction of change of the chemical activity of gene-activating principle, [i], and of the numerical value of the exponential factor A_i by plain mass action.

A cell arrested in the G_0 phase by thus increasing the numerical value of A_2 was employed for investigating the commitment to division, yielding the following results. Commitment to division comprising expression of all the genes specifically involved in proliferation, $OP_3 - OP_5$, was observed when the effective decomposition of the inducing metabolite/gene-activating principle was again suppressed. The biochemical events which may lead to commitment by this mechanism are (cf. Table I)

a) stimulatory regulation of the enzymes providing the inducer/gene-activating principle,

b) inhibitory regulation of the enzymes converting the inducer/gene-activating principle, alternatively

c) a reversal of the biochemical reaction converting the inducer/gene-activating principle either by a shift of equilibrium or with the help of another enzyme catalyzing the backward reaction,

d) levels of inducer/gene-activating principle above steady state,

e) more supply of co-substrates for the enzyme(s) providing the inducer/gene-activating principle if they are rate-limiting, and

f) a drain of co-substrate for the enzyme converting the inducer/gene-activating principle to rate-limiting levels.

When trying to identify biochemical pathways in the machinery of cell proliferation, which may effectuate any of these molecular mechanisms, caution should be paid to that commitment to division from a stationary state may not involve the same trigger mechanisms as the commitment during ongoing cycles of division [34] - [37]. Additional control mechanisms for cellular division may gradually have been overlaid on more fundamental ones during evolution [38, 39]. Accordingly, a higher complexity of regulatory pathways has been found in eucharyotic and mammalian cells than in procharyotic cells. Therefore, the deranged control of cellular proliferation characteristic of tumor cells may render them poor examples of the advanced biochemical mechanisms fine-tuning the progress of the "normal" cell cycle [40, 41]. For example, the oscillatory levels of pathways typical of proliferating normal cells are not always found in tumor cells [42].

The chemical activity of an inducer/gene-activating principle converted by its own pathway, that oscillates in the cell cycle may increase following up-regulation of a primary regulatory pathway. This makes the commitment following metabolic regulation by tyrosine phosphorylation a possible candidate in any of the mechanisms a - f above. Tyrosine phosphorylation is amply involved in the commitment to division of quiescent mammalian cells [46]. Growth regulation by tyrosine kinases is known to involve the mitogen-activated protein kinase (MAPK or ERK) pathway [28, 29, 44, 45]) whereby an exogenous signal to initiate cell proliferation is transmitted via a receptor complex with G-proteins to activate by phosphorylation a MAPK kinase (MAPKK or MEK) which activates the ribosomal protein S6 kinase (pp90rsk) and/or the MAPK leading to

1) activation of transcription-activating factors involved in regulating proliferation such as AP-1, cf. [46]; cjun, cfos, cmyc, cmyb (reviewed in [28, 44]) and

2) post-transcriptional regulation of intermediates in the signaling pathways by phosphorylation, for example fine-tuning of the ubiquitination of the transcription-activating factors by way of phosphorylation, [30].

The kinases which phosphorylate and activate MAPKK-MEK include Raf, MEK kinases and Mos [28] of which the tyrosine kinase-regulated Raf together with the helper protein Ras [33, 47]) constitute a principal path in the signaling pathway downstream of growth factor stimulation of mammalian cells. These complex regulations and phosphorylation cascades importantly converge on the transcription factors regulating the early G_1 phase cyclin D_1 gene [48], whose product in downstream pathways enables a cyclin D_1 -dependent protein kinase, a major function of which is to phosphorylate the Rb protein such as to remove it in its properties of 1) a co-repressor of genes controlled by the E2F family of transcription-activating factors [49, 50] and 2) a linker protein to histone deacetylase [51]. These regulatory features converging on a downstream transcription-activator -controlled (stimulated or inhibited) cyclin D_1 gene which have been identified empirically accommodate general biochemical mechanisms operating according to pattern a - f above, most characteristically by way of activating tyrosine phosphorylations [43, 52] or inactivating tyrosine de-phosphorylations [53], PKA phosphorylations [28, 33], other regulatory phosphorylations [54, 55, 56], and redistributions of cdk -inhibitors[57, 58]. An alternative pathway for commitment, of great potential clinical importance involves hydrogen superoxide acting as a second messenger [59] - [65].

Since the cyclins themselves belong to those proteins which oscillate during the cell

cycle, like illustrated in Fig. 1, and play a most important role in the regulation of cell proliferation it is essential to examine if their biochemistry is compatible with the postulates in Section III. In one plausible molecular scheme involving the cyclins, Eq. 15 - 19 are applied to the enzymatically active cyclin-cdk complexes neglecting excess cdks and the fine-tuning of the latter's activities by phosphorylations-dephosphorylations and cdk-inhibitors, and assuming that the cyclins are saturated with the kinases. Conforming with the general case described by Eq. 12 eq. 15, the phosphorylation of the cyclin (an auto-phosphorylation conferring a bias for ubiquitination and breakdown, ref. [15], cf. also [66] - [70]) is counted per metabolite (cyclin molecule) per active enzyme (cyclin-bound cdk), the probability of which conversion to an inactive form increases asymptotically as a function of the amount of cyclin-cdk -enzyme (left side of Eq. 12). Because of the enzymatic conversion, the probability of the transcription which is dependent on the active cyclin-cdk decreases (due to an autophosphorylation enhancing the tendency for ubiquitin-dependent proteolysis, right side of Eq. 12). The cyclin-cdk may activate the transcription enzymatically as well a by its presence, available for binding to regulatory elements in the gene. If the cyclin is induced by a primary regulatory pathway, the function graph of Eq. 13 (which has a maximum) may gradually be passed from left to right which, in the first place, ensures an increased activation of a cyclin-activated pathway, enzyme, or transcription-activating factor until its maximum level is reached. When further

a) the induction of the cyclin ceases or is inhibited,

b) there is regulatory inactivation of the cyclin-cdk constituting the gene-activating principle,

c) the active cyclin-cdk is sequestered (titrated) by another protein,

d) the cyclin is induced beyond the optimal level of induction indicated by the maximum function value of the transcription it activates, and

e) when other pathways begin to compete efficiently for rate-limiting factors being used in transcription and translation,

the activation of the cyclin-promoted pathway and its transcription are diminished. Hence, cyclins obeying this kinetics may potentially both activate and inhibit ("d" above) an ensuing transcription which depends on either cyclin-dependent phosphokinase activity or the presence of the cyclin (-cdk) as such, available for binding to a promoter region or available for sequestering (titrating) another promoter-binding factor.

Furthermore, when there is an additional distinct substrate-protein for the cyclin-cdk which is slaved to the same kinetics of turnover and which functions as a gene-activating principle, Eq. 13 is valid with corrected proportionality factors and the observed kinetics may also be applicable. This is particularly evident in the cases that the two proteins (the cyclin-cdk and the kinetically slaved protein) are products deriving from the same operon or their amounts are otherwise proportional, for instance, if they are induced by the same master gene or if the cyclin-cdk by binding to another protein marks the formed complex to be a new substrate for the same cyclin-cdk.

In yeast, the G_1 and G_2 cyclins in conjunction with their cdks are capable of self-induction [70] - [74] and self-phosphorylation [15], [66] - [70], ultimately leading to their transient appearance and breakdown by ubiquitination -dependent proteolysis, which are features straightforwardly compatible with the present gene kinetics. Besides self-induction of cyclins, which may not be physiologically important [74], coordinated simultaneous, cf.

[75, 76] and orderly induction of sequentially expressed cyclins [77, 78, 74], cf. [79] also constitute tentative molecular vehicles for the present gene kinetics.

Two features of the cyclin gene metabolism, which can not be overlooked, are the presence of multiple activator and repressor sites in the promoter regions of the cyclin genes [80] - [84] and the involvement of the cyclins with the E2F transcription factors [16, 80], [85] - [87]. In binding to the E2Fs and recruiting them and their complexed proteins in autophosphorylation schemes leading to a removal of the gene-activating principle [16] the cyclins may slave the E2F-dependent pathways and the E2Fs themselves to similar kinetics of expression. In any such gene kinetics the substrate/gene-activating principle (the E2F-cdk-cyclin complex, possibly bound to DNA) may be regarded as distinct from the converting pathway (the cyclin-cdk) to the effect that the variables A (Eq. 4) and n_e (Eq. 15 eq. 18) may be regarded as independent. Such molecular schemes, where the phosphorylation involves a DNA-binding transcription regulator, possibly bound to a cyclin-cdk have the potential of constituting a general mechanism in gene activation, cf. [88] - [91]. Furthermore, the E2Fs may be employed by the early G_1 cyclins for activation of the late G_1 cyclins [76, 80], which contain E2F-binding sites in their promoter regions, thereby slaving ensuing gene expressions to the oscillatory behavior characteristic of the early G_1 cyclins.

The cyclin oscillations characteristic of the cell cycle phases are not expected to be independent of peripherals in the general cellular metabolism. A biochemical process that involves an increased metabolic flux with higher concentrations of intermediates must necessarily increase the chemical activity of inducers. On the other hand, since there is an enhanced flux of reducing equivalents in phosphotyrosine relative to tyrosine -residues, cf. [93], the flux *per se* may play an important role in addition to the regulatory phosphorylations. For example, the commitment to division that is correlated with increased metabolic fluxes [1, 2, 92], may in a more general sense be regarded as an enhanced turnover of the cycling proteins following an enhanced effective decomposition of inducers, even though the primary cause is to increase the effective concentration of gene-activating principle. This can be accounted for by the factor A of Eq. 4. Increased metabolic fluxes first become manifest in the form of an enhanced biological transport, which is one of the earliest signs of a challenge by mitogenic agents [1, 2, 8]. The commitment following bursts of biological transport may be regarded as an out-of-steady state phenomenon whether or not followed by an enhanced protein synthesis and irrespective of type of molecular effector or gene target. In the present simulations, substrate levels above steady state, acting as an inducer for the trigger gene for commitment ($M_2 > 1$) or as inducers for all five operons ($M_n > 1,\ 0 < n < 6$) efficiently took the cell out of the G_0 phase, providing an argument in favor of the hypothesis that plasma membrane derangement accompanied by an uninhibited transport of nutrients into cells residing in the rich internal environment of an organism are an important factor causing the unregulated proliferation of neoplastic cells [2]. Apparently, a stochastic channeling of the excess transported nutrients into all the available pathways is sufficient for commitment in the present case. If, however, only the inducing pathways prior to commitment including the inducer of OP_3 were fed substrate above steady state ($M_2 > 1,\ M_1 > 1,\ M_5 = M_0 > 1$) and the subsequent ones fed normal amounts, the cell did not resume proliferation. This is equivalent of the induction of differentiated functions and pathways of G_0-product synthesis out-competing the cell cycle genes and their auxiliary pathways for the available anabolic capacity and for available common gene-activating factors. Thereby, the empirical evidence

for metabolic channeling between specialized function-differentiation and proliferation being involved in commitment [1] - [4] is being accounted for as an inherent feature of the present gene kinetics. The gross cell-biological evidence for such a metabolic switch is being consolidated by the identification of its molecular effectors in the early G_1 cyclins [94] - [101] and their accessory pathways such as the cdk inhibitors including p53 [102] - [106], Ras [107], and jun [108, 109] and in the cyclic AMP -responsive elements [110]. The overwhelming evidence in favor of a metabolic switch between differentiative and proliferative pathways being functionally linked to the early G_1 cyclins does not preclude the possibility that separate gene activations may be involved in these responses, cf. [46, 102, 111]. The numerous mechanisms that are available to the cell for conveying the metabolic flux into either differentiated function or proliferation suggest that Nature relies on statistical factors rather than a single master key switch. The expression of only a few differentiated functions may thus not be sufficient for counter-balancing a bias for proliferative pathways.

If the operons preceding the trigger gene for commitment of a quiescent cell halted by channeling the metabolic flux into these were deprived of the excess inducer, the cell again resorted to proliferation. An example of this outcome of theory may be the reverse transformation of neoplastic cells induced by cyclic AMP and its derivatives, that is cancelled as soon as the drug is withdrawn (reviewed in [1]). A cell which had been pulled into quiescence by levels of inducer of the G_0 genes above steady state might not again transcribe beyond the operon preceding the trigger operon for commitment unless several of its G_0 genes were again short of inducer. This theoretical result harmonizes with experimental studies in which DNA synthesis was induced in permanently stationary cells, such as nerve cells and striated muscle cells, by exhaustion of metabolism following sustained depolarization [112], adding to the important role of the sodium-potassium balance in explaining these results [112].

Increasing the anabolic capacity, α, did not lead to commitment but instead sustained and enhanced the gene expression prior to the trigger operon of a quiescent cell. This is in accordance with empirical observations that stimulation of protein synthesis *per se* not is a causative event in commitment, although it usually accompanies G_1 entry [8, 113] and may play a permissive role [99, 100]. Apparently, stimulation of protein synthesis is due to pathways which may be parallel with, and not the consequences of, cyclin-cdk activation [113]. In the present simulations, a lower anabolic capacity was associated with a lower threshold for commitment in terms of amount of inducing pathway. These results conform with experimental findings that G_1 cyclin abundance, which regulates and is rate-limiting for the G_1 entry in yeast cells, is positively correlated with the rate of protein synthesis [73, 100]. In the present simulations the expression of the pre-trigger operon of a quiescent cell was typically more than 125 % of that characteristic of maximal induction of the trigger operon whereas in a cycling cell, the expression of the first four operons was typically 55 % of that required for maximal induction of the ensuing operon.

In mammalian cells, cyclin D_1 is the cyclin that most often has been implicated in the commitment to division although it may not play a mandatory role [114] - [117]. Also cyclin E, cyclin E_T and cyclin D_3 may replace cyclin D_1 as a marker for the early G_1 phase transition point of mammalian cells [114] - [117], indicating a certain extent of redundancy among the various early G_1 cyclins of mammalian cells as is the case in yeast [73, 74]. It has been evident for some time though, that the physiological trigger gene for commitment

of mammalian cells should be sought in the context of the cyclin D_1 gene.

VI THE CELL AND MOLECULAR BIOLOGY OF CYCLING CELLS

When gradually down-regulating the inducer/gene-activating principle of the trigger operon for commitment by increasing its effective decomposition, the peak intervals between the pre-trigger operon gene products were lengthened and the transcript amplitudes of those following the inducing pathway of the trigger operon were diminished before they finally disappeared. These results are equivalent of a slow-down of the cell cycle, notably the G_1 phase, as the cells approach quiescence-confluency, in agreement with ample cell culturing data. Specific examples of G_1 phase prolongation during down-regulation of an inducer/transcription-activating factor include the cdk-associated phosphatase KAP which down-regulates cyclin-cdk2 activity in Hela cells [118, 119], cf. [120]. Also the diversification of the metabolic fates of two daughter cells is known to involve phosphorylation schemes, potentially resulting in two distinct species of one G_0 cell expressing differentiated functions and one cycling cell [111]. The observed regulation of the lengths of the peak intervals in the G_1 phase by the effective decomposition of the inducing metabolite of a trigger operon thus also provides a tentative explanation of the experimentally observed different cell cycling times of second generation cells.

Longer half-lives of all the transcripts, equivalent of increasing the numerical values of the factors D_R (Eq. 18 and Eq. 19) resulted in higher amounts of mRNA and enzymes and longer cell cycling times. The effects on the enzyme levels by unspecific mRNAse tended to be proportional to the amount of mRNA relative to basal values. Altering the rate of unspecific decomposition of the enzymes of the inducing pathways by decreasing or increasing the numerical values of the factors D_e (Eq. 19) gave pronounced effects on the levels of mRNA. These simulations were based on the principle of maintained steady state, namely, Eq. 5 is valid and the inducers/gene-activating principles are proportional to their inactivating pathways, conditions that may be applicable to the cyclin-cdks. In the absence of constitutive mRNA competing with the cycling genes (accommodated in the denominator of Eq. 16) a change of the rate of breakdown of protein of merely 1.1 times the basal rate associated with the consecutive gene expression altered the levels of mRNA by a similar magnitude as a 10-fold change of the rate of decomposition of transcripts. In the presence of constitutive mRNA, an increase of the half-lives of all enzymes of merely 1-2 % of the basal value caused a cell cycling prolongation of approximately 20-30 % and increased protein levels by 20-30 %. These proportions were based on numerical half-lives of the enzymes (in the absence of re-synthesis) of approximately 1/20 of the full cell cycling time. The assigned half-lives of transcripts were less than 1 % of the cell cycling time. The numerical values chosen were not critical and could be changed with qualitatively similar results. They correspond to the physiological condition that the enzymes in most cases are more stable than the active transcripts.

The rapidity of cell cycling changed conspicuously upon altering the rates of decomposition of transcript and protein. A generally increased breakdown of transcripts or protein led to shorter peak intervals and acceleration of the cell proliferation, conforming with empirical observations that rapidly proliferating tumor cells often are characterized by enhanced

intra- or extra-cellular hydrolase activities. Various operon- and transcript-specific RNAses (restriction enzymes) and inactivating proteases acting on the individual pathways were also monitored. Besides causing the anticipated effects on the levels of transcript and enzyme (higher or lower product levels due to slower or more rapid breakdown, respectively), these also altered the lengths of the various peak intervals, corresponding to the lengths of the phases of the cell cycle. For example, it was often observed that a more rapid breakdown of the enzyme in addition to decreasing its amount also shortened a preceding peak interval. An increased stability of the mRNA R_3 of the trigger operon lengthened the peak interval following the peak of expression of the trigger gene (late G_1). An increased stability of the proteins of the inducing pathway (e_2) of the trigger gene lengthened the whole cell cycle and notably the entire G_1 phase. An altered stability of R_3 could unambiguously be pinpointed in the pattern of gene expression of OP_3 while an increased stability of e_2 also tended to increase or decrease the levels of expression of other genes than OP_2 and OP_3. The lengthening of the peak interval following the expression of a gene whose product and/or transcript are stabilized and the lengthening of the peak intervals preceding the peak of expression of a gene whose product is stabilized may be regarded as a checkpoint mechanism inherent in this gene kinetics. This is consistent with experimental observations that checkpoint mechanisms involve the stabilization of molecular effectors [13], [121] - [124] and that stabilization of a gene product may lengthen the preceding cell cycle phase [124] - [126].

By altering the numerical values of the pre- or post-ribosomal breakdown of the transcripts the specific fates of the various mRNAs and their effects on the cell cycle kinetics can be monitored, which might be anticipated to contribute to the understanding of the control of cell proliferation, cf. [111, 127, 128]. Pre-ribosomal restriction enzymes inactivating exclusively the transcripts derived from the trigger operon increased the peak intervals preceding the trigger operon and decreased those following. Thus, in agreement with other findings, cf. [73], an inverse relationship could be observed here between the lengths of the G_1 and G_2 phases after some perturbations of the gene metabolism.

The anabolic capacity, α of Eq. 16, is shared by all operons and it may not be necessary to sub-divide the term according to the type of transcript that is being translated. Irrespective of the sequence of decomposition of the macromolecules on this or that side of the nuclear membranes, increasing the anabolic capacity promoted the synthesis of both transcript and protein. The levels of the various transcripts increased asymptotically while the levels of the various pathways increased proportionally to the anabolic capacity. This was also correlated with a lengthening of the peak intervals and a prolongation of the duration of the cell cycle. Typically, a two-fold increase of the numerical value of the anabolic capacity prolonged the peak intervals by 0-25 % depending on the sequence of decomposition of the macromolecules and on whether or not the transcripts were re-utilized after once having been translated. This effect on the rate of cell proliferation by an enhanced protein synthesis is opposite to that of a generally increased breakdown of protein or transcript, which shortens the cell cycle.

Gene amplifications were also amenable for analysis using the present gene kinetics. It is known that amplification of the genes coding for cyclin D_1 and cyclin E leads to G_1 phase shortening [35, 83, 129], cf. [36, 48, 94, 95] and that the combined effects of these cyclins on the G_1 phase length may be additive. This was simulated by multiplying Eq. 15 applied to OP_3 by an integer. A cell that had been taken out of A_2-regulated quiescence by gene

amplification of the trigger operon for commitment displayed G_1 phase lengthening when the gene copies were again gradually removed before its contact inhibition-quiescence was restored. These simulations yielded a G_1 phase shortening of 10-30 % when mRNA synthesis ascribed to gene amplification was increased 4-fold, the magnitude of which agrees with an experimental value of 50 % obtained for cyclin D_1 [35]. Furthermore, the amplification of two consecutively expressed genes, OP_3 and OP_4, tentatively identified as those coding for cyclin D_1 and cyclin B respectively, resulted in additive shortening of the early G_1 phase. The lengthening of the peak intervals noteworthily occurred, not prior to the amplified gene, but one gene earlier such that OP_3 shortened the $OP_1 - OP_2$ interval and OP_4 shortened $OP_2 - OP_3$ interval. These data and the results, cf. [35, 83, 130] that differentiation taking place in the G_0 phase, approach to confluence, and G_1 phase lengthening due to a diminished expression of a trigger gene for commitment are positively correlated suggest that the trigger gene of cycling cells may be tentatively identified as that, or one close to that coding for cyclin D_1. Anyhow, the agreement between theory and experiment is striking. The results suggest that the OP_3, OP_4 and OP_5 genes may be tentatively identified with those coding for respectively cyclin D_1, cyclin E (alternatively cyclin A) and cyclin B whereby intermediaries in the chain of gene activations such as the E2Fs (possibly demanding additional pattern operons for computations) are neglected and the roles of the E2F sites in the promoters of the cyclin genes remain to be accounted for and properly assigned.

The use of the present kinetics with amplified trigger genes also reproduced a constitutive expression in quiescent cells of genes involved in proliferation ($OP_3 - OP_5$), cf. [131], and an increased susceptibility of such cells to transformation (cf. [18]), here equivalent of commitment in consecutive cell cycles) following the concerted action of other factors including gene amplification of other cell cycling genes, cf. [132]. In these simulations, a) a lower trigger level for contact inhibition (the e_2 threshold for cross-induction by e_2 on OP_1) was made inefficient by amplifying the trigger gene for commitment or up-regulating its inducing pathway, leading to ceaseless cell proliferation and b) a quiescent cell which had an amplified OP_3 gene synthesized detectable amounts of OP_4 and OP_5 products in the G_0 phase and underwent commitment following up-regulation of the pathway activating the OP_4 operon or amplification of OP_4.

VII DISCUSSION

The complexity of metabolic pathways and regulatory events in a living biological cell renders a quantitative description of its gene expression difficult. In the present approach the difficulties were partly circumvented by using a probabilistic treatment of the diffusing metabolites based on equating the probabilities of the enzymatic and genomic events and reducing the various metabolic regulations and mass action effects to a single variable, an effective decomposition of a gene-activating principle. Whether regarded as the presence of active inducer/transcription activator or absence of suppressor/transcription repressor, the inactivation of a gene-activating principle to forms which lack the gene expression -promoting faculty are the most commonly encountered, ever since the discovery of the operon concept by Jacob and Monod. The present results show that even the multi-factorial intracellular enzyme-genome kinetics may be analyzed and interpreted in terms of such a theoretical framework and validated by empirical observations. In the computations based on the theory, the cell cycle appeared as a series of peaks of transcript followed by the enzymes coded

for and the G_0 phase as a constant rate of production - differentiated function from genes activated prior to the trigger event in commitment to division. The contact inhibition of proliferation (quiescence) and the release from quiescence (commitment) were observed as reversible switches between these two modes of gene expression, which followed by altering the numerical values of the function variables in accordance with empirical observations. For example, the activation of a trigger operon for commitment could be interpreted quantitatively as metabolic regulation, excess inducer following a burst of biological transport, a shift of the metabolic focus from specialized functions to proliferative pathways, or gene amplification of the trigger operon.

When the trigger gene for commitment was tentatively identified as that, or one temporally close to that coding for cyclin D_1, the experimentally observed G_1 phase shortening following amplification of the cyclin D_1 gene could be simulated based on the theory. An additive effect in G_1 phase prolongation by two consecutively expressed amplified genes, tentatively identified as those coding for cyclin D_1 and cyclin E, as well as a G_1/G_2 phase prolongation caused by checkpoint stabilization of intermediaries, as has been observed experimentally, turned out to be inherent in the gene kinetics. The constitutive expression of cell cycling genes in the G_0 phase of cells having an amplified trigger gene and their increased susceptibility to commitment are also in accordance with laboratory data. The failure of commitment by merely increasing the anabolic capacity, the shorter cell cycling time in the presence of proteases, the slow-down of the cell cycle in cells approaching quiescence, and the all-or-none expression of the genes involved in proliferation of normal (= devoid of gene amplifications, cf. ref. 20) cells constitute additional examples of outcome of theory in accordance with empirical observations. The identification of these kinectic events as being relevant to the cell proliferation is corroborated by the finding that certain aspects of the cyclin and E2F metabolism would be accounted for by the same type of gene kinetics. In particular, cyclin-cdk -tagged gene regulatory proteins turned substrates for the cyclin-cdks constitute probable molecular vehicles for the present gene kinetics.

The kinetics is compatible with commitment being regulated by a variety of stochastic factors operating on a trigger gene and its inducing pathway(s).

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