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PROSPECTS SYMPOSIUM

Multiple Molecular Levels of Cell Cycle Regulation

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Abstract The objective of this brief review is to stress the importance of multiple levels of molecular regulation of complex processes such as cell growth and to illustrate their derangements as they occur in cancer cells. One major research emphasis today is the regulation of transcription by binding of transactivating proteins to promoter motifs. Another focus is on the multiple roles of protein phosphorylations in signal transduction pathways. Evidence is strong, however, that major controls exist at numerous other molecular levels as well (Fig. 1). These include pre-mRNA processing, pre-mRNA degradation, mRNA degradation, control of translation, permanent protein modifications, protein degradation, reversible covalent protein alterations, noncovalent interactions with small molecules and with other proteins, and effects of relocations into cell compartments. These controls are exhibited in all biological processes. A few illustrative examples are briefly discussed, which come mainly from our researches in the area of cell cycle regulation and its derangement in cancer. © 1994 Wiley-Liss, Inc.

Key words: regulation, cell cycle, cancer, transcription, translation, protein modification

Studies of the molecular basis of cell regulation originated in the mid-1950s. Before then, biochemistry was mainly dedicated to discovering metabolic pathways, a great achievement now summarized in the familiar metabolic maps depicting almost all reactions in living cells. But this provided a static picture and next needed determination of the density of travel along these metabolic roads and byways, and explanations of mechanisms by which this flow is regulated. The activities of enzymes catalyzing the reactions then were ascribed to substrate concentrations according to the Michaelis-Menten equation and a few naturally existing inhibitors and activators. Also, secreted proteases were known to be activated by cleavage of their inactive precursors.

The analysis of regulation of cellular processes has its current origins from three developments in the mid-1950s [Pardee, 1959]. These are gene induction and repression [Jacob and Monod, 1961], noncovalent feedback inhibition of enzymes, leading to allostery [Gerhart and Pardee, 1962], and covalent protein modification by phosphorylation [Krebs, et al., 1958]. Recognized at this time were regulatory roles of external factors and entry of molecules into cells by specific transport mechanisms. Many of these studies were done with the bacterium *Escherichia coli.*

The molecular basis of regulation thereafter became an active subject of investigation. Some major steps in the development of current ideas were discoveries of mRNA, systems for synthesis of RNA and protein in extracts, all of molecular biology, signal transduction pathways, recognition of instability of RNA and of proteins (turnover), and ability to culture mammalian cells.

TRANSCRIPTION

The amount of mRNA in a cell depends in part on the number of gene copies. Gene amplifications are often found in cancer cells. For example the cyclin E gene is amplified 8-fold in a breast cancer cell line, accounting for part of the 64-fold increase in the cyclin E mRNA and protein [Keyomarsi and Pardee, 1993].

Control of rates of gene transcription to produce heterogeneous nuclear RNAs (pre-mRNA) have today a major emphasis. Gene activation is by binding of proteins to DNA motifs, most of which are located in 5′ promoter regions. Run-on transcription and promoter-reporter constructs can determine these rates of transcription separately from the subsequent processes.

The original model of one repressor protein binding to regulate β-galactosidase transcription in *E. coli* has been superseded by much

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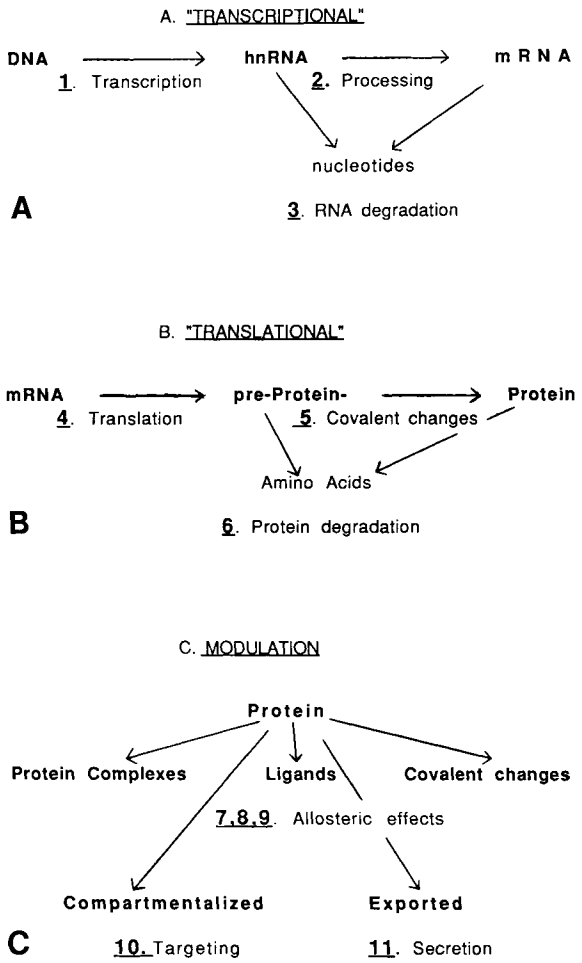

Fig. 1. Regulatory levels of gene expression. A: Transcription; B: translation; C: protein modulation.

more complex systems for eukaryotic cells. Multicomponent complexes determine cycle dependent gene expressions [Devoto et al., 1992]. And these gene activations depend upon phosphorylations of proteins [Pines and Hunter, 1991; Hollingsworth et al., 1993]. For example, the 40-fold increase of murine thymidine kinase (TK) and its mRNA, shortly before the start of S phase, depends upon the same extracellular conditions that initiate DNA synthesis. Transcription accounts for about 1/8 of this mRNA increase. It is activated by successive formations of multi-protein complexes that bind to three DNA consensus sequences at the 5′ end of the TK gene and which include E2F, cyclins E and A, cdk kinases, and retinoblastoma proteins. [Dou et al., 1992].

Other regulatory processes are at least as important quantitatively for TK production as is transcription (Fig. 1A). Processing of pre-mRNA to mRNA appears to account for much of the increase of TK mRNA. The events of 5′ capping, 3′ polyadenylation, splicing, and export from nucleus to cytoplasm all are potentially subject to regulation, but this level of regulation has been little explored.

For example, the pattern of intermediate sized TK pre-mRNAs changes dramatically as cells progress from the quiescent (G0) state to late G1 phase [Gudas, et al., 1988]. Neither highly spliced intermediates nor processed mRNA are initially found in the nucleus (or cytoplasm); they appear only in late G1. These pre-mRNAs are apparently rapidly degraded during most of G1. They appear, in the nucleus, near the restriction point in late G1, when TK mRNA appears in the cytoplasm.

Alternative splicing to produce different mRNAs is another mode of "transcriptional" control. There are many alternative patterns of splicing [McKeown, 1993]. The mechanism depends on SR proteins, a family of splicing factors [Zahler, et al., 1993]. Alternative splicing is observed during differentiation, as for aromatase which utilizes different exons 1 [Harada, 1992]. Some splicing patterns are changed in cancer cells [Rudy et al., 1993].

Degradation of mRNAs has long been known as a major factor determining mRNA concentrations. Thus, degradation of histone mRNAs is strongly activated when DNA synthesis stops [Stein et al., 1984]. In human breast cancer cells, histone H4 and cyclin mRNAs are strongly stabilized relative to the normal cells [Keyomarsi and Pardee, 1993].

TRANSLATION

Synthesis of proteins is subject to several major levels of regulation (Fig. 1B). Rates of translational controls depend upon a dozen initiation and elongation factors, and upon phosphorylation of these proteins [Kozak, 1992]. A dramatic example of the role of translational control is the increased tumorigenic transformation of cells by introduction and over expression of such factors [Tatsuka et al., 1992].

A rapid rate of translation is essential for transit of mouse 3T3 cells through growth regulating restriction point in late G1 of the cell cycle [Rossow et al., 1979]. Moderate inhibition of total protein synthesis by cycloheximide specifically greatly lengthens G1 as compared to the remainder of the cycle. This result can be accounted for by shift of a steady-state balance,

decreased synthesis vs. degradation, of an unstable protein (half life 2–3 h), which creates too low a steady-state concentration to trigger the initiation of DNA synthesis. Pre-existing ability to transit G1 disappeared while protein synthesis was inhibited, which is accounted for by degradation of this labile protein. This requirement for rapid protein synthesis is relaxed in cancerous BP-3T3 cells. This labile restriction point protein most likely is cyclin E, which disappears in the presence of cycloheximide and is overproduced in these murine, and in human, cancer cells [Dou et al., 1993]. It furthermore has a short half-life and appears in late G1 [Pines and Hunter, 1989].

Several covalent, permanent modifications alter activities of proteins. Degradation importantly regulates enzyme levels and is involved in many processes [Goldberg, 1992]. Protein half-lives range from minutes to many days. p53 is a very important short-lived protein in normal cells; its half-life is increased in transformed cells [Martinez et al., 1991].

Other irreversible protein changes include glycosylation [Rudy et al., 1993], myristylation, and prenylation. The oncogene ras is activated by such modifications as well as by deletion of its carboxyl terminal amino acids and carboxy methylation [Clarke, 1992].

A novel finding is that truncations of cyclin E protein are found in human breast cancer cells, but not in the normal cells. This protein appears in several shortened forms and also is increased in amount. Presumably this is due to terminal chain degradation or peptide bond cleaving [Keyomarsi and Pardee, 1993].

Loss of thymidylate synthase (TS) during the cell cycle is an example of specifically modulated translation. Protein and enzyme activity of TS rise and fall dramatically at the start and end of each S phase of the cell cycle [Keyomarsi et al., 1993]. In striking contrast, TS mRNA remains constant. Powerful specific mechanisms must exist for TS activation of translation and its degradation. TS translation is negatively controlled by feedback inhibition by the TS protein which is proposed to bind to the mRNA [Chu et al., 1993]. Evidence for this "detainment" of translation (so named to distinguish it from repression of transcription) is its release by 5-F-uracil or folate analogs, inhibitors that bind to TS and thereby presumably alter the protein's conformation and allosterically block its binding to its RNA [Keyomarsi et al., 1993].

REVERSIBLE PROTEIN MODIFICATIONS

Proteins are reversibly modified by a variety of reactions, as well as being irreversibly modified (Fig. 1C). These changes can alter activities of enzymes, surface receptors, trans-membrane transporters, DNA binding transactivators, structural components, etc., and also locations of these molecules in cells. The modifications can be rapidly or slowly reversed, correspondingly altering biochemical events for different time intervals. Among many variations are covalent and noncovalent interactions with small molecules, and associations with other proteins. Combinations of the above are commonly found.

Kinase catalyzed phosphorylations are central to signal transduction pathways of cells. These covalent modifications are reversed by phosphatases, in contrast to the irreversible alterations of proteins. As examples, the enzyme phosphorylase which is activated by phosphorylation is classical [Krebs et al., 1958]. Growth factor receptors are activated by autophosphorylations of their tyrosines [Fantl et al., 1993]. Hyperphosphorylation of the retinoblastoma protein releases the cell cycle block at the restriction point [Hollingsworth et al., 1993]. Phosphorylation of lamin at mitosis is connected to dissolution of the nuclear membrane [Moir and Goldman, 1993].

Noncovalent binding of small molecules with proteins are important, rapidly acting and reversible mechanism of regulation. The classical example of allosteric control of aspartate transcarbamylase feedback inhibition by CTP [Gerhart and Pardee, 1962] was followed by many others, such as the complex interactions of ribonucleotide reductase with nucleotides that balance the ratio of deoxynucleotides [Stadtman, 1970]. A great variety of enzyme inhibitors are useful for elucidating molecular mechanisms and as drugs for therapies [Pardee and Keyomarsi, 1992].

Protein-protein interactions provide an increasingly observed third mechanism of regulation. Activations of cdk kinases by bound cyclins are important examples [Pines and Hunter, 1989]. Conversely, p53 activity is blocked by combination with MDM2 protein.

Regulation by reactions with both another protein and a small molecule are commonly observed. Often the regulatory protein is modified. Phosphorylations on different amino acids permit or prevent cyclin B activation of cdc2 kinase at mitosis [Kirschner, 1992]. Non-covalent bind-

ing of cyclic AMP to regulatory subunits of kinase A releases catalytic subunits, which can change their locations and function as enzymes [Cho Chung, 1990]. Similarly, Ca^{++} and diacyl glycerol activate kinase C and change this enzyme's binding to the cell membrane [Kikkawa et al., 1989]. GTP vs. GDP binding activates ras and many other G proteins, and is modulated by a variety of other proteins such as GAP [Bokosh and Der, 1993].

Proteins change their locations and thereby their activities, for example, during the cell cycle. At the beginning of S phase several enzymes of DNA precursor biosynthesis move from cytoplasm to nucleus, where they become components of multienzyme DNA synthesis complexes [Reddy and Pardee, 1980].

Coordination of many of these functional regulations is illustrated with the transactivating factor NK-κB [Liou and Baltimore, 1993; Beg et al., 1993]. Its two subunits are made from larger precursor proteins. It is located in the cytoplasm, bound to an inhibitory protein IκB. When cells are stimulated by a variety of cytokines or other factors, a signal transduction chain phosphorylates IκB and releases it from NF-κB; thereafter IκB is rapidly degraded. This process also triggers a rapid resynthesis of IκB. Freed NF-κB moves from cytoplasm to nucleus, where it binds to its motif located in promoters of a number of genes that are involved mainly in immune and inflammatory systems, and also in LTR of the AIDS virus. These activation processes are inhibited by the drug pentoxifylline [Biswas et al., 1993].

SUMMARY

Nature takes advantage of all possible means to control cell functions. The sample of about a dozen such modes mentioned in this brief discussion must represent only a beginning. Understanding these multiple levels of regulation is important for investigating all normal biological processes, such as differentiation and aging, for cancer and for other diseases.

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