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SIMULATION OF PROKARYOTIC GENETIC CIRCUITS

Harley H. McAdams
724 Esplanada Way, Stanford, California 94305;
e-mail: harleymcadams@worldnet.att.net

Adam Arkin
Department of Developmental Biology, Beckman Center, Stanford University School of Medicine, Stanford, California 94305; e-mail: arkin@stalk.stanford.edu

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ABSTRACT

Biochemical and genetic approaches have identified the molecular mechanisms of many genetic reactions, particularly in bacteria. Now a comparably detailed understanding is needed of how groupings of genes and related protein reactions interact to orchestrate cellular functions over the cell cycle, to implement pre-programmed cellular development, or to dynamically change a cell's processes and structures in response to environmental signals. Simulations using realistic, molecular-level models of genetic mechanisms and of signal transduction networks are needed to analyze dynamic behavior of multigene systems, to predict behavior of mutant circuits, and to identify the design principles applicable to design of genetic regulatory circuits. When the underlying design rules for regulatory circuits are understood, it will be far easier to recognize common circuit motifs, to identify functions of individual proteins in regulation, and to redesign circuits for altered functions.

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INTRODUCTION

Biochemical and genetic approaches have identified the molecular mechanisms of many genetic reactions in cells; progress is most advanced for prokaryotic genetics. The Genome Project and advances in experimental techniques will lead to full knowledge of DNA sequences, identification of most genes, and even temporal gene expression patterns for many organisms. A comparably detailed understanding is needed of how groupings of genes and related protein reactions interact to orchestrate cellular functions over the cell cycle, to implement preprogrammed cellular development, or to dynamically change a cell's processes and structures in response to environmental signals. This review addresses the use of simulation models to analyze the dynamical behavior of cellular regulatory networks. Bacterial systems are emphasized since molecular mechanisms of gene expression and regulation are better characterized today in bacteria than in higher organisms. As a result, molecular-level modeling of genetic regulatory networks is also most advanced for bacterial regulation.

Simulations are needed (*a*) to identify design principles for the biochemically based logic, (*b*) to understand the dynamical response of both normal and mutant cells to environmental and internal signals, (*c*) to predict quantitative effects of mutations on regulatory outcomes, and (*d*) to verify consistency and completeness of hypothesized reaction systems. This level of realism requires modeling approximations that have a rationale traceable to physical and chemical mechanisms. Several of the molecular-level simulation models described in a later section treat regulation of systems of coupled intracellular reactions, but either do not involve genetics or have over-simplified models of genetic mechanisms. The challenge now is to develop simulation techniques applicable to cellular processes where genetic regulation is centrally important, such as developmental differentiation, facultative infection processes, and cell cycle control.

Essential features of genetic regulatory systems, as understood today, were recognized 35 years ago. In a prescient report of a 1961 Cold Spring Harbor conference on cellular regulatory mechanisms (68) the importance of regulatory feedback was emphasized, regulatory nets were characterized as "circuits," and regulatory breakdown was postulated as the central initiating event in malignancy, along with numerous other "modern" ideas about cellular regulation.

The operon model of Monod, Jacob, and their coworkers (45–47) stimulated numerous authors to address the integration of genetic regulation into models of enzymatic regulation. In a 1968 review, Rosen (79) summarized the essential methods and approximations that came to be widely used in simulations of genetic regulatory networks: a focus on transcription control, neglect of post-transcriptional control mechanisms, and characterization of protein production as a continuous process modulated by the level of activation or repression of the corresponding operon's operator region.

Genetic regulation is at times characterized using metaphors drawn from the fields of computing and digital electronic circuit design. There is validity to this comparison, but the "hardware" (or "wetware") of cellular logic, chemical reactions in the cytoplasm, is profoundly different from electronic hardware. The next section summarizes organization of genetic regulation in bacterial cells from a regulatory circuit architecture perspective and in comparison with electronic logic.

GENETIC REGULATORY CIRCUITS: ORGANIZATION AND FUNCTION

Bacterial genetic circuits exhibit hierarchical organization: regulons control groups of operons that control gene groupings (32, 69). Global regulons coordinate regulation of operons in multiple metabolic pathways. For example, the σ^{32} heat shock regulator protein (105), a representative sigma factor, is required for RNAP binding at the promoters of a wide spectrum of genes involved in responses to stress. Other global regulators act through control of DNA spatial configuration; integration host factor protein (IHF) is a representative example (23, 31). These global regulators enable the bacterial cell to effect a rapid and coordinated response to threats or opportunities presented by their environment (e.g. heat, cold, presence or absence of essential nutrients, high or low pH) by reconfiguring their biochemical machinery. There are assumptions, explicit or implicit, in every simulation analysis regarding the effects of global regulators. The most common assumption, usually implicit, is that global regulation can be neglected, meaning, in effect, that during the simulation period either the status of global regulators is assumed to be unchanging or the systems changed by global regulators are assumed to be decoupled from the processes under study. Explicit treatment of global regulation will be necessary when we progress to the point of modeling complex cell decision points. For example, regulation of the initiation of sporulation in *Bacillus subtilis* involves two sigma factors, σ_A and σ_H, that compete for binding to core RNA polymerase at promoters of critical genes (42).

The biochemical logic in genetic regulatory circuits provides real-time regulatory control, implements a branching decision logic, and executes stored

programs that guide cellular differentiation extending over many cell generations. In higher organisms, the regulatory algorithms may control sequential execution of developmental processes over many years of the organism's life. The mechanisms that implement bacterial genetic logic functions may be entirely within a single cell, may span many cells (43), or may function across cell generations (7, 50). Genetic circuits may cross species boundaries as in symbiotic relations between bacteria and higher organisms (25, 58, 62). More sinister, perhaps, are the bacterial mechanisms that co-opt the internal logic of target cells to facilitate penetration or evade defensive responses (21, 22, 34).

At any moment, cellular functions are both implemented by and controlled by the network of chemical reactions involving the collection of molecular species in the cell. In a growing cell, the molecular composition is continuously changing as the cell cycle progresses and the instantaneous regulatory control function also changes continuously. In these networks of interconnected reactions, one regulatory protein can control genes that produce other regulators, that in turn control still other genes so that complex branching networks of interactions are formed. Multireagent reactions or genetic mechanisms controlled by multiple input signals are key elements for performing sensor or control-logic functions in these networks.

REGULATORY FEEDBACK Feedback, where the output signal of a network element directly or indirectly influences the value of its input signals, is pervasive in regulatory networks. Autoregulatory feedback loops, where a gene product acts on its gene-expression mechanisms, can lock controlling protein signals on, in turn locking other signals either on or off. In a 1991 inventory of 107 σ^{70} promoters then known in *Escherichia coli* (10), the promoters were organized into 31 regulons, each jointly controlled by one or more regulatory proteins. Twenty-one (68%) of the 31 regulon-controlling proteins are autoregulating, i.e. they repress their own synthesis. Four (13%) of the 31 are autoactivating, that is, they activate their own synthesis.

The complement of distinct molecules in the cell and the state of the DNA (e.g. methylated or not) defines the regulatory logic that establishes how the cell functions at that instant. This logic determines when the cell makes new proteins from DNA-encoded instructions and when existing proteins are destroyed. Specialized enzymes under regulatory control can remove DNA segments, move segments from one site to another, reverse a segment's orientation, or insert foreign segments (14). These DNA changes can lead to temporary or permanent radical changes in the cell morphology, its active metabolic pathways, and importantly, its responses to environmental signals so that future cellular responses to signals differ from current responses. Thus, the cell's stored instructions (the

genetic material) can be dynamically changed according to previously stored instructions.

Mechanisms that sense conditions inside and outside the cell are integrated into the regulatory logic so that the cell can adapt to the needs of the moment. Receptors on the cell surface can respond to specific chemical species and affect the regulatory logic by molecular signaling using signal transduction cascades (17). Other signals may affect reactions in the cascade to change the level or character of the signal and thus act as modulating functions (5, 20, 35). In this way, environmental influences originating both within and outside of the organism can evoke complex regulatory responses. The interconnected networks of protein reactions that connect sensors to response mechanisms are, in a sense, the "nervous system" of unicellular organisms (5). Bray has reviewed interconnected biochemical elements that can form information processing circuits and has assessed their similarity to neural networks (5).

CELLULAR REGULATORY APPARATUS The cellular regulatory apparatus includes both short- and long-term memory mechanisms. The current complement of proteins in the cell and their physical deployment depends on the cell's history, and thus is a memory. Long-term memory mechanisms are implemented by more-or-less permanent changes in the state of the cell (for example, the metabolic enzyme systems that are activated, the global regulators that are active, the complement of surface structures, or major morphological transformations as in sporulation) or in the DNA sequence. In phage lambda and other temperate phage, the integration of the phage DNA into the host chromosome changes the control logic of the phage circuitry. Thus, for example, the state of being either *integrated* or *not integrated* into the host DNA acts as a memory element (61). In higher organisms, successive cellular state changes during organ differentiation are largely irreversible. The self-sustaining, continuous expression of the homeotic genes throughout development and into adulthood is thought to be the long-term memory mechanism recording the differentiated state of each cell as established early in embryonic development (54).

Genetic regulatory networks progress asynchronously through successive reactions, so that biological "time" is based more on the degree of progress along reaction pathways than on clock time. The stochastic pattern of signal protein production (discussed below) may only cause uncertainty in timing of regulatory events, not uncertainty in outcome. (We use the term "stochastic" in this paper in the technical sense of "arising from a random process.") Within broad limits the duration of many cellular functions may be less important to proper cellular function than the proper sequencing of events. For example, cells halt at various checkpoints until conditions (for example, restoration of essential nutrients, completion of precursor cellular events) for further progress

Table 1 Points of similarity between genetic logic and electronic digital logic in computer chips

	Electronic logic	Genetic logic
Signals	Electron concentrations	Protein concentrations
Distribution	Point → point (by wires or by electrically encoded addresses)	Point → point (movement by diffusion or active transport by encoded reaction specificity)
Organization	Hierarchical	Hierarchical
Logic type	Digital, clocked sequential logic	Analog unclocked (can approximate asynchronous sequential logic)
Noise	Inherent noise due to discrete electron events and environmental effects	Inherent noise due to discrete chemical reaction events and environmental effects
Signal/noise ratio	Signal/noise ratio high in most circuits	Signal/noise ratio low in most circuits
Switching speed	Fast ($>10^6$ sec^{-1})	Slow ($<10^{-2}$ sec^{-1})

are satisfied (10, 38, 49, 100). In this case, the indeterminism relates to whether the cell will progress or not along a developmental path at any instant, rather than on the choice of alternate pathways. So, the regulatory decision command sequence is: "HALT until CONDITIONS are met, then PROCEED," where "CONDITIONS" are sensed environmental or cellular signals. The result is dispersion across the cell population in the rate of progression along prescribed pathways rather than dispersion in outcome. However, at developmental switching points (discussed below) stochastic gene expression can lead to random partitioning of the cell population into subpopulations developing on alternative pathways.

Genetic networks have many attributes commonly associated with computing. Table 1 shows points of similarity between genetic logic and the electronic digital logic in chips in today's desktop computers.

Figure 1*a* shows the simplest of genetic circuits, a regulatory cascade capable of initiating events in sequence as illustrated in Figure 1*b*. Figure 1*c* shows a sampling of the control features that are used in cells to create complex control logic structures. The capability to create combinatorial controls with feedback when coupled with memory mechanisms provides every element needed to create a type of asynchronous sequential logic (61). Biological regulatory circuits can have multiparameter combinational control functions at key nodes (for example, promoters controlled by several effectors), reaction cascades that function as "subroutines," the ability to respond conditionally to external

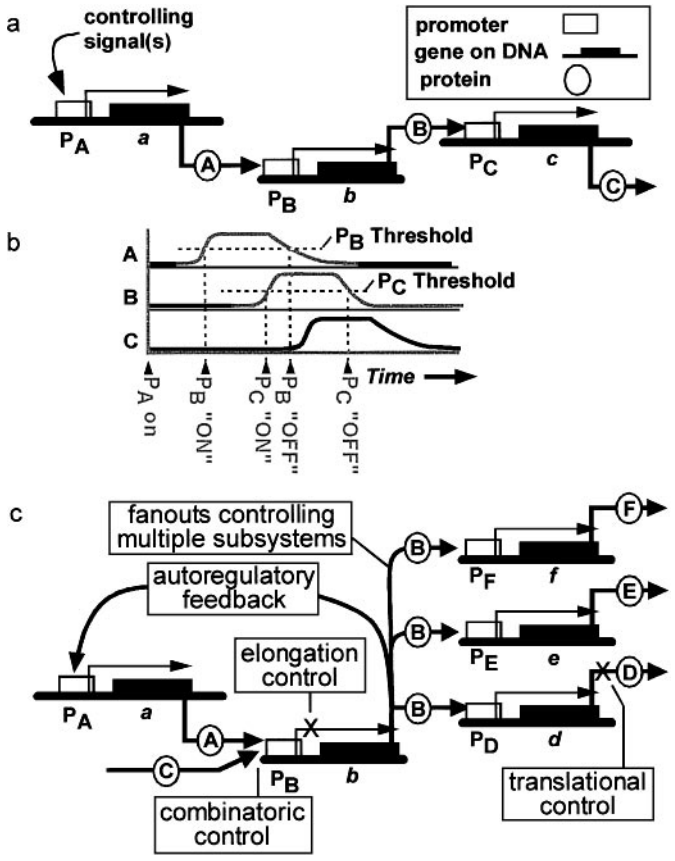

Figure 1 (*a*) A genetic cascade, the simplest genetic circuit. The increasing concentration of signal protein A from transcripts initiated at promoter P_A, turns on the downstream promoter P_B, leading to production of signal B, which similarly activates production of signal C. Signal specificity depends on the addressing created by stereochemically based binding specificity of A, B, and C to the corresponding binding sites in the different promoters. (*b*) The resulting succession of concentration peaks for signals A, B, and C. Decay of the protein signals after the promoters are switched off results from proteolytic degradation of the proteins and also in growing cells from continuous dilution of signal concentration as the cell size increases. (*c*) A small sampling of the numerous additional regulatory mechanisms used in cells to control creation, timing, and decay of protein signals. Posttranscriptional regulatory mechanisms are essential features of regulatory networks and *must* be included in any analysis of the regulatory logic. Small autoregulatory feedback loops are widely used in cells to maintain signals within a concentration range. Switching circuits that control developmental path choices commonly include positive or reinforcing feedback to enhance the commitment to the selected path and negative or repressing feedback to assure that promoters on alternative paths are turned off.

signals, and the ability to read from stored instructions (the DNA). However, the detailed implementation and the manner of processing of information is radically different from the digital information processing paradigm in modern computers. Forcing cellular regulation into the digital information processing paradigm has not been notably productive.

Cellular regulatory circuits implement a complex adaptive control system. A particularly confounding aspect of these cellular control systems is the lack of clear distinction between the mechanisms comprising the *controlling* signals and logic, and the *controlled* process or function. In ordinary engineering control systems, this distinction is usually more apparent. Consider control of traffic lights at an intersection. Buried wires sense cars in turn lanes; a box at the side of the road contains the control logic; and the signal lights over the intersection are the controlled function. In cellular regulation, control reactions and controlled functions are composed of intermingled molecules bumping together, reacting, and forming more-or-less stable assemblages so that identification of roles is more complex.

The genetic regulatory mechanisms of primary concern to this review and the coupled protein reaction-based regulatory networks [reviewed in (5)] together implement the *control system* that enables cells to adapt to their environment. The two systems act with different response speeds: protein reactions govern responses in the range of about 10^{-4} to 10^{2} seconds, while genetic mechanisms govern responses in the range of about 10^{2} to 10^{8} seconds. Additional adaptive capability is provided by (*a*) mutation and selection-driven evolution of the regulatory circuit design and (*b*) acquisition of new functions through horizontal exchange of mobile genetic elements by bacterial conjugation, transformation, and transduction (8, 22).

SIMULATIONS OF CELLULAR REGULATION

Most molecular-level simulations to date have focused on coupled protein reactions with limited treatment of genetic reactions. Where genetics has been included, only control of transcript initiation has usually been considered. Now, however, as details of genetic mechanisms are accumulating rapidly, extension of models to include genetic regulation should be practical. In the following sections we identify several systems where prospects for such extensions are promising.

Metabolic Regulation

Although regulation of the reactions forming the core of intermediary metabolism has been intensively studied, determining how metabolism is regulated has been difficult because there are many complex feedback mechanisms within the pathways. Stress, changes in the environment, or changes in nutrient availability

can all instigate mode changes leading to a revised complement of metabolic enzymes in the cell. Modeling of metabolic control is commonly done by characterizing the coupled chemical reactions with systems of ordinary differential equations based on chemical kinetics. Except in the simplest cases, the resulting equations must be solved numerically; representative software packages supporting metabolic analysis are described in (18, 64, 80).

The principal questions that modeling of metabolic regulation has addressed are: (*a*) How does the cell respond to presence, absence, or changes in nutrient levels? (*b*) How does the cell change pathways to increase (or decrease) production of selected products?

Metabolic engineering of cells to increase the yield of an industrially important product such as lysine or ethanol could have large economic returns. This prospect has stimulated modeling studies and experiments seeking to maximize yield of desirable molecules. Experiment has shown that simple overexpression of a rate-limiting enzyme rarely increases yield of the final product due to the stabilizing effects of feedback and nonlinear dynamics in metabolic control circuits. These complex control mechanisms apparently evolved to maintain optimal flux distributions in the pathways for balanced cellular growth (88). Metabolic control analysis (MCA) (40, 48) techniques show that the biosynthetic flux is usually insensitive to perturbations in any single enzyme; rather, flux control is distributed among many enzymes (101). Thus, expression of many enzymes must be changed to effect a significant change in flux. MCA studies use linear and polynomial models to predict which enzymes to change (40, 48, 81, 85, 86). Detailed enzymological models have been used to analyze pancreatic glycolysis (1, 2), red blood-cell metabolism (55), and glycolytic/gluconeogenic switching (3). Linear and polynomial models are commonly used for deriving local dynamical models to predict changes in flux within a pathway that reflect changes in experimental conditions. Complex enzymological models are used to analyze endogenous cellular control and regulatory circuits that effect large mode changes, such as switching from glycolysis to gluconeogenesis. In these analyses, the kinetics of reactions involved in gene expression are generally assumed to be "enzyme-like" (52, 102) and regulation of transcript elongation or translation is neglected. However, for many pathways these additional genetic mechanisms cannot be neglected. Extension of metabolic modeling methods to include more realistic genetic regulatory mechanisms is a current challenge to the field.

Integrating Environmental Signals into Regulatory Circuits

In the bacterial chemotactic response, attractant or repellent molecules bind to specialized receptors on the cell surface and initiate a phosphorylation cascade that controls the rotary flagellar "motor." The first comprehensive model of the phospho relay–based chemotaxis signaling network (6), constructed

from known reactions and kinetics, reproduced the observed patterns of runs, tumbles, and pauses, matched responses to pulses of chemotactic agents, and matched behavioral changes in chemotactic mutants with altered enzyme activities. With addition of several hypothetical reactions, the model explained both the wild-type strain and over 30 mutants. Later models added adaptation (4, 39, 87). These simulation studies made essential contributions to understanding of the chemotactic response system. The controlled element in chemotaxis is the flagellar motor rather than gene expression, so modeling of genetic mechanisms has not been important in this system. However, other similar sensor-response pathways are widely used in bacteria to control gene expression. Bacteria use the histidyl-aspartyl phosphorelay (the "two-component system") as their predominant mode of signal transduction in regulation of adaptive responses to the environment (17). [In the *E. coli* genome, at least 62 open reading frames have been identified as putative members of the two-component signal transducers (67).] Two-component signal transducers have also been identified in diverse eukaryotic species including plants, fungi, yeast, and slime molds (104).

Cell Cycle Models

The central questions in cell cycle regulation are: (*a*) "What is the cycle of coupled reactions that drives cellular progression through DNA replication and cell division?" and (*b*) "How is genetic regulation coupled to this cycle so that genes active in cell replication, division, creation of cell structures, and other events are expressed at appropriate times?" The large size of the *Xenopus laevis* oocyte and the discovery that cytoplasmic extracts from the oocyte cycle periodically, closely replicating timing of cleavages of intact eggs, facilitated identification of cytoplasmic reactions controlling the early embryonic cell cycle. Modeling studies were undertaken to demonstrate that the known biochemical reactions alone could support oscillations of the correct frequency and with the appropriate chemical concentrations. Hyver & Le Guyader (44) were among the first to propose a rough molecularly based dynamical (differential equation) model for the system that included activation of $p34^{cdc2}$, cyclin/$p34^{cdc2}$ interactions, and cyclin degradation. The only genetic component of this model was the implicit steady production of cyclin through the cell cycle. The model demonstrated the sufficiency of the coupled cytoplasmic reactions for explaining cell cycle oscillations and was used to discriminate between competing hypotheses regarding activation of $p34^{cdc2}$ by cyclin into an active mitosis promoting factor. Later models were more elaborate (29, 70, 73, 97, 98), with the Tyson model (98) providing the most detailed early embryonic cell cycle model. Obeyesekere et al extended the model of M-phase control in embryogenesis to the full cell-cycle in human somatic cells (72). Swanson et al used the Tyson model to analyze calcium control of embryonic cell-division (90). The

mechanisms for coordinate control of cell-cycle regulated genetic mechanisms are only partially identified and have not been integrated into the eukaryotic cell-cycle models yet.

Recent discoveries that progression through the bacterial cell cycle and communication between cellular compartments are mediated by two-component signal transduction systems and signaling pathways involving transcription factor activation by proteolytic processing are reviewed in (82). The coupled reactions controlling cell cycle progression in prokaryotes have not been identified. However, the recent discovery that a member of a phospho relay system that regulates several key cell cycle regulatory proteins in *Caulobacter crescentus* is controlled by both phosphorylation and temporally and spatially regulated proteolysis, suggests that paradigms of cell cycle control have been conserved among prokaryotes and eukaryotes (12, 76). Many of the genetic cascades controlling chromosome replication, cell division, and synthesis of cell structures are well characterized (11, 13, 28, 78) so that an integrated simulation model of bacterial cell cycle regulation may be possible soon.

Developmental Switches

Virtually all bacteria contain genes for many alternative physiological states. The specific metabolic and morphological features expressed are determined by the cell's history and its current environment. The mechanisms for initiating switching between states are composed of transducers on the cell's surface that detect external signals, internal status signals, internal signaling pathways, and the regulatory switching circuitry that turns promoters on or off in response to the signals. The genetic and biochemical details of these integrated switch-sensor subsystems are being identified at an accelerating rate. Two bacterial systems where molecular mechanisms are relatively well identified are sporulation in *Bacillus subtilis* and the lysis-lysogeny switch in phage lambda. Also, in many bacterial pathogens, environmental signals control developmental switches essential to progress of infections (89). Examples include: BvgA/BvgS in *Bordetella pertussis*, ToxR/toxS in *Vibrio cholerae*, *Salmonella* survival within macrophages, and outer membrane porin regulation in *Salmonella* and *E. coli* (22). Integrated simulation models of the molecular mechanisms of environmental sensing, signal transduction, and gene expression that govern global regulation in bacteria are needed now to explain dynamics of these switching mechanisms.

The cell-density-dependent gene expression found in some bacteria is another promising area where simulation models of the regulatory system are needed to explain dynamics of population behavior. The regulatory architecture in many of these "quorum-sensing" systems involves secretion of a pheromone that is detectable by a surface-bound receptor [reviewed in (33, 51)]. Typically, the receptor acts through a two-component response regulator that mediates gene

expression to produce an altered phenotype at differing pheromone concentrations. High cell densities lead to high pheromone concentrations, so this mechanism provides an indicator of local cell concentration. Examples of such quorum-sensing mechanisms as components of genetic switches include the initiation of genetic competence in *Bacillus subtilis* and *Streptococcus pneumoniae*, initiation of sporulation in *Bacillus subtilis*, the virulence response in *Staphylococcus aureus*, and social motility in *Myxococcus*. The regulatory mechanism controlling aggregation of *Dictyostelium discoideum*, which also involves quorum sensing, has been extensively analyzed and simulated (30, 53, 56, 57).

The regulation of the λ phage development immediately after infection provides a paradigmatic model of a switch controlling developmental commitment. Bacteriophage λ phage genes, regulatory mechanisms, and related protein reactions have been intensively studied for forty years. Within five minutes after infection, an *E. coli* cell becomes committed to one of two fates: lysis, where the phage reproduce rapidly and soon lyse the host to release 60–100 new phage particles, or lysogeny, where the λ DNA is integrated into the host chromosome, the cell is immunized against further infection, and many generations may pass before induction of the phage continues the infection. The molecular basis of the regulatory mechanisms controlling the lysis or lysogeny decision are generally known (15, 16, 24, 41, 61, 75). As a result, λ phage regulation has been an attractive model system for studying integrated behavior of multi-gene regulatory subsystems. The central questions in phage λ dynamics are: (*a*) How does the regulatory logic that selects the lytic or lysogenic pathway work? and (*b*) How does the regulatory logic after commitment implement the genetic program leading to lysis or lysogeny?

Thomas et al (95) defined a qualitative λ lysis-lysogeny decision model in 1976 using a Boolean model of promoter regulation that predicted a subsequently constructed mutant phenotype and exhibited the necessary bistable behavior. In 1985 Shea & Ackers (83) modeled the statistical mechanics of the overlapping operator regions of the P_R and P_{RM} promoters [the λ "switch" (75)] and predicted the dynamics of maintenance of lysogeny and phage induction, as well as expected effects on lysogeny maintenance of changes in repressor cooperativity in binding to the operator region. (See discussion of the Shea–Ackers promoter control model in the next section.)

Building on the work of the Ackers group, both Reinitz & Vaisnys (77) and Chung & Stephanopoulos (9) modeled the lambda lysis-lysogeny circuit to analyze the bistability of the switch. Reinitz & Vaisnys defined a differential equation model of the production of CI and Cro based on the Shea-Ackers P_R/P_{RM} promoter model. Though degradation and dilution of the proteins were included phenomenologically in the model, the actions of CII, CIII and the Hfl

proteolytic system as well as the mechanisms of elongation control were ignored due to lack of information about these subsystems and because inclusion of these mechanisms would have made analysis of the circuit too difficult. Reinitz & Vaisnys concluded that the resulting model of the switch, even with the best measured promoter kinetics, was not sufficient to explain the bistability of the reaction mechanism. Rather, they suggested either the promoter kinetics were incomplete or other neglected mechanisms in the pathway caused the bistability. Chung & Stephanopoulos (9) analyzed induction from the lysogenic state, a state in which the only phage protein expressed is the repressor, CI, by defining a simple differential equation model for dynamics of CI concentration. The model includes promoter control, CI monomer and dimer production kinetics, and decay kinetics. In the same paper, Chung & Stephanopoulos show that a similar feedback circuit is found in the lactose operon.

In 1995 McAdams & Shapiro (61) defined the first model of the λ circuit that followed the qualitative dynamics of the phage from infection through the decision to outcome. They concluded that detailed simulation of genetic mechanisms determining the temporal pattern of protein production and of the coupled protein reactions would be necessary to model regulatory circuits quantitatively. While investigating how to simulate molecular level regulatory mechanisms quantitatively, McAdams & Arkin analyzed dynamics of a representative single genetically-coupled link, that is, a one promoter-gene complex whose protein product regulates another promoter (60). In that study, an integrated molecular level model of the effector-operator reactions in the promoter control region, including the closed- to open-complex isomerization reaction and the message-translation control reactions, was developed. Owing to the low intracellular concentrations of the reacting species and the slow reaction rates in these reactions, they concluded that conventional methods of modeling coupled chemical reactions are frequently invalid for modeling the time profile of regulatory protein production. Rather, McAdams & Arkin suggest it is necessary to explicitly include consideration of the randomness in protein production that is inevitable in chemical systems where the reacting species are at low concentrations and reaction rates are slow as is typically the case for genetic regulatory proteins (36, 60). Determining the implications of this observation regarding stochastic regulatory gene expression mechanisms for the dynamics of developmental switches is a current challenge to the field.

MODELING ISSUES

This section discusses several modeling issues central to simulation of genetic regulatory circuits: (*a*) promoter control models, (*b*) stochastic processes in

regulatory kinetics, (*c*) modeling macromolecular complexes, and (*d*) uncertainty in intracellular environment and reaction rates.

Promoter Control Models

The notion that promoter regulation can be modeled using a Boolean threshold logic paradigm is attractive in that it enables algebraic analysis of regulatory networks and rapid simulation algorithms. In a representative example of this approach, Thomas and coworkers analyzed the behavior of different feedback configurations in hypothetical small genetic networks (92–94, 96). Effector concentration is modeled by a differential equation of the form

$$\dot{x}_i = k_i F_i(x_1, x_2, \ldots, x_n) - k_{di} x_i,$$

where the x_i's are concentrations of the ith protein species, $\dot{x}_i$ is the time derivative of x_i, k_i is the rate of protein production when the gene type i is "on," and k_{di} is the degradation rate constant for protein type i. The F_i are step-functions, similar to the limiting form of a Hill function at high levels of cooperativity, assumed to equal 0 or 1 depending on the concentration of the x_i relative to threshold values determined by the kinetics of the promoter sites. The possible "states" of the system are identified with the distinct value ranges of the x_i. Using this formulation, a form of logical analysis is defined that provides qualitative insights into the dynamics to be expected from small genetic circuits with different combinations of positive and negative feedback loops (96). The approach is also applied to analysis of phage lambda immunity control (92). Tchuraev and co-workers use a similar Boolean threshold approximation to promoter control to simulate behavior of genetically controlled systems in an approach they call a "generalized threshold model" (74, 91).

The Boolean threshold approximation to promoter control is also applied to modeling of large networks in so-called Boolean Network models. Assumptions in the Boolean Network approach are summarized in (84): (*a*) the state of each gene or other network element can be characterized as either *on* (one) or *off* (zero), (*b*) the combinational control of gene expression can be reduced to a "wiring diagram" of the network, (*c*) the computation of the interactions indicated by the wiring diagram can be approximated by Boolean combinational logic rules, and (*d*) all elements (to first approximation) update their *on* or *off* states synchronously. A Discrete Dynamics Lab (DDL) software package (103) is available that computes the behavior of hypothetical networks. Proponents suggest that the Boolean Network models provide insight into behavior, evolution, and self-organizing capabilities of large-scale genetic networks and that this paradigm offers an approach to reverse engineering data describing

temporal patterns of gene expression to extract the logical structure of the underlying physical gene regulatory network.

The Boolean characterization of genetic activation and repression is frequently a poor approximation of promoter control functions. For example, the P_{RM} promoter activation function plotted in Figure 2 is distinctly non-Boolean. Furthermore, there are numerous control mechanisms outside of promoter activation control that are equally or more important in regulatory logic. Examples include actively controlled termination sites, the many types of posttranscriptional regulation, and protein-mediated controls (proteolysis, phosphorylation, methylation).

Shea & Ackers (83) define a physically-based promoter control model for the common operator region of the divergent P_R and P_{RM} promoters that are central elements of phage lambda's so-called "switch." This switch is implemented by the intricate biochemistry of the operator region, which includes three closely situated operator sites where homodimers of two phage-encoded molecules, Cro and CI, bind competitively and in sequence, but in opposite order (59, 65, 66, 75). The respective RNA polymerase (RNAP) footprints of promoters P_R and P_{RM} overlap the operator sites. In this well-studied system,

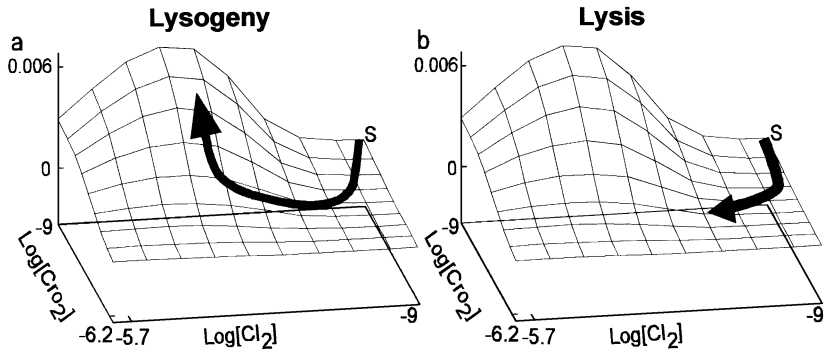

Figure 2 The phage λ P_{RM} promoter is controlled by the concentration of both Cro and CI dimers; the promoter activation level in open complexes per second (OC/s), is plotted in the upward or z-direction versus the log of the molar dimer concentrations plotted in the x–y plane. The temporal pattern of P_{RM} promoter activation immediately after phage infection of an *E. coli* cell depends on the way the concentrations of the controlling effector grows. *Arrows* illustrate the "path" of P_{RM} activation for a lysogenic outcome (*a*) and a lytic outcome (*b*). Each cell starts from the (1 nM Cro_2, 1 nM CI_2) point marked with *S* at the right rear of *a* and *b*. (In an *E. coli* cell, one molecule/cell is roughly equivalent to a 1 nM concentration.) In the lysogenic case, *a*, the system eventually enters a region of positive autoregulation locking repressor production on, while in the lytic case, *b*, repressor production from P_{RM} is never activated.

the 40 possible occupancy configurations (states) of the various binding sites by Cro_2, CI_2, or RNAP are known, as well as transcript-initiation rates for those configurations that are transcriptionally active. Also, the binding free energies for each site and each species binding at the site were measured along with incremental contributions from cooperative binding. Shea & Ackers assumed that the operator binding sites are in near equilibrium with the free concentration of Cro_2, CI_2, and RNAP. Knowing the total binding energy of each state, the fractional occupancy is calculated using the partition function. Then, the transcriptional activity of each promoter is calculated by summing the transcript initiation rates of each transcriptionally active state multiplied by the fraction of time that the system spends in that state. As the concentration of repressor molecules increases, the probability of occupancy of transcriptionally active sites decreases, and that of transcriptionally inactive states increases so that the rate of transcript initiation is repressed. Figure 2 shows the activation surface of the P_{RM} promoter as a function of Cro_2 and CI_2 concentrations calculated using the Shea–Ackers scheme. An advantage of the activation surface representation is that the progression of regulatory decisions implemented by changing concentrations of signal proteins can be visualized as a path on the activation surfaces of the controlling promoters. The differing paths in phage λ-infected cells that commit to lysogeny versus those that become lytic is illustrated schematically in Figure 2.

Cellular regulatory logic is inherently a logic based on continuously variable parameters, that is, it is an "analog" logic. Accordingly, it seems inevitable that the most useful heuristic for simulating promoter control will prove to be the one that best approximates the n-dimensional promoter activation surface rather than one that forces a nonphysical discrete logic approximation. Whenever a Boolean approximation is valid, the analysis and modeling of system behavior can be simplified. However, validity of the Boolean approximation has to be examined critically for each regulatory subelement in a system. Hybrid models with behavior of some elements characterized as Boolean and other elements treated with detailed kinetics may prove to be the most efficient compromise.

Stochastic Processes in Regulatory Kinetics

Numerous studies have analyzed cellular regulation using ordinary differential equations (ODE) to model the macroscopic kinetics of coupled chemical reactions. Analytical or numerical methods are used to solve the equations. Examples include models addressing (*a*) bacterial chemotaxis control (6, 39); (*b*) the oocyte cell cycle (71, 90); (*c*) T7 phage infection (19); (*d*) conditions for lambda lysogen induction (9); and (*e*) the phage λ lysis-lysogeny decision

(77). In defining the differential equations for these models, there is an implicit assumption of continuously varying chemical concentration and deterministic dynamics. For a chemical system to be compatible with these assumptions the number of molecules of each type must be large compared to thermal fluctuations in concentration, and for each type of reaction in the system, the number of reactions per unit time must be large in each observation interval. In vivo genetic reactions usually violate the assumptions of determinism and continuity underlying conventional kinetics because they occur at low concentrations, are isolated spatially, and have slow reaction rates.

McQuarrie (63) analyzes the kinetics of chemical systems with small numbers of participating molecules; the behavior of such "small" chemical systems, predicted using a more exact stochastic formulation, is shown to differ from the prediction using conventional deterministic kinetics. Analytical solution to stochastic reaction equations is only practical for simple reactions. However, numerical solutions can be computed for complex systems of coupled stochastic reactions using the Monte Carlo algorithm described by Gillespie (26). The Gillespie algorithm produces a stochastic realization of the temporal behavior of the system by calculating the probabilistic outcome of each discrete chemical event and resulting changes in the number of each molecular species. If the physical model and its assumptions are valid, and parameter estimates are sound, then this stochastic simulation algorithm produces a more realistic and complete description of the time-dependent behavior of stochastic reaction systems than a deterministic kinetic calculation (26, 27).

The Gillespie stochastic simulation algorithm is based on application of the chemical master equation. The master equation is a stochastic differential difference equation describing the time evolution of the probability densities for the concentrations of the chemical species comprising the reaction system (26, 27, 99). States of the system are characterized by a "state vector." Each component of the state vector represents the number of molecules of a particular molecular species in the chemical system at a given time. Every feasible chemical reaction represents a transition between states and the probability of that transition is determined by the probability of the corresponding reaction. The transition probabilities in these master equations are related to the conventional macroscopic rate coefficients of the reactions that comprise the chemical network.[1]

[1]The stochastic kinetic parameters in (26) are related to conventional deterministic kinetic parameters: For reactions of the form $X \xrightarrow{k} anything, k_{stoch} = k_{det}$; for reactions of the form $X + Y \xrightarrow{k} anything, k_{stoch} = k_{det}/(A_g\, V_{cell})$; for reactions of the form $X + X \xrightarrow{k} anything$, $k_{stoch} = k_{det}/(2\, A_g\, V_{cell})$, where A_g is Avagodro's number and V_{cell} is the cell volume.

In application of the Gillespie algorithm to simulation of bacterial regulation, each simulation run represents a possible evolution of a single cell from a starting condition for that case. Multiple runs are used to develop a statistical characterization of the outcomes expected for a cell population. Statistical sampling theory is used to determine how many simulation runs must be included to achieve a target confidence level in the statistics of outcomes. The Shea–Ackers promoter model can be adapted to the Gillespie simulation algorithm by calculating the instantaneous probability of each distinct transcriptionally active state of a promoter using the partition function, and then using this probability in calculating the reaction probabilities for the transcript initiation reactions.

The predicted temporal pattern of regulatory protein production from a representative activated bacterial promoter is described by McAdams & Arkin (60) and is shown here in Figure 3. They define a detailed model of gene expression mechanisms that includes an integrated molecular-level model of the effector-operator reactions in the promoter control region, the closed- to open-complex isomerization reaction, transcript elongation, and the reactions controlling message translation.

The simulated homodimer concentration growth for three runs is shown in Figure 3*a*; each run exhibits a substantially different pattern of dimer concentration growth and is illustrative of the wide range of regulatory expression patterns that can be expected in a homogenous cell population. Figure 3*b* shows the mean and standard deviation of the expected number of dimers in the cell at each time for gene dosage $=$ 1, 2, and 4. The horizontal lines at 25 and 50 nM delineate a representative range over which switching is effected by bacterial regulatory proteins. These simulations of the pattern of regulatory protein production suggest that proteins are produced in short bursts of variable numbers of proteins that occur at random intervals. As a result, the genetic switching time in growing cells can have considerable uncertainty. Stronger promoters, higher gene dosage (or equivalently in many cases, multiple promoters per gene), and lower signal thresholds all act to reduce timing uncertainty (60). These observations suggest that the validity of using conventional kinetic analysis for in vivo regulatory functions must always be examined critically, particularly for processes in cells that are regulated by low concentrations of short-lived effector molecules.

If we accept McAdams & Arkin's prediction that the temporal pattern of regulatory protein production in individual cells can be quite different for each cell in a population, then this "noise" phenomenon should affect the cellular outcomes for competitively regulated switching mechanisms. As a simple example, consider the situation shown schematically in Figure 4, where an activating and a repressing protein expressed from two independent promoters competitively regulate an operon transcribed from promoter P_G.

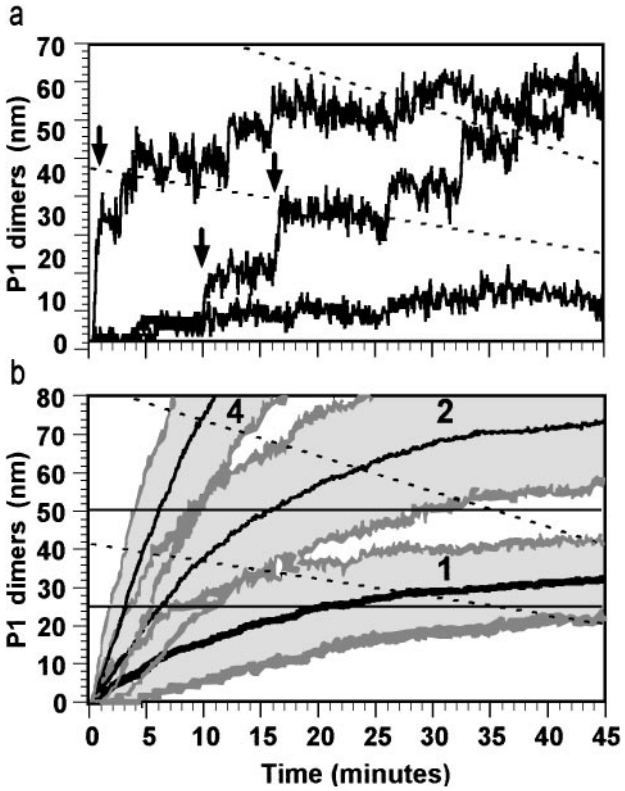

Figure 3 (*a*) Three simulation runs showing a different realization of the pattern of homodimer production from a representative bacterial promoter in a single cell. *Dashed lines*, declining concentrations equivalent to 25 and 50 dimer molecules in the growing cell. Parameters: dimerization equilibrium constant = 20 nM; protein half-life = 30 min. Initial cell volume = 1×10^{-15} l, doubling in 45 min. (*b*) Mean and $\pm 1\ \sigma$ results at gene dosages of 1, 2, and 4. At higher gene dosages, protein P1 is being produced from more genes; the concentration rises more rapidly, and the effective concentration range is reached quicker. Also, the dispersion in time to effectiveness (i.e. the switching delay) is lower for faster-growing signals [from Figure 3 in (60)].

Illustrative contours for P_G activation are shown versus the activator and repressor concentrations. The bold line is the 50% activation contour. Hypothetical successive distributions for the activator and repressor concentrations from the two independent operons are shown at times T_1, T_2, and T_3. The situation at T_3 illustrates how activation of P_G can vary widely from cell to cell because of the statistical differences in activator and repressor production. In developmental switches, such competitively regulated promoters are found as

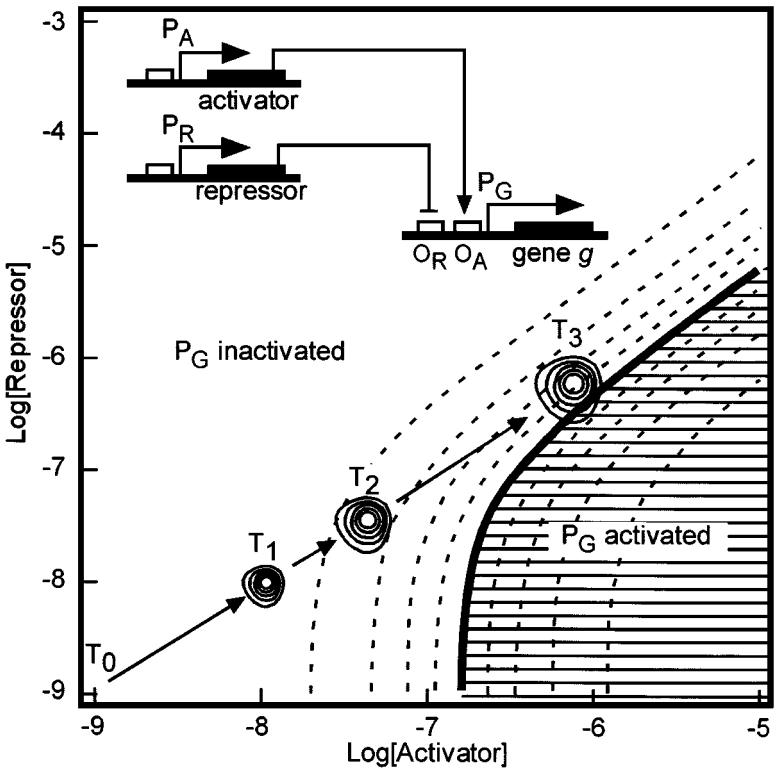

Figure 4 Illustration of differential promoter activation across a cell population resulting from statistical variations in concentrations of competitive effectors. The activator and repressor operons shown are assumed to be independent so that stochastic gene expression mechanisms in each operon produce the respective protein types in a different pattern in each cell. *Small circular contour patterns*, concentration distributions with increasing means at successive times T_1, T_2, and T_3 after activation of P_A and P_R at T_0. Contours of the illustrative activation surface for P_G as a function of activator and repressor concentration are also shown. *Bold line*, 50% activation contour for promoter P_G. This simple example illustrates how normal dispersion of concentrations of controlling proteins can result in differential activation of controlled operons in different cells. Consequences for the cell will depend on the specific kinetic parameters for a particular case and, importantly, on the larger regulatory circuit responding to proteins from the controlled operons.

elements of the regulatory circuitry that determines cell fate. Examples are found in the phage λ and *Bacillus subtilis* cases cited above. In both these organisms, the switching circuits partition the population into subpopulations following different pathways, e.g. fractional commitment to lysogeny or sporulation. Circuits based on bistable genetic regulatory mechanisms are also used in many organisms to produce subpopulations of distinct phenotypes by random inversion of DNA segments (14). Quantitative verification of the role of

molecular-level fluctuations in regulation of these population-level outcomes is a current challenge for experimentalists and modelers.

Modeling Macromolecular Complexes

Multiprotein complexes, such as polymerases, topoisomerases, and ribosomes, are the processing machines that perform the DNA processing and manipulation tasks that are central to execution of genetic regulatory logic. Realistic modeling of the functions of these multiprotein genetic machines is a central challenge in modeling behavior of genetic networks. These complexes, although chemically based and acting to produce chemical changes, behave in many ways more like macroscopic machines than conventional chemical reaction mechanisms. For example, they have to be assembled to function; they can create products from raw materials according to stored instructions (ribosomes and polymerases); they can make macroscopic changes in other cellular objects (integrases and invertases); and many are processive. Heuristic models that capture their dynamical behavior within regulatory networks will differ from conventional chemical reaction models.

Uncertainty of Intracellular Reaction Parameters

The intracellular chemical environment is poorly characterized and probably varies widely from cell to cell in populations and from moment to moment in individual cells. The causes of these variations include the stochastic variations in reaction rate parameters cited above and changes in cellular chemistry as the cell cycle progresses. Except in the best-stirred media, individual cells in a colony can experience wide differences in the local nutrient microenvironment that can affect rates of synthetic reactions or even trigger global regulatory changes within some cells. Furthermore, in growing cells there are significant random components in cell division time, in daughter cell size, and in the partitioning of the molecular endowment between daughter cells. In spite of these perturbations, cells function successfully and regulatory mechanisms have consistent outcomes, suggesting that regulatory circuit designs and the molecular details that determine kinetic parameters are under selective pressure for robust operation in the face of large variations in the intracellular environment. Simulation studies of the signal transduction network in the *E. coli* chemotactic regulatory circuit suggest that that circuit's performance is robust to changes in many parameter values (4, 37). If regulatory circuit designs found in wild-type organisms are indeed selected to have this type of robustness, then construction of adequate simulation models of the circuits may only require rough values for their kinetic parameters. When the "engineering" rules for design of robust regulatory networks are defined, it will be possible to test the proposition that robust performance is a significant source of evolutionary selection pressure.

CHALLENGES AND OPPORTUNITIES

Molecular level models are needed to analyze dynamical behavior of multigene systems, to predict behavior of mutant circuits, and to identify the engineering design principles applicable to design of genetic regulatory circuits. In the near term, the greatest opportunity will be in modeling of bacterial systems. Additional and different mechanisms are present in eukaryotic networks. Key distinctions in eukaryotes include: (*a*) more complex regulatory control regions involving molecular mechanisms that are only beginning to be understood; (*b*) posttranscriptional message processing; and (*c*) compartmentalization of functions, such as separation of transcription and translation, by the nuclear membrane.

Extensive details of many genetic mechanisms are now known in bacterial systems and complete genome sequences are becoming available. Development of simulation models of processes, such as quorum sensing, that involve regulation within the signal transduction networks as well as in the genetic networks will clarify the design principles of cellular regulatory circuits.

In such integrated simulations, the multiprotein complexes that implement genetic regulatory functions (for example, the DNA and RNA polymerase complexes and others that edit and reorganize DNA and RNA) will have to be modeled with heuristic mechanical abstractions that capture their essential regulatory functions. Also, the impact of stochastic intracellular chemistry on regulatory circuit behavior will have to be examined closely. When the underlying design rules for regulatory circuits are understood, it will be far easier to recognize common circuit motifs, to identify functions of individual proteins in regulation, and to redesign circuits for altered functions.

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