QUALITATIVE ANALYSIS OF GENE NETWORKS

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In this paper, we review the qualitative tools developed by our group for the analysis of regulatory networks. Focusing on the dynamical and biological roles of feedback circuits, this method can be applied in the context of both logical and differential formalisms. This approach already led to several interesting results about the relation between the network structure and the corresponding dynamical properties. In particular, it could be shown that at least one positive regulatory circuit is necessary to generate multistationarity (i.e., alternative states of gene expression), whereas at least one negative circuit is necessary to generate a stable oscillatory behavior. Applications to the analysis of complex gene networks, as well as to the synthesis of regulatory models to account for global expression data are discussed.

1. Introduction

This decade will probably be remembered as the "genome decade". Indeed, almost a dozen of microorganism sequences have already been completed, including mainly bacteria but also *S. cerevisiae*. In addition, many other genomic projects are well on their way, including those dealing with Man, Mouse, *A. thaliana*, *C. elegans*, and *D. melanogaster*. However, there is a long way to go from a complete genomic sequence to a functional understanding of the corresponding organism. Even in the case of *E. coli*, the best characterized free-living organism, the recent completion of the DNA sequence let us with a lot of open questions regarding gene function, regulatory mechanisms, or global integration.

Besides genome sequencing, a series of large scale analyses have been initiated, aiming at uncovering the functional organization of cells. In order to disentangle gene regulatory networks at the level of the whole organism, several groups started systematic global studies of gene expression and DNA-protein interactions in different conditions (1, 39, 40). Clearly, such time or space scale snapshots of gene expression in various conditions will be of great help in the delineation of the main regulatory pathways. As a complement to these experimental approaches, there is an increasing need for efficient theoretical tools and formal frameworks to derive regulatory structures from partial expression data (3, 4, 15, 23).

About three decades ago, several groups independently started to develop qualitative tools for the dynamical analysis of gene regulatory networks (5, 8-10). In this paper, we review the work performed at the Université Libre de Bruxelles, leading to the development of a set of theoretical concepts and formal tools which

could be of some help to the global analysis of gene regulatory networks (25-28, 31-38). In the next section, we introduce the key concept of feedback circuit, as well as a description of the classification and the properties of these circuits. The third section is devoted to a brief description of the general logical formalism developed at Brussels. The fourth section briefly discusses the use of the logical formalism vs. the use of the more classical differential formalism. Finally, the fifth section introduces two different uses of our qualitative method: an "analytic" or "deductive" approach which proceeds from the model to its implications, and a "synthetic" or "inductive" approach which proceeds from the experimental data to possible models.

2. Biological and dynamical and roles of feedback circuits

A "feedback circuit" (or "feedback loop") is just a circular chain of interactions. Most often in biology, these interactions have defined positive or negative signs. For any circuit, one can easily check that each element exerts an indirect effect on itself which has the same sign for all elements of the circuit, leading to the definition of the "circuit sign". In fact, this sign only depends on the parity of the number of negative interactions involved in the circuit: if this number is even, then the circuit is positive; if this number is odd, then the circuit is negative.

Biologists have been aware of the interesting properties of specific genetic or biochemical (or mixed) feedback circuits for a long time (e.g., 13, 19). However, the clear delineation of the two classes of feedback circuits and their strikingly different properties is more recent (see Table 1). It has been conjectured (34) and more recently demonstrated (6, 16, 22, 28, 37) that a positive circuit is a necessary condition for multistationarity, and a negative circuit (with two or more elements) for stable periodicity. Biologically, this means that positive circuits are required for differentiative decisions and negative circuits for homeostasis.

Characteristics	Positive circuits	Negative circuits
Number of negative interactions	Even	Odd
Typical dynamical property	Multistationarity	Periodicity
Typical biological property	Differentiation	Homeostasis

Table 1. Main characteristics of positive and negative feedback circuits.

It is essential to clearly realize that appropriate circuits are necessary but not sufficient conditions. Indeed, in order to actually manifest multistationarity or stable periodicity, the system must also display appropriate nonlinearities and proper parameter values. We say that a feedback circuit is "functional" when it actually generates the dynamics corresponding to its sign.

In the context of the logical description, we associate a "characteristic state" to each feedback circuit (or union of circuits), which is defined as the state located at the threshold values involved in the circuit. In fact, it can be shown that the parameter conditions required to have a circuit functional are identical to those required to have the corresponding characteristic state stationary (21).

This concept of characteristic state can be extended to continuous descriptions as follows. When a positive circuit is functional, it usually generates a separatrix. On this separatrix is found one of the steady states of the system, e.g., a saddle point for a two-variable positive circuit. This unstable steady state, always found in association with the property of multistationarity, is thus called the characteristic state of the circuit. Similarly, when a negative circuit is functional, one finds a steady state associated with the periodic motion. This steady state is typically a focus (although it may be an unstable node) in the case of a two-element negative circuit (see also 35 and 38).

3. Kinetic logic and its application to gene networks

Biological regulatory interactions are usually nonlinear, thus rendering analytical approaches problematic. This is why Sugita (24), Kauffman (8-10) and others looked for a qualitative representation of regulatory networks. Our group was led to develop an asynchronous logical formalization whose generalized version can be characterized as follows:

1) Asynchronous updating of the state vector (31).

2) Whenever needed, use of multilevel variables (x_i) and functions (X_i) (33).[1]

3) Explicit consideration of threshold values for the variable and functions (35). Thus, x_i and $X_i \in \{0, s^{(1)}, 1, s^{(2)}, 2, ...\}$; states involving only integer values (0, 1, 2, ...) are called "regular states" (e.g., 00, 01, etc.), whereas states involving one or more threshold values are called "singular states" (e.g., 0 $s^{(1)}$, $s^{(2)}s^{(1)}$, etc.).

4) Use of logical parameters to quantify single interaction or combinations of interactions exerted on a same element (e.g., $K_{i.i}$, $K_{i.ij}$, $K_{i.ijk}$, etc.); these paperameters can take the same values as the corresponding variable x_i (20, 35).

Formally, a regulatory network can be fully described by a set of three matrices, which contains the signs of the interactions, the thresholds associated to these interactions, and the values of the corresponding logical parameters, respectively.

As an illustration, we present below the matrices associated to a simple three-element network, whose concrete concrete nature will be described in section 5. The matrix of interactions is:

[1] Even though logical variables and functions have the same dimension, it is convenient here to assimilate the variables to the presence/absence of the gene products, and the functions to the state 'on' or 'off' of the genes.

	a	**b**	**c**
a	+	+	-
b	+		+
c	-	+	+

in which box 11 (first row, first column) tells us that gene **a** regulates itself positively; box 12 (first row, second column) tells us that gene **b** activates the expression of gene **a**, etc. Just by looking at this matrix, we can identify all the feedback circuits of the system: two positive one-element circuits involving genes **a** and **c**, respectively; three positive two-element circuits involving genes **a** and **b**, **a** and **c**, and **b** and **c**, respectively; plus two negative three-element circuits: **abc** and **acb**. Now, let us consider the following threshold matrix:

	a	**b**	**c**
a	1	1	1
b	2		2
c	1	1	1

in which box 11 tells us that auto-regulation of gene **a** occurs over the first functional threshold of its product; box 21 (row 2, column 1) tells us that the activation of **b** by **a** occurs over the second functional threshold of product **a**.[2] Note that to both genes **a** and **c** are associated three-level logical variables (taking the integer values 0, 1 and 2), whereas a Boolean variable is associated to gene **b**. We use multilevel variables only when it is biologically justified (see section 5). Finally, we introduce the following matrix of logical parameters:

	K_i	$K_{i,1}$	$K_{i,2}$	$K_{i,3}$	$K_{i,12}$	$K_{i,13}$	$K_{i,23}$	$K_{i,123}$
a (i=1)	0	0	0	0	0	1	1	2
b (i=2)	0	1	0	1		1		
c (i=3)	0	0	0	0	1	1	0	2

in which the first column (K_i's) gives the logical weight of the basal expression, i.e., in the absence of activators and in the presence of all repressors (here, all these are considered as null); column 2 ($K_{i.1}$'s) gives the logical weighs of the activation by gene **a** of itself (first row), of **b** (second row), and of **c** (third row), respectively; the other rows define the logical weight of combined actions of the three genes,

[2] In our preceding papers, these first two matrices are compacted into a single one which contains both the interaction signs and the corresponding thresholds.

exerted on a given gene. For example, box 26 (second row, sixth column) gives the logical weight (=1) of the combined contributions of functional levels of the products of genes **a** and **c** on the expression of gene **b**, thus enabling the product of gene **b** to reach its first functional level. Empty boxes correspond to parameters irrelevant in the context of this example. Note that the parameters standing on a same line are not independent. For example, we necessarily have $K_1 \leq K_{1.1} \leq K_{1.12} \leq K_{1.123}$.

Given the three preceding matrices, our simple three-element network is completely defined. On the basis of this matrix, we can fill the state table of the system, which contains the different integer values of the variable vector and the corresponding function values (Table 2).[3]

a b c	A B C
0 0 0	**0 0 0**
0 0 1	**0 0 1**
0 0 2	0 1 1
0 1 0	1 0 1
0 1 1	0 0 2
0 1 2	**0 1 2**
1 0 0	**1 0 0**
1 0 1	0 0 0
1 0 2	0 1 0
1 1 0	2 0 0
1 1 1	0 0 0
1 1 2	0 1 0
2 0 0	1 1 0
2 0 1	0 1 0
2 0 2	0 1 0
2 1 0	**2 1 0**
2 1 1	0 1 0
2 1 2	0 1 0

Table 2. State table for our three-variable network.

The left column ("state" vector) simply lists all possible "regular" or "integer" states of the system. As the system comprises one binary and two ternary variables, there are 3*3*2=18 combinations or rows. The right column ("image" or "function" vector) gives the corresponding values of the functions A, B and C. Whenever the variable and function vectors have identical values, we have a stable state. Thus,

[3] More details on logical parameters and functions are found in 35 and 37, including a detailed explanation of the derivation of the function values from the matrices .

our table contains 5 “regular” stable states, which are written in bold in the table. In addition, the system comprises four “singular” steady states, which are all unstable: $S^{(1)}00$, $00S^{(1)}$, $S^{(2)}S^{(1)}0$, and $0S^{(1)}S^{(2)}$ (not shown). Note that each of these singular steady states are located on the thresholds corresponding to a feedback circuit; e.g., $S^{(1)}00$ is located on the threshold corresponding to the auto-activation of gene **a**.[4]

To any other regular values of the state vector corresponds at least one order of commutation. For example, the image of state 002 is 011. This means that there is an order to express gene **b** and to slow down expression of gene **c**. In such case, we consider that, depending on the corresponding synthesis and degradation delays, the following state can be either 012 or 001. Indeed, it is very unlikely that the two corresponding delays of commutation would be exactly identical. Thus, we opt for a fully asynchronous updating of states, which in fact includes the synchronous approach as a particular case.[5]

At this stage, we have described how to proceed from a defined regulatory system to the corresponding qualitative dynamics. Note that even in this case we need only qualitative data about the regulatory system studied: signs of the interactions, orders of the corresponding thresholds, interaction weights.

However, in most concrete cases, even such qualitative data are partly lacking. In particular, thresholds and weights are poorly estimated. In this case, the logical approach still constitutes a powerful tool to consider alternative models. First because the parameter and variable spaces consist of finite numbers of discrete values, rendering an exhaustive analysis tractable. Second, because it is possible to proceed directly from the matrix of interaction and make a “feedback circuit analysis” of the system.

Indeed, as mentioned earlier, feedback circuits clearly constitute the key dynamical determinants of regulatory systems. In the context of the generalized logical formalism, by hand or using a computer program, one can derive the parameter constraints for any feedback circuit to be functional, i.e., to generate multistationarity if the circuit is positive, to generate one single attractor if the circuit is negative. In addition, compatibility between different functional circuits can be inferred just by checking whether the corresponding sets of parameter constraints are consistent.[6]

In fact, the values included in the matrix of parameters above were chosen such that both one-element and all three two-element positive circuits are simultaneously functional, at least in part of the variable space, whereas the two negative three-variable circuits are not. This parameter set thus maximizes multistability in the

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[4] More information on singular steady states and feedback circuits are found in 21 and 38.
[5] Definitions of commutation delays are included in 33 and 35.
[6] For a detailed explanation about how parameter constraints for circuit functionality are computed, see 35 or 37. For a brief description of the computer algorithm, see 25.

system, leading to the five regular stable states and the four unstable singular steady sates mentioned above.

It is possible to check that the multistable behavior directly depends on the functionality of the positive feedback circuits. Indeed, changing some of the parameter such that the functionality of some of these positive circuits is lost, irremediably leads to the simultaneous loss of some steady states of the system.

Note that, as no negative feedback circuit is functional for the parameter values chosen, there is no trace of homeostatic or cyclic behavior. In fact, we found that there is no parameter combinations such that any of these negative circuits would be functional simultaneously with all five positive circuits.

4. Logical vs. differential modeling

The generalized logical formalism has already been used to model various biological regulatory networks, including gene networks, immunological systems, neural networks, etc. (14, 26, 27, 33, 35, and references herein). In many cases, the logical approach proved to be convenient, first because of the qualitative dimension of the data available, second for the availability of a fully analytic approach. Thus, the logical method and the feedback loop analysis certainly constitute an interesting alternative to differential approaches. On the other hand, the logical approach can also be used to get a first overview of the dynamical properties of a set of differential equations, thus helping to the building and refinement of the corresponding model.

In the differential context, a feedback circuit is formally defined as a combination of terms of the Jacobian matrix of the system, with indices forming a circular permutation. Whenever these terms have fixed signs, a plus or minus sign can be attributed to the circuit, i.e., the sign of the product of the terms involved in the circuit. We have to mention here that the relation between feedback circuits and dynamical properties is perfectly established only in the context of the logical systems or of piece-wise linear differential systems (see, e.g., 20). Nevertheless, even in the case of other type of differential systems (e.g., including linear functions, various non-linearities, etc.), a logical caricature may often be established, leading to a first delineation of the dynamical properties of the corresponding differential system.[7] Dynamical differences are then regularly observed, but these are usually easily understood by analyzing the most striking differences between the original differential system and its logical schematic.

[7] One example of this use of the logical approach can be found in (14), dealing with the modelling of the neuro-endocrine regulation of the immune response.

5. Analytic vs. synthetic approaches

In the section 3, we presented a logical model in terms of predefined matrices of interactions and thresholds. From there, we mentioned two approaches: According to the first one, logical parameter values are chosen in order to perform a simulation and check the dynamical behavior (steady states, commutation orders, etc.). Following the second approach, one first identifies all feedback loops of the system, then computes the constraints on the logical parameter to have each of these circuits functional, to finally check their compatibility. In this latter case, reasoning in terms of dynamical properties and circuit functionality helped us to derive appropriate sets of logical parameter values. Nonetheless, both approaches start from a matrix of interaction to look for some specific dynamical properties, thus falling in the category of "analytic" or "deductive" approaches.

However, it is often the case that one wants to proceed instead from the data to the models, in other words to use a "synthetic" or "inductive" approach (see also 33 and 35). To introduce such a synthetic use of the logical method, let us mention a simplified example coming from plant developmental genetics.

In *Arabidopsis*, flowers are composed of four different types of organs: sepals, petals, stamens and carpels. Mutations studies led to the proposition of a combinatorial model called the ABC model (2). Following this model, each organ would result from one or two combined functions chosen among three: functions A, B and C. Sepals would be produced under function A alone, petals production would depends on both functions A and B, stamens on functions B and C, whereas carpels would result from function C alone.

Now, let us take this idea seriously and ask if it is possible to derive a consistent 3-variable logical model, each variable corresponding to one function or to one representative gene. As we will see, thinking in terms of functional circuits really proves here to be of great help. If we think of the four organs as stable states of the system, we are led to the following propositions:

1) As products of genes A and C are never found simultaneously, we will include a cross-inhibition between them, thus leading to a two-element (minus*minus) positive circuit.

2) Products of genes A and C should be able to maintain themselves at a functional level, to give rise to sepals and carpel, respectively. The easiest way to build such maintenance device is by means of positive auto-regulations, i.e., by means of two one-element positive circuits.

3) We need a cooperative expression of functions A and B on the one hand, of B and C on a other hand, to produce petals and carpels, respectively. The easiest way to obtain it is by mean of positive cross-regulations between these two pairs of genes, thus making two additional (plus*plus) positive circuits.

4) Taking into account that maintenance of products A or C should never occur in the presence of each other, but well even in the absence of B, we are led to propose higher thresholds for the cross-activations.

In fact, it is this reasoning which led us to the matrices of interaction and thresholds presented in the section 3. Now, if we require all positive circuits to be functional, we are led to the parameter values selected in the same section 3. Note that, in addition to the four stable states corresponding to the differentiated states, A (100), AB (210), BC (012) and C (001), we found an additional stable state, 000, which would correspond to the default state, i.e., to the absence of flower organ. For sure, the model derived here is too simple. Indeed, at least eleven regulatory genes have already been involved in the differentiation of *Arabidopsis* flower organs. However, as far as the ABC model remains roughly correct, more realistic models will probably include the corresponding feedback structure. [8]

Note that the logical method can also be used in a mixed analytic-synthetic way, for example to check, modify or complete logical models by comparing them with partial kinetic data. Such mixed approach could be useful for the derivation of models from global gene expression studies.

6. Conclusions and perspectives

In this paper, we meant to review the methodology and the formal tools developed by our group to derive and analyze dynamical models of regulatory networks. Building on qualitative data, this approach can consequently generate only a qualitative dynamical description of the corresponding regulatory system. However, in many cases, such qualitative description constitutes already an important step in the understanding of the complex regulatory networks involved.

Indeed, biological networks usually involve large numbers of elements, which are interconnected in various ways. In the context of molecular genetics for example, only small and specific concrete gene networks have already been thoroughly analyzed (11, 17, 18, 26, 27, 33, 35). However, the whole gene network of an organism ranges from some hundreds (bacteria) to thousands (eukaryotes) of regulatory genes. In addition, when looking at global cellular protein or mRNA snapshots, the regulatory factors are themselves buried among still many more structural products.

In order to be able to disentangle gene networks, one would need experimental tools to distinguish systematically between regulatory and structural products. In this respect, one could possibly use some of the generic properties of regulatory factors, e.g., their affinity for DNA sequences or for other proteins, or the presence

[8] See (12) for the presentation and the analysis of a model taking into account the genetic data available.

of typical domains (HTH, Zn-fingers). In addition, one might look for statistical tools enabling the uncovering of correlation in the expression of clusters of genes, leading to a reduction of the number of variables to be taken into account (4). Finally, efficient integration of regulatory data, e.g., in the context of dedicated interactive databases, could also constitutes an important factor in order to combine the most accurate regulatory data with a synthetic modeling approach (7).

Probably, all these approaches and tools will have to be used simultaneously. At this stage, the remaining amount of work to be done might look titanic. Indeed, we are still very far from being able to derive whole cell regulatory structures from global gene expression data. However, to end with an optimistic note, we would like to mention here two recent results which together suggest that the complexity faced could be lower than expected.

The first result comes from a preliminary analysis of *E. coli* transcriptional regulatory network (29). As about a quarter of this network is already characterized, one can get some idea of its topology. On the basis of an extensive database and literature review, a matrix for transcriptional regulation in *E. coli* has been build and some striking structural properties have been found. First, the connectivity (i.e., the number of regulations per gene) is rather low, lying somewhere between 2 and 3 in the case of the network formed by all known transcriptional regulating/regulated genes. Second, only one-element feedback circuits were found, involving as much as the half of *E. coli* regulatory genes.

On the other hand, the analysis of Boolean networks, including logical parameters as defined above, led to another interesting result: when compared to the gigantic number of possible consistent parameter combinations, the proportion of combinations allowing a circuit of a given length to be functional was found to decrease drastically as the number of element increases (30).

Both results tend to a common interpretation: when looking at the level of whole cell gene networks, the regulatory structures are probably reducible to many small and weakly interconnected regulatory modules, rather than forming intertwined networks. In fact, such an interpretation is certainly consistent with the astonishing productivity of the reductionist approach in molecular genetics. Indeed, if one can so easily isolate mutants for some specific function without affecting irremediably most of the physiological properties of the organism, it means that these properties are controlled by independent regulatory pathways.

Acknowledgments

We wish to thank Luis Mendoza and Roland Somogyi for sharing preprints.

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