To be or not to be active: the stochastic nature of enhancer action

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Summary
Transcriptional enhancers are traditionally considered to regulate the rate at which a linked promoter transcribes mRNA, but recent experiments suggest a reevaluation of this model is necessary. Single-cell assays of transgenes reveal that enhancers increase the probability that a reporter gene will be active, but have little or no effect on the transcription rate once a gene has been activated. These results raise the question of how enhancers affect gene expression in their native contexts. A simple interpretation is that enhancers act in a stochastic fashion to increase the probability that a regulated gene will be transcribed; such a model is compatible with programs of cell differentiation in which multiple similar cells subject to similar environmental stimuli do not respond uniformly. BioEssays 22:381–387, 2000. © 2000 John Wiley & Sons, Inc.

Two different views of enhancer action
Transcriptional enhancers in higher eukaryotes are defined as DNA elements that increase transcription when placed at a distance from and in any orientation relative to a promoter. An enormous body of work has characterized enhancers flanking tissue-specific genes, as well as nuclear factors that bind such enhancers and mediate their function, and much evidence suggests that enhancers play critical roles in determining gene expression in differentiated cells (reviewed in Ref. 1). Two contrasting views of how enhancer elements act have been proposed following early work with the SV40 enhancer. The most popular view is that enhancers function by increasing the rate at which transcription of a linked gene is initiated, and we will refer to this as the “rheostatic” or rate model (Fig. 1). This model proposes that the proteins that bind to enhancer regions increase the rate of transcription by directly contacting the complex of proteins that forms around the TATA box, regardless of the distance of the enhancer from the core promoter. Current textbook discussions of enhancer activity do not address the veracity of the rheostatic model, and it has permeated thinking on transcriptional control to the extent that many are unaware that there is any alternative. Nevertheless, the evidence concerning the actual effect of an enhancer on transcription is complex and subject to multiple interpretations.

An alternative view of enhancer action, which will be discussed here, is that enhancers do not affect transcription rate, but instead act to increase the probability that a linked unit will establish and maintain transcriptional activity (Fig. 1). This may be termed the “binary” or probability model. Enhancers of pol III-transcribed rDNA have long been thought to act in this fashion but it has never been widely accepted for enhancers of promoters that use pol II. The binary model leads to different conclusions about the role of enhancers in gene regulation. Most experimental data are consistent with either model; in fact the two models are not mutually exclusive, so that both mechanisms might apply in some cases. A small body of recent work that has attempted to distinguish between the two interpretations favors the “binary” model, however. Here we review the experimental evidence bearing on this issue, and discuss some of its implications.

Evidence from gene transfer experiments
The concept of the enhancer as a regulator of the rate of transcription initiation arises from studies of the 72 bp repeats in the SV40 early promoter region. A series of experiments found that inclusion of these repeats, which came to be termed the SV40 enhancer, anywhere on a transfected plasmid increased transient expression of a reporter gene. At least three ways by which the SV40 enhancer might increase transcription were considered: a direct increase in transcription from the linked promoter (rheostatic model); an increase in the probability of the promoter becoming active (binary model); and an effect on setting the proper start site of transcription. Enhancers increase the amount of transcript produced by transfected

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cells in nuclear run-on assays; although interpreted as support for the rheostatic model, this observation does not really distinguish between the rheostatic and binary models.\(^{8,9}\) The rheostatic model subsequently became dogma, and numerous sequences with enhancer activity, defined as increased transcript production in transient transfection assays, have been identified within and around genes in higher eukaryotes. All enhancers contain binding sites for transcriptional activators, which are usually defined in the same assays as the enhancers themselves and are assumed to affect transcription by the rheostatic mechanism. Recent work detailing the role of chromatin modification in transcription has not greatly affected this view.\(^{1}\)

In considering the possibility that the “classical” enhancer effect (increased expression from a transiently transfected plasmid) actually occurs through a binary rather than rheostatic mechanism, it is necessary to consider what the assays really tell us. A typical transient assay involves transfection of a plasmid into a dish of cells; after a period of time some or all of the cells are rendered into an extract which is assayed for mRNA or a gene product (such as chloramphenicol transferase, luciferase), or a product secreted into the medium is assayed (such as human growth hormone). Such bulk biochemical assays do not permit distinction between binary and rheostatic modes: either will result in an increase in the total assay product. This is also true of in vitro transcription reactions. As there is no way of knowing how many templates in the reaction are transcribed, any increase could be due either to increased polymerase loading (rheostatic) or to recruitment of otherwise inactive templates (binary). Likewise, bulk biochemical assays of expression from stably integrated transgenes in cell lines or mice cannot differentiate the two modes. It is not possible to know if a control element increases the proportion of cells that express a transgene, or the level of expression in individual cells, unless one analyzes expression in single cells. In fact, when single cell analysis is applied to transgene expression in mice, mosaic expression (variegation) is often found.\(^{10–12}\) Some tissue culture assay systems also permit analysis of expression on a cell-by-cell basis and such studies support the binary model of enhancer action.\(^{13–18}\) with at least one report suggesting that an enhancer influences both the level and the probability of expression.\(^{19}\)

Two of the earliest descriptions of the SV40 enhancer effect used immunostaining of reporter genes to assay expression; both noted heterocellular expression and a sharp increase in the number of stained cells when the transfected construct carried the enhancer.\(^{6,7}\) However, it was not possible in either study to say if this increase was due to an increase in the proportion of transfected cells in which the plasmid became active (binary), or a result of a detection threshold in the immunostaining assay (rheostatic). Later, Weintraub re-examined the effect of the SV40 enhancer using a quantitative method to measure immunostaining of T antigen and concluded that the enhancer had only a slight effect on transcription rate, but greatly increased the probability of a transfected plasmid becoming active.\(^{18}\) As this experiment was published after the point at which a consensus had formed in favor of the rheostatic model, it had no discernible impact on the dominant paradigm, nor did a
later report showing that globin enhancers do not affect expression levels of stably-integrated neoR constructs.\textsuperscript{(20)} In recent years, the issue has been revisited with the aid of newer technology. β-galactosidase (β-gal) expression can be measured with extremely sensitive bulk biochemical enzymatic assays; combination of these with flow cytometry (FACS) permits a clear distinction between cells that express β-gal and those that do not.\textsuperscript{(21,22)} Using this method in a transient transfection assay, we found that the SV40 enhancer greatly increased the probability that a transfected plasmid would express β-gal, but had little effect on the amount of expression in a single cell.\textsuperscript{(16)} In a colony assay (which measures plating efficiency when the reporter is a drug resistance marker), both the SV40 enhancer and a strong globin enhancer (5’HS2) increased the probability of expression after integration, but neither altered the amount of expression from integrated constructs.\textsuperscript{(16)} Deletion of an enhancer from an integrated construct with a site-specific recombinase had only a slight effect on expression level, but greatly decreased the percentage of cells expressing the transgene.\textsuperscript{(17)} Similarly, whilst stimulation of an inducible enhancer maintained transgene expression in a high proportion of cells it had no effect on expression level in individual cells.\textsuperscript{(17)} In transgenic mice, where variegated silencing of transgenes is common,\textsuperscript{(12)} a globin enhancer suppressed silencing of a lacZ transgene in erythroid cells, but had no measurable effect on the level of expression in cells where the transgene was active.\textsuperscript{(10)} Similar results were obtained with a gp91phox transgene, although the elements responsible for suppressing transgene silencing were not characterized.\textsuperscript{(23)}

The evidence above indicates that, when a distinction between the rheostatic and binary modes of enhancer action is possible, enhancers seem to act by the binary mode. Thus it seems likely that, in the assays where they were originally defined, enhancers increase expression by raising the probability that a construct will become transcriptionally active and stay active. This stochastic effect on gene expression illustrates the fact that in two cells with identical transcription factor environments, two completely different outcomes may occur. Even in the presence of an enhancer, a gene will be active in some cells but not others. This fact may have broader significance, as we will discuss later.

The mechanism by which an enhancer exerts an effect is likely to involve the ability of transcriptional activators to disrupt chromatin structure, and there is considerable recent evidence bearing on this issue (reviewed in Ref. 1). However, this does not necessarily require direct contact between enhancer-bound factors and the promoter. The interpretation that enhancers act by increasing transcription rate suggested a direct action by these factors on the polymerase complex, which prompted the idea that enhancer-bound factors directly contact the initiation complex via a “loop” of intervening DNA.\textsuperscript{(24)} Textbook descriptions of enhancers tend to provide a neat cartoon of one or a few proteins binding a distant site, looping out the intervening DNA and contacting the initiation complex to increase the rate at which transcription is initiated.\textsuperscript{(2)} Although there is much biochemical evidence for the interaction of enhancer-binding factors with components of the initiation complex, long-range looping has never been clearly demonstrated in eukaryotes. The complex arrangement of enhancers around many genes makes a simple looping mechanism difficult to envision, and the model less compelling.

If enhancers do not regulate the rate of transcription directly but, rather, effect an on/off switch, other mechanisms, such as chromatin opening or nuclear compartmentalization, become more attractive. It has long been known that active enhancers disrupt chromatin structure. There is, by now, considerable evidence that silent genes are associated with heterochromatin, leading to the model of a nucleus containing a “silent” compartment (heterochromatin) and an “active” compartment in which transcription occurs.\textsuperscript{(25)} The association of the silent genes with telomeres and SIR proteins at the nuclear periphery in yeast\textsuperscript{(25)} as well as the association of the silent brown dominant gene with centromeric heterochromatin in Drosophila.\textsuperscript{(26)} are examples of such compartmentalization. Furthermore, the 5’HS2 globin enhancer, which suppresses transgene silencing, causes the transgene to move away from centromeric heterochromatin in cultured cells.\textsuperscript{(27)} Thus, an enhancer may effect binary gene regulation by removing a gene from heterochromatin, which silences transcription. It might accomplish this by moving a linked gene to an active compartment, or by disrupting non-homologous pairing with heterochromatin, so permitting a gene to enter the active compartment.

At this point, it is worth asking how the rate of transcription is regulated if enhancers do not perform this function. There is considerable evidence that genes are transcribed at different rates, and that these rates can be altered. Reporter genes integrated into different locations in the genome are expressed at modestly different levels when there is only a single template in a clone and all cells express the gene product encoded by that template.\textsuperscript{(28)} This is an example of the “stable position effect” distinguished from variegating position effect (silencing in a proportion of cells) by Lewis.\textsuperscript{(29)} It is not clear how the integration site influences transcription rate. It has been assumed that enhancers are responsible, but their failure to regulate the transcription rate in gene transfer experiments casts doubt on this. Certain genes can be induced from a basal level of expression to a higher level in situations in which the binary and rheostatic models can be distinguished, and these experiments suggest that the promoter and its upstream activator sequences can influence transcription rate.\textsuperscript{(28,30)} Thus, other than the promoter, there

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are no obvious candidates for discrete elements that regulate transcription rate directly. However, the artificial conditions of the in vitro and transgenic assays do not reproduce the normal context of endogenous enhancers, where all elements have evolved to function together in a specific locus. It is necessary to ask if enhancers behave differently in their native contexts than they do in gene transfer assays.

Enhancers in their native contexts
The ability to perform homologous recombination in embryonic stem (ES) cells, and generate mice from those cells, has made it possible to delete transcriptional control elements in their native contexts. Such experiments constitute the most rigorous test of the function of an element, but may be considerably more complex than analysis of a reporter gene. Many genes and cell lineages do not lend themselves to single-cell analysis of gene expression, but this type of analysis is necessary for accurate distinction between an enhancer’s binary and/or rheostatic effect. While several enhancers have been deleted, only a small set provide clear evidence of an enhancer’s effect on transcription rate. Complex multigenic loci such as β-globin, immunoglobulin, and T-cell receptor (TCR) have been the most thoroughly studied by homologous recombination, following initial identification of their enhancers in transfection and transgenic assays.

While it is difficult to draw strong conclusions about the mode of enhancer action from most of the enhancer deletion experiments, some have clearly demonstrated a decrease in gene expression in individual cells. Deletion of the intronic κ enhancer led to a more than tenfold reduction in the number of κ-expressing B-cells; however in those resting B-cells expressing κ, the level of κ expression on the surface was normal. Deletion of the 3′κ light chain enhancer caused only a mild (two to threefold) reduction in surface expression in resting B-cells that expressed κ; even this mild reduction disappeared when the B-cells were stimulated, however. Deletion of the Eκ TCR enhancer led to a 20-fold reduction in the number of αβ T cells, consistent with an effect of reducing the probability of germ line transcription and associated rearrangement. This study found a sixfold decrease in the expression level of the α TCR receptor in sorted αβ T cells that expressed TCR and a fourfold reduction in expression at the closely linked TCR δ locus, but no impairment of δ rearrangement. These experiments suggest that at least some enhancers have both binary and rheostatic effects, but that their influence on transcription rate is not large. The recently published deletion of the entire locus control region (LCR) from the murine β-globin locus provides more evidence of an effect on transcription rate, although in this case the deletion was very large (over 24 kb). The LCR consists of a series of elements that resemble enhancers in structure; some act as enhancers in vitro although they do not increase transcription rate in transfection studies. Surprisingly perhaps, in the absence of the entire LCR the endogenous murine β-globin locus establishes and maintains activity, all of the genes are transcribed, and developmental regulation appears intact: this result defies all current dogma on globin regulation. Of interest to this review, however, is the fact that deletion of the murine LCR results in a decline in the level of globin transcription of from fourfold to greater than 20-fold. Absence of the LCR may result in earlier silencing of the globin locus during terminal differentiation, which would produce an apparent decline in transcription rate. However, there is a fourfold decline in transcription levels in cultured cells, where it can be established that every cell is expressing. Activity of the deleted locus in all erythroid cells, at least in the early phases of differentiation, may reflect the presence of numerous supportive elements elsewhere in the locus.

A recent experiment by Ronai and colleagues provides the strongest evidence to date for a binary mode of action by an enhancer in its native context. They studied hybridomas in which the Eκ core enhancer, and/or the flanking matrix attachment regions (MARs) and switch region (Su), was deleted from the major intron of the immunoglobulin heavy chain locus. Deletion of Eκ, but not of the flanking elements alone, was associated with variegated silencing of the immunoglobulin μ gene, with no apparent decline in the level of μ expression in positive cells. This study is the only one to have used a system permitting an unambiguous test of the binary model, and provides strong support for it.

Taken as a whole, the experimental evidence suggests that the ability of enhancers to suppress silencing of linked genes is autonomous and independent of the context of the integration site, and that their effect on transcription rate (when it occurs) is context dependent. In the native context enhancers may often, if not always, have a role in setting transcription rate, but when placed in ectopic contexts this effect is minimal. Since this rheostatic effect does not occur in all contexts, this implies that it is an indirect effect. This may be contrasted with the binary effect of an enhancer, which is not context dependent: in gene transfer experiments, enhancers integrated at randomly selected sites invariably suppress gene silencing. Many more enhancer knockout experiments are needed, however, to substantiate these initial conclusions.

Stochastic action of enhancers in cell differentiation and development
The evidence discussed above supports the view that enhancer elements that flank many genes increase the probability that the gene will become active or stay active. That the enhancer may change a probability, rather than create a certainty, is a point that may be difficult to grasp, since gene expression is typically considered to be uniform in
cells of a given lineage. However, in some cases a regulatory signal may change the probability of a gene being expressed without raising that probability to 100%. This does not require that the cells in a population differ in any way prior to the switch in gene regulation, merely that the switch occurs in some cells and not others, creating differences between cells that were originally identical. The stochastic activation or silencing of a gene that has a critical effect on cell fate may be a key factor in programs of cell differentiation. For many genes, such as immunoglobulin or TCR, the enhancers associated with the genes must function to create a certainty that the gene will express in the correct cell type at the correct developmental stage, since failure to express will effectively prevent the differentiation of that cell: B cells will not develop without Ig and T cells will not develop without TCR expression. There are many situations, however, in which expression of a particular gene is not mandatory for appropriate development, but rather represents a developmental decision or a graded response to an inductive signal. Thus, the decision to express a gene can differentiate a cell from its neighbors or determine whether an effector cell responds to a stimulus or not. What is the evidence for such mechanisms?

There is considerable evidence that the response of individual cells to inductive signals varies in a way that is consistent with a stochastic response at each inducible allele. A variety of studies have demonstrated situations in which cells, in an apparently homogeneous population and subject to apparently identical stimuli, do not respond with uniform induction of gene expression. The expression of ApoB in the avian liver is responsive to estrogen, and this induction is achieved by recruitment of hepatocytes from a silent to an expressing state. The induction of enzymes in the ornithine cycle (carbamoyl synthetase, phosphoenolpyruvate carboxykinase, and arginase) by glucocorticoids in the ornithine cycle (carbamoyl synthetase, phosphoenolpyruvate carboxykinase, and arginase) by glucocorticoids in cultures of fetal hepatocytes is hetero-
cellular; with induction of the acute phase, hepatocytes are recruited to the expressing pool in a stochastic fashion. Similarly, induction of cytokines by antigen presentation in clonal populations of T cells is stochastic, and most cells express only one of the multiple cytokines expressed by the clonal population.

While compelling, studies such as those cited above are limited by the argument that the equality of the cells and the stimulus can be inferred but not proven. However, recent reports have provided more detailed evidence of stochastic gene regulation. Newlands and colleagues examined expression of both endogenous genes and muscle-specific transgenes in the individual nuclei of multinuclear myofibers, and found that the genes were expressed in only a subset of nuclei despite the fact that all were part of a single cell. Nuclei in which a given muscle-specific gene was not expressed were still transcriptionally competent, and within an individual myofiber the loci were expressed independently, although expression of individual alleles was not assessed. These findings demonstrate stochastic regulation of multiple genes in myofibers, such that, within a given nucleus, a gene is either on or off. During differentiation and development, the proportion of expressing nuclei in a fiber changes, again suggesting that stochastic regulation of a binary switch is used by the fibers to control levels of a gene product.

While the study of Newlands and colleagues is consistent with the notion that expression of some muscle-specific genes is a regulated stochastic process, it might be argued that the nuclei in myofibers are not subjected to uniform stimuli. This objection is answered in a study of IL-4 expression in T cells, by demonstration that individual alleles of a gene are regulated independently and stochastically within the same nucleus. Expression of IL-4 by some T cells after certain stimuli helps to mold the associated immune response, and is not required for the survival or basic function of a T cell. One allele of the IL-4 gene in mice was replaced with a cDNA encoding a cell surface marker, thus permitting independent assessment of the expression of the two alleles in individual T cells. Flow cytometric analysis demonstrated that the two alleles are regulated independently: in many cells expression is monoallelic, and the frequency of biallelic expression can be predicted from the expression frequency of individual alleles. Stimulation of the T-cell receptor (TCR) increases IL-4 expression by increasing the number of cells expressing either of the IL-4 alleles, and the frequency of biallelic expression rose correspondingly. This study suggests that IL-4, a factor that is expressed facultatively by T cells, is regulated in a stochastic fashion, and that the effect of a stimulus applied to all cells in a population is to increase the proportion of the cells that activate one or both alleles of IL-4. A similar finding has recently been reported in a study of Pax5 expression in B cells.

These reports suggest that stochastic regulation of gene expression is a feature of programs of cell differentiation and induction, and there is also theoretical support for this view. While this provides no clue to the regulatory elements controlling the stochastic process, other evidence discussed above suggests that transcriptional enhancers are likely to have a key role since, in situations where individual gene alleles can be assessed, enhancers and the factor which bind them alter the probability of gene expression. This property may be exploited in situations where some, but not all, cells of a given type must adopt a certain lineage fate; the number of such situations is
potentially very large. A theoretical example is the commitment of some stem cells to a specific lineage whilst others are maintained in an uncommitted state (Fig. 2). For example, a gene that is associated with commitment to differentiation has a 70% chance of being active in any particular cell in the pool of pluripotent stem cells, a probability determined by the cis-acting enhancers flanking the gene and the transcription factors binding those enhancers in response to inductive signals. In this case, 30% of the cells will remain pluripotent, maintaining a population of stem cells, but inductive signals may act on the enhancers to alter this probability. This model avoids the need to invoke special niches in the local environment in which conditions are favourable to maintain undifferentiated stem cells. While the “niche” model predicts that committed and uncommitted cells will be found in specific patterns with respect to each other, the stochastic model predicts that committed cells are scattered randomly among uncommitted cells. An example of the latter is the random location of cells fated to form the neural tube in the chick epiblast.\(^\text{[54]}\)

**Conclusion**

A variety of experiments have shown that transcriptional enhancers act on transferred genes to increase the probability of their establishing and maintaining expression. There is good reason to suppose that these results reflect the function of enhancers in their normal contexts. Thus, it seems likely that a major function of enhancers is to ensure, or increase the probability, that genes are transcribed actively in the appropriate lineage. Further experiments to confirm this hypothesis are necessary, and might involve the deletion of enhancers from genes that are expressed in only a subset of cells of a certain lineage, when expression of the gene can be analyzed in single cells. The influence of enhancers on the rate of transcription remains an open question.

The present state of knowledge does allow us to speculate on the biological utility of a stochastic function for enhancers. In some circumstances, these elements may be sufficiently powerful to ensure expression of a gene in all cells of a lineage. In others, however, the elements flanking a gene may be sufficient to establish expression in only a proportion of cells; this property could be used to regulate the behavior of populations of cells in a given lineage, and to drive programs of cell differentiation.

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**References**


