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Determinator–inhibitor pairs as a mechanism for threshold setting in development: A possible function for pseudogenes

(cellular determination/segmentation/pattern formation/sense–antisense RNA/evolution)

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ABSTRACT **Thresholds are frequently thought to be involved in the development of discrete structures in response to a shallow, monotonic gradient of morphogenetic information. We propose a mechanism for threshold setting that incorporates two essential components: (*i*) determinator genes that produce intracellular "determinators" that control cellular differentiation during development and (*ii*) intracellular "inhibitors" that bind tightly and specifically to the determinators to form "determinator–inhibitor pairs" that are inactive with respect to determinator function. The interaction of these components amplifies the intracellular response to an extracellular morphogen, thus producing a sharp transition in determinator gene activity. This system could operate at either the RNA level with the determinator–inhibitor pairs taking the form of sense–antisense RNAs or at the protein level via a competitive inhibition mechanism. In either case this model suggests a possible role for pseudogenes in development as a source of the intracellular inhibitors.**

The process of development in complex multicellular organisms is characterized by a series of quantized decisions as cells become progressively more restricted in their potential (1). Typically these decisions represent choices made at bidirectional branch points in pathways leading to development of different cell lineages and/or discrete structures (2, 3). In many cases, these decisions seem to be based on the interaction of an extracellular source of morphogenetic information and an intracellular mechanism that facilitates an appropriate response to the extracellular morphogen (4–8).

The concept of morphogenetic information in the form of a shallow, monotonic gradient extending over a large distance has frequently been imposed as the extracellular phenomenon governing ordered differentiation in embryogenesis (4–15). The intracellular response is assumed to involve specific macromolecules that have been called "determinators" (16). These determinators establish cellular commitment or determination, by interacting with, and thus predisposing the eventual expression of, an appropriate set of "differentiation" genes that are ultimately responsible for the development of an observable cellular phenotype. In cases where such differentiation involves the development of segments or other discrete structures, it has been proposed that the intracellular response involves thresholds that translate the monotonic gradient into a step function (17). However, a specific mechanism defining this threshold effect at the molecular level, so as to account for specific gene expression, has yet to emerge.

A key requirement of an intracellular threshold mechanism is the production of sharp transitions in response to the monotonic morphogenetic signal. The direct transcriptional response of a determinator gene to a regulatory molecule as described by the mass action equation does not give step functions. For example, transcription of the *lac* operon is proportional to the fraction of *lac* operator free of lac repressor (18). In this example, and others, allostery sharpens the response to the inducer, but the transition is still not sufficiently acute (17, 18). Thus an additional mechanism seems necessary to satisfactorily account for the signal amplification and threshold setting required to control the quantized decisions during development of multicellular organisms. We have found that by imposing the presence of an intracellular macromolecular inhibitor capable of tightly complexing with the determinator gene product, the necessary sharp intracellular transition can be realized. The system then becomes more akin to a buffered acid–base titration. Furthermore, by postulating different determinator–inhibitor pairs (D–I pairs) controlling the development of different discrete structures, the occurrence of multiple thresholds in response to a single gradient is facilitated.

There are two paramount requirements of the inhibitor molecules in this mechanism: each must be highly specific for its paired determinator molecule, and the binding coefficient for each pair must be relatively high. These requirements could be met either at the RNA level via an interaction of sense–antisense RNA or at the protein level, perhaps on the basis of competition between functional and nonfunctional subunits. In either case this suggests a potential function for pseudogenes not previously considered. Thus we propose that pseudogenes could play a significant role in morphogenesis as a source of intracellular inhibitor molecules.

The D–I Model

A fundamental version of our model is shown in Fig. 1. This version assumes an extracellular morphogen present in a monotonic concentration gradient and responsible for bestowing an overall order upon cellular differentiation within a particular morphogenetic field, and an intracellular (or intranuclear) mechanism that mediates the appropriate response to the extracellular morphogen. Central to the intracellular response mechanism are a determinator that is transcriptionally regulated, its paired inhibitor that is produced constitutively, and a cytoplasmic receptor that interacts with the morphogen as it enters the cell. The determinator is the intracellular macromolecule ultimately responsible for setting commitment to a certain cell lineage or differentiation pathway. The inhibitor is an intracellular macromolecule that binds tightly to the determinator forming a complex (D–I) that is inactive with respect to determinator function. The receptor complexes with the morphogen to form a receptor–morphogen complex that, in this version of the model, acts to repress transcription from the determinator gene. Clearly, activation rather than repression could be readily accommodated. The key point is that the rate of transcription of the determinator gene directly reflects the level of morphogen in the extracellular environment. Actual

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Abbreviation: D–I, determinator–inhibitor.

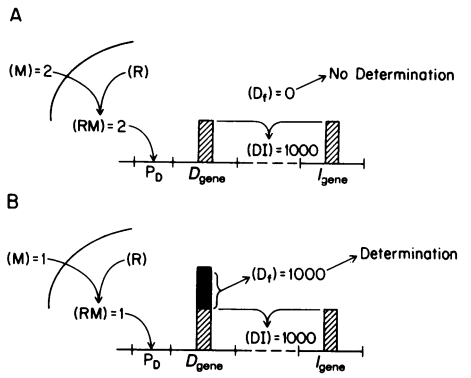

FIG. 1. Schematic representation of threshold setting by D–I pairing. The repressed (*A*) versus the expressed (*B*) states of a particular determinator gene in response to varying levels of external morphogen concentration are schematically represented. (*A*) When the extracellular (or extranuclear in the case of a syncytial system) concentration of morphogen is high, it is reflected in an equally high intracellular concentration of RM that results in a relative repression of determinator gene transcription. As a result the production of determinator gene RNA or protein is at a level equal to or below that of the corresponding constitutively produced inhibitor gene product. Thus the determinator gene product is completely consumed by the formation of D–I complexes and no free determinator is present, so that the determinator function is fully inhibited. (*B*) As the extracellular concentration of morphogen decreases, the intracellular concentration of RM decreases, which in turn results in a relative derepression of determinator gene transcription. At a critical point the determinator gene product rises to a level greater than that of the inhibitor gene product, and there is then a sharp increase in the intracellular concentration of uncomplexed determinator (D_f), and appropriate cellular determination ensues. Thus this mechanism amplifys a small change in extracellular morphogen concentration into a large change in the intracellular concentration of free determinator. P_D, determinator gene promoter; M, morphogen; R, receptor; DI, D-I complexes; D, determinator; I, inhibitor.

determinator activity, however, depends on the presence of free determinator (D_f) that occurs only when total inhibitor (I_t) is less than total determinator (D_t). Therefore, it is the constitutive transcription level of the inhibitor gene that sets the threshold at which transcription from the determinator gene produces sufficient D_t to surpass I_t, thus yielding D_f.

Indeed, since high effective concentrations of determinator and inhibitor relative to the binding constant, *K*, are important for sharp thresholds, intracellular localization or compartmentalization of these components would be advantageous for the model. Intranuclear localization is one obvious example. A second candidate would be membrane localization. Such compartmentalization would lead to high local concentrations even with only a few thousand molecules per cell, and sharp thresholds would then result from D–I titration. It should be noted that even if the active determinator functions at the protein level, threshold setting by D–I pairing could still occur at the RNA level. Thus a possible combination of the compartmentalizations suggested above could occur, with the inhibitor, and thus D–I pairing, confined to the nucleus, while the free determinator could ultimately function in the plasma membrane.

Mass Action Treatment

One attractive feature of a model with the above components and assumptions is that it is amenable to a simple treatment based on mass action laws:

$$\frac{(D_t - DI)(I_t - DI)}{(DI)} = K, \qquad [1]$$

where D_t is the concentration of total determinator, I_t is the concentration of total inhibitor, *DI* is the concentration of determinator–inhibitor complex, and *K* is the equilibrium constant. Eq. **1** can easily be solved for *DI* as a function of D_t, I_t, and *K*. In addition, conservation of mass requires:

$$D_f = D_t - DI, \qquad [2]$$

where D_f is the concentration of determinator free of inhibitor and thus active. Eqs. **1** and **2**, together, then give D_f as a function of D_t. We assume that the rate of transcription of the determinator gene is inversely proportional to the concentration of morphogen. Thus Eqs. **1** and **2** are sufficient to calculate active D_f as a function of the concentration of morphogen.

Fig. 2 illustrates the response of active determinator to the extracellular signal as a function of the binding constant (*K*). When I_t is several orders of magnitude greater than *K*, there is a sharp threshold for the transition to the presence of significant intracellular D_f. By the time *K* is four or five orders of magnitude less than I_t, the transition is virtually a step function, thus:

$$D_f = 0 \quad \text{when} \quad D_t < I_t; \qquad [3]$$
$$D_f > 0 \quad \text{when} \quad D_t > I_t. \qquad [4]$$

There will be essentially no change in D_f in response to the decreasing concentration of morphogen or receptor–morphogen complex until $D_t = I_t$, from which point D_f will increase rapidly. Thus it is I_t that sets the threshold at which D_f will appear and subsequently induce cellular determination.

Consider the following application of this model. Suppose that the constitutive production of a particular inhibitor results in a constant level of approximately 10,000 molecules per cell. This is equivalent to an intracellular concentration of about 10^{-8} M and is similar to the concentration of regulatory molecules (repressor) in *Escherichia coli* (18). Also assume that *K* for this D–I pair is 10^{-14} M or less (a reasonable value for either protein–protein or RNA–RNA interactions). Then as long as the rate of transcription of the determinator gene is such that $D_t < I_t$, there will be essentially no D_f. However, as determinator gene transcription increases in response to a drop in morphogen concentration, a point will be reached where D_t becomes equal to and then surpasses I_t, and from this point on the concentration of D_f will increase rapidly. By using Eqs. **1** and **2** the specific amplification obtained in this example can be calculated. If D_t is 0.96×10^{-8}M, I_t is 1.00×10^{-8} M, and $K = 10^{-14}$ M, then $D_f = 2.40 \times 10^{-13}$ M, or

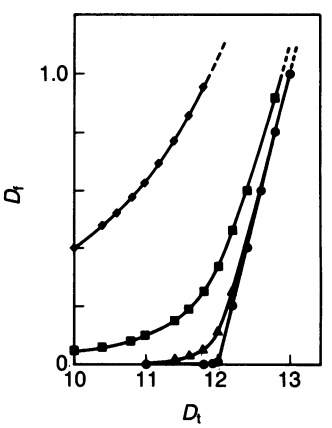

FIG. 2. Effect of D–I binding constant on threshold sharpness. D_f is plotted as a function of D_t. D_t is inversely related to the concentration of morphogen by a proportionality constant, A; $D_t = A/[M]$. Curves are shown for several D–I pair binding constants, *K*, with total inhibitor held fixed at 1.2×10^{-8} M. When *K* is 10^{-14} M or less, the curve is essentially a step function. (◆) $K = 10^{-11}$ M; (■) $K = 10^{-12}$ M; (▲) $K = 10^{-13}$ M; (●) $K = 10^{-14}$ M.

about one molecule per four cells. When D_i increases 10% to 1.06×10^{-8} M in response to a 10% drop in the concentration of morphogen, then D_f increases to 6.00×10^{-10} M, or about 600 molecules per cell. Thus this mechanism converts a 10% change in the extracellular concentration of morphogen into a 2400-fold change in the intracellular concentration of active determinator.

Multiple Segments or Discrete Structures

To account for the development of different segments or discrete structures within a single morphogenetic field, we prefer the possibility that there are different determinator genes responsible for the differentiation of each, although it is clear that more complicated circuits with, for example, feedback loops to ensure stability and allow more flexibility are quite possible (17). Coupled with each determinator would be its specific inhibitor, thus a unique D–I pair would control the development of each segment or structure. The sequential development of structures would then depend on a series of thresholds each resulting in the expression of a new determinator gene product. Fig. 3 shows three different determinator genes being expressed at three different positions in one shallow morphogenetic field. The different thresholds are set by slightly differing constitutive expression levels of each inhibitor gene relative to its paired determinator gene. Implicit in this version of the model is the expectation that once a particular determinator gene has been derepressed, it continues to be expressed in all subsequent structures. Thus there would be a hierarchy of dominance or epistasis existing among the deteterminator genes, such that the ultimate phenotype of the cell will reflect only that prescribed by the highest in the hierarchy of determinator genes expressed in that cell.

Predictions and Potential Experimental Verification

Mutant Phenotypes. A number of specific mutant phenotypes are predicted by the mechanism shown in Fig. 1, regardless of the particular system to which it is applied or whether it functions at the RNA or protein level. Of particular interest with regard to D–I pairs are the following: (*i*) Loss of determinator gene—in the absence of any particular determinator gene function, the phenotype it normally determines would fail to develop where expected and the next subordinate determinator gene phenotype in the dominance hierarchy would prevail. This would represent a step down in the hierarchy and thus would be categorized as a loss of function mutant. (*ii*) Loss of inhibitor gene—in the absence of any particular inhibitor gene function, its corresponding determinator gene phenotype would be inappropriately expressed where it otherwise would not be expected. This would represent a step up in the hierarchy and thus would be categorized as a gain of function mutant. It should be noted that the mutational loss of an inhibitor gene is potentially distinguishable from that of a typical repressor gene, as demonstrated by the effect each would have on determinator gene expression. The former would result in continued inducibility of determinator gene expression with loss of the threshold effect, while the latter would lead to completely uninducible, constitutive expression of the determinator gene.

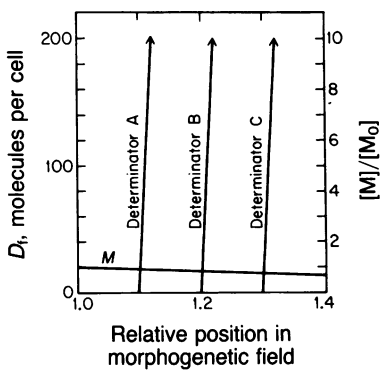

FIG. 3. Generation of multiple thresholds in a single morphogenetic field. Three thresholds are shown to be sequentially generated by three separate D–I pairs, A, B, and C, at three distinct points (1.1, 1.2, and 1.3) in a single morphogenetic field in which the extracellular concentration of M is steadily decreasing. In this example the binding constant K is the same (10^{-14} M) for all three D–I pairs, but the constitutive level of inhibitor gene product increases by 10% for each pair: $I_A = 1.1 \times 10^{-8}$ M (about 10,000 molecules per cell); $I_B = 1.2 \times 10^{-8}$ M; $I_C = 1.3 \times 10^{-8}$ M. Because the concentration of morphogen is inversely related to its position in the morphogenetic field, and transcription from each determinator gene is inversely related to the concentration of morphogen, the increasing levels of expression of each inhibitor gene establish three separate thresholds for the expression of three determinators, D_A, D_B, and D_C, at three separate points in the morphogenetic field. [M], morphogen concentration; [M_0], [M] at field position 1.0.

Functional Pseudogenes. The need for a specific intracellular inhibitor suggests an intriguing possible function for pseudogenes. At the RNA level D–I pairing could involve the formation of sense–antisense RNA duplexes. In this case a pseudogene could be the source of the antisense RNA that would hybridize with the sense RNA from the determinator gene and thereby block its expression. Thus a directly testable prediction of this model would be the presence of antisense RNA homologous to all or part of the message for any determinator gene. Beyond this, we would predict the existence in the appropriate cells of double-stranded RNA representing the bound state of the D–I pairs. Ultimately we would predict a detectable transition from the bound (double-stranded) to the free (single-stranded) state of the determinator gene mRNA taken from the appropriate segments or discrete structures. Finally, with respect to the involvement of pseudogenes as a source of antisense RNA, we would predict that these genes, or portions thereof, are transcribed, but from the opposite strand relative to their functional counterparts.

If the inhibitors act at the protein level, then they should be identifiable, for example, as proteins that modify the electrophoretic migration of determinator proteins. Antibodies directed against determinator proteins coupled with immunoblot techniques could facilitate this identification. The relative timing of expression of such determinator and inhibitor proteins as well as their pairing could be monitored during development.

Specific Applications

The D–I concept was originally conceived independent of any specific biological system. However, in analyzing its potential application to known systems, we have found that various facets of the model can be directly applied with good success to various biological systems.

The differentiation of segments in *Drosophila* is perhaps the best studied system of ordered development of discrete structures and is one of the few systems in which true determinator genes have been described (9, 10, 19). Lewis (9) has described the development of all segments posterior to the second thoracic segment as being under the control of the bithorax gene complex (*BX-C*). On the basis of his studies on several mutants of the *BX-C*, Lewis developed a model describing the genetic control of segment differentiation in *Drosophila* (9). He and others have provided evidence that expression of at least one gene or portion of the *BX-C* is associated with the differentiation of each segment posterior

to the second thoracic segment, and he has proposed that this sequential gene expression occurs in response to an anterior–posterior gradient of repressor. Similar data is now also forthcoming for the antennapedia complex (*ANT-C*) that controls the development of segments anterior to the second thoracic segment (10, 20).

Although we expect the final intricacies of the bithorax system to prove much more complex than the scheme shown in Fig. 1, we find that by equating the *BX-C* genes with our determinator genes, and the repressor gradient with our extracellular morphogen gradient, the model shown comes remarkably close to explaining the following key features: (*i*) sharp intersegmental transcriptional boundaries for each *BX-C* gene; (*ii*) repression or derepression of a given *BX-C* gene mediated by a *cis*-regulatory element; (*iii*) individual derepression of each *BX-C* gene; (*iv*) overall negative control of the *BX-C* maintained by a major regulatory gene; (*v*) continued derepression of each particular *BX-C* gene in all segments posterior to the segment in which each is first expressed; and (*vi*) a sequential increase in the number of *BX-C* genes in the derepressed state in sequentially more posterior segments.

The specific application of our model to segmentation in *Drosophila* is consistent with a scheme in which the morphogen, or a precursor thereof, is laid down maternally in a static gradient maintained in a gel-like state in the ooplasm. Then, embryonic expression of the gene producing the receptor would serve to initiate the binding of morphogen to receptor forming a receptor–morphogen complex and thus facilitate the interpretation of the positional information. This interpretation would involve the translation of the monotonic gradient into a step function via the threshold-setting mechanism, resulting in the stepwise, sequential expression of *BX-C*- or *ANT-C*-like determinator genes. Each active determinator thus produced would, in turn, coordinate the expression of appropriate batteries of differentiation genes within each segment. The recent discovery of the homeobox sequence in the 3′ region of several determinator genes in *Drosophila* (21) would seem to provide a clue to the mechanism by which these genes interact with various differentiation genes. By encoding a protein domain capable of binding DNA, the homeobox sequence apparently enables each determinator protein to interact with appropriate regions of various differentiation genes. Thus the homeobox sequence is apparently involved in regulating expression of differentiation genes, but would have no effect on establishing the pattern of determinator gene expression.

To date, no pseudogenes or other candidates for inhibitor genes in the bithorax system have been reported, and this is one current limitation on the complete application of the model to this system. However, the possibility that such genes do exist represents a testable prediction.

D–I pairs could also be used in development as a counting mechanism, the most important of which would be to count cell divisions. If a stable inhibitor is in excess when its synthesis ceases at a developmental branch point, no effect will be seen until it decreases to a concentration below that of the determinator. For example, if the inhibitor were in 10-fold excess, the cell would differentiate only after the fourth division. This type of counting mechanism could contribute to models such as that of the progress zone proposed to account for proximal–distal pattern formation in vertebrate limb development (22). Similarly, a D–I mechanism could be involved in the onset of transcription in early *Xenopus* embryos where there is evidence that a D–I titration effect initiates RNA synthesis at cleavage cycle 12 (23). Defects in a cell-counting mechanism could lead to heterochronic mutants such as those observed in *Caenorhabditis elegans* (24). These mutants would be essentially analogous to the gain or loss of function mutants discussed above.

We believe the D–I mechanism has wide potential application to various developmental systems and as a general mechanism of gene regulation and threshold setting. However, it is important to note that this mechanism generally does not exclude but rather would enhance most other mechanisms of gene control or threshold setting. Even in the example shown in Fig. 1, signal amplification can be increased by an allosteric interaction of receptor with morphogen and stability can be added by feedback loops. Other models such as the kinetic model of Lewis *et al.* (17) could easily incorporate the D–I pair threshold mechanism as well.

Evolution and Function of Pseudogenes as Inhibitor Genes

The following simply stated evolutionary scheme seems reasonable: (*i*) With the appearance of multicellular organisms the need for cellular specialization and hence differentiation developed. (*ii*) This initially required the evolution of determinator genes to regulate differential gene expression. (*iii*) An overall order was bestowed upon embryonic differentiation by bringing these determinator genes under the control of an extracellular gradient of morphogenetic information. (*iv*) The evolution of the threshold effect was facilitated by the appearance of appropriate inhibitor genes leading to an intracellular amplification of morphogen signal thus producing the necessary sharp transitions. (*v*) The simplest mode of evolution of such inhibitor genes would involve complete or partial gene duplication such that constitutive transcription could occur from either the plus strand, producing a translatable message, or from the minus strand, producing antisense RNA. (*vi*) In either case transcription of a pseudogene could produce the requisite inhibitor substance.

The possibility that inhibitor genes function by producing a protein is a readily feasible option. Often proteins are composed of subunits that bind tightly to each other. Thus evolution must have selected for the appropriate configuration of bonding surfaces such that the active site(s) became correctly formed and/or arranged. If in the evolution of a pseudogene, the sequence coding for the bonding domain remained intact while that coding for the active site was lost or mutated to inactivity, the result would be a specific inhibitor of the original active protein. Thus while this protein would lack function with respect to the activity of the original protein, it could have gained function as an inhibitor. The constitutive–dominant (i^{-d}) mutants of the *E. coli lac* operon provide a precedent for this possibility. The i^{-d} mutants have been shown to result from "killer" subunits (25). Though acceptable from an evolutionary viewpoint, this version of the model does have the disadvantage that the sharpness of the threshold is somewhat reduced unless the killer subunit competes preferentially with the normal subunit.

Because the production of an effective inhibitor at the protein level would seem to require a more involved evolutionary process, the notion of inhibitor genes functioning at the RNA level remains attractive. In addition, D–I pairing in the form of sense–antisense RNA would have the advantage that specificity and high binding affinities are achieved automatically. From an evolutionary standpoint, the periodic occurrence of duplicated genes that are transcribed in opposite directions seems inevitable, and these gene pairs should be conserved as such whenever they interact in an advantageous manner. Thus it is of interest that pseudogenes are relatively prevalent in the genomes of multicellular organisms and scarce in those of unicellular organisms such as yeast. Our hypothesis that pseudogenes could function as the source of inhibitor molecules in a threshold-setting mecha-

nism suggests a potential selective advantage that is unique to multicellular organisms.

An important consideration regarding the feasibility of D–I pairing at the RNA level is whether RNA–RNA hybridization could occur at sufficiently rapid rates *in vivo*. Evidence from both prokaryotic and eukaryotic systems would argue that it can. Mizuno *et al.* (26) found that translation of mRNA for the *omp F* gene in *E. coli* is inhibited by hybridization with homologous, antisense RNA under certain conditions. Coleman *et al.* (27) have demonstrated that similar inhibition can be engineered by expression of antisense RNAs homologous to three *E. coli* genes, lipoprotein, *omp C*, and *omp A*. Simons and Kleckner (28) showed that binding of a small antisense RNA to an homologous mRNA is sufficient to block the transposition function normally encoded by this message. There is also the observation of Saito and Richardson (29) regarding a translational block in a mutant of the bacteriophage T7 produced by the binding of an antisense RNA to the ribosome-binding site of an homologous mRNA. No such evidence has yet to emerge from any naturally occurring eukaryotic system, although the discovery of a short antisense RNA transcribed from the opposite strand of the 5′ flanking region of the mouse dihydrofolate reductase gene (30) is intriguing in this regard. It has been shown that antisense RNA introduced into a host cell is capable of blocking infection of certain strains of virus (31, 32). In addition, Izant and Weintraub (33, 34) showed that the simultaneous injection of sense and antisense RNA-producing herpes *TK* genes into mouse L cells in culture resulted in a significant reduction in TK production and that the introduction of plasmid DNA directing the production of appropriate antisense RNAs can specifically inhibit the expression of endogenous genes as well. Finally, Melton (35) has demonstrated the complete inhibition of β-globin production from mRNA injected into frog eggs by the prior injection of the appropriate antisense RNA (36). In many cases, the level of antisense RNA required to completely inhibit a corresponding functional gene may depend on the intracellular proximity of the sense- and antisense-producing genes.

In considering the production of an antisense inhibitor RNA, it must be remembered that it will not generally contain the sequence-specific signals for posttranscriptional processing that are present in the homologous sense RNA. Thus it will not be spliced to form a direct counterpart of the fully processed sense-strand message. This may not pose any problem for the inhibitory mechanism, but if this is indeed a significant consideration, it would seem that evolution has provided the necessary source for directly homologous antisense RNA in the form of that produced from intronless pseudogenes.

Summary

The model described here provides a partial molecular basis for the differentiation of discrete structures in response to a shallow, monotonic gradient of morphogenetic information. The essence of this threshold-setting mechanism is the proposed existence of tightly-binding D–I pairs, and the corollary idea that pseudogenes could represent a source of the inhibitor. This model seems appealing in that both the evolution of its components, and the proposed mechanism by which they interact are relatively straight forward. A number of testable predictions and potential experimental verifications have been put forth which, along with results from other systems not discussed here, should eventually determine the validity of this model.

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