The Operon: On Its Third Anniversary

Modulation of transfer RNA species can provide a workable model of an operator-less operon.

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In the spring of 1961, F. Jacob and J. Monod of the Pasteur Institute published the review "Genetic regulatory mechanisms in the synthesis of proteins" (*1*) that has exerted a most profound heuristic and dialectic effect on recent physiologicogenetic research. In considering the problem of "information transfer" from DNA, the putative informational repository of polypeptide amino acid sequence, Jacob and Monod proposed, first of all, that each gene, or DNA cistron, acts as a template for synthesis of *messenger RNA* molecules to which the cistronic polynucleotide sequence is transcribed. The nascent messenger molecules enter into temporary union with ribosomes, and the messenger-ribosome complex is competent to synthesize the polypeptide inscribed in the parent cistron as a succession of *codons*, each codon representing a particular amino acid. The messengers have only a limited lifetime, and hence each serves for the construction of only a limited number of polypeptide molecules. The validity of this concept has by now been firmly established, not only by the results of experiments designed to test it directly but also by the many further insights to which its evident predictive value has led. Today, "messenger RNA" is a household word of molecular biology.

Second, Jacob and Monod proposed in their review a coherent model for the regulation of DNA function, based on a large ensemble of observations pertaining to viral and bacterial synthesis of *inducible* and *repressible* enzymes. According to this model, a set of cistrons, all closely linked on the genetic map and hence residing in contiguous sectors of the DNA, form an *operon* by virtue of their coordinate functional control by a common gene, their *operator*. The operator, whose genetic site is closely linked to the operon which it controls, can exist in two states: open and closed. As long as the operator is open, every cistron of the operon synthesizes messenger RNA, and hence gives rise to the polypeptide whose structural information it harbors. As long as the operator is closed, no cistron of the operon synthesizes its messenger RNA. The operator closes whenever it is engaged by a specific cytoplasmic *repressor*; this repressor is itself the product of a *regulator gene*. The activity of different repressors is, in turn, governed by the presence of different specific metabolites, or *effectors*. One kind of repressor (that concerned with the regulation of inducible enzymes) is inactivated by the presence of its effector, and hence prevented from closing the cognate operator. Here the effector induces the otherwise repressed synthesis of the polypeptides coded in all cistrons of the relevant operon. Another kind of repressor (that concerned with the regulation of repressible enzymes) is activated by the presence of its effector and hence is stimulated to close the cognate operator. Here the effector represses the otherwise constitutive function of the operon.

On the whole, the operon concept has fared no less well than the messenger RNA in the intervening 3 years, in that the generality of the notion of coordinate control of closely linked bacterial cistrons by regulator genes has been buttressed by many additional examples. An excellent account of these very extensive results can be found in a forthcoming review by Ames and Martin (*2*). In one respect, however, experimental progress has been rather disappointing: attempts to identify or isolate the postulated repressor, or to understand how it manages to "close" its cognate operator, have so far failed. Possibly, the repressor has proved to be so elusive because the original operon model did not happen to assign to it the proper role in the regulation process. The purpose of this article is to attempt a brief interim appreciation of the operon and to propose an alternative view of the nature of its essential twin features, operator and repressor. This view is but an extension of ideas first developed some months ago by Ames and Hartman (*3*), who showed how the operon concept might be renovated to some profit.

Repressor as an Allosteric Protein

The most significant fact known about the repressor is that it works negatively—in other words, that in its active form it inhibits, rather than fails to promote, enzyme synthesis. This follows from the observation that enzyme synthesis is subject to normal regulation in heterozygous R^+/R^- bacteria carrying both active R^+ wild and inactive R^- mutant alleles of the regulator gene (*4*). First attempts to identify the chemical nature of the repressor suggested that it is a polynucleotide, since the regulator gene appeared to establish its repressive effect in the absence of protein synthesis (*5*). Subsequent reappraisals of this inference, stimulated by the theoretical difficulty of explaining how polynucleotides could manage to recognize and be "inactivated" or "activated" by metabolite-effectors of low molecular weight and also by the discovery of temperature-sensitive (*6*) or effector-insensitive (*7*) repressor mutations and of the phenotypic reversal of R^- mutations by extra-genic suppressors (*8*), led to the latter-day belief that the repressor is a protein. This belief is embraced in the remainder of this article, although it must be admitted that the reasons for so doing still remain more doctrinal than empirical.

Once the proteinaceous nature of the repressor was taken for granted, Jacob and Monod (*9*) showed how the interaction with its effector can be understood. They proposed that the repressor, just like those enzymes whose catalytic function is subject to feedback inhibition by metabolite products of the reaction chain of which the enzyme

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forms a part, possesses two specific, *allosteric* sites. One of these sites possesses an affinity for all or part of the exact nucleotide sequence of the cognate operator gene, and the other site is affined to the specific metabolite-effector. Combination of repressor and effector at the second allosteric site modifies the operator-affinity of the first site. In the case of repressors of inducible enzymes, combination with the effector reduces the operator-affinity of the first site and thus "inactivates" the repressor, whereas, in the case of repressors of repressible enzymes, combination with the effector increases the operator-affinity of the first site and thus "activates" the repressor. Mutations in the regulator gene, which became a cistron specifying the repressor polypeptide, could thus produce different structural modifications of the allosteric protein that bring about changes in the exact conformation, temperature sensitivity, or mode of interaction of the two specific regulatory sites.

Effector Control of Messenger Synthesis

One important prediction by the operon concept of a fact not known at the time of its promulgation has meanwhile been triumphantly confirmed: The presence of a particular effector really does influence the intracellular concentration of the specific messenger RNA produced by the relevant operon. Experimental tests of this prediction became possible when it was found that messenger RNA may form specific molecular hybrids with its homologous DNA, either upon heating and slow cooling of mixtures of the two polymers (*10*) or upon passage of the RNA through special DNA-agar gels (*11*). Such hybrids provided a means by which the amount of messenger RNA corresponding to a particular cistron can be specifically estimated in the presence of the myriad of other messenger RNA species that may also exist in the cell. In this manner, Attardi and his collaborators (*12*) were able to show that growth of bacteria in the presence of the specific inducing effectors greatly increases the quantity of messenger RNA capable of forming hybrids with a particular DNA fraction carrying the relevant operon. These experiments thus appeared to support the view favored by Jacob and Monod that the primary action of the repressor concerns the inhibition of messenger *formation*, rather than the inhibition of messenger *function* as template for protein synthesis (the inhibition of function had originally been put forward by Jacob and Monod as a logically admissible alternative).

First estimates placed the average size of the bacterial messenger RNA molecule in the vicinity of about 10^3 nucleotides (*13*), a size roughly equivalent to the probable length of an average cistron. But as better methods of isolating and characterizing messenger RNA were developed, it was found that some messenger molecules are very much larger than this. In particular, Martin (*14*) could show that an operon comprising the cistrons of ten enzymes appears to give rise to one single *polycistronic* messenger molecule of a size corresponding to about 10^4 nucleotides. It thus transpired that the unit of messenger synthesis is not the individual cistron but the operon. Hence, the messenger-ribosome complex is capable of synthesizing not only one polypeptide but all of the polypeptides encoded in an operon.

Calculation of the approximate number of messenger molecules per bacterium, on the basis of their molecular weight and steady-state amount per cell, now led to a bothersome paradox. As expressed by Watson (*15*), "There . . . must be at least six to eight ribosomes for every messenger molecule. It was very difficult to believe that only 10 to 20 percent of the ribosomes function at any given moment. . . . Instead, it seemed much more likely that almost all of the ribosomes are active." This paradox was resolved with the discovery that single messenger molecules can service more than one ribosome at a time—that the messenger is in simultaneous contact with several ribosomes, which form an aggregate polyribosome (*16*). Since only a short segment of the messenger can be in contact with one ribosome at any moment, messenger and ribosomes must be in constant relative motion during protein synthesis. Having added to its growing nascent polypeptide chain the particular amino acid specified by the actual messenger segment with which it is in contact, the ribosome advances one step along the messenger to the next segment. As soon as a ribosome has reached the end of a cistron, its now complete nascent polypeptide is released and, on advancing to the start of the next cistron, the ribosome lays down the initial, or amino-terminal (*17*) amino acid for a new and different polypeptide chain.

Polarity

But however plausible the idea that the primary control of the synthesis of specific proteins occurs at the level of messenger RNA *formation* may have seemed, and however well supported empirically, some observations made during the last 3 years indicate that there exists, after all, a regulatory mechanism that works through control of messenger *function*. One such observation, made by McAuslan (*18*), is that synthesis of a particular viral enzyme may be turned on or off at a stage of intracellular virus growth when synthesis of the relevant viral messenger RNA has already run its course. Another set of observations pertains to a property of the operon that was set forth by Jacob and Monod (*19*) only some months after publication of their original model: The operon is a polarized unit. This polarity manifests itself in the occurrence of mutations that simultaneously engender loss of activity of one enzyme and reduce the level of other enzymes belonging to the same operon (*3, 19, 20*). Such, by no means uncommon, polarity mutations present a definite hierarchy: The cistron whose enzyme activity has been lost, and in which the mutation can be mapped, is always closer to the operator gene than the other cistrons in whose enzymes a reduction in level has been produced; the mutation does not affect the activity of operator-proximal cistrons.

A further aspect of the polarized nature of the operon was uncovered by Zabin (*21*): Although coordinate control of the synthesis of all enzymes in the same operon results in an invariant ratio in their intracellular amounts under all conditions of repression or induction, that ratio, when computed on a molar basis, may be far from unity. Thus the number of molecules synthesized per unit time can be many times greater for one enzyme than for another enzyme belonging to the same operon. In the case studied by Zabin, the rate of synthesis of one enzyme whose cistron is operator-distal is less than one-tenth

that of another whose cistron is operator-proximal. On first sight, this finding does not seem to fit with the idea that the rate of synthesis of an enzyme set is controlled by the rate of formation of a single, polycistronic messenger RNA molecule that forms the polypeptide chains inscribed into it as it is threaded through the polyribosome complex. For this image would predict not only coordinate control but also equimolar synthesis of all enzymes of the operon.

Ames and Hartman (*3*) offered an explanation of operon polarity by proposing that the polycistronic messenger RNA molecule begins synthesis of its polypeptides with the operator-proximal cistron, and that there exists a finite chance that the translation process comes to a halt somewhere along the messenger before reading of the last cistron has been completed. From this point of view, the chance that on any one messenger molecule moving along the ribosome from its operator end, translation of all cistrons will actually occur represents the compound probability that polypeptide synthesis has not come to a halt at any of the many nucleotides of the messenger chain. Hence, it would naturally follow that the greater the distance from the operator of a cistron, the lower the overall rate of synthesis of its enzyme. Furthermore, the pleiotropic effect of polarity mutations can now be readily understood if it is admitted that the chance that the translation process actually halts anywhere along the messenger RNA molecule depends on the precise nucleotide sequence at that point. Thus, not only could a mutational alteration in messenger nucleotide sequence result in an inactive protein by introducing a pernicious mutant amino acid into the polypeptide, but it could also raise the chance that polypeptide synthesis halts at that point, and could thus reduce the net rate of synthesis of structurally normal enzymes of operator-distal cistrons. Evidently these notions readmit through the back door the idea that some regulation of enzyme synthesis does occur at the level of messenger function.

Modulation

But how *could* the nucleotide sequence at any point influence the chance that the translation process halts there? Itano (*22*), whose ideas came from the study of human hemoglobins rather than bacterial enzymes, proposed what seems to be the most plausible answer: by coding for a particular aminoacyl-transfer RNA. If the right aminoacyl-transfer RNA is readily available within the cell for incorporation into the nascent polypeptide chain, the chance of continuing translation to the next amino acid residue is high; if it is not readily available, the chance is low. Because of the probable degeneracy of the genetic code (*23*), some of the 20 standard amino acids are represented by more than one kind of nucleotide codon, and hence are cognate to more than one species of transfer RNA (*24*). If the intracellular availabilities of different transfer RNA species cognate to the same amino acid are widely different, corresponding to "major" and "minor" representations of that amino acid, then the rate of synthesis of a polypeptide, and of all polypeptides of operator-distal cistrons carried by the same polycistronic messenger, depends on the relative occurrence of major and minor coding representations in the cistronic message. As proposed by Ames and Hartman (*3*), the pleiotropic mutations described above would then correspond to codon changes that call not only for a change in amino acid sequence but also for a switch from major to minor coding representations. Ames and Hartman have used the term *modulation* for this genetic adjustment of relative rates of synthesis through coding for transfer RNA species of different abundance.

Operator-Negative Mutations

One of the two main experimental supports for the original postulation of the operator as a gene of regulation was the discovery of "operator-negative," or O^0, mutations (*1, 25*). These mutations, whose genetic sites cluster at one extremity of the operon, abolish enzyme synthesis of the entire operon. Since in O^0/O^+ heterozygous bacteria carrying both wild O^+ and mutant O^0 alleles the effect of the operator mutation is confined to cistrons *cis* with respect to O^0, it seemed reasonable to suppose that O^0 mutations exert their pleiotropic effect by permanently closing the operator gene and thus averting synthesis of the polycistronic messenger RNA. In apparent confirmation of this view, Attardi and his collaborators (*12*) could later show that O^0 mutant bacteria do not contain detectable quantities of messenger RNA affiliate to the relevant operon, even in the presence of an inducing effector for the O^+ wild type.

But when more detailed studies revealed that the operator, as defined by O^0 mutations, is probably not a separate gene of regulation at all but simply forms part of the first cistron of the operon, it became clear that operator-negative mutants need not be fundamentally different from pleiotropic mutations in other cistrons of the polarized operon (*3, 19*). An O^0 mutation could represent merely a change in nucleotide sequence in the first cistron that calls for an entirely unavailable minor transfer RNA species. Beckwith's (*26*) discovery of the reversal of the phenotype of an O^0 mutant by genetic suppression supports this view. Beckwith found that suppressor mutations at several widely separated loci of the bacterial genome restore full or partial function to the inactive O^0 operon. The nature of these suppressor mutations, particularly their specific effects on the growth of certain mutants of bacterial viruses, is generally thought to reflect alterations in the RNA-protein translation process (*27*), possibly in the specificity of transfer of amino acids to transfer RNA. The functional restoration of the O^0 operon is thus most easily explained by supposing that, despite the apparent evidence to the contrary, operator-negative mutants *can* synthesize the relevant messenger RNA and that the presence of the suppressor mutation produces a perturbation of the normal decoding process that allows polypeptide synthesis to pass over the normally nontranslatable O^0 mutant nucleotide site.

Feedback Control of Messenger Synthesis

How, then, is one to reconcile the supposition that operator-negative mutants can synthesize the relevant messenger RNA with the failure of direct chemical tests to show its presence? A number of reasonable possibilities suggest themselves here. For instance, it can be admitted that unless the operator-negative phenotype is suppressed, the messenger formed by an O^0 operon is highly unstable and thus escapes detection in the DNA–RNA hybrid test (*12, 26*). Another explanation would be that there exists a feedback connection between messenger

synthesis and function: The faster the messenger works in protein synthesis, and hence the faster ribosomes move along it, the faster it is synthesized. This hypothesis can draw support from the finding that nascent messenger RNA formed by the in vitro action of RNA-polymerase does not spontaneously dissociate from its DNA template (*28*), suggesting that, in vivo, an active process, possibly the movement of ribosomes over the nascent messenger, is required for liberation of the messenger molecule from its parent operon. Thus the low level of relevant messenger RNA present in O^0 mutants might reflect only the congestion of the operon with untranslatable, and hence irremovable, messenger molecules. Genetic suppression of the operator-negative phenotype would restore function to the mutant operon by rendering the message translatable at the O^0 mutant site and allowing liberation of the nascent messenger molecules.

The possibility of a feedback loop between messenger formation and function naturally prompts one to reexamine Jacob and Monod's preferred alternative, that the primary action of the repressor is the inhibition of messenger synthesis by operator closure. It now becomes no less plausible to think that the primary action of the repressor is the inhibition of messenger function, and that the increase in messenger RNA observed after administration of an inducing effector is only an epiphenomenon of the relief of the operon congested with an unreadable messenger molecule. Repressor action at the level of messenger function, coupled with secondary feedback control of messenger synthesis, would thus amplify the inhibitory effect on enzyme synthesis of each repressor molecule.

The Repressor as an Enzyme

It is not difficult to imagine some mechanisms through which the repressor could affect messenger function. Here we shall consider only one, an extension of the modulation concept. This hypothesis envisages that the activity of repressors is directed against particular species of transfer RNA required for translation of one or more cistrons of the operons to be regulated. As long as the repressors are active (spontaneously, for inducible operons, or because of effectors, for repressible operons), the necessary aminoacy-transfer-RNA species are in short supply, and translation of particular messengers cannot proceed. As long as the repressors are inactive (spontaneously, for repressible operons, or because of effectors, for inducible operons), the necessary aminoacyl-transfer-RNA species are available and the operon functions. The repressors could exert this control over the availability of particular aminoacyl-transfer-RNA species if they were diesterase or phosphorylase enzymes that remove the terminal cytidylic-cytidylic-adenylate (. . . pCpCpA) residues common to all transfer RNA species (*29*). Since amino acids cannot be transferred to transfer RNA molecules deprived of their terminal . . . pCpCpA sequence, an active nuclease or phosphorylase whose enzymatic specificity is directed against one species of modulating transfer RNA would thus reduce the intracellular availability of the aminoacyl form of that species. The damage wrought by the specific repressor-enzyme would, however, be subject to repair by another enzymatic activity known to be present in bacteria, which specifically restores from the corresponding nucleoside triphosphates the . . . pCpCpA residues to transfer RNA molecules lacking their proper terminal sequence (*29*). This restorative activity could reside either in the repressor proteins themselves (and thus constitute a second specific catalytic function of each of these enzymes) or in a general repair enzyme that can restore the three terminal nucleotides to all incomplete transfer RNA molecules. The essence of this notion is, therefore, that the nucleotide sequence of one or more cistrons of an operon contains one or more modulating codons calling for transfer RNA molecules whose . . . pCpCpA terminus is subject to both removal and restoration by specific regulator enzymes.

Operator-Constitutive Mutants

The second main experimental support for the original postulation of operator genes was the discovery of "operator-constitutive," or O^c, mutations (*1, 19*). These O^c mutations cluster at the same extremity of the operon as the O^0 mutations but result in partially constitutive expression of the whole cistron set. Furthermore, in heterozygous O^+/O^c bacteria carrying also the wild O^+ allele, the constitutive phenotype is confined to cistrons *cis* with respect to O^c. Since the partially constitutive enzyme synthesis of O^c mutants seems to derive from loss of sensitivity of the operon to repressor action, the modulation hypothesis would demand that the mutation $O^+ \rightarrow O^c$ represents either a nucleotide transition that converts a modulating codon in the first cistron of the operon into a nucleotide sequence calling for major species of transfer RNA whose aminoacyl forms are readily available in the cell, or a deletion that removes the modulating codon from the cistronic nucleotide sequence. In this way, mutation would remove the obstacle to messenger translation presented by the wild-type modulating codon. This explanation of the nature of O^c mutations places some restriction on the details of any model involving regulation by modulating transfer RNA. For the number of widely spaced modulating codons cannot be very much greater than perhaps two or three in any operon in which an O^c mutation is known to have generated constitutive enzyme synthesis at a rate approaching that maximally inducible.

The Modulating Codon

Is it actually possible that each operon owes its regulatory individuality to an encumbrance of its messenger RNA with one or more modulating codons? According to present ideas of the genetic code, the codon of each of the 20 standard amino acids is the nucleotide triplet (*23*). Hence, there could exist a maximum of 4^3, or 64, different codons, and hence, 64 transfer RNA species cognate to these codons. Of these 64, at least 20 codons would have to be reserved for one nonmodulating, major representation of each of the 20 standard amino acids. The major representations would be those used for coding the great majority of amino acid residues in all proteins. Only the remaining 44 triplet codons, and hence no more than 44 species of transfer RNA cognate to these codons, would then be available for degenerate minor representations in the regulatory processes imagined here. But since the number of bacterial and viral operons is probably greater than 44, it seems unlikely that regulation occurs through the use of one modulating nucelotide triplet codon per operon.

At least two possibilities can be considered in providing for the regulation of more than 44 operons. One of these is that the modulating codons imagined here contain more than three nucleotides. For instance, the modulating codons might be sextuplets chosen in such a way that the permutation of the first three nucleotides of any modulating sextuplet is never identical with any triplet codon in use as a major, nonmodulating amino acid representation. In this way translation of the messenger could proceed codon by codon, even though the length of the modulating codons exceeded that of the nonmodulating or major nucleotide triplets making up the bulk of the amino acid sequence specification. Such sextuplets would allow a maximum of 44×64, or 2816, different modulating codons, a number more than enough to accommodate the probable number of independently regulated operons.

A second possibility is that though no more than 44 nucleotide *triplet* codons are involved in regulation, some operons contain two or more different modulating codons. This would allow regulation of a virtually unlimited number of operons by use of a limited number of modulating transfer RNA species, but the regulation of most members of such an operon set would not be wholly independent. That is, any two operons sharing the same modulating codons would necessarily manifest a regulatory connection, as the effector-induced activation or inactivation of the repressor-enzyme of one operon alters the availability of the modulating transfer RNA also required for function of the other operon.

Coda

To have set forth here in such detail a hypothesis involving so many untested suppositions may seem quite unwarranted. But since it is not immediately obvious that a reasonable account of operon control can actually be given in terms of modulating transfer RNA species, it may be of some value to have provided a rough outline of at least one apparently workable model of an operator-less operon. It should be noted that the intrinsic complexity of this model is no greater than that of Jacob and Monod's original formulation, since two kinds of regulatory genes still define each operon. Here, the regulator genes code for the transfer-RNA-specific nucleases or phosphorylases, and the operator genes are replaced by genes responsible for synthesis of the corresponding species of modulating transfer RNA. At least one virtue of this model might be that it suggests new biochemical experiments in the search for the hitherto elusive repressor.

Whether or not Jacob and Monod's original notion of the interaction of repressor and operator and its effect on messenger formation turns out to be correct in the end, the magnitude of their contribution in putting forward the messenger-operon concept can hardly be overestimated. Not only did the appearance of their review suddenly bring order into what had hitherto been an enormously confused and complex mass of data, but it provided students of the control of protein synthesis with a badly needed new vocabulary for verbalizing interpretations of their experimental results. Thus, whatever future observations may yet reveal about regulation of cellular functions, the promulgation of the operon in 1961, the same year that saw also the discovery of the general nature of the genetic code, is sure to remain one of the principal milestones of molecular biology.

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