Using Studies on Tryptophan Metabolism to Answer Basic Biological Questions

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In my youth I was overwhelmed by the variety of forms of life around me. Yes, while growing up in New York City! As a student at the Bronx High School of Science my teachers made every effort to convince me that no pursuit could be more exciting or rewarding than searching for explanations for the basic processes common to life. I agreed, but I knew this decision was insufficient, for I would have to choose the area of science that was just right for me. I was aware that major unanswered questions existed in all fields of science, particularly regarding the relationship of biochemistry to genetics, the two subjects that interested me most as a high school student. I decided to major in biochemistry, and enrolled at the City College of New York. I completed a year and a half of college study before being drafted into the army in the spring of 1944. I served in the infantry as a cannoneer during World War II. I fought in the Ardennes in the Battle of the Bulge. Understandably this was an awesome experience. Upon returning to college after the war I was more determined than ever to pursue a career in research. When faced with selecting a Ph.D. program to apply to, I received excellent advice from a knowledgeable professor and textbook author, Benjamin Harrow, chairman of the Biochemistry Department at City College of New York. He suggested exploring gene-enzyme relationships with Neurospora crassa as the ideal project for me. I agreed and applied to do my graduate work with George Beadle at Caltech or Edward Tatum at Yale. I was rejected by Caltech but fortunately was accepted by Yale.

As it turned out, my mentor in graduate school at Yale was not Edward Tatum; it was David Bonner. Bonner had moved with Tatum from Stanford to Yale and had become his research associate. During the year I applied for admission to Yale, Tatum decided to return to Stanford. Fortunately for me, Bonner stayed on at Yale and took over direction of Tatum’s remaining group. Bonner, a wonderful advisor, believed it was in the best interests of both student and advisor to have each student work independently on a well defined project. If successful, he said, we would receive partial credit for our discoveries and would qualify for a faculty position. For most beginning graduate students, selecting a project and deciding how to proceed is relegated to your research mentor and would reflect his or her research preferences. By choosing a specific scientist as your advisor you recognize the importance of his or her contributions. In my initial meeting with Bonner at Yale in June of 1948, as I recall, he handed me a fuzzy culture of a niacin-requiring mutant of Neurospora and gave me advice on how to go about identifying the niacin pathway intermediate this mutant was presumed to accumulate. Our ultimate goal, he said, was identifying all the intermediates in the niacin pathway so this knowledge could be exploited in investigations on gene-enzyme relationships. I was the only laboratory member assigned this type of project, probably because Bonner was aware that my background was principally in biochemistry. This project captured my full attention, and fortunately, I was successful. We identified two intermediates accumulated by niacin-requiring mutants, quinolinic acid and a derivative of kynurenine. The knowledge I
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acquired in these studies served as a valuable resource in decision making throughout the early stages of my career.

Reflections: Questions, Answers, and More Questions

Upon reviewing my research accomplishments and considering what I might emphasize in this article, I was most impressed by the variety of basic biological questions the members of my group have addressed. Early in my career I decided that one of my primary research objectives would be to provide a thorough understanding of all aspects of tryptophan metabolism and to use this knowledge in explaining basic processes of biology. In fact, tryptophan metabolism was the focus of most of my research. However, during the early stages of my career I did not appreciate the variety of scientific questions that I would have the opportunity to address using tryptophan metabolism as my experimental system. Our studies contributed to knowledge on the niacin and tryptophan biosynthetic pathways, enzyme structure/function relationships, organization of genes and operons, the existence of gene-protein colinearity, the molecular basis of suppression, coupling of transcription with translation, regulation of transcription, how tryptophan and tryptophan-tRNA serve as regulatory signals, and the regulatory mechanisms microorganisms use to control tryptophan synthesis and its degradation. The unanticipated role of RNA in regulation, transcription attenuation, was and continues to be one of our major interests. We had no inkling until the 1990s, when bacterial genomes were beginning to be sequenced, that attenuation was so widely used in nature. While we were conducting our investigations on tryptophan metabolism evolutionary questions continually arose. As soon as we understood the features of tryptophan metabolism in one organism we wished to know whether other organisms use the same genes, reactions, and regulatory processes. Despite my personal commitment to tryptophan metabolism, in the early 1980s I returned to studies with *N. crassa* as an experimental organism, addressing other important questions. The lesson to be learned from my experiences, I believe, is to always be on the alert. Important unanswered questions you never anticipated will invariably arise from the results of your current research. It may develop that your chosen experimental system is ideal for answering these questions. Throughout this article I will describe examples taken from my career, where answers led to questions I felt we should address.

The One Gene-One Enzyme Relationship

When I arrived at Yale in 1948 most members of the Bonner group were coping with the most significant question then concerning the *Neurospora* scientific community: how to establish the nature of the gene-enzyme relationship. It was some years after Beadle and Tatum (1) had first proposed the one gene, one enzyme, one biochemical reaction hypothesis. Following the pioneering studies of Garrod in the early 1900s, linking heredity with metabolism, there were numerous observations relating metabolic defects with genetic disorders. Beadle and Tatum cemented this relationship in the early 1940s by selecting an organism, *N. crassa*, that could be used to isolate nutritional mutants. These mutants could then be genetically characterized to establish whether their inability to carry out specific biochemical reactions was because of mutations in specific genes. Most importantly, they observed that there was a one to one relationship between gene and biochemical reaction. Despite these findings, when I was completing my graduate studies in 1951 most scientists were skeptical of the validity of the one gene-one enzyme concept. At this time very little was known about the molecular nature and structure of genetic material or the structure of proteins, and virtually nothing was known about protein synthesis. It was not until the early 1950s that the findings of Hershey and Chase (2) and an earlier finding by Avery et al. (3) convinced most of us that genetic material was most likely DNA, and it was not until 1953 that Jim Watson and Francis Crick (4) described their elegant structure for DNA. Following these major contributions we accepted as proven that the genetic material of most organisms was double-stranded DNA. Furthermore, it was not until the late 1950s that Seymour Benzer’s (5) fine structure genetic analyses with the rII locus of phage T4 equated the genetic map with the structure of DNA. Similarly, it was not until the early 1950s that Sanger’s studies (6) with insulin established that proteins consist of linear sequences of amino acids.

While I was in graduate school the goal considered most important by members of the Beadle-Tatum school was to identify a specific enzymatic reaction for which defective mutants could be isolated and then determine whether these mutants lacked that enzymatic activity. Our hope was that studies like these would provide definitive proof for the one gene-one
enzyme hypothesis. Several members of the Bonner group were following this approach. Naomi Franklin, Otto Landman, Gabriel Lester, and Howard Rickenberg were examining one of the most popular experimental enzymes during this period, \(\beta\)-galactosidase, from both *Neurospora* and *Escherichia coli*. They were hoping to use the knowledge and techniques being provided by Monod, and subsequently by Jacob and Monod and their exceptional coworkers, to explore the Beadle-Tatum gene-enzyme concept more directly. Impressed by this overriding goal of my mentor and the determination of my fellow students, I decided that I too should follow this path. In my third and last year of graduate study, 1950–1951, I abandoned my niacin pathway studies and initiated a search for the ideal "gene-enzyme" experimental system.

No one in our group at Yale was contemplating what today would be considered the most obvious experimental approach: isolating and sequencing a specific gene and comparing this sequence with the amino acid sequence of its polypeptide product. Neither genes nor proteins could be analyzed in this way; we did not yet know that genetic material was DNA or that proteins consisted of linear sequences of amino acids. At this time the prevailing view in the field of genetics was that chromosomes consist of linear arrays of genes arranged like "beads on a string." It was assumed that each gene was indivisible by genetic recombination. If these views were correct how could we determine the relative positions of independent mutational changes in a specific gene, except by structural analysis, which was not possible? We decided that our next step on the gene-enzyme problem should be to demonstrate convincingly that all mutants altered at a single genetic locus lack the specific enzyme that catalyzes the corresponding reaction.

By the late 1940s numerous nutritional mutants of *N. crassa* had been isolated, many requiring the same metabolite. It was evident that amino acids, vitamins, purines, and pyrimidines are all synthesized by sequential enzyme-catalyzed reactions, mostly in separate pathways. However, these pathways were just beginning to be defined. Genetic analyses with these mutants established a very impressive one-to-one relationship between altered gene and loss of a specific biochemical reaction; this was the experimental basis of the Beadle/Tatum concept. It was also evident that a unique set of genes was associated with each metabolic pathway. However, very few of the enzymes in each newly discovered pathway had been identified, and those that were known did not catalyze reactions that were defective in the nutritional mutants that had been isolated. One of the earliest opportunities to examine mutants lacking a specific enzyme was provided by the findings of Umbreit *et al.* in 1946 (7). They demonstrated that extracts of wild type *Neurospora* contain an enzyme they named tryptophan desmolase, which catalyzes the last reaction in tryptophan synthesis, the covalent joining of indole with L-serine to form L-tryptophan. Tryptophan-requiring mutants of *Neurospora* had been identified that could not grow on indole; therefore these mutants should lack this enzyme activity if the Beadle/Tatum hypothesis were correct. Joseph Lein and Dave Hogness, of Hershell Mitchell's laboratory at Caltech, examined extracts of one such mutant, named *td1*, and reported that yes, it did lack tryptophan desmolase activity (8, 9).

Having spent my first 2 years studying niacin and tryptophan metabolism in *Neurospora*, I decided that tryptophan desmolase was promising as a potential subject for gene-enzyme analyses. I initiated my studies by partially purifying and further characterizing the wild type enzyme and confirming the absence of tryptophan desmolase activity in extracts of mutant *td1*. I also examined a second mutant altered at the same locus, mutant *td2*, and showed that it too lacked tryptophan desmolase activity (10). Excited by the simplicity of this enzyme assay and these positive results, members of the Bonner group turned to isolating 20 additional mutants defective in the conversion of indole to tryptophan. We showed that each was genetically altered at the *td* locus and each lacked tryptophan desmolase activity. These initial findings were very encouraging, and they supported the basic assumption of the Beadle/Tatum concept.

In the course of my studies with mutant *td2* one culture grew in media lacking tryptophan. Instead of discarding this culture, we analyzed it genetically and discovered that its ability to grow without tryptophan was due to an unlinked suppressor mutation. The properties of this suppressed *td* mutant raised a new, then unanswerable, question. *How does a suppressor mutation, a mutation in a gene other than the td gene, restore growth without tryptophan?* My enzyme analyses revealed that the suppressor mutation acted by restoring the organism’s ability to form an active tryptophan desmolase (10). Probing still further, I observed that the...
The td2 suppressor gene was allele-specific; it had no effect on mutant td1. Obviously, then, mutants td1 and td2 must have different alterations at the td locus. We next performed "reversion" analyses with all our td mutants and isolated several additional suppressors. Most of these restored tryptophan desmolase activity only when combined with their respective td mutant allele. On the basis of these findings we rephrased our previous question, as follows. If there is a one-to-one relationship between gene and enzyme and only td mutants lack tryptophan desmolase activity, how does a mutation in a gene distinct from the td locus restore this enzyme activity?

My thoughts on possible explanations temporarily diverted attention from my primary objective, establishing the basis of the one gene-one enzyme relationship. I considered our suppression findings to be extremely interesting and believed that their explanation might provide additional insight into this relationship. This experience, I believe, was largely responsible for many of my subsequent decisions on how to proceed in planning future research. I decided then that our knowledge of basic biological processes was so poor it would be foolish to ignore interesting unexplained observations. Following this line of reasoning I set out to compare the properties of tryptophan desmolase isolated from the wild type strain and from several suppressed mutants.

Throughout this period we were frustrated at how little we could do experimentally. The existing molecular technology was clearly inadequate. With a close friend and former member of the Bonner group, Sigmund Suskind, then a postdoctoral fellow performing immunological research at another institution, we designed a different approach that we thought might provide additional insight into the gene-enzyme relationship. The question we set out to answer was the following. Does suppressible mutant td2, but not non-suppressible mutant td1, produce an inactive form of the tryptophan desmolase enzyme? Using my partially purified wild type enzyme as antigen, Suskind prepared a rabbit antiserum that inhibited wild type tryptophan desmolase activity. We used this antiserum in a successful weekend experiment at Yale, analyzing extracts of mutants td1 and td2 for an inactive tryptophan desmolase-like protein that would cross-react with our antiserum (11). Mutant td2 extracts did in fact contain such a cross-reacting material, for which we coined the term "CRM," whereas extracts of mutant td1 did not. Comparable analyses were then performed with extracts of our other td mutants. All our suppressible mutants were shown to be CRM⁺, whereas all our non-suppressible mutants were CRM⁻. These findings implied, incorrectly, that suppression can restore a functional enzyme only if a mutant produces an inactive form of the wild type enzyme. (There are several reasonable explanations for our inability to isolate suppressors of our CRM⁻ Neurospora mutants, which probably had chain termination mutations in the td gene.)

On the basis of our findings I drew a number of interesting conclusions. I presented these at a very exciting symposium entitled "Enzymes, Units of Biological Structure and Function" held at the Henry Ford Hospital in Detroit in 1955 (Fig. 1) (12). My interpretations were of course influenced by new knowledge on DNA and protein structure and the mechanism of protein synthesis. I concluded that "the td locus is the only chromosomal area which directly controls tryptophan synthetase formation" (the accepted name had just been changed from desmolase to synthetase). I also concluded that "the td locus represents a physiologically indivisible unit, damage to any part of which results in a defect in tryptophan synthetase
formation.” I stated that “it would seem likely that different portions of the td locus are concerned with the synthesis of different parts of the tryptophan synthetase molecule.” In attempting to explain how a suppressor mutation restores enzyme activity I postulated that “some product of a suppressor gene cooperates with the altered template in the formation of small amounts of tryptophan synthetase.” Looking back on these interpretations, they were all naive guesses, but they proved to be correct. Unfortunately the experimental tools and approaches needed to establish their molecular validity were not available. These studies on missense suppression preceded the enormous interest in suppression aroused by studies on the genetic code and on nonsense mutations. As is so often the case, the significance of a finding is not appreciated until additional relevant knowledge is acquired.

**Changing my Experimental Organism**

At this stage in my career I was deeply committed to doing everything I could to provide additional insight into the gene-enzyme relationship. I was disappointed at the difficulty I was experiencing attempting to purify the tryptophan synthetase of *Neurospora* and initiated a search for a more suitable experimental enzyme. My first thought was to identify an enzyme in the tryptophan to niacin pathway from *E. coli* or *Bacillus subtilis*, because these organisms were developing as more ideal experimental subjects for biochemical analyses. I performed radioisotope-labeling experiments with these two organisms, hoping to show that one or both synthesizes niacin from tryptophan. My findings provided a disappointing conclusion; neither organism synthesizes niacin from tryptophan (13). This negative result eliminated enzymes of the niacin pathway from my list of possibilities.

While performing these studies I was offered a faculty position in the outstanding Microbiology Department at the Western Reserve University School of Medicine. I decided to accept their offer and left Yale for Cleveland in 1954. As a beginning Assistant Professor I felt it would be wiser to shift my research objectives to a well defined problem, one for which I could foresee obtaining definitive answers. I relied on my prior scientific experience and chose determining the missing reactions in the tryptophan biosynthetic pathway. Although many different classes of tryptophan auxotrophs had been isolated in *Neurospora, E. coli*, and other organisms, only two intermediates in the tryptophan pathway had been identified, anthranilate and indole. I chose an enzymological approach in attempting to identify the intermediates in the pathway and initiated my studies by analyzing extracts of wild type and different classes of tryptophan auxotrophs of *E. coli*.

My efforts focused on unidentified intermediates in the tryptophan biosynthetic pathway were successful. Using an enzymological approach we succeeded where others who had employed *in vivo* approaches had failed. The principal reason for this is that the unidentified intermediates in the tryptophan pathway are all phosphorylated. Phosphorylated intermediates accumulated *in vivo* would have been dephosphorylated and therefore inactive when fed to a mutant. With the aid of my graduate student Oliver Smith, the following intermediates were identified: phosphoribosyl anthranilate, carboxyphenylamino-1-deoxyribulose 5-phosphate, and indole-3-glycerol phosphate (IGP). The initial precursor of the tryptophan pathway, chorismic acid, was isolated and identified by Frank Gibson, working with his own group in Australia. Chorismate also serves as precursor of the other aromatic amino acids. With the identification of these additional compounds, the precursor and all the intermediates in the tryptophan biosynthetic pathway were known.

While conducting these studies I made an unanticipated observation that subsequently proved to be of enormous benefit in our colinearity studies. I observed that many tryptophan auxotrophs of *E. coli*, when cultured on growth-limiting levels of tryptophan, produced 20–50 times more tryptophan synthetase than the wild type strain. I thought that the day might come, as it did, when I could exploit this observation to overproduce mutant proteins for purification and analysis. I was aware of the regulatory significance of this observation and concluded that ultimately we should address the regulatory mechanism(s) responsible for this increase.

Despite this temporary diversion in the mid-1950s, I was still committed to establishing the nature of the gene-enzyme relationship. Knowledge about genes, proteins, and protein synthesis was improving, so much so that the gene-enzyme relationship was redefined. The question had matured to the following. *Is the nucleotide sequence of a gene colinear with the amino acid sequence of the corresponding protein?* During this period we learned many new
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facts about tryptophan synthetase. I thought it might prove to be an ideal enzyme for addressing the colinearity question. Our continuing investigations with this enzyme, from both Neurospora and E. coli, suggested that it may catalyze the last two reactions in tryptophan formation, the cleavage of IGP to indole and the coupling of indole with serine to form tryptophan. However, there were two observations we could not explain: free indole could not be detected as an intermediate in the conversion of IGP to tryptophan, and the rate of conversion of IGP to indole was lower than its rate of conversion to tryptophan (14). We then had to ask the following question. Does the enzyme catalyze a third reaction in which IGP and serine react with one another to form tryptophan, or is indole truly the intermediate, and it remains within the enzyme complex? This puzzle was not satisfactorily solved until the late 1980s. Then, the elegant structural solution for the αβ2 tryptophan synthase (name changed again) enzyme complex of Salmonella by Hyde et al. (15) revealed that there is a physical tunnel in this enzyme complex connecting the active site of one polypeptide subunit, α, where indole is produced from IGP, to an active site of the second subunit, β2, where indole reacts with L-serine to form L-tryptophan (15, 16). As you might imagine, it was comforting to have our confusing early observations explained unambiguously by structural and enzymatic studies.

At this stage in my career everything was going well for me at Western Reserve Medical School. I had quality co-workers and I thoroughly enjoyed my interactions with my fellow faculty members, Howard Gest, John Spizizen, David Novelli, Bob Greenberg, and Abe Stavitsky. However, in 1957 I was contacted by Victor Twitty, chairman of the Department of Biological Sciences at Stanford University, and offered a faculty position. Despite my initial disinterest in considering this appointment, I accepted their offer for a variety of reasons, including my learning that Arthur Kornberg’s department would be moving to the Stanford campus (to the Stanford Medical School, which was being relocated from San Francisco). Of historical interest, when I arrived at Stanford the laboratory space I was provided was in the basement of old Jordan Hall and was the space previously occupied by Ed Tatum and his research team. I truly was treading in Tatum’s footsteps!

Proving or Disproving Gene-Enzyme Colinearity

When setting up my laboratory at Stanford in January of 1958, I decided that the time had come to mount an all out effort to establish or disprove gene-protein colinearity. I was joined in this project by an outstanding young postdoctoral fellow, Irving Crawford, who was recommended to me by Arthur Kornberg. In his exploratory studies with tryptophan synthetase from E. coli, Irving was first to establish that the enzyme is a complex composed of non-identical polypeptide chains. One subunit, TrpA (TSase α), hydrolyzes IGP to indole, whereas the second subunit, TrpB (TSase β2), covalently joins indole and L-serine to form L-tryptophan (17). However, the enzyme from Neurospora is a single polypeptide chain. In what proved to be an extremely valuable observation for our subsequent colinearity studies, Irving found that each E. coli subunit activates the other subunit in the reaction that subunit performs alone. This finding suggested that we might be able to detect and assay each inactive TrpA mutant protein enzymatically by measuring its ability to activate the TrpB subunit in the indole plus serine to tryptophan reaction. This expectation proved to be correct; we routinely assayed each mutant TrpA protein during its purification by measuring its activation of TrpB.

We next prepared a set of pure mutant TrpA proteins, each presumably with a single inactivating amino acid change. Good fortune helped us again, for in 1958 Vernon Ingram described an elegant method, “peptide fingerprinting,” which he had used to detect peptides with single amino acid changes in mutant human hemoglobins (18). This approach seemed ideal for what we wished to do. If we could identify the single amino acid change in each of our mutant proteins we would then only have to compare the positions of these amino acid changes in TrpA with the order of the corresponding altered sites on a fine structure genetic map of the trpA gene to prove or disprove gene-protein colinearit y. I knew that we could construct a fine structure genetic map of trpA using phage P1, based on a previous genetic study I performed with Ed Lennox (19). I was confident that very shortly we would convincingly prove or disprove gene-protein colinearity.

As one’s research accomplishments become better known to the scientific community, increasing numbers of young scientists will apply to join your group. This necessitates making decisions on what size group you consider optimal and how many projects you wish to attack.
Because I enjoyed working at the bench, I felt that I would have sufficient time to serve as advisor to a maximum of about four graduate students and four to six postdoctoral fellows. I had decided sometime earlier to employ two research assistants who would work closely with me, one to perform genetic analyses and the second to carry out biochemical procedures. I was extremely fortunate that an exceptionally bright and competent assistant, Ginny Horn, joined my group in 1958. She performed many of our genetic analyses for over 40 years. A series of talented assistants provided my biochemical "hands."

In the early 1960s the colinearity problem was well publicized. Progress was being made in several laboratories, and it was discussed at many scientific meetings. Outstanding young scientists who joined my group to work on this problem were Don Helinski, Ulf Henning, and Barbara Maling, followed by Bruce Carlton, John Guest, and Gabriel Drapeau. Of considerable aid in our genetic analyses was the use of overlapping trpA deletion mutants for initial localization of primary mutations on our fine structure genetic map of the trpA gene. Thus we exploited the approach used so successfully by Seymour Benzer. We obtained trpA deletion mutants by selecting bacteria resistant to phage T1 and screened for those requiring tryptophan for growth. These arose because the tonB locus is close to the trpA locus, and tonB deletions that confer resistance to phage T1 often extend into trpA. The contributions of the individuals mentioned above and those who replaced them established colinearity of the TrpA protein with the trpA gene in the early 1960s. I first described our findings supporting colinearity at the Cold Spring Harbor Symposium of 1963 (20). Our complete proof was published in 1964 (21) (Fig. 2). Because thoroughness was an essential element of my strategy, we continued our protein sequencing analyses until the entire amino acid sequence of the 268-residue TrpA protein was completed in 1967 (22). This was by no means a trivial feat, given the technology then available. At that time I believe the TrpA protein was the longest polypeptide to have been completely sequenced.

As mentioned, we were not the only group addressing the colinearity problem. Comparable studies were being performed with the alkaline phosphatase of E. coli by Rothman, Garen, and Levinthal, the lysozyme of phage T4 by Streisinger and Dreyer, the rII locus of phage T4 by Benzer and co-workers, and by others working with different gene-protein systems (23). During this period Sydney Brenner and his co-workers also established gene-protein colinearity using a simpler, ingenious strategy (24). They reasoned that the length of a polypeptide chain should be determined by the location of the first in phase stop codon in a coding region. Applying this logic they mapped nonsense mutations to different positions in the head protein gene of phage T4 and demonstrated that the length of the head protein fragments these mutants produced correlated with the locations of the stop codon mutations on the genetic map of the head protein gene.

Despite the many findings in the 1960s supporting gene-protein colinearity, we of course were unaware at the time of the existence of splicing, differential splicing, and trans-slicing, common processes that would have weakened our confidence in our conclusion.
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Turning to the Genetic Code

Technology did not exist in the 1960s that would allow us to determine the nucleotide changes in our mutated genes. Fine structure genetic mapping, a la Benzer, was the only effective strategy to characterize a mutated gene. However, much was being learned about mutagenesis and mutagen specificity, primarily, as I recall, from studies by Seymour Benzer and Ernst Freese. One of their objectives was to use mutagens with differing specificities to help in deciphering the genetic code. If the code is a triplet code, as deduced by Crick and co-workers (25), and if chemical mutagens do induce specific nucleotide changes in DNA, then it should be possible to correlate specific amino acid changes in any protein with presumed induced nucleotide changes in the specifying gene. This indirect approach, if applied to all 20 amino acids, should reveal the nature of the genetic code. We felt that we could use it with our system to solve the genetic code. This basic question was as follows. Can we deduce the genetic code by analyzing the amino acid changes in the TrpA proteins of trpA mutants and their revertants, produced with mutagens with differing known specificities? The following members of my group adopted this strategy: John Guest, Manny Murgola, Hillard Berger, and Bill Brammer. They successfully used specific mutagens to produce multiple classes of revertants from each of our trpA mutants and identified the amino acid changes in many mutant and revertant proteins. This approach also laid the groundwork for impressive subsequent studies on mechanisms of suppression, carried out by Manny Murgola. While these studies were underway the entire scientific world, us included, was startled to learn that Marshall Nirenberg had developed an elegant in vitro method that would allow the complete genetic code to be deciphered quickly and unambiguously. Despite our inability to compete with Nirenberg, we did obtain appreciable in vivo data supporting his deductions for over 45 codons (26). We also performed mutant by mutant crosses with mutants bearing different amino acid changes at the same TrpA position and showed that genetic recombination can occur within a coding triplet and yield a recombinant amino acid (27).

Other Gene-Protein Issues

While performing studies on the proteins of “revertants” of trpA mutants, Don Helinski noticed that some presumed revertants retained the original mutant amino acid change. Prototrophy in these revertants was because of a compensating, second amino acid change. We named this phenomenon “second site reversion” (28). Helinski also observed that the second site amino acid change in one of these “revertants,” when introduced alone in TrpA, also inactivated the protein. Thus, two inactivating single amino acid changes, when combined in the same protein, could restore enzyme activity. These findings could not be explained at the time, and it was apparent that they would have to await structural examination. When three-dimensional structure of the tryptophan synthase enzyme complex of Salmonella was solved in the late 1980s it was observed that the residues altered in the second site mutants were all in close spatial proximity in the active site of the TrpA subunit (15). Computer graphics modeling predicted that the compensating residue changes acted by restoring the proper geometry of the substrate binding site in TrpA (29).

Returning to Suppression

A familiar question resurfaced in the early 1960s in our studies with trpA mutants. How does a mutation in a specific suppressor gene permit a trpA missense mutant to produce a functional enzyme? Stu Brody purified the active TrpA protein of one suppressed missense mutant and used peptide fingerprinting analyses to show that the active protein has the wild type residue, Gly, rather than the mutant residue, Arg, at the critical position in the TrpA protein (30). He postulated that suppression causes translational misreading of the mutant Arg codon, leading to the insertion of the wild type amino acid, Gly, at the critical position in the protein. When Brody became aware of the role of transfer RNAs in protein synthesis, he postulated that his missense suppressors, like previously characterized nonsense suppressors, might produce an altered transfer RNA that incorporates the wrong amino acid. This proposal was confirmed experimentally in beautiful studies with transcripts of synthetic DNAs of defined sequences by John Carbon and Paul Berg (31) and N. Gupta and Gobind Khorana (32). Paul has described our personal interactions that led to these successful in vitro studies (33).
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Opening Pandora’s Box

Following completion of our colinearity studies and our foray into deducing the genetic code, there were many unsolved biological problems begging for our attention. The course I followed was conservative; I decided to exploit the knowledge we had recently gathered and attempt to deducing answer what I considered the next set of important questions including the following. How does each trp enzyme catalyze its respective reaction? What are the three-dimensional structures of the trp enzymes, and how are they related? What are the advantages of forming two enzyme complexes, each containing two different trp polypeptides? What is the purpose of producing two bifunctional trp polypeptides? What is the significance, if any, of the order and organization of the trp genes in the trp operon? What is the explanation for the polar effect of nonsense mutations on downstream gene expression, and what is its significance? What are the important features of transcription, translation, and mRNA degradation for the trp operon of E. coli? What were the ancestral sources of the genes specifying the trp biosynthetic enzymes? Addressing one of these biochemical questions, graduate student Tom Creighton analyzed the subunit structure of the tryptophan synthetase enzyme complex in the mid-1960s. In a collaborative study with Michel Goldberg and Robert Baldwin of the Biochemistry Department at Stanford, they concluded that this enzyme complex has an αβα structure (where α is TrpA and β is TrpB) with α alone existing as a monomer and β alone as a β2 dimer (34). Convinced that structural information was essential if we were to provide a thorough understanding of this enzyme’s action, Ulf Henning grew beautiful crystals of the E. coli α chain hoping they would be suitable for crystallographic analysis. In addition, I spent a summer at the University of California in San Diego exploring with members of Joe Kraut’s group the possibility of growing α chain crystals satisfactory for structure determination. This approach was pursued by Tom Creighton when he moved to Yale. He had some success, but unfortunately satisfactory crystals of the tryptophan synthase α subunit of E. coli could not be grown reproducibly. On a related project, John Hardman of my group initiated studies on the three cysteine residues in the TrpA polypeptide that we thought were essential. His findings on substrate protection of these three cysteines were provocative, but it was evident that without the three-dimensional structure of the protein for reference, these active site studies would be inconclusive. I therefore discontinued work on this project. As I mentioned, the structure of the αβα tryptophan synthetase enzyme complex from Salmonella was eventually solved by Craig Hyde, Edith Miles, David Davies, and their co-workers (15). The structural information they provided served as an invaluable resource for many years, allowing crucial questions to be answered, such as how do the two active sites in the enzyme complex catalyze their respective reactions and how are these sites cross-activated by substrate binding (16, 35).

Ted Cox took a broader view of the consequences of mutations and questioned their impact on organism well being and survival. While with me he began his studies with the mutT mutator gene of E. coli. In 1967 we showed that mutT causes AT to CG mutations preferentially and that continued cultivation of strains with mutT led to a uniform shift in the base composition of their total DNA (36). The changes he detected represented about a 0.2–0.5% increase in GC composition. This observation raised additional questions. What fraction of the residues in each protein is essential? What fraction of the base pairs in the genome of E. coli can be changed without having serious consequences? I decided not to address these questions at this time.

Turning Our Attention to Organization and Expression of the trp Operon

In the mid 1960s the features of the trp operon of E. coli that contributed to its expression were poorly understood. The order of the five genes in the operon had been established, but very little was known about operon transcription or how trp mRNA translation and degradation proceeded or how these processes were regulated. These basic questions were exciting to young molecular microbiologists, and new members of my group were eager to address one or more of these problems. My co-workers on these subjects from the mid-1960s to the early 1970s were Ron Somerville, Dan Morse, Ray Mosteller, Ron Baker, Robert Baker, Jack Rose, Jun Ito, Fumio Imamoto, Ethel Jackson, Jes Forchhammer, and Sota Hiraga. Of significant aid in our mRNA studies was the use of a temperate bacteriophage, φ80, characterized by A. Matsushiro. This phage genome integrates adjacent to the trp operon, allowing one to obtain improperly excised transducing phage that carry different segments of the trp operon. The DNAs of these trp transducing phage could then be used to detect and measure the relative amounts of
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labeled mRNA derived from any segment of the operon. A very important, but unrelated project, was carried out with this phage by Naomi Franklin, who was then in my laboratory, with Bill Dove at our Medical School. They provided genetic evidence indicating that during lysogenization the φ80 genome is inserted into the bacterial chromosome. I believe this was the first experimental evidence supporting the Campbell integration model of lysogenization (37).

Using the isolated DNA of trp transducing phage bearing different segments of the trp operon, RNA hybridization data were gathered for different genes of the operon. It was shown that the operon specifies a single polycistronic trp mRNA encoding all five of the trp polypeptides and that the transcript was translated as it was being synthesized. It was also observed that nascent trp mRNA was generally attacked before its synthesis was completed. Thus most trp transcripts isolated from growing cultures were less than full length (38). The last coding region of the trp operon transcript, trpA mRNA, was found to be degraded in the 3′ to 5′ direction (39). Most nonsense mutations in the first four genes of the operon had a negative, polar effect on downstream gene expression, reducing both trp mRNA and protein levels for the downstream genes (40). This “polarity” was a common observation with many bacterial systems. We also found that the untranslated mRNA segment immediately downstream of each introduced nonsense codon was particularly labile (41), consistent with Rho-mediated transcription termination in the untranslated region of the messenger and 3′ to 5′ degradation of each untranslated mRNA segment. Ron Somerville observed continued synthesis of the TrpA polypeptide, but not the TrpB polypeptide, upon prolonged tryptophan starvation (42). His findings were consistent with the presence of a single Trp residue in TrpB but none in TrpA (43). The location of the internal promoter within the trp operon, previously identified by Bauerle and Margolin in the Salmonella trp operon (44), was determined for E. coli by Ethel Jackson of my group by preparing and examining internal deletions in the operon (45). Ultimately its nucleotide sequence (for E. coli) was established by Terry Platt’s group when Terry had his own laboratory (46). In other studies with the TrpA protein, Dave Jackson observed that he could complement (restore activity to) a mutant TrpA polypeptide in vitro by unfolding and refolding the polypeptide in the presence of a second mutant TrpA polypeptide that had an amino acid change elsewhere in the protein (47). Refolding of a mixture of mutant polypeptides allowed this normally monomeric protein to occasionally form an active dimeric species. Restoration of enzyme activity also was observed upon refolding a mutant polypeptide in the presence of a short fragment of wild type polypeptide that corresponds to the mutated segment (47). A model has been proposed explaining these examples of in vitro complementation (16). These studies suggested interesting approaches that could be used in studying the mechanism of protein folding.

On to Operon Regulation

Despite these advances, we had not yet begun to address what was becoming the most challenging question for most bacterial physiologists. How is transcription of your operon regulated? In early regulatory studies with the trp operon of E. coli, Georges Cohen and Francois Jacob identified a presumed repressor locus, trpR, that appeared to negatively regulate expression of the trp operon. In the early 1960s the only additional regulatory observation that concerned tryptophan biosynthesis was the finding that the enzyme catalyzing the initial reaction in the pathway, anthranilate synthase, was feedback-inhibited by tryptophan. Feedback inhibition of the enzyme performing the first reaction in a pathway is common to most biosynthetic pathways. At this time we were reasonably comfortable with the belief that repression plus feedback inhibition for the trp operon could deal with all the regulatory needs of the bacterium. To analyze repression more thoroughly, Cathy Squires and Jack Rose of my group partially purified the trp repressor and (with the aid of Geoffrey Zubay and H. L. Yang) performed in vitro analyses showing that the trp repressor is tryptophan-activated and that the repressor does inhibit transcription initiation at the trp operon promoter. Follow-up studies by Jack Rose, Cathy Squires, Frank Lee, Rick Kelley, George Bennett, and Rob Gunsalus developed the trp repressor-trp operator into an excellent experimental system. They showed that the repressor is a dimer, that it has two helix-turn-helix DNA binding domains, and that crucial base pairs in the palindromic trp operator are required for repressor binding (48–52). With the aid of Andrzej Joachimiak from Paul Sigler’s group, the trp repressor was purified and initially characterized. Sigler’s group then initiated their elegant studies culminating in determination of the three-dimensional structures of the trp
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A Surprise: the trp Operon Is Also Regulated by Transcription Attenuation!

In the early 1970s we were well aware of the findings by other groups who were conducting regulatory studies with amino acid biosynthetic operons of bacteria. The experimental results of Bruce Ames and his co-workers at the University of California, Berkeley, were of particular interest to us because the his operon of Salmonella they were studying and our trp operon had many similarities. Ames showed that transcription of the his operon was not regulated by a histidine-responsive his repressor; rather, histidinyl-tRNA was implicated as the molecule that was sensed in the regulatory decision (58). Furthermore, the leader region of the his operon, not its promoter, appeared to be the site of regulation. Graduate student Ford Doolittle of my group was persuaded to consider these findings seriously, and he performed a series of regulatory studies with slightly defective E. coli tryptophanyl-tRNA synthetase mutants. His results demonstrated that tryptophanyl-tRNA is not involved in trp repressor action; thus his findings put our concerns to rest, at least for the moment (59). However, measurements of trp mRNA levels carried out during this period by Ron Baker of my group suggested that there may be a second regulatory mechanism, distinct from repression, that regulates transcription of the trp operon of E. coli. Baker observed that mutants lacking a functional trp repressor still responded to tryptophan starvation by increasing their rate of synthesis of trp mRNA. Consistent with this observation was the finding by Fumio Imamoto, then back in his own laboratory in Japan, that transcription in progress in the initial segment of the trp operon was stopped prematurely upon addition of tryptophan to a tryptophan-starved culture. We wondered: what is the significance of these regulatory findings?

Explaining Transcription Attenuation

In the early 1970s Ethel Jackson made the key observation that convinced me to search for a regulatory mechanism distinct from repression that regulates transcription of the trp operon (60). As mentioned, Ethel developed a procedure that allowed her to isolate deletions with both end points within the trp operon. Her initial objective was locating the internal promoter precisely. During these studies she made the unexpected observation that a class of internal deletions with one end point in the leader region of the operon, the region just following the promoter and before the first structural gene, trpE, increased operon expression 6-fold. This increase also was observed in a repressor minus strain! This suggested that there may be a second regulatory site, possibly a site of regulated transcription termination, that can influence trp operon expression (60). At about the same time, A. Kasai, at Johns Hopkins University, was performing similar analyses of the effects of deletions that ended in the leader region of the his operon of Salmonella. Kasai also concluded that the his operon leader region may contain a regulated site of transcription termination (61). He introduced the term “transcription attenuation” to describe the mechanism of transcription regulation that presumably occurs at this site. I adopted this term in our studies with the trp operon because I felt it was entirely appropriate.

Attenuation Proves to Be a Complex, Multistep Process

In the early and mid-1970s most members of my group were studying features of the trp operon and tryptophan metabolism that we believed contributed to operon expression or regulation. We did not appreciate that each was analyzing an event that was crucial to transcription attenuation. The trp operon leader region (the genetic segment responsible for transcription attenuation) was isolated and characterized. It is ~160 bp in length and is
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located between the promoter and the first major structural gene of the operon. This “leader region” was sequenced, first as RNA, and then, when DNA sequencing technology became available, as DNA. This sequence raised several new questions requiring our immediate attention. What features of the leader region are responsible for transcription attenuation? Is tryptophan or tRNA\textsuperscript{Trp} the signal that is recognized during attenuation in the trp operon? Does the leader region sequence provide any clues that would help us to explain how one of these molecules could act as a regulatory signal? A potential transcription termination site was located in the leader region just before trpE. It had all the features now ascribed to intrinsic transcription termination sites. The members of my group who performed these initial studies were: Kevin Bertrand, Craig Squires, Cathy Squires, Frank Lee, Morley Bronson, Terry Platt, Laurence Korn, George Bennett, Iwona Stroynowski, and Giuseppe Miozzari. Terry Platt had been attempting to identify all the ribosome binding sites in trp operon mRNA. He detected one ribosome binding site that was unanticipated; it was located in the leader segment of the transcript. This was an exciting discovery because this site was associated with a 14-residue coding region with two adjacent tryptophan codons. This coding region was located just prior to the terminator sequence. To explore the function of this leader peptide coding region we added the following questions to our “list” of those we felt must be addressed. What is the role of the transcript’s leader peptide coding region, is it regulatory, and does it allow regulation of transcription termination? If it is regulatory, do the Trp codons in the leader peptide coding participate in the regulatory decision? Is the regulatory signal that is sensed uncharged tRNA\textsuperscript{Trp}? It was evident we were dealing with unfamiliar events in a complex process. Fortunately for us, previous findings with the his operon addressed several of these questions; therefore they were extremely helpful. In retrospect, every member of my group contributed to answers to one or more of these questions. We were aided in these studies by Larry Soll, a former student of Paul Berg, who was then at the University of Colorado; Larry performed some of the crucial early experiments implicating tRNA\textsuperscript{Trp} as the regulatory signal (62). Dan Morse, after leaving my laboratory, independently contributed findings establishing the role of uncharged tRNA\textsuperscript{Trp} in trp operon attenuation (63). It was becoming clear that transcription attenuation in the trp operon involved several sequential events, each dependent upon specific sequences in the transcript of the leader region.

Of particular significance during this period was the finding by graduate student Frank Lee that the trp leader transcript could fold to form alternative hairpin structures, each of which plays an essential role in determining whether transcription termination will occur (64). One RNA hairpin serves as a transcription terminator; it directs RNA polymerase to terminate transcription. The second, alternative RNA hairpin functions as an antiterminator. Inspection of the sequence revealed that prior formation of the antiterminator would prevent formation of the terminator. Which of the alternative hairpin structures would form would depend on the cell’s ability to translate the two Trp codons in the 14-residue leader peptide coding region. When these two tryptophan codons are translated, the antiterminator would not form; this would allow the terminator to form and terminate transcription (65, 66). When cells are deficient in charged tRNA\textsuperscript{Trp} the translating ribosome would stall at one of the Trp codons. This stalling would promote antiterminator formation, which would then prevent formation of the terminator (65, 66).

Studies over the past 30 years have shown that transcription attenuation is a common regulatory process; variations are used by many bacterial species and their viruses (67). Several transcription attenuation mechanisms are described in a recent review by Henkin and myself (68). In the earliest studied example of regulation by transcription termination/antitermination, the N protein of bacteriophage λ was shown to prevent Rho-dependent transcription termination during transcription of a region of the phage genome. Attenuation in the his operon of Salmonella, as mentioned, also was an early studied example; most of its features closely resemble those of attenuation in the E. coli trp operon. More recently it has been learned that in addition to ribosome and protein-mediated transcription attenuation decisions, uncharged tRNA (69) and various metabolites can interact directly with leader RNA and regulate transcription termination (70). For example, many of the genes encoding aminoacyl-tRNA synthetases in B. subtilis and other Gram-positive bacteria have been shown by Frank Grundy and Tina Henkin and their co-workers to be regulated by direct tRNA-mediated transcription attenuation (71). There are related translational examples where translation of an upstream mRNA coding region influences translation initiation at the adjacent down-
stream coding region (72). Often translation initiation at the downstream coding region is blocked by an appropriate RNA secondary structure; an event occurring during translation of the upstream coding region, such as chloramphenicol binding to the translating ribosome, then exposes the downstream translation initiation region to ribosome loading and translation initiation (72).

Following this initial period of our investigations on transcription attenuation, it was obvious that we would have to establish the role of each segment of the trp leader transcript and explain the many events participating in this process. Gerard Zurawski, George Stauffer, and Dirk Elseviers, and sabbatical visitors Keith Brown and Dale Oxender performed important studies that provided a thorough understanding of many of the features of transcription attenuation in the trp operon of E. coli (65, 66). One obvious, crucial concern that we had not yet addressed was as follows. How are transcription and translation of the trp leader region coordinated and coupled, as they must be if translation of the leader peptide coding region is to serve as the decision-making event regulating transcription termination? Postdoctoral fellows Malcolm Winkler, Bob Fisher, Bob Landick, and Jannette Carey answered this question. They established the role of a third hairpin structure that can form in the trp leader transcript, a structure that precedes and is an alternative to the antiterminator. This structure, which also serves as an anti-antiterminator, causes the transcribing RNA polymerase to pause during transcription of the leader region (73–76). This pause allows sufficient time for a ribosome to bind to and initiate translation of the leader peptide coding region. Landick and Carey in fact showed that it is this translating ribosome that releases the paused RNA polymerase, allowing transcription and translation to proceed simultaneously (77). This coupling is essential to allow charged or uncharged tRNATrp to be recognized by the translating ribosome and serve as the regulatory signal. Progress has been made in explaining polymerase-transcript interactions that are responsible for transcription pausing, thanks to thorough studies on this subject by Bob Landick and his group (78) and detailed structural analyses on RNA polymerases and their action provided by Roger Kornberg, Seth Darst, and their co-workers. A simplified view of most of the stages in regulation by transcription attenuation in the trp operon of E. coli is presented in Fig. 3.

In vivo analyses were also performed to assess the relative contributions of repression and transcription termination in regulating trp operon expression (55). We concluded that repression regulates transcription initiation in the trp operon about 80-fold, with repression at a minimum during growth with little or no tryptophan. In contrast, transcription attenuation in the trp operon of E. coli allows only 6-fold regulation, with termination being relieved only when cells are virtually depleted of charged tRNATrp. The relative insensitivity of attenuation
regulation of the trp operon to the accumulation of uncharged tRNA^{Trp} reflects the presence of only two Trp codons in the trp leader peptide coding region. In the his operon of Salmonella, for example, where attenuation is the major transcription regulatory mechanism, there are seven contiguous His codons in the leader peptide coding region. This organization makes the his operon particularly sensitive to a deficiency of charged tRNA^{His}.

DNA microarray analyses have been performed with wild type E. coli and several regulatory mutants under a variety of growth conditions that influence tryptophan metabolism (79). In general the changes in mRNA abundance observed are consistent both qualitatively and quantitatively with expectations based on years of studies of tryptophan metabolism. As expected, many indirect effects were also observed.

Miscellaneous Important Developments

A major advance in conventional cloning was the development of plasmid ColE1 for this purpose by Don Helinski, Herb Boyer, and their co-workers in the early 1970s. We provided the trp operon for these studies and analyzed its expression in their classic plasmid cloning/overexpression paper (80). When DNA cloning and sequencing procedures became available, my group collaborated with several of my former students in determining the complete 7000-base pair sequence of the trp operon of E. coli; this sequence was published in 1981 (43). Inspection of the nucleotide sequence of the operon revealed many unsuspected features. Among these was the presence of overlapping stop and start codons, UGAUG, joining trpE and trpD, and trpB and trpA (43). This punctuation arrangement was intriguing because we already knew that both the TrpE and TrpD polypeptides, and the TrpB and TrpA polypeptides, associate to form enzyme complexes. We wondered, what is the significance of these stop/start overlaps? Do they allow some form of translational coupling that ensures that the cell synthesizes equal numbers of polypeptides that will form an enzyme complex? Dan Oppenheim and Anath Das addressed these questions and concluded that translation of these adjacent coding regions is coupled, i.e. equal numbers of the two polypeptides encoded by adjacent regions are produced only when the upstream coding region is translated to completion at its overlapping stop codon (81, 82). Translation initiation and termination obviously are very complex processes; transcript sequences can have profound effects on these events.

Studies on Regulation of Tryptophan Degradation

E. coli and many other bacteria have the ability to degrade tryptophan. They produce the enzyme tryptophanase, which degrades tryptophan to indole, pyruvate, and ammonia. Pyruvate and ammonia can be used as carbon and nitrogen sources. Indole’s role, other than serving as a tryptophan precursor, is not clear, although recent evidence suggests that it may act as a volatile signal molecule during biofilm formation and quorum sensing (83, 84). The latter observations raise an additional unexpected question. Is the purpose of the tunnel connecting the two active sites of tryptophan synthase to prevent biosynthetic indole from escaping into the environment?

Because degradation of tryptophan would be expected to influence regulation of its synthesis, I decided in the early 1980s that it was essential that we thoroughly investigate the tryptophanase (tna) operon and how it is regulated. Our initial questions were as follows. How is the tna operon organized? How is this operon regulated? What are the effects of tna operon expression on trp operon expression and regulation? Studies on these questions were initiated by Mike Deeley; he was followed on this project by Valley Stewart, Paul Gollnick, Kurt Gish, Ajith Kamath, Vincent Konan, and most recently, Feng Gong. The tna operon of E. coli has two structural genes, one encoding tryptophanase and the second specifying a tryptophan permease. Transcription initiation in this operon had been shown by others to be regulated by catabolite repression. In our investigations we discovered that transcription of the structural genes of this operon is regulated by a novel mechanism of transcription attenuation. This mechanism is based on features of the nucleotide sequence of the operon’s ~300-bp leader region. Tryptophan is the signal molecule that leads to relief from transcription termination. When cultures are growing without excess tryptophan, Rho factor binds to the nascent tna operon leader transcript. Bound Rho then contacts the transcribing polymerase that is paused at one of several pause sites in the leader region, and it instructs it to terminate transcription (85, 86). If cultures are growing with high levels of tryptophan, Rho factor’s ability to bind to the leader RNA is prevented. Therefore the paused polymerase resumes transcription into the structural genes of the operon (85, 86). Synthesis of a 24-residue tryptophan-containing leader
peptide, TnaC, as well as high levels of free tryptophan are required for induction (86). It is thought that the combined action of the nascent uncleaved TnaC-peptidyl-tRNA and bound tryptophan inhibits peptidyl transferase cleavage of the TnaC-peptidyl-tRNA (87, 88). The uncleaved TnaC-peptidyl-tRNA therefore remains associated with the translating ribosome, preventing it from dissociating from the transcript. The stalled ribosome then blocks Rho factor's access to the transcript, thereby allowing the paused polymerase to resume transcription (87). Recent studies suggest that the tryptophan binding induction site may be the site normally occupied by the aminoacyl moiety of a charged tRNA during translation (88). These findings raise challenging questions about the functional flexibility of the ribosome, questions that become more interesting when they are related to recent exciting structural studies with the ribosome. What are the structural features of the tryptophan binding site created in the ribosome? How does the tryptophan residue at position 12 of the leader peptide create or modify this binding site? How does bound tryptophan inhibit peptidyl transferase? The presence of active tryptophanase in a growing culture reduces the tryptophan concentration, which increases trp operon expression (89).

Evolutionary Issues

As our gene structure and function studies progressed, many evolutionary questions arose. Do other organisms use the same genes, proteins, operon organization, and regulatory processes as E. coli in performing tryptophan biosynthesis and its regulation? Can homologous segments of a trp polypeptide from two species be exchanged without loss of enzyme activity? What were the ancestors of the present day genes and proteins of tryptophan biosynthesis? Members of my group who addressed these questions were Steven Li, Iwona Strzybonska, Bill Schneider, Brian Nichols, Richard Denney, Mike Manson, Joan Hanlon, Eric Selker, and Giuseppe Miozari. To begin with, the trp genes of many organisms were cloned and partially or completely sequenced; the sequences and genetic locations then were compared. Many other laboratories provided comparable information for their favorite genes and operons. Comparative studies with the trp genes revealed that all organisms that synthesize tryptophan use the same seven catalytic enzyme domains. The enzymes of the tryptophan biosynthetic pathway therefore probably evolved just once. However, within each polypeptide, when one compares different species, there is appreciable sequence variation. This is typical for most protein evolutionary comparisons. In one beautiful study performed in my laboratory by Bill Schneider and Brian Nichols, segments of the TrpAs of E. coli and Salmonella were exchanged, generating recombinant TrpA polypeptides. All the recombinant TrpA proteins produced were fully functional despite the fact that 40 of the 268 amino acid residues in the parental homologous TrpA proteins differ (90). This result implies that most of these amino acid differences are tolerable when inserted individually or in clusters.

Evolutionary comparisons of the organization of the seven trp genes in different species revealed appreciable variation. In some species the trp genes are split in several operons, and often two or more trp genes are fused to form multifunctional enzymes. Regulatory mechanisms also vary considerably, possibly reflecting differences in operon organization and participation of one or more pathway intermediates in a second pathway. Nucleotide sequence divergence for the trp genes correlated well with predictions of species relatedness based on analyses of ribosomal RNA sequences although there are some hints of horizontal transfer.

Understandably, I was particularly interested in knowing whether the features of repression and transcription attenuation observed with the trp operon of E. coli are conserved in unrelated species. Among the enteric bacteria we examined the major features were retained, although there was considerable leader sequence variation, presumably reflecting slightly different species-specific objectives (91). Mitzi Kuroda of my group initiated comparative regulatory studies with the trp operon of a second well studied prokaryote, B. subtilis. We were joined in this effort by Dennis Henner and his group at Genentech when we learned that he too was concerned with this problem. We already knew that there were significant differences in trp gene and operon organization in B. subtilis versus E. coli. B. subtilis has seven distinct trp genes, only six of which are clustered as a trp operon. Furthermore the six-gene trp operon is located within an aromatic supraoperon, which has three additional upstream genes and three additional downstream genes, each concerned with some aspect of aromatic amino acid metabolism (92). The seventh trp gene, trpG, is located in the folate biosynthetic operon (92). Its location is logical because its polypeptide product, TrpG, is a glutamine amidotransferase.
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That is a component of two similar enzyme complexes, one catalyzing para-aminobenzoate synthesis in the folate pathway and the second catalyzing ortho-aminobenzoate (anthranilate) formation in the tryptophan pathway. The studies by Mitzi Kuroda and the Henner group were followed by investigations in my laboratory by Paul Gollnick, Paul Babitzke, Joe Sarsero, Enrique Merino, and most recently, by Angela Valbuzzi and Guang-nan Chen. The basic features of attenuation regulation of trp operon expression in B. subtilis were established; they were quite different from those used for the trp operon of E. coli. Although alternative antiterminator and terminator hairpin structures also participate in the transcription termination decision, an 11-subunit tryptophan-activated RNA-binding protein, named TRAP, is used by B. subtilis to disrupt the antiterminator and promote terminator formation (93–95). We selected TRAP as the name for this protein because it is a trp RNA-binding attenuation protein (93). Tryptophan-activated TRAP also binds trpG mRNA and several other RNAs concerned with tryptophan metabolism. The TRAP binding sites in these transcripts all overlap translation start sites; thus TRAP binding also regulates translation initiation (93). Furthermore, TRAP binding to trp operon leader RNA indirectly regulates translation initiation at the trpE start site (96). A most interesting recent discovery in my laboratory is that B. subtilis contains a previously unidentified regulatory operon, rtpA-ycbK, that is designed to sense (and respond to) uncharged tRNA^{Trp} (97). We named the rtpA protein AT (Anti-TRAP) because it is designed to bind to and inactivate tryptophan-activated TRAP (98, 99). Transcription of the rtpA-ycbK operon is regulated by tRNA^{Trp}-mediated transcription attenuation by the T box antitermination mechanism discovered by Grundy and Henkin (69). We recently observed that uncharged tRNA^{Trp} accumulation has a second regulatory effect on the rtpA-ycbK operon; it increases translation of rtpA, thereby providing higher levels of AT protein (G. Chen and C. Yanofsky, manuscript in preparation). Thus B. subtilis employs two independent mechanisms of sensing uncharged tRNA^{Trp} in this operon; one is transcriptional and the second translational; both regulate AT synthesis (G. Chen and C. Yanofsky, manuscript in preparation). DNA microarray analyses have also been performed with wild type and regulatory mutants of B. subtilis to analyze the total genome’s transcriptional response during growth under nutritional conditions that affect tryptophan metabolism (R. M. Berka, X. Cui, and C. Yanofsky, manuscript in preparation). The genes we expected to respond did; however, many additional genes responded comparably, suggesting that their expression is closely tied to the genes involved in tryptophan synthesis.

The knowledge we have gathered in our studies on tryptophan metabolism in E. coli and B. subtilis raise the very tough “why” question. Why do E. coli and B. subtilis use such dissimilar mechanisms to sense tryptophan and tryptophan tRNA as regulatory signals? I would love to know the answer!

Enzyme Structural Questions

One particular set of challenging questions was always on my wish list, but I left these questions for other scientists to answer. What are the three-dimensional structures of the seven protein domains required for tryptophan synthesis? Are any of these domains evolutionarily related? What are the likely ancestral sources of the seven catalytic domains? I am delighted to report that the three-dimensional structures for all seven protein domains required for tryptophan synthesis have been determined. This knowledge should permit investigators to consider structural as well as catalytic issues when attempting to deduce possible evolutionary origins for these domains. Interestingly, the structures of three of the tryptophan pathway enzymes are 8-fold αβ barrels. Particularly exciting in this regard is the recent demonstration that an enzyme catalyzing a reaction in histidine biosynthesis (a reaction similar to one catalyzed by an enzyme of the tryptophan pathway) was converted into an active trp enzyme by introducing a single amino acid change (100). Given the extraordinary wealth of information being provided by sequence analyses, evolutionary exploration of enzyme origins should be an interesting subject for future investigations.

Returning to Neurospora

Whenever I selected a research project for one of my graduate students I was well aware that this individual might prefer to work on some other problem as a member of my group. Eric Selker, an exceptional graduate student who joined me in the late 1970s, decided that the project I had assigned him, characterizing the genes of the trp operon of Salmonella typhimurium, would not break new ground, and therefore he preferred to work on a project that was
more challenging. He proposed reintroducing N. crassa into my laboratory as an experimental organism. As I recall, I resisted Eric’s proposal to switch his project only modestly. I knew this organism well, and most importantly, one of my closest colleagues, David Perkins, whose laboratory is just down the hall, was a major contributor to Neurospora research. Eric convinced me that the time had come to apply the procedures, technology, and concepts developed in studies with bacteria and yeast to the superb experimental eukaryote selected by Beadle and Tatum as their experimental organism. At a minimum, I thought, we should be able to compare the genes and proteins of tryptophan metabolism and their regulation in N. crassa with those in E. coli. A few years later a second bright graduate student, Vivian Berlin, also with my approval, switched from her initial bacterial studies to apply modern molecular approaches in analyzing an excellent model developmental process in Neurospora, asexual spore formation. Many talented graduate students and postdoctoral fellows subsequently joined my group to perform fungal studies. Their work greatly improved the technology that could be applied in investigations with Neurospora. Most of these individuals, after leaving my group, continued to make significant scientific contributions in fungal biochemistry and genetics.

My Treasures

Reflecting on what I would consider our two most impressive contributions, I would select proving gene-protein colinearity and determining the stages and features of regulation by transcription attenuation. The first required our identifying the amino acid changes in a set of TrpA mutant proteins and comparing the relative locations of these amino acid changes with the order of the corresponding mutational changes on the genetic map of the trpA gene. Essentially, we verified a relationship, which, at the time, we believed existed. Transcription attenuation, by contrast, was a poorly understood process initially thought to be used only rarely. We were required to break new ground and perform step by step analyses of the roles played by tandem overlapping segments of a transcript, as well as explaining how ribosome stalling at either of two Trp codons selects between alternative RNA structures. I had no reason to suspect that transcription attenuation was such a common regulatory strategy or that so many different mechanisms of attenuation existed. I have illustrated in this article how the answers we obtained while focusing on some specific questions invariably raised new unanswered questions. More often than not, these questions were so challenging they could not be ignored.

Despite the enormous satisfaction I feel personally from what we have accomplished scientifically, I believe my greatest pleasure in practicing science has come from the give and take of daily interactions with members of my group and from thoughtful and stimulating discussions with fellow scientists. My journey in science has been great fun! I was very fortunate to have had Dave Bonner as my mentor and lucky that I “grew up” with a wonderful group of smart graduate students. Learning biochemistry from Joseph Fruton was an extraordinary experience. At Western Reserve, Howard Gest, Bob Greenberg, Abe Stavitsky, and John Spizizen were all special friends who contributed to my development. At Stanford, because of our personal friendship and frequent discussions, Paul Berg put his stamp of approval on virtually everything I have done. I have had many other close friends and colleagues at Stanford, including Dave Perkins, Don Kennedy, Norm Wessells, Paul Ehrlich, Bob Schimke, Phil Hanawalt, Bob Simoni, Dale Kaiser, Dave Hogness, Lucy Shapiro, and Bob Lehman. Scientists at other institutions, some of whom spent a sabbatical in my laboratory, also were great friends, including Howard Zalkin, Frank Gibson, Dale Oxender, Ron Bauerle, Kasper Kirschner, Edith Miles, Stan Mills, Michael Chamberlin, and Paul Sigler. Stan Prusiner, an outstanding scientist with completely different interests, became a very close friend. Finally, throughout the past 40+ years of my career Arthur Kornberg's wisdom and commitment have served as models guiding my behavior. There are many other “treasured” individuals who I did not get to mention in this article; I thank you all.

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