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PHAGE RNA AS GENETIC MATERIAL*

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IN 1922, with remarkable prescience, H. J. Muller, in discussing the potential impact of the newly discovered bacteriophages on gene theory, wrote as follows:

> It would be very rash to call bodies genes and yet at present we must confess that there is no distinction known between the genes and them. Hence we cannot categorically deny that perhaps we may be able to grind genes in a mortar and cook them in a beaker after all. Must we geneticists become bacteriologists, physiological chemists, and physicists, simultaneously with being zoologists and botanists? Let us hope so.

Since 1940 geneticists have been engaged in fulfilling that hope.

The development of the gene theory of inheritance created an enormous problem—the nature of the gene. While many geneticists were content to follow by analysis of crosses the formal aspects of the science, others such as Muller pondered the nature of these remarkable entities. Genes had two properties that were almost mutually exclusive. All genes had to reproduce themselves with an enormous degree of fidelity and could modulate this fidelity to reproduce with equal fidelity the occasional mutation that occurred within them. In this sense, they were all the same. Contrariwise, in some unknown fashion, each gene resulted in a different character in the organism, and there were myriads of such characters; all genes were different.

It is not my purpose to develop this evening the solution to this problem. It is a well known tale and has been recounted in these lectures, as it developed, on many evenings. It is summarized in what has been called the "central dogma" of molecular genetics.

$$\overset{\curvearrowleft}{\text{DNA}} \rightarrow \text{RNA} \rightarrow \text{protein}$$

* Lecture delivered September 22, 1966.

Even as the dogma was promulgated, it was known that there were a few exceptions to this dogma, in that for certain viruses RNA was the genetic material rather than DNA. These exceptions in no way embarrass the central dogma, as the composition and structure of DNA and RNA are sufficiently similar that it is probably only for the sake of efficiency that these materials have in general taken on different roles. However, because the same material element is used in the auto- and the heterocatalytic function, RNA viruses provide the opportunity to study directly both these aspects of template function.

It is to the description of the exploitation of some of these opportunities which presented themselves when an RNA-containing bacteriophage was discovered some years ago (Loeb and Zinder, 1961) that this lecture is directed. It has been the work of many. Within my laboratory, there have been the students T. Loeb, S. Cooper, H. Lodish, D. Engelhardt, Fran and Jim Schwartz; also my colleagues Drs. R. Valentine, K. Horiuchi, G. Notani, and R. Webster. In addition, we have had the able collaboration of Drs. Dan Nathans and William Konigsberg. Many other laboratories have been involved in the study of RNA phages and have provided critical insights.

Although RNA-containing viruses are the oldest known viruses and include such medically important forms as polio and influenza, the study of their basic biology was long hindered by the difficult technologies necessary. An RNA-containing bacteriophage immediately made available all the sophisticated genetic and biochemical probes that have so aided the studies of the other bacterial viruses.

In no sense were we searching for an RNA-containing bacteriophage. Loeb and I were seeking to study further the mysteries of conjugating *Escherichia coli*. It was felt that a phage which could differentiate male from female bacteria would aid these endeavors. When the *f* phages were isolated from sewage (Loeb, 1960), they proved to be useless for the kinds of experiments we had in mind. Although they grew only on male bacteria due to the fact that they adsorbed only to male bacteria, they would kill at best 80 per cent of the males. They were almost put aside

except for their diagnostic use in defining coli mating types which hitherto had required a cross with standard and well marked *E. coli* strains.

The property that really insisted that we carry the analysis further was the enormous yield from each infected bacterium. Some 10,000 to 30,000 particles were made, two orders of magnitude larger than any other phage. It could only be a small phage, and there were not very many of these. The phage proved very easy to purify by ammonium sulfate fractionation and differential centrifugation. Finally the infectious material was isolated from a band in CsCl density centrifugation. The band contained the infectivity, some protein, some RNA, and little detectable DNA. Apparently we had an RNA-containing bacteriophage.

It is at this stage in an investigation that the scientist, when faced with a new phenomenon, pulls out all the stops of his imagination and, playing the devil's advocate, develops the most gratuitous set of hypotheses and experiments to test them in order to prove that the straightforward and simplistic view is probably wrong or wishful thinking. Some on hindsight look childish; others so interesting that one is almost tempted to look specifically for phenomena of that kind. As hard as we would wiggle, the genetic material of the phage proved to be RNA. It is now very simple to prove. During that brief period when phages are added to host bacteria and before they penetrate to initiate infection, they are sensitive to destruction specifically by the enzyme ribonuclease. Since that time many other RNA-containing phages have been isolated all over the world by the use of the same screening procedure. As an aside, it should be mentioned that screening for male specific coli phages also yields another creature—a long filamentous phage containing a single strand of DNA (Marvin and Hoffmann-Berling, 1963; Zinder *et al.*, 1963). It is not clear why two such unusual creatures should be isolated in this manner, nor is it clear why they are so ubiquitous, for their laboratory hosts, *E. coli* male bacteria, are extremely hard to find in nature.

In Table I we have listed the properties of the phage particles. The most relevant datum is the size of the RNA. It is 1,000,000 molecular weight, which means some 3000 nucleotides. On cur-

rent coding theory, it would suffice to code for only 1000 amino acids. This fact has influenced in part almost all our thinking about this phage.

We had arrived, in another way, in Hershey heaven. Al Hershey once told Garen that his idea of scientific happiness was having one experiment which works and to keep doing it all the time. When I told Al about RNA phage, he remarked, "It is marvelous; all of these experiments to do without having to think."

Had we stopped to think, our thinking might have gone as follows. It will probably take us a few months to repeat for this

TABLE I
PHYSICAL PROPERTIES OF THE BACTERIOPHAGE f2

Molecular weight	3,500,000
Diameter of phage particle	200 Å.
$s_{20,w}$	76 S.
Per cent RNA	28–29%
Molecular weight of RNA	1,000,000
$s_{20,w}$ of RNA	27 S.
Base ratios of RNA	
Adenine	0.227
Guanine	0.272
Uracil	0.237
Cytosine	0.264

phage all the basic biology of known phages, such as latent period, eclipse period, effects on host metabolism, etc. What then?

There were three points which should have influenced our thinking. First, as mentioned previously, the small size of the RNA which must mean that it had a small information content. Second, since the year was 1961 and the messenger RNA hypothesis was in full bloom—a key insight into the real meaning of the central dogma—the RNA from the phage should be pure messenger with which one could directly measure gene function. Last, when pondering on just how we should proceed with the analysis of the genetic functions of the phage RNA, we should be making use of the then not quite crystallized concept relating to conditional-lethal mutations.

Through the work of Epstein *et al.* (1963) with phage T4, Campbell (1961) with the phage lambda, Benzer and Champe (1962) with T4r 2 mutants, and Garen and Siddiqi (1962) with alkaline phosphatase, it had become apparent that there was a class of mutants which could be obtained in many genes without necessarily knowing the function of the gene. Conditional lethals are just what the name implies; whereas wild-type genes produce functional product when tested at either of specified conditions, mutant genes function only with one of these conditions. As currently used, such mutants fall into two categories: temperature-sensitive mutants and host-dependent or amber mutants. Temperature-sensitive (*ts*) mutants can be understood in terms of mutant proteins which are more thermolabile than the wild-type protein either in their function or in their synthesis. Host-dependent mutants require some special components of cell to produce a functional product. We will discuss in more detail one particular kind of host-dependent mutant later on.

After mutagenesis of phage, a series of both temperature-sensitive and amber-type mutants were obtained (Zinder and Cooper, 1964; Horiuchi *et al.*, 1966). The screening for the amber mutants was done on strains isolated by Garen which could suppress a number of specific mutants in the alkaline phosphatase gene of *E. coli.* Subsequently these same strains were shown to suppress the T4 amber mutants.

The methodology of study of conditional-lethal mutants runs as follows: cells are infected under nonpermissive conditions. An attempt is then made to determine which of the normal events that occur after phage infection fails to occur. The simple assumption is that the genes function independently of each other. However, with phage infection one must always be aware of the fact that there can be contingent processes which can lead the analysis astray. Ultimately we must resort to a number of different criteria to define any particular gene.

The ingenuity, if any, is in measuring the effect of a particular mutant by as many criteria as possible. For RNA phage, which have only minor effects on normal host metabolism, this means looking for phage specific components in the midst of many bacterial ones. In Fig. 1 we have illustrated the usual events that occur

after infection with wild-type phage or with mutant phage under permissive conditions. There appear a phage-induced polymerase, double-stranded RNA, and finally the phage itself.

Infection with amber mutants is a static experiment in that one can only assay "presence-absence" kinds of things, as the conditions for the experiment have been defined at the time of infection. Temperature-sensitive mutants allow for alteration of the conditions during the experiment, and one can determine the time when

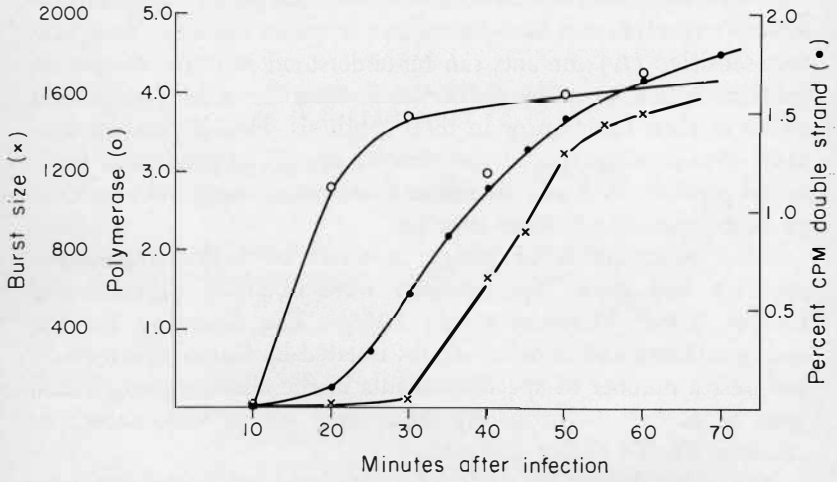

FIG. 1. Elements seen after infection with f2. The open circles represent the phage RNA polymerase activity; the filled circles, double-stranded RNA. The X's represent the phage yield. After Lodish and Zinder (1966a).

the needed gene product functions. Figure 2 illustrates temperature shift-down experiments for an early gene. Note that the latent period for phage production is delayed for as long as the temperature is kept up. Contrariwise, phage production by genes affecting late functions is about normal upon shift-down (Fig. 3). Shift-up experiments aid in analysis of whether something that functions or is made early is also needed late in infection.

Table II (p. 9) lists the three known genes for the RNA phage. It is not known whether these three are all of the phage's genes. This will not be known until such time as the size of the three pro-

teins specified are known so that a correlation of number of nucleotides in the phage with the total number of amino acids in the proteins can be made. In addition, we lack the formal genetic procedure of recombinational analysis to define the position of the genes

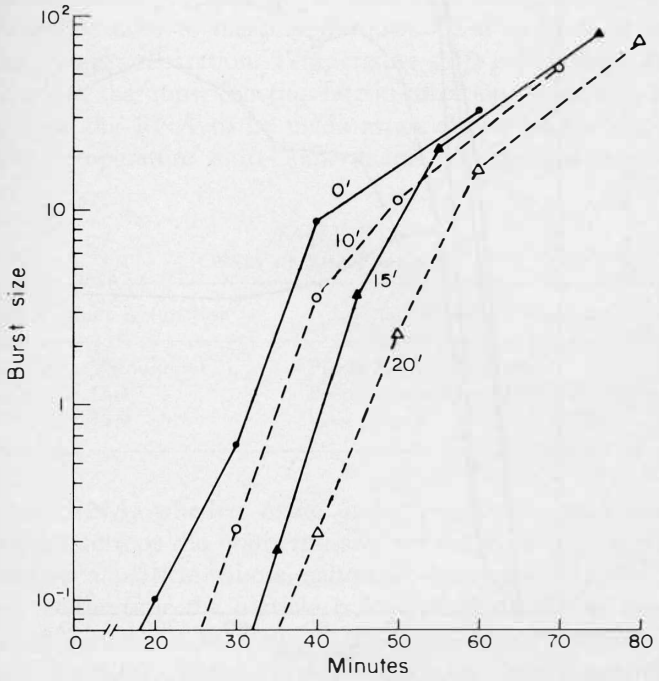

FIG. 2. Temperature shift-down of an "early" mutant. Cells infected at 43°. At the intervals indicated on the figure, the infected cells were shifted down to 35° and the phage yields were measured. After Horiuchi, Lodish, and Zinder (1966).

vis-à-vis each other, or, for that matter, to position sites within a gene. Thus the independently isolated mutants with the same physiological properties could conceivably all be at the same site.

I will organize this part of the discussion about the individual genes, for each has made possible certain kinds of experiments to probe the biosynthesis of the phage.

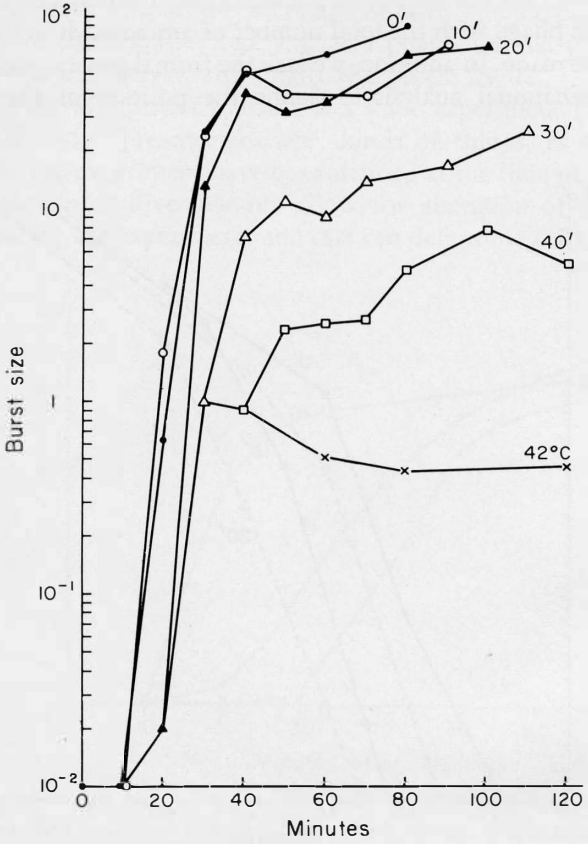

FIG. 3. Temperature shift-down of a "late" mutant. Cells were infected at 43°. At the intervals indicated on the figure, the infected cells were shifted down to 35° and the phage yields were measured. After Horiuchi, Lodish, and Zinder (1966).

Gene 2, or the assembly gene, is the simplest to define operationally, but its exact nature is still unknown. When bacteria are infected under nonpermissive conditions with either *ts* or amber mutants in this gene, there ensues a normal sequence of events including the lysis of the cells. A full complement of phagelike particles are produced (20,000), but these are defective. These

particles do not attach to bacteria. Wild-type f2, like ribosomes, can be absorbed out by Millipore filters whose pores are ten diameters of the particles; however these defective particles also do not attach to Millipores (Lodish and Zinder, 1965). When one has another procedure to measure the total number of particles such as antiserum precipitation or acid insolubility, Millipore filtration can be used to measure the proportion of such defective particles in any preparation. Temperature shift experiments define gene 2 as one that must function late in infection. Infectious RNA assays show the RNA to be made at an almost normal rate. In addition, temperature shift experiments during phage growth

TABLE II
GENES OF THE PHAGE f2

Gene No.	Time of function	Presumed function
1	Throughout	Phage RNA polymerase
2	Late	Proper assembly of RNA and coat protein
3	Late	Coat protein

show that RNA, whether made under permissive conditions of low temperature or the nonpermissive condition of high temperature, enters a particle whose nature is determined by the temperature at the time the particle is formed. Thus RNA, made at high temperature but entering a particle at low temperature, is in infective particles. These defective particles, when isolated by CsCl gradient centrifugation, often are found to contain less RNA (Lodish *et al.,* 1965). However, recent work by Heisenberg (1966), using a similar mutant and ribonucleaseless host, has shown that all the particles originally have normal RNA contents, but that, unlike wild-type, when such particles are exposed to nuclease their RNA can be partially digested even when in the particle. The most plausible interpretation of these results is that gene 2 specifies the formation of a protein which plays a keystone role in the proper assembly of phage RNA and coat protein. However, a strictly morphogenetic and nonstructural role for gene 2 is still possible.

Gene 1, we believe, specifies a phage RNA polymerase. Since we have not purified this enzyme from the mutants and shown it to be different, our arguments are somewhat indirect.

Amber mutants in this gene produce no detectable effects. None of the elements associated with phage infection appear.

Temperature-shift experiments with the temperature-sensitive mutants show that the product of this gene has an early and continuing function as would be expected for an RNA synthesizing enzyme. Because f2 infection has but little effect on the RNA metabolism of the infected cell—all the RNA continues to be made—it is difficult to follow in detail the RNA precursors in the cell. However, as first shown by Weissmann *et al.* (1964), bacterial cells contain double-stranded RNA only when infected. Although there is still some dispute as to the functional role of this material, we will assume its relevance in discussing RNA replication.

When cells are infected at high temperature, the imput RNA does not become double-stranded until the temperature is shifted down. When cells are infected at low temperature and shifted up late in infection, double-stranded RNA synthesis ceases while single-stranded RNA synthesis proceeds (Lodish and Zinder, 1966a). Last, the fate of imput RNA has some relevance. In Fig. 4 we see the time course of conversion of parental label to double strand. Usually there is a slow displacement of input RNA. If, as suggested by Weissmann and others, replication is semiconservative, then the slowness of the displacement is due to recycling. However, if we shift a *ts* enzyme mutant up, then recycling should be impossible, and, as we see, the label rapidly chases out (Lodish and Zinder, 1966c).

The detailed structure of the replicative intermediate is still being studied. Recently Franklin (1966) has isolated material in preparative amounts which seems to be double-stranded RNA with tails of single strands attached.

The outer shell of the virus particle is composed of 180 units of a single protein. Gene 3 specifies the sequence—recently obtained by Weber *et al.* (1966)—of the 129 amino acids in this protein (Fig. 5).

It is necessary at this point to digress for a moment to discuss our current understanding of the nature of the amber and similar nonsense mutants. This analysis is based on work done in Brenner's, Garen's, Watson's, and my own laboratory (*Cold Spring Harbor Symp.*, 1966). An amber mutant occurs whenever there is mutation to the codon UAG. In nonpermissive hosts or, as they

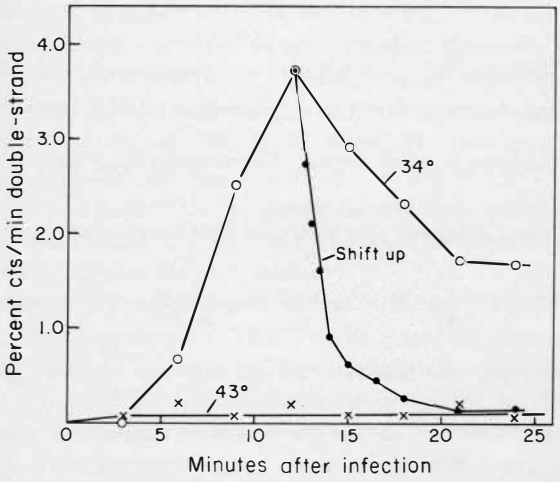

FIG. 4. Fate of labeled imput RNA with a *ts* mutant in the polymerase gene. Cells were infected at temperatures indicated with P^{32}-labeled phage, and the fraction of the label that was RNase-resistant was measured as a function of time and temperature. After Lodish and Zinder (1966c).

are more properly called, suppressor-negative bacteria, premature termination of growing protein chains occurs at this point. Brenner has suggested that the codon UAG and the other closely connected codon UAA (ochre) are the natural chain-terminating codons. Such mutants would be lethal were it not for the fact some bacteria contain suppressor genes which can translate these codons with some efficiency. For the amber codon, there are three suppressor genes; Su-I, which inserts serine, Su-II, which inserts glutamine, and Su-III, which inserts tyrosine. The serine and tyrosine suppressors are relatively efficient, while the glutamine is less efficient.

It is now known that the serine strain contains a special seryl sRNA which translates this codon (Capecchi and Gussin, 1965; Engelhardt *et al.*, 1965). Probably the other strains have similar special sRNA's.

Two different amber mutants in the coat protein are now known, one at site 6 from the N-terminus, and the other at site 70. The

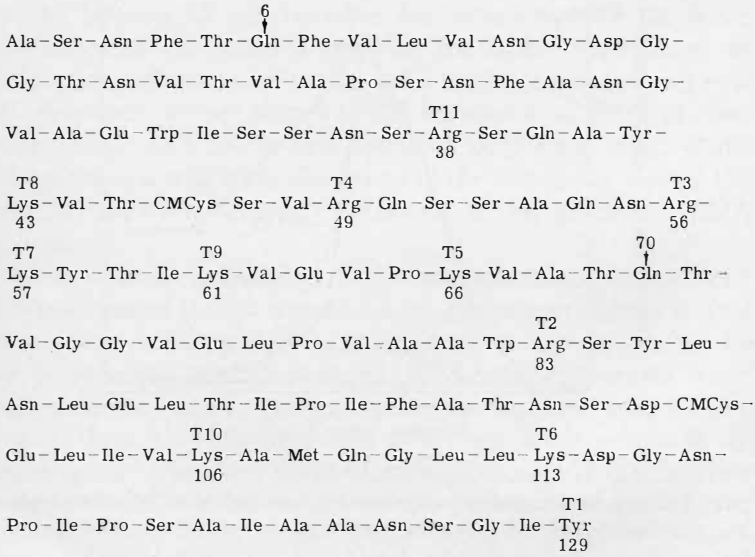

FIG. 5. Sequence of amino acids in the coat protein of f2. After Weber *et al.* (1966).

wild-type phage has the amino acid glutamine at these positions. It is only because of the fortunate fact that the Su-I suppressor gene causes the insertion of serine that we are aware of the sites of these mutations. The physiological effects of these amber mutants when infecting Su^- bacteria were such that, until the protein chemistry was complete, we were uncertain that they were in gene 3. With the brilliance in analysis always afforded by hindsight, it is perhaps not surprising that mutations in this gene should have profound effects. During the course of normal infec-

tion. there is synthesized in each cell some two to four million molecules of coat protein. Other gene products must certainly be made in lesser amounts, and yet all gene products have the same RNA template. Some control must exist for varying the translational frequency of the various genes. In Fig. 1, we saw that the synthesis of viral polymerase ceases about half way through the latent period, long before any significant amounts of phage have been synthesized (Lodish and Zinder, 1966b).

A priori, mutants defective in coat protein synthesis would be expected to initiate synthesis normally and accumulate large amounts of viral RNA. However, when Su^- bacteria are infected with phage containing the mutation at site 70, two anomalies are found: (1) that the RNA accumulated is double stranded, and (2) that the bacterial extracts contain severalfold excess of polymerase activity. Both double-stranded RNA and enzyme are hyperproduced (Lodish and Zinder, 1966b).

Contrariwise, when Su^- bacteria are infected with phage containing the mutation at site 6, none of the elements seen in normal infection appear. It is hard to differentiate this infection from that with a mutation in the polymerase gene, as the primary manifestation is a deficiency in RNA synthesis. However, when this same phage infects a suppressor strain with only a small amount of suppressor activity, such as Su-II (I illustrate this point with Su-II as it results in the insertion of glutamine, an amino acid which we know would be adequate to form phage particles), it also hyperproduces enzyme and double-stranded RNA (Lodish and Zinder, 1966b).

We can ask whether this excess double-stranded RNA is a consequence of enzyme synthesis or merely a consequence of the absence of coat protein. Experimental analysis shows that late protein synthesis is also required (Lodish and Zinder, 1966b).

The picture that emerges is as follows: In the course of normal infection, the amount of coat protein regulates the frequency of translation of the other genes, perhaps simply by condensing with the nascent RNA molecules to begin to form a particle. In the absence of coat protein, the other genes on the molecule can be translated, producing more enzyme which, in turn, doubles up the RNA. This latter finding provides a major support for our

hypothesis that the primary role of the phage-specific enzyme is to make a complementary copy of the viral RNA. However, there also exist other controls on the frequency of translation of the various genes on the RNA molecule, as will become apparent in a discussion of the *in vitro* system. Recall that gene 2 controls a late function. It is unlikely that as many molecules of its product are made as of coat protein. Control in this instance is probably through modulation (Ames and Hartman, 1963) of gene translation.

When the mutant at site 6 infects Su^- bacteria, we encounter another phenomenon—that of polarity. Polarity as with many other regulatory processes, has been described by Jacob and Monod (1961) for the *lac* operon in *E. coli*. Mutants now known to be nonsense mutants at the operator end of the lactose gene not only prevent the synthesis of galactosidase, but also the synthesis of the other enzymes specified by this operon. This phenomenon of polarity and the insight it provides in controlling the translation frequency of genes in an operon is currently the subject of intensive investigation. Discussion of the coat protein gene provides a natural entrée to a discussion of the *in vitro* system. To date, the coat protein is the only phage-specific product that we have isolated or analyzed.

The experiment is to take phage RNA and add to it an *in vitro* protein-synthesizing extract and compare the product with the known material. Since there is no net synthesis of protein, radioactive amino acids are used as tracers. Tryptic digests are prepared, and the "fingerprints" are radioautographed. It is hard to resurrect, in this day and age of the obvious, the excitement generated when D. Nathans first found that phage RNA would stimulate protein synthesis and we waited impatiently for the radioautograms prepared by Gur Notani and Jim Schwartz to be developed. Today perhaps our questioning and diffidence would be greeted by a "Well, what did you expect?"

The fingerprints showed two things: (1) that the majority of coat protein peptides were made; the viral RNA was indeed template or messenger RNA; and (2) despite the fact that only 15 per cent of the RNA molecule was needed to code for the coat protein, it was the major product (Nathans *et al.*, 1962).

It was possible to probe more deeply into the happenings in the *in vitro* protein-synthesizing system by analyzing the amber mutants in the coat protein. Recall that amber mutants cause premature chain termination at the site of the mutation. This predicts that mutant RNA, when translated in an Su^- extract, should give the appropriate peptide fragment. The fragments which are smaller than the whole molecule should be resolvable by chromatography on the basis of size. Gel filtration, through Sephadex, is the procedure we chose. We isolated the fragment from the mutant at site 70 (Engelhardt *et al.,* 1967). Since this fragment contains more than half of the amino acid residues of the coat protein, definitive proof that it is the right fragment requires almost as much effort as analyzing the whole coat protein. However, analysis of its peptide composition shows it to contain the peptides proximal to the mutant site and to be lacking the peptides distal to this site. More convenient to study is the fragment obtained from the mutant site 6, only six amino acids long (Engelhardt *et al.,* 1965).

If instead of an Su^- extract, we use an Su^+ extract, little of either fragment is made relative to complete chains, so suppression occurs *in vitro*. The next question to which we addressed ourselves was which of the components of protein synthesis is different in an Su^+ extract. Most readily added to extracts is additional transfer RNA. Transfer RNA from Su^+ cells suppresses the production of fragment. A more convenient assay is based on the fact that the major product of the *in vitro* system is the coat protein. Since in general protein synthesis is measured by the conversion of labeled amino acids into hot acid-insoluble material, and since the little fragment is acid soluble, then mutant RNA should appear to be a poor template of protein synthesis. The little fragment made would not be apparent in this assay. Should the addition of a component allow for translation of the nonsense triplet, it would appear to stimulate total protein synthesis. Addition of sRNA from Su^+ bacteria did so. Since all the other components of the system derive from Su^- bacteria, obviously there is a special seryl transfer RNA.

Although we were early interested in the problem of initiation of protein chains by natural template RNA, there did not appear

to be much that we could do to study this process. Our feeling that there was something special arose from the fact that the phage RNA seemed to be translated with a high degree of fidelity. The paradox was that any random polyribonucleotides also stimulated protein synthesis. They could all start, and yet somehow the phage RNA message always started at the right point. Our analysis of the problem might be entitled "The Case of the Missing Peptide." Let us again look at the N-terminal sequence of the coat protein (Ala-Ser-Asn-Phe-Thr-Gln-Phe-Val). Fingerprints of chymotryptic digests should give rise to two small peptides, Ala-Ser-Asn-Phe and Thr-Gln-Phe. Fingerprints from phenylalanine-labeled *in vitro* product give the tripeptide Thr-Gln-Phe, but the tetrapeptide was missing. Another peptide did appear at an unexpected position. Similarly, digests of the site 6 fragment gave only this latter peptide. We proceeded with an amino acid analysis of the small fragment, and it contained the right amino acids (Fig. 6) and was about the right size, and yet was uncharged at pH 1.9. Part of our problem was that at that time we really were not certain that there was not another peptide proximal to the tetrapeptide, for although I have presented the arguments as though we knew the N-terminal sequence, it was the analysis from the *in vitro* system which aided the peptide chemistry in developing the sequence. Anyhow, we had looked at this for several months when at one of those lab seminars where ideas fly thick and fast the idea arose that the N-terminus might be masked. Proceeding on that assumption, we guessed that either an acetyl or formyl group would do. Were it a formyl group, this could be readily tested since formyl groups can be removed by mild acid hydrolysis. We had some peptide at hand which was labeled at both ends with C^{14}-Ala and H^3-Phe. These two amino acids would provide controls for any hydrolysis of the peptide. Mild acid hydrolysis restored the missing peptide, indicating the presence of a formyl group. We were attempting to isolate formyl-alanine from the peptide when Watson arrived to tell us that, based on the finding by Marcker and Sanger (1965) of a special Met sRNA upon which methionine can be N-formylated, they had found that phage RNA would stimulate the incorporation of F-Met into protein (Adams and Capecchi, 1966). We had not analyzed our peptide fragment for methionine as there is

only one methionine in the f2 coat, and that at residue 108. Methionine was found in the fragment and also at the N-terminus of the complete protein chains (Webster *et al.*, 1966). The mystery of the missing peptide was solved. Apparently proteins

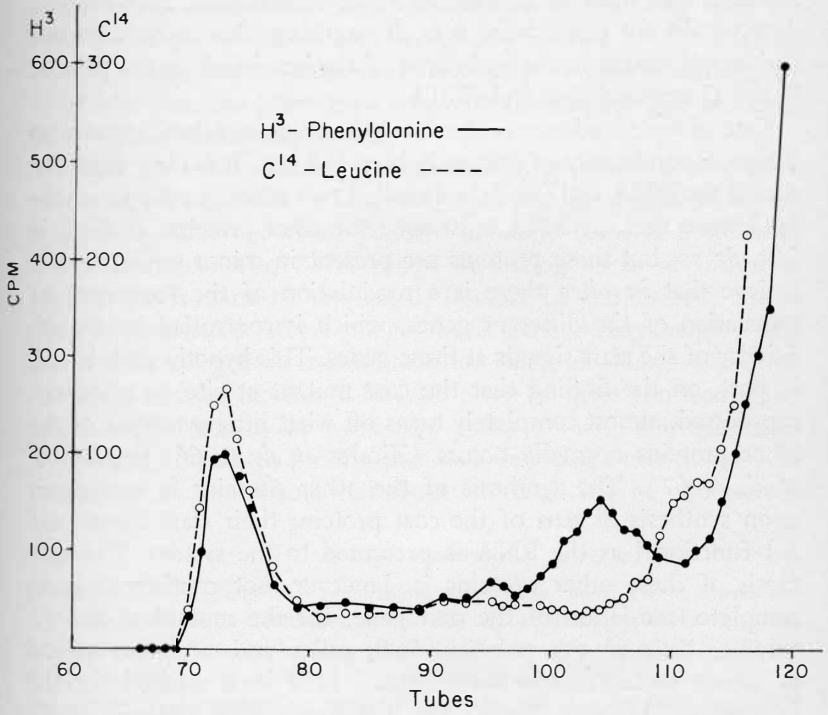

FIG. 6. Absence of leucine in the N-terminal fragment. Fragment is in tubes 100 to 110 and is labeled with phenylalanine-H^3.

are synthesized in *E. coli,* starting with F-Met, which, *in vivo,* is subsequently removed. Our protein-synthesizing extracts were fortunately deficient in this activity and left us the intermediate to be found. The detailed mechanism by which start signals work is still under investigation, but the existence of such signals is clear (see *Cold Spring Harbor Symp.*, 1966).

Also necessary for proper protein synthesis are proper "stop" signals so as to release the growing protein chains from peptidyl-sRNA. The amber (UAG) and the ochre (UAA) codons have been suggested for this role. We have found, using the amber mutant specifying site 6 in the coat protein, that the N-terminal fragment is released free of sRNA (Webster *et al.,* 1967). Operationally this must be considered chain termination. However, to date we do not know what it is, if anything, that recognizes this codon and results in the hydrolysis of the bond linking the peptide to the C-terminal threonyl-sRNA.

One of the paradoxes of the *in vitro* protein-synthesizing system is the preponderance of coat protein as product. It is clear from the size of the RNA and from the fact that two other genetic functions are known that the RNA must code for other proteins. It does, in fact, do so, but these proteins are present in minor amounts. We believe that *in vitro* there is a modulation of the frequency of translation of the different genes, which is controlled by the efficiency of the start signals at these genes. This hypothesis is based, in part, on the finding that the coat mutant at site 6, when not suppressed, almost completely turns off what little synthesis of the other proteins normally occurs (Zinder *et al.,* 1966; Engelhardt *et al.,* 1967). The synthesis of the other proteins is contingent upon synthesis of part of the coat protein; their start signals are not functional as the RNA is presented to the system. The synthesis of these other proteins is, however, not contingent upon complete translation of the coat gene, for the mutant at site 70 exhibits little if any polarity. Such polar and nonpolar effects of amber mutants in *in vivo* systems have been studied for the *lac* operon (Malamy, 1966), the histidine operon (Martin *et al.,* 1966), and the tryptophan operon (Imamoto *et al.,* 1966). What was not clear from the *in vivo* studies was whether a purely translational polarity existed. The findings described above show that there is translational polarity. However, *in vivo* this might well feed back to give transcriptional polarity (Stent, 1965).

There are two models for translational polarity; the conformational model and the gliding model. In the conformational model, it is assumed that phage RNA, when added to the *in vitro* system, has a single binding site available for attachment to ribosomes—

that at the N-terminus of the coat protein gene. With the initiation of protein synthesis, the RNA becomes unfolded so as to reveal start signals of various efficiency buried in the RNA structure. In the glide model, the single attachment site must be at the 5′ OH terminus of the messenger RNA. Protein synthesis proceeds down the RNA until a chain-terminating signal is reached, at which point the ribosomes have some probability of dissociating from the template, a probability which is directly related to the distance from the next start signal. Both models provide mechanisms by which the different genes on a single RNA template can be translated with differing frequencies.

I have given a broad and perhaps rambling picture of our work on the RNA phage. It is, however, clear that the problems which arise using RNA genomes parallel in many respects those with DNA genomes. The RNA phage is analogous to an infective operon. RNA must be replicated, it must be transcribed, and it must be translated. RNA phages have some disadvantages, such as the lack of genetic recombination. They also have some advantages, such as the direct *in vitro* assay of gene function. Considering the intensity of work now going on with this system, it would not be rash to predict that in a few years this phage will be one bit of genetic material about which all will be understood.

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