in the uterus and masks the response of induced protein (Noteboom and Gorski, 1963). The lag phase of about 40 min probably corresponds to the time necessary for the synthesis, transport to cytoplasm, and translation of the specific mRNA for the induced protein; since actinomycin D will block the synthesis of induced protein only if injected before estradiol. Actinomycin D injected 30 min after estradiol was no longer effective in blocking the synthesis of induced protein, suggesting a lag due to some posttranscriptional event (DeAngelo and Gorski, 1970).

These data support previous conclusions (Noteboom and Gorski, 1963), based on the use of protein synthesis inhibitors, that estrogen brings about specific protein synthesis which in turn brings about the numerous changes in uterine metabolism due to estrogen. Whether one or more of such induced proteins are involved is not known. The early glucose response to estrogen is also cycloheximide sensitive (Smith and Gorski, 1968); therefore, it would appear likely that increased rates of synthesis of other uterine proteins will be found. However, the synthesis of induced protein presently represents the only available system for such study.

In summary, a single administration of 17β-estradiol to immature rats or mature ovariectomized rats induces *de novo* synthesis of a specific uterine-soluble protein. This effect of estradiol is detectable under *in vivo* conditions within 45–60 min after injection of estradiol.

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Initiation of ϕX-174 RF-Primed Protein with *N*-Formylmethionine*

Robert N. Bryan and Masaki Hayashi

ABSTRACT: Using the replicative form of deoxyribonucleic acid from the bacteriophage ϕX-174 to prime a cell-free system, we coupled transcription to translation and synthesized phage-specific protein. The coupled system incorporated formate from the formyl donor, N^{10}-formyltetrahydrofolic acid.

When the *in vitro* protein product was analyzed by polyacrylamide gel electrophoresis using ϕX-174 wild-type *in vivo* protein as an internal marker, its components comigrated with some of the phage specific proteins. All of the *in vitro* proteins incorporated formate. High-voltage electrophoresis of pronase-digested *in vitro* protein demonstrated that the formate was incorporated into *N*-formylmethionine.

Phage-specific protein can be synthesized in a cell-free system which couples transcription to translation (Bryan *et al.*, 1969). RF[1] from the bacteriophage ϕX-174 primes the "coupled system" which uses DNA-dependent RNA polymerase, ribosomes, and a soluble fraction prepared from uninfected *Escherichia coli*. During concomitant RNA and protein synthesis the entire genome is transcribed. The principal product, which is precipitable with anti-ϕX-174, seems to be a phage structural protein with a molecular weight of about 20,000 (Gelfand and Hayashi, 1969a).

This system provides an excellent opportunity to study the initiation of protein synthesis. The experiments which showed that fMet is an initiator of protein synthesis (Adams and Capecchi, 1966; Capecchi, 1966; Clark and Marker, 1966; Viñuela *et al.*, 1967; Webster *et al.*, 1966) used only RNA as the template, either RNA from RNA bacteriophages or endogenous *E. coli* mRNA. We now extend these observations and show that the initiation codons of a DNA phage are correctly transcribed and translated *in vitro* resulting in the incorporation of fMet into genome-specific protein.

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[1] The following abbreviations are used: RF, replicative form DNA; EtSH, mercaptoethanol; SRM, standard reaction mixture (0.13 ml).

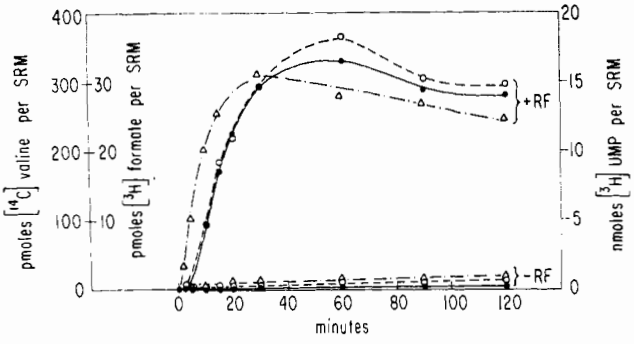

FIGURE 1: Kinetics of RNA and protein synthesis in the coupled system with formyl donor. The composition of an SRM is described in Experimental Section. Four parallel reaction mixtures were used, two to measure RNA synthesis (with and without RF) and two to measure protein synthesis (with and without RF). [^{3}H]UTP was used in the former. N^{10}-[^{3}H]Formyl-H_4-Folic acid, and [^{14}C]-valine were used in the latter. RNA synthesis was measured as nmoles [^{3}H]UMP incorporated into cold trichloroacetic acid insoluble material (△-·-△). Protein synthesis was measured as picomoles of [^{14}C]valine (○---○) and picomoles of [^{3}H]formate (●—●) incorporated into alkaline-insoluble material (Adams and Capecchi, 1966).

Experimental Section

N^{10}-[^{3}H]Formyl-H_4-folic acid was synthesized by the method of Rabinowitz and Pricer (1962). The reaction mixture contained, per milliliter: 100 μmoles of triethanolamine (pH 8.0), 10 μmoles of $MgCl_2$, 5 μmoles of *dl*-H_4-folic acid, 5 μmoles of ATP, 5 μmoles of [^{3}H]formate (Tracerlab, 3.74 Ci/mmole), and 200 units of formyl-H_4-folic acid synthetase. After 30 min at 37° the mixture was acidified with 1 N HCl and adsorbed onto a 3 × 4 cm microgranular cellulose column (Whatman CC31). Then, according to the procedure of Huennekens *et al.* (1963), the methenyl-H_4-folic acid was eluted with 0.1 N formic acid–0.01 M 2-EtSH, and lyophilized. The resulting yellow residue was dissolved in 0.1 N HCl–0.1 M 2-EtSH and stored at −20°.

Coupled system components were prepared as previously described (Bryan *et al.*, 1969).

In vitro protein was synthesized in a 5× SRM containing, per milliliter: 12 μmoles of Tris (pH 7.9), 11.5 μmoles of magnesium acetate, 69 μmoles of ammonium chloride, 7 μmoles of 2-EtSH, 5 μmoles of phosphoenolpyruvate, 2 μmoles of ATP, 0.4 μmole each of GTP, UTP, and CTP, 0.01 μmole of N^{10}-formyl-H_4-folic acid, 0.03 μmole each of 20 amino acids, 38 μg of pyruvate kinase, 24 μg of RF, 19 μg of DNA-dependent RNA polymerase, 38 OD_{260} units of *E. coli* Q13 ribosomes, and 5.2 OD_{260} units of *E. coli* Q13 soluble fraction. (The radioactive compounds are indicated in the figure captions.) After 60-min incubation at 33° the proteins were separated from the nucleic acids by two treatments with 0.22 ml of phenol saturated with 0.1 M Tris (pH 8.4), 0.01 M EDTA, and 1% (v/v) 2-EtSH. The phenol layers were pooled and dialyzed against tenfold concentrated sample buffer for 24 hr followed by three changes of sample buffer for 8 hr each. Sample buffer is 0.01 M Tris-acetate (pH 9.1), 0.1% (w/v) sodium dodecyl sulfate, 0.001% (w/v) EDTA (pH 8.0), 0.5 M urea, and 0.1% (v/v) 2-EtSH (Gelfand and Hayashi, 1969b).

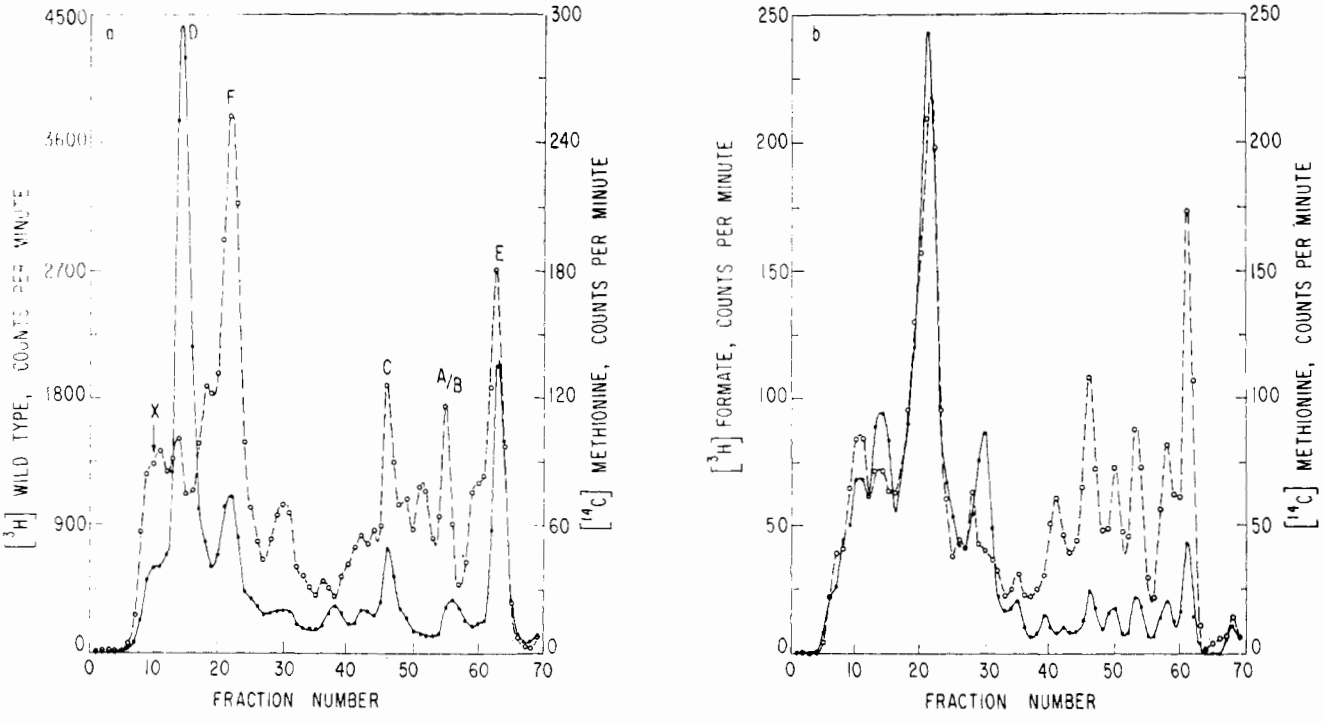

FIGURE 2: Polyacrylamide–sodium dodecyl sulfate gel electrophoregram of (a) *in vivo* wild-type φX-174 protein labeled with [^{3}H]amino acids (●—●) and *in vitro* protein labeled with [^{14}C]methionine (○---○); and (b) *in vitro* protein labeled with [^{3}H]formate (●—●) and [^{14}C]methionine (○---○). Migration is toward the anode, from right to left. X, F, A/B, and E are phage proteins. D and C are found in infected cells. Identification of these proteins with φX-174 complementation groups has been described by Gelfand and Hayashi (1969b).

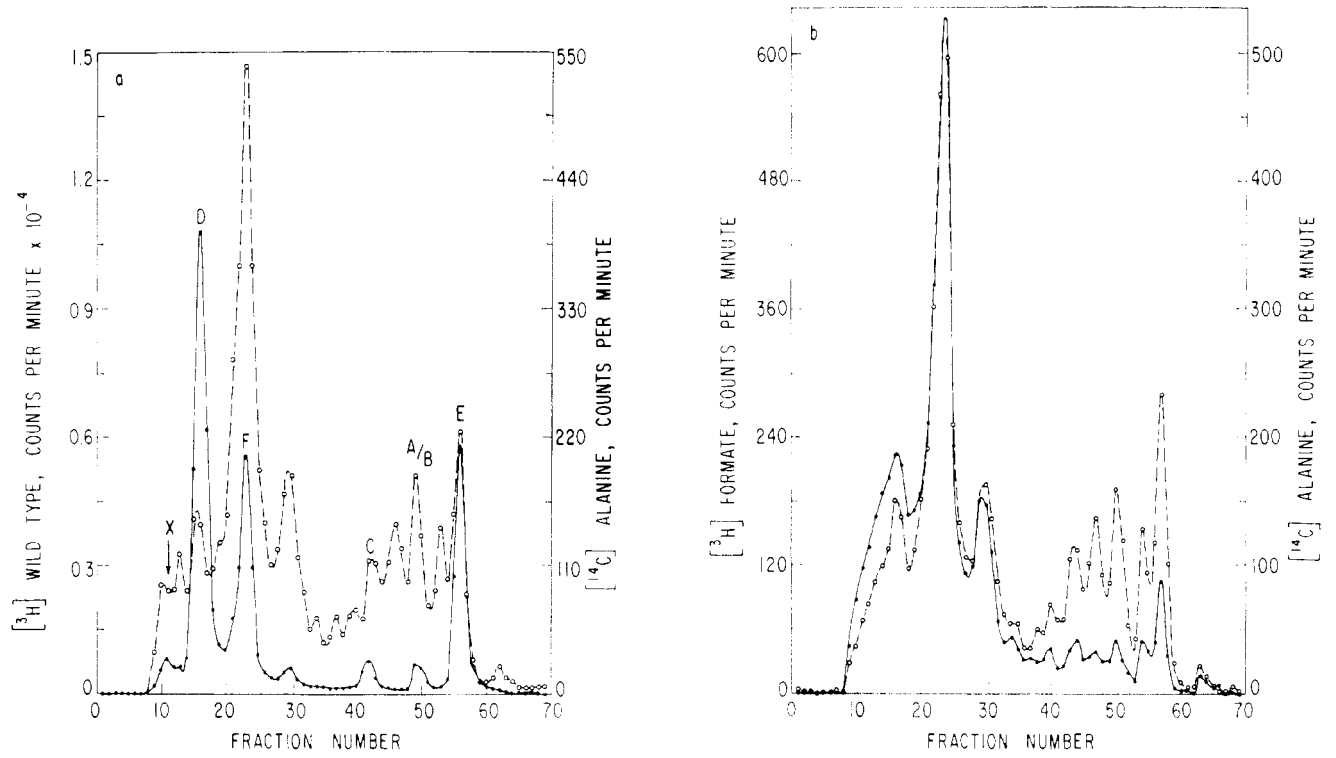

FIGURE 3: Polyacrylamide–sodium dodecyl sulfate gel electrophoregram of (a) *in vivo* wild-type ϕX-174 protein labeled with [^{3}H]amino acids (●—●) and *in vitro* protein labeled with [^{14}C]alanine (○- - -○); and (b) *in vitro* protein labeled with [^{3}H]formate (●—●) and [^{14}C]alanine (○- - -○). Migration is toward the anode, from right to left.

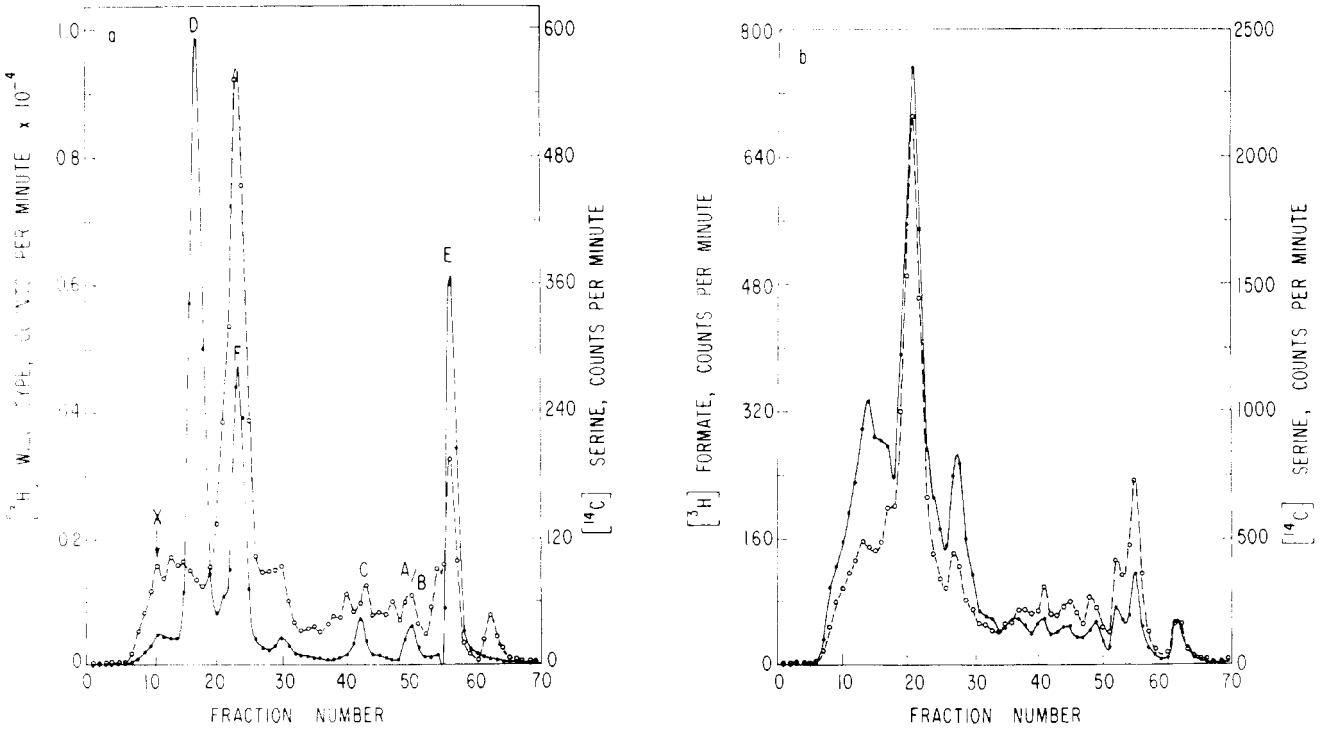

FIGURE 4: Polyacrylamide–sodium dodecyl sulfate gel electrophoregram of (a) *in vivo* wild-type ϕX-174 protein labeled with [^{3}H]amino acids (●—●) and *in vitro* protein labeled with [^{14}C]serine (○- - -○); and (b) *in vitro* protein labeled with [^{3}H]formate (●—●) and [^{14}C]serine (○- - -○). Migration is toward the anode, from right to left.

Polyacrylamide gel electrophoresis has been described elsewhere (Gelfand and Hayashi, 1969b).

Pronase digestion of in vitro protein was carried out in 0.1 M ammonium bicarbonate. *In vitro* protein in sample buffer was dialyzed against 0.1 M ammonium bicarbonate. Pronase (Calbiochem, grade B) was added to a final concentration of 1 mg/ml, and the mixture was incubated at 37° for 24 hr with two further additions of pronase at 8-hr intervals. The digest was cleared by centrifugation and adsorbed onto a 6-ml column of Dowex 50 (Bio-Rad, AG 50W-X4, hydrogen form), and the formylated amino acids were eluted with water and concentrated by flash evaporation (Capecchi, 1966; Viñuela *et al.*, 1967). The extent of protein hydrolysis by pronase was not determined; however no ^{3}H or ^{14}C radioactivity was lost during the digestion and subsequent centrifugation. Eighty per cent of the [^{3}H]formyl label applied to the Dowex was recovered in the eluate.

High-voltage electrophoregrams were developed at pH 6.4 pyridine–acetic acid–H_2O, 200:8 to (2 l.), on a water-cooled plate. L-Met-L-Ser and L-Met-L-Ala (Mann) were formylated according to the procedure of Sheehan and Yang (1958). fMet (Sigma) and the formylated dipeptides were spotted with the pronase-digested samples on Whatman No. 3MM paper. After 3.25 hr at 1500 V the paper was dried at 80° for 10 min, and the standards were visualized with the platinic iodide stain (Toennies and Kolb, 1951). Strips 3 cm wide were cut into 1-cm pieces and counted in a liquid scintillation counter.

Results

The kinetics of RNA and protein synthesis in the coupled system are illustrated in Figure 1. The close correspondence between the incorporation of formate and valine into an alkaline-insoluble fraction suggests that new protein chains were initiated throughout the first 30 min. Although formate was readily incorporated the system did not seem to be dependent on the addition of formyl donor (data not shown).

We used electrophoresis in polyacrylamide–sodium dodecyl sulfate gels to identify the protein products from the coupled system. In an earlier report from this laboratory, Gelfand and Hayashi (1969b) identified six of the gene products from ϕX-174 in this gel system. They analyzed the proteins from *E. coli* infected with various amber mutants of ϕX-174. They were able to associate certain peaks with specific complementation groups and thus with known functions. We analyzed the *in vitro* products in these gels using protein from cells infected with wild-type ϕX-174 as an internal marker. The *in vivo* protein was labeled with [^{3}H]amino acids, but the quantity of radioactivity applied to the gels was much higher than that of the *in vitro* [^{3}H]formyl-protein. So even though the *in vivo* and *in vitro* samples were applied to the same gel, the ^{3}H radioactivity of the *in vitro* protein had no appreciable effect on the *in vivo* profile. *In vitro* proteins were labeled with [^{3}H]formate and either [^{14}C]methionine (Figure 2), [^{14}C]alanine (Figure 3), or [^{14}C]serine (Figure 4).

On the basis of migration with the internal markers, it seems that the coupled system synthesized two of the ϕX-174 proteins which have been identified in infected cells, F and E. *In vitro* proteins may also migrate with X, D, C, and A/B, but these peaks are not as well resolved as the others. The

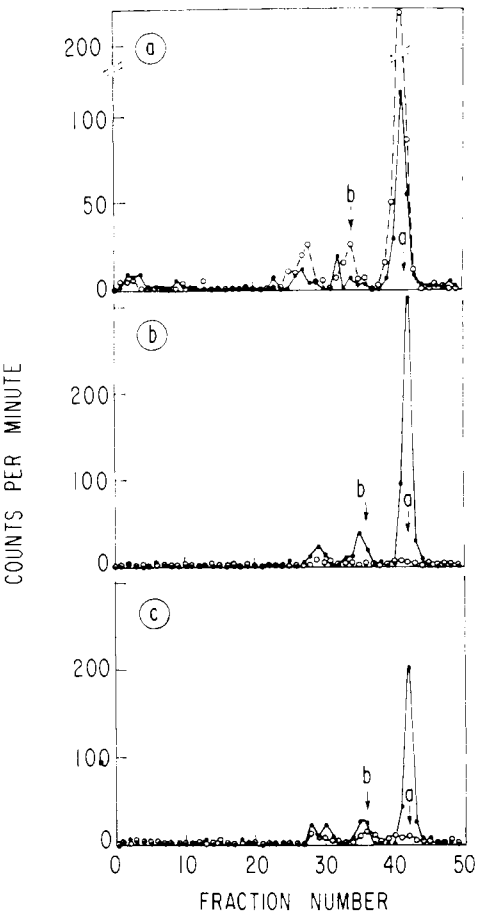

FIGURE 5: High-voltage electrophoregrams of pronase digested *in vitro* protein: [^{3}H]formate (●—●) and (a) [^{14}C]methionine (○---○), (b) [^{14}C]alanine (○---○), or (c) [^{14}C]serine (○---○). Standards: fMet, a; fMetSer, b. The origin is at the left, and migration is from left to right.

in vitro proteins do not occur with the same frequencies as the *in vivo* proteins.

All of the *in vitro* proteins incorporated formate. Notice that the ratio of formate:amino acid decreases with increasing molecular weight (from left to right) in the gels. This is to be expected if formate is incorporated as formylated amino acid during initiation of protein chains.

While it was clear that formate was incorporated into protein synthesized *in vitro*, it was necessary to ask which amino acids were formylated. Poljak (1968) reported that methionine and serine are the N-terminal amino acids in the phage proteins. In view of the recent discovery of *N*-fSer-tRNA in *E. coli* (Kim, 1969), this question had special relevance for our work. We digested the *in vitro* protein, which was labeled with [^{3}H]formate and either [^{14}C]methionine, [^{14}C]alanine, or [^{14}C]serine, with pronase and analyzed the fragments by high-voltage electrophoresis (Figure 5). [^{3}H]Formyl-labeled compound(s) migrated with the fMet standard; but of the three [^{14}C]amino acids, only [^{14}C]methionine migrated with fMet. Small amounts of [^{3}H]formate, [^{14}C]methionine, and [^{14}C]serine were detected with the dipeptide standard, fMetSer. In this buffer system fMetSer is not resolved from fMetAla although fMetSer runs slightly

ahead of fMetAla. fMet was the only formylated amino acid detected in the digest. Occasionally a small peak of ^{3}H was detected slightly ahead of fMet. This peak was also labeled with ^{14}C when the reaction mixture contained [^{14}C]methionine suggesting that it was the oxidation product of fMet, *N*-formylmethionine sulfoxide (Lodish, 1968, 1969).

Discussion

All of the *in vitro* proteins, those that migrate with ϕX-174 proteins and those that are unidentified, incorporate formate. It may be argued that these proteins are the result of initiation at internal AUG or GUG codons or initiation out of phase. However, if this were the case, we would expect many polypeptides of various lengths to be synthesized. These would give a heterogeneous profile in the gels. But we see only discrete peaks, some of which comigrate with phage-specific markers, and one of which is precipitable with anti-ϕX-174 (Gelfand and Hayashi, 1969a). The *in vitro* proteins which do not migrate with an *in vivo* marker may be the result of ϕX-174 proteins which are terminated prematurely.

Paper electrophoresis of lyophilized performic acid oxidized phage particles yields four components (Poljak, 1968). One of these, α, corresponds to F on the basis of per cent of total phage weight (Tonegawa and Hayashi, 1970). The N-terminal amino acid of α is methionine. Since the major product of the coupled system migrates with F, the high-voltage electrophoregrams suggest that F is initiated with fMet. This result is in agreement with Poljak's N-terminal analysis. Other *in vitro* proteins do not occur in a large enough proportion to definitely exclude their initiation by other formylated amino acids.

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