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*THE IN VITRO TRANSLATION OF A MONOCISTRONIC MESSAGE**

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Analyses of the translation of RNA into protein are ideally carried out with the aid of defined RNA messages directing the *in vitro* synthesis of proteins.[1] The most convenient sources of homogeneous translatable messages are the RNA viruses. In the cases thus far reported,[2–5] the RNA messages employed have been polycistronic,[5, 6] i.e., they contain information for the coding of several proteins. This feature permits the study of the factors governing the order and frequency of translation of several cistrons linearly arrayed in the same RNA molecule. Multiple cistrons, however, introduce an unwanted complexity if the intent is to relate the detailed chemistry of a message to that of the final polypeptide product produced. For such purposes, one would prefer a monocistronic message containing information for only one protein.

Recent investigations[7] with Satellite Tobacco Necrosis Virus (STNV) suggest that the RNA of this virus is 400,000 in molecular weight and that its coat protein contains approximately 400 amino acid residues. Assuming a nonoverlapping triplet code, it is evident that the genome of STNV contains just enough information to code for its coat protein. The RNA molecule must consequently be a monocistronic message.

The present paper reports the message properties of STNV-RNA. The tryptic

peptides obtained from the C^{14} product produced *in vitro* in response to STNV-RNA confirm the prediction[7] that it directs the synthesis solely of STNV coat protein.

Materials and Methods.—(1) *Preparation of cell-free extracts:* *E. coli* B cells were grown at 37°C in a well-aerated medium containing 0.8% casamino acids, 0.5% glucose, 10% tap water, 0.0005 *M* $MgSO_4$, and 0.01 *M* potassium phosphate, pH 7.0. Growth was stopped at early log phase (OD_{660} of 0.2) by the rapid addition of crushed ice. The cells were then harvested, washed two times in standard buffer (0.04 *M* Tris-Cl, pH 7.8; 0.01 *M* magnesium acetate; 0.03 *M* KCl; 0.008 *M* β-mercaptoethanol) and suspended in an equal volume of standard buffer before storage at −20°C

Cell-free extracts were prepared from these frozen preparations by rupture in a Hughes press. Twenty μg of crystalline pancreatic DNase and 2 ml of standard buffer per gram of original cells were added, followed by centrifugation at 10,000 × *g* for 10 min. The supernatant was then centrifuged at 30,000 × *g* for 30 min. To 16 ml of the resulting 30,000 × *g* supernate were added 2.0 ml of 0.10 *M* phosphoenolpyruvate (PEP), 1 ml of 0.05 *M* ATP, 0.6 ml of 0.01 *M* GTP, 0.80 ml of a solution 0.001 *M* in each of the 20 L-amino acids found in proteins, 0.10 ml of pyruvic kinase (10 mg/ml), and 0.10 ml of a 20-fold concentrated standard buffer. All the organic phosphates were used as the K^+ salts. This mixture was incubated at 35°C for 40 min, dialyzed for 10 hr at 2°C against three 2-liter changes of standard buffer, and finally clarified by centrifugation at 20,000 × *g* for 20 min before freezing in a dry ice bath. Such preparations, designated as S-30, are stored as small aliquots at −70°C. They retain full activity for at least one month if not thawed and refrozen.

(2) *Incorporation assay:* C^{14}-amino acid incorporation studies were run at 37°C in 0.05-ml volumes containing standard buffer and 0.006 *M* PEP; 0.0015 *M* ATP; 0.003 *M* GTP; 0.5 μc of algal C^{14}-protein hydrolysate (1.8 μc/μg); 4×10^{-5} *M* in each of the following C^{12}-L-amino acids: asparagine, glutamine, tryptophan, and L-cysteine; 2.5 μg of crystalline pyruvic kinase; 0.01 ml of S-30; and 0–40 μg of STNV-RNA isolated from STNV by phenol extraction. Reactions were stopped by the addition of 3 ml of cold 10% TCA. The resultant precipitates were then resuspended in 0.4 ml of 0.2 *M* glycine-NaOH buffer, pH 10.0, and incubated for 1 hr at room temperature. This was followed by four further precipitations and resuspensions in cold 10% TCA as above. The precipitates were finally collected on membrane filters, washed 3 times on the filters with 4-ml washes of cold 10% TCA, and then dried (15 min at 90°C). The radioactive material retained was then measured in a liquid scintillation spectrometer.

(3) *Peptide fingerprint assays:* C^{14}-protein used for fingerprint studies was prepared using the incubation conditions of the incorporation assay. The incubation was stopped after 10 min by the

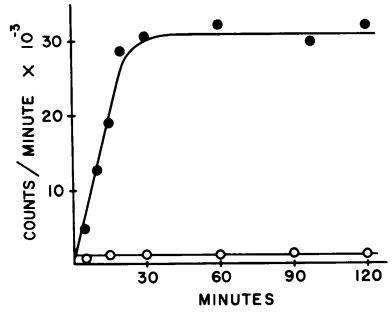

FIG. 1.—Kinetics of STNV-RNA dependent incorporation of C^{14}-amino acids into acid-insoluble material. The reaction conditions are as given in *Materials and Methods*. Incubations contain 35 μg STNV-RNA per 0.05 ml (closed circles) or lack STNV-RNA (open circles). All assays were preincubated for 3 min at 37°C before initiation of reactions with added STNV-RNA and/or S-30 enzyme.

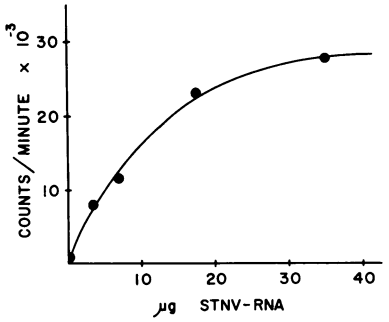

FIG. 2.—Dependence of amino acid incorporation upon STNV-RNA concentration. The reaction conditions are as given in *Materials and Methods*. All assays were incubated at 37°C for 90 min.

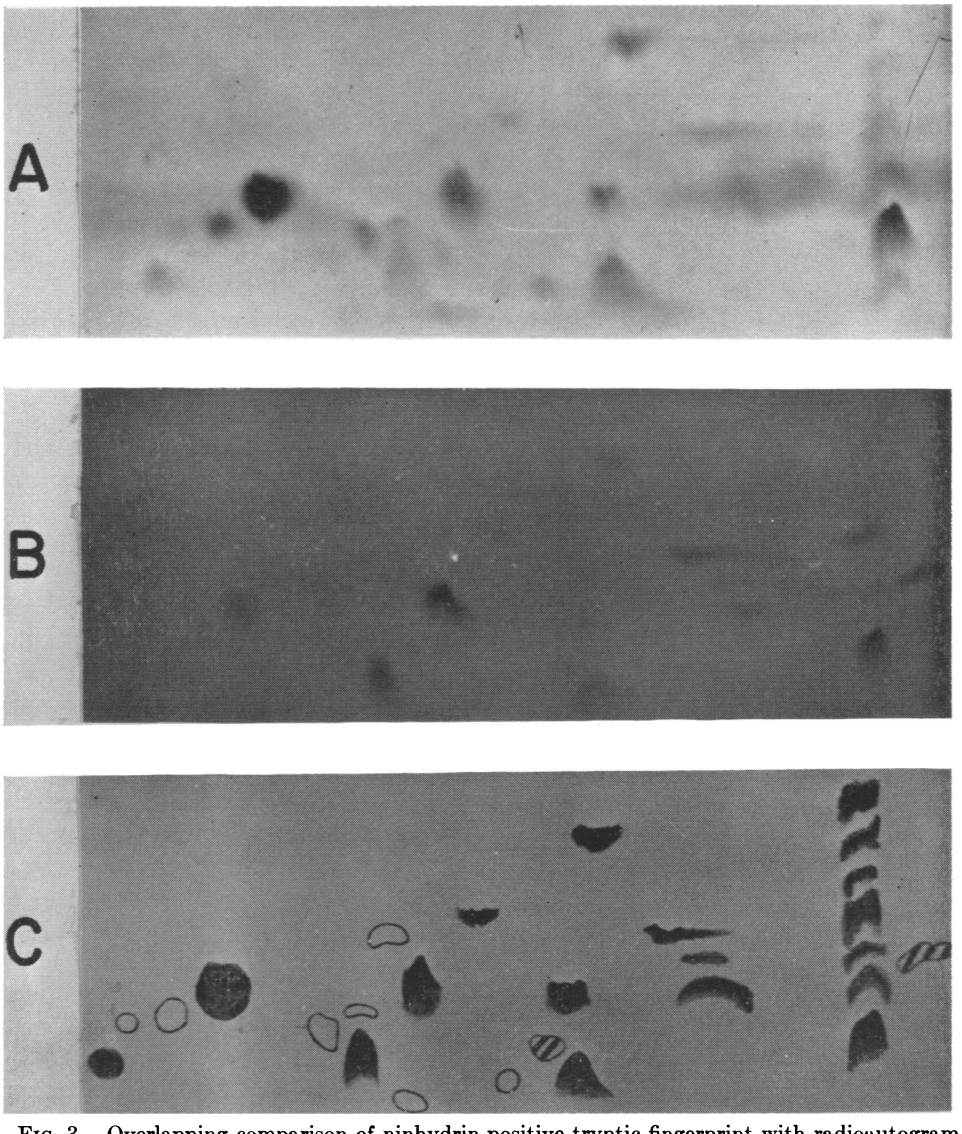

FIG. 3.—Overlapping comparison of ninhydrin-positive tryptic fingerprint with radioautogram positive tryptic fingerprint. Radioautograms were allowed to develop for five weeks. Tryptic digestion was carried out for 1 hr at 37°C. Electrophoresis was in 10% aqueous pyridine containing 0.4% HAc and chromatography in n-butanol:HAc:H_2O(4:1:5). (*A*) Ninhydrin-positive cationic peptides. (*B*) Radioautogram-positive cationic peptides. (*C*) Artist's tracing of coincidence between ninhydrin-positive and radioautogram-positive peptides (dark spots), peptides detected only with ninhydrin (open spots), and peptides detected only by radioautogram (hash-lined spots).

addition of equal volumes of cold 0.5 *M* NaOH. These preparations were then dialyzed for 24 hr at 5°C against three 1-liter changes of 1% $(NH_4)_2CO_3$ as described previously.[7] Tryptic digests and peptide fingerprints were prepared following the method of Rothman and Byrne.[8] Unless otherwise specified, 3 mg of unlabeled coat protein was added to the C^{14}-synthesized product prior to processing and digestion. For each 3 mg of protein, 0.2 mg of 2 × crystallized salt-free trypsin (Worthington Biochemical Corp., Freehold, New Jersey) was used.

Results.—The ability of STNV-RNA to stimulate the incorporation of amino

acids in extracts of *E. coli* was found to be extremely sensitive to the method employed in cell rupture. Although responding to synthetic polynucleotides, preparations derived by sonication or alumina grinding were completely inactive with TNV-RNA. A similar situation had been observed[9] with RNA from the coliphage MS-2 and a systematic investigation led to the conclusion that the Hughes press yielded extracts containing less nuclease activity. In any event, this procedure was used and gave preparations which uniformly responded to STNV-RNA as described in Figures 1 and 2. The amount of amino acid incorporated increases with the level of STNV-RNA until a concentration of 40 μg is reached.

Character of the product: Evidence supporting the conclusion that STNV-RNA codes only for STNV coat protein is found in the radioautogram obtained from a tryptic fingerprint of the STNV-RNA-dependent-C^{14}-labeled product (Fig. 3). There is excellent agreement between the positively charged, ninhydrin-reactive peptides obtained upon tryptic digestion of STNV coat protein, and the positively charged C^{14}-labeled product. That this correlation is not due to adsorption of C^{14}-materials to the tryptic peptides of STNV coat protein was established by controls run in the absence of added coat protein which yielded similar radioautographic patterns. Overlapping comparisons show exact agreement in 18 out of 25 positively charged, ninhydrin-reactive peptides obtained from STNV coat protein. Two faint radioautogram spots, possibly due to protease activity in the reaction mixture, are detected that do not correspond with any of the known tryptic peptides of STNV coat protein. Five of the ninhydrin-positive spots detected from STNV coat protein do not appear as clear radioautogram spots under our assay conditions and time of radioautogram exposure.

The 10 negatively charged tryptic peptides of STNV coat protein also similarly mimic the radioautogram spots. Here the agreement is not as obvious due to the previously observed[7] weak reaction of these materials with ninhydrin.

Discussion and Summary.—The data presented offer supporting evidence for the earlier conclusion that the approximately 1200 nucleotides of STNV-RNA code only for the 400 amino acid residues of the coat protein. The numerical correspondence and the *in vitro* data are clearly in agreement with a nonoverlapping triplet code. The relative freedom of the radioautographs from nonspecific peptides indicates that the *in vitro* system is not introducing significant confusion by incorrect or fragmentary translations. The comparatively accurate translations of a plant virus genome by a bacterial system lends further support for the universality of the translational system.

The availability of STNV-RNA as a monocistronic message makes possible a host of informative experiments which can hope to illuminate various aspects of the translational mechanism. These include, among others, the accurate identification of RNA codons and of initiating and terminating sequences of cistrons.

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*SPECIFIC DEGRADATION OF THE COLLAGEN MOLECULE BY TADPOLE COLLAGENOLYTIC ENZYME**

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Recently the production of a collagenolytic enzyme by anuran tadpole tissues in culture was demonstrated by lysis and degradation of reconstituted mammalian collagen gels upon which tissue fragments were cultivated *in vitro* under physiologic conditions.[1, 2] The culture chamber acts as a closed reservoir in which the enzyme accumulates over a period of time long enough to reach detectable levels. There is no appreciable amount stored in the tissues at any one moment,[1, 3] nor has the enzyme been obtained by extraction of fresh living or autolyzing dead tissues.

By incubating thin strips of sterile living tadpole tail fin or back skin on filter paper disks floating in amphibian Tyrode solution at 37°C for several days, relatively large amounts of a collagenolytic enzyme appear in the culture medium. The details of preparation, purification, and description of some of the properties of this enzyme will appear elsewhere. In brief, the enzyme has been purified 300-fold from the lyophilized dialyzed culture medium by sequential ammonium sulfate fractionation, Sephadex filtration, starch gel electrophoresis, and DEAE-cellulose chromatography. It has a pH optimum of 6–8, loses activity at 50–60°C after 10 min, is inhibited by ethylenediaminetetraacetate and cysteine, but not by diisopropylfluorophosphate, and has very little activity against casein.[4]

In this report we describe some aspects of the effect of this enzyme on the collagen molecule, the products of which are far different from those resulting from the action of bacterial collagenase.

Methods.—Several different purified acid-extracted calf skin collagen preparations and neutral extracted collagen from the skin of normal and lathyritic guinea pigs and rats and from tadpole tail fins were used in these experiments. Two samples of acid-extracted calf skin collagen were prepared as described by Gross and Kirk[5] and another sample obtained from the laboratory of