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PHYSICAL PROPERTIES OF MESSENGER RNA OF BACTERIOPHAGE T4

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The finding of rapidly labeled RNA fractions in many systems[1–6] has led to the general acceptance of the messenger hypothesis. Most of the predictions made by Jacob and Monod[7] have been experimentally verified[8–11] through investigations carried out by specifically labeling the messenger fractions with radioisotopes. Such studies, however, have not required the separation of messenger RNA from the bulk of ribosomal and soluble RNA, and since optical methods such as the measurement of melting profiles could not be applied to such mixtures of RNA species, little could be said about the physical state of the messenger RNA.

With the selective technique of complex formation between strands of complementary sequences,[13, 14, 18] it has become possible to isolate T4 msRNA* in a highly purified form on a T4 DNA-cellulose column.[15] The biological activity of T4 msRNA isolated in this fashion was established with cell-free extracts of *E. coli*.[12]

The purpose of this paper is to present recent studies on the physical state of T4 msRNA and to discuss possible correlations between its structure and its biological activity.

Materials and Methods.—*Escherichia coli* B, grown in one-liter batches of minimal medium to a titer of 5×10^8 cells/ml were infected with a total of 3×10^{12} T4 wild type phage particles in the presence of 5 μg/ml of tryptophan. The bacteria

were harvested 8 min after infection as described previously,[15] and the washed cells were frozen in dry ice-methanol. RNA was extracted with sodium dodecylsulfate (SDS) and hot phenol in the presence of polyvinyl sulfate (PVS) as described by Scherrer and Darnell.[6] The T4 DNA-cellulose column and the incubation and elution steps have already been described.[12] Measurements of optical density were performed with a Zeiss spectrophotometer equipped with a heatable cell holder. Melting profiles were measured at 260 mμ, in quartz cells of 3-ml capacity stoppered with special short-stem thermometers purchased from Paracell Inc., N. Y. Pancreatic ribonuclease was purchased from Worthington Biochemicals. Poly A and poly U were purchased from Miles Chemical Company, Clifton, N. J.

Sucrose density gradient centrifugation: The RNA samples were layered on top of a linear gradient ranging from 5–20 per cent sucrose (Merck) solution. The samples and the sucrose solutions were in 1×10^{-2} *M* Tris buffer pH 7.4, containing 1×10^{-3} *M* Mg^{++}. The tubes were spun at 38,000 rpm for 5 hr in a Spinco SW 39 swinging bucket rotor, and after conclusion of the run the tubes were punctured at the bottom and fractions of 10 drops each were collected through a 20-gauge hypodermic needle. To each fraction 1 ml of 1×10^{-2} *M* Tris buffer pH 7.4 was added, the optical density was read, and the RNA was precipitated with 10 per cent trichloroacetic acid (TCA). The precipitates were collected on millipore filters, dried, and counted in a Packard Model EX liquid scintillation counter. The RNA used was labeled with H^3-uracil (New England Nuclear Corporation, specific activity 1 c/m*M*) from 5–8 min after infection with T4 phage particles as described before.

The assays for amino acid incorporation in cell-free extracts contained per ml: 14 μM Mg acetate, 0.1 ml of a preincubated[16] 30,000 $\times$ *g* supernatant fraction from *E. coli* B cells (OD_{260} = 240), 1 mg *E. coli* B S-RNA, 0.2 μc C^{14} L-threonine (specific activity = 164 mc/m*M*). The other additions were the same as described by Nirenberg and Matthaei.[16] The samples were incubated for 60 min at 35° and the radioactivity of acid-insoluble protein was determined the following way: After hot TCA (5 per cent) hydrolysis and repeated washing with 5 per cent TCA and ethanol ether (1:1), the precipitates were dissolved in 0.4 ml formic acid, mixed with 15 ml *p*-dioxane scintillation liquid,[17] and counted as described above.

Results.—Size distribution of T4 msRNA before and after infection: Figure 1*a* shows the distribution of H^3-uracil-labeled T4 msRNA after sucrose density gradient centrifugation. When a sample of the total RNA, isolated 8 min after infection, was applied, a substantial portion of the T4 msRNA sedimented with S values considerably higher than those observed by other investigators under similar conditions.[18–20] Centrifugation in the absence of Mg^{++} results in a similar distribution of radioactivity, and the fractions of high molecular weight thus obtained seem not to result from artificial association between smaller molecules brought about by the action of bivalent cations. We attribute this relatively high yield of high-molecular-weight messenger RNA to the hot phenol isolation procedure, which seems to be superior to such methods as the use of lysozyme or SDS plus cold phenol. It also yields messenger RNA of higher activity as judged by amino acid incorporation in cell-free extracts. Figure 1*b* shows the molecular size of purified T4 msRNA as it is eluted from the T4-DNA cellulose column. It shows less heterogeneity in size since the components having S-values above 23 are almost entirely missing. The

question of whether the large-size T4 msRNA, lost during hybridization with T4 DNA, represents a complex between several messenger RNA molecules or between messenger RNA and ribosomal RNA molecules is under current investigation.

Hyperchromicity of T4 msRNA: Incubation of 5 mg of RNA from T4-infected cells for 6 to 8 hr at 51°C on the DNA-cellulose column yielded approximately 100 μg of an RNA fraction which appeared to consist mainly of T4 msRNA. This material shows positive orcinol and negative diphenylamine reactions, it has a ratio of OD 260/280 = 2.04 and OD 240/260 = 0.62, and it shows a hyperchromic effect upon heating in standard saline citrate (Fig. 2). The amount of hyperchromicity

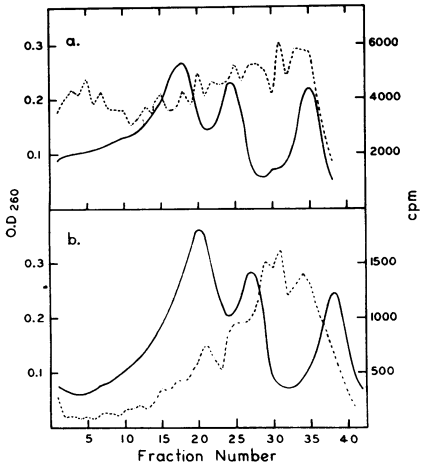

FIG. 1.—Size distribution of T4 msRNA labeled with H^3-uracil from 5–8 min after infection: (*a*) before fractionation on the T4 DNA-cellulose column, (*b*) after fractionation on the T4 DNA-cellulose column. To (*b*) 0.4 mg of *E. coli* RNA was added as carrier. —— = absorbance at 260 mμ. ---- = radioactivity, given as acid-precipitable counts.

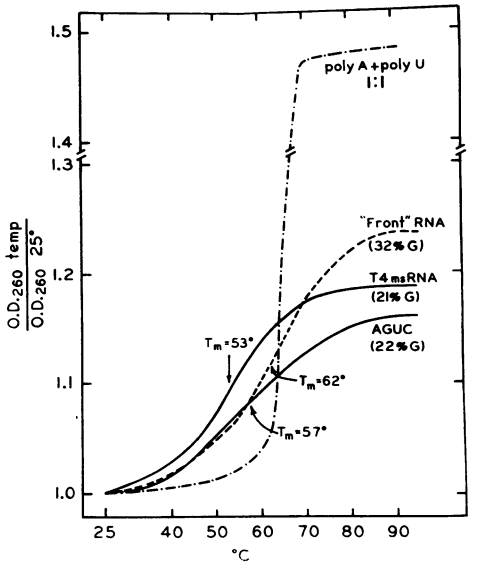

FIG. 2.—Increase in optical density with temperature for the following polynucleotides in 0.15 *M* NaCl + 0.015 Na citrate, pH 7: Purified T4 msRNA as obtained after passage through the DNA-cellulose column. *E. coli* RNA = Front RNA not retained by the DNA column. A random AGUC copolymer, prepared by O. W. Jones, National Institutes of Health, with polynucleotide phosphorylase. A 1:1 mixture of poly A + poly U. The samples were at a concentration of 15 to 30 μg/ml. Upon cooling of the heated samples, the absorbancies returned to the original values. No correction was made for thermal expansion of the solvent.

varies for different RNA preparations (Table 1), but the temperature-midpoint of the helix-coil transition (T_m) proved to be relatively constant for several preparations, especially for those isolated at similar times after infection. Other experiments showed that samples of RNA tested before passage through the DNA-cellulose column and samples not adsorbed onto the column (their major constituent being *E. coli* ribosomal RNA), under the same conditions, show between 23 and 24 per cent hyperchromicity with T_m values of between 62 and 63°C. In comparison, an AGUC copolymer having the base composition: A: 22 per cent, G: 22 per cent, U: 27 per cent, C: 29 per cent (O. W. Jones, personal communication),

TABLE 1

INCREASE IN OPTICAL DENSITY AT 260 mμ FOR SEVERAL T4 msRNA SAMPLES IN 0.15 *M* NaCl + 0.015 *M* Na CITRATE

Preparation	Isolated min after infection	Treatment after elution from DNA column	Per cent hyperchromicity	T_m
a	8	ethanol precipitation followed by dialysis against 1×SSC	19	53
a	8	dialysis against 1×SSC	20	55
b	8	EtOH precipitation	15	52
b	8	dialysis against 1×SSC	16	52
c	8	EtOH precipitation heated and slow cooled	20	52
c	8	EtOH precipitation heated and fast cooled	20	52
d*	16	dialyzed against H_2O	18	55
e*	25†	dialyzed against H_2O	20	54

* Found to be as active in stimulating incorporation of C^{14}-threonine in a cell-free system as the RNA isolated 8 min after infection.
† At 5 minutes after infection, chloramphenicol was added at a concentration of 100 μg/ml.

shows 16 per cent hyperchromicity with T_m = 57°C (Fig. 2). It was observed furthermore that, with all T4 msRNA samples tested, the optical density profiles obtained by heating and cooling are identical.

Kinetics of ribonuclease digestion: The hyperchromic effect on RNA upon heating can also be observed following digestion with ribonuclease. Figure 3 shows the change in optical density of T4 msRNA and *E. coli* RNA as a function of time of incubation with 0.2 μg/ml of pancreatic ribonuclease. In the presence of monovalent cations, T4 msRNA is degraded at a rate slightly greater than is ribosomal RNA. At 10^{-2} *M* Mg^{++}, the difference is much more pronounced.

Heat inactivation of T4 msRNA: When tested in the Nirenberg system,[16] T4 msRNA, isolated on the DNA column, actively stimulates amino acid incorporation into acid-insoluble proteins.[12] The specific messenger activity of this material is about eight times as high as the activity of the unfractionated RNA. The melting profile and also the kinetics of ribonuclease digestion indicate that the T4 messenger in its active form consists of a single polynucleotide chain. Heat-inactivation studies given in Figure 4 confirm this indication, since the T4 msRNA can be heated for short periods of time well above the apparent midpoint of helix-

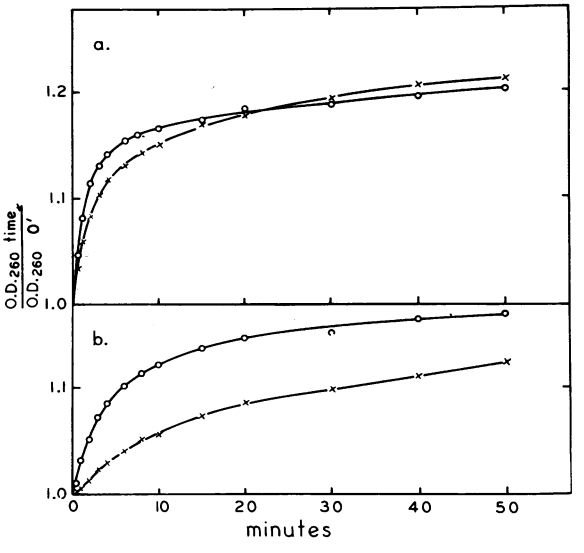

FIG. 3.—Kinetics of digestion by ribonuclease of RNA isolated 8 min after T4 infection = X, and of purified T4 msRNA = 0; (*a*) in 0.08 *M* phosphate buffer pH 7.0, (*b*) in 0.01 *M* Tris Mg^{++} buffer pH 7.4. At zero time 0.2 μg/ml ribonuclease was added.

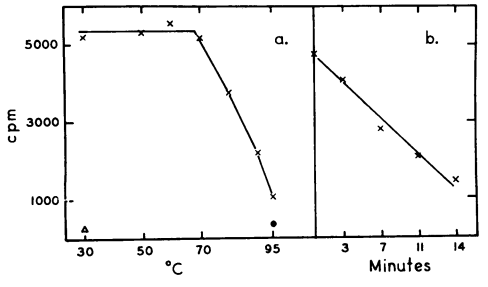

FIG. 4.—Heat-inactivation of T4 msRNA. (*a*) Samples of 15 μg of T4 msRNA were incubated for 10 min at a given temperature in 0.1 *M* Tris pH 7.6 + 0.01 *M* Mg^{++} and after rapid cooling the promotion of C^{14} L-threonine incorporation into acid-insoluble protein was determined as described in *Materials and Methods*. × = incorporation with RNA added. △ = blank (no RNA added). ⊙ = same as X, but cooled slowly. (*b*) Samples of 12 μg of T4 msRNA were heated at 90°C for given times. Experimental conditions as in 4*a*.

coil transition. Since the time curve of inactivation at 90°C follows roughly a single-hit curve (Fig. 4*b*), the decrease in activity above 70°C (Fig. 4*a*) seems to be caused by thermal scission of the polymer. Heating of a T4 msRNA sample to 95°C for 10 min, followed by slow cooling, inactivates the RNA to a greater extent than heating followed by fast cooling (Fig. 4*a*), a result which again indicates the active messenger to be a single-stranded polynucleotide.

Discussion.—Doty *et al.*[21] have found that upon heating of polynucleotides in solution the observed increase in absorbance shows complete correlation with the decrease in optical rotation, a finding that could only be explained by the transition from an ordered helix to a random coil. The helical content of a polynucleotide was found to vary between 0 per cent for poly U and 100 per cent for an equimolar mixture of poly A + poly U or for DNA. The helical content of TMV RNA was estimated to be of the order of 60 per cent, while polynucleotides of random sequences could have a helix content as high as 40 per cent.[21, 22] These high values suggest that there need not be a perfect matching of complementary base sequences for the establishment of short helical regions. There seems to be a wide distribution of size and strength of the helical regions, indicated by the broad transition curves obtained for single-stranded polynucleotides (see also Fig. 2). Although the maximal increase in absorbance (Δ OD_{max}) should be a direct measure of helix content, we can only give an estimate in the case of T4 msRNA preparations. However, the frequently observed 20 per cent increase in absorbance seems to represent an upper limit which, taken tentatively as a correct value, would then attribute to T4 msRNA a helix content of

$$\frac{\Delta OD_{max}\ \text{T4 msRNA}}{\Delta OD_{max}\ \text{Poly A + poly U}} = \frac{0.20}{0.48} = 42\ \text{per cent.}$$

It is likely that the relatively low value of T_m for T4 msRNA compared with the T_m of the *E. coli* RNA is mainly due to the difference in guanine content. A similar dependence upon guanine content seems to exist for the susceptibility towards digestion by ribonuclease. T4 msRNA is much more readily attacked than is ribosomal RNA by ribonuclease at a magnesium concentration at which *in vitro* protein synthesis proceeds at maximal rate. Such conditions might be very similar to the physiological, and it therefore seems worth stressing this point in view of the rapid metabolic turnover of msRNA. The relative resistance of ribosomal RNA to attack by nuclease is perhaps one of the factors which have played a strong evolutionary role in selecting for the unique base composition of ribosomal RNA in the most unrelated species.

The question of whether one or both strands of DNA are utilized as templates

for synthesis of msRNA is still unanswered. Studies on DNA-dependent RNA synthesis, catalyzed by a purified enzyme system from *Micrococcus lysodeikticus*, clearly show that, *in vitro*, both DNA strands do function as primers. The product, a high-molecular-weight RNA with DNA-like base composition and complementary base sequences, assumes (possibly during the isolation procedure) the structure of a double-stranded helix.[23] If one accepts the *in vitro* synthesis of RNA as a model system, it seems likely that both strands of DNA may at least potentially serve as templates for msRNA synthesis. On the other hand, there are two findings which could be interpreted best by assuming that *in vivo* only one DNA strand is utilized. One is the fact that in T4 msRNA guanine does not equal cytosine[15] and the other is the observation that only about one-half of the A-T mutant sites in the T4 rII region show an effect with 5-fluorouracil.[24] Whereas the former observation indicates that only one of the two DNA strands mediates RNA synthesis, the latter suggests that only one RNA strand is the active messenger for either the rIIa or the rIIb protein. Although these two arguments support each other, they do not settle the question decisively.

Provided that the complementary strand was not lost during the isolation and purification steps, the results reported here support the view that *in vivo* only one of the two DNA strands serves as a primer for messenger RNA synthesis. However, it should be emphasized that the single-strandedness established for purified T4 msRNA does not eliminate the possibility that the reading alternates between the two DNA strands or that both strands are read, but at different times.

While the solution of these problems will require quantitative studies on the biosynthesis of messenger RNA, from our results it can be concluded that the *active* T4 messenger seems to be a single polynucleotide chain with a helix content of roughly 40 per cent, its melting profile being similar to that of a random copolymer of comparable base composition.

Summary.—Parallel studies on heat denaturation, digestion by ribonuclease, and hyperchromicity of T4 messenger RNA indicate that the messenger RNA molecule in its biologically active form is a single-stranded polynucleotide with a degree of secondary structure similar to that of a random copolymer.

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* Abbreviations used throughout this paper: T4 msRNA = phage T4 messenger RNA. (We have found it necessary to designate a two-letter symbol for the term "messenger" since the symbol *m* is already being used in T_m = temperature midpoint of helix-coil transition.) A = adenylate; G = guanylate; U = uridylate; C = cytidylate.

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*INSULIN—A PROBABLE GROSS MOLECULAR STRUCTURE**

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In an earlier communication,[1] we described two alternative models for the gross structure of the insulin molecule. The models were based on our evidence for rodlike regions of high electron density in the orthorhombic crystal forms and were derived from transform and Patterson studies.

One of the crystal forms of insulin (a shrinkage stage, type C) has the space group $I2_12_12_1$ with one molecule per asymmetric unit. In this form, the two molecules of the dimer are related by a crystallographic twofold rotation axis parallel to *a*, whereas in the other forms (space group $P2_12_12_1$) the twofold axis is noncrystallographic. The restrictions imposed by the body-centering on the models and on their intermolecular arrangement have been discussed, and the arrangements of the models in the *a*-plane projection of the type C form have been defined.[1]

This paper describes our further studies with the C form in an attempt to refine the models and to choose between them. The models were described as arrays of rodlike units parallel or nearly parallel to *a* and, in one model, of overlapping units of unspecified configuration, which give rise to comparable regions of high density in the *a*-plane projection. In the model termed arrangement (1), there are two rods (A + B) per molecule; the projected positions of the rodlike units on the *a*-plane are shown in Figure 1*a;* a complete unit-cell projection is shown in Figure 2*a*, and an illustration in three dimensions of the model is shown in Figure 3*a*. The model termed arrangement (2) consists of one rodlike unit, B, and two units of unspecified configuration, A and C. The projected positions of these units are shown in Figure 1*b;* a complete unit-cell projection is shown in Figure 2*b* (unit C omitted for clarity of presentation); an illustration in three dimensions of the model is shown in Figure 3*b* (unit C again omitted).