STUDIES ON THE MESSENGER RNA OF BACTERIOPHAGE λ, I. VARIOUS SPECIES SYNTHESIZED EARLY AFTER INDUCTION OF THE PROPHAGE*

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Our purpose in undertaking the study of the *in vivo* transcription of the temperate phage λ has been to characterize the various identifiable species of messenger RNA (mRNA) synthesized during vegetative growth following induction of the prophage. It is expected that such an approach will provide information on the clustering of genes in operons and on the regulation mechanisms that control the various transcription units. The λ chromosome has some peculiarities that warrant such an approach: while most of the late genes are situated in the left half of the λ chromosome, all the early ones are clustered in the right half (for review, see ref. 1). In fact, it appears that "early" λ mRNA is transcribed exclusively from the right half and from both DNA strands, whereas "late" mRNA is transcribed from both halves and predominantly from one strand.[2-7]

In this paper, we shall describe five species of λ mRNA found *early after induction* of the prophage, i.e., before the onset—or in the absence—of DNA replication. These early species have been characterized by two properties; their molecular weight, as estimated from either sucrose gradient sedimentation[8, 9] or gel electrophoresis, and the DNA strand with which they hybridize.

Materials and Methods.—Strains: Strains C_{600}, C_{600} (λ_{857}), C_{600} (λt_{11}), C_{600} (λi_{434}), and Hfr 1858 (λ_{857}) (Thy^-) were obtained from Dr. F. Jacob. X_{5213} ($\lambda_{857}bio_{11}$) (pyr C^-) carries $\lambda_{857}bio_{11}$, a biotin transducer lacking in integrase, exonuclease, and β protein, isolated by Dr. E. Signer. W_{3350} ($\lambda_{857}susR_5$), W_{3350} ($\lambda_{857}susN_7N_{53}$) were kindly given to us by Drs. H. Eisen and L. Pereira da Silva.

Bacterial growth, pulse labeling, and RNA extraction: Cells were grown in medium 63 supplemented with glycerol (0.4%), vitamin-free charcoal-treated Difco casaminoacids (0.2%), vitamin B_1 (1 μg/ml), and thymine (20 μg/ml) or uracil (5 μg/ml) when required. Cultures were pulse-labeled for 1 min, at a density of 2.10^8 cells per ml, with tritiated uracil (H^3-5-uracil, CEA, 24 c/mole, 3 μc/ml). Harvest of the labeled bacteria, RNA extraction, and purification were performed as previously described.[8]

Induction: C_{600} (λt_{11}), grown at 37°C, was induced with mitomycin (2 μg/ml). All the other strains were lysogenic for thermoinducible prophages carrying the CI_{857} mutation.[10] They were grown at 33°C and induced by heating to 42°C, the temperature change being achieved in less than 30 sec. The time course of phage development is precise and reproducible: λ exonuclease is detectable as early as 1–2 min after induction,[11] lysozyme at 6–7 min,[12] and DNA replication at 5 min;[13] λ-specific RNA synthesized up to 5 min hybridizes only with the right half of the λ chromosome.[7, 13] Heat-induced cells were labeled between 3 and 4 min after heating, unless otherwise stated.

Phage and DNA preparations: Phage λ_{857} was obtained by thermoinduction of C_{600} (λ_{857}), and $\lambda_{857}bio_{11}$ by infection of C_{600}. Phages were concentrated by using a two-phase separation system:[14] polyethylene glycol, 6.9%; dextran sulfate, 0.2%, NaCl, 0.3 M. After standing overnight at 4°C, the phage is found on the dextran phase, and is then freed of bacterial debris and dextran sulfate by precipitation with KCl at a final concentration of 0.4 M. Further purification of the phages by differential centrifugation, band-

ing in a CsCl gradient, and DNA extraction were performed according to Kaiser and Hogness.[15] The DNA halves and λi_{434} DNA were generous gifts of Dr. S. Naono.

λ DNA strands: λ DNA strands were prepared as described by Hradecna and Szybalski,[16] Cohen and Hurwitz,[5] and Sheldrick.[7] The preparations used in this study were at least 90% pure with respect to contamination with the complementary strand.[7] The "heavy strand" (H) is the DNA strand that has the greater buoyant density in the CsCl gradient in the presence of poly IG (66%), whereas the "light strand" (L) is approximately 15 mg/cm^3 less dense under the same conditions.[5-7]

Sucrose gradient sedimentation: RNA preparations were centrifuged in linear, preformed (5–30%, w/w) sucrose gradients containing Tris (pH 7.4), 10^{-2} *M*, KCl, 0.05 *M*. Runs were either for 4$^1/_2$ hr at 35,000 rpm in the SW 39 rotor (4.5-ml gradients), or for 20 hr at 20,000 rpm in the SW 25 rotor (25-ml gradients) of a Spinco model L centrifuge. Gradients were collected in drops, and the fractions were hybridized as described below.

Gel electrophoresis: Gels (7 × 0.5 cm) were prepared as described by Loening,[17] except that 10% glycerol was added to all buffers and 0.5% SDS was present in the buffer used for electrophoresis.[18] Acrylamide concentration in the gels was 2.7% unless otherwise specified. Electrophoresis was performed at room temperature (10 v/cm at 5 mA/gel). Gels were then frozen in a hexane dry ice bath and cut into 1- or 2-mm slices. RNA was quantitatively eluted from the slices (95% of the counts) after a 6-hr incubation in 1 ml (per slice) of 5 × SSC at 66°C (SSC = 0.15 *M* NaCl, 0.015 *M* Na citrate). The eluates were used for subsequent hybridization.

Hybridization: We have used the Gillespie-Spiegelman technique[19] with several minor modifications. Membrane filters (Sartorius MF 50) were loaded by filtration with alkali-denatured DNA (or the DNA strands) (1 μg/ml in 2 × SSC), washed with a few milliliters of 2 × SSC, air-dried for 1 hr, and dessicated overnight *in vacuo* at room temperature over $CaCl_2$. The standard hybridization conditions were: 2 μg of λ DNA or 2 μg of one strand per filter; 1 or 1.5 ml of 5 × SSC; 48 hr at 66°C. Up to three filters (the two DNA strands and a blank filter), labeled with a pencil, were incubated in the same vial. Filters were then washed by immersion in a large volume of 2 × SSC before and after RNAse treatment (10 μg/ml, 3 ml of 2 × SSC, 1 hr at 20°C), dried, and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Results.—We shall present evidence for the existence of five different species of early λ mRNA synthesized after induction of various λ prophages. All of them hybridize exclusively with the right half of λ DNA, where all the known early genes are clustered. We shall describe separately two "high"- and three "low"-molecular-weight (mol wt) species; the latter have been detected under conditions where the former are not synthesized.

(*a*) *"High"-molecular-weight mRNA species:* Two classes of fast-sedimenting λ mRNA, with sedimentation constants around 30*S*, can be observed early after thermoinduction of $\lambda_{857}susR_5$ (Fig. 1): one hybridizes with the heavy strand, the other with the light strand. These two classes have been further characterized by using appropriate mutants, λt_{11} and $\lambda_{857}bio_{11}$.

λt_{11} is a defective mutant of region x[20] which hyperproduces exonuclease and β protein,[21] fails to replicate DNA,[20, 22] and synthesizes mRNA that hybridizes only with the right half of λ DNA[4] and almost exclusively with the light strand.[5-7] The sedimentation pattern of the λ-specific mRNA synthesized by C_{600} (λt_{11}) 40 minutes after addition of mitomycin shows a broad peak of mRNA with a mean sedimentation constant of 27*S* (Fig. 2*a*). Values of 29*S* and 31*S* were obtained in other experiments. Gel electrophoresis of the same mRNA preparation (Fig. 2*b*) reveals a sharper peak, the mobility of which corresponds to a molecular weight of 1.7 × 10^6,[23] which is in good agreement with a sedimentation constant

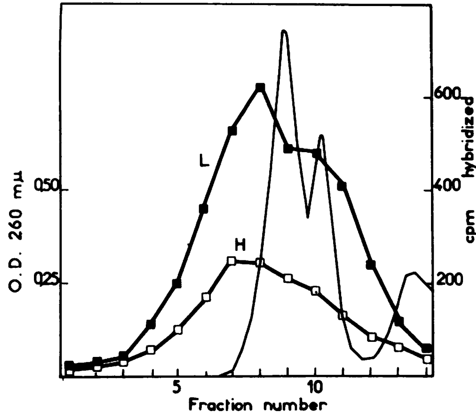

FIG. 1.—Sedimentation pattern of λ_{857} sus R_5 early mRNA. 400 μg of RNA extracted from a culture of W 3350 (λ_{857} sus R_5) pulse-labeled 3 min after thermoinduction was sedimented in a 4.5-ml sucrose gradient. Specific activity of the RNA preparation, 3.5×10^6 cpm/mg; hybridization level with λ DNA, 4.8%; strand ratio of light/heavy, 65/35.

(■——■) Cpm hybridized with the light strand (*L*).

(□——□) Cpm hybridized with the heavy strand (*H*).

(——) OD at 260 mμ.

of 27*S*.[24] The significance of the minor peaks in the gel pattern remains unknown.

It has been possible to localize this mRNA species on the λ chromosome by using the DNA of phage $\lambda_{857}bio_{11}$, a biotin transducer deleted for genes into A, exo, and β (see Fig. 5). The different fractions of the sucrose gradient of Figure 2*a* were hybridized with λ and $\lambda_{857}bio_{11}$ DNA. It is clear (Fig. 2*a*) that the fast-sedimenting material hybridizes much less with $\lambda_{857}bio_{11}$ than with λ DNA, a fact which demonstrates that it is transcribed in great part from the DNA segment deleted in phage $\lambda_{857}bio_{11}$.

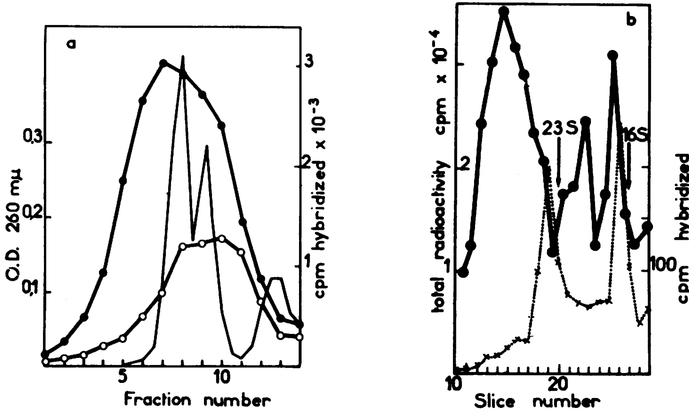

FIG. 2.—Sedimentation and electrophoretic patterns of λt_{11} mRNA. C_{600} (λt_{11}) was pulse-labeled 40 min after addition of mitomycin (2 μg/ml).

(*a*) 1 mg of the RNA preparation (spec. act., 20×10^6 cpm/mg; hybridization level with λ DNA, 4%; strand ratio of light/heavy, 97/3) was sedimented in a 25-ml sucrose gradient.

(*b*) 40 μg was submitted to a 3-hr polyacrylamide gel electrophoresis. The 4*S* bacterial RNA migrates out of the gel under these conditions.

(●——●) Cpm hybridized with λ DNA.

(○——○) Cpm hybridized with λ bio_{11} DNA.

(——) OD at 260 mμ.

(×...×) Total radioactivity.

As could be expected, the above mRNA species is not synthesized after heat induction of $\lambda_{857}bio_{11}$; as shown in Figure 3, no λ mRNA hybridizable with the light strand is found in the 30*S* region of a $\lambda_{857}bio_{11}$ early mRNA sucrose gradient. The peak observed at 15*S* might correspond to a truncated portion (one fourth) of the intact species.

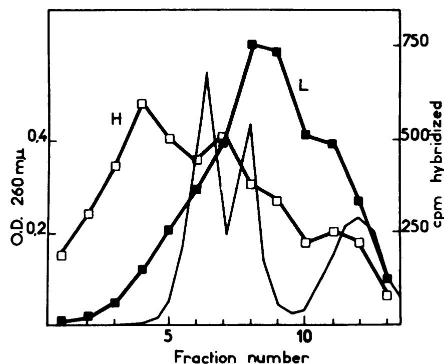

FIG. 3.—Sedimentation pattern of λ bio_{11} early mRNA. 1 mg of RNA extracted from X_{5213} ($\lambda_{857}bio_{11}$) pulse-labeled 3 min after heat induction was sedimented in a 25-ml sucrose gradient. Specific activity of the RNA preparation, 4×10^6 cpm/mg; hybridization level with λ DNA, 2.5%; strand ratio of light/heavy, 50/50.
(■——■) Cpm hybridized with the light strand (*L*).
(□——□) Cpm hybridized with the heavy strand (*H*).
(——) OD at 260 mμ.

However, $\lambda_{857}bio_{11}$ synthesizes another mRNA species with a high sedimentation constant (30–32*S*, 1.8×10^6 mol wt) transcribed from the heavy strand (Fig. 3). A molecular weight of 2×10^6 was found by electrophoresis.

Thus, there appear to be two species of "high"-molecular-weight mRNA synthesized early after induction, one transcribed from the light, the other from the heavy strand.

(*b*) *"Low"-molecular-weight mRNA species:* Under certain conditions—induction of λ_{857}sus NN, or induction of λ_{857} in the presence of high concentrations of chloramphenicol[25]—the two "high"-molecular-weight mRNA species described above are not produced, even as late as 25 minutes after induction of a λ_{857}sus NN mutant. Their absence, which allows easy detection of lower-molecular-weight species, could account for the low hybridization levels (0.4%) observed with respect to early wild-type mRNA (4%).

Similar sedimentation and electrophoresis patterns were obtained for λ_{857} induced in the presence of chloramphenicol and for λ_{857}sus NN induced either with or without chloramphenicol. Figure 4*a* shows an acrylamide gel of a λ_{857}sus N_7N_{53}RNA preparation hybridized with the two DNA strands. Three species of λ mRNA can be seen; one, which hybridizes with the heavy strand, has a mobility that indicates a molecular weight of about 900,000. The other two are transcribed from the light strand and have molecular weights of about 225,000 and 25,000, respectively. Electrophoresis on concentrated acrylamide gel (7.5%) provides a more accurate estimation of the molecular weight of the latter species (30,000), which migrates as a single and rather symmetrical peak between the 4*S* and 5*S* bacterial RNA's (Fig. 4*b*). Analogous patterns are obtained by sucrose gradient sedimentation, but the two species that hybridize with the light strand are not separated.

In contrast with the mRNA found in noninduced lysogenic cells, which does not hybridize with λi_{434} DNA,[6, 27] the three "low"-molecular-weight species

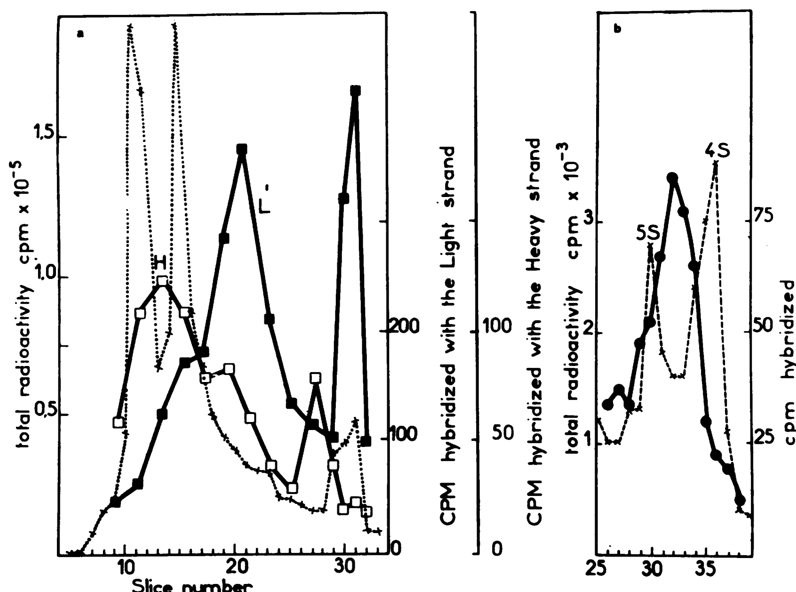

FIG. 4.—Electrophoretic patterns of λ_{857} sus N_7N_{53} mRNA. 40 μg of RNA extracted from pulse-labeled thermoinduced cells (spec. act., 20 × 10^6 cpm/mg; hybridization level, 0.40%; strand ratio of light/heavy, 70/30) was submitted to electrophoresis (*a*) on a 2.7% acrylamide gel for $1^1/_2$ hr and (*b*) on a 7.5% gel for 2 hr.

(■——■) Cpm hybridized with the light strand (*L*).
(□——□) Cpm hybridized with the heavy strand (*H*).
(●——●) Cpm hybridized with whole λ DNA.
(×...×) Total radioactivity.

described here are hybridized equally well with λ and λi_{434} DNA. Their template must therefore be outside the immunity region.

Discussion.—In this report, we have described five species of λ mRNA synthesized early after induction of various λ prophages. Appropriate controls, which show that the techniques used for the characterization of these mRNA's are reasonably reliable, will be published elsewhere. The results are summarized in Table 1. The three mRNA species hybridizing with the light DNA strand have been called l_1, l_2, and l_3, whereas the two species hybridizing with the heavy strand have been designated as h_1 and h_2.

No attempt to detect "low"-molecular-weight species under conditions where those of "high" molecular-weight are synthesized has been undertaken so far, because it would be difficult to distinguish unambiguously the former species (l_1, l_2, h_1) from the degradation products and growing chains of the latter species (l_3, h_2). However, the "low"-molecular-weight species are present in early λ_{857} RNA, as judged by competition experiments,[12] and we assume that their appearance corresponds to a normal early stage of development, rather than to an abnormal physiological condition due to the N-mutation or to the addition of chloramphenicol.

The five species described here are not found in the uninduced lysogens.[27] The possibility that more than five λ mRNA species are synthesized early after induc-

TABLE 1. *The different early λmRNA species.*

mRNA species	DNA strand with which they hybridize	Mol Wt Estimated by: Sedimentation	Electrophoresis
l_1	Light strand	—	30,000
l_2	Light strand	—	2.25×10^5
l_3	Light strand	1.8×10^6	1.7×10^6
h_1	Heavy strand	0.85×10^6	0.9×10^6
h_2	Heavy strand	1.8×10^6	2×10^6

Conditions in Which These mRNA Species Have Been Characterized

mRNA species	λt_{11}	$\lambda_{857}bio_{11}$	λ_{857}	λ_{857} + CAP*	$\lambda_{857}susN_7N_{53}$	$\lambda_{857}susN_3N_{53}$ + CAP*
l_1	−	−	+†	+	+	+
l_2	−	−	+†	+	+	+
l_3	+	−	+	0	0	0
h_1	0	−	+†	+	+	+
h_2	0	+	+	0	0	0

The symbols used are: (+) present; (0) absent; (−) not tested.
* CAP stands for chloramphenicol.
† Presence inferred from competition experiments.[14]

tion cannot be excluded. However, if the molecular weights given in Table 1 are not biased by systematic degradation, and if the different species do not overlap, they cover at least two thirds of the right half of the λ physical map. Since it is known that some late mRNA (which presumably corresponds to the lysozyme gene[12]) is transcribed from the right half[3] and that, on the other hand, the b_2 region does not seem to be transcribed early after induction,[12] it seems unlikely that other early mRNA species of *high* molecular weight could have escaped our notice.

If mRNA of high molecular weight is polycistronic, as is generally assumed, then species l_3, h_2, and h_1 correspond to *large* operons. Species l_3 has been shown to be the transcription product of at least some of the genes deleted in λbio_{11}: the int A, exo, and β genes which probably are part of a large operon, transcribed from right to left on the left side of C_I (see Fig. 5). Although no direct evidence is presented here, it is possible to make some reasonable predictions on the localization of the other four species. Increasing evidence indicates[28] that, after inactivation of the repressor, transcription starts from both sides of C_I on each of the DNA strands. If this is so, h_1 might correspond to

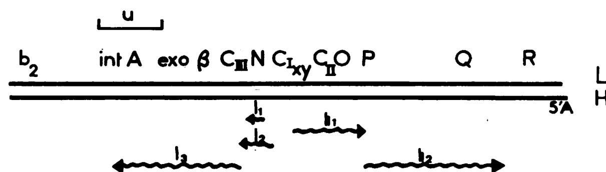

FIG. 5.—Right half of the vegetative map of λ chromosome. The length unit on the figure corresponds to 3000 base pairs (equivalent molecular weight of mRNA, 1×10^6). Genes N, C_I, O, P, and Q have been positioned according to Hogness.[32] The various mRNA species have been drawn to scale. Their direction of transcription and their tentative localization are also indicated. Species l_1 and l_2 are not supposed to overlap; their order is not known.

genes C_{II}, O (and P ?), which are known to be functional in sus N mutants,[29] while l_1 and/or l_2 might correspond to gene N (and/or C_{III}). It seems likely then that h_2 is the transcription product of the region of gene Q.†

Transcription of the three "low"-molecular-weight species (l_1, l_2, h_1) appears to be directly controlled by the λ repressor since they are the only ones synthesized in the presence of chloramphenicol. How three species can be directly controlled by the C_I product remains an unanswered question. It appears that protein synthesis is required for the transcription of the two "high"-molecular-weight species (l_3 and h_2). The fact that they are not synthesized by sus N mutants strongly suggests that protein N is required. The product of gene N therefore controls the expression not only of a group of genes to the left of C_I, as has been found for exonuclease and β protein (species l_3),[30, 31] but also of some other genes, which are presumably localized to the right of C_I and might include gene Q (species h_2).

Although protein N is required for the transcription of species l_3 and h_2, it appears not to be sufficient. If it were, x mutants like λt_{11}, which are able to complement N,[20] should synthesize species h_2, even if they fail to synthesize h_1. On the other hand, it has been shown by one of us[13] that exonuclease synthesis is transinducible in heteroimmune superinfection *only* when the repressor is inactivated. These two observations suggest that, in addition to the N control, there may be another kind of early control which has a *cis* effect at the level of transcription: such an effect would prevent transcription of species l_3 and h_2 unless species l_1, l_2, and h_1 have been *previously* transcribed.

The nature and function of species l_1 are still unknown. Whether or not it is a λ-specific transfer RNA is being studied.

Summary—Five species of early λ mRNA have been identified on the basis of their molecular weights, determined by zone sedimentation or gel electrophoresis, and their direction of transcription, inferred from the strand with which they hybridize. Three species, l_1, l_2, and l_3 (approximate molecular weights of 3×10^4, 2.25×10^5, and 1.7×10^6, respectively), are transcribed from the light strand; the other two, h_1 and h_2 (molecular weights of 0.9×10^6 and 2×10^6), are transcribed from the heavy λ DNA strand. Evidence for the localization of l_3 on the left of C_I in the β-exonuclease-integrase region has been obtained, and a tentative localization of the templates of the other species on the λ chromosome is proposed. Transcription of h_1, l_1, and l_2 is under direct control of the repressor. Transcription of the two high-molecular-weight species, l_3 and h_2, most probably requires synthesis of protein N.

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The abbreviations used are: mRNA, messenger RNA; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; SSC, 0.15 *M* NaCl, 0.015 *M* Na citrate.

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† *Note added in proof:* The proposed locations of these different mRNA species have been confirmed by hybridization with DNA of appropriate deletion mutants.

[1] Dove, W., *Ann. Rev. Genet.*, **2** (1968).
[2] Naono, S., and F. Gros, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966), p. 363.
[3] Skalka, A., these PROCEEDINGS, **55** 1190 (1966).
[4] Skalka, A., B. Butler, and H. Echols, these PROCEEDINGS, **58**, 576 (1967).
[5] Cohen, S. N., and J. Hurwitz, these PROCEEDINGS, **57**, 1759 (1967).
[6] Taylor, K., Z. Hradecna, and W. Szybalski, these PROCEEDINGS, **57**, 1618 (1967).
[7] Sheldrick, P., manuscript in preparation.
[8] Kourislky, P., and D. Luzzati, *J. Mol. Biol.*, **25**, 357 (1967).
[9] Kourilsky, P., D. Luzzati, and F. Gros, *Compt. Rend.*, **265**, 89 (1967).
[10] Sussman, R., and F. Jacob, *Compt. Rend.*, **254**, 1517 (1962).
[11] Luzzati, D., manuscript in preparation.
[12] Kourislky, P., unpublished data.
[13] Naono, S., and F. Gros, *J. Mol. Biol.*, **25**, 517 (1967).
[14] Lederman, R., *Virology*, **30**, 341 (1966); Salser, W., personal communication.
[15] Kaiser, A. D., and D. S. Hogness, *J. Mol. Biol.*, **2**, 392 (1960).
[16] Hradecna, Z., and W. Szybalski, *Virology*, **32**, 633 (1967).
[17] Loening, U. E., *Biochem. J.*, **102**, 251 (1967).
[18] Weinberg, R. A., U. E. Loening, M. Willems, and S. Penman, these PROCEEDINGS, **58**, 1088 (1967).
[19] Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).
[20] Eisen, H. A., C. R. Fuerst, L. Siminovitch, R. Thomas, L. Lambert, L. Pereira da Silva, and F. Jacob, *Virology*, **30**, 224 (1966).
[21] Radding, C. M., *J. Mol. Biol.*, **18**, 251 (1966).
[22] Brachet, P., personal communication.
[23] Bishop, D. H. L., J. L. Claybook, and S. Spiegelman, *J. Mol. Biol.*, **26**, 373 (1967).
[24] Kurland, C. G., *J. Mol. Biol.*, **2**, 83 (1960).
[25] Chloramphenicol was added 5 min before heating, either at a concentration of 100 μg/ml or 500 μg/ml. In the first case, the cells were labeled early after thermoinduction (2–3 min); in the second case, the cells were labeled later (15–16 min). If cells induced in the presence of 100 μg/ml are labeled late (15–16 min), significant amounts of "high"-molecular-weight RNA are detected:[8] one of the two "high"-molecular-weight species is then predominantly produced.[9] With lower concentrations of chloramphenicol (30–100 μg/ml), one can even observe all the early mRNA species, as judged by competition experiments.[12] The action of this drug on λ development therefore seems to be time- and concentration-dependent, as was already noticed for transcription[4] and replication.[26]
[26] Young, E. T., and R. L. Sinsheimer, *J. Mol. Biol.*, **30**, 165 (1967).
[27] Kourislky, P., manuscript in preparation.
[28] Ptashne, M., and N. Hopkins, these PROCEEDINGS, **60**, 1282 (1968); Szybalski, W., personal communication.
[29] Eisen, H. E., L. Pereira da Silva, and F. Jacob, personal communication.
[30] Protass, S. S., and D. Korn, these PROCEEDINGS, **55**, 1089 (1966).
[31] Radding, C. M., and H. Echols, these PROCEEDINGS, **60**, 707 (1968).
[32] Hogness, D., *J. Gen. Physiol.*, **49**, 29 (1966).