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GENETIC TRANSCRIPTION DIRECTED BY THE b_2 *REGION OF* λ *BACTERIOPHAGE**

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It has been well established that, in phages, some genes are transcribed at different times than others.[1,2] The analysis of transcriptional regions in phage chromosomes has been most successful in phage λ. Such experiments essentially were based on the isolation of left and right halves of λ DNA[2] and upon the separation of the complementary DNA strands.[3] However, as a consequence of the technical limitations involved, these previous studies did not include the analysis of transcription directed by the central part of the λ chromosome—namely, the b_2 region which occupies 18 per cent of the total chromosome[4] and is located between the early and late gene clusters.[5]

The b_2 region is "mutationally silent" with respect to vegetative growth. In fact, the only phenotypic trait accompanying b_2 deletion is the inability of the mutant phage to integrate into the host chromosome, thus causing abortive lysogenization.[4,6] Campbell has shown that λb_2 is missing a region required for physical recognition of the proper site in the host DNA.[7] However, this did not exclude the possibility that the b_2 region could code for enzyme(s) required for stable lysogenization or, alternatively, that the region might contain some other genes that are not obligatory for the lytic cycle. It is in these contexts that we became interested in studying transcriptional activity of the b_2 region.

Materials and Methods.—Phage, bacteria, and media: The stocks of phages λc_{71} and $\lambda b_2 c_I$ (both received from Dr. J. Weigle) were prepared on *E. coli* W3110 in K medium. The stock of $\lambda susN_7 c_{I,90}$ (from Dr. H. Echols) was prepared on *E. coli* C600 (thr^- leu^- B_1^-) in K medium supplemented with 1 μg/ml of thiamine. Phage was purified by two cycles of differential centrifugations and a treatment with pancreatic DNase (Worthington Biochemical) followed by two equilibrium zonal centrifugations in a preformed CsCl gradient. For preparation of the P^{32}-labeled phage, the phosphate concentration in K medium was reduced to 10^{-3} *M* (5 μc/ml) and tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), pH 7.2, was added to a final concentration of 0.1 *M*. H^3 thymidine was used, (0.5 mc/mg/l) to prepare H^3-labeled phage.

Buffers: The following were used: TM buffer (Tris-HCl, pH 7.3, 0.02 *M;* $MgSO_4$, 0.1 *M*), TS (0.1) buffer (Tris-HCl, pH 7.3, 0.02 *M;* NaCl, 0.1 *M*), TS (0.01) buffer (Tris-HCl, pH 7.3, 0.02 *M;* NaCl, 0.01 *M*), BS buffer ($Na_2B_4O_7$, pH 9.5, 0.03 *M;* Na_2SO_4, 0.1 *M*), SSC buffer (NaCl, 0.15 *M;* Na-citrate, 0.015 *M*), and TE buffer (Tris-HCl, pH 8.5, 0.005 *M;* EDTA, 0.001 *M*).

DNA extraction: Phage DNA was prepared by the method of Mandell and Hershey.[8] DNA's isolated from λc_{71} phage and $\lambda b_2 c_I$ phage are designated as λ^+ DNA and λb_2 DNA, respectively.

Preparation of DNA fraction rich in b_2 *region:* A 30-ml solution of λ^+ DNA at 50 μg/ml in BS buffer was stirred in a shearing apparatus (as described by Hogness and Simmons[9]) at a speed of 3950 rpm for 5 hr at 4°C. (The average relative length of the sheared DNA was 10% of a whole molecule when measured by sucrose gradient analysis using the whole λ^+ DNA as a sedimentation marker.[10]) A nitrocellulose tube for a Spinco no. 40 angle rotor[11] was filled with mineral oil and 5 ml of a mixture of 120 μg sheared DNA, $HgCl_2$[12] (0.22 mole per mole of phosphorus in DNA), and 43% (w/w) Cs_2SO_4 (Harshaw Chemical Co.) in BS buffer and spun at 36,000 rpm for 40 hr at 6°C in a

Beckman model L-2 ultracentrifuge. The fractions containing the two major bands were separately pooled, concentrated by flash evaporation, and dialyzed against 0.1 *M* EDTA, pH 8 and then against TS buffer. These DNA fragments were used in hybridization experiments as the AT-rich half DNA (the heavier major band) and the GC-rich half DNA (the lighter major band). The fractions containing the heaviest minor band were also pooled, concentrated to 43% (w/w) in Cs_2SO_4, and again spun in a no. 40 rotor. The fractions containing the main band were pooled (see Fig. 1*b*). This DNA solution is called M DNA.[25]

Isolation of complementary strands of λ *DNA:* The complementary strands of λ^+ DNA were isolated by the procedures described by Hradecna and Szybalski.[13] The strand which forms the heavier band in CsCl when combined with poly G is called the H strand; the lighter band is called the L strand.

RNA preparation: *E. coli* W3110 grown to 2.5×10^8 cells/ml in 20 ml of K medium were collected by a low-speed centrifugation, washed twice in TM buffer, and resuspended in the same buffer at 1×10^{10} cells/ml. Phage was then added at moi = 5, and the mixture was incubated without aeration at 37°C for 20 min. Infection was started by diluting the *E. coli*–phage complex tenfold with 10 ml of prewarmed K medium. Incubations were carried out at 37°C with aeration. The latent period under these conditions was approximately 40 min. At the desired time, the culture was pulsed for 2 min with 200 μc of H^3 uridine (New England Nuclear Corp., 1 mc/10 μg) and then poured onto crushed ice containing 0.01 *M* NaN_3. RNA extraction was carried out according to Sagik *et al.*[14] RNA's isolated from λc_{71}-infected cells and $\lambda b_2 c_I$-infected cells are designated as λ^+ RNA and λb_2 RNA, respectively.

RNA-DNA hybridization: RNA-DNA hybridization experiments were performed according to the method of Gillespie and Spiegelman.[15] The two-step hybridization technique was originally suggested by Skalka *et al.*[16] The procedures employed in our work were as follows: To enrich the H^3-labeled RNA in b_2 RNA, 20–50 μg of M DNA were fixed on a Millipore membrane filter (HAWP, 24 mm), and the annealing was carried out in 2 × SSC for 12 hr at 65°C. The ratio of the input amount of RNA to the denatured DNA was kept constant, except in the case specifically noted, and DNA was in excess. After the annealing, the filter was extensively washed with 2 × SSC and a portion (4 mm in diameter) was cut from the center region in order to directly measure the counts in the hybrid. The major portion of RNA in the hybrid was eluted from the remaining "doughnut" by being heated at 100°C for 15 min in 1/100 × SSC and rapidly cooled to 0°C.[16] (The efficiency of the elution of RNA was 80–95%.) The DNA that was partially detached from the filter during heating was digested by being treated with 10 μg/ml of pancreatic DNase at 37°C for 30 min in 0.01 *M* $MgCl_2$. The DNase was inactivated by being heated at 95°C for 10 min, and $MgCl_2$ was removed by dialysis against 2 × SSC. These H^3-RNA samples, enriched in b_2 RNA by hybridization with and elution from M DNA, are called M RNA.[26] These RNA's were hybridized either with excess of λ^+ DNA or with λb_2 DNA (the second hybridization). The difference of the maximal hybridizable counts was taken as b_2 RNA. The hybridization "noise" was measured for each set of experiments with a minus DNA filter. Results expressed are after noise subtraction.

Synthesis and purification of RNA in vitro: The purification procedure for the *E. coli* C122–DNA-dependent RNA polymerase was that of Chamberlin and Berg.[17] The ingredients of the reaction mixture and the purification procedures for the synthesized RNA were as previously described.[18] The ratio of the amount of the template DNA employed to that of the enzyme was such that DNA was in two- to threefold excess of that amount required for maximum incorporation.

Results.—*Isolation of the* b_2 *region of* λ *DNA (M DNA):* A mixture of H^3-(thymidine)-labeled λ^+ DNA and P^{32}-labeled λb_2 DNA was sheared to produce approximately one-tenth fragments. After being complexed with $HgCl_2$, the DNA fragments were banded in a Cs_2SO_4 density gradient. The H^3 and P^{32} profiles are shown in Figure 1*a*. As reported earlier by Skalka *et al.*,[19] H^3 counts

in λ^+ DNA are distributed in two major bands and in one denser, minor band. It has been shown that the two major bands, heavy (AT-rich) and light (GC-rich), are composed of fragments from the right and left half of the λ DNA molecule,[19] respectively. It also has been shown that the minor band, the lowest in GC content, originates from the middle portion of the λ DNA.[19] The distribution of P^{32} counts (λb_2 DNA) is completely superimposed on that of H^3 counts (λ^+ DNA), except in the minor band where almost no P^{32} counts are seen. Therefore, this minor band must be composed of the b_2 region. When the fractions corresponding to the shaded area in Figure 1*a* were pooled and rebanded in a Cs_2SO_4 gradient (Fig. 1*b*), a single band with a small shoulder on the lighter side of the gradient was obtained. Fractions indicated by the shaded area in Figure 1*b* were pooled, and DNA fragments thus prepared are called M DNA.

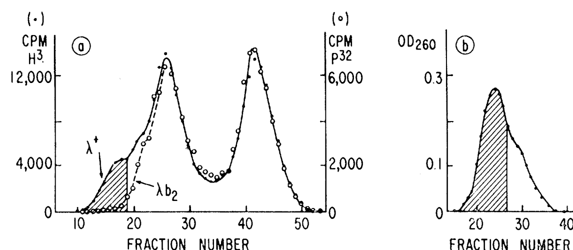

FIG. 1.—(*a*) Cs_2SO_4 density gradient profiles of mercuric complexes of one-tenth fragments of λ^+ and λb_2 DNA. A mixture (1:7 on weight basis) of P^{32}-labeled λb_2 DNA and H^3-thymidine-labeled λ^+ DNA was sheared to approximately one-tenth fragments, then complexed with $HgCl_2$ (0.2 mole per mole of phosphate in DNA), and spun as described in *Materials and Methods*. H^3 counts were corrected for the heterogeneity in GC content along the density gradient assuming that the GC content is 38% for fractions 10–19, 45% for fractions 20–35, and 57% cent for fractions 36–53.[19] (*b*) Enrichment of b_2-region fragments by second banding in Cs_2SO_4. The fractions corresponding to the shaded area in (*a*) were pooled, concentrated, and rebanded in Cs_2SO_4.

Synthesis of b_2 RNA in vivo and in vitro: To be used directly in RNA-DNA hybridization experiments for assaying the b_2 RNA synthesis, the M DNA preparation must be highly pure in b_2 DNA. In particular, if the contamination is restricted to a specific region of λ DNA other than the b_2 region, the hybridization experiments could give misleading results. In order to test the purity of the M DNA preparation, the levels of hybridization to M DNA were compared between H^3 RNA isolated from λb_2c infected cells and those isolated from λc_{71} infected cells. As shown in Table 1, a considerable fraction of λb_2 RNA hybridizes with M DNA, indicating contamination. To overcome this problem, the two-step hybridization method described in *Materials and Methods* was employed in assaying b_2 RNA synthesis.

In Table 1, the kinetics of the b_2 RNA synthesis are shown as well as those of the RNA syntheses directed by the right (AT-rich) and left (GC-rich) halves of λ DNA. The kinetics of RNA synthesis on the AT- or GC-rich half are similar to those reported previously by others[2] and are shown here for comparison with the b_2 RNA synthesis. Early synthesis (2–4 min after infection) of b_2 RNA is at

about one-tenth the rate of the AT half RNA. The rate of b_2 RNA synthesis decreases by more than one half between 3 and 14 minutes and then gradually increases as the total rate of the λ RNA synthesis increases. On the basis of template size (see below), the ratio of the rates of RNA syntheses in the three regions of λ DNA (the AT half, the b_2 region, and the GC half) is 12.0:3.8:1.2 at three minutes after infection and 4.7:2.8:12.0 at 37 minutes. (The ratio of the amounts of nucleotide bases in the AT half, the b_2 region, and the GC half was calculated from Fig. 1*a* as 45:13:42.) Table 1 also contains the results of RNA synthesized *in vitro* on λ^+ DNA by *E. coli*–DNA-dependent RNA polymerase. In this case, the ratio on the basis of template size is 4.2:4.0:0.73.

TABLE 1. *Synthesis of b_2 RNA.*

H^3 RNA Sample		Denatured DNA Fixed on Filters						
Host-phage	Pulse (min after infection)	None	λ^+ whole (10γ)	AT half (3γ)	GC half (3γ)	M (1γ)	$\frac{\lambda^+ - \lambda b_2}{\lambda^+}$ M (%)	b_2 RNA (% of input)
		(% of input)						
W3110-c_{71}	2–4	0.02	2.83	2.65	0.25	0.99	25	0.25
W3110-c_{71}	4–6	0.04	2.61	2.45	0.40	—	—	—
W3110-c_{71}	13–15	0.02	2.48	1.55	1.12	0.33	30	0.10
W3110-c_{71}	27–29	0.02	5.02	2.15	2.82	0.50	45	0.23
W3110-c_{71}	36–38	0.04	8.04	2.10	5.01	0.80	45	0.36
W3110	2	0.02	0.20	0.12	0.08	0.12	—	—
In vitro RNA on λ^+ DNA		0.28	25.0	19.0	3.1	8.2	63	5.2
W3110-b_2c_I	2–4	0.02	2.64	2.40	0.24	0.99	—	—
W3110-b_2c_I	36–38	0.01	6.93	1.01	5.88	0.24	—	—

In vivo H^3 RNA samples were prepared from λc_{71}- or λb_2c_I-infected W3110 or uninfected W3110 as described in *Materials and Methods*. The incubation mixture for the *in vitro* RNA synthesis contained in 2 ml, 200 μg of λ^+ DNA and 160 μg of enzyme (DNA excess). The incubation was carried out at 37°C for 10 min. Hybridization reactions were carried out in 2 × SSC at 65°C for 6 hr in a total volume of 0.15 ml. The amount of denatured DNA fixed on filters was well in excess. $(\lambda^+ - \lambda b_2)/\lambda^+$ refers to the relative difference in the saturation levels in the second step of hybridization. The hybridization level of b_2 RNA in the original H^3 RNA sample was calculated by multiplying the per cent of counts hybridized to M DNA by $(\lambda^+ - \lambda b_2)/\lambda^+$ and listed in the final column. All numbers are an average of at least two hybridization experiments of two different sets of RNA samples.

Two examples of the saturation curves obtained in the second hybridization step are shown in Figure 2*a* and *b*. These were used in Table 1 in order to calculate the levels of the b_2 RNA synthesis. Figure 2*c*, which serves as a control for Figure 2*a* and *b*, shows the saturation curves of RNA synthesized *in vitro* on λb_2 DNA. The difference in the saturation levels obtained by λ^+ DNA and λb_2 DNA in Figure 2*a* and *b* is due to b_2 RNA. This is independently confirmed by the competition experiments shown in Figure 3. Excess unlabeled RNA isolated from λb_2c-infected cells was used to prevent λ RNA (except b_2 RNA) from hybridizing with λ^+ DNA. Figure 3 shows that, with both H^3-early M RNA (2–4 min)[20] and H^3-late M RNA (36–38 min), 50 per cent of the H^3 counts can not be competed for by unlabeled λb_2 RNA. Figure 3 also shows that these noncompetitive fractions of the H^3 RNA are now effectively excluded from the hybrid upon the addition of unlabeled RNA isolated from λc_{71}-infected cells. This supports the fact that the plateaus obtained in the presence of excess unlabeled λb_2 RNA are due to b_2 RNA. When λb_2 DNA was used instead of λ^+

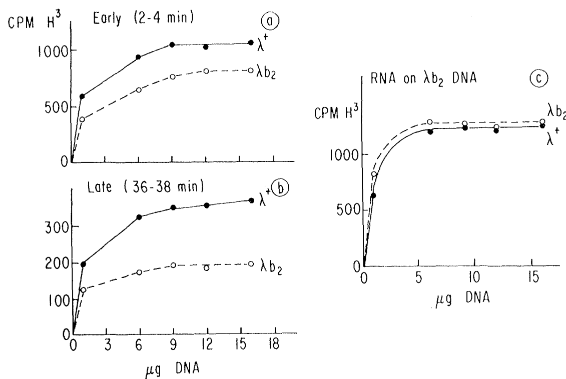

FIG. 2.—Saturation curves by λ^+ and λb_2 DNA. The M RNA samples described in Table 1 were hybridized with varying amounts of denatured λ^+ or λb_2 DNA: (*a*) 2–4 min RNA, input counts 2660 cpm; (*b*) 36–38 min RNA, input counts 7050 cpm; (*c*) H^3 RNA synthesized (10 min at 37°C) by *E. coli* RNA polymerase on whole λb_2 DNA was also hybridized with λ^+ or λb_2 DNA; input counts 3895 cpm.

DNA as a control, H^3 RNA in the hybrid was completely excluded from the hybrid by the excess amount of unlabeled λb_2 RNA.

Analysis of template strand of b_2 RNA: To determine the template strand in the b_2 region, competition experiments were performed with the isolated strands of λ^+ DNA. The results are shown in Figure 4. Early M RNA hybridizes almost exclusively (99%) with L strand (3209 cpm vs. 40 cpm with H strand) even in the absence of the competitor, unlabeled λb_2 RNA. Since 50 per cent of early M RNA is b_2 RNA (see Figs. 3*a* and 4*a*), this indicates that only L strand is the active template for the synthesis of early b_2 RNA. The competition experiments with L strand DNA (Fig. 4*a*) confirm this conclusion, since the plateau value was near 50 per cent. In contrast, late M RNA hybridizes both with L strand (290 cpm) and H strand (508 cpm) in the absence of unlabeled λb_2 RNA, and the competition experiments reveal that approximately 40 per cent of each are b_2-specific RNA (Fig. 4*b*). Therefore, on the actual count basis, 64 per cent of the late b_2 RNA is synthesized on H strand and 36 per cent on L strand. In all the above cases, additions of unlabeled λ^+ RNA reduce the counts in the plateaus, confirming the conclusion that the plateaus are due to b_2 RNA.

Figure 5 shows the results of experiments which were intended to find any qualitative difference between early b_2 RNA and late b_2 RNA, both synthesized on L strand. The experiments are similar to those for Figure 4 except that unlabeled λ^+ RNA, isolated early (4 min) or late (40 min) after infection, was separately added to the annealing mixture in the presence of excess unlabeled λb_2 RNA. As seen in Figure 5*a*, unlabeled λ^+ late RNA is a poor competitor for early λb_2 RNA, i.e., out of the 40 per cent of the plateau level obtained on addition of excess unlabeled λb_2 RNA, at least one half is not competed for by λ^+ late RNA. This, together with other controls presented in Figure 5, demonstrates that the synthesis of approximately 50 per cent of the early b_2 RNA is turned off during late transcription. The late L strand b_2 RNA is synthesized at early times also, as demonstrated by the complete elimination of late H^3 RNA from the plateau on the addition of unlabeled early λ^+ RNA (Fig. 5*b*).

Effect of the gene N on the synthesis of the early b_2 RNA: It has been reported that gene *N* controls at least part of the early transcription.[16] The effect of gene *N* on the synthesis of early b_2 RNA was studied by comparing the synthesis of b_2

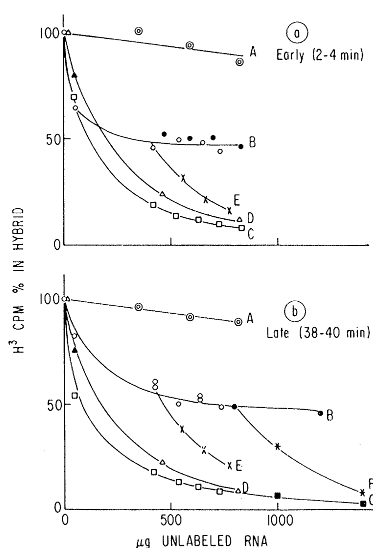

FIG. 3.—Competition experiments with M RNA. The M RNA samples similar to those described in Fig. 2*a* and *b* were hybridized with λ^+ or λb_2 DNA (2 μg) in the presence of unlabeled RNA. To prepare unlabeled λ^+ or λb_2 RNA, the infected W3110 cells were harvested in an equal volume at 3, 6, 10, 25, 35, and 40 min after infection, and the combined six portions were subjected to RNA isolation. *A*, λ^+ DNA-*E. coli* RNA; *B*, λ^+ DNA-λb_2 RNA; *C*, λb_2 DNA-λb_2 RNA; *D*, λ^+ DNA-λ^+ RNA; *E*, λ^+ DNA-λb_2 RNA 421 μg + λ^+ RNA; *F*, λ^+ DNA-λb_2 RNA 800 μg + λ^+ RNA.

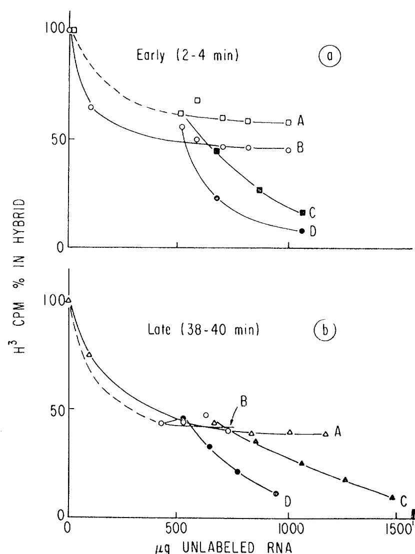

FIG. 4.—Competition experiments with λ DNA strands and M RNA. The M RNA samples used in Fig. 3 were hybridized with λ^+ DNA (2 μg), H strand (1 μg), or L strand (1 μg) in the presence of unlabeled RNA.

(*a*) *A*, L strand-λb_2 RNA; *B*, λ^+ DNA-λb_2 RNA; *C*, L strand-λb_2 RNA 465 μg + λ^+ RNA; *D*, λ^+ DNA-λb_2 RNA 465 μg + λ^+ RNA.

(*b*) *A*, H strand-λb_2 RNA; *B*, L strand-λb_2 RNA; *C*, H strand-λb_2 RNA 670 μg + λ^+ RNA; *D*, L strand-λb_2 RNA 421 μg + λ^+ RNA.

RNA between the λsusNc_I-infected permissive host C600 and the λsusNc_I-infected restrictive host W3110. As shown in Table 2, in the restrictive host W3110, the total early λ messenger RNA synthesis was reduced by 40 per cent, as reported by others.[16] In the same host there was no detectable b_2 RNA synthesis at either one to three or three to five minutes after infection.

Discussion.—Using the isolated left and right halves of λ DNA, Skalka[2] studied the temporal pattern of λ RNA synthesis. Later, Taylor *et al.*[3] analyzed the template strand as a function of time after infection and came to a conclusion that there were three separate regions of transcription in a molecule of λ DNA. In these works, however, the study of the transcription directed by the b_2 region was not included.

The results presented in this paper prove that the b_2 regions of λ DNA is physiologically functional, i.e., directs the synthesis of RNA in the lytic cycle of λ development. They further provide evidence that the transcriptional control of early-to-late transition exists even within the b_2 region. b_2 RNA can be grouped into three classes from the present work: class 1 transcribed mostly early

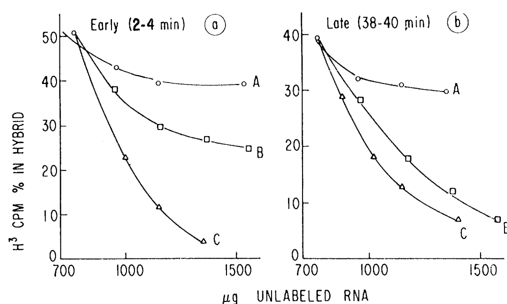

FIG. 5.—Competition experiment with L strand. H^3-labeled M RNA and unlabeled λb_2 RNA were prepared as described in the legend to Fig. 3. Early and late unlabeled λ^+ RNA were prepared from λc_{71}-infected cells harvested at 4 min and 40 min, respectively, after infection. The total volume of the annealing mixture, 0.21 ml; L strand DNA fixed on a filter, 1 μg; H^3 counts hybridized to DNA in the absence of unlabeled RNA were taken as 100%. *A*, λb_2 RNA; *B*, λb_2 RNA 770 μg + λ^+ late RNA; *C*, λb_2 RNA 770 μg + λ^+ early RNA.

on L strand, class 2 transcribed early and late on L strand, and class 3 transcribed mostly late on H strand.

The assay for b_2 RNA synthesis is dependent on the isolation of λ DNA fragments enriched in the b_2 region and the use of two-step hybridization. The former is a prerequisite for the quantitative assay because the direct use of the H^3-uridine-pulsed RNA, in which a small proportion is b_2 RNA, would not result in a detectable difference in the saturation levels between λ^+ and λb_2 DNA, nor in the plateau levels obtained in the competition experiments.

According to the current view[21] on the mechanism of the integration of λ DNA into the host chromosome, there may exist within the b_2 region at least a part of the section homologous to the host chromosome. This raises a question on the origin of the detected b_2 RNA: is it from the phage or the bacteria? The possibility that the detected b_2 RNA is synthesized on the homologous region of the host chromosome seems excluded by the results reported in Figure 3 which shows that RNA able to compete with b_2 RNA exists only in λ^+ phage-infected cells but not in λb_2 phage-infected cells.

The function(s) of b_2 RNA is unknown. It is not obligatorily required for the vegetative growth of λ phage because, as far as phage production is concerned, λb_2 mutant shows no defectiveness[4, 6] and is subjected to the early and late switch of transcription.[22] At least two λ-directed protein products are known to be dispensable for the development of λ phage: a λ specific nuclease and β protein. These, however, have been mapped between gene c_{III} and the b_2 region and out-

TABLE 2. *Effect of gene N on the synthesis of early b_2 RNA.*

H^3 RNA Sample: Host	Pulse (min after infection)	Denatured DNA Fixed on Filters: None	λ^+ whole (10γ)	AT half (3γ)	GC half (3γ)	M (1γ)	$\frac{\lambda^+ - \lambda b_2}{\lambda^+}$ (%)	b_2 RNA (% of input)
		(% of input)						
C600	1–3	0.03	3.10	2.64	0.27	0.77	23	0.18
C600	3–5	0.04	2.85	2.14	0.37	0.88	15	0.13
W3110	1–3	0.03	1.70	1.66	0.25	0.76	<5	<0.04
W3110	3–5	0.03	1.51	1.32	0.22	0.53	<5	<0.03

H^3 RNA samples were prepared from $\lambda susN_7c_{I,90}$-infected C600 or W3110. The level of b_2 RNA synthesis was calculated as described in Table 1.

side of the latter.[23] It is of interest that the synthesis of both these dispensable proteins[23] and early b_2 RNA is controlled by gene *N*.[24] It may be that undiscovered genes are located within the b_2 region.

Summary.—A fraction of λ DNA fragments rich in the b_2 region was isolated and used to assay the RNA synthesis directed by this region. b_2 RNA is synthesized both early and late in the λ lytic development. The synthesis of early b_2 RNA occurs on the light strand only, whereas both strands are used for the synthesis of late b_2 RNA. Gene *N* controls the synthesis of early b_2 RNA. b_2 RNA is also synthesized *in vitro* on λ DNA by purified *E. coli* DNA-dependent RNA polymerase.

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[25] This fraction of λ DNA is called Middle DNA (M DNA) because it has been shown in ref. 19 that this fraction comes from the middle section of the λ DNA molecule.

[26] M RNA is operationally defined as RNA eluted from the M DNA filter which was pre-annealed with any RNA sample.