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NUCLEASE ACTIVITY IN DEFECTIVE LYSOGENS OF PHAGE λ, II. A HYPERACTIVE MUTANT

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Defective lysogens are bacterial cells bearing mutant, defective prophages; they offer an opportunity to study lethal mutations of the bacteriophage whose genome can be propagated as prophage along with the host genome but is unable to lead to the production of mature phage particles.[1] Jacob *et al.*,[2, 3] and Brooks,[4] studying defective lysogens of λ, have described and characterized mutants falling into two classes: (1) early mutants which are blocked prior to vegetative replication of the phage genome, and (2) late mutants which are blocked in some subsequent maturative function. To explore biochemical aspects of early mutants, we have examined defective lysogens for the increase in nuclease activity associated with induction of lysogens of phage λ.[5] Two altered biochemical phenotypes have thus been found: (1) one which is characterized by a deficiency in nuclease activity

after induction, i.e., a failure to show an increase of the same magnitude as shown by a wild-type lysogen, K12(λ^+),[6] and (2) one which is characterized by a tenfold greater increase in activity than is shown by the wild type.[7] The latter was found among some defective lysogens which were generously sent to us by Dr. François Jacob, and is the subject of this report.

Materials and Methods.—(a) *Media and biochemicals:* Media were prepared as described by Kaiser and Hogness.[8] Their H medium was supplemented with 0.05% vitamin-free casamino acids (Difco), 0.05% bacto-peptone (Difco), and 0.5% glucose, and was termed H_s medium. Pancreatic DNase was purchased from Worthington Biochemical Corp., *E. coli* soluble RNA from General Biochemicals, and mitomycin C from Nutritional Biochemicals Corp. Streptonigrin was a gift of Chas. Pfizer & Co., Inc., Maywood, N. J.

(b) *Bacteriophage:* The following strains were obtained from Dr. A. D. Kaiser: λc_{71} (c_I) and λc_{68} (c_{II})[9]; λi^{434} (ref. 10); and λ^+.

(c) *Strains of E. coli K12:* (1) Sensitive strains: C600 and W3350 (cf. ref. 11). (2) Wild-type lysogens: C600(λ^+) and W3350(λ^+) were isolated from the survivors of cultures infected with λ^+ on an agar plate. (3) Defective lysogens: (*a*) T11(λ_{T11}). The lysogen was designated T11 by Dr. F. Jacob.[12] (*b*) C600(λ_{T11}), W3350(λ_{T11}). The defective λ genome, λ_{T11}, was transferred from T11(λ_{T11}) by a method similar to those of Appleyard[13] and Jacob *et al.*:[3] T11(λ_{T11}) was exposed to an inducing dose of ultraviolet light and superinfected with λc_I. The resulting lysate, which was treated with $CHCl_3$ to kill surviving bacterial cells, was assumed to contain the defective phage, λ_{T11}, in addition to λc_I, and was used to prepare new defective lysogens. C600 (λ_{T11}) and W3350(λ_{T11}), like T11(λ_{T11}), were immune to infection by λ but could not be induced to lyse, or produce plaque-forming phage with a frequency greater than 2×10^{-5} (Table 1 and Fig. 1*b*). (*c*) C600(λc_I, λ_{T11}) and W3350(λc_I, λ_{T11}). These isolates were also produced by infec-

TABLE 1

PROPERTIES OF DEFECTIVE AND WILD-TYPE LYSOGENS

Lysogen	Induction: Inducer	Induction: Lysis	Induction: Fraction induced	Relative Efficiency of Plating: λ^+	Relative Efficiency of Plating: λi^{434}
C600(λ^+)	SN	+	0.86	2×10^{-9}	0.95
	MC	+	1.2		
T11(λ_{T11})	SN	−	1.8×10^{-5}	2.5×10^{-9}	0.88
	MC	−	9.0×10^{-6}		
C600(λ_{T11})	UV	−	1.0×10^{-5}	10^{-9}	0.98
	SN	−	1.3×10^{-5}		
	MC	−	1.0×10^{-5}		
C600(λc_I, λ_{T11})	UV	+	0.81	$<10^{-9}$	0.87
	SN	+	0.79		
	MC	+	0.88		
W3350(λ^+)	MC	+	0.84	$<10^{-9}$	1.1
W3350(λ_{T11})	UV	−	1.8×10^{-5}	10^{-9}	1.2
	MC	−	1.1×10^{-5}		
W3350(λc_I, λ_{T11})	MC	+	1.0	$<10^{-9}$	1.1

The lysogens were induced as described in *Methods*. SN, streptonigrin; MC, mitomycin C; UV, ultraviolet light. Fraction induced refers to the fraction of cells which produced infective centers when plated on strain C600 of *E. coli* K12. Efficiency of plating is relative to the titer on C600.

tion with the lysate containing λc_I and λ_{T11} (cf. sect. 3*b*, above). In addition to the data shown in Table 1, the lysogens were characterized as follows: the lysates of W3350(λc_I, λ_{T11}) and C600-(λc_I, λ_{T11}) contained predominantly clear plaques, less than 0.3% turbid plaques in either. The phage forming clear plaques in both lysates were identified as c_I by failure to complement with c_I while control tests with c_{II} demonstrated complementation.[9] While the isolated plaques from the lysates were c_I, a spot of either lysate on C600 was turbid with a thin, clear halo, indicating the presence of a complementing defective phage. Ten isolated colonies from each strain were induced with mitomycin C and the lysates were tested; all isolates produced λc (clear plaques) and a complementing defective phage. The lysate of C600(λc_I, λ_{T11}) was used at low multiplicity to infect C600, and that from W3350(λc_I, λ_{T11}) to infect W3350. Immune, defective derivatives of C600 and W3350 were obtained which, phenotypically, were C600(λ_{T11}) and W3350(λ_{T11}) as

measured by hyperactivity with respect to λ nuclease (cf. *Results*). The presence of λ_{T11} in the above lysates was thus proved. By replica-plating on C600, 1–2% of colonies of C600(λc_I, λ_{T11}) and W3350(λc_I, λ_{T11}) were found to lose the ability to produce phage; those isolates were still immune to λ, indicating that λc_I was lost by segregation.

(d) *Preparation of extracts:* Cells were induced, at a density of 2×10^8/ml, by: (*a*) mitomycin C (6 μg/ml) or streptonigrin (*ca.* 6 μg/ml), in tryptone broth;[8] (*b*) ultraviolet light, in H_s medium. At intervals, 100-ml aliquots of cultures were removed and quickly chilled by pouring onto crushed ice in a 250-ml centrifuge cup. The pellets obtained by centrifugation were resuspended in 1.0 ml of a buffer at pH 7, containing 0.05 *M* glycylglycine, and 0.001 *M* glutathione; the resuspended pellets were stored at −20°. For purification of nuclease, up to 10 liters of induced cultures were chilled by pouring into stainless steel vats immersed in a bath of ethylene glycol at −30° to −40°. The cells were harvested by centrifugation and stored at −20°. Extracts of thawed 1-ml aliquots were made by sonication with four 15-sec pulses in the MSE ultrasonic disintegrator. The extracts were centrifuged at 10,000 rpm for 15 min prior to assay for protein and nuclease activity. For purification of nuclease, extracts were made with a Nossal shaker.

(e) *DNase assay:* DNase activity was assayed at pH 10 as described previously.[6] One unit of activity was defined as 10 mμmoles of nucleotide made acid-soluble in 30 min.

(f) *Purification of nuclease from T11*(λ_{T11}): λ nuclease was partially purified by modifications of the method of Korn and Weissbach.[5] Table 2 shows the results of our purification procedure which will be described in a later publication.

TABLE 2

PURIFICATION OF NUCLEASE FROM INDUCED T11(λ_{T11})

Fraction	Step	Units/ml	Units/mg	Stepwise % recovery
I	Nossal extract	9,580	463	100
II	100,000 × *g* supernatant	11,450	594	102
III	Streptomycin eluate	12,200	4,660	50.4
IV	Autolyzed streptomycin eluate	5,150	4,520	84.0
V	$(NH_4)_2SO_4$	20,080	4,770	104.0
VI	DEAE (peak fraction)	1,810	10,590	24.0
	DEAE (all fractions summed)			53.9

(g) *Viscosity measurements:* Viscosity was measured at 37° in an Ostwald-type viscometer. The reaction mixture for studying the degradation of DNA by the partially purified nuclease from T11(λ_{T11}) contained the following in a final volume of 3 ml: 10 μmoles of $MgCl_2$, 10 μmoles of 2-mercaptoethanol, 200 μmoles of an equimolar mixture of tris and 2-aminoethanol at pH 10, 0.5 μmoles of H^3-DNA from *E. coli*, containing 3.97×10^6 cpm, and 4.5 units of fraction VI (cf. Table 2). The initial specific viscosity was 0.185. The effect of pancreatic DNase on the same substrate was measured in a similar reaction mixture with the substitution of 200 μmoles of tris at pH 7.4 as the buffer. The initial specific viscosity was 0.180. In both experiments, 0.01-ml aliquots were removed from the well of the viscometer for measurement of the liberation of acid-soluble radioactivity. It had previously been determined that removal of up to 0.1 ml from the 3.0 ml of reaction mixture introduced less than 2% error in the determination of specific viscosity.

Results.—(a) *Nuclease activity of induced wild type versus T11*(λ_{T11}): The enzymatic hyperactivity of the defective lysogen, T11(λ_{T11}), is shown in Figure 1*a*. In this experiment, the specific activity of nuclease was measured in extracts of aliquots taken after treatment of various lysogens with an inducing dose of mitomycin C. Under these conditions, the wild-type lysogen, C600(λ^+), lysed at about 60 min (Fig. 1*b*). One hundred per cent of the cells were induced with an average burst of 129 phage. The defective lysogen, T11(λ_{T11}), did not lyse and less than 0.001 per cent of cells formed infective centers (cf. Table 1). Increase in nuclease activity appears to have begun at 20–30 min after induction in both strains, but the maximum rate of increase of activity was about five times as great for the

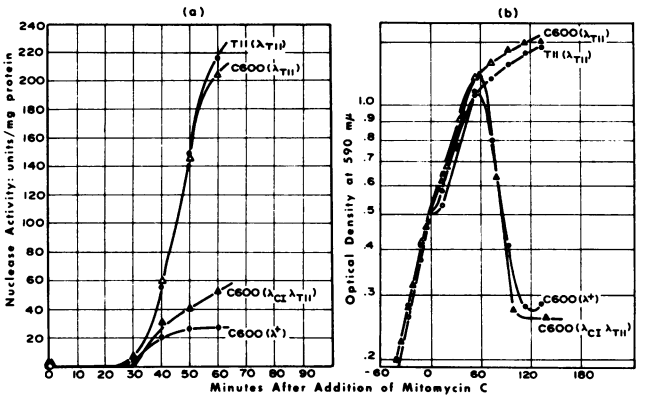

FIG. 1.—(*a*) Specific activity of nuclease versus time after induction by mitomycin C; (*b*) optical density of the cultures versus time.

mutant as for wild type. In this and other experiments, nuclease activity continued to increase in the defective lysogen as long as the optical density of the culture continued to increase, resulting in a final nuclease specific activity at least ten times greater than in the wild-type lysogen.

(b) *Association of hyperactivity with the mutated* λ *genome:* In order to relate the enzymatic hyperactivity specifically to the mutated λ genome, as opposed to some host factor such as a suppressor mutation which might have been produced in the course of the production and isolation of the defective lysogen, we have transferred the prophage from T11(λ_{T11}) to strains C600 and W3350 of *E. coli* K12, producing the lysogens C600(λ_{T11}) and W3350(λ_{T11}). Like T11(λ_{T11}), the new strain C600(λ_{T11}) did not lyse or produce phage after treatment with inducing agents (Table 1, Fig. 1*b*), and was similarly hyperactive with respect to nuclease activity (Fig. 1*a*).

(c) *Inhibition of the expression of* λ_{T11} *hyperactivity in a double lysogen:* When the lysogen C600(λc_I, λ_{T11}) was treated with mitomycin C, 88 per cent of cells produced phage and the culture lysed like a wild-type lysogen (Table 1 and Fig. 1*b*). The increase in nuclease activity was greater than in wild type, but strikingly reduced as compared to T11(λ_{T11}) and C600(λ_{T11}) (Fig. 1*a*). On the basis of rate or yield (and subtracting the expected contribution of a T11$^+$ locus to enzymatic activity), the lysogenic combination of $\lambda c_{IT11}{}^+$ and $\lambda c_I{}^+{}_{T11}$ inhibited the enzymatic hyperactivity due to the latter by about 85 per cent.

(d) *Nuclease activity in lysogenic derivatives of W3350:* The behavior of the lysogens W3350(λ_{T11}) and W3350(λc_I,λ_{T11}), with respect to nuclease activity, was similar to that observed for the corresponding lysogens of C600: the rate of increase and yield of nuclease activity for W3350(λ_{T11}) were comparable to those parameters for T11(λ_{T11}); and in W3350(λc_I, λ_{T11}), nuclease hyperactivity due to λ_{T11} was 85–95 per cent inhibited. These results provide a confirmation of the observations on the lysogenic derivatives of C600.

(e) *Nuclease activity as a phenotypic test:* Part of the proof of the structure of C600(λc_I,λ_{T11}) and W3350(λc_I,λ_{T11}) was to use lysates of induced cultures to produce "secondary derivatives" of C600 and W3350 which were immune and defective (cf. *Methods*). Since we had shown that hyperactivity of nuclease was transferred

with the λ_{T11} genome, it was possible to use hyperactivity as a phenotypic test for λ_{T11}. The "secondary derivative," C600(λ_{T11})', at 60 min after induction with mitomycin C had a nuclease specific activity of 160 units/mg as compared with a control value of 26.3 units/mg for C600(λ^+). The secondary derivative W3350-(λ_{T11})' had 130 units/mg as compared with 24.7 units/mg for W3350(λ^+).

(f) *Mixing experiment: Extracts of W3350(λ^+) and W3350(λ_{T11})*: To test for a possible stimulating substance in extracts of W3350(λ_{T11}) or an inhibitor in extracts of W3350(λ^+), the extracts were assayed individually and in mixtures. A mixture of 0.16 units of activity from W3350(λ^+) with 0.10 units from W3350-(λ_{T11}) had 0.26 units of activity; 0.16 units of the former plus 0.25 of the latter gave a mixture with 0.46 units of activity. Similar results have been obtained with extracts from W3350(λ^+) and T11(λ_{T11}).

(g) *Purification of nuclease from T11(λ_{T11})*: To investigate the specific enzymatic basis for the hyperactivity of λ_{T11}, nuclease has been purified 23-fold from T11(λ_{T11}), to a specific activity of 10,600 units/mg of protein (106 μmoles of acid-soluble nucleotide produced in 30 min/mg of protein) (Table 2). Achievement of a similar specific activity from the wild-type lysogen would require 230-fold purification.

The results of DEAE-cellulose chromatography of nuclease activity from induced T11(λ_{T11}) are shown in Figure 2. Most of the recovered activity was found in a sharp peak eluted in less than one "column volume" by 0.30 *M* NaCl in 0.01 *M* tris, pH 7.4. A second apparent peak occurred when the concentration of NaCl in the eluant was raised to 0.4 *M*.

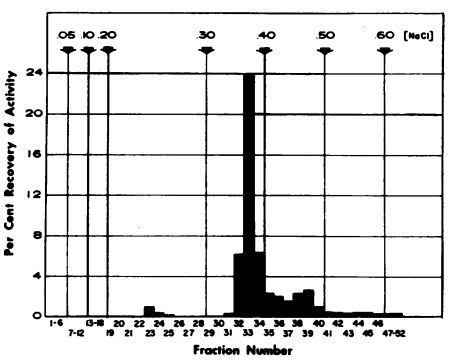

FIG. 2.—Fractionation of nuclease from induced T11(λ_{T11}) by DEAE-cellulose chromatography using stepwise elution as cited in *Methods*. Arrows indicate change of eluant, and numbers above the arrows are the molar concentrations of NaCl in 0.02 *M* tris buffer, pH 7.4. Each fraction contained 4 ml; the volume of the column of DEAE was 20 ml.

Of the total nuclease activity, only 54 per cent was recovered after fractionation on a DEAE column. The reason for this loss is not known, but it was subsequently found that the pH optimum for fraction VI is lower than 10, the pH of our standard assay. Recombination of DEAE fractions 23–46 did not improve the recovery.

(h) *Properties of partially purified nuclease:* In Figure 3 are illustrated two properties of the nuclease from induced T11(λ_{T11}): (1) a pH optimum at about

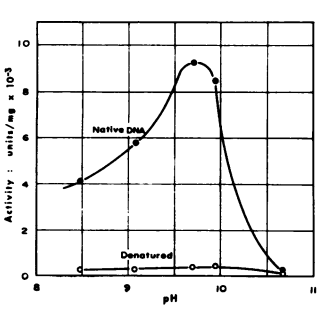

FIG. 3.—pH optimum of nuclease in fraction VI, tested with native and denatured DNA as substrate. DNA at a concentration of 1 μmole/ml in 0.01 *M* tris, pH 7.4, and 0.01 *M* NaCl was denatured by heating for 10 min at 100° followed by rapid cooling in ice. The conditions were those of the standard assay except that glycine buffer was replaced by 10 μmoles of equimolar mixtures of tris and 2-aminoethanol.

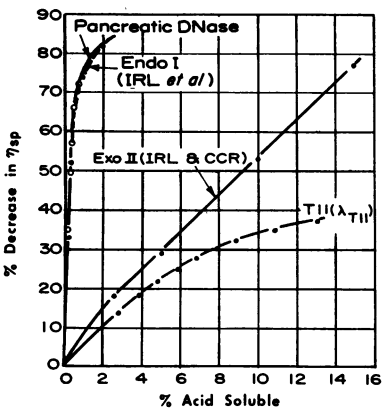

FIG. 4.—Per cent decrease in specific viscosity versus per cent acid-soluble radioactivity: data obtained from experiments with partially purified nuclease from induced T11(λ_{T11}) (fraction VI, Table 2) and pancreatic DNase compared with data replotted from Lehman *et al.* for endonuclease I[15] and exonuclease II,[16] both from *E. coli*.

9.7, and (2) a 25-fold more rapid hydrolysis of native DNA than denatured DNA.

No inhibition was observed when 0.1, 1.0, or 10 mg of soluble RNA from *E. coli* were added to the assay mixture. The nuclease activity in DEAE fraction 39 (Fig. 2) had an identical pH optimum and a tenfold preference for native DNA.

An exonucleolytic mode of action is indicated by the data shown in Figure 4. In experiments with fraction VI and pancreatic DNase, two parameters were measured in each reaction mixture: the specific viscosity of the DNA substrate, and the production of acid-soluble nucleotides. In 1 hr, the partially purified nuclease from T11(λ_{T11}) reduced the specific viscosity 37 per cent and liberated 13 per cent of the labeled substrate as acid-soluble nucleotides. By contrast, pancreatic DNase, which is an endonuclease,[14] reduced the viscosity 72 per cent and produced less than 1 per cent acid-soluble nucleotides. At the end of each experiment a large excess of nuclease from T11(λ_{T11}) and/or pancreatic DNase was added as a control to demonstrate that the viscosity measured was attributable to DNA. After incubation overnight with excess enzyme, 93 per cent or more of the radioactivity was recoverable as acid-soluble material. In Figure 4, the percentage decrease in specific viscosity is plotted as a function of percentage production of acid-soluble nucleotides. Along with our data for the nuclease from T11(λ_{T11}) and pancreatic DNase, we have replotted, for comparison, the data of Lehman *et al.* on endonuclease I[15] and exonuclease II[16] of *E. coli*. The similarity by these parameters of the nuclease from T11(λ_{T11}) to exonuclease II and dissimilarity to endonuclease I and pancreatic DNase are consistent with an exonucleolytic mode of action by the λ enzyme.

Discussion.—Those "early" mutants of λ which we have examined by studying the defective lysogens have been classifiable as belonging to one of three phenotypes with respect to nuclease activity after induction: (1) nuclease$^+$, the wild phenotype; (2) nuclease$^-$, a class characterized by little or no increase in activity; (3) nuclease^{T11}, a class, containing only one member, characterized by a fivefold more rapid increase in activity and a tenfold or greater yield of activity than shown by the wild type. Table 3 summarizes these observations, some of which have been reported previously.[6] The existence of early mutants which are nuclease$^+$ is an important control on the specificity of the nuclease$^-$ and

TABLE 3

PHENOTYPIC CLASSIFICATION OF EARLY MUTANTS OF λ

Phenotype	Mutants
Nuclease$^+$	sus_{029}, sus_{P3}, T5
Nuclease$^-$	sus_{N7}, sus_{N96A}, sus_{N53}, T27, P22
Nuclease ^{T11}	T11

The *sus* mutants are those of Campbell.[11] The subscript letters identify different complementation groups. The other mutants are those of Jacob *et al.*[3, 12] P22 (also designated d22)[3] is very close to sus_N on the genetic map, and is in the same complementation group,[19, 12] as is T27;[12] T11 and T5 are in different complementation groups.[12]

nuclease^{T11} mutants; alterations in enzymatic activity appear to be associated with specific mutations. Moreover, the genetic classification of these mutants into complementation groups[11, 12, 19] is thus far consistent with the phenotypic classification (cf. legend, Table 3). The aim of our studies is to determine the molecular basis for the phenotypes nuclease$^-$, and nuclease^{T11}, in order to explore the nature of some of the "early" functions which are required for the autonomous replication of λ[2-4] and perhaps are involved in the control of lysogeny.

The experiments on the transfer of λ_{T11} from the original host to two other strains of *E. coli* K12, demonstrate that the enzymatic hyperactivity is specifically associated with the mutated λ genome. The significance of this point derives from the production and isolation of λ_{T11} as a defective prophage, in other words, as a segment of the genetic material of the complex comprised of host and latent virus (cf. refs. 2 and 3). The transfer of the capacity for enzymatic hyperactivity with the mutated λ genome renders improbable that the increased activity is a property of a mutation or other factor peculiar to the original host, although the experiments do not rigorously exclude transduction of such a factor. Inhibition of the hyperactivity in the double lysogens, C600(λc_I,λ_{T11}) and W3350(λc_I,λ_{T11}) is evidence against a transduction since one would expect such a transduced host factor to be dominant over $\lambda c_{I\ T11}{}^+$ as well.

From our studies on the partial purification of nuclease, we conclude that the hundredfold or greater increase in nuclease activity observed after induction of T11(λ_{T11}) is assignable to an enzyme or enzymes whose chromatographic mobility, pH optimum, preference for native versus denatured DNA, insensitivity to inhibition by soluble RNA, and apparent exonucleolytic mode of action are all similar to the properties reported for nuclease purified from a wild-type lysogen.[5] These results do not prove that the nuclease associated with induction of T11(λ_{T11}) is identical to that associated with induction of a wild-type lysogen. Current work is aimed at establishing the identity or nonidentity of nuclease purified from a hyperactive lysogen and from a wild-type lysogen. The availability now of lysogenic derivatives of a single strain of *E. coli*, W3350, which are individually nuclease$^-$, nuclease$^+$, and nuclease^{T11}, should augment the interpretability of studies on purified enzyme by eliminating possible changes in the primary structure of the enzyme caused by different suppressor loci.[17, 18]

Neither mixing of crude extracts from wild-type and hyperactive strains, nor the data on purification of nuclease from T11(λ_{T11}) have given any evidence that the hyperactivity of λ_{T11} is due to the synthesis of a stimulating factor, or the absence of an inhibitor.

The experiments with the double lysogens C600(λc_I,λ_{T11}) and W3350(λc_I,λ_{T11}) have shown that the phenotypic expression of λ_{T11} is inhibited 85 per cent or more by the presence of $\lambda c_{I\ T11}{}^+$. This result, by itself, is consistent with either (*a*) preferential synthesis of a normal nuclease or nuclease component by $\lambda c_{I\ T11}{}^+$ which replaces a hyperactive enzyme made by λ_{T11}, or (*b*) repression of nuclease synthesis by$\lambda\ c_{I\ T11}{}^+$, a function which λ_{T11} fails to carry out.

The two mutant loci which alter nuclease activity, N and T11, are in different complementation groups[12] (cf. Table 3). Mutants in cistron N are lacking or deficient in nuclease; the one mutant at the T11 locus is markedly hyperactive. A simple hypothesis which accounts for these data is that cistron N is the structural

gene for λ nuclease, while T11 exerts its stimulatory action on nuclease activity by a mechanism which causes increased synthesis of λ nuclease. Although this is an attractive hypothesis, which we are testing, an important reservation should be made: the mutant P22, of Jacob, which is in the same cistron as the sus_N mutants of Campbell[11, 12, 19] has been extensively characterized by Jacob *et al.*[3] Of the early mutants described, it is the most defective; the only evidence that it performs any λ function is its killing action on the cell after either induction or infection. It is possible that its failure to synthesize nuclease is a manifestation of inability to direct the synthesis of any proteins.

One of the striking aspects of the behavior of λ_{T11} is that the *rate* of increase of nuclease activity is more rapid than in the wild type; the enzymatic hyperactivity of K12(λ_{T11}), therefore, cannot be assigned solely to a failure to turn off enzyme synthesis. Either λ_{T11} makes a hyperactive nuclease, presumably by modifying the primary structure of the enzyme, or the T11 gene must function before or concomitantly with the structural gene for nuclease so that nuclease synthesis is stimulated from its inception.

The early mutants of λ, including those in the N cistron and T11, are unable to replicate.[2-4] For both N and T11, respectively, a unitary mechanism must be found to explain the effect on nuclease activity and on the autonomous replication of λ.[20] If N is the structural gene for λ nuclease, it follows that this enzyme is required for autonomous replication of λ, or some step leading to it. While an obvious explanation for the block in DNA synthesis in the hypernuclease strain would be the degradation of λ DNA by increased nuclease activity, preliminary experiments on the yield of superinfecting λ in UV-induced lysogens which are $nuclease^-$, $nuclease^+$, and $nuclease^{T11}$ do not support that idea.

While further biochemical and genetic studies are required to determine the molecular basis for the increased nuclease activity associated with λ_{T11}, it is more probable that the increased activity reflects the synthesis of more enzyme rather than the synthesis of an altered, hyperactive enzyme. This conclusion would ascribe regulatory properties to the T11 locus and dictate an examination of the interaction of the T11 locus with other λ genes or products.

Summary.—(*a*) Among the early defective lysogens of Jacob, one, T11(λ_{T11}), has been found which is five times more active than wild type with respect to the rate of appearance and at least ten times more active with respect to the total yield of nuclease activity which appears after induction. (*b*) The capacity for increased enzymatic activity was transferred to other strains of *E. coli* K12 with the defective prophage. (*c*) In the double lysogens C600(λc_I, λ_{T11}) and W3350-(λc_I, λ_{T11}) the phenotypic expression of λ_{T11} was found to be inhibited 85 per cent. (*d*) Studies on the partial purification of nuclease from T11(λ_{T11}) indicate that the increased activity is assignable to an enzyme or enzymes whose properties are similar to those reported for nuclease purified from a wild-type lysogen.[5]

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SEQUENTIAL REPLICATION OF THE BACILLUS SUBTILIS CHROMOSOME, III. REGULATION OF INITIATION

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Evidence for the sequential replication of the *Bacillus subtilis* chromosome has been presented for strain W23 in our previous reports. The experiments were based on the comparison of marker frequencies in DNA preparations from the exponential and stationary growth phases, using genetic transformation. If the chromosome replicates in a single sequential order, the frequency of each marker in the exponential phase should be a function of its location on the chromosome. On the other hand, the nonreplicating or completed chromosomes in the stationary growth phase should give uniform frequency of all markers, and DNA from stationary phase cells should provide the standard for measuring marker frequencies in the exponential growth phase by genetic transformation. Previous results[1] indicated that such a polarity does exist in strain W23 of *B. subtilis*. Based on these results, a genetic map was constructed in which the adenine (*ade*) marker is located near the point of origin of chromosome replication, and methionine (*met*) and isoleucine (*ileu*) markers near the terminus. This work also indicated that chromosomes in the stationary phase were in the completed form. The possibility of other models of replication which, although less likely, could account for these results has previously been discussed in detail.[1] All of these conclusions were confirmed more directly by using isotopic transfer experiments.[2] However, the same type of experiments carried out on another strain of *B. subtilis* (W168) revealed an apparent absence of replication polarity of the chromosome.[3] This communication reports experimental results showing that the nonpolarity of strain W168 is only an apparent one. Thus, unlike strain W23, chromosomes of strain W168 in stationary phase cells are not in the completed form but are in various stages of replication.