HEAT-INDUCIBLE λ PHAGE. V. INDUCTION OF PROPHAGES WITH MUTATIONS IN GENES *O*, *P*, and *R*

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MUTATIONS in the C_i gene of λ bacteriophage which cause the repressor (immunity substance) to be inactivated by heat can be divided into two groups which have been designated $C_{it}A$ and $C_{it}B$ (LIEB 1966a). $C_{it}A$ mutants are heat inducible under either growing or nongrowing conditions. However, heat induction of tB mutants (measured by loss of immunity or by killing of the lysogen) can be blocked by nongrowing conditions and by agents that inhibit protein synthesis. Heat induction of the latter group of mutants can also be inhibited by addition to the λ genome of a mutation in a closely neighboring gene (gene N) (LIEB 1966b; CROSS and LIEB 1967). Heating of λ lysogens having a mutation in gene N combined with a tA mutation may result either in the "conversion" of the host phenotype (LIEB 1967) or in killing of the cell (LIEB 1966b; CROSS and LIEB 1967); in other words, induction is not blocked in this group. These findings have been used to generate the hypothesis that the repressor of λ may be, in fact, a repressor complex, containing the products of gene C_i and gene N (LIEB 1966b; CROSS and LIEB 1967).

Recently, PEREIRA DA SILVA and JACOB (1967) and DAMBLY, COUTURIER and THOMAS (1967) have suggested that the product of C_i directly prevents the transcription of not only gene N but also gene O (on the opposite side of C_i from N). Thus, one might expect to find that mutations in gene O would mimic the effect of mutations in gene N and would affect inducibility of λC_{it} mutants. However, we find that mutations in O , P or R do not inhibit heat induction. Thus, we have no evidence for the direct interaction of the C_i product with the products of these latter genes. In the course of these studies, we have observed extensive production of nonlysogenic cells ("curing") from heat-induced lysogens carrying mutations in either gene O or P , but only limited curing following heat induction of R mutants. We present evidence that the events leading to curing cannot be completed while λ is derepressed.

MATERIALS AND METHODS

Media and culture methods have been described previously (LIEB 1966a; CROSS and LIEB 1967).

Bacteria and bacteriophage stocks: The sources and characteristics of the bacteria and most of the mutant λ phages used in this study have been described previously (LIEB 1966a, b). *O* *sus* 29, *P* *sus* 3, and *R* *sus* 5 are conditional lethal ("amber") mutations described by CAMPBELL (1961). In bacteria lacking the appropriate suppressor, phages carrying any of these mutations are defec-

tive. Phages with *sus* mutations were obtained from DR. A. CAMPBELL and DR. J. WEIGLE. To simplify the text, we shall omit the mutant identification number in most references to the *sus* mutations.

O λ 2 is a mutation in gene *O* which results in a defective prophage, unable to replicate. It was isolated following a cross of λ t1 with a defective lysogen believed to contain λ "X" τ 11. The prophage was initially thought to have the genotype t1-"X" τ 11. However, the mutation causing defectiveness has been mapped at a site midway between *C*_{II} *c*68 and *O* *sus* 29 and apparently was present in one of the original stocks involved in the cross.

Escherichia coli strain M3 carries a suppressor and is permissive (pm^+) for λ *sus* mutants. Strain 594 (obtained from DR. J. WEIGLE) is nonpermissive (pm^-) for the *sus* mutants. Although the *sus* mutants cannot replicate in pm^- cells, they will lysogenize forming defective lysogens. λ C_It mutants lysogenize at 36°C, but the prophages are induced when the lysogens are placed at 42° to 45°. The relative positions of the phage mutations on the λ prophage map are shown in Figure 1 of the accompanying paper (CROSS and LIEB 1967). The prefix "C_I" will be omitted in most subsequent references to the heat-inducible mutants; the C_I site number will be listed first and will be separated from *sus* designations by means of a dash, i.e., λ t2-0 *sus*.

Isolation of double-mutant phages and preparation of lysogens: Lysogens of double-mutant phages containing a mutation in both C_I and *O*, *P* or *R* were obtained according to the method outlined previously (LIEB 1966b; CROSS and LIEB 1967).

Measurement of induction: We have previously defined λ induction as any alteration in the repressor (or immunity substance) which results in derepression of λ mRNA synthesis with concomitant loss of immunity (CROSS and LIEB 1967). Under normal circumstances, induction gives rise to a series of λ -dependent events which culminate in the release of mature phage from a lysogenic bacterium. However, phage production or cell lysis are not appropriate indicators of induction when prophage are defective, as in the case with most of the phages used in this study. Killing of the lysogen is a conveniently measured phenotypic change associated with induction. In the absence of killing, we consider a loss of immunity to superinfecting λ (CROSS and LIEB 1967) or the production of nonlysogenic progeny (see subsequent discussion of curing) to be indicators of induction.

Heat induction was initiated by diluting lysogens 1:100 into prewarmed tryptone broth (TB). Samples for assay were removed from the warmed medium at intervals. For ultraviolet (UV) induction, the bacteria were diluted 1:100 into buffer at 0°C and irradiated with constant shaking. The UV source was either one or two General Electric Co. Germicidal lamps each delivering 4 ergs/mm²/sec to the target area. After irradiation, the bacteria were kept chilled and all plating was done under yellow light.

The assay plates from induction experiments were replicated to tryptone agar (TA) plates spread with λ c47. Replicas of colonies composed largely, or entirely, of sensitive cells appear as watery, nearly transparent colonies in contrast to the normal opaque replicas of immune colonies.

RESULTS

*Heat induction of pm^- (λ C_It-0 *sus* 29):* Pm^- (λ t2-0 *sus*) is typical of pm^- (λ tA-0 *sus*) cultures and is killed rapidly at either 43° or 45°C; it thus resembles pm^- (λ t2-0⁺) (Figure 1). Bacteria that form colonies of nonlysogenic cells ("cured") are recovered after only brief heating and the maximum frequency (about 6% of the input cells) is observed after 12 minutes at 43°C. Continued heating results in a loss of colony-forming bacteria.

Multiplication of nonlysogenic cells originally present in the culture is believed to be responsible for the increase in the number of survivors between 40 and 60 minutes. Curing of pm^- (λ t2-0⁺) lysogens has not been observed in our induction experiments.

FIGURE 1.—Response of a $pm^- (\lambda C_i tA-O \text{ sus } 29)$ lysogen to heating. Zero and 3-minute points estimated for curve of cured fraction. No curing observed in $pm^- (\lambda t2-O^+)$ cultures. Procedure in MATERIALS AND METHODS.

FIGURE 2.—Response of a $pm^- (\lambda C_i tB-O \text{ sus } 29)$ lysogen to heating. Curing observed in $pm^- (\lambda tU9-O^+)$ is abnormally high; nondefective lysogens carrying a t mutation at other sites in C_i region B show no curing or only slight curing. Zero points estimated for curves of cured fraction.

The killing and curing behavior of $pm^- (\lambda tU51-O \text{ sus})$ lysogens is not significantly different from $pm^- (\lambda t2-O \text{ sus})$. The kinetics of the survival curves of $pm^- (\lambda tU37-O \text{ sus})$ are similar to $pm^- (\lambda t2-O \text{ sus})$ to approximately 10% survival. We have been unable to obtain cultures of $pm^- (\lambda tU37-O \text{ sus})$ containing fewer than about 10% sensitive cells; the cultures appear to undergo "spontaneous" curing at a relatively high rate.

$Pm^- (\lambda tU9-O \text{ sus})$, a typical $pm^- (\lambda tB-O \text{ sus})$ culture, is able to survive 25 to 30 minutes at 43°C before a significant decrease in viable cells is seen (Figure 2). After 24 minutes at 43° approximately 80% of the lysogens still form colonies. This behavior is in sharp contrast to $pm^- (\lambda tU9-O^+)$ cultures which die very rapidly and contain only 0.03% survivors after 24 minutes of heating. However, the delay in killing of the double-mutant lysogens does not reflect lack of induction. After 12 minutes of heating at 43° , approximately 95% of the input lysogens produce colonies of sensitive cells. As with the λtA lysogens, the number of cured colonies that appear after plating declines with continued heating. Similar results were obtained with other λtB mutants, e.g., $pm^- (\lambda t1-O \text{ sus})$, $pm^- (\lambda tU46-O \text{ sus})$ and $pm^- (\lambda tU50-O \text{ sus})$.

Heat induction of M3 ($\lambda t1-O \text{ i}2$): The combination of $\lambda t1$, a C_i region B muta-

FIGURE 3.—Response of an $M3(\lambda C_t B-O L2)$ lysogen to heating. Zero points estimated for curing curves.

FIGURE 4.—Response of a $pm^-(\lambda C_t A-P sus 3)$ lysogen to heating. Zero point estimated for curve of cured fraction. No curing observed in $pm^-(\lambda t2-P^*)$ cultures.

FIGURE 5.—Response of a $pm^-(\lambda C_t B-P sus 3)$ lysogen to heating. Zero point estimated for curve of cured fraction. No curing observed in $pm^-(\lambda t1-P^*)$ cultures.

tion, with the defective $\iota 2$ mutation of gene O creates a double-mutant prophage which is induced rapidly at 43°C , but following induction, causes very slow killing of the host (Figure 3). After ten or more minutes at 43° , the majority of the colonies formed by heated lysogens contain sensitive cells. The still undefined curing events appear to be very tolerant of prolonged inducing conditions in these lysogens, since approximately 50% of $M3(\lambda\iota 1-O \iota 2)$ bacteria can survive and produce colonies of cured cells even after 40 minutes at 43°C , and 10% can survive 80 minutes of heating. It is possible that these lysogens survive heating at 43° for a longer time than do O *sus* lysogens because the $\iota 2$ defective mutation is not leaky. The killing of $\lambda\iota B-O$ *sus* lysogens with continued heating could then be attributed, in part, to the synthesis of a small quantity of O product.

Heat induction of pm^- ($\lambda C_{\iota}t-P$ *sus* 3): The heat killing curves of a λtA lysogen, pm^- ($\lambda\iota 2-P$ *sus*), and a $\lambda\iota B$ lysogen, pm^- ($\lambda\iota 1-P$ *sus*) (Figures 4, 5), resemble closely those of the corresponding heat-inducible O *sus* lysogens. Both prophages are induced rapidly, as shown by the increase in cured cells, but the killing curves of pm^- ($\lambda\iota 1-P$ *sus*) lysogens have a shoulder which is not found on killing curves of pm^- ($\lambda\iota 2-P$ *sus*). Curing is again more extensive in the lysogen containing the $\lambda\iota B$ mutant, with potentially cured cells increasing to 85% of the input pm^- ($\lambda\iota 1-P$ *sus*) cells. The proportion of heated lysogens that give rise to sensitive colonies is higher in pm^- ($\lambda tA-P$ *sus*) than in pm^- ($\lambda tA-O$ *sus*) cultures.

FIGURE 6.—Response of a pm^- ($\lambda C_{\iota}tA-R$ *sus* 5) lysogen to heating. Zero point estimated for curve of cured fraction. No curing observed in pm^- ($\lambda\iota 2-R^+$) cultures.

FIGURE 7.—Response of a pm^- ($\lambda C_{\iota}tB-R$ *sus* 5) lysogen to heating. Zero point estimated for curve of cured fraction. No curing observed in pm^- ($\lambda\iota 1-R^+$) cultures.

Heat induction of pm⁻ (λ C₁t-R sus 5): The *R sus* mutation has little effect on the heat-induced killing of λ C₁t lysogens. The survival curves at 43°C of the double mutant lysogens pm⁻ (λ t2-*R sus*) (Figure 6) and pm⁻ (λ t1-*R sus*) (Figure 7) closely match those of the respective single-mutant lysogens. Some curing occurs after brief heating, but less than 1% of the input cells become cured when the λ tA mutant, t2, is present and only about 5% cure with the λ tB mutant, t1. Gene *R* is the structural gene for endolysin (CAMPBELL and CAMPILLO-CAMPBELL 1963) which performs a "late function". Phage replication is not impaired by the *R sus* mutation (JOYNER *et al.* 1966). Extensive phage production presumably kills the *R sus* lysogens, and thus accounts for the low frequency of cured cells.

UV induction of heat-inducible lysogens: The sensitivity of λ C₁t lysogens to killing by UV is not altered by the presence of a mutation in gene *O*, *P*, or *R*. The UV sensitivity of a particular double-mutant lysogen reflects the sensitivity to UV induction associated with the C₁t mutation of the prophage. After UV irradiation, an intracellular inducer is formed that apparently combines with the part of the λ repressor corresponding to C₁ region A. Thus, UV induction of *any* λ lysogen is analogous to heat induction of a λ tA lysogen (LIEB 1966b; Cross and LIEB 1967).

DISCUSSION

All C₁t lysogens containing an unsuppressed *O sus*, *P sus*, *R sus* or an *O* defective mutation are induced at 43°C. Thus, it appears that mutations which alter the *N* product may be unique in their ability to *prevent* heat induction. However, we have again observed that prophages with *t* mutations in C₁ region A differ from those with mutations in C₁ region B in their response to heating: extensive curing occurs only in λ tB lysogens.

Lysogens carrying heat-inducible λ tA mutants are killed when heated, and mutations in genes *O*, *P* or *R* do not markedly affect the rate of killing. Previous experiments (Cross and LIEB 1967) have shown that lysogens are also killed following the induction of λ tA-*N* mutants. λ DNA synthesis as well as all "late" functions are blocked by mutations in *O*, *P* and *N*; thus, killing can be attributed to λ replication only in the case of *R* mutants. The elegant experiments of EISEN, SIMINOVITCH and MOHIDE (1967) suggest that killing of λ lysogens defective in prereplicative functions (*N*, *O*, and *P* mutants) occurs as the result of interruption of the bacterial chromosome. Scission of the chromosome thus appears to be one of the "earliest" known λ functions.

In lysogens carrying λ tB mutants, *sus* mutations in genes *O* and *P* permit the cells to survive heating at 43°C for about 25 minutes, and delay the rate of killing when heating is continued. However, induction (as we define it) occurs at a normal rate. After 10 to 12 minutes at 43°, most previously lysogenic cells produce colonies of sensitive bacteria, indicating that phage-dependent functions were derepressed. Curing of lysogens containing prophages mutated in genes *O* and *P* was first observed after UV induction by EISEN *et al.* (1966). We have shown that over 90% of lysogens contained a λ tB mutation combined with *O* λ 2, *O sus* 29, or *P sus* 3 form colonies of sensitive cells after a few minutes at 43°C.

Thus, the products of genes *O* and *P* are not required for curing. Conversely, curing is rare in *N sus* lysogens (EISEN *et al.* 1966; CROSS and LIEB 1967) suggesting that one or more products of the *N* operon participate in repair of the host chromosome after scission. FRANKLIN (1967) has observed that nonlethal deletions of λ , mapping to the left of *N*, are associated with the loss of curing capacity.

Some curing is also observed in λtA lysogens containing *O sus* or *P sus* mutations. Although brief heating (6 to 12 minutes) kills a large fraction of these lysogens, the majority of bacteria that do survive form colonies containing sensitive cells. Continued heating results in additional killing. Since nonlysogenic bacteria are not killed at 43°C, we must conclude that curing is not complete by 12 minutes. The decline in the frequency of cells as heating continues indicates that an event (or events) leading to curing must occur after the cells are cooled. A slow decrease in the surviving fraction, which consists almost entirely of cured cells, is also observed after extended heating of λtB lysogens having a mutation in *O* or *P*.

The mechanism of curing: We assume that both lysogenization and curing require a sequence of events which bring about the cutting of chromosome strands and their reunion. Evidence that lysogenization and curing involve site-specific recombinational events has been presented by SIGNER and BECKWITH (1966). In the APPENDIX, we present a detailed model for pairing of homologous regions, scission of the DNA, and repair and reunion of DNA strands. The manner in which the required steps are coordinated is not understood, and the genes required for the various steps remain to be identified. Scission and repair of the host chromosome appear to occur normally after induction. Curing is observed in heat-inducible, nondefective lysogens that have been heated for only a short time (WEISBERG and GALLANT 1966; Figures 2 and 3, this publication). Moreover, repair of the host chromosome seems to be possible even in a lysogen that has entered the lytic cycle. In recombination studies, chromosomes were transferred to *F*⁻ cells from *Hfr* (λtB) lysogens that had previously been heated for an extended period. These chromosomes had lost the heat-inducible prophage, but genes on either side of the λ attachment region remained linked (OPPENHEIM 1967).

The role of the repressor in curing: The difference in the relative frequencies of cured and killed cells when defective λtA and λtB lysogens are heated can be explained by assuming that the re-establishment of repression is required for repair of the host chromosome. Following scission of the chromosome, the bacterial cell is presumed to be unable to replicate until repair enzymes heal the break (EISEN, TALLAN and SIMINOVITCH 1967). Both the host and the phage have genes that affect DNA synthesis, and may function in the repair of breaks in the DNA. However, the continued synthesis of phage "scission" enzyme(s) would prevent effective repair. If repair is delayed, an increasingly large fraction of the lysogens may undergo irreversible changes resulting in the inability to form colonies. When repair is successful and the cell survives, the defective prophage is rarely reincorporated into the host chromosome, and the cell is cured.

We have shown that at 43°C, defective lysogens containing λtB mutants retain their potentiality to be cured much longer than do λtA lysogens. In lysogens containing λtB mutants, λ mRNA synthesis is repressed, at least in part, within a few minutes after cooling (NAONO and GROS 1966). Re-establishment of repression is also indicated in experiments reported by OGAWA and TOMIZAWA (1967). They found that immunity produced in abortive lysogens by λtB phages reappears rapidly when heated cells are chilled; slow or limited re-repression after cooling was observed with λtA mutants. Both groups of workers have suggested that the C_I product forms an aggregate which, in the case of λtB mutants, is only partially or reversibly disassociated by heat. Thus, cooling would result in rapid re-repression of λ functions in λtB lysogens and may permit prompt repair of host chromosomes, resulting in extensive curing. Curing would also be expected to occur in those λtA lysogens in which repression was re-established before irreversible lethal events had occurred in the cells, and we indeed find that most of the heat-survivors are cured. In lysogens carrying a prophage *not* defective for a prereplicative function, only a small proportion of the heat-survivors produce sensitive progeny. In the majority of the survivors of this class, we may assume that the host chromosome had not yet been cut, or that the induced phage reincorporated as a prophage. When repression is re-established, the probability of relysogenization *vis a vis* curing may be proportional to the multiplicity of phage genomes present.

Functioning of certain phage genes, followed by repression, appears to be the normal sequence of events in lysogenization. For example, in phage P22, the product of a gene corresponding to C_I of λ inhibits phage replication after certain lysogenization steps have occurred (SMITH and LEVINE 1964). Thus, early events in curing or phage integration proceed while certain phage genes are derepressed. When the repressor becomes effective, following its synthesis or reactivation, it represses most of the phage genome, including the unknown gene(s) producing the endonuclease needed to cut the host chromosome. *One or more repair enzymes must remain active after repression is established, in order to complete the curing process.* In the case of λ , the phage does not appear to depend on the host for any of the enzymes required in lysogenization, curing or vegetative recombination. These events, as well as heat-induction of prophages, occur in recombination-deficient cells (BROOKS and CLARK 1967) and we have observed extensive curing in λ lysogens of the *rec*⁻ strain AB2492 (kindly supplied by DR. P. HOWARD-FLANDERS).

The hypothesis that repression is required for curing is also proposed by SLX, EISEN and SIMINOVITCH (1967) to account for the curing curve of $\lambda tB-O$ *sus* and $\lambda tB-P$ *sus* lysogens. They present data from F-duction experiments which indicate that chromosomes containing these prophages are cut after heating, and are repaired upon subsequent cooling.

We have considered the possibility that the decline in the number of viable (cured) cells during continued heating might be due to a *direct* effect of the heated C_I product. For example, host DNA synthesis might be blocked by inacti-

vated repressor. Arguing against this hypothesis is the fact that certain *N sus* lysogens continue to grow although the repressor is inactivated (LIEB 1967).

Lysogenization by λC_I^+ can be prevented by holding infected cells at 42°C, indicating that a temperature-sensitive step is involved (LIEB 1953; ZICHICHI and KELLENBERGER 1963). If curing and lysogenization are essentially identical processes, there may also be a temperature-sensitive step in curing. However, heating does not kill prospective lysogens, it causes elimination of the phage genome. This suggests that the temperature-sensitive step in lysogenization precedes DNA scission, and might be involved in circularization of the λ genome, or its pairing with the host chromosome.

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SUMMARY

Heat induction was studied in lysogens containing λ prophages with two mutations—one in region A or B of gene C_I , and one in a neighboring gene. Genes *O* and *P* function in λ DNA replication, and *R* is the structural gene for λ endolysin. Induction of defective lysogens was indicated by loss of the ability to form a colony, or by the production of nonlysogenic progeny ("curing"). The heat-killing curves of lysogens containing $\lambda C_{I,t}A$ prophages were not significantly altered by the simultaneous presence of a mutation in gene *O*, *P* or *R*. When the prophage contained a $C_{I,t}B$ mutation and also a mutation in *O* or *P*, the majority of lysogens were not killed after 20 to 30 minutes at 43°C. However, the survivors produced nonlysogenic colonies, indicating that induction had occurred but had resulted in curing. Little curing was observed in lysogens carrying prophages with mutations in gene *R*, which permits λ replication.—It is suggested that at 43°C the loss of repression in a lysogen containing a $\lambda C_{I,t}$ mutation results in the scission of the prophage from the host chromosome. Curing can then occur, provided that the prophage does not replicate, and that the host chromosome is repaired. Curing requires that repressor function be re-established before some unidentified event leads to killing of the host. Rerepression occurs slowly in lysogens containing $\lambda C_{I,t}A$ prophages, but is very efficient in $C_{I,t}B$ lysogens, resulting in the curing of over 90% of all lysogens in some experiments. A model of prophage excision is presented.

Note added in proof: Recent experiments by Dr. A. GUHA and Dr. W. SZYBAŁSKI (unpublished) indicate that at 43°C, the rate of production of "early" mRNA by a $pm^-(\lambda C_{I,t}B)$ lysogen is 20% of the rate observed in a $pm^-(\lambda C_{I,t}A)$ lysogen. Thus, $pm^-(\lambda C_{I,t}B)$ lysogens may produce significantly lower amounts of certain "early" gene products than do $pm^-(\lambda C_{I,t}A)$ lysogens. This deficiency of gene products might be responsible, at least in part, for the slow killing of $C_{I,t}B-O_{sus}$ and $-P_{sus}$ pm^- lysogens.

FIGURE 8.—Diagram of prophage excision. See text of APPENDIX for details. The letters shown along the strands are not intended to represent individual genes, but are included only to permit a given region to be followed through the diagram.

APPENDIX: PROPHAGE EXCISION

Figure 8 outlines a model for prophage excision which is based on the CAMPBELL model for lysogenization (CAMPBELL 1962). The *b2* region of λ is homologous to the λ attachment site of the bacterial chromosome (CAMPBELL 1965). Lysogenization by recombination within the homologous regions results in a bacterial chromosome in which the prophage is included between two identical regions of DNA. In Figure 8a, these homologous regions are in close proximity. An exchange of hydrogen bonding between complementary bases in adjacent strands is assumed to occur next (8b), followed by cleavage of internucleotide phosphoester bonds (8c). This reaction requires an endonuclease, presumably one which is able to recognize the unique molecular configuration presented by the strand exchanges and/or some unique sequence of bases. After the strands have been broken in the vicinity of the exchanges, the λ and bacterial chromosomes are functionally separate. In 8d we have drawn λ and the host chromosome as physically separate but with the strands not yet realigned; 8e shows the strands realigned, but not yet rejoined. It is possible that bases are removed from cut ends by exonuclease activity, and that DNA synthesis may be required to replace them. The breaks in the λ and bacterial chromosomes must then be repaired if the phage is to replicate or the cell is to survive. The action of one or more additional enzymes may be required to rejoin the final phospho-ester link in each strand.

The scissions probably occur in single strands at different nucleotide levels, somewhat as diagrammed, rather than through all four strands at the same nucleotide level. If there are base sequences particularly vulnerable to enzymatic attack, their location would differ in the

complementary strands of DNA. Furthermore, recombination *via* breaks at the same site in all four strands requires the postulation of an enzyme system that switches and rejoins the strands with high efficiency, without the loss of any genetic material. We suggest that the recombinational events involved in lysogenization and excision are similar to those allowing recombination between λ phage genomes and involve the formation of hybrid regions (MESELSON 1964). A detailed theoretical discussion of recombination at the DNA level is given by WHITEHOUSE (1963) and by MESELSON (1967).

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