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Genetic Specificity of DNA Synthesized in the Absence of T4 Bacteriophage Gene 44 Protein

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Upon infection of *Escherichia coli* B with T4 phage with DO amber mutation in gene 44, a minimal amount of phage DNA is synthesized. This progeny DNA is, for the most part, covalently attached to the parental DNA. Analysis of the genetic representation of this DNA was performed by hybridization to cloned genetic segments. It was shown that areas preferentially replicated differ from origins observed in "normal" replication: under normal conditions, there is a strong origin in the genetic area of genes 50-5 and lack of initiation within the group of genes 40-43 and 35-52. In contrast, in the absence of the gene 44 protein, the genetic area of 50-5 is underrepresented, genes 35-36, tRNA, and genes 40-41 are the most prominent among progeny DNA, and the area of gene 39 is least represented. Since the area of gene 35 is known from the genetic data of others to be a high-frequency recombination area, and since the area of gene 39 is known to display a low frequency of recombination, we postulate that the observed uptake of label occurs at the site-specific recombinational intersections.

Initiation of DNA replication in wild-type T4 bacteriophage-infected cells was followed in this laboratory by using hybridization of early progeny DNA to cloned T4 DNA segments. We had localized two areas of initiation of DNA replication in T4 phage: the more predominant one in the area of gene 50-5, and the other, less pronounced, in the area of genes W-29 (3).

In the present paper we will analyze the physicochemical properties and genetic specificity of the DNA produced in the absence of the gene 44 protein.

MATERIALS AND METHODS

Bacterial and phage strains. *Escherichia coli* B23 was used in all of the experiments. The phage strains employed were the osmotic shock-resistant strain $T4BO_1^r$ and the T4*am*N82 strain, defective in gene 44. The pBR322 recombinant plasmids and the T4 genes they contained were described by Ling et al. (9). The PCR1 recombinant plasmid, 46, contained the $tRNA^{Arg}$ gene (15). In addition, newly cloned genetic segments were used: lambda, B48, gene 39; lambda, A47, genes 44-46; TFH 3051, gene 55; TFP 2006, genes 8-9; lambda, B28, genes 19-20; TFH-3052, gene 33. Those plasmid-carrying strains and lambda phages (14) were kindly provided by J. Abelson.

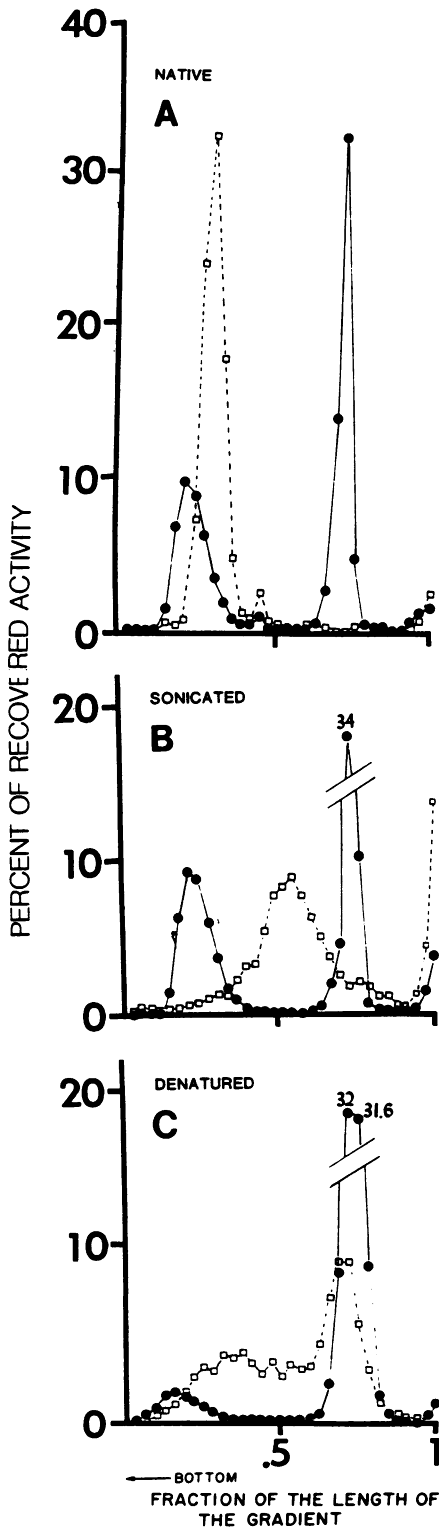
Growth medium and preparation of phage stocks, DNA extraction from infected cells, isotope labeling, CsCl and Cs_2SO_4 density gradients, the cloning and transfer of cloned T4 DNA segments to nitrocellulose filters, and the hybridization of the experimental DNA to the cloned DNA were described extensively in one of our previous papers (9).

RESULTS

In the experiments described here, we will first demonstrate some of the physicochemical properties of DNA synthesized in the absence of gene 44. Second, we will analyze genetic representation of this DNA.

Purification and physicochemical properties of progeny DNA. *E. coli* B (nonpermissive host for amber mutants) was grown in TCG medium (8a). When the density of cells reached 3×10^8/ml, the suspension was divided and infected with a multiplicity of infection of 8 of either: (i) light-density DNA phage T4*am*N82; (ii) heavy-density DNA (5-bromodeoxyuridine substituted) phage T4*am*N82; or (iii) light-density DNA wild-type phage. At 4 min postinfection, a radioactive package of [^{3}H]thymidine ([^{3}H]TdR) (specific activity, 150 mCi/mg of TdR) was added. At later times, samples were withdrawn for the extraction of intracellular DNA. From cells infected with wild phage (iii above), the samples were taken at 6 or 7 min (in two different experiments described here); samples were taken at 30 min postinfection from cells infected with T4*am*N82.

We measured the rate of [^{3}H]TdR uptake to *E. coli* B infected with T4*am*N82 and discovered that uptake to phage DNA was initiated at some 15 to 20 min postinfection and reached a plateau at 30 to 35 min postinfection. Until about 10 min postinfection there was an observable ^{3}H up-



take. This uptake, however, was restricted, as demonstrated by hybridization assay and Cs_2SO_4 isopycnic analysis, to residual host DNA synthesis. After this time, this DNA decomposed, and then uptake to phage DNA began. By about 20 min postinfection, little or none of host ^{3}H-labeled DNA was left within infected cells, presumably due to degradation by phage-coded enzymes. Interestingly (see below), it was at those late times when interparental recombination could be observed.

All extracted DNA was subjected to analysis and purification by isopycnic centrifugation. Extracts from part of the experiment where no density label was used were fractionated in Cs_2SO_4 density gradients. Purified DNA was used for hybridization to cloned genes as described below. Extracts from bacteria infected with heavy-density (HH) T4*am*N82 were preparatively fractionated in CsCl to isolate ^{3}H-labeled DNA. Isolated DNA was subject to further analysis.

Preparative CsCl runs (data not shown but very similar to those in Fig. 1A) of labeled DNA revealed that the incorporated label banded at the near-heavy density location, and no label banded at the light density location. Thus there was firm association of the light-density ^{3}H label with the heavy parental molecule, and no amplified DNA, which would band at the light location, was present. ^{3}H-labeled moieties were isolated, dialyzed against 10^{-3} M EDTA, and reanalyzed in CsCl in native, sonicated, and denatured form. Light- and heavy-density ^{32}P-labeled reference DNAs were added. In an independent run, it was proved that the absorbance at 260 nm of experimental (parental) DNA coincided with that of the ^{32}P-labeled reference DNA used here; the reference [^{32}P]DNA exactly represents, therefore, the density of the parental heavy DNA. This is important since the uptake of 5-bromodeoxyuridine might vary by 5%. Results of CsCl analysis revealed that light ^{3}H-labeled DNA contributed some 10% to parental heavy DNA, as the incorporated label banded at about 10% of the distance between HH and light (LL) ^{32}P-labeled references (Fig. 1A). After sonication, the ^{3}H-labeled DNA assumed the hybrid location (Fig. 1B). Thus stretches of new DNA must be larger than the sizes of the sonicated DNA (which were 5×10^5 to 1×10^6 molecular weight). Moreover, since there was no sequestration of light-density subunits after

FIG. 1. CsCl isopycnic analysis of [^{3}H]TdR-labeled progeny T4*am*N82 DNA. The ^{3}H-labeled moiety (open squares) was first purified in a preparative CsCl run. After dialysis against 10^{-3} M EDTA, moieties were supplemented with heavy and light ^{32}P-labeled reference DNA (closed circles) and reanalyzed.

sonication, there were no frequent (if any) reinitiations. Finally, analysis of denatured DNA (Fig. 1C) revealed two classes of single-stranded fragments: one was a minority of some 25% of the total recovered label of light density, and the other banded at intermediate densities. This indicates that most of the incorporated label is covalently joined to parental heavy strands.

DNA was also analyzed in alkaline sucrose gradients. Results (not shown) had revealed a broad distribution of sizes with median length of one-quarter of the T4 genome. The observed distribution of sizes was very similar to the distribution of sizes of the parental T4*am*N82 phage DNA observed upon infection of *E. coli* B (see Fig. 4 in reference 7). Since the median length of fragments carrying the label corresponded to 2×10^7 daltons, and since newly synthesized DNA contributed 10% of the mass of such fragments (Fig. 1A), the length of the newly added progeny strand should be some 2×10^6 daltons. Considering that sonication reduces the size of strands to 2×10^5 to 5×10^5, it is nearly certain that the newly synthesized stretches are sheared multiply. Indeed, denaturation of the sonicated moiety leads to separation of progeny strands of pure light density. In summary, in the absence of gene 44 protein, the [^{3}H]TdR label is incorporated in relatively long stretches of semi-conservatively replicated DNA. Most progeny strands are covalently joined to the parental molecule, suggesting that observed synthesis might be occurring at recombinational intersections.

Genetic representation of newly synthesized DNA. In this part, we will document results of two independent experiments performed without the use of density label. In each experiment, part of the suspension was infected with T4*am*N82 and part was infected with wild-type phage (see description of the experiment above). Cs_2SO_4-purified, ^{3}H-labeled DNA was hybridized to nitrocellulose filters charged with cloned genes. Genetic representation of initiative areas in cells infected with wild phage is shown in Fig. 2B. The obtained patterns resemble very well those documented previously (3). Specifically, we observed predominant sites of initiation in the genetic area 50-5. Furthermore, what is important when comparing with Fig. 2A, there was minimal initiation near the group of genes 40 and 35-36, located at two distal parts of the map. It should be noted that the number of genes tested was increased as compared with earlier results (3). We also tested genes 39, 44-46, 55, 8-9, 19-20, and 33, thus covering, more representatively, the length of the genetic map. Among those, however, no additional sites of initiation could be located.

Figure 2A illustrates the results of hybridization of progeny DNA produced in the absence of gene 44 protein. Values obtained in two independent experiments were remarkably similar, which encouraged us to treat the obtained patterns with confidence. It should be observed that the genetic representation of newly synthesized DNA was not random, nor did it resemble initiative patterns observed with wild phage. On the contrary, whereas there was a predominant initiative peak (genetic area 50-5) in the pattern of the wild-type phage, a flat, low valley occurred in the absence of gene 44 protein. The lowest points were located in the areas of genes 39 and 30. Conversely, the normal low areas of initiation in the area of genes 35-36 were the highest peaks in progeny synthesized in the absence of gene 44 protein.

DISCUSSION

When *E. coli* B is infected with a ^{32}P- and density-labeled amber mutant in gene 44 (T4*am*N82), no change in the density of the parental DNA is observed, even after prolonged incubation. This is true for heavy phage infecting light bacteria and vice versa (8). In fact, of all DO amber mutants tested, only T4*am*N82 revealed no change in the density of parental DNA, whereas the remaining DO phages, in similar experiments, reveal different extents of partial replication (C. C. Howe, Ph.D. dissertation, University of Pennsylvania, Philadelphia, 1972). Even though upon infection with T4*am*N82 there is abundant synthesis of both ligase and polymerase, the molecular interparental recombinants resulting from the coinfection with heavy and light phages are not repaired (joined) covalently (5).

Mathews and co-workers (12, 13) observed, upon infection of *E. coli* B with T4*am*N82, a minute uptake of [^{3}H]TdR to phagelike DNA. (The exact calculation of average net DNA synthesis is difficult due to minimal uptake of the label, which might be, therefore, severely affected by the size of the precursor pool and host breakdown products.) To reconcile our data with the apparent lack of the replication of parental molecules, we postulate that either (i) the extent of replication is limited to short segments of the parental molecule, making it unrecognizable by the density analysis performed in our laboratory, or (ii) incorporation is limited to a minority of parental molecules or cells infected, whereas the majority might not experience any replicative event. The results in the present paper favor this last possibility. We observed (Fig. 1A) that [^{3}H]TdR-labeled light-density progeny was incorporated in such a proportion to the parental heavy molecules as to result in a partially replicated structure in which the progeny-to-parent ratio was approximately

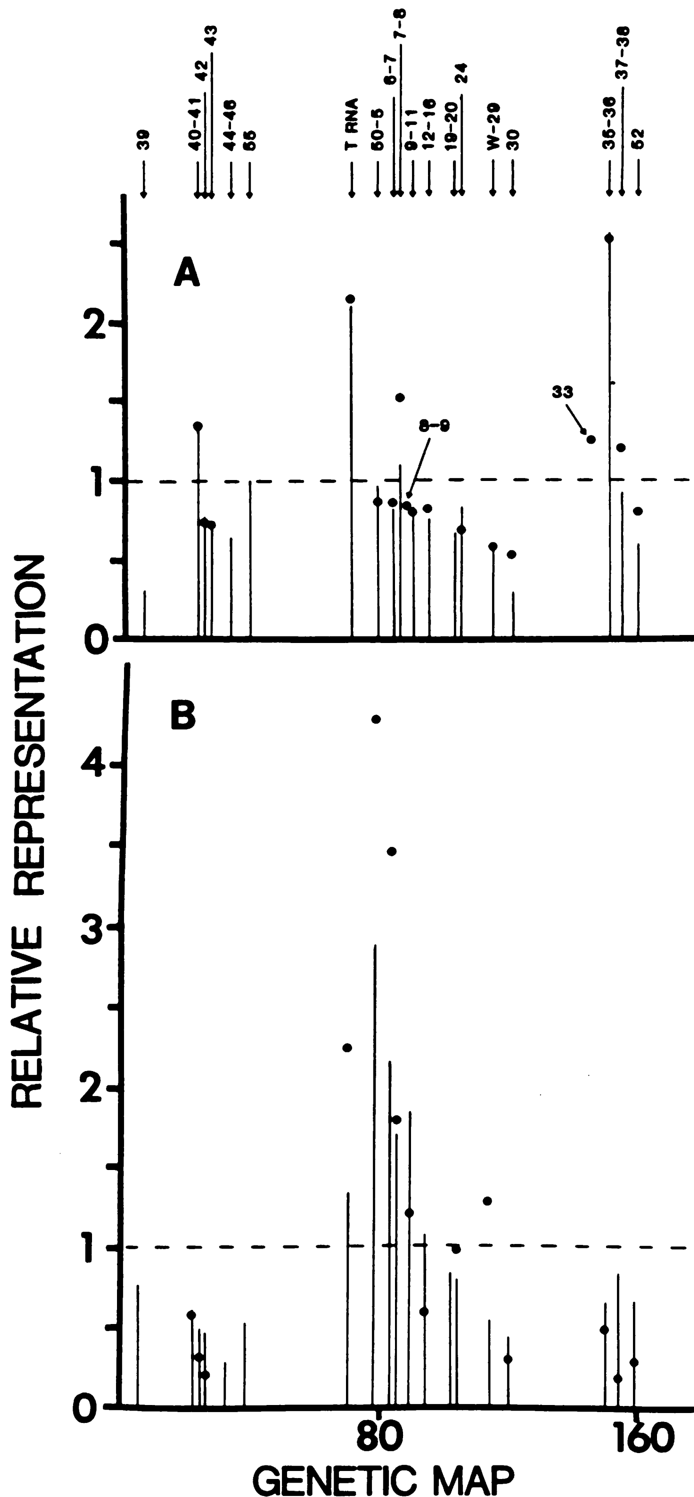


FIG. 2. Hybridization of progeny DNA to cloned genes. The results of hybridization are expressed as relative representations (RR) of genetic segment x in the progeny DNA. The $^{3}H/^{32}P$ ratio observed for a filter charged with a given genomic area, x, was divided by the ratio of the sums of ^{3}H and ^{32}P hybridized to all of the nitrocellulose filters in a set: $RR = (^{3}H_x/^{32}P_x)/(\Sigma^{3}H/\Sigma^{32}P)$. (A) Progeny produced in the absence of gene 44 protein (*E. coli* B infected with T4*am*N82). (B) Progeny produced in the presence of gene 44 protein (*E. coli* B infected with wild phage). Dots and bars represent results obtained in two separate experiments. In (B), dots indicate samples taken at 6 h and bars indicate samples taken at 7 h. Cells infected with T4*am*N82 (A) were sampled at 30 min in both experiments. Note the "typical" pattern of initiation in cells infected with wild phage (B) and the drastically different pattern in cells infected with T4*am*N82 (A). Observe coincidence of hybridization values obtained in independent experiments.

1:9. Thus, those infrequent parental molecules that do incorporate label do so to a measurable extent. Since the strand length (in alkaline sucrose gradient) of moieties containing [^{3}H]TdR is approximately one-quarter of the genome (which is similar to the previously described strand length of the T4*am*N82 parental DNA in *E. coli* B [5]), then if every infected bacterium incorporated ^{3}H label to one-quarter of the parental subunits, a displacement of 25% of the parental label away from the original nonreplicated location should be oberved. This was, however, not observed in the past; parental DNA invariably cobanded in CsCl gradients with reference DNA without even a trace of a skew in the specific activity of parental versus the reference label.

When we followed the uptake of [^{3}H]TdR in *E. coli* B infected with HH amber phage in gene 44, we observed that all of the incorporated label banded close to the heavy location and no ^{3}H label would band at the light location. This contradicts the possibility that the lack of change in the density of a majority of parental DNA might be due to the detachment, in vivo or upon extraction, of the progency DNA. Such detachment, of course, should result in an accumulation of ^{3}H progeny label at the light location in CsCl gradient.

Sonication of the replicated moiety resulted in the shift of the ^{3}H label away from the near-heavy location to an HL location. Therefore, replication was of a semiconservative nature, and the length of replicative areas exceeded the length of the sonication product. Moreover, since sonication did not release LL subunits, there were few if any reinitations. Upon denaturation, most of the progeny label assumed density intermediate between heavy and light; thus, the label is covalently attached to the parental strand. This suggests, in agreement with Mathews and co-workers (12, 13), that the observed replication might be of a "repair type." Such replication might occur while gaps are being repaired or recombinants are being joined, or might result from initiation of replication at the 3′ end of the recombinational subunits, as proposed previously (2). We favor this last possibility, because uptake is late and coincidental with belated interparental recombination (5, 8). In the past, we have disproved repair (joining) of interparental recombinants (5) in *E. coli* B infected with T4*am*N82. However, mechanisms calling for initiation at recombinational intersections, leading not to joining but to strand displacement, would reconcile both the lack of observed joining of recombinants, as reported previously, and the covalent attachment of progeny to parental strands documented in this paper. The reason why such replication is limited to only a small proportion of recombinational intersections demands further investigation.

We will now turn our attention to the genetic site specificity of the observed synthesis. It should be recalled that "normal" initiation of T4 DNA replication occurs predominantly at the genetic area of 50-5 and to a lesser extent at area W-29 (3). There is minimal initiation at the areas of genes 40-43 at one extreme of the linearized map (starting at rII) and, what is important here, genes 35-36 on the other end (see also Fig. 2B). In the absence of recombination, reinitiation, occurring even at late times after infection when a large amount of progeny DNA has accumulated, resembles the initiative area on the parental molecule (2). At late times after infection, when recombination and joining occur, reinitation is found to be nonspecific (2). It was postulated that at late times 3′-hydroxyl ends of the recombining subunits act as randomly located primers. From such primers, according to the proposed model, newly synthesized DNA elongates. In contrast to the conventional view, in which progeny elongating from intersections merely joins the participating recombinational fragments, we proposed displacement of strands resulting in elongation far beyond areas of annealing of recombinational subunits by mutual complementarity (2). This last mechanism is, most likely, relevant for discussion of the results described in the present paper. The idea that initiation might occur at the recombinational intersections was expressed previously. In the past, even though neither group proposed specifics of molecular mechanisms nor elaborated on the relevance for the phenomenon of high negative interference, both we (5) and Mosig et al. (11) have presented the concept of initiation at recombinational intersections. The difference between these two groups in assigning the time of the occurrence of such initiations should not be overlooked. We had shown that such initiations occur relatively later after infection, when enzymes involved in recombination are expressed (some 9 min postinfection at 37°C) and a large amount of progeny has accumulated in the cell (2). Mosig et al., on the other hand, suggest that, at high multiplicities of infection, interparental recombination precedes initiation and provides primers (11). As we elaborated in a previous paper (2), we do not consider the documented data (11) as supportive of those conclusions.

In addition, there is much evidence against interparental recombinations leading to initiation, as follows. (i) It should be emphasized that (at high multiplicity of infection) early produced progeny is not covalently attached to parental strands (1, 4). (ii) If, indeed, putative interparen-

tal recombination intersections were responsible for initiation, then differences in the genetic specificity of early progeny should be expected, depending on whether the experiment was performed at a high or low multiplicity of infection. This was not observed (6). (iii) Loops resulting from interparental recombination should have 3′-ending strands in *cis* configuration (see Fig. 5 in reference 2). In contrast, early replicative loops observed in our laboratory had 3′ termini in *trans* configuration (1). (iv) Finally, normal initiative events do not occur at locations which would correspond to those of high frequency of recombination.

Before describing the genetic makeup of the progeny produced in the absence of gene 44 protein, we will comment on the control experiment in which we followed initiation in the presence of the gene 44 protein (i.e., upon infection with wild-type phage). The main reason, apart from providing immediate comparison with DNA synthesized in the absence of gene 44 protein, is to document that site specificity of initiation without the use of density label is similar to that described before in the density experiment. This contradicts the frequently vocalized possibility of "5-bromodeoxyuridine artifact." We expanded in this study, the number of genetic areas tested (namely, we added genes 39, 44-46, 55, 8-9, 19-20, and 23). We take this opportunity to update the list of genes tested. In summary, the observed pattern of initiation was similar to that described before (3). We did not discover new initiative areas corresponding to additionally tested cloned segments (see Fig. 2B). It should be observed that in this study we tested, among others, genes 39 and 41-40 and found them to be negative in normal initiation. These areas are included in the large *Sal*I restriction fragment spanning genes rII and 42. We are aware that Mosig and co-workers published autoradiographs interpreted to indicate predominant initiative areas within this span (11). We are not able to explain this apparent discrepancy.

Genetic representation of progeny DNA produced in the absence of gene 44 protein differs dramatically. There was little uptake to the area of genes 50-5. In contrast, the most pronounced uptake occurred in the area of genes 35-36, which normally is one of the most underrepresented in the initiation. Major peaks of the uptake in the absence of gene 44 protein were very sharp (i.e., did not have a tendency to taper gradually on both sides, as observed for normal initiation). One should note also that the values of relative gene representations were, in two independent experiments, very similar (see Fig. 2B).

Considering covalent joining of progeny to parental DNA, delayed replication coinciding in time with the previously described interparental recombination, and a dramatically changed site specificity of ^{3}H incorporation, we postulate that the observed uptake is restricted to initiation at site-specific recombinational intersections. The high representation at genes 35-36 might be due to the high frequency of recombination at this area. Indeed, it was shown by Mosig (10) that the area of genes 34-35 displays a very high frequency of genetic recombination relative to its physical size. In this same paper, Mosig showed that the region from rII to 56 shows low recombination for its physical length. This region contains gene 39, which we found here, in the absence of gene 44 protein, to be very underrepresented. This strengthens our interpretation that, in the absence of gene 44 protein, incorporation of label is restricted to the recombinational intersections. Our results suggest further that areas of tRNA, and to a lesser extent genes 40 and 7-8, should also display a high frequency of genetic recombination. We do not want to imply that the observed "atypical" initiations are caused by the absence of the gene 44 protein. It is more likely that, due to the absence of normal replication and the decrease in the total uptake of ^{3}H, the infrequent and aborted initiations at locations shown in this paper become unmasked. One can reason that priming and elongation from the origins stringently requires the presence of gene 44 protein, whereas elongation from the 3′ end of the recombinational intersection is less demanding of its presence.

We feel that these results might offer a novel physicochemical approach for analysis of the site specificity of recombination.

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