JOURNAL OF VIROLOGY, June 1971, p. 749-752
Vol. 7, No. 6
Printed in U.S.A.
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Source of the Nonlinear Dependence of Bacteriophage SP82 Transfection on Deoxyribonucleic Acid Concentration

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Received for publication 21 December 1970

Extracts of competent cells of *Bacillus subtilis* exhibited nuclease activity on radioactively labeled cell deoxyribonucleic acid (DNA). The activity was not decreased when Mg was omitted from the reaction mixture but was decreased to zero by addition of ethylenediaminetetraacetic acid. Of the other metals tested, only Ca increased nuclease activity more than Mg. Addition of 1.5×10^{-3} M Ca to transfection mixtures increased the nonlinearity of the relationship between number of transfectants and DNA concentration. The inferred role of the Ca-activated nuclease was checked by showing that the ultraviolet enhancement of transfection is reversed by addition of Ca. It was also shown, by testing in three different laboratories, that the Ca ion in water is the likely source of the different nonlinear relationships found in different laboratories.

When bacteria are made competent for transformation, the number of transformants is linearly proportional to the concentration of deoxyribonucleic acid (DNA) to which the bacteria are exposed. If the competent cells are exposed to DNA from phages which normally attack those cells, progeny phages are formed, and their number is usually linearly proportional to the DNA concentration, as for phage SP02 transfection of *Bacillus subtilis* (17). However, a small number of subtilis phages (7, 8, 18, 20) have been found whose transfection is proportional to a power of the DNA concentration greater than unity. Green (8) showed that phage SP82 transfection is sometimes proportional to as much as the fourth power of the DNA concentration, although usually a second- or third-power curve is obtained. He traced the nonlinear transfection to the inactivation of the DNA after its entry into competent bacteria (9, 10), resulting in very high recombination frequencies for crosses done by transfection. Recently (19) it was shown that DNA uptake is proportional to the first power of the DNA concentration in experiments in which transfection was at least second power in DNA concentration, yielding the direct interpretation that some DNA taken up did not function.

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It was also shown (5) that the numerical value of this power varied during the development of competence by the bacteria. SP82 transfection was of a lower power if done before peak competence had developed. Surprisingly, transfection by SP02 DNA was of a higher power if done before the time of peak competence for SP82 transfection. It would seem that at least two enzymes are required to discriminate between two different DNA species since the effects on the respective DNA species were affected oppositely during the development of competence. Because SP82 DNA contains hydroxymethyluracil (HMU) in place of thymine (Kahan and Kahan, quoted in reference 3), it was postulated (5) that heterologous DNA species are attacked by an enzyme whose activity increases as competence develops, whereas there is a decreased attack on DNA species like that of SP02 which is a temperate phage whose DNA is, physicochemically, homologous to that of the bacteria.

These inferences about enzymes in competent cells can be studied by examining extracts of such cells. In this paper, the results of examining nuclease activities of extracts are reported. A Ca-activated nuclease attacking only denatured DNA is shown to be capable of effecting the observed nonlinear dependence of transfection on DNA concentration.

MATERIALS AND METHODS

Descriptions of the general materials and methods of preparation and transfection of competent cells have been published previously (5, 6). DNA was denatured by exposure to boiling-water temperature for at least 5 min followed by quick cooling in an ice-water mixture. Protein was determined by the method of Lowry et al. (13).

Cell extracts were prepared as described by Okasaki and Kornberg (16) except that, on the advice of H. V. Aposhian, the Mg concentration was one-seventh that used in that reference. Cells for extracts were centrifuged, washed once in 0.1 M Tris buffer (*p*H 8), and taken up in that buffer plus 10^{-4} M 5-mercaptoethanol. Cells were then sonically treated in a Branson Sonifier, and extracts remained fully active for several weeks if stored at 4 C. Nucleotide triphosphates were obtained from P-L Biochemicals, Inc. ^{3}H-thymidine triphosphate (7 Ci/mmole) was obtained from New England Nuclear Corp. (Boston, Mass.).

Reaction mixtures (0.3 ml) normally contained 0.1 ml of extracts (0.5 mg of protein/ml), 0.02 ml of ^{3}H-labeled DNA (20 μg/ml), and 0.05 ml of water or water solutions of metal ions or ethylenediaminetetraacetic acid (EDTA) as described. Mixtures were incubated for 30 min at 37 C, 5 to 6 ml of iced trichloroacetic acid was added, and the tubes were left in ice for at least 15 min before being filtered through Whatman GF/C filters and washed with a similar volume of iced trichloroacetic acid. Filters were dried in an oven, placed in scintillation vials in 10 ml of toluene (containing 42 ml of liquifluor/liter), and counted in a Packard liquid scintillation counter whose ^{3}H background was 20 counts/min. The ^{3}H-DNA from *B. subtilis* was that described in reference 19. To give an idea of the experimental numbers on which conclusions are based, the untreated *B. subtilis* DNA gave a counting rate of more than 1,000 counts/min on the filters. Typical solubilizations of 65 and 85% of the starting count means that the counting rates were, respectively, 350 and 150 counts/min. The data given in this report were all taken ftom total counts of at least 1,000 so the theoretical standard deviations are about 3% or less.

RESULTS

Measurements of enzyme activity were carried out on three kinds of cells: broth-grown cells at concentrations between 10^{8} and 10^{9}/ml, cells growing for 4 hr in the precompetency medium of Anagnostopoulos and Spizizen (1), and cells diluted from the precompetency medium into the competency medium in which they became maximally competent after 60 to 90 min of incubation.

Table 1 gives the results of 4 experiments typical of the 15 performed with extracts of competent cells. The units are per cent counts per minute in DNA made acid-soluble during 30 min at 37 C.

The observation that activity exists without added Mg and is entirely inhibited by EDTA could indicate the existence of another activating metal in cells which have been centrifuged and washed before being resuspended in a tris (hydroxymethyl)aminomethane-mercaptoethanol buffer. Various monovalent and divalent ions were tested at 10^{-3} M. A lower activity level was obtained with Mn and no activity was found with Na, K, or NH_4. But, 10^{-3} M Ca raised the solubilization percentage to 85% of the DNA (which is the maximum value obtained with the particular batch of denatured DNA).

TABLE 1. *Nuclease activities in competent cell extracts*

Determination	Per cent counts/min solubilized			
	Expt 1	Expt 2	Expt 3	Expt 4
Heat-denatured DNA plus				
0.0014 M $MgSO_4$	64	66	71	53
Water	71	67	70	81
0.001 M EDTA		0		
0.001 M $CaCl_2$	85	87	82	92
Native DNA under all conditions	Zero to 11, averaging about 3			

If a Ca-activated enzyme is involved in the nonlinear transfection process, it should be possible to predict the results of transfection experiments in which Ca has been added. The increased activity of this enzyme should lower the transfection level and increase the requirement for recombination to obtain plaques. Figure 1 shows the result of a typical experiment of this type.

Transfection mixtures containing Mg yielded a typical (for our laboratory) second-power curve. When Ca was added to 1.5×10^{-3} M, the transfectant number dropped and the curve became a third-power one. At about half this Ca concentration, the effect on transfection was barely detectable.

Two other experiments were done to verify the implication of this Ca-activated enzyme in nonlinear transfections. First, experiments with Ca at various *p*H values gave a slightly greater solubilization rate at *p*H 6.2 than at *p*H values above 7. Correspondingly, transfection at *p*H 6.2 was decreased and a 2.4-power curve was obtained.

The second experiment revealed a striking role of the Ca-activated enzyme in transfection enhancement by ultraviolet light (UV). It was previously (4) shown that transfection is increased and becomes linear with DNA concentration if competent cells are irradiated with UV before adding the transfecting DNA. This effect was interpreted as showing that photoproducts of the

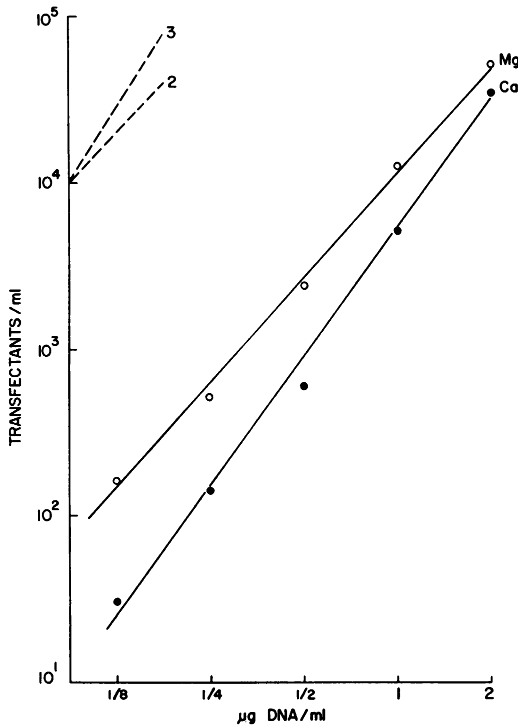

FIG. 1. *Plaques formed as a result of infecting 10^8 competent cells per ml with various concentrations of SP82 transfecting DNA, with Mg or Ca ions added to 10^{-3} M. The dashed lines are theoretical second- and third-power curves.*

irradiation bind the enzyme responsible for the nonlinear relation. If so, addition of Ca should increase the effectiveness of whatever enzyme activity is available, so there should be a shift of the transfection back toward a lower level and a nonlinear dependence on DNA concentration. Figure 2 shows the results of a typical experiment on transfection, its UV enhancement, and the "enhancement" in the presence of 1.5×10^{-3} M Ca.

Transfection enhancement by UV was reversed by the addition of Ca to the transfection medium. Thus, this Ca-activated enzyme is capable of producing the nonlinear dependence on DNA concentration, although the possibility that another metal or another enzyme might be the one whose effect is normally measured is not excluded.

Next, we measured DNA solubilization by extracts of broth-grown cells, precompetent cells, and cells which were maximally competent. The solubilization percentages in a typical experiment were, respectively, 29, 41, and 74%. Making cells competent roughly triples the enzyme activity The average relative activity in 11 experiments was just under 3, but the variability was great, with ratios lying between 1.3 and 4; the majority, of course, were near 3.

Finally, it was possible to look into the question of why different transfection powers are obtained in different laboratories and, occasionally, in one laboratory at various times. Experiments on transfection were done at Tel Aviv University, at the Marine Biological Laboratory in Woods Hole, and at Brandeis University. All experiments used the same DNA preparations and the same strain of bacteria.

At Woods Hole, transfection with and without Ca gave the same results as obtained at Tel Aviv University. At Brandeis University, third-power transfections were obtained whether Ca was added or not Since all of the chemicals and the cells were carried directly from Woods Hole to Brandeis, the only obvious source of difference

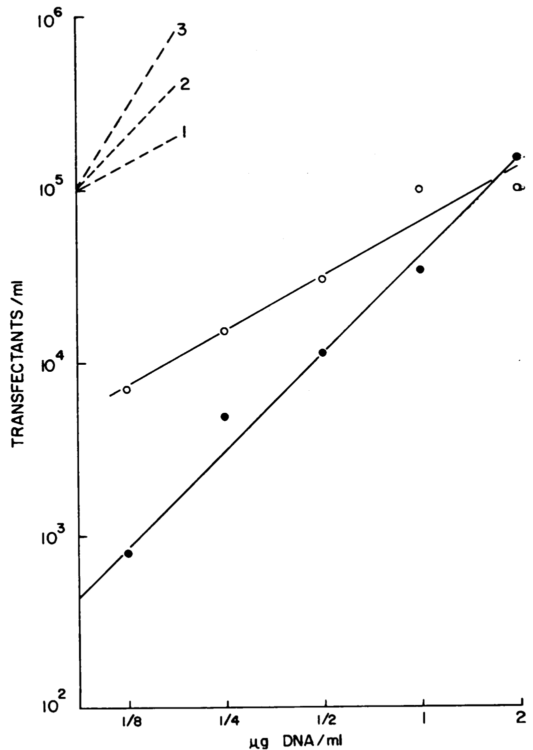

FIG. 2. *Plaques formed as a result of infecting 10^8 competent cells per ml with various concentrations of SP82 transfecting DNA. Open circles refer to transfection of cells irradiated with 10^4 ergs/mm^2 of ultraviolet light. Closed circles refer to transfection of the same irradiated cells in the presence of 10^{-3} M Ca. The dashed lines are theoretical first-, second-, and third-power curves.*

was in the distilled water. Accordingly, triply distilled water was used to make up new media, and, with these solutions, the transfections were the same as in the other two laboratories. The inference is that the standard distilled water at Brandeis contained appreciable amounts of Ca which were removed by the extra distillation steps.

DISCUSSION

The working hypothesis about competence development presented in an earlier report (5) supposed that cells become binucleate, cease DNA synthesis but go through at least one more cell division, thereby become less dense, take up DNA, and examine it (by enzymes) for homology to cell DNA. There is a lowered activity of enzymes attacking homologous DNA, whereas there is an increased activity of enzymes attacking heterologous DNA. Except for the decrease of activity of enzymes attacking homologous DNA, this entire picture is now supported by experiments done before and after its proposal (2, 3, 11, 14, 21). We are turning to a more sensitive assay of nuclease activity to determine if the initial stages of enzyme action on heterologous and homologous DNA species can exhibit the postulated differentiation.

An extracellular Ca-activated nuclease has been recently reported by Tevethia and Mandel (23). Its connection with competence cannot be inferred from the published information.

It should be kept in mind that, in all of the work reported herein, the entire culture was used. Several workers (12, 15, 22) have estimated the maximum fraction of competent cells in competent *B. subtilis* cultures at between 15 and 20% of the total. Thus, the conclusions with respect to the state of the competent cells are extrapolations from the altered properties of the entire culture.

However, transfections followed the expectations based on enzyme activities measured on extracts of the whole culture. Thus, there is evidence that these enzymes are acting in the cells which actually exhibit competence.

Therefore, the inferences about nuclease activity, though inferred from properties of entire cultures, are in fact valid, especially for the cells exhibiting biological competence. Accordingly, the nuclease changes correlate with changes leading to establishing competence. Since both competent and noncompetent cells have undergone these changes, there is clearly at least one more factor that determines the ultimate biological functioning of the absorbed DNA, for otherwise the entire culture would be competent. It should be of interest to carry out similar studies on cultures of pneumococci which are believed, for genetic and cytological reasons, to be 100% competent.

ACKNOWLEDGMENTS

The author is pleased to acknowledge the skilled aid and cooperation of Anita Gottlieb.

This work was done during the tenure of a Guggenheim Fellowship.

LITERATURE CITED

1. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. 81:741–746.
2. Bodmer, W. F. 1965. Recombination and integration in *Bacillus subtilis* transformation: involvement of DNA synthesis. J. Mol. Biol. 14:534–557.
3. Cahn, F. H., and M. S. Fox. 1968. Fractionation of transformable bacteria from competent cultures of *Bacillus subtilis* on renografin gradients. J. Bacteriol. 95:867–875.
4. Epstein, H. T. 1967. Transfection enhancement by ultraviolet light. Biochem. Biophys. Res. Commun. 27:258–262.
5. Epstein, H. T. 1968. Factors affecting bacterial competence for transfection and transfection enhancement. Bacteriol. Rev. 32:313–319.
6. Epstein, H. T., and I. Mahler. 1968. Mechanisms of enhancement of SP82 transfection. J. Virol. 2: 710–715.
7. Foldes, J., and T. A. Trautner. 1964. Infectious DNA from a newly isolated *Bacillus subtilis* phage. Z. Vererbungslehre 95:57–65.
8. Green, D. M. 1964. Infectivity of DNA isolated from *Bacillus subtilis* bacteriophage, SP82. J. Mol. Biol. 10:438–451.
9. Green, D. M. 1966. Intracellular inactivation of infective SP82 bacteriophage DNA. J. Mol. Biol. 22:1–14.
10. Green, D. M. 1966. Physical and genetic characterization of sheared infective SP82 bacteriophage DNA. J. Mol. Biol. 22:15–22.
11. Hadden, C., and E. W. Nester. 1968. Purification of competent cells in the *Bacillus subtilis* transformation system. J. Bacteriol. 95:876–885.
12. Javor, G. T., and A. Tomasz. 1968. An autoradiographic study of genetic transformation. Proc. Nat. Acad. Sci. U.S.A. 60:1216–1222.
13. Lowry, D. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
14. McCarthy, C., and E. W. Nester. 1967. Macromolecular synthesis in newly transformed cells of *Bacillus subtilis*. J. Bacteriol. 94:131–140.
15. Nester, E. W., and B. A. D. Stocker. 1963. Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis*. J. Gen. Microbiol. 49:267–275.
16. Okasaki, T., and A. Kornberg. 1964. Enzymatic synthesis of deoxyribonucleic acid. XV. Purification and properties of a polymerase from *Bacillus subtilis*. J. Biol. Chem. 239:259–268.
17. Okubo, S., and W. R. Romig. 1965. Comparison of ultraviolet sensitivity of *Bacillus subtilis* bacteriophage SP02 and its infectious DNA. J. Mol. Biol. 14:130–142.
18. Okubo, S., B. Strauss, and M. Stodolsky. 1964. The possible role of recombination in the infection of competent *Bacillus subtilis* by bacteriophage deoxyribonucleic acid. Virology 24:552–562.
19. Oostindier-Braaksma, F., and H. T. Epstein. 1970. DNA fixation and development of transformability and transfectability in *Bacillus subtilis*. Mol. Gen. Genet. 108:23–27.
20. Reilly, B. E., and J. Spizizen. 1965. Bacteriophage deoxyribonucleate infection of competent *Bacillus subtilis*. J. Bacteriol. 89:782–790.
21. Singh, R. N., and M. P. Pitale. 1968. Competence and deoxyribonucleic acid uptake in *Bacillus subtilis*. J. Bacteriol. 95:864–866.
22. Somma, S., and M. Polsinelli. 1970. Quantitative autoradiographic study of competence and deoxyribonucleic acid incorporation in *Bacillus subtilis*. J. Bacteriol. 101:851–855.
23. Tevethia, M. J., and M. Mandel. 1970. Nature of the ethylenediaminetetraacetic acid requirement for transformation of *Bacillus subtilis* with single-stranded deoxyribonucleic acid. J. Bacteriol. 101:844–850.