Mechanisms of DNA Transformation

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INTRODUCTION

Plasmid transformation of *Escherichia coli* is now a cornerstone of modern molecular biology, being widely utilized for cloning and amplifying DNA sequences. Its origins were set in the early 1970s with the discoveries that treatment of *E. coli* and *Salmonella typhimurium* (official designation, *Salmonella enterica* serovar Typhimurium) at 0°C in a solution of divalent cations, notably calcium chloride, rendered those cells competent for DNA uptake and establishment of episomal replicons (11, 37, 41, 46, 63, 73, 74, 79) or integrative recombinants (12, 13, 28, 53, 82). Subsequent research (4, 5, 14, 24, 33, 40, 50, 64, 65, 78, 84, 85) has further characterized the transformation process and led to the development of conditions that produce high-efficiency DNA transformation, whereby about 1% of the plasmid DNA molecules effect a transformed cell and 10% of all cells are competent. More recently, techniques for electroporation have been introduced (9, 18), in which a mixture of *E. coli* cells and DNA is subjected to a transient high-voltage electric field, thereby inducing transfer of that DNA and establishment of the transformed state. Ten percent of the plasmid DNA molecules produce transformed cells when this technique is used, and up to 95% of the cells are competent for transformation. In contrast to the obligatory involvement of divalent or multivalent cations for the "classical" procedure, electroporation does not require treatment with multivalent cations and in fact is best performed at very low ionic strength. These two methods for inducing competence for transformation are distinct in terms of their optimal physiological conditions and will be referred to as chemical competence and electrocompetence. Moreover, the conditions for inducing high-efficiency competence vary in both methods with the genetic constitution of the *E. coli* strain, providing some insight into mechanism. The methodology involved in producing chemically competent and electrocompetent *E. coli* has been presented in depth in two recent reviews (26, 26a). In this chapter, we will consider the parameters of the two methods and their implications for the mechanisms of DNA transformation.

A PERSPECTIVE ON THE PHYSICAL PROCESS OF TRANSFORMING *E. COLI* CELLS WITH DNA

The *E. coli* and *S. typhimurium* cell envelopes are composed of an outer membrane, a rigid cell wall, and an inner membrane. The two membranes are fused through holes in the cell wall, called zones of adhesion (3, 47). A variety of experimental evidence supports the view that the zones of adhesion are channels through which macromolecules are transported. There are about 400 zones of adhesion in a typical *E. coli* cell. The outer membrane is composed of a phospholipid bilayer and a variety of proteins, into which is anchored a lipopolysaccharide (LPS) array. The LPS consists of a phosphate-linked saccharide core with long polysaccharide side chains extending out from the cell. Both DNA and the cell can be considered polyanions, the DNA with its phosphate-rich backbone and the cell with a phospholipid surface and the phosphate-rich LPS core. These two large polyanions must associate, which could be considered to be electrically unfavorable. The sizes of these two interacting structures are a factor as well. The typical *E. coli* cell is a rod 0.2 μm in diameter and 1 μm long. The length of common plasmids ranges from 2 to 20

μm. It is therefore evident that inside the *E. coli* cell plasmids must organize into compact minichromosomes, much as the bacterial chromosome is condensed (chapter 12, this volume). Thus, not only must plasmid DNA be introduced into the cell but also it must be organized into a minichromosome and established as a replicon. These characteristics undoubtedly impact on the empirical identification of factors that influence transformation and should be kept in mind as the parameters are discussed.

PARAMETERS OF CHEMICAL COMPETENCE

The feature which has endured throughout continuing investigations of DNA transformation of *E. coli* and *S. typhimurium* with chemical treatments is the requirement for incubation of cells and DNA in a solution of multivalent cations at temperatures near 0°C. Calcium ions are not strictly required for competence induction but, rather, are among the most efficient cations at eliciting susceptibility for DNA uptake and transformation. A number of multivalent cations are capable of effecting DNA transformation when the cells and DNA are incubated with the salt at 0°C (25). Manganese(II) (D. Hanahan, unpublished data) and barium(II) (75, 76) are each actually better than calcium(II) for some strains. A variety of other compounds and conditions have been found to improve transformation frequencies, including freeze-thaw cycles (17, 31; J. Jessee and F. R. Bloom, U.S. patent 4,981,797, January, 1991), organic solvents (24, 40), and sulfhydryl reagents (24). It is expected that most of these compounds influence DNA association and transport across the cell envelope, since none enhance transformation when electroporation is used to effect DNA uptake (see below). There are currently two alternative methods for inducing high-efficiency chemically competent *E. coli* cells, each of which is most effective on different lineages of *E. coli,* which are distinguishable by mutations in genes affecting the structure of the cell envelope. The conditions are compared in Table 1 and discussed below.

Chemical Modification of the Cell Surface

The original observation (46) that treatment of *E. coli* with calcium chloride at 0°C induced a state of competence for DNA transformation has been followed by a series of investigations that have collectively identified chemical compounds and combinations thereof (termed transformation buffers) capable of enhancing transformation efficiency. In previous reports (24, 26, 26a), we have described the methodology and the approaches used to identify compounds and optimize their levels and combinations. To summarize, the compounds that are most essential for chemical competence induction are di- and multivalent cations: calcium, manganese, magnesium, and hexamine cobalt(III). In addition, competence of most *E. coli* strains is improved by addition of dimethyl sulfoxide (DMSO) and dithiothreitol. The two formulations of chemical competence-inducing conditions that produce high-efficiency transformation are termed “simple” and “complex.” The simple conditions involve a combination of divalent cations and a pH buffer, whereas the complex procedure includes in addition the monovalent cation potassium, the trivalent cation hexamine cobalt, and DMSO and dithiothreitol (Table 1).

Growth of *E. coli* in elevated levels of magnesium ions (e.g., 20 mM) improves subsequent competence for cells being treated with the chemical transformation conditions. The presence of magnesium in the growth medium is thought to alter the cell surface by reducing the number of protein-LPS bonds by increasing the number of ionic bonds mediated by the divalent cation (42). The substitution of ionic for covalent bonds results in increased fluidity of the LPS on the cell surface, which we infer then renders it more susceptible for reorganization or removal during the competence-induction process involving multivalent cations and low temperatures. The use of rich growth media (digested casein and yeast extract) is quite important for efficient transformability; the use of minimal media results in cells of considerably lower competence (at least 100-fold reduced). Results of transformation experiments with chemically defined rich media suggest that there is no special ingredient but, rather, that rapidly cycling cells are most susceptible to transformation (Hanahan, unpublished).

TABLE 1 Comparison of conditions used in high-efficiency DNA transformation procedures that involve chemical induction of competence

Simple conditions	Complex conditions
Calcium(II) chloride	Calcium(II) chloride
Manganese(II) chloride	Manganese(II) chloride
Magnesium(II) chloride	Potassium chloride
	Hexamine cobalt(III) chloride
Potassium acetate (pH 7.0)	MES buffer (pH 6.0)
Incubate at 0°C	Incubate at 0°C
	Add DMSO plus dithiothreitol
Incubate with DNA (0°C)	Incubate with DNA (0°C)
Heat pulse (approx. 90 s): (0°C → 42°C → 0°C)	Heat pulse (approx. 90 s): (0°C → 42°C → 0°C)

Temperature Effects on Transformation

Incubation of *E. coli* at 0°C in buffers containing multivalent cations is crucial for inducing competence. Moreover, a rapid temperature transition (a heat shock) further improves transformation frequency (46). Typically, following incubation of cells and DNA in a transformation buffer, the mixture is transferred to a water bath at 37 to 42°C for 30 to 120 s and then quickly returned to 0°C. The heat shock is effective only after the competent cells are incubated with the DNA; therefore, it appears to act on a later phase of the the transformation process (see below). Since membrane fluidity is dramatically affected by temperature, one can infer that transient relaxation of the quasi-crystalline membrane state achieved at 0°C facilitates completion of the uptake process for the long DNA molecules.

The temperature at which *E. coli* is grown before the cells are collected for competence induction can also influence the efficiency of transformation. We (Jessee and Bloom, U.S. patent) and others (31) have observed that growth of *E. coli* cells at reduced temperatures (25 to 30°C) improves their subsequent transformability compared with that of cells grown at 37°C, especially when the competent cells are frozen and thawed prior to performing the actual transformation. *E. coli* cells growing at 25 to 30°C have an increased proportion of unsaturated membrane phospholipids relative to cells growing at 37°C (45), which probably increases membrane fluidity and hence susceptibility to reorganization during the competence induction process in the presence of the transformation buffers at 0°C.

Further, there is a clear correlation between the growth temperature of the cells and the optimal time and temperature of the heat shock. Van Die et al. (81) found that growing cells at 22°C resulted in a downward shift in the optimal heat shock temperature by 5°C. Alternatively, we have found that cells grown at 25 to 30°C require a shorter heat shock time at the standard temperature of 42°C (80; F. R. Bloom and J. Jessee, unpublished observations). Taken together, these results suggest that cells grown at lower temperatures synthesize membranes with fewer saturated lipids, resulting in increased fluidity. The data suggest that more-fluid membranes require less thermal energy to transiently reorganize (or relax) the cell envelope (or, more specifically, its uptake channels), thus enhancing DNA transfer. In contrast to chemically induced transformation, electroshock efficiencies are not improved by growth of cells at lower temperatures (J. Jessee, unpublished observations), consistent with the notion that the chemicals and heat shock are in part affecting structural changes in the membranes that are unnecessary for DNA transfer by electroporation.

Genetic Factors Influencing Chemical Competence Induction

The two high-efficiency chemical transformation protocols (Table 1) have proved to be differentially effective on genetically distinct strains of *E. coli.* The procedure involving the complex transformation buffer produces the most efficient transformation of many strains of *E. coli* K-12, including several that are widely used in molecular cloning experiments: HB101 and strains in the MM294 lineage (DH1, DH5α, and JM109) (see reference 26 and references therein). In contrast, strains in the lineage of MC1061 (10), including DH10B (23) and DH12S (43), are much more susceptible to transformation under the simple conditions with only divalent cations. This difference probably resides in the constitution of the LPS component of the outer membrane. MC1061 is *gal*, which results in LPS molecules lacking much of their lengthy O side chains. If strain DH5α, which is most susceptible to the complex conditions involving DMSO, dithiothreitol, and hexamine cobalt, is rendered *gal*, the resultant strain (DH5αE) is now preferentially transformed by the simple conditions of divalent cations (J. Jessee and F. Bloom, unpublished observations). DMSO, dithiothreitol, and hexamine cobalt(III) no longer improve transformation, much as for strains in the MC1061 lineage. Therefore, we infer that during competence induction, dithiothreitol, DMSO, and hexamine cobalt(III) are reorganizing the long O side chains of the LPS, likely to facilitate interaction of the transforming DNA with the uptake channels into the cell (see below). Magnesium during growth and divalent cations plus 0°C temperatures may therefore act both on the LPS core and on the phospholipid surface of the cell, under both simple and complex transformation conditions.

These results are consistent with previous studies that investigated the effects of varying the composition of the LPS on the susceptibility for plasmid transformation. So-called “rough” strains, which lack the O-linked polysaccharide that extends out from the cell surface, transform considerably more efficiently than smooth strains, which have an extensive O-linked LPS (76, 77). Similar studies with *S. typhimurium* confirm the generality of this effect (44) as, again, mutations which produce short LPS molecules confer improved transformability on the strains which harbor them. Interestingly, however, complete removal of the LPS is not beneficial to transformation. Strains totally deficient in LPS are much less susceptible to transformation than those with short LPS. Comparisons of a series of both *E. coli* and *S. typhimurium* mutants that lack increasing amounts of the LPS have demonstrated that removal of the first few residues actually reduces transformation drastically (44, 76, 77). This may reflect the participation of the LPS core in the organization of the outer membrane, which includes a variety of protein-LPS complexes (see, e.g., reference 7). The data suggest, therefore, that removal of the long O side chain extensions improves access of the DNA to the cell surface. These side chain extensions probably evolved to keep large structures such as antibodies at a distance and probably have the same effect on approaching DNA molecules, thus necessitating their removal or reorganization to allow efficient uptake. However, the core of the LPS may serve positive functions at the cell surface in terms of facilitating productive DNA interactions. Thus, the chemical and genetic factors identified by empirical investigation may be balancing both positive and negative effects of LPS on the outer membrane structure and accessibility of the uptake channels.

FACTORS INFLUENCING ELECTROSHOCK TRANSFORMATION

The treatment of both prokaryotic and eukaryotic cells with a brief pulse of high-voltage electricity has been found to permeabilize them toward entry of a variety of macromolecules (36, 49). It is presumed that the discharge of a voltage potential across a field which includes cells transiently depolarizes their membranes and induces pores which can be entry points for these macromolecules. In particular, electroshock treatment is applicable to DNA transformation of *E. coli* and other bacteria. For *E. coli,* electroshock transformation is the most efficient method available and approaches the theoretical maximum frequency of 100% cell transformation (9, 18, 71).

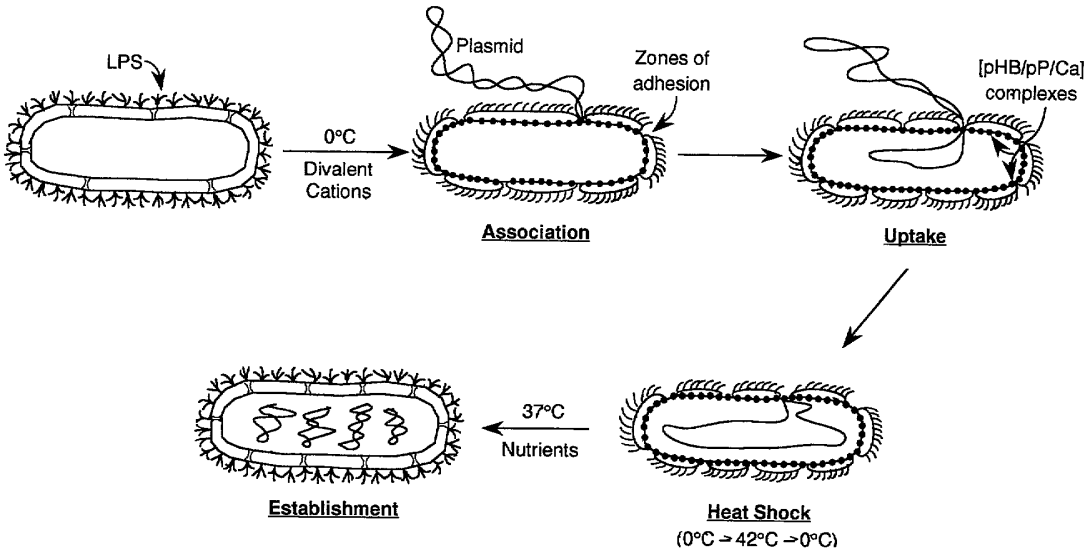

FIGURE 1 Schematic view of the stages of plasmid transformation of *E. coli*. Transfer of *E. coli* cells to 0°C in a solution of divalent cations induces reorganization of the LPS superstructure and solidification of the inner and outer membranes, rendering the zones of adhesion (macromolecular transport channels) accessible to DNA association; these conditions also induce the formation of [poly(HB):poly(P):Ca^{2+}] complexes (a putative DNA transporter), which are localized to the inner membrane. Over a period of minutes, DNA is taken up into the cell, perhaps in the context of these complexes. A heat shock that transiently melts the membranes completes the uptake process. Addition of medium and incubation at 37°C result in establishment of the plasmid, a process that includes its replication and organization into an episome (a minichromosome).

An electroshock is generated by the discharge of a high-voltage capacitor through a mixture of bacterial cells and DNA suspended between two electrodes. The pulse length of the capacitor discharge can be varied by increasing the capacitor size or the resistance in the circuit itself, which includes the mixture of cell suspension and DNA. A parallel resistor can also be used to modulate the resistance of the circuit. The time of the electric current pulse (the shock) is described by a decay time constant τ, which corresponds to the time at which the voltage has dropped to ~37% of its original value. The time constant of the electroshock is determined by the product ($\tau = R \times C$) of the resistance (both of the cell-DNA mixture and of any parallel resistor) and the capacitance of the circuit through which the electric field is being discharged. Field strengths used for optimal electroshock transformation of *E. coli* range from 12.5 to 16.7 kV/cm (18, 71). Several other parameters in addition to those of the electroshock itself influence transformation efficiencies. *E. coli* MC1061 (10) and its derivative DH10B (23) give the highest frequencies of electroshock transformation. Growth of cells in medium without added Mg^{2+} produces the highest competence. In addition, cells must be washed extensively to remove all salts, and the final cell slurry should be at a density of 5×10^{10} to 1×10^{11} cells per ml, with an optical density at 550 nm of >250 U.

Comparison with the parameters of chemical competence provides some insight into both processes. It is crucial to minimize the ionic strength of the cell-DNA mixture for electroshock transformation. Since the transformation frequencies are even higher than those achieved with chemical competence, it can be concluded that none of the multivalent cations or other modifiers of the LPS, including calcium(II) itself, are essential for inducing "physiological competence" for transformation. However, the use of *gal* mutant strains also improves electroshock transformation. Therefore, we can infer that here again, the O side chains interfere with association between DNA and the cell. This conclusion is supported by experiments

defining optimal *Salmonella* strains for electroshock transformation (52), as well as by recent experiments wherein the *gal* region of DH10B was transferred into DH5α, producing a strain, DH5αE, with improved electroshock transformation efficiency (B. Donahue and F. Bloom, unpublished observations). Thus, one common denominator between the simple chemical transformation method and the electroshock transformation method is the increase in efficiencies as a result of absence of the long O side chains of the LPS.

EVALUATION OF TRANSFORMATION EFFICIENCY

The efficiency of transformation can be evaluated from two perspectives, that of the DNA and that of the cell. The probability that a DNA molecule will give rise to a transformed cell is commonly used to measure transformation efficiency. Values are often given as the number of transformed colonies (cells) that would be formed per microgram of plasmid DNA (XFE). This is readily converted to a molecular probability, given the mass of the DNA molecule. For example, plasmid pBR322 is often used as a standard. Transformation efficiencies ranging from 1×10^6 to 2×10^9 CFU/μg of supercoiled pBR322 DNA are characteristic values, which means that the probability of an individual plasmid molecule transforming a cell ranges up to 1%, given that there are about 2×10^{11} molecules of pBR322 per μg. With electroshock transformation, efficiencies exceed 2×10^{10} CFU/μg, giving molecular probabilities of 10%.

Transformation probability (or transformation efficiency) represents the interaction of one DNA molecule with one cell, as it indicates how efficiently that DNA molecule can enter the cell and become established as a stable transforming agent (whether a multicopy episome, a bacteriophage infection, or a chromosomal integration). For this reason, transformation probability is best measured under conditions of cell excess, in which the number of cells far exceeds the number of DNA molecules.

The second criterion for evaluating transformation is the ability of individual cells to become transformed. In other words, given a population of cells, what fraction of those cells are capable of becoming transformed? This measure is generally assessed under conditions of DNA excess, in which the number of DNA molecules far exceeds the number of cells, such that all cells capable of transformation will have sufficient DNA molecules to give rise to that event. For chemical transformation procedures, the fraction of competent cells (F_c) ranges up to about 12% of the viable cells in a population, depending on the genetic background of the strain and the method of preparation. For electroshock transformation with strains such as DH10B, up to 90% of the viable cells can be transformed under conditions of DNA excess.

CHARACTERISTICS OF THE TRANSFORMATION PROCESS

The response of various forms and types of DNA, both as transforming molecules and competing with transforming molecules, has provided some perspective on possible mechanisms of transformation. Plasmid transformation experiments have addressed the possible involvement of specific sequences in the process. When plasmids are mixed with various types of DNAs and then combined with competent cells, retardation in the transformation probability is observed. These reductions follow a standard saturation curve. All DNAs tested were found to compete similarly with plasmids for transformation; these DNAs included distinguishable plasmids, *E. coli* DNA, and human DNA (24). The fact that all DNAs compete similarly indicates that there is probably not a sequence-specific interaction of DNA with the cell. Such questions have been motivated by the observations that DNA transformation of *Haemophilus parainfluenzae* is mediated by a specific DNA sequence, which binds to a protein on the *H. parainfluenzae* envelope (69). A second distinction is that single-stranded DNA is transferred during transformation of *H. parainfluenzae* (19, 29, 70). Transformation of *E. coli* with bacteriophage DNA carrying thymine dimers created by UV irradiation has shown that the dimers are excised and correctly replaced, which argues strongly that double-stranded DNA is transferred as such into the *E. coli* cell (72). Taketo (73) has shown that both single- and double-stranded bacteriophage DNAs can be transfected into *E. coli*, with their

relative efficiencies dependent on the strain and conditions used.

The effects of plasmid size on transformation efficiency provide some insight into the pores or channels in the cell envelope through which the DNA is entering. When the plasmid size is varied from 2 to 66 kb, the molecular probability that a plasmid molecule will give rise to a transformed cell declines linearly with increasing size (24). Furthermore, there is no significant difference between relaxed and supercoiled forms of plasmids. These characteristics indicate that the pore or channel size is not of the order of the average three-dimensional size of (or space occupied by) the DNA molecule. If plasmids were diffusing through "holes" in the cell, one would expect to see a difference between compact supercoils and the larger relaxed forms, and, furthermore, there should be an abrupt reduction in transformation probability as the plasmid size exceeds the channel size, such that it can no longer comfortably diffuse into the cell. When one considers that the persistence length of a 66-kb plasmid is 20 μm, while the dimensions of an *E. coli* cell are roughly 0.2 by 1 μm, it is clear the channel size cannot be "bigger" than such large plasmids. This observation strongly argues for DNA uptake through some deliberate action, which includes the organization or compaction of that DNA into a chromatin-like condition as it enters the cell, so that it will fit both into any conceivable pore diameter and into the volume of the cell.

A similar inverse correlation between increasing size and transformation probability is observed with electroshock transformation, although one study reported a more rapid falloff (~eightfold) in efficiency when comparing transformation probabilities of a 65- and an 85-kb plasmid (71), suggesting a possible size threshold for efficient DNA transfer. However, there is not a strict size limit, since libraries of bacterial artificial chromosomes in low-copy-number plasmid vectors carrying human DNA sequences of 130 to 150 kb (32) or 300 kb (68) have been successfully established in E. coli DH10B by electroshock transformation.

When increasing numbers of plasmids are used in a transformation, the transformation probability begins to decline when the number of plasmids exceeds the number of cells, and all the competent cells become transformed when the plasmid-to-cell ratio reaches about 200:1. When *recA* and *recA*$^+$ cells are compared, the *recA* cells, which are scored as viable cells rather than total cells (of which the viable cells are about one-half), require twice the levels of DNA before nonlinearity and saturation are observed (24). The simplest interpretation of such experiments is that all cells must compete for DNA (whether those cells are viable or not and competent or not) and that every cell has on the order of 100 channels through which DNA can be taken up. Cotransformation experiments (24, 37), in which distinguishable plasmids are frequently taken up even when supplied in disparate ratios, again indicate that there must be about l00 independent uptake pathways.

In summary, experiments with DNAs with different forms and from different sources have revealed several qualities of the transformation process: (i) there are about 100 independent channels through which DNA can be taken up; (ii) the interactions involved in uptake do not appear to require specific DNA sequences; and (iii) uptake is not likely to occur by passive diffusion through channels whose dimensions are large relative to that of the average volume occupied by the DNA molecule.

THREE STEPS IN THE TRANSFORMATION PROCESS

Comparison of the statistics of chemical and electroshock transformation suggests that the process of plasmid DNA transformation can be divided into three steps: (i) DNA association with the cell (binding); (ii) DNA uptake into the cell; and (iii) establishment of a stable episome. If chemically competent cells are treated with a vast excess of plasmid molecules (measuring F_c), about 10% can be transformed. Alternatively, if the cells are in excess (measuring XFE), only 1% of the plasmid molecules give rise to a transformed cell. In comparison, electrocompetent cells are virtually all susceptible to transformation; when plasmid molecules are in excess, about 90% of the cells become transformed. However, when the cells are in excess, only 10% of the plasmid molecules produces a transformation event. These comparisons suggest that there are three steps in the process. First, all cells associate with DNA, whether competent or not (see

the previous section). Second, the DNA is transferred into the cell (uptake). When chemically competent cells are used, about 10% of the cells are competent for uptake, whereas for electrocompetent cells, the fraction approaches unity. However, in each case, only about 10% of the plasmids taken up produce transformed cells, a third step referred to as establishment. Thus, for example, when virtually all electroshocked cells are competent, every plasmid molecule should produce a transformed cell, but the observation is that only 10% of plasmids produce a transformed cell under nonsaturating conditions. When chemically competent cells are used, every cell binds DNA (as seen in the *rec*$^+$/*rec* comparisons, etc.) but only 10% are competent, such that 10% of the plasmid molecules should produce a transformation. However, only 1% do, which again suggests that only 10% of the plasmids taken up by the 10% subpopulation of competent cells establish a stable transformation, resulting in the observed 1% XFE. Additional data supporting the proposition that transformation includes discrete uptake and establishment steps are discussed below.

When equimolar ratios of pBR322 and pACYC184 were supplied under saturating conditions (24), 70 to 90% of the cells transformed by one of these plasmids were actually doubly transformed, in that they carried both plasmids (pBR322 and pACYC184 are compatible and distinguishable). This result argues that chemically competent cells are capable of taking up multiple DNA molecules, a conclusion supported by a previous study with less-efficient competent cells (37). A similar characteristic of frequent cotransformation is observed when electroshock transformation is used (M. Smith, J. Jessee, S. Li, T. Landers, and F. Bloom, unpublished observations). Thus, we infer that the uptake process is quite efficient for cells that are competent, i.e., 10% of the viable cells treated by the chemical induction protocols and 90% of those treated with an electroshock.

When the number of plasmid molecules is varied over a very wide range, from plasmid-to-cell ratios of 1 plasmid per 6×10^4 cells up to ~500 plasmids per cell, the plasmid transformation probability is more or less constant until the number of plasmids exceeds the total number of cells, at which time the molecular probability declines. By this we infer that every cell (whether competent or not; indeed, even whether viable or not) competes for DNA by binding or association. At the equimolar point in the high-efficiency chemical transformation methods, 1% of the plasmids effect a transformed cell and 1% of all cells are transformed. Increasing the number of plasmids 100-fold results in a 10-fold increase in the number of transformed cells, arguing that increasing the number of plasmids being taken up does indeed improve establishment. In electroshock transformation, at the equimolar point where cell number equals plasmid number, 10% of the plasmids cause a transformation and 10% of all cells will become transformed. Increasing the plasmid-to-cell ratio 100-fold increases the fraction of cells transformed to 90%. The simplest interpretation of these data is to propose that in each case uptake is quite efficient and that multiple plasmids can be transferred into a cell by both methods. Then, in each case, there is an establishment phase with a probability of 0.1, which is modestly improved by supplying increasing numbers of plasmid molecules to every cell. Figure 1 presents a schematic view of the stages in plasmid transformation of *E. coli* cells rendered competent by divalent cations and low temperatures; the data underlying this scheme are discussed in the sections below.

DNA Association

The observation that all DNAs, whether mammalian or bacterial, of low or high complexity, of linear, relaxed-circular, or supercoiled forms, all compete for plasmid transformation when at 100-fold mass levels (relative to plasmid DNA) and at number excess over the available cells suggests that DNA readily associates with *E. coli* cells in a nonspecific but potentially productive fashion, probably at or near the major channels for macromolecular transport into (and out of) the cell. Both electroshock and chemical transformations require 0°C temperatures, which suggests that crystallization of the membranes (and perhaps the LPS) may be necessary for this association to occur efficiently. We propose that the initial association of DNA occurs at holes in the LPS network that are stabilized by low temperatures to allow access of the DNA macromolecule to uptake channels in the outer membrane. However, in lieu of chemical

or electroshock treatments, those channels are closed and not competent to proceed with DNA uptake.

DNA Uptake

For the chemical transformation procedure, multivalent cations and 0°C temperatures are obligatory for induction of competence for transformation. Multivalent cations are adept at shielding phosphates through the formation of relatively stable association complexes. Temperatures near 0°C in conjunction with millimolar concentrations of divalent cations will crystallize membranes, rendering the normally fluid structure relatively rigid and probably more susceptible both to stable phosphate shielding and to DNA association with the cell. Thus, one can postulate that low temperatures solidify the distribution of phospholipids, proteins, and LPS on the cell surface and that the cations act to shield the phosphates on the cell surface as well as on the DNA, thereby creating favorable conditions for DNA transfer across the cell envelope. For some strains, DMSO facilitates this reorganization and shielding. DMSO probably acts to organize and stabilize the charge space at the hydrophobic/hydrophilic boundaries produced by the phospholipids. For other strains such as DH10B, DMSO actually reduces transformation, perhaps because the *gal* LPS structure renders the cell excessively sensitive to its actions. The reorganization or relaxation of the cell envelope barrier functions by divalent cations at 0°C is also evident in the increased accessibility of periplasmic ampicillin-binding proteins to interaction with the antibiotic and by increased osmotic lability (21).

Some experiments have suggested that DNA enters the cell through large channels forming the zones of adhesion. Cobalamin (vitamin B_{12}) and several analogs associate with the zones of adhesion and are transported into the cell through binding to a specific transmembrane receptor (2, 7, 67). Addition of increasing amounts of cobalamin along with the DNA has revealed its ability to inhibit the transformation process (Hanahan, unpublished). A standard saturation curve can be produced with increasing levels of cobalamin, in which the molecular transformation probability declines in a manner indicative of competitive inhibition. Bacteriophage BF23, which enters cells by binding to the cobalamin receptor, also competes for DNA uptake (Hanahan, unpublished). Moreover, crude cell membrane preparations (79) and isolated outer membrane proteins competitively interfere with DNA transformation (8). The most likely explanation for these observations is steric exclusion by the prior occupancy of competing molecules in the uptake channels used by DNA.

Reusch et al. have implicated another type of channel in DNA uptake, which is composed of poly-β-hydroxybutyrate [poly(HB)], polyphosphate [poly(P)], and Ca^{2+} (59–61). Competent *E. coli* cells have elevated levels of poly(HB) in their cytoplasmic membrane (60). Synthesis of poly(HB) is induced upon transfer from exponential aerobic growth to 0°C and incubation in transformation buffer, as is formation of the [poly(HB):poly(P):Ca^{2+}] complexes. The appearance of [poly(HB):poly(P):Ca^{2+}] complexes coincides with a distinctive physical property: a membrane phase transition occurs at approx. 55°C as the temperature is raised, and both the complex and the phase transition correlate with competence for plasmid transformation. When acetaldehyde, an inhibitor of poly(HB) synthesis, was added as the cells were collected for incubation in transformation buffer at 0°C, competence for transformation was abolished whereas cell viability was unaffected (60). Thus, synthesis of poly(HB) correlates remarkably with susceptibility for DNA uptake, suggesting involvement in the process. Recently, Huang and Reusch (30) have presented evidence for distinct requirements for this complex and for exogenous divalent cations in the transformation process. Incubation of *E. coli* cells at 0°C in a variety of divalent cations induced competence and increased levels of the complex. However, when the [poly(HB):poly(P):Ca^{2+}] complex was purified, it contained only Ca^{2+}, arguing that endogenous stores of intracellular Ca^{2+} are sufficient to contribute to complex formation, provided that millimolar concentrations of mono- or multivalent cations and 0°C temperatures are present to induce poly(HB) synthesis and complex formation. However, induction of [poly(HB):poly(P):Ca^{2+}] complex formation, for example by 50 mM Na^{+} and 0°C temperatures, was not sufficient to induce competence, which required the presence of divalent cations,

apparently to perform other functions in the uptake process.

In a recent investigation of the poly(P) component of this putative uptake channel, Castuma et al. have shown that a special low-molecular-weight form of poly(P) is synthesized when cells are incubated in complex transformation buffer at 0°C (10a). This form is composed of 60 to 70 phosphate residues, in contrast to the 500- to 1,000-residue long form found in the cytosol. This special poly(P)$_s$ is also found associated with the cytoplasmic membrane, in association with p(HB) at a poly(HB)-to-poly(P)$_s$ ratio of 2:1. As with poly(HB), the level of this short-chain poly(P)$_s$ in cells also correlates with their degree of competence for DNA transformation. It is remarkable that incubation of *E. coli* cells in millimolar concentrations of cations at 0°C induces synthesis of two novel molecules and their assembly into a membrane-associated complex with Ca^{2+}, the prototypical competence-inducing factor.

Molecular modeling suggests that the [poly(HB):poly(P):Ca^{2+}] complex forms a transmembrane channel, in which poly(HB) forms a cylindrical barrel with a lipophilic exterior and a hydrophilic interior that is interlocked by Ca^{2+} ions, in association with the poly(P)$_s$ that fills the center of the barrel (61). A DNA uptake model has been proposed wherein DNA is transferred into the periplasmic space in a process that may be mediated by divalent cations (including Mn^{2+}, Ca^{2+}, and Mg^{2+}), where it then associates with the poly(HB) cylinder, entering the cell by displacing the poly(P) (59, 61). Interestingly, Taketo (74) noted 21 years ago that poly(P) inhibited Ca^{2+}-mediated bacteriophage DNA transfection. In the current model, poly(HB) is envisioned to wrap around the double helix of DNA, itself a poly(P), in the context of bridges formed by calcium ions, so as to produce a shield that facilitates DNA transfer across the membrane. It will be of interest to determine whether the [poly(HB):poly(P):Ca^{2+}]complex is associated with the zones of adhesion, in addition to its prevalence in the cytoplasmic membrane. Another intriguing question is whether free poly(HB), or the poly(P) channel it forms with poly(P)$_s$ and Ca^{2+}, plays additional roles in the cell. Since both poly(HB) and poly(P)$_s$ are present at very low levels in cells growing at 37°C and are synthesized only in response to low temperatures, other putative activities would seem likely to relate to the special physiological requirements for cell survival and function at low temperature. It is notable that the experiments of Reusch and Kornberg have been performed with fresh preparations of competent *E. coli* at relatively modest overall transformation efficiencies, of 10^7 CFU/μg of plasmid DNA. Currently it is possible to achieve 100-fold-higher efficiencies and to do so with frozen competent cells. Thus, it will be of interest to further investigate the relationships between levels of poly(HB) and poly(P)$_s$ that are synthesized and of the Ca^{2+}-dependent complex they form, as a function of increasing competence up to levels at which >10% of the cells are highly competent for plasmid DNA uptake. Finally, reconstitution of the [poly(HB):poly(P)s:Ca^{2+}] channels in synthetic liposomes may provide a method to directly test the ability of this putative channel to transport DNA across membranes, as well as to investigate its physiochemical properties and other possible functions.

A genetic factor that may regulate some aspect of the uptake process has been revealed by a mutational inactivation of a transcriptional repressor protein encoded by the *deoR* gene, which was originally described in the early 1970s as a regulator of nucleoside catabolism (1, 48). More recently, inactivation of *deoR* has been found to improve plasmid transformation of *E. coli* K-12 (D. Hanahan, U.S. patent 4,851,348, July, 1989). The phenotype of *deoR* mutant strains includes the ability of these strains to grow preferentially in a minimal medium containing inosine as the sole carbon source. This property was used by Hanahan to select a *deoR* derivative of *E. coli* DH1. The *deoR* strain, designated DH5, was found to be approximately three- to fourfold more competent than DH1 by using test plasmids pBR322, pUC8, and pUC9. Moreover, DH5 was ~30-fold more transformable than DH1 with a 66-kb plasmid. The *deoR* mutation therefore enhances the uptake of larger plasmids and may thereby facilitate the construction of gene libraries containing large inserts. The *deoR* mutation maps at min 19 of the *E. coli* K-12 chromosome (chapter 109, this volume) and is found in strain DH5 and its derivatives DH5α and DH5α MCR (23) as well as in strain DH10B (23). The beneficial effect of the *deoR* mutation on plasmid transformation efficiency is most pronounced when using chemical competence induction methods and the complex transformation buffer (Table 1), suggesting that a gene normally repressed by *deoR* is contributing to the uptake process, perhaps by altering the architecture of the cell envelope, since that is where the additives in

the complex conditions seem to be acting. It is notable, however, that loss-of-function mutations in three *deoR*-regulated genes that encode nucleoside transporters do not impair DNA transformation. Thus, strains defective in *nupC* and/or *nupG*, whose products are resident in the cytoplasmic membrane, can be efficiently transformed (Hanahan, unpublished), as can strains mutated in *tsx*, which encodes a porin localized to the outer membrane (76). Identification of the putative *deoR*-regulated gene that improves transformation could shed new light on the uptake process.

The ability of *E. coli* to take up DNA molecules with a relaxed length larger than the dimensions of the cell presents a case for active transport. However, there is no consistent genetic or physiological evidence to support this possibility. Regarding energy-dependent transport processes, Taketo (74), Sabelnikov and Domaradsky (64), and Reusch et al. (60) have collectively (and reproducibly) shown that azide, cyanide, arsenate, and 2,4-dinitrophenol fail to inhibit transformation, arguing strongly against an active transport mechanism, despite one report that the membrane potential is obligatory for transformation (66). Strains deficient in the cobalamin transporter (unpublished data) and nucleoside transport (76; Hanahan, unpublished) show normal ability to take up DNA, which indicates that these “pumps” are not involved. However, since cobalamin can compete with DNA for uptake, it is presumed that the two molecules share a common channel but utilize distinctive “binding” factors. Perhaps poly(HB) and divalent cations make up the DNA receptor in a channel that also contains other receptors, including the one for cobalamin. A genetic strategy to isolate mutants will be necessary to identify and clarify components of the transport system involved in DNA uptake; genetic inactivation of poly(HB) synthesis would seem a logical beginning.

Whatever the DNA uptake mechanism, it seems to frequently leave the DNA only partially transferred into the cell, as revealed by the beneficial impact on transformation efficiency of the heat pulse. Experiments testing DNase sensitivity before and after heat shock support its involvement in completing DNA transfer into the cell (4, 84). One can envision that the heat pulse transiently melts the crystalline membrane, thereby causing conformational changes that facilitate continuation of the DNA uptake process. However, the heat shock improves chemical transformation procedures only 10-fold, which indicates that the DNA uptake process can be completed without this treatment. However, the final step in all procedures is addition of medium and transfer to 37°C, which also melts the membrane structures and thus may facilitate completion of DNA transfer, albeit less efficiently than the transient heat pulse.

There is also evidence for similar association and uptake steps in the transformation of plasmid DNA by electroshock treatment. Eynard et al. (20) examined transfer of plasmid DNA into *E. coli* by using a square-wave electric pulse. Following a pulse of 4 ms at an electric field strength of 2 kV/cm, visual examination of the cells revealed a rapid alignment along the field lines. The pulse increased the amount of light transmitted through the cell suspension and reduced electrical resistance, suggestive of changes in cell shape and surface charge distribution of the LPS. Plasmid DNA added before (but not after) the electric pulse was taken up. When DNase was added before the electric pulse, transformation was abolished. Moreover, the DNA remained susceptible to DNase degradation for up to 3 s after the pulse. If the cells were immediately diluted into medium and transferred to 37°C after the electroshock, the transformation frequency was 10-fold higher than if the cells were first incubated on ice for 10 min, suggesting that a heat shock-like phase transition may also facilitate completion of the uptake process initiated by an electroshock.

The observations are consistent with the hypothesis that the electric pulse disrupts or reorganizes the cell envelope, thereby enhancing productive association and inducing partial uptake of the plasmid DNA. Eynard et al. (20) argue that the cell participates in the uptake process and indeed completes the transfer of the DNA into the cytoplasm. This latter step is slow relative to the electroshock itself and is influenced by temperature. It will be of interest to conduct similar experiments with the standard electroporation devices that produce exponentially decaying electric fields, to determine whether these separable steps, of initial association and uptake induced by the electroshock followed by completion of uptake by the cell facilitated by increased temperature, are involved in this higher-efficiency version of electroshock transformation.

Establishment of the Transforming DNA

The characteristics of the process suggest that transformation of *E. coli* (and *S. typhimurium*) by both chemical and electroshock protocols involves alteration in the envelope to allow productive interaction and uptake of DNA into the cell. The facts that (i) there are multiple independent channels for uptake of DNA molecules, as evidenced by cotransformation experiments, and (ii) providing a cell with more than one identical DNA molecule only modestly improves the prospects of that cell for becoming transformed suggest that establishment is not a simple probabilistic event. If establishment were, for example, a probability of completing an uptake process through 1 of 200 independent channels, the probability should be additive with the number of plasmid molecules occupying independent channels. Rather, it is necessary to supply a 100-fold excess of plasmid molecules to increase the transformation probability F_c 10-fold by either electroshock or chemical transformation. This comparison instead argues for the establishment stage occurring after the DNA molecules have been transferred inside the cell.

There are no genetic or biochemical hints to the molecular mechanism of the establishment step. It seems clear that plasmids cannot exist as naked DNA but, rather, must be organized and compacted into chromatinlike structures of protein and DNA, much like the cellular chromosome. It is possible that organization of chromatin proteins on the naked DNA is more difficult than the semiconservative transfer or duplication of chromatin complexes on established episomes during replication, such that the initial assembly is rate limiting and perhaps dependent on factors that are not continuously present throughout the cell cycle. Thus, nonfunctional minichromosomes might be produced frequently, with only 10% of the transferred plasmids correctly assembled, thereby allowing initiation of replication and subsequent establishment of a stable episome. Correctly assembled chromatin could also be the appropriate substrate for the recombination events which occur during homologous integration of transferred chromosomal DNA, thus producing similar parameters of inefficient establishment (4, 5). Another possibility is that the cell cycle is involved, perhaps in the form of replication initiation proteins with low abundance and short half-life, such that multiple replicons increase the probability of assembling a functional replication complex, which then becomes self-sustaining. The future may see an intersection of studies on *E. coli* chromatin, cell cycle, and plasmid replication with those on establishment of transferred plasmid DNA, leading to clarification of the mechanism.

METHYLATION-DEPENDENT RESTRICTION DURING DNA TRANSFORMATION

E. coli K-12 contains two general classes of genetic restriction functions that serve to monitor DNA entering the cell: (i) the classical K-type restriction-modification system (*hsdRMS*), which restricts only unmodified DNA, and (ii) a class currently comprising three methylation-dependent restriction activities (referred to as the methylation-dependent restriction systems [MDRS]). While it seems likely that the K-type restriction system has evolved to control bacteriophage infections, this second class may represent a component of a natural mechanism of DNA transformation designed to allow limited genetic diversification; its existence is of considerable importance for molecular cloning experiments involving genomic DNAs from other organisms. In contrast to the K-type system, the restriction specificities making up the MDRS are not balanced by cognate sequence-specific methylases. These restriction systems are also under distinct physiological controls (35), belying separate functional purposes.

There are presently four genes known to be involved in genetic restriction of transforming DNA if and only if that DNA is methylated (51, 54, 56). Incoming DNA containing methylcytosine can be degraded by restriction endonucleases specified by the *mcrA*, *mcrBC*, or *mrr* genes. In contrast, if the incoming DNA contains methyladenine, only the *mrr* gene product is capable of restricting that DNA (27). The *mcrA* gene is located at 25 min on the *E. coli* linkage map (57; chapter 109, this volume), while the *mcrBC* and *mrr* genes are closely linked at 98.5 min, near the *hsdRMS* genes (27, 57; chapter 109, this volume). While the *mrr* locus was originally thought to specifically restrict methyladenine-containing sequences, recent work

has revealed that the *mrr* gene product can also restrict certain sites modified with methylcytosine (34, 38, 58, 83); this activity was formerly suggested to be a gene closely linked to but distinct from *mrr*, called *mcrF* (34, 58).

The genotypes of *E. coli* strains for the *mcrA, mcrBC,* and *mrr* loci can be determined by methylating appropriate plasmids in vitro or in vivo and transforming the test strain with both methylated and unmethylated plasmids. For example, methylation with either the *Alu*I or *Hae*III methylases is diagnostic for the *mcrB* gene (56). Transformation of an *mcrB*$^+$ strain with an *Alu*I- or *Hae*III-methylated plasmid results in a 100-fold decrease in transformation efficiency relative to that obtained with an unmethylated plasmid. Both methylated and unmethylated plasmids transform an *mcrB* strain with similar efficiencies. Fine-structure mapping of the *mcrB* region has revealed that this locus is actually composed of both the *mcrB* gene, which encodes a 51-kDa protein, and an additional gene (*mcrC*), encoding a 39-kDa protein (62). Current evidence suggests that *mcrB* encodes a restriction endonuclease while the *mcrC* gene product acts to broaden the range of methylated DNA sites that are recognized and restricted by *mcrB.* For example, restriction of an *Sst*I- or *Msp*I-methylated plasmid requires only the *mcrB*$^+$ gene product, whereas restriction of an *Hae*II- or *Alu*I-methylated plasmid requires both the *mcrB*$^+$ and *mcrC*$^+$ gene products (16, 34). Thus, the *mcrC* gene product can be envisioned as a protein which alters the DNA sequence specificity of the *mcrB* restriction endonuclease. The *Hpa*II methylase can be used as a diagnostic test for *mcrA* (56), and the *Hha*II or *Pst*I methylase can be used as a diagnostic test for *mrr* (27). DNA methylated with the *Sss*I methylase has been shown to be restricted by *mcrA, mcrB, mcrBC,* and *mrr* (34). The *Sss*I methylase therefore provides a very stringent test for the presence of restriction activity against methylated CpG sites. The *mcr* and *mrr* genotypes (and phenotypes) of a number of commonly used host strains are discussed in several recent reports and reviews (39, 54, 55, 58, 86, 87).

These three methylation-dependent restriction activities can significantly impair molecular cloning in *E. coli* of genomic sequences from a wide variety of eukaryotic organisms that methylate their genomes, including most higher eukaryotes and many bacteria. Plant DNA may contain up to 25% of all cytosine as methylcytosine. In mammalian DNA, the values are 4 to 10% (references cited in reference 6). The existence of the MDRS in *E. coli* and the widespread methylation characteristic of other genomes argue that genomic DNA from most sources, prokaryotic or eukaryotic, should be cloned into a host strain that is deficient in all of the MDRS genes. Numerous examples of cloning experiments which resulted in recovery of clones only in MDRS-deficient strains are given in reference 55. Plasmid and bacteriophage rescue experiments, in which integrated bacterial vector sequences are recovered from eukaryotic cells, are also best performed with MDRS-deficient strains (22, 23, 38, 86). Woodcock et al. (87) have evaluated a number of *E. coli* strains for their ability to tolerate methylated cytosine sequences. The strains were analyzed by using primary genomic libraries in a bacteriophage λ vector prepared from either petunia or a mouse cell line DNA. The highest methylation tolerance was obtained with *E. coli* K-12 strains which were *mcrA* and contained a deletion eliminating the *mrr, hsdRMS,* and *mcrBC* genes. This conclusion is consistent with the analysis of a series of MDRS mutants for their ability to rescue plasmids from transgenic mouse DNA (23).

E. coli C, which lacks most if not all of the MDRS (15), also has a high methylation tolerance. At least a 10-fold improvement in the titer of a λ phage library containing methylated sequences can be expected when MDRS-deficient host strains are used (87).

It is intriguing to contemplate the possibility that the ability of *E. coli* to restrict DNA from divergent genomes during experimentally induced transformation reflects an evolutionary selection to perform a similar role in nature. *E. coli* and *S. typhimurium* reside in mammalian intestinal tracts. If DNA transformation is a process which can occur (albeit inefficiently) in its natural habitat, it could be that these organisms have developed a way to distinguish related DNA (self) from distant, foreign DNA (nonself). In evolution, it might have proved useful for bacterial cells to be able to exchange information by direct DNA transformation, but mammalian genes, with their coding information interrupted by intervening sequences and foreign control mechanisms, would not be likely to contribute, and the ability to distinguish between

the two DNAs could be beneficial. The mammalian intestinal tract is rich in divalent cations, and one could speculate that traumatic events such as death of the host organism (with consequent development of low temperatures) might set in motion genetic exchange mechanisms such as direct DNA uptake, so as to provide new diversity to the population of *E. coli* or *S. typhimurium* in an attempt to adapt to the new environmental challenges.

ACKNOWLEDGMENTS

We thank the following for communication of unpublished results and for comments on the manuscript: Joel Jessee, Life Technologies, Inc.; Arthur Kornberg, Stanford University; Elizabeth Raleigh, New England Biolabs, Inc.; and Rosetta Reusch, Michigan State University. We also thank Brooks and Elise Low for assistance with Fig. 1.

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