Calcium-Mediated DNA Adsorption to Yeast Cells and Kinetics of Cell Transformation by Electroporation

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ABSTRACT Detailed kinetic data suggest that the direct transfer of plasmid DNA (YEp 351, 5.6 kbp, supercoiled, $M_r \approx 3.5 \times 10^6$) by membrane electroporation of yeast cells (*Saccharomyces cerevisiae*, strain AH 215) is mainly due to electrodiffusive processes. The rate-limiting step for the cell transformation, however, is a bimolecular DNA-binding interaction in the cell interior. Both the adsorption of DNA, directly measured with [^{32}P]dCTP DNA, and the number of transformants are collinearly enhanced with increasing total concentrations [D_t] and [Ca_t] of DNA and of calcium, respectively. At [Ca_t] = 1 mM, the half-saturation or equilibrium constant is $\bar{K}_D = 15 \pm 1$ nM at 293 K (20°C). The optimal transformation frequency is $TF_{opt} = 4.1 \pm 0.4 \times 10^{-5}$ if a single exponential pulse of initial field strength $E_0 = 4$ kV cm^{-1} and decay time constant $\tau_E = 45$ ms is applied at [D_t] = 2.7 nM and 10^8 cells in 0.1 ml. The dependence of TF on [Ca_t] yields the equilibrium constants $K^0_{Ca} = 1.8 \pm 0.2$ mM (in the absence of DNA) and K'_{Ca} (at 2.7 nM DNA), comparable with and derived from electrophoresis data. In yeast cells, too, the appearance of a DNA molecule in its whole length in the cell interior is clearly an after-field event. At $E_0 = 4.0$ kV cm^{-1} and $T = 293$ K, the flow coefficient of DNA through the porous membrane patches is $k^0_f = 7.0 \pm 0.7 \times 10^3$s^{-1} and the electrodiffusion of DNA is approximately 10 times more effective than simple diffusion: $D/D_o \approx 10.3$. The mean radius of these pores is $r_p = 0.39 \pm 0.05$ nm, and the mean number of pores per cell (of size $\varnothing \approx 5.5$ μm) is $N_p = 2.2 \pm 0.2 \times 10^4$. The maximal membrane area that is involved in the electrodiffusive penetration of adsorbed DNA into the outer surface of the electroporated cell membrane patches is only 0.023% of the total cell surface. The surface penetration is followed either by additional electrodiffusive or by passive (after-field) diffusive translocation of the inserted DNA into the cell interior. For practical purposes of optimal transformation efficiency, 1 mM calcium is necessary for sufficient DNA binding and the relatively long pulse duration of 20–40 ms is required to achieve efficient electrodiffusive transport across the cell wall and into the outer surface of electroporated cell membrane patches.

GLOSSARY

[Ca_t]	total concentration of Ca
[Ca]	concentration of free Ca
[D_t] = c^0	total concentration of DNA
[D_b]	concentration of bound DNA
[P_2]	concentration of DNA permeable pores
a	cell radius
β_D	degree of DNA binding or adsorption to cell surface
D_0	diffusion coefficient of DNA
D	electrodiffusion coefficient of DNA
E_0	initial field strength
E	effective field strength
e_0	elementary charge
ε_0	vacuum permittivity
ε_w	dielectric constant of water
ε_L	dielectric constant of lipids
F	Faraday constant
f_T	degree of cell transformation
$f(\lambda_m)$	conductivity factor
$\overline{\Delta\varphi_m}$	mean electrical potential difference across the electroporated membrane patches
$\bar{K}_D$	equilibrium constant of overall DNA binding
K^0_{Ca}, K'_{Ca}	equilibrium constants of Ca binding in the absence of DNA and in the presence of DNA, respectively
k_B	Boltzmann constant
k_b	rate coefficient [mol^{-1} s^{-1}]
k_p	electroporation rate coefficient [s^{-1}]
k^0_f	flow coefficient [s^{-1}]
λ	wavelength
λ_m	transmembrane conductivity
λ_0	conductivity of bulk solution
λ_i	conductivity of cell interior
N_p	number of pores per cell
n^0_b	initial amount of cell components binding the penetrated DNA
n^{in}	amount of DNA in all cells
n^{in}_c	amount of DNA in one cell
n^{out}	amount of DNA in the bulk solution
r_p	pore radius
ρ_c	cell density
S_c	cell surface area
S_0	maximal electroporated area of the cell surface
S	electroporated area of cell surface
S_p	surface area of the average pore
t_E	electrical pulse duration
τ_E	decay time constant of the exponential field pulse
U	electrophoretic mobility
v	volume of sample
V_c	cell volume
z_{eff}	effective charge number (with sign) of the DNA-phosphate group

Received for publication 16 January 1996 and in final form 6 May 1996.

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0006-3495/96/08/868/10 $2.00

INTRODUCTION

The direct transfer of DNA into cells and microorganisms by membrane electroporation has become the method of

choice in cell biology and medicine. Since the early documentations of direct gene transfer by electroporation producing stable transformants of mammalian culture cells (Neumann et al., 1982; Wong and Neumann, 1982; Falkner et al., 1984; Evans et al., 1984; Potter et al., 1984), intact yeast cells and yeast spheroplasts have also been electrotransformed (Karube et al, 1984; Hashimoto et al, 1985; Meilhoc et al., 1990; Ganeva and Tsoneva, 1993). Among the various electrical and biological parameters characterizing electrotransformations of cells, the electroporative gene transfer is greatly facilitated by the adsorption of DNA to the cell surface (Wolf et al., 1989; Xie and Tsong, 1990a,b, 1992, 1993; Xie et. al., 1992; Neumann, 1992). Interestingly, alternating electric fields of low intensity promote DNA transfer into *Escherichia coli* bacteria, presumably by the electrical stimulation of DNA permeases (Xie and Tsong, 1990b). Evidence for the dominant electrodiffusive or electrophoretic contributions to the electroporative gene transfer of the polyelectrolyte DNA has accumulated (Klenchin et al., 1991; Sukharev et al., 1992; Spassova et al., 1994). Electroosmotic contributions (Dimitrov and Sowers, 1990) and membrane invagination facilitated by electroporation (Pastushenko and Chizmadzhev, 1992) have also been advocated.

Despite the numerous examples for the application of the electroporation technique, the molecular mechanisms of the DNA transfer into cells are not yet clarified to the extent that goal-directed and reliable directives can be given (Dimitrov, 1995). Model calculations usually cover only selected aspects such as the electrical properties and line tension (see, e.g., Glaser et al., 1987). Here we report on characteristic DNA parameters such as the electrophoretic mobility and the binding equilibrium constants of DNA and Ca^{2+} ions. The correlations between the electrotransformation of yeast cells and the DNA and Ca binding are quantified along the lines developed previously (Wolf et al., 1989; Neumann, 1992). The dependencies of the extent of electrotransformation on the electric field strength and the duration of the electroporation pulse are analyzed in terms of the Nernst-Planck (electrodiffusion) equation yielding estimates for the rate coefficient of membrane electroporation, for the average number of pores per cell, and for the mean radius of the electropores in the membrane patches permeable to the DNA. The relatively long pulse durations of 20–40 ms are required to guarantee an effective entrance of the DNA molecules into the surface of the electroporated membrane patches.

MATERIALS AND METHODS

Cells and plasmid DNA

Yeast cells of *Saccharomyces cerevisiae* AH 215 strain (MATa, *leu* 2–3, 2–112, *his* 3–11, 3–15) were used. The strain was cultivated in YEP (1% yeast extract (Difco, Detroit, MI) 2% peptone (Difco), and 2% glucose) media up to an optical density of OD = 1.3–1.4 at the wavelength $\lambda = 650$ nm, corresponding to a cell density of $\rho_c \approx 10^8$ cells/ml. The reversion rate of *leu* 2 was less than 10^{-9}, which is extremely low. The plasmid YEp 351 (5.6 kbp, relative molecular mass $M_r = 3.6 \times 10^6$) (Hill et al., 1986) compensates *leu* 2 mutation of *S. cerevisiae* (Ganeva and Tsoneva, 1993). The isolation and purification of the plasmid DNA in the supercoiled form was performed using the method of Maniatis et al. (1982). The DNA/Ca titrations and the electropulsing experiments were performed with cell suspensions of 1 M sorbitol, 1 mM Tris-HCl at pH 7.2. The condition [Ca] = 0 was achieved by 1 mM EDTA, pH 7.2. The conductivity of the medium at $[Ca_t] = 1$ mM is $\lambda_0 = 0.076$ S m^{-1}. The temperature of all experiments was $T = 293$ K (20°C).

Electrotransformation protocol

Because it is known that the treatment of yeast cells with thiol compounds enhances the electrotransformation frequency (Meilhoc et al., 1990), samples of 10^9 cells/ml, after washing the cells twice in water, were incubated in 25 mM 2-mercaptoethylammonium chloride for 30 min at $T = 303$ K (30°C), washed twice, and resuspended in 1 M sorbitol. The electrotransformation studies were performed either at a constant plasmid DNA concentration, varying the Ca^{2+} concentration or at different plasmid concentrations at a constant [Ca].

The sample chambers (Bio-Rad Laboratories, Richmond, CA) were equipped with flat parallel electrodes; the electrode distance is $d = 0.2$ cm. The chambers have to be used with alternate voltage polarity to avoid major anodic oxidation of the aluminum electrode material. The counterpotentials due to galvanic electrochemical surface reactions cannot exceed 3 V, which is negligibly small compared with the initial pulse voltage 600 V $\leq U_0 \leq$ 1200 V. In this voltage range and at the low medium conductivity ($\lambda_0 = 0.075$ S m^{-1}), corresponding to a low current density, the actual field strength E in the solution between the electrodes is estimated to $E \approx E_0 \times 0.90 \pm 0.05$ (see Pliquett et al., 1996).

To permit a straightforward data analysis in terms of a defined electroporated cell surface area, a single exponential pulse of initial electrical field strength $E_0 = U_0/d$ and decay time constant τ_E was applied. After the pulsing, 900 μl of 1 M sorbitol solution was added to the electroporation chamber and two samples of 100 μl were grown on minimal medium (2.5% agar, 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, 30 μg/ml histidine, 1 M sorbitol). The number of colonies, typically 500–800 (with ≤5% error margin) was counted after 5 days, although already after 2 days the colonies were clearly observable. The regeneration (viability) of the yeast cells after the various electrical treatments (in the absence of DNA) was measured by counting the colonies in the media containing 2.5% agar, 1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol. Each data point reflects five different experiments. The error margin is ≤15%.

DNA binding to the cell surface

The adsorption of DNA to the cell surfaces was determined by using radioactively labeled plasmid DNA, prepared with [^{32}P]dCTP (3000 mCi/mmol) by the nick translation method (Feinberg and Vogelstein, 1983). The binding experiments were performed with samples of volume $v = 0.1$ ml at $\rho_c = 10^9$ cells/ml. The samples were incubated for 5 min with 1 μg of plasmid DNA at different total concentrations ($[Ca_t]$) of Ca. The concentration $[D_b]$ of bound DNA was determined either at $[Ca_t] = 1$ mM or at [EDTA] = 1 mM (zero Ca concentration). The Ca^{2+} was applied as calcium acetate. The samples were centrifuged at 1000 × g for 10 min. The radioactivity of the supernatant was measured by a LKB-1215 RackBeta II analyzer.

Microelectrophoresis

The electrophoretic mobility of the yeast cells was measured at 298 K (25°C) using a Rank Brothers apparatus. The samples were filled into a rectangular quartz cell equipped with Pd electrodes and incorporated in a video monitor system. Cell movement was observed over $d_c = 175$ μm in both directions (reversing the field direction), and the cell velocity was

calculated from 20 observations in every set of experiments. The electrophoretic mobility (U) was calculated, using the expression $U = |\vec{v}|/|\vec{E}|$, where the electrical field strength $E = U/d_c$ is calculated from the measured voltage U.

RESULTS

DNA/Ca binding and cell electrotransformation

In Fig. 1 it is seen that the concentration $[D_b]$ of bound DNA and the density T of transformants TC in $v = 0.1$ ml run parallel (on a log-log scale) with increasing total concentrations $[D_t]$ of DNA at two different total Ca concentrations. DNA/Ca adsorption and gene transfer can be described with one and the same half-saturation constant $\bar{K}_D$. The DNA/Ca-binding data are consistent with the overall scheme:

$$D + Ca + B \overset{(K_D^0)}{\leftrightarrow} DB + Ca \overset{(K_{Ca}')}{\leftrightarrow} DCaB, \quad (1)$$

where B denotes a DNA/Ca-binding site on the cell surface and D_b is the overall bound DNA, both as DB and DCaB. In terms of total concentrations $[D_t]$ and $[B_t]$, the binding data are represented by the concentration $[D_b] = [DB] + [DCaB]$:

$$[D_b] = \frac{1}{2}\{([D_t] + [B_t] + \bar{K}_D) - \sqrt{([D_t] + [B_t] + \bar{K}_D)^2 - 4[D_t][B_t]}\}. \quad (2)$$

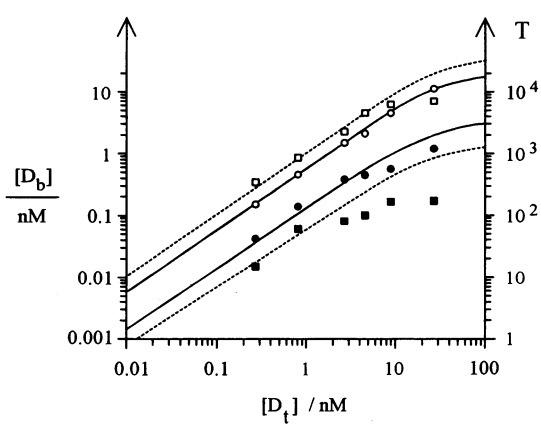

FIGURE 1 Collinearity of DNA adsorption and electroporative transformation of yeast cells. Concentrations $[D_b]$ of bound DNA (D) and density T of transformed cells TC in $v = 0.1$ ml solution, both as a function of the total concentration $[D_t]$ of DNA in the presence of Ca^{2+} and in the absence of Ca^{2+} (i.e., in the presence of EDTA) (logarithmic scales): ○, $[D_b]$, and □, T, at $[Ca_t] = 1$ mM; ●, $[D_b]$, and ■, T, at [EDTA] = 1 mM. Cell density $\rho_c = 10^8$ per 0.1 ml. The solution contained 1 M sorbitol, 1 mM Tris-HCl, pH 7.2, and T = 293 K (20°C). Plasmid DNA was YEp 351 (5.6 kbp). Binding/adsorption data were performed with [^{32}P]dCTP DNA. Electroporation conditions were one exponential pulse of the initial electric field strength $E_0 = 4$ kV cm^{-1} and the decay time constant $\tau_E = 25$ ms. Each data point is the mean of five separate experiments; the error margin is ≤5% for the binding data and ≤10% for the transformation density. Note that the effective field strength is $E = (E_0/\sqrt{2}) \times (0.9 \pm 0.05)$, because of the use of Bio-Rad aluminum electrodes in low conductivity medium ($\lambda_0 = 0.076$ S m^{-1}).

See Eq. A2 of the appendix. Note that $\bar{K}_D = [D]([B] + [CaB])/([DB] + [DCaB])$, $[D] = [D_t] - [D_b]$, and $[B] + [CaB] = [B_t] - [D_b]$, where [D] and [B] are the concentrations of the free DNA and the free binding sites, respectively.

In the range of $[D_t]$, where $[D_t] \leq \bar{K}_D$ holds, i.e., $[D_t] \leq 10$ nM for $[Ca_t] = 1$ mM and $[D_t] \leq 2$ nM for [EDTA] = 1 mM and where the electroporative transfer of (adsorbed) DNA may be considered independent of the other (adsorbed) DNA molecules, the density of transformants T is proportional to $[D_b]$. Hence, the degree of transformation f_T may be written as

$$f_T = T/T_\infty = [D_b]/[D_{b,\infty}], \quad (3)$$

where T_∞ and $[D_{b,\infty}]$ are the respective values at infinitely high DNA concentration. From Eq. 2 we obtain 1) at zero Ca^{2+} concentration, $\bar{K}_D([Ca] = 0) = K_D^0 = 25 \pm 2$ nM, $[D_{b,\infty}] = 4.0 \pm 0.2$ nM, $T_\infty = 1.3 \pm 0.1 \times 10^3$/0.1 ml; and 2) at [Ca] = 1 mM, $\bar{K}_D = 15 \pm 1$ nM, $[D_{b,\infty}] = 20 \pm 1$ nM, $T_\infty = 3.5 \pm 0.3 \times 10^4$/0.1 ml.

Note that Eq. 2 yields a practically very useful relationship for the half-saturation of DNA adsorption: $[D_t]_{0.5} = \bar{K}_D + 0.5[B_t]$. In the presence of EDTA, $[D_t^0]_{0.5} = 27 \pm 1.5$ nM (i.e., >2 nM); and at $[Ca_t] = 1$ mM, $[D_t]_{0.5} = 25$ nM (i.e., >10 nM) (see also Table 1).

In Fig. 2 we see that up to $[Ca_t] = 1$ mM the density of transformed cells and the fraction (β_D) of bound DNA run parallel with increasing $[Ca_t]$. The presence of Ca^{2+} not only increases the DNA adsorption but also enhances the concentration of the transformants (Figs. 1 and 2).

TABLE 1 Characteristic electrotransformation parameters (derived from data in Figs. 1 and 4) of yeast cells (*Saccharomyces cerevisiae*, strain AH 215)

	[Ca]	
	1 mM	0 ([EDTA] = 1 mM)
$\bar{K}_D$ (nM)	15 ± 1	25 ± 2
$[D_{b,\infty}]$ (nM)	20 ± 1	4.0 ± 0.2
T_∞ 0.1 ml	$3.5 \pm 0.3 \times 10^4$	$1.3 \pm 0.1 \times 10^3$
T_{max} 0.1 ml	$8.0 \pm 0.5 \times 10^3$	$2.0 \pm 0.2 \times 10^2$
TF ($[D_t] = 2.7$ nM)	$3.6 \pm 0.2 \times 10^{-5}$	
S_v	0.70 ± 0.05	0.70 ± 0.05
TF ($[D_t] = 0.8$ nM)		$1.4 \pm 0.1 \times 10^{-6}$
TF_{opt} ($\tau_E = 45$ ms)	$4.1 \pm 0.4 \times 10^{-5}$	

The sample volume is $v = 0.1$ ml, the cell density is $\rho_c = C_t/v = 10^8$ cells/0.1 ml, where C_t is the total number of cells both at [Ca] = 1 mM and at [Ca] ≈ 0 by [EDTA] = 1 mM, respectively. Single-pulse electroporation at the initial field strength $E_0 = 4.0$ kV cm^{-1}, field decay time constant $\tau_E = 25$ ms. Electroporation medium contained 1 M sorbitol, 1 mM Tris-HCl, pH 7.2, at 293 K (20°C). T is the transformant density, the number of transformed cells in $v = 0.1$ ml. T_∞ is the (extrapolated) limit value for $[D_t] \to \infty$; T_{max} is the actually obtained maximal value (here at $[D_t] = 10$–30 nM). The viability $S_v = RC/C_t$ of the cells is the fraction of regenerated cells RC after electroporation. The transformation frequency is defined as $TF = T/(S_v \cdot \delta_c)$ per μg of DNA in the volume $v = 0.1$ ml. TF_{max} is the respective maximal value obtained at the specified $[D_t]$ values, and TF_{opt} is the respective optimal value of TF. Every numerical value is the mean value of five separate experiments.

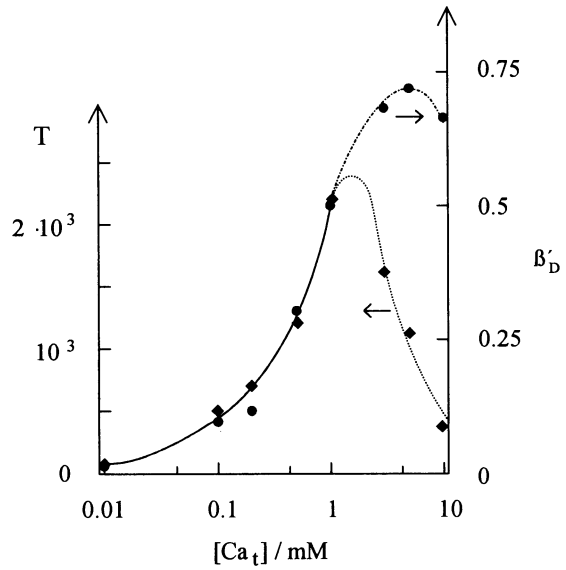

FIGURE 2 Transformation density T (TC in $v = 0.1$ ml) (♦) scaled with the fraction $\beta'_D = [D_b]/[D_t]$ of bound DNA (●) both as a function of total Ca^{2+} concentration (calcium acetate). $[D_t] = 2.7$ nM; other conditions are as in the legend of Fig. 1. Notice the apparent collinearity of T and up to $[Ca_t] = 1$ mM. Data points are mean values of five separate experiments. Error margins are 10% for T and 5% for β'_D, respectively.

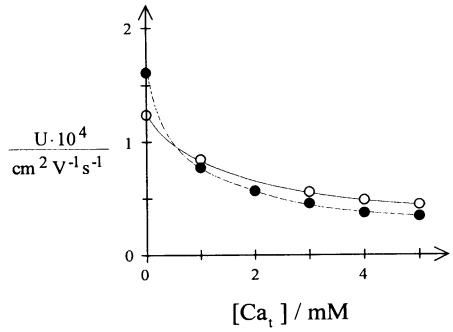

FIGURE 3 Electrophoretic mobility U_c of single yeast cells as a function of the total concentration $[Ca_t]$ of calcium, in the absence of DNA (○) and in the presence of 0.027 nM DNA (●), corresponding to $[D_b] = 0.0035$ nM. Cell density $\rho_c = 10^6$ per ml. The solution contained 1 M sorbitol, 1 mM Tris-HCl, pH 7.2, and $T = 293$ K (20°C). The data points are means of 20 experiments per point. The error margin is 8%.

For additional analysis it is practical to define the degree of DNA binding or adsorption by

$$\beta_D = \frac{[D_b]}{[D_{b,\infty}]} = \frac{[DB] + [DCaB]}{[B_t]} = \frac{[D]}{[D] + \bar{K}_D}, \quad (4)$$

where Eq. A1 of the Appendix was applied and where $[D] = [D_t](1-\beta_D)$ is the concentration of free DNA. Substitution of Eq. 4 into Eq. A1 yields

$$\bar{K}_D = \frac{(1-\beta_D)}{\beta_D}([D_t] - \beta_D[D_{b,\infty}]) = K_D^0 \frac{1 + [Ca]/K_{Ca}^0}{1 + [Ca]/K'_{Ca}}. \quad (5)$$

The values of β_D (and $[D_{b,\infty}]$) at the various Ca^{2+} concentrations may be calculated from the data in Fig. 2, where $\beta'_D = \beta_D[D_t]/[D_{b,\infty}]$; the respective $[D_{b,\infty}]$ values may be estimated by interpolation between the experimental values $[D_{b,\infty}] = 4$ nM at $[Ca_t] = 0$ and $[D_{b,\infty}] = 20$ nM at $[Ca_t] = 1$ mM. In the range where $[Ca] \ll K_{Ca}^0$, Eq. 5 reads $\bar{K}_D = K_D^0/(1 + [Ca]/K'_{Ca}$ and we obtain $K'_{Ca} = 0.8 \pm 0.1$ mM. For the condition $[Ca] \gg K_{Ca}^0$, hence $[Ca] \gg K'_{Ca}$, Eq. 5 reduces to $\bar{K}_D = K_D^0 \times K'_{Ca}/K_{Ca}^0$, yielding $K_{Ca}^0 = 1.8 \pm 0.3$ mM if $K_D^0 = 25$ nM (Fig. 1) is used. The results of the chemical/thermodynamic analysis of the data in Figs. 1 and 2 are summarized in Table 1.

Electrophoretic mobility of yeast cells

As shown in Fig. 3, the electrophoretic mobility of yeast cells decreases with increasing concentrations of Ca, in the presence as well as in the absence of DNA. At pH 7, yeast cells as well as DNA are negatively charged. The electrophoretic mobility of the cells in the absence of DNA and at $[Ca_t] = 0$ is $U_C^0 = 1.25 \pm 0.1 \times 10^{-4}$ cm^2 V^{-1} s^{-1}. In the presence of 0.027 nM DNA at $[Ca_t] = 0$, we obtain $U_{DC}^0 = 1.65 \pm 0.1 \times 10^{-4}$ cm^2 V^{-1} s^{-1}.

If we assume that U_C is proportional to the free anionic sites (B), we may apply a simple mass action law $K_{Ca} = [Ca][B]/[CaB]$ with $\beta_D = [CaB]/[B_t]$ and use the relationship

$$\frac{U_C}{U_C^0} = \frac{[B]}{[B_t]} = 1 - \beta_{Ca} = \frac{K_{Ca}}{K_{Ca} + [Ca]}, \quad (6)$$

where U_C^0 refers to the Ca^{2+}-free surface. Data evaluation according to Eq. 6 yields $K_{Ca}^0 = [Ca_t]_{0.5} = 2.3 \pm 0.3$ mM and $K'_{Ca} = 1.0 \pm 0.2$ mM. These estimates are consistent with the thermodynamic binding data of Fig. 2 ($K_{Ca}^0 = 1.8 \pm 0.3$ mM; $K'_{Ca} = 0.8 \pm 0.1$ mM).

Dependence of electrotransformation on the field strength and pulse duration

In Fig. 4 it is seen that the transformation frequency (*TF*) and viability (S_v) both depend on the choice of electrical parameters (field strength E_0 and pulse time constant τ_E). With increasing field strength, the viability decreases and the *TF* values first increase and then decrease.

Fig. 5 represents the data evaluation of Fig. 4 *B* in terms of a strength-duration relationship according to a polarization mechanism with $E_0^2 \times \tau_E = W_p \times$ constant, where W_p is the polarization energy (see, e.g., Neumann, 1992).

KINETICS AND TRANSPORT THEORY

The number or the density of transformants *T* and the classical transformation frequency *TF* are related by the degree of transformation:

$$f_T = \frac{T}{T_\infty} = \frac{TF}{TF_\infty}, \quad (7)$$

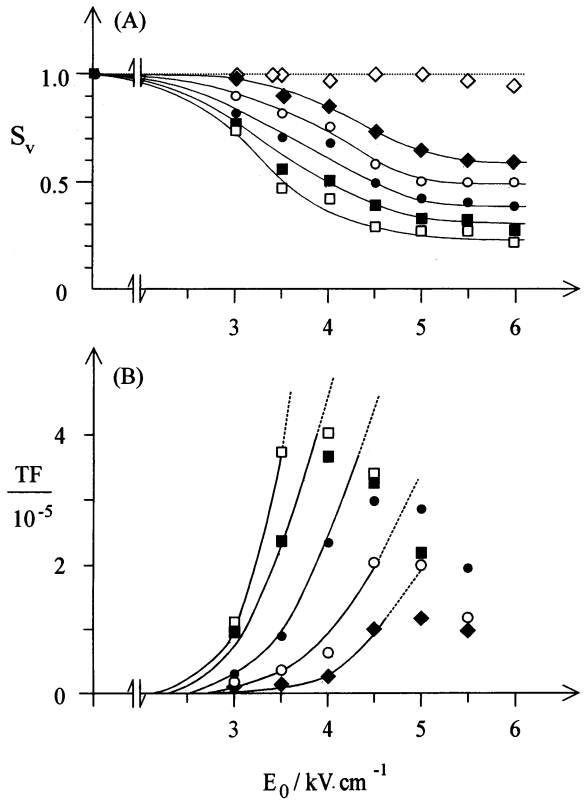

FIGURE 4 (*A*) Fraction (S_v) of regenerated cells (*RC*) relative to the total number C_t of cells ($S_s = RC/C_t$). (*B*) Transformation frequency $TF = T/(S_v\delta_c)$ per μg of DNA in the volume v = 0.1 ml, both as a function of the initial field strength E_0 at various pulse time constants τ_E/ms = 3.6 (◇), 10.7 (◆), 17.9 (○), 25 (●), 35 (■), and 45 (□). Solution conditions are 1 M sorbitol, 1 mM Tris-HCl, pH 7.2; T = 293 K; [Ca_t] = 1 mM.; [D_t] = 2.7 nM; $TF_\infty \approx 5.10^{-5}$. Note that E = ($E_0/\sqrt{2}$) × (0.9 ± 0.05).

where $TF_\infty = 5 \times 10^{-5}$ (see Fig. 4 *B*). The kinetic data suggest that f_T, presented in Fig. 6 as a function of the pulse time, does not directly reflect the extent of membrane electroporation although electroporation is a prerequisite for the cell transformation. In particular, the long delay phase followed by a rather steep increase of f_T with τ_E cannot be described solely in terms of simple membrane state transitions (Neumann et al., 1982). In addition to state transitions,

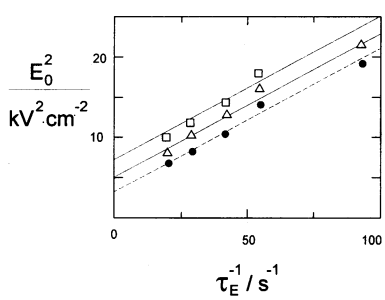

FIGURE 5 Field strength (E_0^2) as a function of the reciprocal (τ_E^{-1}) of the pulse duration at a few selected low *TF* values from Fig. 4 *B*: ●, 0.5 × 10^{-5}; ◇, 1.0 × 10^{-5}; □, 1.5 × 10^{-5}; consistent with $E_0^2\ \tau_E = W_p \times$ constant, where W_p is the polarization energy.

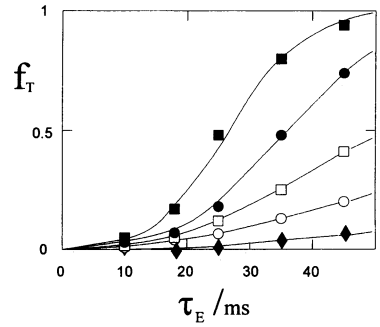

FIGURE 6 The degree of transformation $f_T = T/T_\infty$, where $TF_\infty = 5 \times 10^{-5}$ (Fig. 4) as a function of the pulse time constant (τ_E) at various initial field strengths E_0/kV cm^{-1} = 2.5 (◆), 3.0 (○), 3.25 (□), 3.5 (●), or 4.0 (■). The lines are calculated with Eq. 11 of the text and with the effective field strength $E = (E_0/\sqrt{2}) \times (0.9 \pm 0.05)$.

a bimolecular binding step is necessary. The data indicate that the transformation is rate limited by the association of the DNA molecules (D), which have entered the cell interior, yielding the complex Db with a cell component b, perhaps the nucleus. The data are described in terms of the simple scheme

$$D + b \xrightarrow{(K_b)} Db, \tag{8}$$

where the reverse reaction step is neglected.

The rate equation of the binding process can be written in terms of the amount of substances (n). The rate (dn_b/dt) of the decrease in the moles n_b of free binding sites upon binding of DNA available as n^{in} moles within the cell volume is given by

$$-\frac{dn_b}{dt} = k_b n^{in} n_b, \tag{9}$$

where k_b is the bimolecular rate coefficient (in mol^{-1} s^{-1}). The degree of transformation as a function of the pulse time t_E may then be approximated by the degree of integral DNA binding $f_b = n(Db)/n_b^0 = (n^{in}(t_E) - n^{in})/n_b^0$:

$$f_T(t_E) = f_b = \frac{n^{in}(t_E) - n^{in}}{n_b^0}, \tag{10}$$

where n^{in} (t_E) is the amount of DNA transported into the cell after an electroporation pulse of field strength E and duration t_E; n_b^0 is the total amount of cells.

The characteristic time constant (τ_T) of cell transformation (colonies visible as products of cell divisions) is approximately 2 h. The actual transport time of DNA through the long-lived electroporated cell membrane patches is estimated to be on the order of seconds (Eynard et al., 1992). We may, therefore, consider $n^{in}(t)$ to be practically constant for the time interval $t > t_E$, independent of time. Hence, the integration of Eq. 9 within the boundaries $t = 0$ and $t = \tau_T$ yields

$$f_T = \frac{n^{in}(t_E)\{1 - \exp[k_b\tau_T(n^{in}(t_E) - n_b^0)]\}}{n_b^0 - n^{in}(t_E) \cdot \{1 - \exp[k_b\tau_T(n^{in}(t_E) - n_b^0)]\}}. \tag{11}$$

The derivation of Eq. 11 is given in the Appendix. For the initial phase $n^{in}(t_E) \ll n_b^0$, the fraction of transformed cells $f_T \approx n^{in}(t_E)/n_b^0$ (see Eq. A5). In this limiting case, f_T is directly proportional to the DNA transported into the cells after the pulse (E, t_E) was applied. In the other limiting case, $n^{in}(t_E) \gg n_b^0$, the maximal value $f_T \approx 1$ is reached although the transport of DNA into the cells still continues (see Eq. A6).

The electrodiffusive transport of DNA into and across the electroporated membrane of the average cell is described by the Nernst-Planck equation for the mole flow density vector of the amount (n_c^{in}) of DNA in the direction of the external field E:

$$\frac{dn_c^{in}}{S \cdot dt} = -\frac{D_o}{d}\left[\frac{n_c^{in}}{V_c} - \frac{n^{out}}{v}\left(1 - \frac{|z_{eff}| \cdot e_0}{k_B \cdot T}\overline{\Delta\varphi_m}\right)\right], \quad (12)$$

where S is the electroporated surface area and V_c is the volume of the average cell; d is the membrane thickness, D_o is the apparent diffusion coefficient of DNA across the electroporated surface patches (in interactive contact with DNA), $|z_{eff}|$ is the absolute value of the effective charge number (with sign) of the DNA-phosphate group, e_0 the elementary charge, k_B the Boltzmann constant; T is the absolute temperature, $\overline{\Delta\varphi_m}$ the mean electrical potential difference across the electroporated membrane surface, n^{out} the amount of DNA, and v the volume of the bulk solution, respectively.

If we assume spherical geometry for the slightly ellipsoidal yeast cell, the ionic-interfacially-induced membrane potential difference $\Delta\varphi(\theta)$ depends on the positional angle θ relative to the electric field vector E. The stationary value for spherical cells of radius a is given by

$$\Delta\varphi(\theta) = -\frac{3}{2} aEf(\lambda_m)|\cos\theta|. \quad (13)$$

The choice of sign, consistent with the Maxwell definition of the electric field vector, implies that cations move in the direction of E and anions opposite to the direction of E.

The conductivity factor $f(\lambda_m)$ permits one to describe the changes in the membrane conductivity during the electroporation process (Kakorin et al., 1996). As DNA is a polyanion, the electrodiffusive part of the transport refers to only one hemisphere of the average cell. Therefore, the average potential difference term operative for the DNA-anion flux is defined by

$$\overline{\Delta\varphi_m} = \frac{1}{2}\int_0^{\pi/2} \Delta\varphi_m(\theta)\sin\theta\, d\theta = -\frac{3}{8} aEf(\lambda_m). \quad (14)$$

The description of the $f_T(t_E)$ data (Fig. 6) in terms of closed and open membranes states (Neumann et al., 1982) requires that the simple electroporation/annealing cycle C↔P (Neumann and Boldt, 1989), where C is the closed membrane state and P denotes electroporated states, has to be extended. The rather long sigmoid phase of $f_T(\tau_E)$ requires a description in terms of at least a two-step process:

$$C \xrightarrow{k_p} P_1 \xrightarrow{k_p} P_2, \quad (15)$$

where the state P_1 denotes pore structures of negligible permeability for DNA; P_2 is the porous membrane state of finite permeability for DNA. The electroporation rate coefficient k_p is assumed to be the same for both steps. Pore resealing, i.e., the reverse reaction steps ($P_2 \rightarrow P_1 \rightarrow C$), may be neglected in the presence of the external field.

Conventional kinetic analysis applied to the reaction sequence Eq. 15 yields the integrated rate equation (see Eq. A8 of the Appendix). Assuming that [P] is proportional to the surface S, we have $[P_2]/[P_2]_0 = S/S_0$ and we obtain for the build-up of the electroporated surface

$$S(t) = S_o(1 - (1 + k_p t)e^{-k_p t}), \quad (16)$$

where S_o is the maximal electroporated cell surface. In the range where the field strength/duration relationship $E_0^2 \times \tau_E = W_p \times$ constant holds (Fig. 5), $[P_2]_0$ and hence S_o are constants, independent of E.

We now substitute Eq. 16 into Eq. 12, integrate within the boundaries $t = 0$ and t_E and obtain

$$n_c^{in}(t_E) = \frac{n^{out}V_c}{v} \cdot \left[1 - \exp\left(-\frac{D_0}{V_c \cdot d}\left(1 - \frac{|z_{eff}| \cdot e_0}{k_B \cdot T}\overline{\Delta\varphi_m}\right)\int_0^{t_E} S(t)\, dt\right)\right], \quad (17)$$

where the surface integral is given by

$$\int_0^{t_E} S(t)\, dt = \{t_E + k_p^{-1}[(2 + k_p \cdot t_E)e^{-k_p t_E} - 2]\}.$$

If the total amount $n^{in} = n_c^{in} \times N_c$ of DNA inside all of the N_c cells is small compared with n^{out}, i.e., $n^{in} \ll n^{out}$, Eq. 17 reduces to

$$n^{in}(t_E) \approx k_f^0 c^0 N_c V_c \cdot \{t_E + k_p^{-1}[(2 + k_p t_E)e^{-k_p t_E} - 2]\}, \quad (18)$$

where $c^0 = n^{out}/v$. The conventional flow coefficient of non-equilibrium thermodynamics is given by

$$k_f^0 = \frac{DS_0}{dV_c}. \quad (19)$$

The (electrodiffusion) coefficient D involves the diffusion coefficient D_o and the electrical part, given in Eq. 14. Hence,

$$D = D_0\left(1 + \frac{3|z_{eff}|e_0 af(\lambda_m)E}{8k_B T}\right). \quad (20)$$

We now return to Eq. 11 and use the ratio $r_b = n^{in}/n_b^0$.

The substitution finally yields the explicit expression for the degree of electrotransformation of the cell ensemble as

a function of the pulse time t_E:

$$f_T(t_E) = \frac{r_b(t_E)[1 - y(t_E)]}{1 - r_b(t_E)[1 - y(t_E)]}, \quad (21)$$

where the term $y(t_E)$ is given by

$$y(t_E) = \exp(k_b \tau_T n_b^0 [r_b(t_E) - 1]). \quad (22)$$

If exponential pulses are applied, the pulse characteristic $E_0 \times \exp(-t/\tau_E)$ necessitates the introduction of a kind of an effective field strength E and an effective pulse time $t_E = \tau_E$ via the condition of equal polarization energies of the exponential and equivalent rectangular pulses: $(1/2)E_0^2 \times \tau_E = E^2 \times t_E$ (see Neumann, 1992). Here, due to aluminum electrode oxidation (Pliquett et al., 1996), the initial field strength in the solution is only $0.9 \times E_0$ (see the electrotransformation protocol). Therefore, at $t_E = \tau_E$, the effective field strength is $E = E_0/(1.11 \times \sqrt{2})$. The dependence of f_T on E yields the field strength dependence of the rate constant of electroporation according to (Kakorin et al., 1996):

$$k_p = k_p^0 \exp(b^* f^2(\lambda_m) E^2), \quad (23)$$

where k_p^o is the (poration) rate coefficient at zero field strength. The exponential term b* refers to the replacement of lipids by water during electric pore formation and is given by

$$b^* = \frac{9\pi\epsilon_0 a^2(\epsilon_w - \epsilon_L)\bar{r}_p^2}{8dk_BT}. \quad (24)$$

In Eq. 24, ϵ_o is the vacuum permittivity, ϵ_w and ϵ_L are the dielectric constants of the solution in the pore ($\epsilon_w = 80$) and of the lipid phase ($\epsilon_L = 2.5$), respectively, and $\bar{r}_p$ is the mean pore radius.

The data in Fig. 6 are evaluated in terms of Eqs. 18 and 21, yielding $k_p(E)$ (Fig. 7). The analysis of the data in Fig. 7 with Eqs. 23 and 24 gives the mean pore radii $\bar{r}_p(P_1) = 0.28 \pm 0.04$ nm and $\bar{r}_p(P_2) = \sqrt{2} \times \bar{r}_p(P_1) = 0.39 \pm 0.05$ nm of the P_1 and P_2 porous membrane states, respectively.

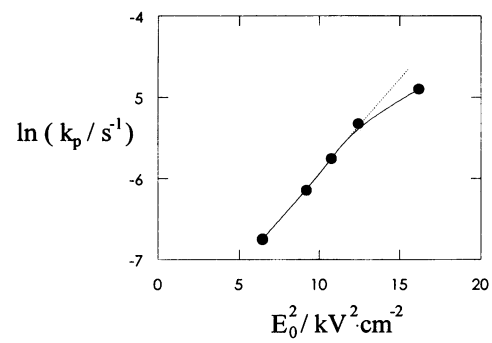

FIGURE 7 Rate constant k_p/s^{-1} of electroporation (logarithmic scale) as a function of E_0^2 for the evaluation of the mean pore size, using Eqs. 23 and 24 of the text and the effective field strength $E = (E_0/\sqrt{2} \times 0.9 \pm 0.05$.

DISCUSSION

It is recalled that, when properly scaled, the transformation frequency and the degree of DNA binding to yeast cells run parallel at the various Ca^{2+} concentrations (Figs. 1 and 2). Therefore, at low DNA concentrations ($[D] \leq K_D$) the degree f_T of transformation expressed in terms of the transformation efficiency TF, normalized to TF_∞, can be described with the same set of characteristic distribution constants (K_D, K_{Ca}^0, K'_{Ca}) as the degree (β_D) of DNA binding or adsorption, measured independently (see Table 1). The experimentally observed field strength/duration relationship $E_0^2 \times \tau_E \approx W_p \times$ constant suggests that the electrotransformation is consistent with membrane electroporation initiated by ionic interfacial polarization (Neumann, 1992).

At higher DNA concentrations, TF is reduced compared with the predictions derived from the lower DNA concentrations. One possible reason for the observed smaller effects may be that at high numbers of plasmid DNA molecules in the cell, DNAse activity or concentration inhibition of DNA replication may reduce the transformation frequency.

The addition of DNA to the cell suspension immediately after the pulsing only occasionally leads to transformants, typically the density is $T = 10 \pm 5$ in 0.1 ml as compared with $T = 2.0 \pm 0.2 \times 10^3$ in 0.1 ml at $E_0 = 4$ kV cm^{-1} and $\tau_E = 25$ ms. Under these conditions, the initial concentration of DNA in the bulk solution is $[D_t] = 2.7$ nM, corresponding to an initial amount of outside DNA of $n_0^{out} = 2.7 \times 10^{-13}$ mol in $v = 0.1$ ml. If we assume that ≤ 10 DNA molecules per cell, with $N_c = 10^8$ cells in $v = 0.1$ ml, are sufficient for the transformation of this cell, we have $n_c^{in} \leq 1.7 \times 10^{-23}$ mol and the total amount n^{in} of DNA inside all of the cells is $n^{in} = n_c^{in} \times N_c \leq 1.5 \times 10^{-15}$ mol, which indeed is small compared with $n_0^{out} = 2.7 \times 10^{-13}$ mol. Hence, for this case ($n_0^{out} \gg n^{in}$), Eq. 18 holds and $n^{out}/v \approx c^o = [D_t] = 2.7 \times 10^{-9}$ M is practically constant.

The volume $V_c = 4\pi a^3/3$, where $a = 2.7$ μm is the estimate for the radius of the average cell is $V_c \approx 10^{-13}$ dm^3 and $n_c^{in}/V_c \approx 1.7 \times 10^{-10}$ M. Hence, in Eq. 12 we may consider n_c^{in}/V_c as small compared with the term $(n^{out}/v)(1 - |z_{eff}| \times F \times \overline{\Delta\varphi_m}/RT)$. Under this condition, Eq. 12 is rewritten as mole flow equation

$$\frac{dn^{in}}{dt} = \frac{D_0 S}{dV_c} c^0 V_c N_c \left(1 - \frac{|z_{eff}| F}{RT} \overline{\Delta\varphi_m}\right). \quad (25)$$

Integration of Eq. 25 within the boundaries $t = 0$ and $t = t_E$ yields Eq. 18, yet in a more indirect way. Therefore, for practical purposes, the most useful expression for the fraction of transformed cells is

$$f_T \approx n^{in}(t_E)/n_b^0 \quad (26)$$

using $n^{in}(t_E)$ given in Eq. 18.

When the data in Fig. 6 were analyzed with Eqs. 18 and 21, we obtain $k_b n_b^0 \tau_T = 7.1 \times 10^{-2}$, a quantity that will not be made use of. As D_o of DNA in the electroporated

membrane area is not known, we apply the free diffusion coefficient $D_o \approx 2.5 \times 10^{-10}$ m^2 s^{-1} as derived from the Einstein relationship $D_0 = U \times k_B T/(|z_{eff}|\, e_0)$ using $z_{eff} = -1$ (phosphate) and $U \approx 10^{-8}$ m^2 V^{-1} s^{-1} (see Results). If the conductivities of the outside bulk solution $\lambda_0 = 0.076$ S m^{-1}, of the internal medium $\lambda_i = 1.0$ S m^{-1}, and of membrane $\lambda_m = a \times G_m \approx 2.7 \times 10^{-5}$ S m^{-1} ($G_m \approx 10$ S m^{-2} is the specific membrane conductance) are used, we estimate $f(\lambda_m) \approx (1 + \lambda_m \times (2 + \lambda_i/\lambda_0)/(2\lambda_i d/a))^{-1} \approx 0.9$ (see Neumann, 1989). The electrodiffusive part in Eq. 20 is estimated to be $3\,|z_{eff}|\, e_0 a f(\lambda_m) E/(8k_B T) \approx 9.3$ at $E = 2.57$ kV/cm. Thus, the electrodiffusion is approximately 10 times more effective than simple diffusion: $D/D_o \approx 10.3$. With $V_c \approx 10^{-16}$ m^3, the data fit yields the flow coefficient $k_f^0 = 7 \times 10^3$ s^{-1}, and hence from Eq. 19 we obtain the maximal electroporated cell surface: $S_o = 2.1 \times 10^{-14}$ m^2 per cell.

As the total surface of the average cell is $S_c = 4\pi a^2 \approx 10^{-10}$ m^2, the fraction of the maximal electroporated area conditioning the electrodiffusive DNA flow is $S_o/S_c \approx 0.023\%$. From the data in Fig. 7, using Eqs. 23 and 24, we obtain $b^* = 0.7 \pm 0.1 \times 10^{-10}$ (m/V)^2. Hence the mean radius of the pore in the DNA-permeable patches is estimated to be $\bar{r}_p(P_2) = 0.39 \pm 0.05$ nm. The mean number of pores in the membrane patches permeable to DNA is given by $N_p = S_o/S_p = 2.2 \pm 0.2 \times 10^4$ per cell, where $S_p = \pi \bar{r}_p^2 = 0.9 \pm 0.1$ nm^2 is the surface area of the average pore.

The mean pore radius $\bar{r}_p(P_2)$ is in the same order of magnitude $\langle r_p \rangle$ as of electroporated lipid vesicles (Neumann et al., 1992; Kakorin et al., 1996) and of planar membranes (see, for instance, Glaser et al., 1987). These pore sizes appear to be too small for free diffusion of DNA molecules. The pore size is not sufficient for the entrance of a free end of a linear DNA molecule (diameter of the double helix $\varnothing \approx 3$ nm) and not at all for the entrance of the more bulky circular DNA. The theoretical proposals of Pastushenko and Chizmadzhev (1992) cannot yet be checked experimentally. Therefore, as in the case of planar membranes (Spassova et al., 1994), the transport of DNA appears to be a thermally diffusive migration of the DNA molecule that transiently interacts with many small pores of an electroporated membrane patch. Adsorbed DNA appears to be drawn into the surface, thereby pushing lipid molecules transiently aside. The inserted DNA (the complex DP in Fig. 8) is at least partially pulled through the electroporated bilayer patches by the electric field. The longevity of the electroporated membrane state facilitates after-field diffusion of DNA into the cell (Neumann et al., 1982). Due to the small size of the pores, contributions of electroosmotic drag of the DNA across the electroporated membrane may be only small (Dimitrov and Sowers, 1990) and thus appears to be not essential. If there is an electroosmotic transport of DNA on the cell surface, it will be of minor importance for DNA transfer across the membrane.

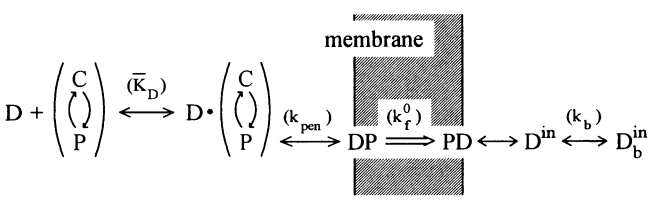

FIGURE 8 Scheme for the coupling of DNA binding, electrodiffusive penetration (rate coefficient k_{pen}) into the outer surface of the membrane and translocation across the membrane, in terms of the Nernst-Planck transport equation (k_f^0) and the binding of the internalized DNA (D^{in}) to a cell component b to yield the interaction complex Db as the starting point for the actual genetic cell transformation.

CONCLUSION

Our data and the results of the theoretical analysis clearly indicate that the direct gene transfer by membrane electroporation is a sequence of interactive, electrodiffusive and passively diffusive events. The electric field strength dependence of the electrotransformation (Fig. 4) clearly indicates that the DNA penetration into the membrane surface requires a minimal field intensity. For yeast cells and pulses of 20–40 ms duration, the threshold field strength is $E_{th} \approx 2.5$ kV cm^{-1}. On the other hand, the electrically induced membrane state transitions of the type C $\leftrightarrow$ P, as identified in lipid vesicles, proceed steadily with increasing field strength (Neumann et al., 1992; Kakorin et al., 1996). The electroporative gene transfer involves the coupling between an interactive, probably Ca-mediated, contact formation of DNA with the cell surface and cell membrane and the electroporation-resealing cycle (C $\leftrightarrow$ P). Our data confirm, in terms of a quantitative model, that the DNA adsorption is coupled to (at least two) electroporated membrane states (P_1 and P_2). The penetration of DNA into the outer surface of electroporated membrane patches (forming the surface complex DP in Fig. 8) is probably a highly interactive electrodiffusional process. The actual cross-membrane transport (or translocation DP $\rightarrow$ PD in Fig. 8) does not require the presence of an external field (Eynard et al., 1992) but is certainly accelerated by a membrane field.

Clearly, the data suggest a sequence of events involved in electroporative gene transfer as modeled in Fig. 8. Surprisingly, the (after-field) process of cell transformation is rate limited by an intracellular interaction process that is bimolecular in nature. In particular, this chemical binding feature is crucial for the description of the rather long delay phase in the kinetics of transformant formation $f_T(\tau_E)$ (see Fig. 6). This essential observation cannot be modeled with DNA-cell surface binding and (electro)diffusive transport alone.

For practical purposes of gene transfer, the condition of 1 mM Ca concentration is necessary for DNA adsorption to the cell surface, and the long pulse durations of 20–40 ms are required to achieve efficient electrodiffusive transport across the cell wall and into the surface of the electroporated cell membrane patches.

We thank J. Doncheva, M. Süss, and A. Gieselmann for technical help and M. Hofer for processing the final form of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, grant 227/9–2 to E. Neumann and the Bulgarian National Fund (grant K-411) to I. Tsoneva.

APPENDIX

DNA/Ca binding to cell surfaces

The overall equilibrium constant $\bar{K}_D$ for the reaction Eq. 1 of the text is defined as

$$\bar{K}_D = [D]\frac{[B] + [CaB]}{[DB] + [DCaB]} = K_D^0 \frac{1 + [Ca]/K_{Ca}^0}{1 + [Ca]/K'_{Ca}}, \quad (A1)$$

where $K_D^0 = [D][B]/[DB]$ is the equilibrium constant for the DNA binding in the absence of Ca^{2+}, $K_{Ca}^0 = [Ca][B]/[CaB]$ refers to Ca^{2+} binding in the absence of DNA; and $K'_{Ca} = [Ca][DB]/[DCaB]$ is the equilibrium constant for the Ca^{2+} binding to the complex DB.

If we define the overall quantities $[D_b] = [DB] + [DCaB]$, $[B] + [CaB] = [B_t] - ([DB] + [DCaB])$, and $[D] = [D_t] - ([DB] + [DCaB])$, Eq. A1 takes the form

$$\bar{K}_D = ([D_t] - [D_b])\frac{([B_t] - [D_b])}{[D_b]}, \quad (A2)$$

which leads to a quadratic equation in $[D_b]$. The solution is Eq. 2 of the text.

Integration of bimolecular rate equations

Equation 9 of the text can be rewritten in terms of $x = n^{in}(t_E) - n^{in}$ and $n_b = n_b^0 - x$ as

$$\frac{dx}{[n^{in}(t_E) - x][n_b^0 - x]} = k_b dt. \quad (A3)$$

The integration of the standard differential equation within the boundaries $t = 0$ and $t = \tau_T$ yields

$$\frac{1}{[n_b^0 - n^{in}(t_E)]} \ln \frac{n_b^0 - x}{n^{in}(t_E) - x}\Big|_0^{n(Db)} = k_b t\Big|_0^{\tau_E}. \quad (A4)$$

Substitution of Eq. A4 into Eq. 10 of the text yields Eq. 11.

Note that for the initial phase of DNA uptake, $n^{in}(t_E) \ll n_b^0$, and Eq. 11 reads

$$f_T \approx [n^{in}(t_E)/n_b^0](1 - e^{-k_b \cdot \tau_T n_b^0}) \approx n^{in}(t_E)/n_b^0. \quad (A5)$$

In the other limiting case, $n^{in}(t_E) \gg n_b^0$, and Eq. 11 takes the form

$$f_T \approx 1 - e^{-k_b \cdot t_T \cdot n^{in}(t_E)} \approx 1. \quad (A6)$$

Kinetics of electropore formation

The rate equations for the scheme in Eq. 15, involving extremely small reverse rate coefficients ($k_{-p} \approx 0$), are straightforwardly given by

$$\frac{d[C(t)]}{dt} = -k_p[C(t)],$$

$$\frac{d[P_1(t)]}{dt} = -k_p([P_1(t)] - [C(t)]), \quad (A7)$$

$$\frac{d[P_2(t)]}{dt} = k_p[P_1(t)].$$

The boundary conditions for the membrane states at $t = 0$ are $[C(0)]/[C_o] = 1$ and $[P_i(0)]/[C_o] = 0$, where, according to the mass conservation condition, the total number of membrane states is given by $[C_0] = [C(t)] + \Sigma_{i=1}^2 [P_i](t)]$. Integration of the rate equations (A7) within these boundaries yields the relationship for the build-up of the DNA-permeable pore state P_2 according to

$$[P_2(t)] = [C_0](1 - (1 + k_p t)e^{-k_p \cdot t}). \quad (A8)$$

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