Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 422–426, January 1982
Cell Biology

Mechanisms of DNA uptake by mammalian cells: Fate of exogenously added DNA monitored by the use of fluorescent dyes

(gene transfer/calcium phosphate coprecipitation/pH dependency/DNA concentration)

ABRAHAM LOYTER*†, GEORGE A. SCANGOS*‡, AND FRANK H. RUDDLE*§

Departments of *Biology and §Human Genetics, Yale University, New Haven, Connecticut 06511

Contributed by Frank H. Ruddle, August 12, 1981

ABSTRACT **Coprecipitation of DNA with calcium phosphate is a commonly used method of gene transfer in mammalian cells. We have found that DNA forms a tight complex with Ca P_i and that DNA in this complex is resistant to nucleases present in serum or added externally. Under optimal conditions, virtually all of the recipient mouse Ltk$^-$Aprt$^-$ cells take up Ca P_i–DNA complexes, as determined by fluorescent dyes specific for DNA (4′,6-diaminilo-2-phenylindol dihydrochloride) or for calcium salts (chlorotetracycline). However, only a small proportion of the cells have detectable Ca P_i–DNA complexes in the nucleus. Uptake of the Ca P_i–DNA complexes was highly dependent upon the pH at which the Ca P_i–DNA complex was formed and upon the concentration of DNA in the complex.**

It has been known for many years that under certain conditions, eukaryotic cells can take up large amounts of DNA and transport it into the cell nucleus (1, 2). This phenomenon has been exploited to achieve transformation of eukaryotic cells with both selectable and nonselectable genes (1, 2,¶). Addition of polycations, such as polyornithine (3) or DEAE-dextran (3, 4), to the DNA solution or precipitation of the added DNA on the cell surface by calcium phosphate (5) have proven useful in increasing both the uptake of the DNA and the frequency of cell transformation. Despite the large amount of high molecular weight DNA taken into cells in the presence of certain facilitators (3, 6), at best only about one in 10^4 cells eventually becomes transformed (7, 8). This has limited transfer to those genes for which there exists a good selective system and to certain cell lines, such as mouse L cells, that function as efficient recipients (8).

The detailed mechanisms by which external DNA is integrated into a foreign chromosome and expressed in a foreign environment are as yet poorly understood. However, a large body of recent work (9, 10) has begun to elucidate the process. On the other hand, essentially nothing is known about the way by which DNA molecules cross the cellular membrane and are transported to the nucleus. The studies described here were designed to study the quantitative aspects and the mechanisms of entry of DNA into cells after coprecipitation with Ca P_i. We have used the specific fluorescent dye 4′,6-diaminilo-2-phenylindol dihydrochloride, (DAPI), which specifically stains double-stranded DNA (11), and chlorotetracycline, which stains complex salts of Ca (12). We show that virtually all recipient cells take up Ca P_i–DNA complexes, which appear in the cytoplasm in a characteristic structure, and that only a few of the cells have detectable Ca P_i–DNA complexes in the nucleus. Additionally, DNA uptake was highly dependent on the pH at which the Ca P_i precipitate was formed and upon the concentration of DNA in that precipitate.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cell Culture. The murine cell line Ltk$^-$Aprt$^-$ was cultured in minimum essential medium, α modification (GIBCO), supplemented with 10% (vol/vol) fetal calf serum. In most cases, cells were plated in 35-mm or 60-mm Petri dishes 24 hr before each experiment, and the culture medium was supplemented with penicillin/streptomycin and kanamycin (exceptions are mentioned in figure legends).

Preparation of ^{3}H-Labeled DNA. HeLa cells were grown in spinner culture in α minimum essential medium supplemented with 5% fetal calf serum, penicillin/streptomycin, and kanamycin. Logarithmic phase cells (3.5×10^7) were added to 50 ml of fresh medium to which 10 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]thymidine per ml was added. The cells were harvested 24 hr later, and the DNA was isolated as described by Pellicer *et al.* (13).

Measurement of [^{3}H]DNA Entry into Ltk$^-$Aprt$^-$ Cells. ^{3}H-labeled (or unlabeled) HeLa DNA was precipitated with Ca P_i essentially as described (14). Briefly, 80–100 μg of DNA (45–50 μl of a solution containing 2 mg of DNA per ml) was dissolved in 1 ml of 250 mM $CaCl_2$ buffered with 25 mM Hepes (pH 7.12). This solution was added to 1 ml of 280 mM NaCl/1.5 mM Na_2HPO_4/25 mM Hepes, pH 7.12. After 30 min at room temperature, 0.25 ml (containing 10–12.5 μg of DNA) was added to each 35-mm Petri dish containing 2.5 ml of medium and 2–6 $\times 10^5$ cells. When 60-mm Petri dishes were used, 0.5 ml of Ca P_i–DNA (containing 10–15 μg of DNA) was added to 4 ml of medium. After incubation under CO_2 at 37°C, medium was removed, external Ca P_i was dissolved, and DNA was digested as follows. After removal of medium, cells were washed successively with 1 ml of Ca/Mg-free phosphate-buffered saline (referred to as saline), 1 ml of 5 mM EDTA or ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) in saline, and 1 ml of complete saline and then were incubated with 1 ml of complete saline containing 60 units of micrococcal nuclease (Boehringer Mannheim) and 60 g of DNase I (Sigma). After 20 min at 37°C with gentle shaking, the cells were washed twice with saline and lysed by the addition of 0.5 ml of 10 mM EDTA/0.6% $NaDodSO_4$/10 mM Tris, pH 7.5 (lysis buffer). After the addition of 0.2 ml of unlabeled salmon sperm DNA (2 mg/ml) per 0.5 ml of lysed cells, the DNA was precipitated

Abbreviations: DAPI, 4′,6-diaminilo-2-phenylindol dihydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

† Present address: Dept. of Biological Chemistry, Life Science Institute, Hebrew University of Jerusalem, Jerusalem, Israel.

‡ Present address: Dept. of Biology, The Johns Hopkins University, Baltimore, MD 21218.

¶ Szybalski, W. (1963) *Proceedings of the 12th Annual Session of the National Poultry Breeders Roundtable*, Kansas City, MO, pp. 90–109.

with 10% (wt/vol) trichloroacetic acid onto Whatman GF/A filters, and the amount of acid-precipitable [^{3}H]DNA was determined.

Fluorescence Microscopic Observations of DAPI-Stained DNA and of Chlorotetracycline-Stained Ca P_i. HeLa DNA was added to a buffered $CaCl_2$ solution as just described, except that the concentration of the DNA was 30–40 μg/ml; then 10 μl of DAPI (Sigma) at 1 mg/ml in water was added to give a DAPI/DNA ratio of 1:2 to 1:4. When chlorotetracycline (United States Biochemicals, Cleveland, OH) was used, it was added to the $CaCl_2$/DNA solution to give 40 μg/ml (chlorotetracycline/DNA ratio of 1:1). The $CaCl_2$/stained DNA solution was added to phosphate buffer as described. After 30 min at room temperature, 0.5 ml of Ca P_i–DNA complex was added to 60-mm Petri dishes containing 4 ml of medium and 3–8 × 10^5 cells seeded on coverslips the previous day. After the incubation with Ca P_i–DNA, the cells were washed once with 5 ml of saline and once with 5 ml of 5 mM EDTA or EGTA in saline and were viewed directly with fluorescent microscopy or digested with the combination of nucleases described above and then examined. For observation under fluorescent illumination, washing with EDTA, or preferably EGTA, alone was sufficient to remove detectable external DNA complexes.

RESULTS

Properties of the Ca P_i–DNA Complex. Incubation of DNA in cell growth medium containing either native or heat-denatured serum resulted in rapid degradation, as monitored by the appearance of ^{3}H-labeled hydrolytic products (not shown) and by analysis of hydrolytic products by gel electrophoresis (Fig. 1, lanes D and F). Incubation of DNA in medium in the absence of serum did not cause any substantial changes in its electrophoretic pattern (Fig. 1, lane B). DNA molecules were protected to a high degree from serum nucleases after coprecipitation with Ca P_i under conditions commonly used for gene transfer (Fig. 1, lanes C and E). Degradation of added DNA by serum nucleases also was inhibited by the addition of $CaCl_2$ alone to the concentration used in gene transfer (12.5 mM) (not shown).

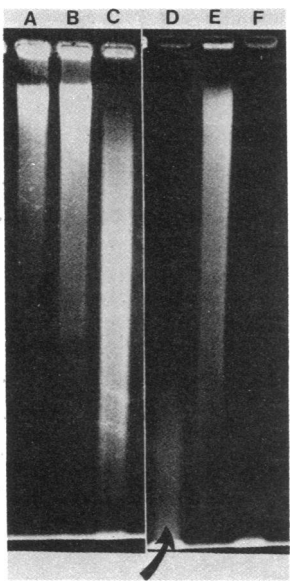

FIG. 1. Gel electrophoresis analysis of DNA molecules incubated in a cell growth medium: effect of serum and of Ca P_i. HeLa cell DNA (150 μg) was dissolved in 1 ml of minimum essential medium, and 100 μl was removed immediately for analysis (lane A). The DNA was then incubated for 6 hr at 37°C in that medium (lane B) or at the same concentration (150 μg/ml) in minimum essential medium containing 10% heated (lane D) or nonheated (lane F) fetal calf serum. At the end of the incubation period, a volume of 100 μl was loaded in agarose gel. For precipitation with Ca P_i, 150 μg of HeLa DNA was dissolved in 1 ml of $CaCl_2$ (250 mM) and mixed with phosphate buffer. After 30 min at room temperature, 0.5 ml of the Ca P_i–DNA suspension was added to Petri dishes containing 4 ml of MEM media with 10% heated (lane C) or nonheated (lane E) serum. After 6 hr of incubation (CO_2 incubator, 37°C), the medium was aspirated, and the Ca P_i–DNA precipitates were collected and suspended in 1 ml of complete phosphate-buffered saline. The suspension was centrifuged for 15 min at 500 × *g*, and the pellet obtained was dissolved in about 100 μl of lysis buffer and loaded in the gels. Heated serum was obtained by incubating fetal calf serum at 56°C for 60 min. Gel electrophoresis on agarose gel and ethidium bromide staining of DNA were as described (15).

Coprecipitation of [^{3}H]DNA with Ca P_i caused significant quenching of the tritium emissions (Table 1). The quenching was due to the formation of the Ca P_i–DNA complex because it could be reversed by solubilization of the complex by EDTA (Table 1). The extent of quenching was dependent on both time and temperature, as was formation of the Ca P_i precipitate. For example, after 4 hr of incubation, only 9% of the radioactivity present in the DNA could be detected (Table 1). Addition of EDTA solubilized the complex and allowed the detection of 76% of added radioactivity. Some quenching was seen after addition of $CaCl_2$ alone to the DNA but significantly less than that observed with Ca P_i.

Essentially the same phenomenon was seen when the DNA was complexed with another common facilitator of gene transfer, DEAE-dextran (Table 1). Blocking of the formation of the DEAE-dextran–DNA complex by heparin (4) allowed detection of most radioactivity. These two experimental systems suggest that a tight complex was formed between DNA and these facilitators.

Table 1. Reduction in the measurable radioactivity of [^{3}H]DNA after complexing it to Ca P_i or to DEAE-dextran

Exp.	37°C incubation, min	[^{3}H]DNA treatment	Detectable radioactivity cpm	%
A	0	—	18,310	100
		Ca P_i	11,837	64
		Ca P_i, EDTA	14,584	80
	240	—	20,631	100
		Ca P_i	1,892	9
		Ca P_i, EDTA	15,502	76
B	30	—	9,100	100
		DEAE-dextran	3,204	35
		Heparin, DEAE-dextran	8,892	98
		DEAE-dextran, heparin	4,862	53

Experiment A: 25 μl of [^{3}H]DNA (2 mg/ml) were dissolved in either 2 ml of 280 mM NaCl/1.5 mM Na P_i/25 mM Hepes, pH 7.12 (control system) or first in 1 ml of 250 mM $CaCl_2$/25 mM Hepes, pH 7.12 and then mixed with the above Na P_i-containing solution to form DNA–Ca P_i complex. The DNA-containing solutions were incubated at 37°C, and samples (0.5 ml) were removed either immediately or after 4 hr of incubation at 37°C. The radioactivity of the [^{3}H]DNA was determined on samples of 25 μl either directly or after solubilization of the Ca P_i precipitate by EDTA (pH 8.0; final concentration, 125 mM). Incubation of [^{3}H]DNA in the NaCl/Na P_i/Hepes buffer did not cause any change in its measurable radioactivity, and the amount of cpm in these samples was considered as 100%. Experiment B: 3 μl of [^{3}H]DNA (2 mg/ml) were mixed with 6 μl of DEAE-dextran (Sigma; M_r, ≈500,000; 2 mg/ml) in phosphate-buffered saline and then diluted with saline to give a final volume of 0.5 ml. Heparin (15 μl from a solution of 2 mg/ml) was added either before DEAE-dextran or after it. Only when heparin was added before DEAE-dextran could most of the [^{3}H]DNA radioactivity be detected, indicating that heparin prevented the formation of the DEAE-dextran complex.

Uptake of Ca P_i-Precipitated [^{3}H]DNA. Uptake of DNA by cells has been measured by determining the fraction of added [^{3}H]DNA that was nuclease resistant (3, 6). Because we showed that coprecipitation of DNA with Ca P_i causes both quenching of radioactive emissions and protection from nuclease digestion (Table 1 and Fig. 1), it was important to determine conditions for differentiating the amount of DNA which actually entered cells from that which remained adsorbed to the cell surface in a nuclease-resistant form. As can be seen in Table 2, 30% and 38% of added tritiated DNA precipitated onto the surface of Petri dishes in the absence of cells and in the presence or absence of serum, respectively. Neither solubilization of the Ca P_i complex with EDTA nor digestion with DNase I or micrococcal nuclease completely removed DNA from the precipitate (Table 2). Only when the Ca P_i precipitate was first solubilized with EDTA (or EGTA, not shown) and the DNA was then digested with combinations of nucleases was the DNA completely removed from the precipitate (Table 2, experiment A). When the Ca P_i–DNA coprecipitate was added to Petri dishes containing 6×10^5 cells, approximately 7.5% of the DNA could not be removed by the EDTA/combined nuclease treatment (Table 2, experiment B). We feel that this figure represents the amount of DNA that actually was taken up by the cells. If this figure actually represents uptake, then the amount of EDTA- and nuclease-resistant DNA should increase linearly with cell concentration. Fig. 2*A* demonstrates that, indeed, this is the case, and uptake of [^{3}H]DNA did increase linearly with cell concentration, whereas the level of adsorption did not. When EDTA treatment was omitted, only partial linearity was seen (Fig. 2*A*), confirming that not all external DNA was susceptible to nuclease digestion.

Fig. 2*B* shows that uptake of DNA was not linear with DNA concentration. Optimal uptake was seen when 10–20 μg of DNA was added to each Petri dish. Significantly less DNA was taken up at higher concentrations (Fig. 2*B*). On the other hand, adsorption of DNA increased to approximately 24 g of DNA and then decreased only slightly.

Uptake of Fluorescently Labeled DNA After Precipitation with Ca P_i. To determine the proportion of cells that took up DNA, we made use of the dye DAPI, which becomes fluorescent after binding to double-stranded DNA (11). DNA complexed to Ca P_i could be visualized as a small precipitate, both adsorbed to the cells and to the Petri dish in which the cells were grown (Fig. 3*A*). In Fig. 3*B*, the same field is shown under fluorescent illumination. The precipitate is seen as small fluorescent spots. After removal of the adsorbed complex by washing with EDTA or by EDTA/nuclease digestion, no Ca P_i precipitate could be seen between or adsorbed to cells (Fig. 3*C*), but many fluorescent particles could be visualized inside the cells (Fig. 3*D*). The nuclei of all cells were brightly fluorescent due to staining of recipient cell DNA by free DAPI that enters cells under our conditions. In contrast to the diffuse and homogenous nuclear fluorescence, the fluorescence in the cytoplasm was confined to small spots of characteristic shape and size. These spots represent entry of exogenous DAPI-stained DNA into

Table 2. Coprecipitation with Ca P_i and uptake of [^{3}H]DNA

Exp.	Sample	Treatment*	Cl_3 CCOOH-insoluble [^{3}H]DNA, % of total DNA added: With serum	Without serum
A (In the absence of cells)				
	1	—	30	38
	2	EDTA	15.2	27
	3	DNase	16.2	20.5
	4	Nuclease	18	22
	5	EDTA + DNase	5.6	6.8
	6	EDTA + nuclease	7.4	8.7
	7	EDTA + DNase + nuclease	<1	1
B (6×10^5 Ltk$^-$Aprt$^-$ cells)				
	1	EDTA	20.3	
	2	EDTA + DNase + nuclease	7.4	

[^{3}H]DNA (80 μl of DNA at 2 mg/ml was precipitated in 4 ml of $CaCl_2$ and phosphate solutions. After 30 min at room temperature, a volume of 0.25 ml of the DNA–Ca P_i suspension was added to each 35-mm Petri dish containing 2.5 ml of medium without (experiment A) or with (experiment B) plated cells. After 6 hr of incubation, the medium was aspirated and the DNA–Ca P_i precipitate was lysed in a lysis buffer for determination of radioactivity after the following treatments: (*i*) no further treatment—after aspiration of the medium, the precipitate was dissolved and counted; (*ii*) samples 2 in experiment A and 1 in experiment B were washed once with 1 ml of Ca/Mg-free saline and then with 1 ml of 5 mM EDTA; (*iii*) precipitate was washed once with 1 ml of complete phosphate-buffered saline and then incubated for 20 min with 1 ml of complete saline containing 60 μg of DNase I; (*iv*) as sample 3 but with 60 units of micrococcal nuclease (Boehringer Mannheim); (*v*) precipitate was washed first with EDTA and then incubated with DNase; (*vi*) as sample 5 but with micrococcal nuclease; (*vii*) sample 7 in experiment A and sample 2 in experiment B—precipitates were washed with EDTA and incubated with DNase I and micrococcal nuclease.

* Of Ca P_i–[^{3}H]DNA precipitate.

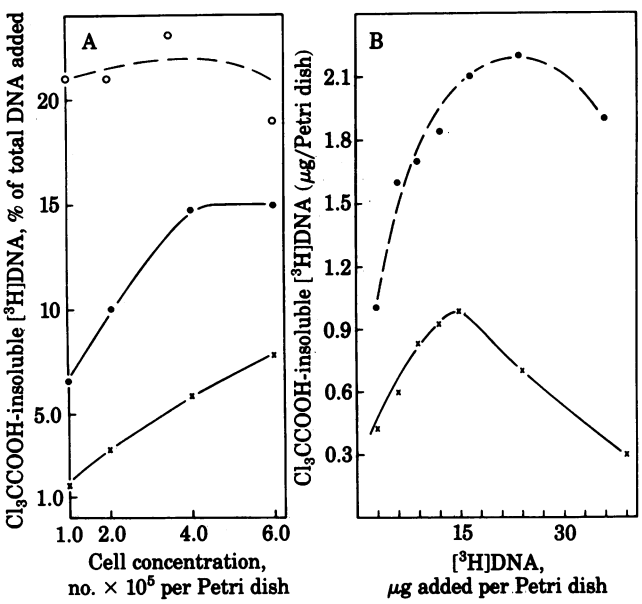

FIG. 2. Adsorption and uptake of [^{3}H]DNA by Ltk$^-$Aprt$^-$ cells. (*A*) The effect of cell concentrations. Ltk$^-$Aprt$^-$ cells at increasing concentrations were plated in 60-mm Petri dishes. The amount of [^{3}H]DNA was determined 6 hr after incubation with the cells. x——x, Uptake of DNA: [^{3}H]DNA was determined after washing the cells with EDTA and incubation with DNase and micrococcal nuclease. ●——●, Cells were washed once with complete phosphate-buffered saline and incubated with DNase and micrococcal nuclease. ○---○, Adsorption of [^{3}H]DNA: after the incubation period, the medium was aspirated, the Ca P_i–[^{3}H]DNA was dissolved in lysis buffer, and the amount of [^{3}H]DNA was determined. (*B*) Effect of DNA concentration. Increasing concentrations of DNA were precipitated by Ca P_i, and 0.5 ml was added to 60-mm Petri dishes containing 5×10^5 cells. The concentration of DNA in the figure represents the amount of DNA per 0.5 ml of suspension. ●---●, Adsorption of [^{3}H]DNA (amount of [^{3}H]DNA left on the cells' surface after the Petri dishes were washed once with complete phosphate-buffered saline); x——x, uptake of [^{3}H]DNA (EDTA- and nuclease-resistant [^{3}H]DNA). Each point in these figures is the average of two independent experiments.

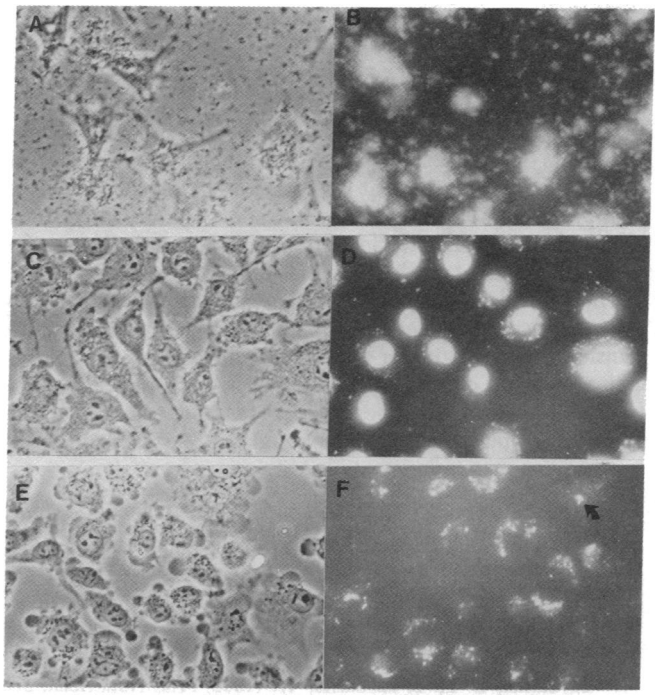

FIG. 3. Adsorption and uptake of Ca P_i–DNA complexes by Ltk^-Aprt^- cells as visualized by phase and fluorescence microscopy: DNA was stained by DAPI or chlorotetracycline, precipitated by Ca P_i and added to Ltk^-Aprt^- cells (6×10^5 cells plated on coverslips in 60-mm Petri dishes). All samples were incubated at 37°C in a CO_2 incubator before examination. For microscopic observation, a drop of 50% (wt/vol) glycerol in isotonic buffered NaCl was added to the cells. (*A* and *B*) Precipitation and adsorption of DAPI-stained DNA–Ca P_i precipitates. After incubation with the Ca P_i–DNA, the medium was removed and the cells were immediately examined either in phase (*A*) or in fluorescent microscopy (*B*). Note that fluorescent precipitates in *B* can be seen on the surface of the cells and also in the space between the cells. (*C* and *D*) Phase (*C*) and fluorescence (*D*) microscopy of Ltk^-Aprt^- cells from which Ca P_i–DNA complexes were removed by EDTA/nuclease treatment. No Ca P_i precipitate (*C*) or fluorescent dots (*D*) can be seen in the space between the cells, whereas many fluorescent dots (DAPI-stained DNA) can be seen inside the cells. All the cell nuclei are fluorescent due to staining of chromosomal DNA by free DAPI. (*E* and *F*) The same as *C* and *D*, but the dye chlorotetracycline was added to the $CaCl_2$/DNA solution instead of DAPI. Nuclei are not stained. Many intracytoplasmic fluorescent dots can be seen. Only a few of them appear to be in the nucleus (arrow in *F*). Cells underwent partial lysis (E) because of the long exposure needed to photograph these cells. (×900.)

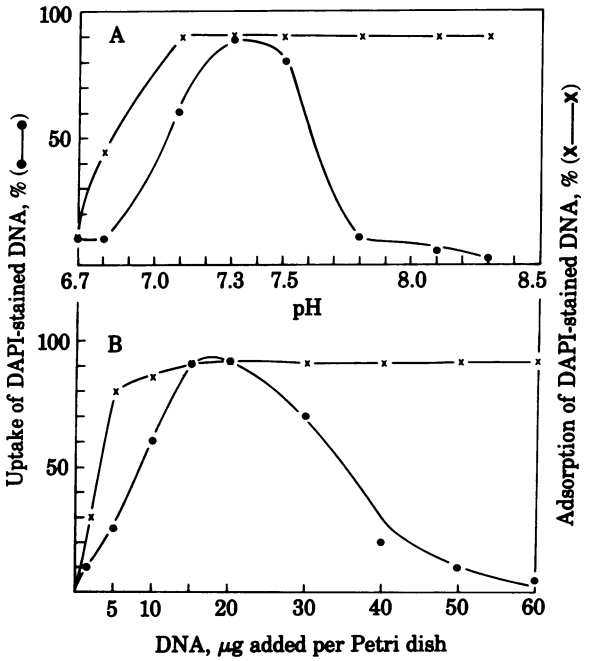

FIG. 4. Adsorption (cells containing adsorbed fluorescent dots) and uptake (cells containing more than 10 intracellular fluorescent dots) of DAPI-stained DNA as a function of the pH of the Ca P_i–DNA complex (*A*) and DNA concentration in the Ca P_i–DNA complex (*B*). In *A*, a 2-ml sample of buffered $CaCl_2$ solutions (250 mM $CaCl_2$/25 mM Hepes, pH 7.12) and of phosphate solution (280 mM NaCl/25 mM Hepes/1.5 Na P_i, pH 7.12) were adjusted to the different pHs by addition of concentrated (10 M) NaOH. HeLa DNA and DAPI were added to the buffered $CaCl_2$ solution to give a concentration of 40 μg/ml and 10 μg/ml, respectively. The addition of 0.5 ml of the Ca P_i–DNA precipitate, which was formed at the different pH values, to 60-mm Petri dishes containing 5 ml of medium buffered with 20 mM Hepes (pH 7.12) did not significantly change the pH of the medium. After 6 hr of incubation, the medium was removed, and the coverslips containing cells were observed immediately in the fluorescence microscope (absorption) or after solubilization of the external Ca P_i with EDTA (uptake). Increasing amounts of DNA and DAPI (DNA/DAPI, 4:1, wt/wt) were added to a buffered $CaCl_2$ solution, which was then precipitated. The DNA concentration in the figure represents the amount of DNA in 0.5 ml of the Ca P_i–DNA complex that was added to 5 ml of medium in 60-mm Petri dishes. In both experimental systems (*A* and *B*), the number of cells containing intracellular DAPI-stained DNA dots was estimated from fluorescence micrographs; 20–30 cells were counted in each of five to seven photographs that were taken of each system. Thus, each point is based on counting a total of 100–200 cells.

recipient cells. No fluorescent spots were seen in the cytoplasm of ATP-depleted cells or cells incubated with Ca P_i at 4°C, although under these conditions, the Ca P_i precipitates could be seen to be adsorbed to cells and to the Petri dishes in a fashion similar to that seen in Fig. 3*B* (not shown; unpublished data).

It was of interest to determine whether the entire Ca P_i–DNA complex or only the DNA itself entered the cells. To differentiate between these possibilities, we made use of the fact that the fluorescent molecule chlorotetracycline specifically stains calcium complexes (12). Fluorescent spots of the same shape and at similar numbers could be seen in the cytoplasm of recipient cells after incubation with the chlorotetracycline-stained Ca P_i complexes in the presence (Fig. 3*F*) or absence (not shown) of DNA. This result showed that Ca P_i was able to enter the cells even in the absence of DNA. In contrast to DAPI, chlorotetracycline did not stain recipient cell nuclei, allowing the detection of Ca P_i complexes in recipient cell nuclei. Although Ca P_i–DNA complexes were visible in the cytoplasm of most cells, complexes were seen in the nuclei of only a small proportion (arrow in Fig. 3*F*).

Uptake Is Dependent on the pH and DNA Concentration in the Ca P_i–DNA Precipitate. The pH of the formation of the Ca P_i–DNA complexes and the concentration of DNA in those complexes are crucial for successful gene transfer (5, 16). Studies on the effect of pH and DNA concentration upon the entry of DAPI-labeled DNA into cells showed that only when the Ca P_i complexes were formed between pH 7.1 and 7.5 could fluorescent spots be visualized in the cytoplasm of recipient cells (Fig. 4*A*). Although large Ca P_i precipitates were formed above pH 7.5, no entry of these complexes into cells could be detected.

The appearance of fluorescent spots adsorbed to cells increased with DNA concentration to about 20 μg per dish (Fig. 4*B*). When higher concentrations of DNA were complexed at the same concentration of Ca P_i, adsorption was not affected, whereas the appearance of cytoplasmic fluorescence was drastically reduced (Fig. 4*B*). At high DNA concentration (50 μg

per dish), no cytoplasmic fluorescent spots could be detected, confirming the results of the previous section measuring the uptake of radiolabeled DNA (Fig. 2*B*).

DISCUSSION

Our results clearly show that a portion of DNA previously believed to have entered cells on the basis of DNase resistance remains adsorbed to the surface complexed with Ca P_i in a nuclease-resistant form. Similar observations have been made after the addition of DNA to plant protoplast—i.e., that bivalent metals such as copper or zinc protect the DNA from nuclease digestion (17). An accurate determination of DNA uptake can be obtained only when the Ca P_i–DNA complex is first solubilized by chelators such as EDTA or EGTA, thus exposing the DNA to nuclease activity.

That we measured actual uptake is supported by our finding that DNA can be observed intracellularly with specific fluorescent dyes such as DAPI. The appearance of the intracellular fluorescent spots varied with the pH at which the Ca P_i precipitate was formed and with the concentration of DNA in the complex in a similar fashion as has been reported for gene transfer (16), indicating that the uptake of DNA as measured by DAPI fluorescence is correlated with ultimate gene expression. Interestingly, it was not the concentration of DNA on the cell surface but the concentration in the precipitate that governed the extent of uptake.

The use of chlorotetracycline as a probe suggested that DNA entered the cell together with the Ca P_i. The same technique demonstrated that Ca P_i alone entered cells in a similar fashion in the absence of DNA, suggesting that there are no specific surface groups that bind DNA and facilitate entry into cells. Out of the many cells that had chlorotetracycline fluorescence in the cytoplasm, only a few (approximately 1–5%) had detectable fluorescence in the nucleus. Also, electron microscopic data indicate that DNA enters the nucleus as part of Ca P_i complex (unpublished data). Interestingly, introduction of plasmid pTKx-1 (containing the herpes simplex virus thymidine kinase gene) directly into cell nuclei by microinjection results in $<1\%$ of cells that grow to express stably the TK gene (unpublished data). Because Ca P_i–DNA precipitates are detectable in only 1–5% of nuclei and, by extrapolation from microinjection, $<1\%$ of these should go on to stably express genes contained on that DNA, the frequency of gene transfer after Ca P_i–DNA coprecipitation should be on the order of 10^{-4}. This frequency is consistent with the best gene transfer frequencies (8, 10, 16). These results suggest that movement of DNA from the cytoplasm to the nucleus and processing of DNA in the nucleus are the most significant barriers to gene transfer.

It is likely that Ca P_i is a relatively efficient facilitator of gene transfer for a number of reasons. First, it increases the concentration of DNA on the cell surface by precipitation. At the same time, it protects the DNA from digestion by serum nucleases. Also, we have obtained evidence that Ca P_i induces phagocytosis, thus facilitating entry of DNA into cells, and protects the DNA from degradation by intracellular nucleases (unpublished data). The observations that Ca P_i–DNA complexes formed at high pH or with high DNA concentrations did not enter cells efficiently suggest that the induction of phagocytosis is a specific process dependent on the form of the Ca P_i precipitate.

Thus, our data indicate that the extent of DNA uptake into the cytoplasm is an important parameter for successful gene transfer. However, under optimal conditions, most of the recipient cells do contain cytoplasmic DNA. To increase gene-transfer frequency, it will be necessary to facilitate entry of DNA from the cytoplasm to the nucleus and its integration and maintenance in a manner compatible with gene expression.

A.L. is a recipient of an Eleanor Roosevelt International Cancer Fellowship and an American Cancer Society Fellowship. This work was supported by National Institutes of Health Grant GM09966 (to F.H.R.) and by the National Council for Research and Development, Jerusalem, Israel, and the G.S.F., Munich, Federal Republic of Germany (to A.L.).

1. Szybalska, E. H. & Szybalski, W. (1962) *Proc. Natl. Acad. Sci. USA* **48**, 2026–2034.
2. Bhargava, P. M. & Shannugan, G. (1971) *Nucleic Acids Res.* **11**, 103–192.
3. Farber, F., Melnick, J. L. & Butel, J. S. A. (1975) *Biochim. Biophys. Acta* **390**, 298–311.
4. Pagano, J. S. (1970) *Prog. Med. Virol.* **12**, 1–48.
5. Graham, F. L. & Van der Eb, A. J. (1973) *Virology* **52**, 456–467.
6. Groneberg, J., Brown, D. T. & Doerflev, W. (1975) *Virology* **64**, 115–131.
7. Pellicer, A., Wigler, M., Axel, R. & Silverstein, S. (1978) *Cell* **14**, 131–141.
8. Lewis, W. L., Srinivasan, P. R., Stoke, N. & Siminovitch, L. (1980) *Somatic Cell Genet.* **6**, 333–348.
9. Pellicer, A., Robins, D., Wold, B., Sweet, R., Jackson, J., Lowy, I., Roberts, J. M., Sim, G. K., Silverstein, S. & Axel, R. (1980) *Science* **209**, 1414–1422.
10. Scangos, G. & Ruddle, F. H. (1981) *Gene* **14**, 1–10.
11. Williamson, D. H. & Funnel, D. J. (1975) *Methods Cell. Biol.* **12**, 335–351.
12. Tan, A. T. (1975) *J. Neurochem.* **24**, 127–134.
13. Pellicer, A., Wigler, M. & Axel, R. (1978) *Cell* **14**, 133–141.
14. Wigler, M., Pellicer, A., Silverstein, S. & Axel, R. (1978) *Cell* **14**, 725–731.
15. Huttner, K. M., Scangos, G. & Ruddle, F. H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5820–5824.
16. Graham, F. L., Bacchetti, S. & McKinnon, R. (1980) in *Introduction of Macromolecules into Viable Mammalian Cells*, eds. Baserga, R., Croce, C. & Rovera, G. (Liss, New York), pp. 3–25.
17. Behki, R. M. & Lesley, S. M. (1979) *In Vitro* **11**, 851–856.