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Quantitative Analysis of the Fate of Exogenous DNA in *Nicotiana* Protoplasts[1]

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ABSTRACT

After a 5-hour incubation of protoplasts of *Nicotiana tabacum* L. 'Xanthi' with ^{3}H-DNA (7.26 μg/ml) from *N. tabacum* L. 'Xanthi nc' 3.5% of the initial radioactivity was found in acid-insoluble substances of the protoplasts. The addition of DEAE-dextran and poly-L-lysine to the incubation medium nearly doubled radioactivity absorption. The absorption was inhibited by 2,4-dinitrophenol, KCN, and low temperature (0 C); this inhibition could not be reversed by exogenous ATP. About 500 tobacco plants established from protoplasts of a normally tobacco-mosaic virus-susceptible cultivar that had been allowed to absorb DNA prepared from a resistant cultivar did not show transfer of the virus-resistant gene.

A detailed analysis was performed of the disposition of exogenous DNA in plant protoplasts, by employing *Escherichia coli* ^{3}H-DNA and *Nicotiana glutinosa* protoplasts. In 5 to 20 hours, about 10% of the ^{3}H-DNA entered the protoplasts. Competition experiments between the ^{3}H-DNA and unlabeled DNA or thymidine showed that the entry occurred as undegraded ^{3}H-DNA. Examination of intraprotoplast fractions revealed that 60 to 80% of the absorbed radioactivity resided in the "soluble" fraction of the cytoplasm and 20% in the nuclear fraction. The mitochondrion fraction also contained measurable radioactivity. Sizing on sucrose density gradients showed that the bulk of the absorbed *E. coli* DNA had been depolymerized. Of the incorporated radioactivity, 15% was accountable as DNA, exogenous as well as resynthesized, and 15% as RNA, protein, and other cell constituents. DNA/DNA hybridization test indicated that 17.6% of the re-extractable ^{3}H-DNA retained homology with the *E. coli* DNA; this was equivalent to 2.6% of the absorbed radioactivity. Resynthesized receptor protoplast DNA was represented by a fraction at least 1.7% of the total absorbed radioactivity. The amount of bacterial DNA remaining in protoplasts suggests that each protoplast retained 2.3 × 10^{-15}g donor DNA, or approximately half of the *E. coli* genome.

The major methods explored in the use of somatic cells to modify higher plants genetically have included fusion between protoplasts of different genomes and transfer of DNA and similar genetic materials from donor to receptor protoplasts. The latter approach is studied in this laboratory and previous papers described steps of isolating protoplasts from cell cultures (31) and requirements associated with the regeneration of dividing cells from protoplasts (32). We now describe our efforts to introduce genetic material in the form of purified DNA into protoplasts.

Other investigators have already shown that a small but nevertheless significant quantity of exogenous DNA from diverse sources is absorbed by plant protoplasts (11, 30). Nevertheless, on theoretical grounds alone, it may be unreasonable to expect that the DNA absorbed by plant cells or protoplasts will remain undegraded and become a functional part of the recipient cell's genome. Gleba *et al.* (11) as well as Lurquin and Behki (28) and Lurquin and Hotta (29) noted that the foreign DNA was first degraded and then reutilized in synthesis of the recipient cell's DNA. Kleinhofs *et al.* (21) were unable to demonstrate the integration and replication of exogenous DNA in plant cells as claimed by Ledoux and Huart (23, 24).

Several alternatives may be anticipated with respect to the fate of exogenous DNA in protoplast feeding experiments, and these are shown schematically in Figure 1. DNA could enter protoplasts either intact or degraded. Following entry, it could be translocated to cytoplasmic genetic apparatus, it might serve immediately as template for transcription, it could be incorporated and integrated into the nucleus, or it is possibly degraded and its nucleotides reutilized in DNA synthesis. This investigation has attempted to assess quantitatively some of these alternatives.

MATERIALS AND METHODS

ABSORPTION OF HOMOLOGOUS DNA BY *NICOTIANA TABACUM* PROTOPLASTS

Preparation of *N. tabacum* ^{3}H-DNA. Cells of *N. tabacum* L. 'Xanthi nc' were cultured as a suspension in a nutrient solution containing 2 μCi/ml of ^{3}H-thymidine (83.8 Ci/mmol; ICN). The basic cell culture procedure was the same as described previously (31). Unlabeled thymidine was included in the medium in a concentration 1 mg/l. After 6 days, the cell suspension was filtered through Miracloth, and the collected cells were rinsed three times with further deionized distilled H_2O.

Sixty g of cells were incubated in a solution containing 1% cellulase Onozuka and 0.2% Macerozyme (both enzymes obtained from Calbiochem, San Diego, Calif.) for 3 hr at 27 C. The incubated mixture was centrifuged 5 min at 480*g* (Sorvall RC2-B centrifuge). To the pellet fraction were added 15 ml of tris-SSC buffer (10 mM tris, 0.15 M NaCl, and 0.015 M trisodium citrate [pH 7]), 5 ml SLS (5% sodium lauryl sulfate), and 60 ml phenol. The mixture was homogenized 7 min at high speed (VirTis S "45," Scientific Products, Evanston, Ill.). The homogenate was centrifuged 10 min at 7700*g*. Two volumes of cold 95% ethanol were added gently to the supernatant. The nucleic acids were sedimented by centrifugation at 7700*g* for 10 min. The pellet was dissolved in tris-SSC buffer and dialyzed 3 to 4 hr in the buffer. The dialyzed sample was treated with an equal volume of 4 M $LiCl_2$ for 4 hr, then centrifuged at 7700*g* for 20 min to remove the ribosomal RNA. An equal volume of 95% cold ethanol was added to the supernatant and the fibrous DNA was wound out on a glass rod. The DNA was redissolved in tris-SSC and dialyzed overnight in the same buffer. After dialysis, the DNA fraction was incubated in a solution containing 10 μg/

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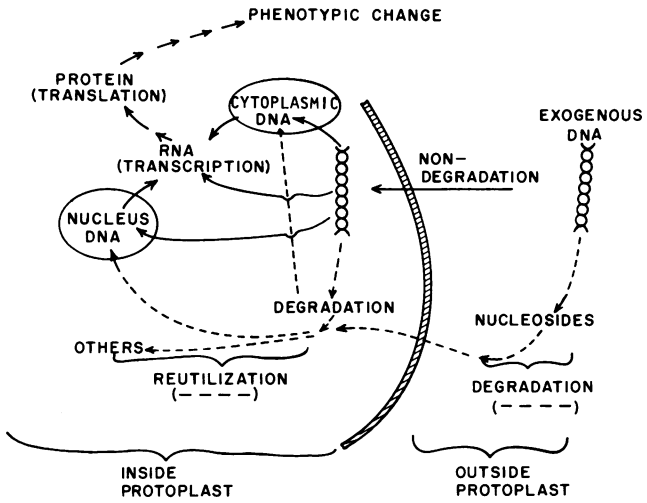

FIG. 1. Schematic diagram showing alternative events in uptake of exogenous DNA by protoplasts, fate of absorbed DNA, and gene expression.

ml heat-treated RNase A (Worthington Biochemical Co.) at 37 C for 30 min to degrade residual RNA. Two volumes of cold 95% ethanol were added and the DNA was again wound out and dialyzed in tris-SSC buffer 4 to 5 hr. The RNase treatment was repeated twice. Finally, the purified DNA was placed in SSC (tris-SSC lacking tris) solution and stored at 4 C. Treatment of the DNA preparation with a 10 μg/ml solution of RNase-free DNase for 30 min at 37 C resulted in total loss of radioactivity in the trichloroacetic acid precipitate, indicating that the DNA preparation was free of ^{3}H-RNA contamination.

The DNA yield was determined by Burton's diphenylamine test (3). The specific radioactivity of the prepared DNA was found to be 1.9×10^3 cpm/μg. The counts were made on the precipitate obtained with 5% cold trichloroacetic acid and collected on a 0.45-μm Millipore filter.

INCUBATION OF PROTOPLASTS WITH ^{3}H-DNA AND MEASUREMENT OF ABSORPTION

Protoplasts of *N. tabacum* L. 'Xanthi' were obtained from cells cultured in liquid nutrient by the method described earlier (31). They were suspended in the tobacco protoplast culture medium (32) in a density of 5×10^6 protoplasts/ml. The ^{3}H-DNA in SSC solution obtained from the cultivar 'Xanthi nc' cells was added to the nutrient medium in a concentration 7.26 μg/ml. The total volume of the incubation mixture was 300 μl (270 μl protoplast suspension plus 30 μl ^{3}H-DNA is SSC), and the incubation was carried out in a culture tube (10 × 75 mm) sealed with Parafilm. The incubation temperature was 27 C. After prescribed periods, the protoplasts were collected by centrifugation (480*g*, 5 min) and washed with fresh culture medium lacking ^{3}H-DNA. The washing was repeated twice.

The protoplasts were resuspended in 450 μl of nutrient medium. A 50-μl quantity of DNase solution (Worthington Biochemical Co.); 1 mg/ml stock solution prepared with 0.03 M $MgCl_2$) was added and the suspension was incubated at 37 C for 5 min to degrade any DNA that might be adhering to the protoplast surface. The DNase-treated protoplasts were washed three times with culture medium, centrifuging each time to remove the rinse medium. Other experiments showed that the DNase treatment was unnecessary if the protoplasts were washed four or more times with 0.7 M mannitol.

The protoplasts were again resuspended in 450 μl of SSC and then lysed by adding 50 μl of 10% solution of SDS. Equal volumes of cold 10% trichloroacetic acid and BSA were added to the lysate. The precipitate was collected on a Millipore membrane filter, 0.45 μm, and washed in order with 30 ml cold 5% trichloroacetic acid and 5 ml cold 95% ethanol. The collected precipitate was dried and its radioactivity was measured with a Beckman LS-100C liquid scintillation counter. The carrier consisted of 5 ml toluene containing 4 mg/ml Omnifluor (New England Nuclear). The process of incubating protoplasts with ^{3}H-DNA and measuring the absorbed quantity was repeated on a second sample.

In some experiments, poly-L-lysine, spermine, DEAE-dextran, amphotericin B, 2,4-dinitrophenol, KCN, and ATP were included in the ^{3}H-DNA uptake media and their influence on DNA absorption was determined. Absorption rates at incubation temperatures of 0, 27 and 37 C were also compared. Aseptic conditions were maintained throughout and sterilization of test addenda was accomplished by Millipore filtration.

REGENERATION OF CELLS AND PLANTS FROM PROTOPLASTS FED HOMOLOGOUS DNA

Preliminary attempts were made to observe possible phenotypic change in plants arising from protoplasts that had been incubated with homologous exogenous DNA. The DNA was obtained from *N. tabacum* L. 'Xanthi nc' cell cultures and purified according to the procedure described above for ^{3}H-DNA, except no labeling was involved. This cultivar of *Nicotiana* was selected because it possessed a dominant single gene trait for resistance to TMV[3] (7). Infection by the virus results in localized necrotic lesions, in contrast to the mosaic pattern that characterizes the susceptible cultivars. During the course of this investigation approximately 400 to 500 g of cells were used as a source of DNA. The extraction was carried out on 40- to 50-g samples and the yield of DNA was about 4 μg/g cells.

The protoplasts were isolated from *N. tabacum* 'Xanthi' cell cultures by the procedure described earlier (31). This cultivar has been susceptible to TMV. The protoplasts were placed in culture medium and allowed to absorb DNA prepared from the resistant cultivar, for 5 to 10 hr at 27 C. Aliquots of 2 to 3 ml culture solution containing 10^5 to 10^6 protoplasts and 20 to 30 μg DNA/ml nutrient were employed. Centrifuge tubes (15 × 115 mm) sealed with aluminum foil were used as incubation vessels.

The protoplasts were centrifuged at 300*g* for 2 min, then washed with fresh nutrient solution three times to remove adsorbed DNA. They were transferred to 50-ml DeLong flasks containing 5 ml protoplast culture medium (32); in each flask were approximately 5×10^5 protoplasts. They were incubated without agitation at 27 C and under 16-hr daily exposure to 1,000 lux illumination from Gro Lux lamps. After 1 to 2 weeks, the cultured protoplasts were mixed with freshly prepared agar-containing (1.6% Difco Bacto-agar) nutrient medium and transferred to Petri dishes for further culture. The protoplast culture was mixed with agar medium in a 1:1 ratio, and distributed at a rate of 2 to 3 ml/dish (10 × 55 mm sterile, disposable plastic dishes). The agar medium was prepared without mannitol, but with the sucrose concentration increased to 0.3 M. They were incubated a further 1 to 2 months, after which time colonies of 3 to 5 mm callus massess were attained. The callus masses were transferred individually to culture tubes (25 × 150 mm) containing 25 ml shoot-inducing nutrient agar (Murashige and Skoog salts, 0.3 M sucrose, 0.4 mg/l thiamine·HCl, 100 mg/l *myo*-inositol, 2 mg/l kinetin, 2 mg/l IAA, 80 mg/l adenine sulfate-$2H_2$0, 170 mg/l $NaH_2PO_2 \cdot H_2O$, 50 mg/l L-tyrosine, and .08% Bacto-agar). During the course of culture, the shoots that arose were separated and placed in a rooting medium (Murashige and Skoog salts, 3% sucrose, 0.5 mg/l thiamine·HCl, 100 mg/l *myo*-inositol, 2 mg/l IAA, and 0.8% Bacto-agar) and recultured under 10,000 lux illumination for a further 2 to 4 weeks. The

[3] Abbreviation: TMV: tobacco mosaic virus.

rooted plants were placed in soil in the greenhouse, and when about 10 to 15 cm tall, assayed for TMV resistance.

Aqueous suspensions containing 100 μg/l TMV were kindly furnished by W. O. Dawson of our plant pathology department. The upper surfaces of the youngest two to three leaves of each plant were swabbed with the TMV suspension, and the appearance of mosaic symptoms was recorded after 2 to 3 weeks.

FATE OF BACTERIAL DNA ABSORBED BY *N. GLUTINOSA* PROTOPLASTS

Preparation of *Escherichia coli* ^{3}H-DNA. *E. coli* B5-1,thy$^-$ was used to prepare ^{3}H-DNA. The medium for bacterium culture contained the following in g/l: NH_4Cl, 1; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 2; KH_2PO_4, 3; Na_2HPO_4, 6; glucose, 5; and thymidine, 0.002. Twenty ml nutrient medium contained in 125-ml DeLong flasks were inoculated with several colonies of *E. coli* and incubated overnight in darkness at 35 C. The cultures were agitated continuously at 150 rpm on a gyrotory shaker. A 10-ml sample of culture was transferred to 250 ml nutrient medium, contained in a 1-liter flask, and incubated a further 5 hr. A 250-μCi sample of ^{3}H-thymidine (7 Ci/mmol; Schwarz/Mann) was then added. After 12 to 16 hr, the bacteria were harvested by centrifuging at 3000*g* for 15 min. The pellet was washed by resuspending in 50 ml NET buffer (0.1 M NaCl, 0.01 M Na_2EDTA, and 0.01 M trizma base) and recentrifuging. The pellet was resuspended in a 2 ml NET and, following addition of 0.2 ml lysozyme (1 mg/ml), incubated 20 min at 37 C. Subsequently, 0.2 ml pronase (10 mg/ml) and 0.2 ml Sarkosyl (1%) were added to the lysate and the mixture was incubated 30 min at 60 C. To the viscous lysate were added 2 ml NET and 6 ml Sevag reagent (9:1, chloroform-octanol). The lysate was homogenized in a Vortex junior mixer for 5 min at its highest speed. To the supernatant obtained by centrifugation at 6000*g* for 10 min were added 2 volumes of cold 95% ethanol. The fibrous DNA was wound out on a glass rod, dissolved in 0.1 × SSC, and dialyzed in SSC solution overnight. Following treatment with 10 μg/ml RNase A and 100 units RNase T_1 for 30 min at 37 C, 2 volumes of cold 95% ethanol were added. The fibrous DNA was rewound, dissolved in 0.1 × SSC, and dialyzed overnight in SSC solution. The yield of purified DNA was 600 μg; it was stored at 4 C. The specific activity of the DNA was 9800 cpm/μg.

Competition in DNA Absorption. *N. glutinosa* protoplasts were incubated 5 hr in a medium (32) containing 4.4 μg/ml ^{3}H-DNA from *E. coli*, together with varying concentrations of calf thymus DNA or thymidine, and the absorption of radioactivity was measured. The incubation conditions were the same as described above, except the incubation volume was 250 μl (225 μl protoplast suspension plus 25 μl ^{3}H-DNA in SSC)/tube.

Intraprotoplast Localization of Absorbed ^{3}H-DNA. *N. glutinosa* protoplasts, following treatment with *E. coli* ^{3}H-DNA, were suspended in SET buffer (0.25 M sucrose, 3 mM EDTA, and 25 mM tris-MES ([tris(hydroxymethyl)aminomethane]-[2-(N-morpholino)]ethanesulfonic acid); pH 7.2) and homogenized manually by 50 plunges in a Teflon tissue grinder. The homogenate was centrifuged at 1000*g* for 10 min. The pellet was resuspended in SET buffer and recentrifuged 10 min at 1000*g*. Triton X-100 was added to the pellet in a 0.7% final concentration, mixed, and the mixture was centrifuged again for 10 min at 1000*g*; the pellet was saved for further study. The supernatant fractions from the first and second centrifugations were pooled and centrifuged further at 10,000*g* for 15 min; the pellet obtained was set aside as the mitochondria fraction. The supernatant was centrifuged for an additional 30 min at 40,000*g*, using a Spinco SW 27.1 rotor; the pellet from this centrifugation was designated as the membrane fraction and the supernatant was retained as the "soluble" fraction. Absence of nuclei in the 10,000 and 40,000*g* fractions was confirmed by using aceto-orcein stain. ATPase, Cyt *c* oxidase and NADH Cyt *c* reductase were assayed of each subcellular fraction according to methods of Leonard *et al.* (26).

Sucrose Density Gradient Centrifugation. A 200-μl sample of protoplast lysate containing 0.5% SDS was layered onto a 16.5-ml linear gradient, ranging from 5 to 20% (w/w) sucrose in SSC at pH 7, and 0.5 ml of 50% sucrose was layered under the 5 to 20% sucrose. Centrifugation was performed at 25,000 rpm for 16 hr at 8 C, using a Spinco SW 27.1 rotor. The gradient was fractionated with ISCO model 640 density gradient fractionator, equipped with a UV monitor.

Radioactivity in DNA, RNA and Protein. Radioactivities were measured of the nucleus and cytoplasm portions of protoplasts that had been fed *E. coli* ^{3}H-DNA. The nucleus and cytoplasm fractions were separated by following the same steps as described above in examining the subcellular fractions. The pellet from the 1000*g* centrifugation was considered as the nuclear fraction and the supernatant, the cytoplasm fraction. The RNA, DNA, and protein was extracted and purified by following the method of Hamilton *et al.* (13). The precipitates obtained by treating the nucleus and cytoplasm fractions with cold 5% trichloroacetic acid were resuspended in cold 95% ethanol and centrifuged at 1000*g* for 5 min. This rinsing process was repeated once. The pellet was resuspended in 0.25 M $HClO_4$ and centrifuged a further 5 min at 1000*g*. After adding 5 ml of 0.3 N NaOH, the pellet was incubated 2 hr at 37 C. The mixture was adjusted of its pH to 1 to 2 with 5 N $HClO_4$ and placed in ice for 30 min. It was then centrifuged at 7700*g* for 5 min and the supernatant was saved as the RNA fraction. The pellet, after addition of 5 ml of 0.5 N $HClO_4$, was kept at 70 C for 20 min, then centrifuged at 7700*g* for 15 min. The supernatant was retained as the DNA fraction. A 5-ml aliquot of 1 N NaOH was added and the pellet was further centrifuged at 7700*g* for 15 min; the supernatant was set aside as the protein fraction. The final supernatant, combined with the initial 1000*g* supernatant, was designated as the fraction of "unknown" substances. RNA was measured spectrophotometrically at 260 nm. The DNA content was determined by the diphenylamine method and the protein by Lowry's method (27). Radioactivity was assayed in 2:1, toluene-Triton X-100, containing 4 mg/ml Omnifluor.

DNA/DNA Hybridization. The ^{3}H-DNA was re-extracted from the *N. glutinosa* protoplasts by following the steps described earlier in the extraction of *N. tabacum* ^{3}H-DNA, except the phenol was replaced by a 9:1 mixture of chloroform-octanol. The re-extracted DNA was purified by employing HAP (hydroxylapatite) column chromatography, according to the method of Lurquin and Hotta (29). It was first dialyzed against 0.005 M PB (equal volumes of 0.005 M NaH_2PO_4 and 0.005 M $Na_2HPO_4 \cdot 7H_2O$) and then loaded onto a HAP column (0.7 × 2 cm) that had been previously equilibrated with 0.005 M PB. The loaded column was washed with 3 ml of 0.005 M PB and the DNA was eluted with 3 ml of 0.5 M PB. The eluate was dialyzed overnight in SSC.

Nonisotopic DNA preparations from *E. coli* and *N. glutinosa* were sonicated in a MSE Mullard ultrasonic power unit for 10 sec at its highest speed. The sonicated DNA was denatured by exposure to 0.1 M NaOH for 5 min, then was neutralized with 1 M KH_2PO_4. An equal volume of 10 × SSC was added to the DNA solution and the sheared and denatured DNA was loaded with gentle suction onto Selectron B-6 membrane filters (25 mm diameter, 0.45 μm pore size; Scientific Products, Irvine, Calif.) that had been washed previously with SSC. The DNA-loaded filters were washed with 5 × SSC and incubated in a mixture composed of 0.2 g/l each Ficol, PVP (mol. wt. 360,000), and BSA for 6 hr at 60 to 65 C (9). The amount of DNA/filter was 10 μg according to the diphenylamine test. After incubation, each filter was transferred to a glass vial and the ^{3}H-DNA, re-extracted from protoplasts, sonicated and denatured, was added. Following a further 12-hr incubation, each side of the filter was

washed with 30 ml of SSC. The filters were dried and their associated radioactivities were determined.

RESULTS

EXPERIMENTS WITH HOMOLOGOUS DNA

Time Course of Radioactivity Absorption. The time course of absorption of ^{3}H-labeled homologous DNA by *N. tabacum* protoplasts is shown in Figure 2. Note that radioactivity present in only the acid-insoluble fraction of the protoplasts has been measured. There was a progressive uptake of the exogenous radioactivity and after 5 hr, when the absorption was deliberately terminated, over 3% of supplied radioactivity could be observed in the protoplasts.

Influence of Some Addenda and Temperature on Rate of Radioactivity Absorption. As apparent in Table I, the content of radioactivity in tobacco protoplasts after a 4-hr incubation was doubled by poly-L-lysine and DEAE-dextran. Spermine also enhanced absorption, but not substantially. A slight stimulation by amphotericin B was observed.

In a preliminary exploration of the mechanism underlying the DNA absorption process, the influence of temperature, metabolic inhibitors such as DNP and KCN, and ATP were studied. As can be seen in Table II, at the 27 C temperature that has been standard for protoplast and cell culture, a substantial incorporation (3.5%) of exogenous radioactivity occurred. Nevertheless, a higher temperature, 37 C, enabled significantly more absorption, 4.7% of the supplied radioactivity. DNP, KCN, and low temperature decreased incorporation of radioactivity. Exogenous ATP did not reverse the inhibitory effect of KCN or DNP.

Regenerated Tobacco Plants. About 500 *N. tabacum* plants were regenerated from protoplasts of the virus-susceptible cultivar that had been fed DNA prepared from the resistant cultivar. None of the plants showed phenotypic change.

FATE OF BACTERIAL DNA IN *N. GLUTINOSA* PROTOPLASTS

Absorption of Undegraded DNA. Competition experiments between unlabeled calf thymus DNA or thymidine and ^{3}H-DNA from *E. coli* showed that the calf thymus DNA inhibited absorption of radioactivity by the *N. glutinosa* protoplasts, whereas the thymidine did not (Fig. 3). Thymidine in concentrations of 30 μg/ml and higher promoted radioactivity absorption slightly. The inhibition of ^{3}H-DNA absorption by calf thymus DNA was as much as 83% at the 1000 μg/ml level.

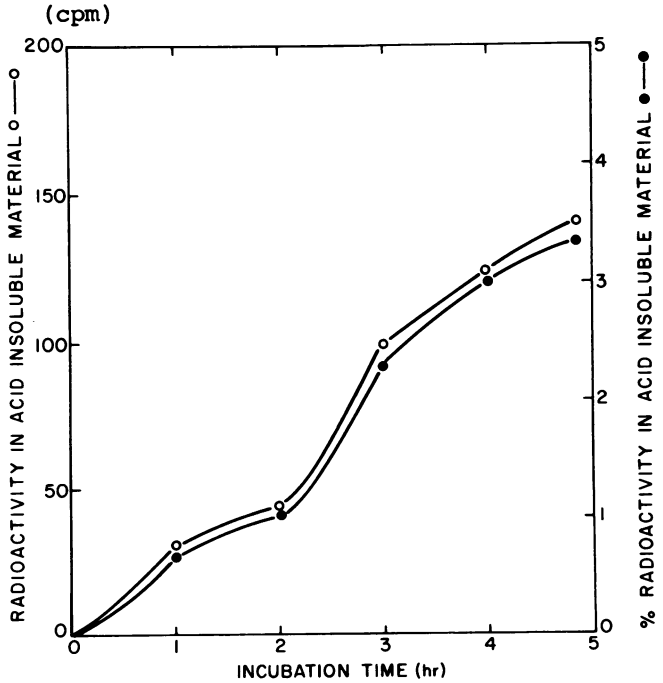

FIG. 2. Time course of increase of radioactivity in acid-insoluble materials of tobacco protoplasts treated with homologous ^{3}H-DNA. *N. tabacum* L. 'Xanthi' protoplasts were incubated in a medium (32) containing *N. tabacum* L. 'Xanthi nc' ^{3}H-DNA (7.26 μg/ml; 1.9 × 10^3 cpm/μg DNA).

Table I. Effects of Poly-L-lysine; DEAE-dextran, Spermine, and Amphotericin B on Radioactivity in Acid-insoluble Materials of Tobacco Protoplasts Treated with Homologous ^{3}H-DNA. Poly-L-lysine (mol wt 86,000), DEAE-dextran (mol wt 2,000,000) and spermine (mol wt 398) were obtained from Sigma Chemical. Amphotericin B was purchased from Calbiochem.

Addendum	Incorporation	
μg/ml	(cpm)	(%)
None	73	1.74
Poly-L-lysine (5)	145	3.45
DEAE-dextran (5)	166	3.95
Spermine (0.367)	118	2.81
Amphotericin B (200)	96	2.29

Table II. Effects of Temperature, KCN, DNP and ATP on Radioactivity in Acid Insoluble Materials of Tobacco Protoplasts Treated with ^{3}H-DNA.

Treatment	Incorporation	
	(cpm)	(%)
Temperature (C):		
0	0	0
27	146	3.5
37	196	4.7
Components added (at 27 C):		
DNP (x 10^{-4}M)	14	0.33
KCN (x 10^{-3}M)	0	0
ATP (x 10^{-4}M)	175	4.16
KCN + ATP	0	0

Intraprotoplast Localization. The activities of marker enzymes of the subcellular fractions are summarized in Table III. Of the total Cyt *c* oxidase activity, 83.9% was observed in the mitochondrion fraction. NADH Cyt *c* reductase, the marker enzyme of endoplasmic reticulum, was not evident in the nuclear fraction. The outer membrane of the nucleus was apparently dissolved completely by Triton X-100; in other experiments where the Triton X-100 treatment was omitted about 2.7% of the NADH Cyt *c* reductase activity was located in the nuclear fraction. ATPase, the marker enzyme for plasma membrane, was observed in the membrane fraction in a proportion 2.5% of the total cell activity.

In Table IV can be seen the intraprotoplast distribution of radioactivities. In the experimental sample, 60.9% of the radioactivity within the protoplasts was associated with the "soluble" fraction and 28.4% with the nuclear fraction. In contrast, in the control sample, only 8.6% of the radioactivity was in the nuclear fraction, but as much as 79.2% in the soluble fraction. Similar data were obtained with the acid-insoluble substances. By subtracting the control radioactivities from the experimental samples, it is apparent that the nuclear fraction of the latter contained 18 to 19% more radioactivity than the control sample; the mitochondrion fraction had 6.6% greater radioactivity in the experimental sample. The membrane and soluble fractions showed the reverse trend.

Degradation of DNA into Smaller Units. After a 5-hr incubation of the *N. glutinosa* protoplasts with *E. coli* ^{3}H-DNA, the peak of radioactivity was seen to shift from large molecules to smaller units (Fig. 4). For control, ^{3}H-DNA was centrifuged in combination with the lysate from protoplasts that had not been fed ^{3}H-DNA. No change of the position of radioactivity peak

occurred, when samples of *E. coli* ^{3}H-DNA and the *E. coli* ^{3}H-DNA plus protoplast lysate were compared.

Reutilization of degraded *E. coli* ^{3}H-DNA. After a 5-hr incubation with *E. coli* ^{3}H-DNA, 56.4% of the cytoplasmic radioactivity was found as cytoplasmic DNA and 51.2% of the nuclear radioactivity as nuclear DNA (Table V). The remainder was distributed in RNA, protein, and other substances. A 20-hr incubation resulted in a decrease of radioactivity in cytoplasmic DNA to 37.6% of the total cytoplasmic activity and an increase in the nuclear DNA fraction to 65% of the nuclear radioactivity.

Foreign DNA Molecular Sequences Remaining in Protoplasts. The DNA/DNA hybridization experiment revealed that a 17.6% homology existed between the ^{3}H-DNA re-extracted from *Nicotiana* protoplasts and the *E. coli* DNA on the membrane filter (Table VI). The homology between re-extracted ^{3}H-DNA and *N. glutinosa* DNA on the filter was 11.6%. Other experiments showed that the degree of hybridization between *E. coli* ^{3}H-DNA and *N. glutinosa* DNA, or between *N. glutinosa* ^{3}H-DNA and *E. coli* DNA, was negligible and equal to the blank controls.

DISCUSSION

Previous investigations have shown that higher plant protoplasts are able to absorb measurable quantities of foreign DNA. Ohyama *et al.* (30) reported that as much as 1% of *E. coli* DNA (1.09×10^{-14}g DNA/protoplast) was found in the acid-precipitable fraction of *Glycine max* protoplasts after 4 hr; *Ammi visnaga* protoplasts absorbed 0.11% of the bacterial DNA (0.1×10^{-14}g DNA/protoplast. Gleba *et al.* obtained an incorporation of 0.25% (0.33×10^{-14}g DNA/protoplast) of the radioactivity from *Bacillus subtilis* ^{3}H-DNA into *N. tabacum* mesophyll protoplasts in 4.5 hr (11). Our experiments with *N. tabacum* revealed an uptake in 5 hr of about 3.5% (5.08×10^{-14}g DNA/protoplast) acid-insoluble radioactivity from homologous DNA; the absorption associated with ^{3}H-DNA from *E. coli* was as high as 10% (8.8×10^{-14}g DNA/protoplast) of total input radioactivity in 5 to 20 hr. Our experiments also confirmed the enhancement of absorption by DEAE-dextran and poly-L-lysine (11, 30). Presumably, the enhancement was related to the substances stabilizing the tertiary structure of the DNA (5). Amphotericin B was also tested because it was reported to stimulate DNA absorption in mammalian cells *in vitro* (22); only a slight, if any, promotion of nucleic acid intake by tobacco protoplasts was observed. Inclusion of DNP or KCN in the medium and use of a low temperature reduced drastically the absorption of DNA by tobacco protoplasts. This observation agrees with the notion that the entry of large molecules into cells or protoplasts occurs by pinocytosis (2, 8), the process having an energy requirement. Exogenous ATP was unable to negate the inhibitions.

The entry of exogenous DNA into protoplasts should not be surprising, in view of reports of exogenous DNA absorption by cultured cells, seeds and seedlings. Bendich and Filner (1) described the absorption by tobacco cell cultures of as much as 0.5% bacterial DNA in 17 hr. In a series of investigations, Ledoux and Huart (23, 24) described incorporation as well as integration of DNA from several bacteria by *Hordeum vulgare* and *Arabidopsis thaliana*, following immersion of seeds or roots of seedlings in nucleic acid solutions. *A. thaliana* was reported as having absorbed 0.7 ng *E. coli* DNA/seed after 96 hr. Hess (14) also noted that the DNA from red flowering petunia could be incorporated into seedlings of white flowering petunia.

The seemingly low rates of DNA absorption by protoplasts reflect the relatively short exposure period after which measure-

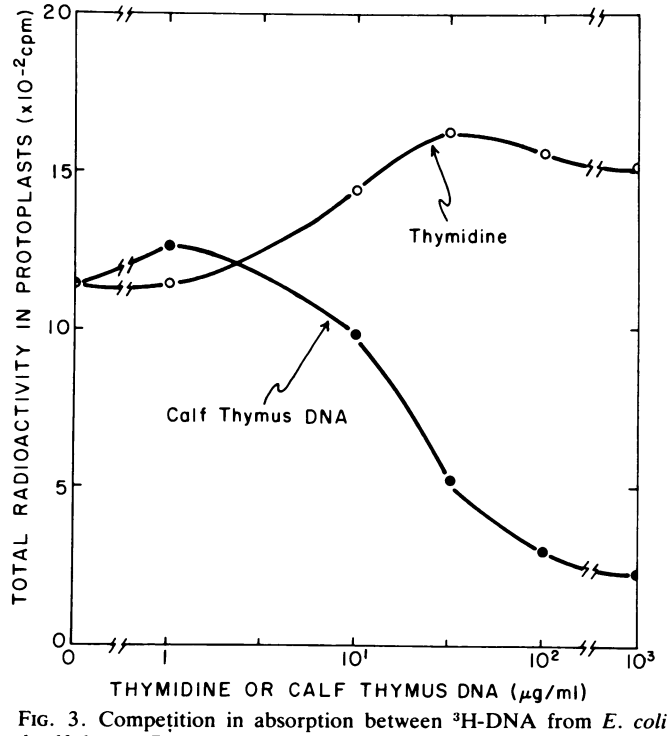

FIG. 3. Competition in absorption between ^{3}H-DNA from *E. coli* and calf thymus DNA or thymidine. Protoplasts were incubated 5 hr in the medium (32) containing *E. coli* ^{3}H-DNA together with various concentrations of either calf thymus DNA or thymidine. The input of ^{3}H-DNA was 1.12 μg (11,000 cpm).

Table IV. Subcellular Distribution of Radioactivity in Protoplasts Treated with E. coli ^{3}H-DNA.
As a control, the ^{3}H-DNA of E. coli was mixed with N. glutinosa protoplasts lysates which had not been fed ^{3}H-DNA, and the subcellular components were fractionated.

Subcellular fraction	Radioactivity total		acid insoluble	
	Experimental	Control	Experimental	Control
	%			
Nuclei	28.4	8.6	25.9	7.3
Mitochondria	7.6	5.6	28.1	21.5
Membrane	3.1	6.6	9.4	14.6
"Soluble"	60.9	79.2	36.6	56.6

Table III. Activity Distribution of Marker Enzymes in Subcellular Fraction of N. glutinosa Protoplasts.

Fraction	(μmoles Cyt c/min) Cyt c oxidase	NADH-Cyt c Reductase	ATPase (pH 6.5)	(μmoles Pi/hr) (pH 9)
Nuclei	0.21 (4.2)[1]	0.00 (0)[1]	63.3 (9.8)[1]	40.0 (8.5)[1]
Mitochondria	4.22 (83.9)	1.37 (38.6)	69.9 (10.8)	112.7 (23.8)
Membrane	0.15 (3.0)	0.34 (9.6)	16.0 (2.5)	12.4 (2.6)
"Soluble"	0.45 (8.9)	1.84 (51.8)	495.3 (76.9)	307.5 (65.1)

[1]percentage of total in parenthesis

ments have been made. The short exposures were deliberate and intended to minimize errors associated with DNA synthesis from recycled nucleotides.

Whereas, the incorporation of radioactivity from exogenous ^{3}H-DNA into plant protoplasts has been demonstrable repeatedly, major questions of interpretations have remained. Part of our investigation with homologous DNA feeding yielded no evidence of phenotypic alteration in the receptor tobacco. The negative results suggest several alternative conclusions. Possibly the DNA was being degraded prior to its entry into protoplasts. If the absorption involved intact DNA molecules, perhaps degradation occurred after entry, or other failures of integration into the receptor protoplasts' genome could have resulted.

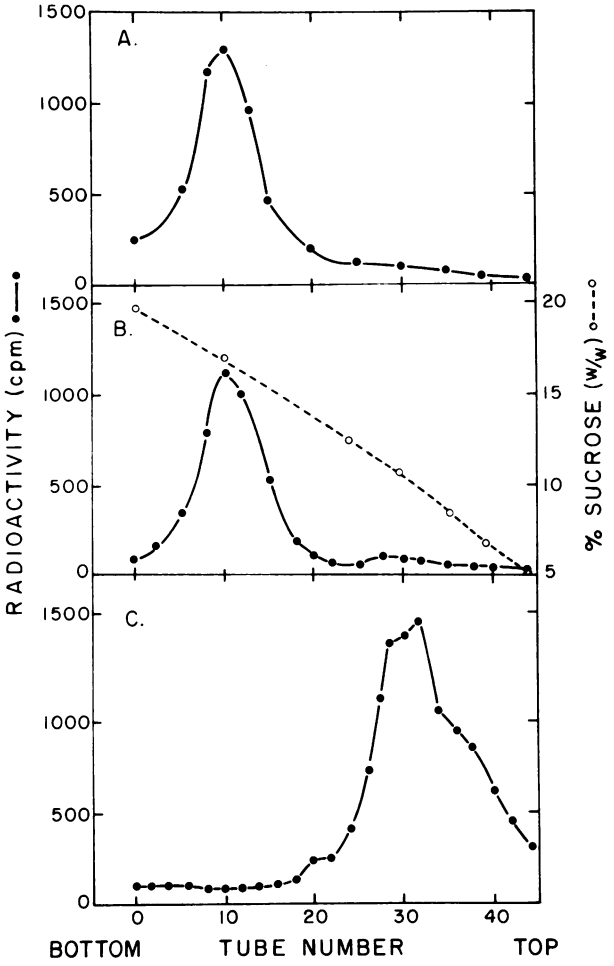

FIG. 4. Sucrose density gradient (5-20%) of *N. glutinosa* protoplast lysate treated with *E. coli* ^{3}H-DNA. A: *E. coli* ^{3}H-DNA alone; B: *E. coli* ^{3}H-DNA plus lysate from protoplasts which had not been fed *E. coli* DNA; C: lysate from protoplasts fed 10 hr ^{3}H-DNA of *E. coli*. Radioactivity was measured in the cold 5% trichloroacetic acid precipitates of each tubes.

Evidently, the predominant fraction of radioactivity entered the protoplasts in the form of undegraded supplied DNA. A significant decrease of isotope uptake resulted when *E. coli* ^{3}H-DNA was supplied together with calf thymus DNA. Incubation of *N. glutinosa* protoplasts with *E. coli* ^{3}H-DNA in the presence of thymidine did not repress radioactivity entry. The degradation of DNA prior to its incorporation is dependent on DNase released by the protoplasts. Holl (17) reported low levels of DNase activity in cotyledons and protoplasts of *G. max* that were undergoing little or no cell divisions.

According to Hoffman (15), a 12-hr incubation of petunia protoplasts with homologous DNA resulted in 0.04% of the absorbed material being located in the nucleus and 0.007% in the cytoplasm fraction. Our investigation with tobacco protoplasts showed that the radioactivity of ^{3}H-DNA absorbed in 5 hr was distributed in a 1:4 ratio between nucleus and cytoplasm. Hotta and Stern (18) found that the nuclear membrane of *Vicia faba* adsorbed bacterial DNA, and the rate of binding could be decreased by treatment with Triton X-100. After dissolving and removing the nuclear membrane with Triton X-100, we were still able to observe 18 to 19% of the absorbed radioactivity in the nucleus fraction. Furthermore, we have found significant radioactivity, 6.6% of the acid-insoluble radioactivity, in the mitochondrial fraction.

Our sucrose density gradients showed that the *E. coli* ^{3}H-DNA was degraded within the *N. glutinosa* protoplasts. Lurquin and Behki (28) reported a similar observation in experiments with *Chlamydomonas reinhardi*. Employing the method of molecular sieving with Sepharose 4B, they found that *E. coli* ^{3}H-DNA was depolymerized into nucleotides after absorption by the *Chlamydomonas*.

An analysis of cell constituents showed that 50% of the radioactivity from absorbed ^{3}H-DNA was relocated in substances other than DNA after 5 to 20 hr. In a related investigation, *N. glutinosa* protoplasts have been observed to transform ^{3}H-uridine and ^{3}H-thymidine into acid-insoluble components, implicating progress of nucleic acid synthesis and probably reutilization of the degraded foreign DNA. Lurquin and Behki (28) similarly attributed the finding of labeled *Chlamydomonas* DNA to a reutilization of ^{3}H-thymidine derivatives released from ^{3}H-DNA degradation. In another investigation, Lurquin and Hotta (29) observed that *A. thaliana* callus cells that had been cultured for 7 days in the presence of *E. coli* or *Micrococcus lysodeikticus* DNA contained, as evident through isopycnic centrifugation, only *A. thaliana* DNA; the bacterial DNA was presumably degraded and reutilized in *Arabidopsis* DNA synthesis. These authors studied the reassociation kinetics and were unable to find bacterial DNA sequences in the re-extracted DNA. In exploring plant tumors associated with *Agrobacterium*, Chilton *et al.* (6) performed a renaturation kinetic study and discovered negligible or no bacterial DNA in the host cells; Kado and Lurquin (20) attempted DNA/DNA hybridization and were also unable to find significant quantities of microbial DNA. Goebel and Schieb (12) discovered that plasmid DNA from *E. coli*,

Table V. Distribution of Radioactivity in Various Intercellular Components of N. glutinosa Protoplasts Treated with E. coli ^{3}H-DNA.

Time	Fraction	Acid Insoluble	Acid-Insoluble Count				
			DNA	RNA	Protein	Other	Total
(hr)		cpm			%		
5	Cytoplasm	7.9×10^3	56.4	9.0	18.2	16.4	100
	Nucleus	1.7×10^4	51.2	22.0	17.0	9.8	100
20	Cytoplasm	1.3×10^4	37.6	30.0	11.3	21.1	100
	Nucleus	4.1×10^3	65.0	12.7	12.7	9.6	100

Table VI. DNA/DNA Hybridization of Re-extracted DNA from N. glutinosa Protoplasts Fed ^{3}H-DNA of E. coli.

Re-extracted ^{3}H-DNA was supplied on a membrane filter which was bound either E. coli DNA or N. glutinosa DNA. The amount of DNA loaded on a filter was 10 μg and the input of re-extracted DNA on a filter was 2.3 μg quantity. Radioactivity of blank membrane filter was subtracted from the radioactivity of experimental filters.

DNA Bound on filter	Input cpm	cpm hybridized	% DNA[1] annealed
E. coli	4,315	760	17.6
N. glutinosa	4,315	268	11.6

[1]Under the same hybridization condition, about 35% of N. glutinosa ^{3}H-DNA (1,800 cpm/μg DNA) annealed with N. glutinosa DNA on the membrane filter, and less than 0.1% with E. coli DNA.

when introduced into cultured mammalian cells, was disintegrated into acid-soluble fragments after a few cell generations. Our DNA/DNA hybridization experiments also showed that 17.6% of the ^{3}H-DNA re-extracted from *N. glutinosa* protoplasts after about a 10-hr feeding still retained *E. coli* DNA sequences.

The fate of *E. coli* DNA in *N. glutinosa* is summarized schematically in Figure 5. Within 5 to 20 hr, about 10% of the bacterial DNA was absorbed by the *Nicotiana* protoplasts. Of the incorporated DNA, only 2.6%, or a minority fraction, was found as *E. coli* DNA. This value is the product of the 17.6% of annealed DNA of the hybridization experiment and the 15% of ^{3}H-DNA found in recipient cells. The larger proportion, 70%, of the absorbed DNA was degraded. Of the remaining 30%, comprised of acid-insoluble substances, 15% was accountable as DNA, exogenous as well as resynthesized, and 15% as RNA, protein, and other cell constituents. At least 1.7% of the absorbed *E. coli* DNA was reutilized in *Nicotiana* DNA synthesis. It has not been possible to account for approximately 10% of the absorbed DNA. The proportion 2.6% was equal to a quantity 2.3×10^{-15}g of *E. coli* DNA. Assuming 4.7×10^{-15}g DNA/*E. coli* genome, half of the bacterial genome could still be found in the *Nicotiana* protoplast at the conclusion of the experiment.

Failure to impart TMV resistance to the susceptible tobacco cultivar does not preclude genetic modification of higher plants via the transfer of DNA into protoplasts. Observations in other investigations suggest strongly that gene transfer through somatic cells is distinctly possible. Ledoux *et al.* (25) noted that the thiamine requirement of certain *Arabidopsis* seedlings could be alleviated by allowing seeds to absorb bacterial DNA; nearly 1% of treated plants showed the phenotypic change. Hess (14) described the development of red flowers in 18% of white flowering petunia seedlings that had been allowed to imbibe a solution containing DNA extracted from the former. Both Ledoux's group and Hess were able to observe transmission of the modifications to the progenies.

This investigation has confirmed that the plant cell is endowed with mechanisms that are destructive of foreign DNA. Hence, successful gene transfer will require that the DNA be inserted suitably protected against the recipient protoplast's destructive mechanism. Possibilities of bacteriophages as intermediary have been examined by some investigators. Doy *et al.* (10) and Johnson *et al.* (19) claimed the apparent transfer of the *lac* operon from bacteria to tomato, *Happlopappus*, and sycamore cells following their infection by phage that had previously parasitized the bacteria. Activities of the *S*-adensylmethionine-cleaving enzyme and T3 RNA polymerase were observed at low levels following infection of protoplasts with bacteriophage T3 (4). Plasmids also remain as potentially useful vehicles in gene transfer into higher plants.

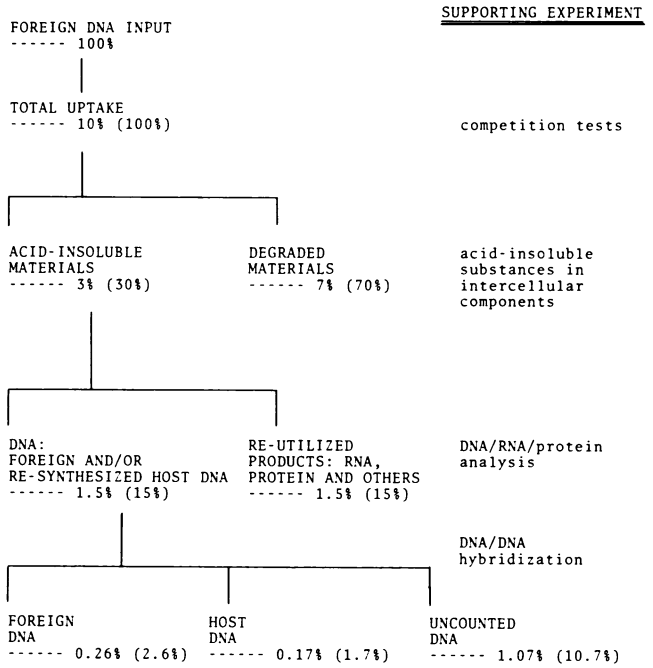

FIG. 5. Summary of quantitative estimates of the fate of exogenous DNA in plant protoplasts. The amount of each components relative to total radioactivity was found in protoplasts, is expressed as percentage, given in parentheses.

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