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Strontium Phosphate Transfection of Human Cells in Primary Culture: Stable Expression of the Simian Virus 40 Large-T-Antigen Gene in Primary Human Bronchial Epithelial Cells

DOUGLAS E. BRASH,[1]* ROGER R. REDDEL,[1] MYRA QUANRUD,[1]† KE YANG,[1] MICHAEL P. FARRELL,[2] AND CURTIS C. HARRIS[1]

Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20892,[1] and Lombardi Cancer Center, Georgetown University Medical Center, Washington, D.C. 20007[2]

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Strontium ion formed DNA-phosphate precipitates analogous to those formed by calcium but lacking the lethal and differentiation-inducing effects of calcium on many epithelial cell types in primary culture. Human primary bronchial epithelial cells were transiently and stably transfected by using strontium phosphate; the frequency of stable transformation with a plasmid carrying the simian virus 40 large-T-antigen gene was greater than 10^{-4}.

Calcium phosphate transfection has been widely used to introduce cloned and genomic DNAs and RNAs into cultured cell lines and intact animals (2–4, 6, 8, 12, 26, 30). This technique results in transient expression and stable integration of cloned or genomic DNAs at high copy numbers by using a simple procedure and results in efficient expression of stable integrants. Recent improvements in techniques for growing differentiated cells in primary culture (9, 20, 23) have made accessible a new range of questions in cell biology. However, primary cultures of normal human bronchial epithelial (NHBE) cells (14) are lysed by calcium phosphate precipitates, and at lower concentrations, calcium ion in the presence of serum induces NHBE cells and certain rat, mouse, and human keratinocytes to undergo squamous differentiation (10, 21). In addition, calcium inhibits the growth of primary human prostate cells (E. Kaighn, personal communication). The biological regulatory activity of Ca^{2+} ions may also limit the use of calcium phosphate precipitates in other cell types.

We report here that it is possible to substitute the strontium divalent cation for calcium in forming a physically similar DNA-phosphate precipitate, that both transient expression and stable integration occur in simian and murine cell lines and in primary NHBE cells, and that the simian virus 40 (SV40) large-T-antigen gene transfected into primary NHBE cells is stably expressed in these cells.

Strontium phosphate precipitates. NHBE cells were established as primary cultures from explants and grown as previously described (14) on collagen-fibronectin-coated 100-mm dishes in semidefined serum-free medium LHC-9 (pH 7.3 to 7.4) in a 4.5% CO_2 humidified incubator. At 18 h prior to transfection, the incubator CO_2 level was checked and cells were passaged by using 0.02% trypsin–0.02% EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]–1% polyvinylpyrrolidone at room temperature and seeded in 25-cm^2 flasks at 2×10^4 to 4×10^4 cells per cm^2. At 3 h prior to transfection, cells were refed with 5 ml of medium which had been equilibrated in the incubator overnight. CV-1 monkey kidney cells and NIH 3T3 cells were grown in Dulbecco minimal essential medium with high glucose (GIBCO Laboratories, Grand Island, N.Y.) plus 10% fetal calf serum at a pH of 7.3 to 7.4. At 18 h prior to transfection, cells were passaged and reseeded at 10^4 cells per cm^2. At 3 h prior to transfection, cells were refed with 5 ml of medium.

The 2 M $SrCl_2$ solution was prepared in distilled water and filter sterilized. Sources were BDH, Poole, England (0.05% Ca; distributed by Gallard-Schlesinger, Carle Place, N.Y.) and Johnson Matthey Chemicals, Royston, Hertfordshire, England (Puratronic; 0.0001% Ca; distributed by AESAR, Seabrook, N.H.). It was important to rinse filters with distilled water prior to filtering these solutions to avoid subsequent aggregation of precipitate. A 2× HEPES balanced salt solution (HBS; 2× HBS is 8.18 g of NaCl–5.95 g of HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]–0.2 g of $Na_2HPO_4 \cdot 7H_2O$–distilled water to 500 ml) (5, 30) was pH adjusted to 7.7 to 7.9 with NaOH, filter sterilized, and stored at 4°C. For calcium phosphate, 2 M $CaCl_2$ (BDH) was used and 2× HBS was adjusted to pH 7.08. Glycerol was 15% (wt/vol) in HBS or 11.8% (vol/vol); the solution was filter sterilized and stored at 4°C.

To prepare precipitates, solutions were warmed to room temperature. For a 25-cm^2 flask, 5 μg of DNA in x μl of solution was transfected by consecutively mixing $220 - x$ μl of sterile distilled water, 31 μl of 2 M $SrCl_2$, and x μl of DNA (cesium chloride banded and supercoiled) in a sterile nonwettable plastic tube (Falcon 2059; Becton Dickinson Labware, Oxnard, Calif.). This solution was added dropwise to 250 μl of 2× HBS, with mixing between drops to prevent high local concentrations of phosphate. Mixing was done by adding the solution to the depression created in the 2× HBS by a nitrogen gas stream. As the mixture stood, the precipitate increased in size, first forming small dustlike threads and then forming progressively larger networks. The size of the precipitates was monitored by phase-contrast microscopy immediately after successive samples were transferred to a slide without a cover slip. At the stage of fine dust (5 to 10 min), the precipitates were pipetted into the flasks. The efficacy of transfection could be predicted from the quality of the precipitate. After 90 min (NHBE), 2 h (CV-1), or 3 h (NIH 3T3), the cells were rinsed twice with serum-free medium at 37°C, incubated with 1.5 ml of 15% glycerol for 30 s at room temperature (NHBE), 3 min at 37°C (CV-1), or 1.5

* Corresponding author.
† Present address: Concordia College, Moorhead, MN 56560.

min at room temperature (NIH 3T3), rinsed three times with serum-free medium, and refed (11, 29).

With 2× HBS of pH 7.08 to 7.5, Sr did not form a visible DNA-phosphate precipitate. At pH 7.7 to 7.9, however, a dustlike precipitate formed which was indistinguishable from the calcium phosphate precipitate at pH 7.08. Coarser precipitates were formed up to pH 8.7. In a narrow pH range near 7.6, the precipitate consisted of uniform dark oval particles when viewed by phase-contrast microscopy. As the pH 7.7 to 7.9 precipitates were allowed to stand, the dustlike threads increased in size and formed large networks. This process was faster at pH 7.9 than at 7.7 and was accelerated when filters used for sterilizing solutions had not been prerinsed with distilled water.

On addition to CV-1 or NHBE cells with a cell medium pH of 7.3 to 7.4, the precipitates with 2× HBS at pH 7.7 to 7.9 settled onto the cells and dish to form an adherent dustlike layer which was not removed by rinsing with medium. In contrast, the large networks which formed at long incubation times did not adhere to the cells. At cell medium pHs greater than 7.5, the precipitate became refractile and nonadherent. Below a cell medium pH of 7.2, the precipitate redissolved. The oval particles that were precipitated with 2× HBS at pH 7.6 either redissolved in pH 7.3 to 7.4 medium or failed to adhere. Many of these properties were found with calcium phosphate precipitates.

Transfection. To determine whether the strontium phosphate precipitates allow DNA to enter the cell and become expressed, CV-1 cells were transfected with pRSVcat and pSV2cat, DNA plasmids which carry the chloramphenicol acetyltransferase (CAT) gene (7). CAT activity was detected in CV-1 cells transfected with pRSVcat by using Sr at pH 7.8 and 7.9 but not at pH 7.7 (Fig. 1A). CAT activity was not detected following Sr transfections at pH 7.2 to 7.6 (data not shown). The level of expression was somewhat greater with Sr at pH 7.8 than with Ca at pH 7.1. The precipitate at pH 7.7 was initially very fine but had redissolved by the time of glycerol shock; it was found that the efficiency of the pH 7.7 precipitate varied between experiments, probably due to slight variations in actual pH. The accuracy of pH meters and the change of pH upon filtration were sufficiently variable that we prepared pH 7.7 and 7.8 solutions and used the stock giving the best precipitates. Expression of CAT activity was 10-fold greater after transfection of supercoiled plasmid than after transfection of plasmid linearized at the unique *Acc*I site (data not shown).

Calcium phosphate precipitates lysed NHBE cells within minutes after addition to the culture. The strontium phosphate precipitate, on the other hand, was effective in transfection at pH 7.6 to 7.9, as determined by an assay of CAT activity 3 days after transfection of the pSV2cat plasmid (Fig. 1B). The efficacy of transient expression following transfection of NHBE cells was less than that of CV-1 cells but was somewhat greater than that of NIH 3T3 cells (data not shown).

The strontium phosphate precipitate, like calcium phosphate in calcium-transfectable cells, was not entirely nontoxic. This reduction in cell number occasionally approached 30% in serum-free medium and appeared to be due to internalized precipitate, as overnight incubation of cells with strontium chloride in the absence of 2× HBS was not toxic and toxicity was greatest at those pHs most effective in transfection. Toxicity could be nearly eliminated by adding 0.1% fetal calf serum at the time of refeeding prior to the experiment and removing it during the glycerol shock.

Since some techniques, such as DEAE-dextran, give transient expression but rarely stable integration, CV-1 cells were transfected with pSV2neo and selected for resistance to geneticin. At 11 days, colonies appeared at the same pHs giving transient expression of the CAT gene (7.7 to 7.9 but not 7.5 or 7.6) and at the same frequency as with calcium phosphate transfection, about 0.4% (Fig. 2). Supercoiled and linearized DNA yielded the same numbers of large colonies; supercoiled plasmid also gave small pseudocolonies which proliferated for several doublings before dying.

Strontium transfection of NHBE cells also generated stable transfectants. Primary NHBE cells were transfected with the plasmid pRSV-T, which carries the gene for SV40 large T antigen (generous gift of Bruce Howard and John Brady; N. Z. Xu and B. Howard, unpublished data). Foci of morphologically altered cells were obtained after 28 days at a frequency of 2.5 colonies per 10^4 cells transfected. One of these foci was passaged repeatedly. Genomic DNA isolated after eight passages was digested with the restriction enzyme *Eco*RI (which cuts twice within pRSV-T, at sites flanking the T-antigen gene) or with *Bam*HI (which cuts the plasmid once, outside the T-antigen gene). After Southern blotting to Gene Screen Plus (New England Nuclear Corp., Boston, Mass.), the DNA was probed with the 1.2-kilobase (kb) *Hin*dIII fragment of the T-antigen gene, which was labeled by nick translation (Lofstrand Laboratories, Silver Spring, Md.). The expected 3.5-kb T-antigen *Eco*RI band was detected (Fig. 3). Three *Bam*HI bands were detected at approximately 6, 12, and 23 kb, indicating that the transfected plasmid had integrated at three or more genomic sites. Indirect immunofluorescence for SV40 large T antigen

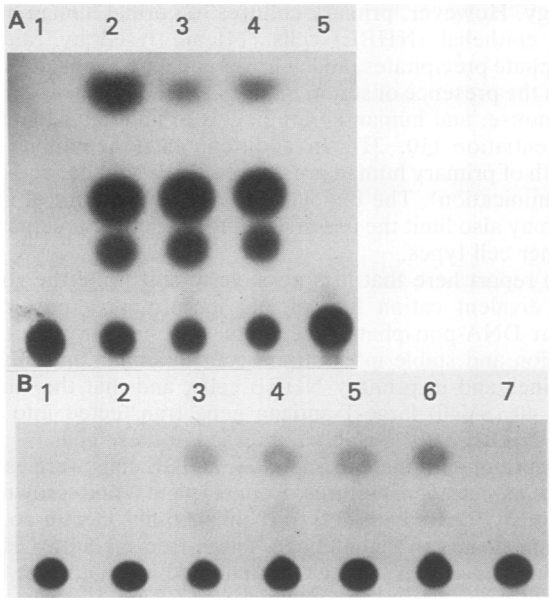

FIG. 1. (A) Transient transfection of the CAT gene (pRSVcat) into CV-1 monkey kidney cells by using strontium and calcium ions. Since calcium phosphate precipitate formation is extremely pH dependent (8), various pHs of 2× HBS were used. Lanes: 1, Sr at pH 7.7; 2, Sr at pH 7.8; 3, Sr at pH 7.9; 4, Ca at pH 7.1; 5, Ca at pH 7.1 without plasmid. (B) Transient transfection of pSV2cat into NHBE cells in primary culture by using strontium. Lanes: 1, pH 7.4; 2, pH 7.5; 3, pH 7.6; 4, pH 7.7; 5, pH 7.8; 6, pH 7.9; 7, pH 7.8 without plasmid. Cells were harvested and assayed for CAT activity 2 to 3 days after transfection (7). NHBE cells were harvested by incubating the cells with 0.2% EGTA for 10 min at room temperature before scraping, as these cells were prematurely lysed by the standard buffer.

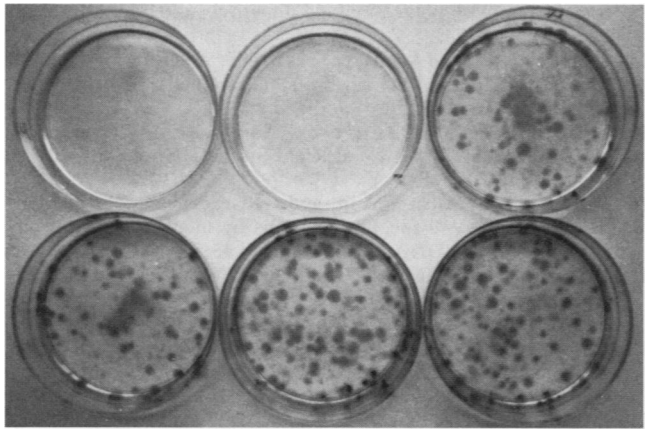

FIG. 2. Stable expression of Neo^r in the genome of CV-1 monkey kidney cells. Top row, left to right: pHs of 7.5, 7.6, and 7.7. Bottom row, left to right: pHs of 7.8, 7.9, and (calcium phosphate) 7.1. At 2 to 3 days after transfection of pSV2neo plasmid by the method described in the text, CV-1 cells were trypsinized and reseeded at 2×10^3 cells per cm^2 and selected with 800 μg of geneticin per ml (GIBCO; 27). After 11 days, colonies were fixed, stained with crystal violet, and counted.

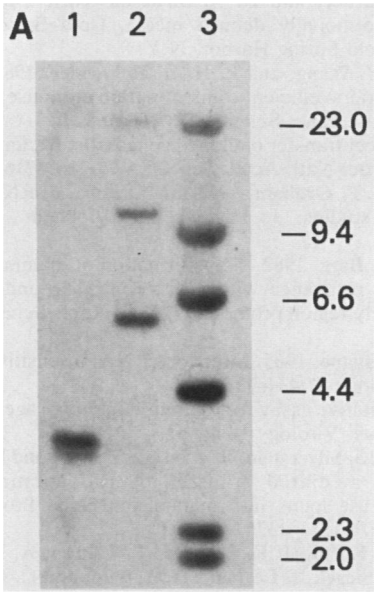

FIG. 3. (A) Stable integration of the SV40 large-T-antigen gene into the genome of primary NHBE cells. NHBE cells (5×10^5 per 10-cm dish) were transfected with 10 μg of pRSV-T, an SV40 Ori$^-$ plasmid in which the Rous sarcoma virus long terminal repeat directs expression of the SV40 large-T-antigen coding region. Cells were passaged 60 h after transfection. One colony of morphologically transformed cells was trypsinized and expanded by repeated subculturing, usually at a subculturing ratio of 1 to 4. Cells from passage 8 of the T-antigen-transfected clone were lysed in sodium dodecyl sulfate–proteinase K, phenol extracted, and purified by two cycles of centrifugation to equilibrium on CsCl gradients (15). Samples (10 μg) were digested with *Eco*RI in the presence of 1 mM spermidine or with *Bam*HI, electrophoresed on a 0.7% agarose gel, and Southern transferred to Gene Screen Plus by using the procedure recommended by the manufacturer. The 1.2-kb *Hin*dIII fragment of the T-antigen gene was labeled by nick translation and used to probe the blot by the procedure suggested for Gene Screen Plus. Lanes: 1, genomic DNA digested with *Eco*RI; 2, genomic DNA digested with *Bam*HI; 3, *Hin*dIII lambda digest radiolabeled marker. (B) Restriction map of plasmid pRSV-T.

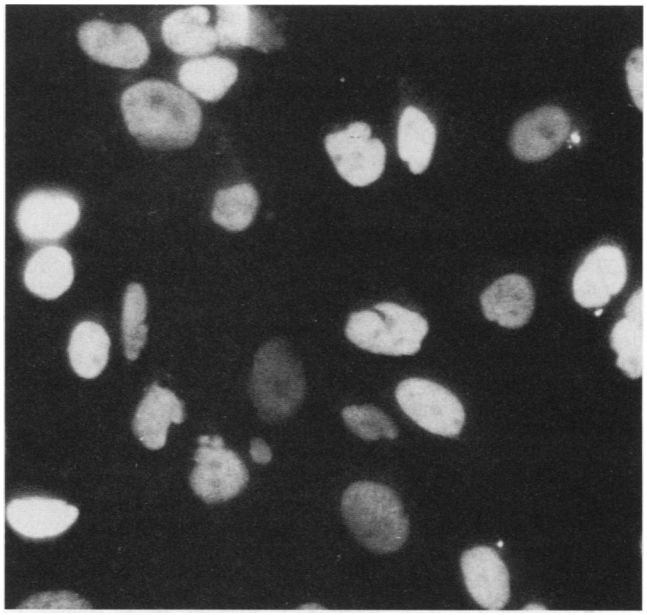

FIG. 4. Expression of SV40 large T antigen in NHBE cells transfected with pRSV-T. Cells were grown on tissue culture chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) four passages after selection of the transformed colony and fixed in absolute methanol at 4°C. Indirect immunofluorescence was obtained by using a mouse monoclonal antibody for SV40 large T antigen (Oncogene Science, Mineola, N.Y.), followed by a fluorescein isothiocyanate-labeled anti-mouse immunoglobulin antibody (Miles).

showed that after four passages of this focus, greater than 99% of the cells were expressing T antigen (Fig. 4).

Strontium phosphate precipitates thus enabled transient and stable introduction of genes into primary cultures of NHBE cells, which would have been lysed by calcium phosphate precipitates, and also into the CV-1 monkey kidney cell line and NIH 3T3 fibroblasts.

Phosphate precipitation methods have two useful features in addition to simplicity: first, that DNAs can be introduced into cells without first being cloned, thus permitting transfection of genomic DNAs; and second, that DNAs are integrated into the recipient chromosome at high copy numbers, thus permitting screening of stable cells for an altered phenotype. In contrast to the phosphate precipitation methods, protoplast fusion (25, 31) and retrovirus vectors (1) require the DNA to be cloned, and DEAE-dextran (18, 19, 22, 28) rarely gives stable integration. Electroporation (17) and encapsulation of DNA in liposomes (24) are limited in usefulness for transfecting large DNAs such as genomic DNA. Microinjections (16) must be performed individually and thus are not practical for screening genomic DNAs for particular genes. DEAE-dextran and Polybrene (13) were also toxic to NHBE cells (unpublished data).

Strontium phosphate precipitation is also able to transfect other cell types in primary culture, including rat and mouse keratinocytes (John Harper, Scripps Clinic, personal communication), human mesothelial cells (R. R. Reddel, unpublished data), and human prostate epithelial cells (E. Kaighn, J. Lechner, and R. R. Reddel, unpublished data). We anticipate that its use will make a wide variety of differentiated cells in primary culture accessible to transfection of cloned and genomic DNAs.

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ADDENDUM IN PROOF

The procedure can be simplified by adding reagents, prewarmed to 37°C, in one tube in the order: 2× HBS, H_2O, DNA, and $SrCl_2$. Agitate gently, hold at 37°C for 30 s, and add to cells. The precipitate forms in the dish during the first hour, with the characteristics described. The 2× HBS is changed to 0.188 g of $Na_2HPO_4 \cdot 7H_2O$, 1.08 g of glucose, 8.0 g of NaCl, 0.372 g of KCl, 4.76 g of HEPES, and H_2O to 500 ml (pH 7.3).

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