PLASMID DNA TRANSFECTION INTO FIBROBLASTS OF ATHYMIC RATS

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The FK-7 line of athymic rat fibroblasts, obtained from the stroma of a human tumor, after passage through athymic animals, was described by the writers previously [1]. Analysis of the tumorigenicity of a cell population of this line yielded 18 cell clones. Injection of these clones into athymic mice revealed four clones not possessing tumorigenicity. This led us to test the possibility of using these cells in the DNA transfection procedure.

The cells most frequently used for this purpose are immortalized aneuploid cell lines of rodents, such as NIH 3T3, C3H $10T_{1/2}$ and REF, because of their relatively high frequency of genetic transformation. However, if these lines are bred, spontaneous transformation of the cells is observed [5, 8], which makes it necessary to reclone the lines to be used. Also, on introduction only of plasmid PSV2 NEO into Chinese hamster embryonic fibroblasts [7] and into NIH 3T3 cells [9], foci of transformation appear in vitro and genetically resistant clones exhibit tumorigenicity when injected into athymic mice; more than 50% of the clones, moreover, were tumorigenic [9]. It has also been shown that the actual procedure of cell treatment with calcium phosphate alone may lead to changes in the tumorigenic and metastatic properties of the cells, as has been shown in particular on the SP1 cell line of mouse mammary gland adenocarcinoma [6].

We accordingly decided to test the possibility of spontaneous transformation during the longest possible duration of monitoring tumor formation after injection of the cells: the possibility of introducing plasmid DNA and expression of the phenotypic properties determined by it, especially cell transformation.

EXPERIMENTAL METHOD

Cell cultures were grown on MEM medium with a double set of amino acids and with 8% fetal calf serum, 2 mM L-glutamine, and 0.08 mg/ml gentamicin at 37°C.

The cells were cloned by the limiting dilutions method on conditioned medium with 20% fetal calf serum, followed by transfer of the suspension into 96-well plates and counting the number of cells per well after 2 h. Wells which each contained one cell were used for the subsequent work. The medium was changed next day and thereafter on the 3rd-4th day for semiconditioned medium with 20% fetal serum. The growing clones were transferred to 24-well plates with fresh medium containing 8% of fetal serum.

Karyologic analysis was carried out on the cell culture in the logarithmic phase of growth, after preliminary incubation for 2 h in medium containing colchicine in a concentration of 0.2 μg/ml. The cells were subjected to hypotonic treatment with 0.56% KCl solution for 35 min followed by fixation with a mixture of acetic acid and methyl alcohol (1:3). The preparations were stained with Giemsa's dye. The modal class of chromosomes was determined among 100 metaphase plates.

To test for their tumorigenicity, cells ($1 \cdot 10^7$) were inoculated subcutaneously in the subscapular region of nude mice. Observation on tumor formation was carried out for not less than 8 weeks.

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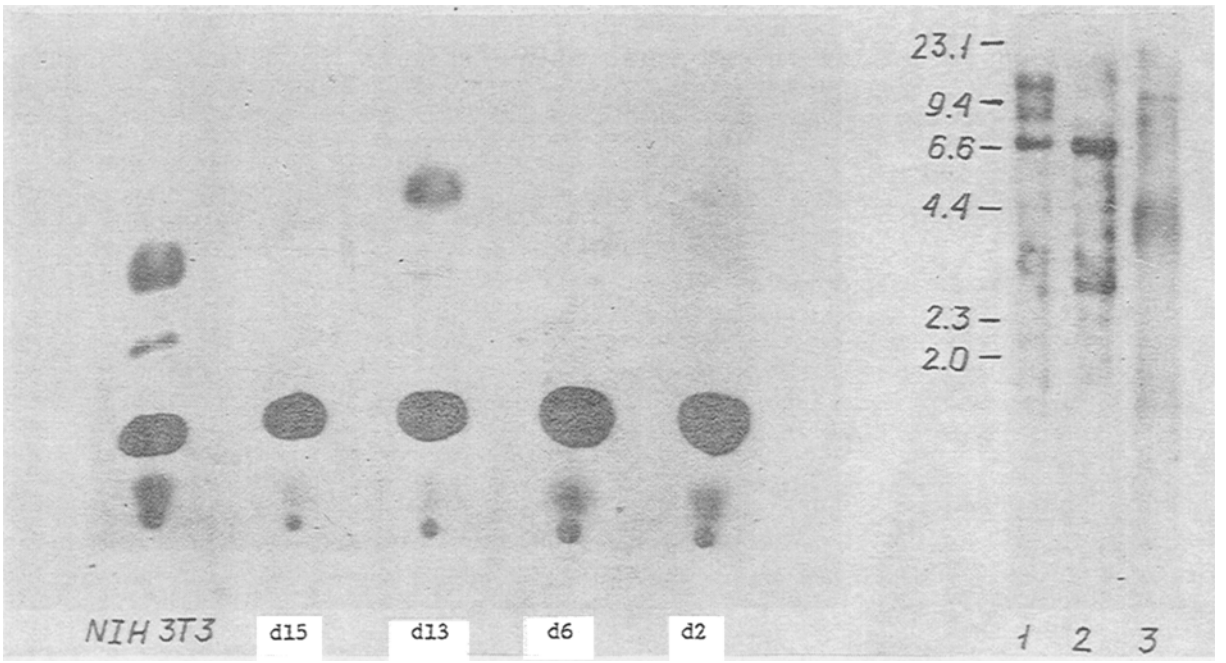

Fig. 1. Determination of CAT activity in extracts from $1 \cdot 10^6$ cells of four clones nontumorigenic for nude mice from strain FR-7. As positive control NIH 3T3 cells were used. The highest level of accumulation of acetylated forms of chloramphenicol of the four clones tested was observed on testing extract from clone cl 13.

Fig. 2. Hybridization of tumor DNA with ^{32}P 6.6 kb fragment of pEI DNA. 1-2) Restriction by Bam HI; 1) tumor obtained after introduction of pEI cells transfected with pSV2neo, followed by selection with G418 and combined introduction of clones; 2) tumor obtained after introduction of transfected pEI cells, without selection; 3) DNA of intact FR-7 cl 13 cells, restricted by Eco RI. Exposure of tracks 1-2 was 5 days, track 3 20 days.

Plasmid DNA was transfected into FR-7 cl 13 cells by the calcium phosphate method [3]. Plasmid DNA was added in the composition of the precipitate without DNA carrier in a dose of 5 μg per 60 mm Petri dish. The cells numbering $(20\text{-}25) \cdot 10^4$ were seeded 24 h before the beginning of the experiment and maintained with the DNA–calcium phosphate coprecipitate for 18 h, followed by a change of medium. After 24 h the cells were plated out in the ratio 1:4, and with effect from next day selection was carried out with gentamicin (400 μg/ml medium). Resistant colonies were separated with the aid of titanium cylinders and grown in plastic flasks.

Chloramphenicol acetyltransferase activity was determined [4] on the 3rd day after transfection with plasmid pSV2cat (10 μg per 60 mm Petri dish). Efficiency of transfection was estimated by studying autoradiographs for the relative quantity of ^{14}C-labeled chloramphenicol converted into the monoacetate form.

Isolation of DNA for blot hybridization was carried out by lysis of the cells with 1% SDS solution followed by deproteinization with phenol/chloroform. Restriction, electrophoresis, blotting, and immobilization of DNA were carried out as described previously [2]. Hybridization was carried out in the presence of 50% formamide with a 6.6 kb plasmid pEI, with specific activity of $2 \cdot 10^8$ cpm/μg DNA, labeled with ^{32}P in the nick translation reaction. Prehybridization, washing, and autoradiography were carried out as described previously [2].

EXPERIMENTAL RESULTS

The fibroblastlike cell line FR-7, which we described previously, was isolated from the third passage of a xenografted human colonic carcinoma RTK-7 cl in nude rats, and exhibited tumorigenicity when injected into nude

mice, was used for cloning by the limiting dilutions method in 96-well plates. As a result we obtained 18 clones which differed both in growth characteristics and in morphology. All these clones were tested for tumorigenicity by subcutaneous injection of $1 \cdot 10^7$ cells into nude mice. The times of observation for the appearance of a tumor were not less than 2 months, and two animals were used in each experiment. In the absence of appearance of a tumor, the corresponding experiment was repeated. As a result we discovered four clones which did not possess tumorigenicity for nude mice.

As the first screening test to select a cell clone which may be suitable for determination of the transforming sequences during transfection we used determination of expression of chloramphenicol acetyltransferase after introduction of plasmid pSV2cat into the cells. Relative comparison of expression in four nontumorigenic clones and in NIH 3T3 cells taken as the control showed that expression of the marker enzyme in one of the four clones was significantly higher than in the other three and was comparable with that of the NIH 3T3 cells (Fig. 1). We used this clone (FR-7 cl 13) for the subsequent work.

The FR-7 cl 13 cell culture consists of elongated fibroblast-like cells, growing to a continuous monolayer [$(75\text{-}85) \cdot 10^4$ cells/25 cm^2 area of growth] on medium MEM with a double set of amino acids and 8% fetal calf serum, with a multiplicity of seeding of 1:(4-5) after 5-7 days. To obtain more detailed characteristics of the clone we used, karyologic analysis was undertaken. This showed that the modal class consisted of 41 chromosomes in 76% of the cells analyzed. The modal region, with a range of variation of 41-42 chromosomes accounted for 90% of the cells. Polyploids accounted for 0.5% of the population, when counted in 500 metaphase plates.

During introduction of this strain (over 40 passages) we never observed the formation of any foci of multilayered growth. To determine the possible appearance of tumorigenic properties, FR-7 cl 13 cells after 6, 18, and 32 passages were injected subcutaneously in a dose of $1 \cdot 10^7$ cells into nude mice, followed by a period of observation of not less than 2 months. In no case were tumors seen to be formed.

To determine quantitative characteristics of stable transformation, cells of the FR-7 cl 13 clone were transfected by the calcium phosphate method with plasmid pSV2neo, followed by selection with the antibiotic G418 in a concentration of 0.4 mg/ml medium for 2 weeks. In control flasks with selective medium the cells died by the 10th-12th day. The number of colonies was counted in each of five flasks and the efficiency of transfection determined as the ratio of the number of stable colonies to the initial number of cells per microgram plasmid, and for cells of clone FR-7 cl 13 it was $5.4 \cdot 10^{-5}$.

Considering the possibility of transformation of cells as the result of introduction of plasmid pSV2neo into them, the 14 clones obtained as a result of selection and isolated were grown up to $3 \cdot 10^6$ cells and were injected subcutaneously in combinations of 3-4 clones into nude mice. Tumor formation was not observed in any single case during periods of 2-6 months. These results also indicate that the phosphate itself has no effect on modification of the tumorigenic properties of FR-7 cl 13 cells.

Cotransfection of plasmid pEI with pSV2neo into FR-7 cl 13 cells followed by selection and combined injection of the clones into nude mice (3-4 clones, $1 \cdot 10^6$ cells of each) led to the appearance of relatively slowly growing tumors (compared with transformed NIH 3T3 cells) after 11-14 days. In the case of similar transfection but without subsequent selection of the cells and injection of $1 \cdot 10^7$ cells into nude mice, we observed the appearance of tumors after 21-25 days. Histologic investigation revealed a morphological picture corresponding to fibrosarcomas. Autoradiographs of hybridization of the ^{32}P 6.6 kb fragment of pEI DNA with Bam HI-restricted DNA from tumor formed in nude mice after receiving an injection of FR-7 cl 13 cells, transformed by plasmid pEI in the experiments described above, are given in Fig. 2. The principal hybridized band on the tracks is the 6.6 kb fragment, excised by Bam HI from the intact plasmid. The presence of additional bands of different sizes and intensities on the tracks is evidence of rearrangements of the plasmid genome, evidently taking place mainly during the process of insertion into the cell genome. It may also indicate both the polyclonal nature of the population of transformed FR-7 cl 13 cells, although the clone with the unarranged plasmid also is predominant, and also the multiplicity of integration.

Hybridization with DNA from untransformed cells revealed a band with the endogenous murine Ha-ras genome, but after much more prolonged autoradiography.

Thus after long-term introduction, and also by the use of the calcium phosphate method and of plasmid pSV2neo the appearance of tumorigenic properties in the FR-7 cl 13 cells was not observed on injection into nude mice, in which respect they are superior to NIH 3T3 cells usually used in transfection experiments. Transfection of FR-7 cl 13 cells with pSV2neo, pSV2cat, and pEI DNA showed that the cells are able to receive and express foreign

genetic information and, in particular, these cells may develop tumorigenic properties as a result of introduction of oncogenes. This cell clone can accordingly be recommended for use as target cells with which to study the properties of an introduced foreign DNA

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DYNAMICS OF CHEMILUMINESCENCE REACTION OF SYRIAN HAMSTER BLOOD NEUTROPHILS TO GROWTH OF TEN DIFFERENT TUMOR CELL STRAINS

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One of the most important characteristic features of the blood neutrophils, responsible through phagocytosis for their bactericidal and cytotoxic properties, is their ability to generate an oxygen burst, i.e., to release active forms of oxygen [4, 12, 14].

Products of the oxygen burst of neutrophils (O_2^-, OH, and, especially, H_2O_2) have been shown to be highly toxic for tumor cells [12]. Since tumor cells can secrete factors inhibiting activity of neutrophils (prostaglandins of the E_2 type, for example) [1], it is not yet clear how that cytotoxic activity of neutrophils is modified and, in particular, how active forms of oxygen are secreted during growth of different types of malignant tumors.

The aim of this investigation was to use the luminol-dependent chemiluminescence test to discover what effect growth of experimental subcutaneous tumors, differing in their origin and level of malignancy, has on the process of secretion of hydrogen peroxide (H_2O_2) by peripheral blood neutrophils of tumor-bearing hamsters.

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