Quantitative Characteristics of the Transformation of Hamster Cells by PARA (Defective Simian Virus 40)-Adenovirus 7

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An in vitro method for the quantitative measurement of transformation in hamster embryo fibroblasts by the PARA [defective simian virus 40 (SV40)]-adenovirus 7 hybrid has been developed. Transformation by PARA particles followed one-hit kinetics with a ratio of 1 focus-forming unit per 250 plaque-forming units. The method of viral adsorption had a direct effect upon the total number of foci which developed but not on the quantitative aspects of this assay. A fluorescent-focus assay was developed which provided a direct correlation of the observed morphological transformation and the presence of the PARA genome. This fluorescent-focus assay utilized detection of the SV40 tumor antigen, which was present in all foci transformed by PARA. Single foci induced by PARA were isolated and grown into cell lines. Two types of foci were observed and isolated; the first contained cells having a cuboidal or SV40-type morphology, and the second consisted of epithelial or adenovirus-type transformed cells. Both types contained the SV40 tumor and SV40 surface antigens as determined by the indirect fluorescence technique; however, only the epithelial cells contained the adenovirus 7 tumor antigen. All five cell lines which were injected into weanling Syrian hamsters were found to be oncogenic. These cell lines induced antibodies to both SV40 and adenovirus 7 tumor antigens in tumor-bearing animals.

Among the several oncogenic deoxyribonucleic acid (DNA) viruses, only the PARA [defective simian virus 40 (SV40)]-adenovirus 7 (Ad7) hybrid population is known to contain a defective particle which is responsible for the oncogenic potential of the virus stock (2, 6). It has been proposed that the presence of incomplete or defective virus particles in the population of any DNA virus may be responsible for the transforming or tumorigenic ability (12). If this hypothesis is correct, PARA particles should induce transformation at a higher frequency than other DNA viruses when the number of particles needed to induce transformation is calculated against the number of infective particles in the virus population.

Several reports describing quantitative in vitro transformation by DNA viruses have been published. Adenovirus 12 (Ad12) and simian adenovirus 7 have been shown to transform hamster embryo fibroblasts, with the number of transformed foci dependent upon the dose of infecting virus (5, 13-15). The transformation potential of

SV40 and polyoma virus has also been determined in vitro (7, 18, 19). Although in vitro transformation of cells by PARA has also been reported (3, 8, 20), the studies were not concerned with establishing the quantitative aspects of this virus property.

This report describes the transformation of hamster embryo fibroblasts by PARA in quantitative terms. Some of the properties of selected cell lines, each arising after a single transformation event induced by PARA, were also investigated.

MATERIALS AND METHODS

Virus. The PARA-Ad7 hybrid population was described previously (10). Virus was used from the SP2 stock, which had been passaged three times in green monkey kidney (GMK) cells. All virus stocks were grown in cell cultures in 16-oz (40 cm) bottles. After the infected cells exhibited 75 to 100% cytopathic effects (CPE), the cells were disrupted by two cycles of rapid freezing and thawing, sonic oscillation, and clarification by low-speed centrifugation. The virus stocks were then quick-frozen and stored at -90°C .

Cells. Hamster embryo fibroblasts (HEF) were pre-

pared from 13-day-old Syrian hamster embryos. Embryos from a single litter were pooled and dispersed with 0.4% trypsin. Sixteen-ounce (40 cm) bottles were seeded with 10^7 cells and incubated at 37 C. All HEF cells were grown in Eagle basal medium containing 10% fetal calf serum, 10% tryptose phosphate broth, and 0.08% NaHCO_3 .

Plaque assay. Primary GMK cell monolayers were inoculated with 0.1 ml of the virus suspension to be assayed. The virus inoculum was diluted with 0.5 ml of tris(hydroxymethyl)aminomethane (Tris) buffer after addition to the cultures in 60-mm petri dishes. After virus adsorption for 1 hr at 37 C, the GMK monolayers were overlaid with Eagle basal medium containing 10% fetal calf serum, 1% agar, and 0.23% NaHCO_3 . The infected cells were kept in a 5% CO_2 incubator at 37 C for 7 days. At this time, a second overlay containing a 1:20,000 dilution of neutral red was added. Plaques were counted 10 days after initial virus adsorption.

Focus assay. The methods of virus adsorption are described under Results. After virus adsorption, the cell cultures were grown in 60-mm plastic petri dishes containing Eagle basal medium (CaCl_2 concentration reduced to 0.1 mM) with 10% fetal calf serum, 10% tryptose phosphate broth, and 0.08% NaHCO_3 . Six days after infection, the concentration of NaHCO_3 was increased to 0.23%. Cell cultures were maintained at 37 C in a 5% CO_2 incubator and the medium was changed at 3- to 4-day intervals. Transformed foci generally appeared 12 days postinfection. Prior to counting of foci, the cell cultures were stained with Wright stain.

Isolation of transformed foci. Transformed foci were removed from the cellular monolayer by aspiration with a capillary pipette. Foci were isolated 17 to 19 days after viral infection of the HEF cells. Each focus was placed in a 35-mm plastic petri dish containing Eagle low-calcium basal medium. The medium was changed every 3 to 4 days. After cellular growth for 10 to 15 days, each focus isolate was trypsinized, and cells were transferred to 15-mm cover slips in 35-mm plastic petri dishes and directly to a separate 60-mm petri dish.

Immunofluorescence techniques. PARA-transformed HEF cells were grown on 15-mm cover slips; 10^4 cells per cover slip were seeded initially. One day after seeding the cells, the cover slips were washed three times in warm Tris buffer (pH 7.4) and air dried. After drying, the cells were fixed for 3 min in acetone. Sera from hamsters bearing tumors induced by the injection of SV40 tumor cells were adsorbed to the cells on each cover slip. Anti-Ad12 T antigen serum, which cross-reacts with Ad7 T antigen, was obtained from Microbiological Associates, Inc., Bethesda, Md., and used in an identical manner. After adsorption of the T antibody for 1 hr, the cover slips were again washed three times in warm Tris buffer. Anti-hamster gamma globulin labeled with fluorescein isothiocyanate was then adsorbed to the cells for 1 hr. Specific fluorescence was detected by use of a Zeiss fluorescence microscope. Human embryonic kidney (HEK) cells infected with Ad7 and GMK cells infected with SV40 were used to demonstrate the presence of T antibodies in sera from

tumor-bearing hamsters. The method of staining the virus-infected cells was identical with that described for transformed cells.

The method of detecting SV40-specific S antigen has been described previously (17). This method was used for the experiments described in this paper with the following exception. The cells were not reacted with the anti-S serum and fluorescein-conjugated serum while in suspension. They were reacted with the serum while attached to a cover slip at a cellular density of low magnitude.

In vivo techniques. Weanling Syrian hamsters (4 to 5 weeks old) were injected with 10^6 transformed cells per animal. Hamsters in which a tumor appeared were bled by heart puncture, and the serum was collected. All injected animals were kept for 10 weeks after cell injection and were observed weekly for the appearance of tumors.

RESULTS

Transformation of HEF by PARA-Ad7. Confluent HEF monolayers attached to 60-mm plastic petri dishes were inoculated with varied concentrations of PARA-Ad7 in 1 ml. Infected monolayers were trypsinized after virus adsorption and reseeded into 60-mm petri dishes, at a concentration of 2×10^5 HEF cells per plate. Transformed colonies, which first appeared 12 days postinfection, were stained with Wright stain and counted microscopically 17 to 19 days after viral infection. Two distinct types of transformed colonies were observed in virus-infected plates; uninoculated cultures were negative for transformed colonies. The first type of transformed colony (type A) consisted of cells with a cuboidal morphology (Fig. 1). Such a morphology has been described for SV40-transformed cells. Dispersed among the cuboidal cells in this type of transformed focus were numerous giant cells. A second type of transformed focus (type B) contained small rounded cells which were densely packed into a multilayered focus (Fig. 2).

The number of foci appearing in individual petri dishes was directly related to the concentration of the PARA particles in the infecting inoculum (Table 1). The ratio of focus-forming units (FFU) to plaque-forming units (PFU) was calculated to average approximately 1 to 300. This ratio is lower than similar values which have been reported for other DNA viruses with transforming potential. At high concentrations of virus (1 PARA PFU per HEF), the efficiency of transformation was reduced, probably due to the toxic effect of the Ad7 fiber antigen. However, at lower virus concentrations, this ratio was consistently close to 1 to 300. Furthermore, each twofold virus dilution resulted in a twofold reduction of transformed foci (Table 1). When the viral dilution is plotted against the number of transformed foci,

FIG. 1. Photomicrograph of transformed focus (type A) induced by PARA-adenovirus 7 after exposure of hamster embryo fibroblast cells. $\times 40$.

FIG. 2. Photomicrograph of transformed focus (type B) induced by PARA-adenovirus 7 after exposure of hamster embryo fibroblast cells. $\times 40$.

TABLE 1. *Quantitative transformation of hamster embryo fibroblasts by PARA (defective SV40)*

Dilution of PARA	PARA PFU ^a	PARA FFU ^b per dilution			Avg FFU per dilution	FFU:PFU
1:5	1.2×10^5	198	190	207	198	1:606
1:10	6.0×10^4	172	196	171	180	1:333
1:20	3.0×10^4	94	110	112	105	1:286
1:40	1.5×10^4	44	54	51	50	1:300
1:80	7.5×10^3	24	28	28	27	1:278
1:160	3.8×10^3	14	14	11	13	1:292
1:320	1.9×10^3	6	8	8	7	1:271
1:640	1.0×10^3	3	4	3	3	1:333
1:1,280	5.0×10^2	2	2	2	2	1:250
1:2,560	2.5×10^2	1	1	1	1	1:250
1:5,120	1.3×10^2	0	0	0	0	

^a PFU (plaque-forming units) determined on GMK cells infected with additional Ad7.

^b FFU (focus-forming units) or number of transformed HEF colonies.

a linear relationship between viral concentration and transformation is evident (Fig. 3). This linearity confirms earlier observations (2) that the transformation induced by the PARA virus particle is the result of a single virus particle.

Effect of method of virus adsorption on the appearance of transformed foci. PARA-Ad7 particles were adsorbed to the same pool of HEF cells by three methods, and the comparative transforming frequency obtained with each method was measured. The first method (A) is described in the preceding section. In the second method (B), the virus was adsorbed in suspension with the HEF cells. A 1-ml amount of each virus dilution was mixed with 1 ml of a cell suspension. Each cell suspension contained 8×10^5 cells. The virus-cell mixture was shaken for 3 hr at 37 C. This mixture was then divided into four parts, and each part containing 2×10^5 cells was plated in a 60-mm petri dish.

The third method (C) was initiated by plating 2×10^5 HEF cells into a 60-mm petri dish. After the cells had settled for 1 hr, the medium was removed and replaced by 1 ml of virus dilution. Virus was adsorbed to the attached cells for 3 hr at 37 C before additional medium was added to each culture. The procedure after virus adsorption (described in Materials and Methods) was identical for all three methods.

All cultures were stained with Wright stain 17 to 19 days after virus infection, and the transformed colonies were counted. Transformation induced by PARA adsorbed under the three different conditions are compared in Fig. 4. HEF cell cultures transformed by the hybrid virus under the conditions of method B (adsorption in suspension) contained twice as many transformed foci as cells transformed as a result of virus adsorbed by method A. Furthermore, transformed

colonies, which appeared when method C was used, were approximately fourfold fewer than transformed foci observed in method B cultures and approximately twofold less than method A. However, all methods yielded a twofold reduction in the number of transformed colonies for each twofold dilution in virus concentration.

Fluorescent focus assay for SV40 tumor antigen in transformed colonies. It has been previously shown that all cells transformed by PARA contain the SV40 T antigen (2). This antigen was therefore used as the marker for a fluorescent focus assay of the transformed colonies induced by PARA-Ad7.

Hamster embryo fibroblasts, infected with the hybrid virus under the conditions of method A, were inoculated into 35-mm plastic petri dishes. Each petri dish contained a cover slip with a diameter of 25 mm. All petri dishes were inoculated with 8×10^4 cells. Each 25-mm cover slip, which had one-half the surface area of a 35-mm petri dish, therefore received 4×10^4 cells. Seventeen days after viral infection, the cultures were stained for the SV40 T antigen.

In a parallel experiment, infected cells from the same embryonic pool were seeded into 60-mm petri dishes, and resulting foci were counted under a light microscope, after staining with Wright stain. The number of transformed foci was compared to the number of foci positive for SV40 T antigen on the 25-mm cover slips. Since the 60-mm petri dish received five times as many infected cells as were inoculated onto the 25-mm cover slips, the number of foci on each cover slip was multiplied by 5 (Fig. 5). A comparable number of viral particles induced the same number of foci as measured by both assays. All fluorescent foci were found to be of a transformed morphology and, furthermore, all foci exhibited the

FIG. 3. Transforming potential of PARA-adenovirus 7 compared to virus concentration. The virus was adsorbed to HEF monolayers for 3 hr, and the cells were trypsinized and plated into 60-mm plastic petri dishes at a cellular concentration of 2×10^5 cells per plate. Four plates of HEF were plated for each dilution of PARA. Seventeen days after virus inoculation, the cultures were stained with Wright stain, and the transformed foci were counted. The data presented are the average number of foci in the four plates of each dilution.

loss of contact inhibition expected from transformed cells. No transformed areas were found which did not give a positive reaction for the SV40 T antigen, and SV40 T antigen was rarely detected in cells not comprising a focus.

Morphology of HEF transformed in vitro. Individual transformed foci (19 days after virus infection) were removed from the parent culture by aspiration with a capillary pipette and placed into 35-mm petri dishes containing low Ca medium. With frequent changes of medium, the viable cells from these foci multiplied and were transferred 10 to 15 days after initial isolation. At

the time of transfer, some of the transformed cells were placed on cover slips and stained with hematoxylin and eosin. Two types of foci were identified by the morphology of individual cells within each culture. The first type (type A) was essentially cuboidal (Fig. 6) but still retained some of the morphological features of the fibroblastic parent HEF cells (Fig. 7). Dispersed throughout these cells were significant numbers of giant cells. When compared to normal HEF cells, the transformed cells were larger, contained greater numbers of nucleoli, and exhibited increased nuclear size.

The second morphological type of transformed cells (type B) was epithelial, with a few giant cell forms (Fig. 8). These transformed cells also exhibited a chain-like pattern of growth. The amount of cytoplasm within these cells was small when compared to the nuclear size. Both types of transformed foci exhibited a high rate of cellular division.

Viral-specific antigens in PARA-transformed HEF. Transformed cell cultures were grown on cover slips as described in the preceding section. After acetone fixation, each cell line was examined

FIG. 4. Effect of the method of adsorption on HEF transformation by PARA-adenovirus 7. Method A ($\square$): PARA-adenovirus 7 was adsorbed to HEF monolayers and the cells were trypsinized after virus adsorption. Method B ($\bullet$): PARA-adenovirus 7 was adsorbed to HEF for 3 hr, while the cells were in suspension. Method C ($\circ$): cells were plated and, after cellular attachment to the petri dish, the virus was adsorbed to the cells. All plates received 2×10^5 cells. Cell cultures were grown for 17 days and, after staining with Wright stain, the transformed foci were counted microscopically.

FIG. 5. Comparison of fluorescent-focus assay and transformed focus assay. ○, PARA-infected cells (4×10^4) were plated onto 25-mm cover slips and examined for SV40 T antigen in discrete foci by the indirect fluorescent method after cell growth for 14 days. The number of foci counted was multiplied by 5. ●, PARA-infected cells (2×10^6) were plated into 60-mm petri plates. Fourteen days after infection, the cultures were stained with Wright stain, and the transformed foci were counted microscopically.

by the indirect fluorescent antibody test for both the Ad7 and SV40 T antigens, and SV40-specific S antigens. The results of this experiment are shown in Table 2. All 30 cultures which were tested contained the SV40 T antigen. Furthermore, all 30 cell lines contained the SV40 S antigen. Six of the 30 cultures examined were positive for the Ad7 T antigen. Cultures which contained only the SV40 T antigen were of cuboidal morphology. The epithelial, or chain-forming, cell cultures were positive for both SV40 T antigen and Ad7 T antigen. No cultures containing both viral-induced T antigens, as determined by the use of anti-Ad12 T antigen serum, were of the cuboidal type.

Cells from five transformed cultures, four of the cuboidal type and one of the epithelial type, were injected into weanling Syrian hamsters. Each hamster received 10^5 viable cells. All cell lines were oncogenic in hamsters (Table 3). The sera from

these tumor-bearing animals were collected and tested for antibodies against SV40 T antigen and Ad7 T antigen. Most sera tested contained antigens against both T antigens (Table 3). These results are in conflict with those described previously in this section for the antigenic makeup of the cells, and this discrepancy will be considered.

DISCUSSION

This paper presents data which demonstrate that transformation of HEF by PARA is a quantitative event. This transformation shows typical one-hit kinetics. In a virus population which contains two virus genomes, PARA and Ad7, it is necessary to determine which genome is responsible for the observed transformation. Earlier results have demonstrated that PARA, rather than Ad7, is responsible for transformation (2). This paper confirms these earlier results and demonstrates that nearly all, if not all, transformation events are at least partially dependent upon the SV40 portion of the PARA genome. Such a conclusion is based upon the failure to find any transformed foci which contain only the Ad7 T antigen; however, all transformed foci were found to contain the SV40 T antigen. The FFU to PFU ratio of Ad12 has been reported to be approximately $1:10^6$ (5). Furthermore, the highly oncogenic simian adenovirus 7 has been reported to have a ratio of approximately 1 FFU to 10^5 PFU. The ratio of FFU to PFU reported when using PARA can be as low as 1:250, which is significantly lower than the values reported for the oncogenic adenoviruses. SV40, which was previously reported to be the best transformer among the DNA viruses, requires at least 10^3 infectious units for one transformation event (1, 19). When comparing the results of quantitative transformation by PARA with the reported values obtained for other oncogenic DNA viruses, it can be concluded that the defective PARA transforms HEF more efficiently than do other oncogenic DNA viruses. It is pertinent to note that this efficiency far exceeds that of both the parental Ad7 and SV40 and suggests either selection of a highly oncogenic variant, elimination of an inhibitor of transformation in that portion of the genome lost from the defective virus, or synergism between the adeno- and SV40-defective genomes found in PARA.

A problem with many DNA virus transformation assays has been the failure to directly relate the observed cellular transformation with the presence of the virus genome within the transformed cell. The fluorescent focus assay provides direct evidence of virus involvement. In this manner, the observed morphological transformation can be quantitatively correlated with the pres-

FIG. 6. Photomicrograph of transformed cells (type A) induced by PARA-adenovirus 7 after exposure of hamster embryo fibroblast cells. $\times 400$.

FIG. 7. Photomicrograph of normal hamster embryo fibroblasts. $\times 400$.

FIG. 8. Photomicrograph of transformed cells (type B) induced by PARA-adenovirus 7 after exposure of hamster embryo fibroblast cells. $\times 400$.

TABLE 2. Characteristics of distinct clonal cell lines^a

Cellular morphology	No. of clones	No. of clones with SV40 T antigen ^b	No. of clones with Ad7 T antigen ^c
Cuboidal	24	24	0
Epithelial	6	6	6

^a All cell lines were tested in the second passage after isolation.

^b Cells were tested for SV40 T antigen by the indirect fluorescent technique. Antiserum against SV40 T antigen was obtained from hamsters bearing tumors induced by cells transformed by SV40.

^c Cells were tested for Ad7 antigen by the indirect fluorescent technique. Antiserum against Ad7 T antigen was obtained from Microbiological Associates, Inc.

ence of the infecting virus genome. This technique should be useful when establishing the oncogenicity of other DNA viruses.

It has been demonstrated that the method of virus adsorption is an important step in the induction of virus transformation (14). The present report shows that the transforming frequency of PARA can be improved by adsorbing the virus to the cell while both are in suspension. However,

TABLE 3. *In vivo* characteristics of clonal cell lines^a

Clone	In vitro morphology of cells	No. of hamsters injected	No. of hamsters with tumors	No. of hamsters with SV40 T antibody ^b	No. of hamsters with Ad7 T antibody ^c
1e	Epithelial	8	4	4	4
2d	Cuboidal	8	5	5	5
3f	Cuboidal	8	6	6	6
9d	Cuboidal	8	7	7	6
9e	Cuboidal	8	6	6	5

^a All cell lines were injected in the seventh passage after isolation.

^b Sera were tested for SV40 T antibodies by the indirect fluorescent technique. GMK cells infected with SV40 were used as test cells.

^c Sera were tested for Ad7 T antibodies by the indirect fluorescent technique. HEK cells infected with Ad7 were used as test cells.

it is interesting that the assay remained quantitative regardless of the method used.

The failure to directly demonstrate the Ad7 T antigen in all transformed cells tested is in accord with previous results which indicated that all PARA-transformed cells do not contain the Ad7 T antigens (11); however, all cell lines tested in these experiments induced Ad7 T antibody in

hamsters which developed a tumor as a result of the injection of these cells. Of the five *in vitro*-transformed cell cultures tested in hamsters, only one was directly positive for Ad7 T antigen when tested before injection into hamsters. An explanation for these results is not available at the present time; however, it is possible that the Ad7 T antigen induced in HEF during PARA transformation is specific for Ad7 and does not cross-react with Ad12. Since the antiserum used for the demonstration of Ad7 T antigen was prepared against Ad12 T antigen, a lack of cross-reactivity could explain the results. A second explanation is that the PARA-transformed cells contain only small amounts of the Ad7 T antigen which is not detectable by the techniques used in the present study; however, the prolonged exposure of the animal to this antigen during tumor development is then sufficient to induce an antigenic response. Experiments are now in progress to determine which, if either, of these explanations is correct.

It has been previously reported that hamster cells transformed by the PARA-Ad7 hybrid have either the morphology of SV40-transformed cells or of adenovirus-transformed cells. Furthermore, some tumors isolated from animals may contain both cellular types (4). These earlier observations are in accord with the results presented in this paper. No evidence is available to explain the precise role of the Ad7 genome in cells transformed by PARA which have the morphology of an adenovirus-transformed cell. Several possibilities are being investigated. (i) A separate Ad7 genome in addition to the PARA genome has been incorporated into this cell type and has assumed the dominant role for directing the morphology of the transformed cell. The resulting cuboidal cell could then be called a double transformant, such as the retransformation of SV40-transformed cells by polyoma (16). (ii) The adenovirus portion of the PARA genome is more fully expressed in the cuboidal type of transformed cell than in the SV40 type of transformed cell. Differences in oncogenic potential of PARA which had been transcapsidated with different adenovirus types have been demonstrated and illustrate differences in genome expression among PARA particles (9). (iii) The normal host cell which has been transformed by PARA is different and plays a role in determining the alternate morphology after transformation. Which alternative is correct cannot be ascertained; however, it is clear that both morphological types of cells contain the SV40 T antigen, the SV40 S antigen, and some portion of the Ad7 T antigen. Furthermore, both cell types are oncogenic in the host animal.

Recently a variation of oncogenic potential among plaque-purified clones of PARA was reported (11). The reasons for this variation have not been established; however, correlation of *in vivo* oncogenicity with ability to transform *in vitro* by the assay system described in this report is now in progress.

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