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Adenovirus Type 12-Induced Rat Tumor Cells of Neuroepithelial Origin: Persistence and Expression of the Viral Genome

HORST IBELGAUFTS,[1,2]† WALTER DOERFLER,[1]* KARL H. SCHEIDTMANN,[1]‡ AND WOLFGANG WECHSLER[2]§

Institute of Genetics, University of Cologne,[1] and Max-Planck-Institute for Brain Research,[2] Cologne, Germany

Four cell lines derived from adenovirus type 12-induced rat brain tumors were studied. The polyploid cells displayed neuroepithelial characteristics and were transplantable into syngeneic rats and nude mice. In tissue culture the cells grew in monolayers and multilayers. A very high saturation density was reached, and the cells plated in agar and were easily agglutinated with low concentrations of concanavalin A. Between 2 and 11 copies of the viral genome per diploid cellular genome were detected by reassociation kinetics analysis in the different lines. The patterns of distribution of viral DNA sequences in these lines, as revealed by blot analysis, suggest colinear integration of the intact viral genome into the cellular DNA. The patterns of integration were stable after more than 15 months of prolonged tissue culture and after animal reimplantation. Integration patterns were identical in three of the tumor lines and different in another line. Viral sequences were transcribed. The extent of homology found toward adenovirus type 12 DNA in polyadenylated polysome-associated mRNA isolated from the tumor lines suggests that the early and some of the late genes of adenovirus type 12 DNA are transcribed in these tumor cells. Infectious virus was not rescuable from these lines.

Human adenovirus type 12 (Ad12) is capable of transforming a variety of cells in vitro (28, 29; J. D. Levinthal and W. Petersen, Fed. Proc. **24**: 174, 1965) and induces tumors in a number of rodents (21, 31, 32, 34, 45, 46). Neuronal tumors in different animals demonstrate a remarkably uniform phenotype (for a review, see reference 30). All attempts to reisolate infectious virus from adenovirus-transformed and tumor cells have been unsuccessful so far (17, 47). However, it has been shown that viral DNA sequences persist in Ad12-transformed and Ad12-induced tumor cells (11, 17, 18, 24) and that some of these viral sequences are also transcribed (14, 37, 38). Data from our laboratory have demonstrated covalently linked adenovirus DNA sequences in transformed and infected cells (for a review, see reference 7). Several lines of evidence demonstrate that Ad12 DNA persists in transformed hamster cells in an integrated form (18, 19, 43). In some of these adenovirus-transformed cells, the patterns of integration of the viral genome have been studied in detail (43). It has been found that the sites of integration of the viral genomes were different for each line studied (43). The integration pattern was identical in all cells of one line and remained stable for at least 12 months of continuous cell culture (43).

In the present communication we describe the establishment of cell lines derived from Ad12-induced rat brain tumors and report on some of their biological properties. The detection of viral DNA sequences in these tumor lines will be reported. Data of the pattern and stability of integration of the viral genome after long-term tissue culture and repeated animal transplantation of the tumor will be presented. We also will report on the expression of viral DNA sequences in the rat brain tumor cell lines.

MATERIALS AND METHODS

Animals. BDIX rats (9) used in the experiments came from a colony which has been maintained as a closed brother-sister-mated colony for more than 2 years at the Max-Planck-Institute for Brain Research in Cologne, Germany. Athymic nude mice (BALB/c *nu/nu*) were obtained specific pathogen-free from Bomholtgard, Ry, Denmark.

Virus propagation and purification. Human Ad12, Huie strain, and Ad2 were propagated in sus-

† Present address: Institute of Animal Genetics, University of Edinburgh, Edinburgh, Scotland.

‡ Present address: Institute of Immunobiology, University of Freiburg, Freiburg, Germany.

§ Present address: Department of Neuropathology, University of Düsseldorf, Düsseldorf, Germany.

pension cultures of KB cells as described previously (8, 37). The virus preparations used for injections of animals were purified free from incomplete particles by the method described by Tjia et al. (44). Plaque assays were carried out on primary human embryonic kidney (HEK) cells as described previously (26).

Injection of newborn animals with virus. A 10-μl amount of highly purified virus suspension (optical density at 260 nm = 7.2 in the case of Ad12; optical density at 260 nm = 23 in the case of Ad2) which had been dialyzed against phosphate-buffered saline (PBS) (10) for 2 h was inoculated into the right hemisphere of the brains of newborn BDIX rats with a 10-μl Hamilton syringe with a LUER lock to which a fine canula (N733, type 4) had been fitted.

Tissue culture. Animals showing neurological symptoms were sacrificed. The brains and spinal cords were screened for the presence of tumors. Tumors were excised from the animal, washed thoroughly with prewarmed PBS, and then minced into fine pieces with sterile forceps and scissors. Explant cultures were grown in Dulbecco medium (1) supplemented with 10% calf serum (Flow Laboratories, Inc., Rockville, Md.). The petri dishes were incubated at 37°C in a humidified atmosphere containing 12% CO_2. Before cloning, the cells were subcultured without trypsinization by pipetting the growth medium up and down several times in a sterile Pasteur pipette, thus detaching the cells. Cloned cells were trypsinized with 0.25% trypsin in PBS. Clones were obtained by growing single cells on small sterile glass splinters prepared from glass cover slips and transferring single splinters to individual petri dishes containing prewarmed conditioned medium. Conditioned medium was filter-sterilized medium in which uncloned cells had been grown previously for at least 72 h.

Growth experiments in agar. Growth experiments in agar were carried out as described elsewhere (27).

Growth rates. Growth rates were measured by seeding equal numbers of cells into petri dishes in triplicate and by counting cells from one dish on consecutive days with a Coulter Counter.

Efficiency of plating. The efficiency of plating was determined by seeding a known number of cells into petri dishes in quadruplicate and counting colonies 14 days later after the cells had been fixed with ethanol and stained with Giemsa.

Agglutinability by concanavalin A. Agglutinability of the cells with the plant lectin concanavalin A (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was tested as described previously (2, 20). Briefly, the cells were washed with PBS deficient in Ca^{2+} and Mg^{2+}, treated with 0.001 M EDTA to yield single cells, and resuspended in PBS to a titer of 10^6 cells per ml. A 10-μl volume of the cell suspension was mixed with 10 μl of a concanavalin A solution (30 to 1,000 μg/ml) at room temperature. Agglutination was observed in a phase-contrast microscope.

LETS protein (fibronectin). For the detection of the large external transformation-sensitive protein (LETS protein) (22), the cells were fixed at room temperature with formaldehyde, incubated with fibronectin antiserum, washed, and counterstained with fluorescein-isothiocyanate-labeled fibronectin antiserum essentially as described earlier (20). Fibronectin antiserum was a gift of A. Vaheri, University of Helsinki, Helsinki, Finland. Fluorescein-isothiocyanate-labeled serum was obtained from Progressive Laboratories, Baltimore, Md.

Karyotype analysis. The rat brain tumor cells were arrested in metaphase by treatment overnight with 10^{-4} μg of colchicine per ml. For the preparation of metaphases, the cells were collected, treated in hypotonic medium (0.025 M KCl) for 20 min, fixed in ice-cold Carnoy solution (methanol-acetic acid [3:1]), and spread on clean glass slides. Metaphase chromosomes were stained with conventional Giemsa stain.

Fusion of cells. Cells were fused by the polyethylene glycol method as described previously (5).

Nucleic acid preparations. Isolation of viral DNA, preparation of ^{3}H-labeled viral DNA, purification, and separation of the strands of viral DNA were carried out as detailed earlier (37). The *Eco*RI restriction endonuclease fragments of Ad12 DNA were prepared by electrophoresis in 0.8 to 1% cylindrical agarose gels as described previously (43). Highly ^{32}P-labeled viral DNA and restriction enzyme fragments of viral DNA with specific activities of 2×10^8 cpm/μg were prepared by the nick translation procedure of Rigby et al. (39). DNA for reassociation kinetics analysis was extracted from tumor cells grown in tissue culture by published methods (11). The amount of viral DNA persisting in transformed cells was calculated by procedures published elsewhere (16). Cellular DNA for blotting experiments was extracted as described earlier (43). Nuclear and polysomal RNAs from tumor cells grown in tissue culture were isolated as described previously (37), and the polyadenylated mRNA was selected as published elsewhere (25).

Hybridization experiments. Reassociation kinetics analysis was performed as detailed before (11). DNA-DNA hybridizations were carried out as described by Denhardt (6) with minor modifications (43). DNA from agarose slab gels was transferred to nitrocellulose filters by the technique described by Southern (42) with some modifications (23, 43). DNA-RNA hybridizations were carried out as outlined in a previous paper (37).

RESULTS

Frequency of tumor induction. The tumor incidence in animals injected intracranially with Ad12 at birth was 50% after latency periods ranging from between 40 and 430 days. Control animals, which had received an injection of the supernatant culture fluid of KB cell cultures, did not develop tumors during an observation period of 2 years. No tumors were obtained in animals injected with either Ad2 or incomplete particles of Ad2 or Ad12 (44). The brain tumors described in this study were designated RBT12/3 (RBT12 standing for rat brain tumor induced by Ad12), which arose in the lateral ventrical of one animal at 150 days after injection, and RBT12/6, RBT12/13, and RBT12/15, which arose as multiple tumors at different locations (in the frontal

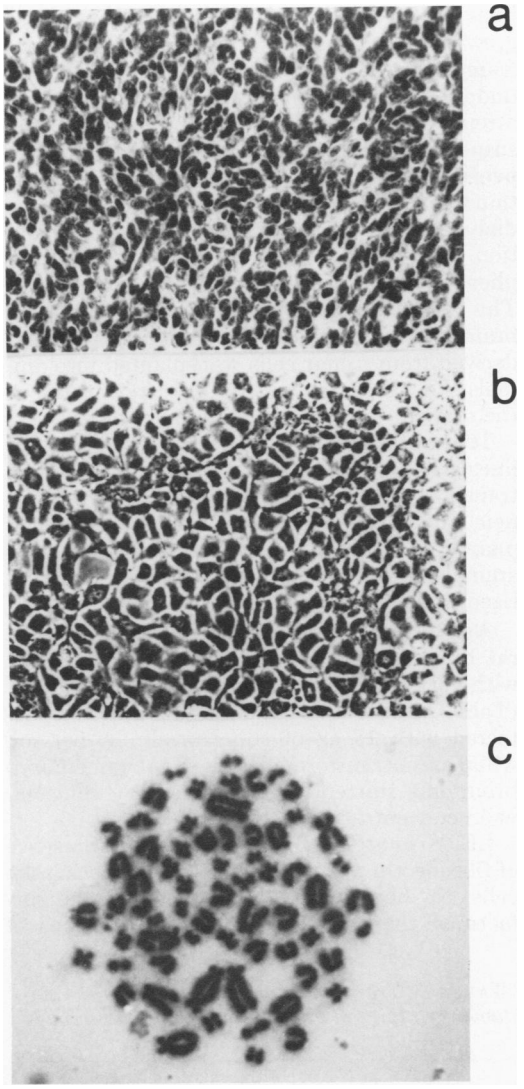

FIG. 1. *Morphology of Ad12-induced rat brain tumors and tumor cells. (a) Typical appearance of Ad12-induced rat brain tumor. Histological section. Cresol violet staining; original magnification, ×512. (b) Ad12-induced rat brain tumor cells in tissue culture grown in Dulbecco medium supplemented with 10% calf serum. The cells are epithelioid in shape. Phase-contrast micrograph; original magnification, ×160. (c) Metaphase from rat brain tumor cells. The cells were treated with 10^{-4} μg of colchicine per ml. One dicentric chromosome, one chromosome with a gap in one arm, and several chromosome fragments are visible. Giemsa staining; original magnification, ×400.*

lobe, the medulla oblongata, and the olfactory bulb, respectively) in the brain of another animal after a latency period of 278 days. The histology of the tumors was that of undifferentiated neuroepithelial neoplasms Fig. 1a). Our data confirm the histological observations made by other authors (30, 32, 36) who described the Ad12-induced tumors as undifferentiated neuroepithelial tumors.

Establishment of cell lines and behavior of the cells in tissue culture. Outgrowth of fibroblasts and epithelial cells from tumor explants was observed after 14 days for RBT12/3 and after 6 days for RBT12/6, RBT12/13, and RBT12/15. In all instances the epithelial cells rapidly outgrew the fibroblasts and formed either multilayered colonies or monolayers (Fig. 1b) on the culture dish. Very often, colony morphology was characterized by the formation of ball-like aggregates or cell clumps floating freely in the medium or by the formation of incomplete monolayers or multilayers with large areas devoid of cells. Similar observations have been made with Ad2- or Ad5-transformed rat cells. Their biological properties have been studied previously (15). The same morphological peculiarities of the rat brain tumor lines were also observed in medium with low calcium concentrations (0.2 mM instead of the usual 1.8 mM) and in medium supplemented with 10% dialyzed bovine serum. The tumor cells have been kept in culture for more than 300 cell doublings and thus can be considered to have acquired the ability to grow indefinitely in tissue culture.

Serum requirements. All cell lines studied exhibited a strong serum dependence. None of the cell lines was able to grow in medium without serum. Line RBT12/13 did not grow in medium supplemented with 1% serum. Lines RBT12/3 and RBT12/15 grew markedly faster in medium supplemented with only 1% serum than was observed for the other lines with doubling times of between 38 and 50 h, as opposed to 72 h for line RBT12/6. In medium supplemented with 5 and 10% serum, all cell lines studied showed a similar growth rate, with doubling times being between 22 and 29 h. The data on serum requirements of the rat brain tumor cells are summarized in Table 1, part 1.

Saturation density. All cell lines grew to high saturation densities of 1×10^6 to 2×10^6 cells per cm^2, whereas nontransformed cells from rat embryo brain grew to saturation densities of about 8×10^4 cells per cm^2 (Table 1, part 2).

Efficiency of plating. The efficiencies of plating of the different cell lines at 1 and 10% serum concentrations differed widely among the individual lines and increased with rising serum concentrations (Table 1, part 3). RBT12/6 and RBT12/15 showed the highest values, with 20 and 42%, respectively, at a serum concentration of 10%.

Growth in agar or liquid suspension. All

TABLE 1. *Biological characteristics of rat brain tumor cell lines and of rat embryo brain cells*[a]

Cell line	(Part 1) Time of doubling (h) at following serum concn:			(Part 2) Saturation density (cells per cm^2) at 10% serum concn	(Part 3) Efficiency of plating[b] at following serum concn:		(Part 4) Efficiency of plating in agar suspension[b] at 10% serum concn	(Part 5) Growth in suspension at 10% serum concn	(Part 6) Agglutinability by concanavalin A[c] at following concn (μg/ml):					(Part 7) Presence of fibronectin (LETS protein)
	0%	1%	10%		1%	10%			500	250	125	62.5	30	15	0	
RBT12/3	0	50	27.6	1×10^6	1.0	2.6	0.02	No growth	++++	++++	+++	+++	++	−	−	−
RBT12/6	0	72	25	2×10^6	3.2	20	0.025	No growth	++++	+++	++	+	+	+	−	−
RBT12/13	0	72	24	1×10^6	0	2.4	0.14	No growth	++++	+++	+++	++	+	+	−	−
RBT12/15	0	37.2	21.6	1×10^6	6.5	42	0.22	No growth	++++	+++	++	++	++	+	−	−
REB[d]	0	ND[e]	ND	8×10^4	0	ND	0.00	No growth	+++	++	+	−	−	−	−	+
HEK	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+

[a] Experimental details are described in the text.
[b] Expressed in percentage of cells plated.
[c] Symbols: −, 100 to 95% single cells; +, 95 to 70% single cells; ++, 70 to 50% single cells; +++, 50 to 30% single cells; ++++, 30 to 5% single cells. This graded scheme was suggested by Becker et al. (2).
[d] REB, Rat embryo brain cells.
[e] ND, Not determined.

lines plated well in agar suspension and also grew colonies on the agar surface, whereas cells from untransformed rat brain did not plate at all under these conditions (Table 1, part 4). Several attempts were made to grow the tumor cells in suspension culture, using Eagle medium for suspension. Neither lowering of the Ca^{2+} concentration to 0.2 mM and using serum, which had been dialyzed against calcium-free PBS, nor incubation of the suspension culture in a CO_2 atmosphere resulted in cell growth (Table 1, part 5). The same behavior was found with rat embryo brain cells. The growth characteristics of lines derived from tumor cells transplanted into animals did not differ significantly from those of the original tumor lines (data not shown).

Transplantability. The cells from all four lines grew as tumors in nude mice and could be transplanted intracranially into newborn syngeneic rats. The lines were, however, not transplantable subcutaneously into adult syngeneic animals. The transplantation data are summarized in Table 2.

Agglutinability with concanavalin A. All rat brain tumor cells were easily agglutinable with very low concentrations of concanavalin A (Table 1, part 6). Agglutination could be detected with 15 μg of concanavalin A per ml, whereas untransformed cells from rat embryo brain only started to agglutinate at a concanavalin concentration of 125 to 250 μg/ml.

LETS protein (fibronectin). The presence of fibronectin was assayed 24 h after plating the cells. No fibronectin was detected on the surfaces of the cells or at the cell junctions of

TABLE 2. *Transplantation behavior of rat brain tumor cell lines in syngeneic animals and in nude mice*[a]

Cell line	No. of positive takes/no. of animals injected				
	Syngeneic rats				Nude mice (s.c.)
	s.c.		i.c.		
	Newborn	Adult	Newborn	Adult	
RBT12/3	1/9	0/17	ND	0/8	2/3
RBT12/6	5/9	0/9	6/6	4/4	4/6
RBT12/13	0/7	0/7	6/6	0/3	2/3
RBT12/15	ND	0/5	4/4	0/9	2/2

[a] A total of 10^7 cells was injected subcutaneously (s.c.) into syngeneic animals; 10^5 cells were used in intracranial (i.c.) injections. The cells were trypsinized and adjusted to the appropriate titer after a trypan blue test for viability. Counting was done in a Coulter Counter. Animals were observed for a period of 2 years. ND, Not determined.

confluent monolayers of cells when cells of passage 8, 32, or 40 were investigated by indirect immunofluorescence. Tumor lines derived from transplanted tumor cells were also found to be fibronectin negative. HEK cells, which were used as controls, and rat embryo brain cells were found to be fibronectin positive (Table 1, part 7).

Karyotype analysis. All lines, including lines derived from transplanted tumor cells, showed a high degree of polyploidy and numerous atypical chromosomes but no marker chromosomes (Fig. 1c). The distributions of chromosome numbers in the four tumor lines are shown in Fig. 2.

T-antigen expression. All four lines were tested repeatedly for the expression of viral T-antigen in passages 3, 19, 24, and 32 by using confluent cultures and were found to be negative for the expression of T-antigen by immunofluorescence. Control cells, i.e., KB cells at 24 h postinfection with Ad12, however, showed positive fluorescence.

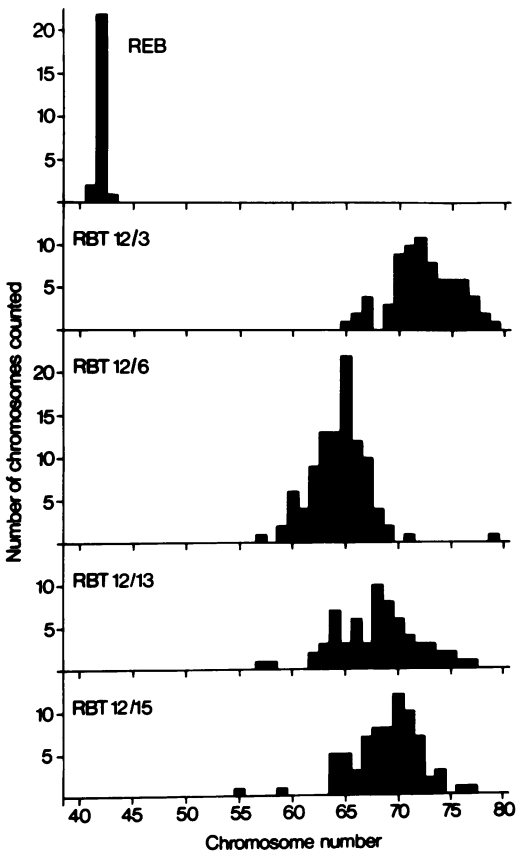

FIG. 2. *Distribution of chromosome numbers in rat embryo brain cells (REB) and cells from the four rat brain tumors induced by Ad12, RBT12/3, RBT12/6, RBT12/13, and RBT12/15. Metaphases from colchicine-treated cells were prepared as described in the text.*

Attempts to rescue infectious virus. All attempts to rescue infectious virus particles from the tumor cells were negative. No infectious virus particles were found either in supernatants of confluent monolayers of the tumor cells or in tumor cell extracts prepared from 10^7 cells by ultrasonic treatment, as judged by plaque assay on HEK cells. Cocultivation of the tumor cells with HEK cells and fusion of tumor cells to HEK cells with polyethylene glycol (5) did not cause cytopathic effects in HEK cell cultures.

Quantitation of persisting Ad12 genomes. The presence of multiple copies of Ad12 DNA persisting in the tumor lines was demonstrated by reassociation kinetics analysis. ^{3}H-labeled Ad12 DNA was reassociated in different experiments in the presence of 2 mg of cellular DNA per ml extracted from the four tumor lines. The time of reassociation was for 80 to 100 h. Figure 3 shows the results of these experiments, and the data obtained in several experiments are summarized in Table 3. In lines RBT12/3, RBT12/6, RBT12/13, and RBT12/15, 4, 2, 11, and 6 copies of Ad12 DNA, respectively, were detected per cellular genome.

The data shown in Fig. 3 were also plotted in the form of $1/f_{ss}$ versus $t/t_{1/2p}$, where f_{ss} is the fraction of input DNA remaining single stranded at time t, to determine whether the entire Ad12 DNA molecule was represented in the DNA of the rat brain tumor lines. These plots (data not shown) indicate that in lines RBT12/3, RBT12/6, and RBT12/15 small segments of the viral genome are possibly missing, whereas in line RBT12/13 the entire viral genome is represented.

Patterns of persistence and integration of Ad12 DNA in rat brain tumor cells. The patterns of persistence and integration of Ad12 DNA in rat brain tumor cells were investigated by blot analysis. A 10-μg amount of DNA from each of the four lines was cleaved with restriction endonucleases *Eco*RI and *Bam*HI. The fragments were separated by electrophoresis in 0.5 or 1% agarose slab gels, and the DNA was transferred to nitrocellulose filters by the blot technique of Southern (42) and subsequently hybridized to intact Ad12 DNA or to each of the *Eco*RI fragments of this DNA which had been ^{32}P labeled by nick translation (39). Figure 4 shows the results of hybridization experiments with ^{32}P-labeled Ad12 DNA of *Eco*RI-cut cellular DNA. A comparison of the *Eco*RI-cut cellular DNA from lines RBT12/6, RBT12/13, and

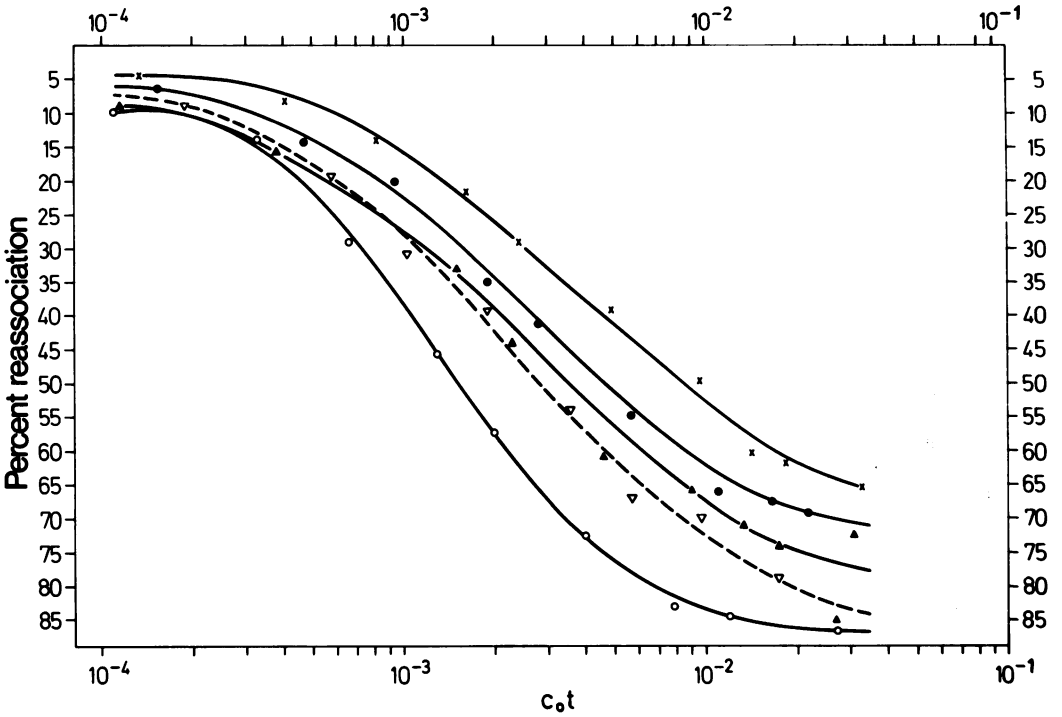

FIG. 3. *Quantitation of Ad12 DNA persisting in rat brain tumor cells by reassociation kinetics analysis. Reassociation of 0.055 μg of ^{3}H-labeled Ad12 DNA (specific activity, 5.8 × 10^5 cpm/μg) in the presence of 2 mg of salmon sperm DNA or of DNA isolated from rat brain tumor cells per ml as indicated. The total reaction mixture of 2 ml was incubated at 68°C for 80 to 100 h. Samples of 0.1 ml were withdrawn and stored at −20°C until the reaction was completed. All samples were subjected to S1 nuclease treatment and acid precipitated, and the amount of radioactivity was determined in a liquid scintillation counter. Details of the technique have been described previously (11). Symbols: ×, DNA from salmon sperm; ▽, DNA from line RBT12/3; ●, DNA from line RBT12/6; ○, DNA from line RBT12/13; ▲, DNA from line RBT12/15.*

TABLE 3. *Summary of C_0t experiments with DNA extracted from rat brain tumor lines*

Source of DNA	Concn of DNA (OD_{260}[a]/ml)	Concn of DNA probe[b] (OD_{260}/ml)	$C_0t_{1/2}$ (mol × liter^{-1} × s) experimentally measured	$C_0t_{1/2}$ of control[c]/$C_0t_{1/2}$ of experimental[d]	Ad12 DNA copies per diploid genome[e]
RBT12/3	14.6	4.79 × 10^{-4}	4.67 × 10^{-3}	1.75	4
RBT12/6	14.6	4.71 × 10^{-4}	5.89 × 10^{-3}	1.39	2
RBT12/13	14.6	3.32 × 10^{-4}	2.11 × 10^{-3}	3.88	11
RBT12/15	14.6	3.80 × 10^{-4}	3.50 × 10^{-3}	2.34	6

[a] OD_{260}, Optical density at 260 nm.

[b] The specific activity of the ^{3}H-labeled DNA used in these experiments was 5.8 × 10^5 cpm/μg.

[c] $C_0t_{1/2}$ value of ^{3}H-labeled Ad12 DNA reassociated in the presence of salmon sperm DNA was 8.19 × 10^{-3} mol/liter × s.

[d] $C_0t_{1/2}$ value of ^{3}H-labeled Ad12 DNA reassociated in the presence of DNA extracted from tumor cells can be calculated from the values listed.

[e] These numbers were calculated by the method described by Gelb et al. (16). The values shown in this table are not derived from the same experiment as those shown in Fig. 3.

RBT12/15 revealed that these three lines were identical in their *Eco*RI patterns. All bands hybridizing to ^{32}P-labeled Ad12 DNA comigrated with the corresponding marker fragments of virion DNA. A banding pattern similar to the ones obtained for RBT12/6, RBT12/13, and RBT12/15, showing comigration of bands with the corresponding marker fragments of virion DNA, was also observed in the DNA of line RBT12/3. There was, however, one exception: with *Eco*RI-cut cellular DNA, the Ad12-specific band comigrating with the *Eco*RI marker fragment C of

virion DNA was missing in the DNA from line RBT12/3 (Fig. 4). The absence of a band at the position of the terminal *Eco*RI C fragment of virion DNA (for map position, see Fig. 4, bottom) might indicate that the left-hand end of Ad12 DNA was covalently linked to cellular DNA.

Analysis of the distribution of viral DNA sequences, using *Eco*RI restriction enzyme fragments of Ad12 DNA as probes for hybridization. Figure 5a to e show the distribution of the *Eco*RI restriction enzyme fragments A to E of Ad12 DNA in the DNA from the four rat brain tumor cell lines.

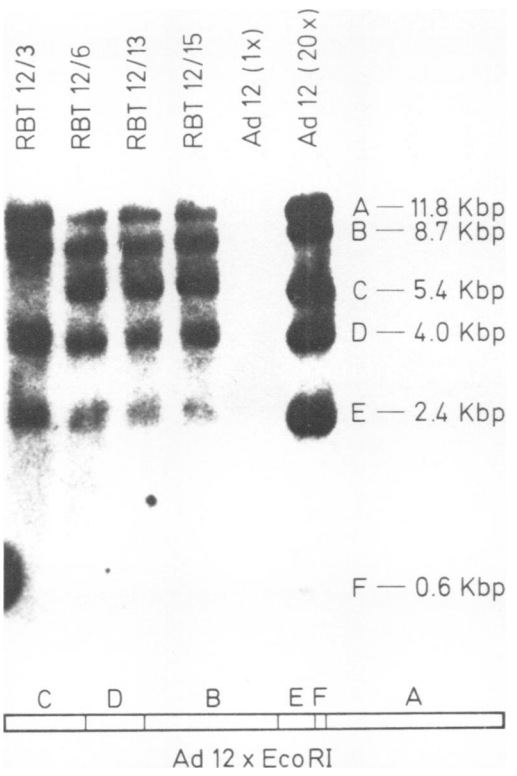

FIG. 4. *Analysis of the patterns of persistence and integration of viral DNA with EcoRI-cut cellular DNA from the four rat brain tumor lines. A 10-μg amount of cellular DNA isolated from tumor lines RBT12/3, RBT12/6, RBT12/13, and RBT12/15 was cut with the restriction enzyme EcoRI. After transfer of the DNA fragments from agarose slab gels to nitrocellulose filters, the DNA on the filters was hybridized with ^{32}P-labeled Ad12 DNA (specific activity, 2 × 10^8 cpm/μg). Lanes 5 and 6 (from the left) contain 1 and 20 copies of viral DNA per diploid genome, respectively, corresponding to 5 × 10^{-4} μg and 10^{-2} μg of Ad12 DNA, respectively. The molecular weights of the resulting fragments hybridizing to the ^{32}P-labeled Ad12 DNA are given in kilobase pairs (Kbp). The EcoRI map of Ad12 DNA is indicated at the bottom (35).*

The map locations of these fragments are indicated in the scheme at the bottom of Fig. 5. The essential results derived from these patterns can be summarized as follows.

(i) Hybridization with *Eco*RI fragment A, the right-hand terminal fragment of Ad12 DNA, revealed one band containing viral sequences in the DNAs of all lines tested. These bands comigrated with virion *Eco*RI fragment A. Multiple bands were not observed in the cellular DNA of each individual line tested (Fig. 5a). In this particular experiment the fragment A probe was slightly contaminated with fragment B of Ad12 DNA. However, this contamination did not affect the analysis.

(ii) Multiple bands were not observed either upon hybridizations with *Eco*RI fragment B, D, or E of Ad12 DNA. All bands in the cellular DNAs from the four tumor lines, which hybridized to each of these fragments, comigrated with the corresponding fragment of the Ad12 virion marker DNA (Fig. 5b, d, and e). Fragment F was not used in these experiments.

(iii) In hybridization experiments with *Eco*RI fragment C, the left end of Ad12 DNA, DNA from the RBT12/6, RBT12/13, and RBT12/15 lines yielded identical results, and the adenovirus-specific bands comigrated with the corresponding *Eco*RI marker fragment C of virion DNA. DNA from line RBT12/3 clearly gave different results. RBT12/3 DNA showed an Ad12-specific band in a molecular weight region well above that of *Eco*RI fragment C of Ad12 DNA (Fig. 5c). This finding would explain the absence of a band in the region corresponding to *Eco*RI fragment C, when *Eco*RI-cleaved cellular DNA from the RBT12/3 line was hybridized to intact Ad12 DNA (Fig. 4).

The hybridization experiments using the *Eco*RI fragments of virion DNA as probes revealed apparently perfect comigration of virus-specific bands in the cellular DNAs of lines RBT12/6, RBT12/13, and RBT12/15 and almost perfect comigration in the cellular DNA of line RBT12/3. This finding did not support the notion of integrated viral DNA in these lines, unless one assumed that the *Eco*RI sites in the cellular DNA were very near the virus-cell DNA junctions. *Eco*RI fragment A of Ad12 DNA is rather large (11.8 kilobases); therefore, small differences in molecular weight, indicative of a junction between viral and cellular DNA, could have been missed by gel electrophoresis if the cellular DNA sequences were very short. There was evidence, however, for integration of viral DNA in the cellular DNA of line RBT12/3 since

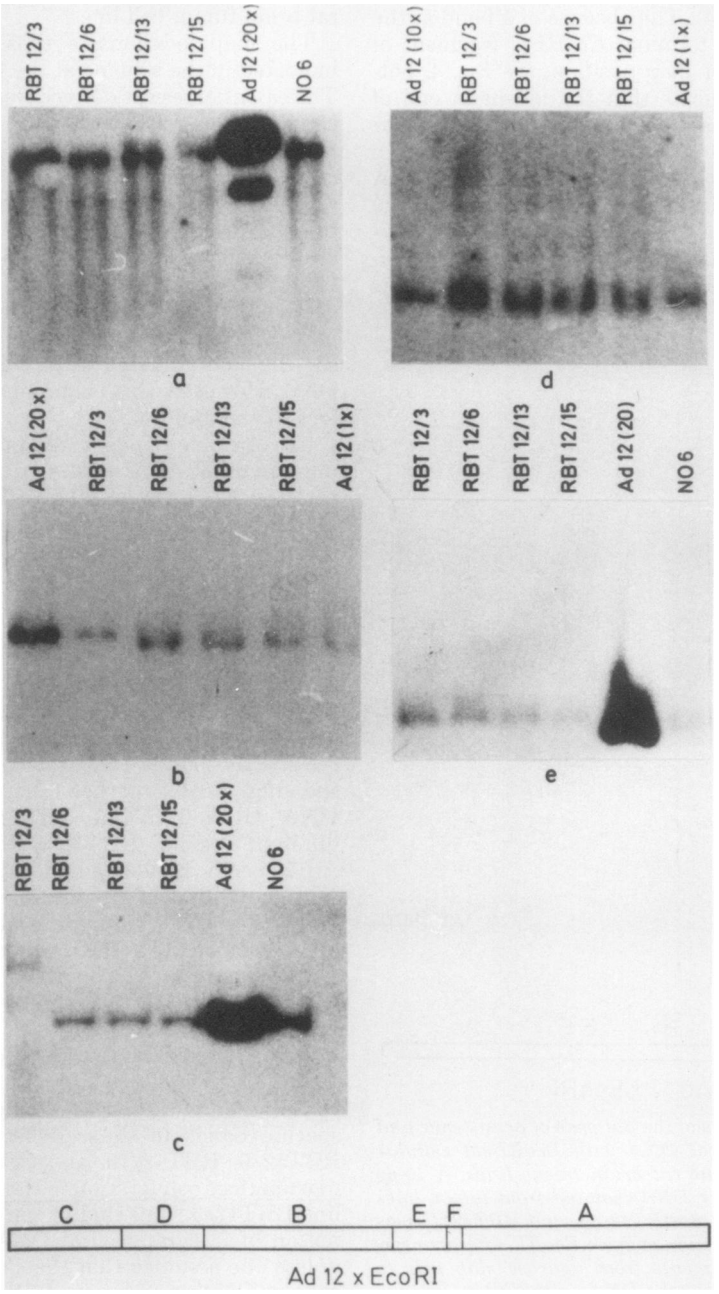

FIG. 5. *Analysis of the patterns of persistence and integration of Ad12 DNA in rat brain tumor cells, using EcoRI restriction endonuclease fragments A to E of Ad12 DNA as specific probes. Experimental details were similar to those described in the legend to Fig. 4, except individual ^{32}P-labeled fragments of Ad12 DNA were used as probes in hybridization experiments. (a) to (e) show the results of hybridization experiments with the EcoRI fragments A, B, C, D, and E, respectively. The map at the bottom indicates the positions of the EcoRI fragments on the Ad12 DNA molecules (35). EcoRI fragment A (a) was contaminated with EcoRI fragment B, but this contamination did not interfere with the interpretations of the results. In (a), (c), and (e), the DNA from transplantation line NO6 (RBT12/6 into nude mice) was also analyzed.*

the virus-specific band hybridizing to *Eco*RI fragment C was "off-size" (Fig. 5c).

Patterns of persistence and integration of Ad12 DNA in rat brain tumor cells derived from *Bam*HI-cut cellular DNA. Lines RBT12/6, RBT12/13, and RBT12/15 yielded identical hybridization patterns also when the cellular DNAs were cut with the restriction enzyme *Bam*HI (Fig. 6). The internal *Bam*HI fragments of Ad12 DNA comigrated with the corresponding Ad12 marker fragments (see Fig. 6, scheme on the bottom). In *Bam*HI-cut cellular DNA, bands were observed which were of higher molecular weight than *Bam*HI fragment A of Ad12 DNA (Fig. 6). Bands comigrating with *Bam*HI fragments A and E of virion DNA were absent in all four lines tested (Fig. 6). Line RBT12/3 was different from the other three lines in that there was only one band in the molecular weight region larger than *Bam*HI fragment A of Ad12 DNA, and this band was in a position different from the off-size fragments in the DNAs derived from the other three lines. Figure 6 shows that *Bam*HI fragments A and E represent the terminal fragments of the virion Ad12 DNA.

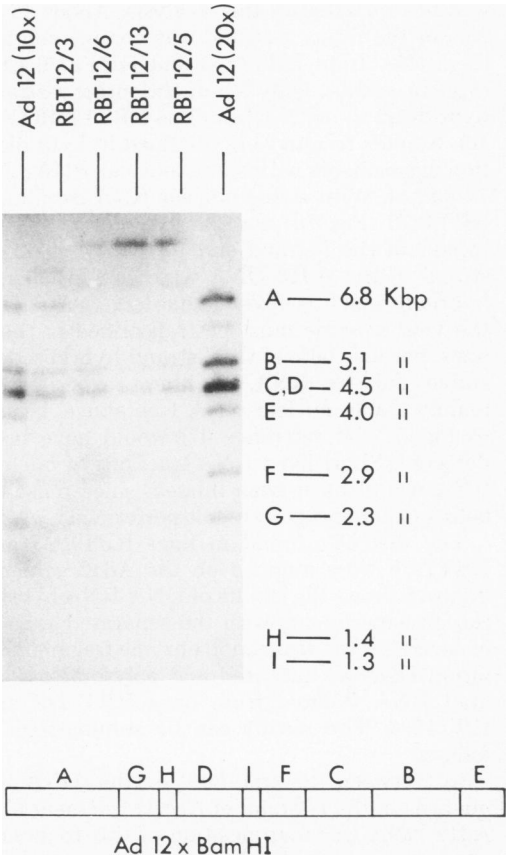

FIG. 6. *Analysis of the patterns of persistence and integration of viral DNA, using BamHI-cut cellular DNA isolated from the four rat brain tumor lines. The experimental conditions were identical to those described in the legend to Fig. 4, except the cellular DNA was cleaved with the BamHI restriction endonuclease. The sizes (in kilobase pairs [Kbp]) of the BamHI Ad12 marker DNA fragments are indicated in the right margin. The BamHI map of Ad12 DNA is also given.*

Thus, the patterns of distribution of virus-specific DNA bands in *Bam*HI-cut cellular DNAs from the four rat brain tumor lines provide direct evidence for the integrated state of Ad12 DNA in these lines. These findings were not at variance with the results of the hybridization experiments shown in Fig. 4 with *Eco*RI-cut cellular DNA. It is likely that the cellular *Eco*RI sites closest to the viral DNA of lines RBT12/6, RBT12/13, and RBT12/15 and one of the cellular *Eco*RI sites in the DNA of line RBT12/3 were very near the virus-cell DNA junctions. Upon cleavage with the *Bam*HI restriction endonuclease, only two virus-specific off-size bands were detected in the DNAs of lines RBT12/6, RBT12/13, and RBT12/15, and only one off-size band was detected in line RBT12/3. This finding suggested that only one or very few sites existed for the integration of viral DNA into the cellular genome.

Taken together, the data support a model in which the viral DNA is colinearly integrated into the DNA of the host. In line RBT12/3, the first *Eco*RI site in cellular DNA adjacent to the right terminal *Eco*RI fragment A of Ad12 DNA and the first *Bam*HI site in cellular DNA adjacent to the left terminal *Eco*RI fragment C of Ad12 DNA were very near the site of virus-cell DNA junctions. In lines RBT12/6, RBT12/13, and RBT12/15, the *Eco*RI sites adjacent to either terminus of the integrated Ad12 DNA molecules were very near the virus-cell DNA junctions. There was no evidence for the occurrence of tandemly integrated Ad12 DNA which actually can be ruled out (see below).

The patterns of distribution of virus-specific bands were different with respect to the terminal fragments of Ad12 DNA in at least two independent tumor lines, namely, RBT12/3 as compared with RBT12/6, RBT12/13, or RBT12/15 (Fig. 4 and 6). Since each of the rat brain tumor lines contained multiple copies of Ad12 DNA (Table 3) and apparently only one site of junction between virus and host cell DNA, it will be interesting to determine whether integration occurred mainly in redundant cellular DNA sequences. Lines RBT12/6, RBT12/13, and RBT12/15 were tumors derived from the same animal. It is therefore possible that these tumors

either arose as independent tumors or originated from a single transformation event. The latter assumption would explain why the patterns of distribution of viral DNA in these three tumors were identical. The finding that these three lines contained different amounts of viral DNA (Table 3) might be due to an unequal amplification during growth as separated tumors in the brain of the animal.

All hybridization experiments reported here were repeated using increasing amounts of restriction endonucleases in order to cleave the cellular DNA isolated from the rat brain tumor lines to completion. The results observed were identical to the ones presented here (date not shown). Therefore, the results obtained were not due to incomplete digestion of cellular DNA.

Stability of the patterns of integration of Ad12 DNA in rat brain tumor cell DNA. The stability of the patterns of persistence and integration of Ad12 DNA in rat brain tumor lines after long-term tissue culture passage and after repeated transplantation of the cells was investigated. For line RBT12/3, the pattern of hybridization shown in Fig. 4 remained stable after 34 weeks of continuous tissue culture passage. For line RBT12/6, the patterns shown in Fig. 4 and 6 remained stable after 30 weeks in tissue culture (data not shown). For line RBT12/6, a stable pattern identical to those shown in Fig. 4 and 6 was also obtained after two passages of the cells either in nude mice or in syngeneic animals and upon reestablishment of new transplantation lines (data not shown). Since the original tumor lines and the transplanted lines were more than 60 weeks apart, it can be concluded that the patterns of distribution of viral DNA in two independent tumor lines, namely, RBT12/3 and RBT12/6, remained stable for more than 60 weeks. The results of an extension of this work are shown in Fig. 5. DNA isolated from line NO6 was cleaved with the restriction enzyme *Eco*RI and was found to hybridize to Ad12 *Eco*RI fragments A (Fig. 5a), C (Fig. 5c), and E (Fig. 5e). NO6 was a transplantation line derived from RBT12/6 cells transplanted into nude mice. It is apparent that the pattern of hybridization in the DNA of this line remained stable. Neither were additional bands observed nor was there a shift in the positions of the virus-specific bands as compared with the parent line.

Absence of Ad12-specific DNA in nontumor tissues derived from rats. In some hybridization experiments, intact Ad12 DNA was hybridized to *Eco*RI-cleaved cellular DNA derived from fetal rat brain and from a rat kidney fibroblast line, which was established from the kidneys of BDIX rats. No bands were detectable in several independent hybridization experiments, demonstrating that nontransformed tissues from these rats were devoid of Ad12-specific DNA sequences (data not shown).

Transcription of the viral genome in rat brain tumor cells. It was of considerable interest to determine which of the persisting viral DNA sequences were transcribed in rat brain tumor cells. Figure 7 shows that some of the viral DNA sequences which were detected in the Ad12-induced rat brain tumor lines were also transcribed into mRNA and that more than 40% of the intact *l*-strand sequences of Ad12 DNA were complementary to polysome-associated mRNA isolated from rat brain tumor lines RBT12/3 (Fig. 7a), RBT12/6 (Fig. 7b), and RBT12/13 (Fig. 7c). RNA from line RBT12/15 was not included in this analysis. About 15 to 20% of the intact *r*-strand was complementary to mRNA from RBT12/3 and RBT12/6 cells (Fig. 7a and b). Only 5% of the intact *r*-strand hybridized to mRNA from line RBT12/13 (Fig. 7c). Similar results were obtained in hybridization experiments with stable nuclear RNA (Fig. 7d and e). With stable nuclear RNA from lines RBT12/3 (Fig. 7d) and RBT12/6 (Fig. 7e), up to 60% of the *l*-strand and 10 to 15% of the *r*-strand of the Ad12 DNA were converted into hybrid forms. It is obvious that large portions of the viral genome must be transcribed in these lines, because 60% of the *l*-strand hybridized to stable nuclear RNA. In these experiments, mainly stable nuclear RNA (see above, legend to Fig. 7, and reference 37) would have been detected. Short-lived RNA fractions of nuclear RNA would have been missed, since pulse-labeling experiments were not performed.

The mRNA's found in lines RBT12/3 and RBT12/6 were mapped on the Ad12 genome. Figure 8 shows the results of DNA-RNA hybridization experiments with the separated strands of several of the restriction enzyme fragments of virion DNA as indicated and polysome-associated RNA isolated from lines RBT12/3 and RBT12/6. The results can be summarized as follows.

(i) Between 40 and 50% of the DNA sequences in the *r*-strand of *Eco*RI fragment C of Ad12 DNA (for map position of this fragment, see Fig. 8a) hybridized to polysome-associated RNA isolated from lines RBT12/3 (Fig. 8a, upper panel) and RBT12/6 (Fig. 8a, lower panel).

(ii) The most striking observation was the homology found for the polysome-associated RNA isolated from both lines toward *Eco*RI fragment D and *Bam*HI fragment D of Ad12 DNA. Since these parts of the viral genome have been shown to code for late mRNA (40), it

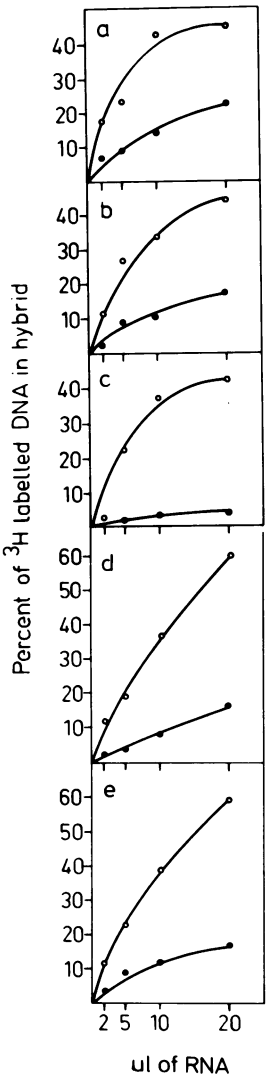

FIG. 7. *Hybridization of polysomal and nuclear RNAs from rat brain tumor cells to the isolated strands of Ad12 DNA. Polysome-associated mRNA (a, b, and c) and nuclear RNA (d and e) were isolated as described in the text and hybridized to the separated r and l strands of ^{3}H-labeled Ad12 DNA as described previously (37). At the end of the annealing reaction, the DNA-RNA mixture was treated with S1 nuclease to degrade single-stranded DNA which was not annealed to complementary RNA. The S1 nuclease-resistant ^{3}H-labeled DNA-RNA hybrids were acid precipitated, and the percentage of the S1 nuclease-resistant ^{3}H activity was calculated. Percentage of ^{3}H-labeled l-strand (○) and percentage of labeled r-strand of Ad12 DNA (●) rendered S1 nuclease resistant by increasing amounts of RNA. The units given on the abscissa represent microliters of RNA preparation added. The exact amounts of RNA used were not determined. RNA used in these experiments came from lines RBT12/3 (a and d), RBT12/6 (b and e), and RBT12/13 (c). RNA from line RBT12/15 was not tested in this set of hybridizations. Short-lived nuclear RNA with high turnover would not have been analyzed completely in this experiment, since the total nuclear RNA was extracted and pulse-labeling experiments were not performed.*

appears that in rat brain tumor lines RBT12/3 and RBT12/6, even some late mRNA sequences were synthesized.

(iii) *Eco*RI fragment E and *Bam*HI fragment E were transcribed into mRNA to a considerable extent.

(iv) The patterns of transcription in the two lines (RBT12/3 and RBT12/6) were very similar, although the extent of transcription varied slightly.

DISCUSSION

Biological properties. The Ad12-induced rat brain tumors described in this communication arose with rather long latency periods which exceeded those described in reports from other laboratories (32, 33). It is not known whether differences in rat strains or Ad12 preparations may have played a role.

The failure of incomplete particles to induce tumors might have been due to the lack of stability of these particles or their decreased content of viral DNA.

The cell lines established from some of the tumors displayed characteristic neuroepithelial morphology (Fig. 1b). The cells grew in monolayers and multilayers and were also observed to float freely in the form of ball-shaped aggregates in the tissue culture medium. Freeman et al. (13) reported similar findings and a marked calcium dependence of these phenomena in cultures of Ad12-transformed cells and Ad12-induced tumor cells, a finding which we did not observe. The growth properties of the tumor lines in tissue culture were similar to the ones generally observed in transformed cells; i.e., the cells had acquired an unlimited life-span, grew to very high saturation densities, plated in agar suspension, and were easily agglutinated by low concentrations of the plant lectin concanavalin A.

Cell line RBT12/13 showed no correlation between the ability to grow in agar suspension and the efficiency of plating (Table 1). The cells were not substrate independent, as could be seen by a comparison of the plating efficiencies and doubling times at different serum concentrations (Table 1). The finding that two of the cell lines (RBT12/13 and RBT12/15) plated rather efficiently in soft agar also correlated with the fact that they were tumorigenic in nude mice. For

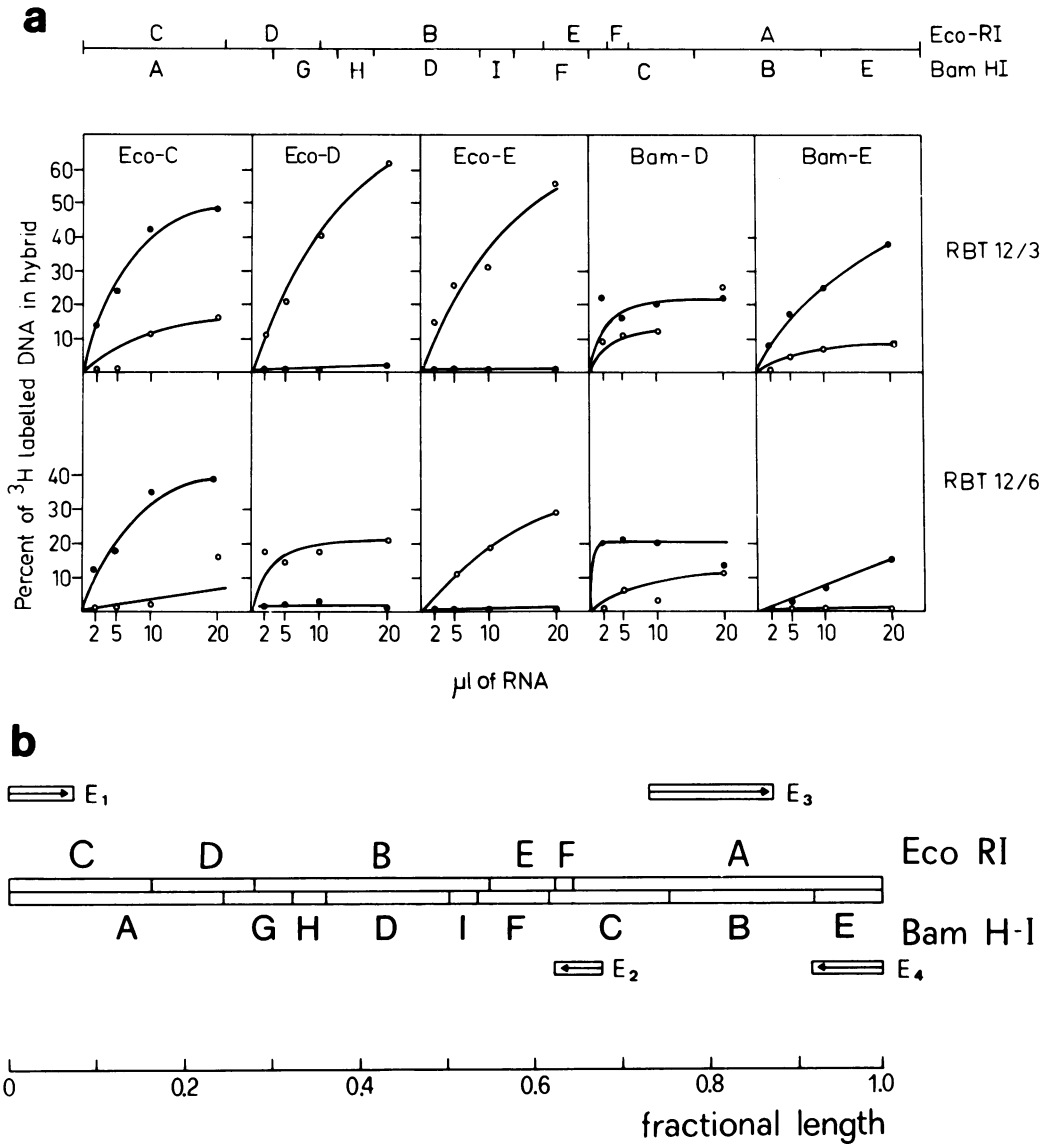

FIG. 8. *Hybridization of polysomal RNA isolated from rat brain tumor lines to the isolated strands of EcoRI fragments of Ad12 DNA. Experimental details were similar to those described in the legend to Fig. 7, except separated strands of the EcoRI and BamHI fragments of Ad12 DNA were used in the hybridization experiments, as indicated (a). The symbols used are described in the legend to Fig. 7. (b) EcoRI and BamHI maps of Ad12 DNA (35) and the location of the early genes of Ad12, E_1 to E_4 (37).*

lines RBT12/3 and RBT12/6, this correlation did not hold (Tables 1 and 2). A correlation of tumorigenicity in nude mice and growth in soft agar has been proposed by several authors (12, 41), but may not generally be found. Fibronectin was not detected in any of the tumor cell lines or transplanted lines studied. Since the rat brain tumor cells were only transplantable intracranially into newborn syngeneic animals and not into adult syngeneic animals, the absence of fibronectin does not seem to correlate with tumorigenicity in general in the lines studied, although a correlation between tumorigenicity and fibronectin has been suggested in the past (4).

T-antigen could not be detected in the rat brain tumor lines by indirect immunofluorescence. We have demonstrated, however, that at

least part of *Eco*RI fragment C, which is thought to code for the Ad12 T-antigen, is present in the rat brain tumor lines (Fig. 5c) and appears to be transcribed (Fig. 8). Line HA12/7, an Ad12-transformed hamster cell line (11), also expresses only low levels of T-antigen which cannot be detected by indirect immunofluorescent assays.

Persistence and integration of the viral genome. The data presented in this communication have demonstrated that the viral DNA in all four rat brain tumor lines is colinearly integrated into the cellular DNA. We have detected multiple copies of the viral genome in the cellular DNA of the four rat brain tumor lines studied (Table 3). However, it was apparent from the data shown in Fig. 2 that all lines studied were highly polyploid. Since we do not know whether the chromosomes containing the viral sequences were present in more than two copies, the calculated numbers could be underestimates. The intriguing observation, therefore, was that only one or two distinct virus-specific bands were seen in all hybridization experiments (Fig. 4 and 6). Moreover, there were only two Ad12-specific off-size bands observable in the DNAs of all four rat lines when cellular DNA was cut with the *Bam*HI endonuclease, with the exception of line RBT12/3 (Fig. 6). These findings may be explained by assuming identical or very similar cellular DNA sequences in the immediate vicinity of the integrated viral DNA. The interpretation of multiple copies of Ad12 DNA being integrated at identical or similar sites was further supported by the results of hybridization experiments with isolated *Eco*RI restriction endonuclease fragments of Ad12 DNA as probes.

In these experiments, hybridization of *Eco*RI-cut DNA from all four rat brain tumor lines on Southern blots with each of the isolated ^{32}P-labeled *Eco*RI fragments A, B, C, D, and E gave rise to single Ad12-specific bands (Fig. 5a to e). The positions of these bands coincided with the positions of the corresponding Ad12 marker DNA bands, with the exception of the DNA from line RBT12/3 when hybridized to *Eco*RI fragment C (Fig. 5c). The fact that even the terminal Ad12 DNA fragments A and C yielded only one band of homology identical or nearly identical in size to the virion marker DNA fragments ruled out the possibility of tandemly integrated Ad12 DNA molecules abutting each other directly. In this case, tandem fragments would have been found consisting of covalently linked *Eco*RI fragments A+C or A+A or C+C, depending on the type of tandem. There was clearly no evidence for the occurrence of such fragments (Fig. 5a and c), i.e., for the existence of tandems. Perhaps, it is more likely that viral DNA sequences were integrated into repetitive sequences of cellular DNA. An arrangement of multiple copies of viral DNA molecules integrated at the same sites, but separated from one another by short stretches of cellular DNA carrying an *Eco*RI site, was also possible. This arrangement could not be considered a real tandem, which is defined by one of a kind followed immediately by another one of the same kind.

It was conceivable that the apparent integration into repetitive sequences could also be explained by amplification of a single copy of viral DNA and the adjacent cellular sequences at some time after the integration event. Amplification could have been unequally efficient in lines RBT12/6, RBT12/13, and RBT12/15, and, in fact, these lines contained different copy numbers of Ad12 DNA. The finding that the internal fragments of Ad12 DNA were distributed in identical patterns in all lines investigated indicated colinearity of integration of intact Ad12 genomes. One must be cautious in comparing the patterns of integration of Ad12-specific DNA in lines RBT12/6, RBT12/13, and RBT12/15, because these tumors arose at different sites of the brain in one animal. Thus, these tumors may be derived from one transformation event.

Several laboratories have reported differences in the patterns of distribution of virus-specific sequences in a number of different lines of Ad12-transformed hamster cells (43), simian virus 40-transformed hamster cells (3), or simian virus 40-transformed rat cells (23). First, it might turn out to be very difficult to compare results obtained with cells transformed by different viruses. Moreover, the Ad12-induced rat brain tumor lines described in the present report were selected under conditions very different from those employed for in vitro transformation. Selection conditions in the intact organ of an animal may be much more stringent than those in cell culture or in soft agar. Similarities or identities in integration patterns of viral DNA might therefore reflect this stringency in the selection of certain transformed cells. Further work will be required to resolve these intriguing problems.

Stability of the pattern of integration of the viral genome. Earlier work on the stability of the viral DNA in Ad12-transformed hamster cells has demonstrated that prolonged tissue culture of up to 11 months did not alter the patterns of integration in these lines (43). We have extended this study to the Ad12-induced rat brain tumor cells and have shown that after prolonged tissue culture of cells for up to 9 months and after repeated animal passage and reexplantation into cultures, the patterns of persistence of viral DNA remained stable over a total period of more than 15 months. To our

knowledge this type of analysis of persisting viral genomes was not reported before. We conclude that the different types of selective pressures in tissue culture and in animals do not interfere with the patterns of persistence and integration of Ad12 DNA in the rat brain tumor cell lines.

Expression of the viral genome in Ad12-induced rat brain tumor lines. Surprisingly, a high degree of homology was detected between mRNA's isolated from the tumor lines and the two strands of the viral genome. In earlier work on the transcription of Ad12 DNA sequences in transformed hamster cells, transcription of the late region of the viral genome was not observed (37). Investigations of the transcription of Ad12 DNA in rat brain tumor cells, however, indicated that fragments of the viral genome which were considered to yield late mRNA in the productive infection cycle (40) were transcribed. The same patterns of transcription were observed in at least two independent tumor cell lines (Fig. 8). We cannot decide whether this finding was a reflection of the fact that the distribution of the virus-specific DNAs in these two lines was similar (Fig. 4, 5, and 6) or whether this was a specific property of these tumors. At the present time, it is unknown which parts of the late mRNA are translated. Virus particles were, however, not observed in the tumor cells, and virus production could not be induced by fusion of the tumor cells to permissive HEK cells. As in other lines of cells transformed by DNA tumor viruses or of tumor cells induced by these viruses, the way in which the expression of integrated viral sequences is regulated remains unknown.

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