MOLECULAR AND CELLULAR BIOLOGY, May 1990, p. 2027–2034
0270-7306/90/052027-08$02.00/0

Vol. 10, No. 5

Suppression of the Progression Phenotype in Somatic Cell Hybrids Occurs in the Absence of Altered Adenovirus Type 5 Gene Expression

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Received 27 September 1989/Accepted 23 January 1990

In the present study we have analyzed the genetic regulation of increased expression of transformation-associated traits, a process termed progression, in adenovirus type 5 (Ad5)-transformed secondary rat embryo cells. Somatic cell hybrids were formed between a highly progressed neomycin-resistant Ad5-transformed cloned cell line (E11-NMT^{neo}) and an untransformed chloramphenicol-resistant rat embryo fibroblast cell line (CREF^{cap}). Parental E11-NMT^{neo} cells grew with high efficiency in agar, exhibited reduced ^{125}I-epidermal growth factor (EGF) binding, and were tumorigenic in nude mice. Parental CREF^{cap} cells exhibited phenotypes opposite to those of E11-NMT^{neo} cells. A high proportion (84%) of the presumptive hybrid cell types obtained after fusion and genetic selection (G418 and chloramphenicol) displayed a flat morphological phenotype intermediate between CREF^{cap} and E11-NMT^{neo} cells, suggesting that a *trans*-dominant extinction phenomenon had occurred. Two hybrids with a round morphology (R), which still exhibited the progressed phenotype, and two hybrids with a flat morphology (F), which had lost the progressed phenotype, were chosen for detailed analysis. Both R hybrids grew efficiently in agar, exhibited low ^{125}I-EGF binding, and were tumorigenic in nude mice, whereas both F hybrids grew poorly in agar, displayed increased ^{125}I-EGF binding in comparison with E11-NMT^{neo} and R hybrids, and were nontumorigenic in nude mice. An analysis of the viral DNA integration patterns and the rates of transcription, steady-state mRNA accumulation, and relative levels of the Ad5 E1A and E1B gene products revealed no differences among the parental and hybrid cells. These studies indicate that normal CREF cells may contain a suppressor gene(s) which can inhibit the expression of specific traits of the progression phenotype in Ad5-transformed cells and that this suppression is not associated with changes in the expression of Ad5 transforming genes.

Although recent advances have led to a better understanding of the potential role of defined genetic elements in the etiology of cancer, the molecular details by which a cell becomes transformed and ultimately evolves into a population of cells possessing tumorigenic and metastatic potential have not been delineated (reviewed in references 9, 24, and 27). Transforming genes (oncogenes) have been isolated and characterized from viral and mammalian tumor cell genomes which when transferred and expressed in appropriate recipient cells display a dominant-acting phenotype (reviewed in references 2 and 4). In addition to these dominant-acting transforming genetic elements in the genomes of certain tumor cells, several lines of experimental evidence indicate that specific genes exist in normal eucaryotic cells which may function as inhibitors of expression of the transformed phenotype and tumor formation, and these have been referred to as suppressor genes, repressor genes, antioncogenes, or emerogenes (1, 3, 18, 23, 28, 36). Evidence supporting the existence of suppressor genes has come from (i) the analysis of somatic cell hybrids generated by fusing nontransformed and transformed cells (19, 22, 31); (ii) genetic analysis of specific human tumors, such as neuroblastoma, retinoblastoma, and Wilms' tumor, indicating a correlation between the loss of specific DNA sequences and the development of neoplasia (5, 7, 18, 23, 32, 35); (iii) the isolation of morphological (flat) revertants of RNA tumor virus-transformed cells which display a selective resistance to retransformation by specific oncogenes (3, 26); (iv) the ability to reverse, by insertion of a cloned retinoblastoma gene, the tumorigenic and transformed cell phenotype in osteosarcoma and retinoblastoma cells not expressing the retinoblastoma gene (20); (v) the identification of human DNA sequences, such as Krev-1, which induce a reversion of the transformed cell phenotype in Kirsten *ras*-transformed rodent cells (21, 25); and (vi) studies indicating that progression of the transformed phenotype in certain virally transformed cells is reversible (1, 7). From these considerations, it is clear that a complete understanding of the carcinogenic process will require not only the identification and characterization of dominant-acting oncogenic elements, but also a detailed molecular investigation of suppressor genetic elements.

Studies involving somatic cell hybridization and microcell hybrid systems, in which a single human chromosome has been transferred, indicate that suppressor genes are located on specific chromosomes in the human genome (32, 33, 35). Intraspecific and interspecific somatic cell hybrids formed between normal (early-passage or established cell lines) and transformed cells have been shown to lose the tumorigenic potential of the transformed parental cell as long as specific chromosomes from both parental cells are retained (22, 30–33). In hybrids which display an instability in chromosome content, segregates can develop which have regained expression of the tumorigenic phenotype, and this process is often associated with the loss of specific chromosomes (22, 32, 33). Therefore, like cellular oncogenes, it is possible that

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a family of different suppressor genes exists. Whether these suppressor genes exert their effects on expression of the malignant phenotype through common or distinct biochemical pathways remains to be determined.

Evidence from both in vivo and in vitro studies indicates that carcinogenesis is a multifactor and multistep process which may involve the cooperative interaction of several genetic elements and which is affected by diverse environmental factors (reviewed in references 9, 27, and 34). In previous studies we have demonstrated that adenovirus type 5 (Ad5) transformation of early-passage rat embryo cells is a multistep process (12, 13, 20). By selecting Ad5-transformed cells after growth in agar or reisolating Ad5-transformed cells from a tumor induced in a nude mouse, clonal populations of Ad5-transformed cells have been obtained which display an enhanced expression of the transformed phenotype (a process termed progression). In the present study we have analyzed the effect of somatic cell hybridization on expression of the transformed and malignant phenotypes of hybrids formed between progressed E11-NMT cells and untransformed normal rat embryo fibroblast (CREF) (10) cells. We demonstrate that expression of the transformed phenotype and expression of the malignant phenotype are under separate genetic controls and that the state of progression of the transformed phenotype is not correlated with the rate of transcription of the viral E1A and E1B genes of Ad5, steady-state levels of RNA encoded by the E1A and E1B transforming genes of Ad5, or the loss of expression of the polypeptides encoded by the E1A and E1B genes of Ad5.

MATERIALS AND METHODS

Cell cultures. A specific single-cell clone of the Fischer 2408 established rat embryo fibroblast cell line, referred to as CREF (10), which exhibits traits normally attributed to nontumorigenic cells was used as the nontransformed parental cell for cell hybridization experiments. CREF cells were selected for resistance to chloramphenicol by a stepwise selection, without prior mutagenesis, in increasing concentrations of chloramphenicol. $CREF^{cap}$ cells resistant to 100 μg of chloramphenicol per ml were used for generating somatic cell hybrids. E11 is a single-cell clone of benzo[*a*]pyrene-pretreated Ad5 temperature-sensitive mutant (H5ts125)-transformed Sprague-Dawley secondary rat embryo cells (12). Liquid and filter hybridization analysis indicates that E11 cells contain a single integrated copy of the Ad5 genome (6). These cells display similar biological properties to later-passage wild-type Ad5- and H5ts125-transformed secondary Sprague-Dawley rat embryo cells not exposed to carcinogens (1). E11-NMT is a subclone of E11 cells isolated from a nude mouse tumor (1). A culture of E11-NMT cells was transfected by using the standard calcium phosphate-mediated DNA transfection procedure with DNA from a pSV2-neo plasmid (29), and a G418-resistant (1,000 μg/ml) clone, $E11\text{-}NMT^{neo}$, was isolated and used for somatic cell hybridization experiments. Cultures were grown in Dulbecco modified Eagle medium containing 5% fetal bovine serum supplemented with penicillin and streptomycin and 100 μg of chloramphenicol per ml ($CREF^{cap}$) or 1,000 μg of G418 per ml ($E11\text{-}NMT^{neo}$). All cultures were maintained at 37°C in a 5% CO_2-humidified air incubator.

Formation of somatic cell hybrids. $CREF^{cap}$ cells (2×10^5) were mixed with 3.5×10^5 $E11\text{-}NMT^{neo}$ cells, pelleted, and fused with polyethylene glycol (PEG 1500; Boehringer Mannheim Biochemicals) warmed to 37°C for exactly 1 min. The PEG 1500 is a 50% ready-to-use solution buffered with 75 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 8.0), which has been filter sterilized to prevent the formation of oxidation products during autoclave sterilization and which has been fusion tested by the manufacturer. After the pellet was exposed to PEG for 1 min, it was immediately rinsed with 10 ml of normal culture medium containing serum and centrifuged. This wash was repeated two additional times, and the fused cells were plated in 10-cm dishes at 10^4 cells per dish in normal culture medium. As a control, identical numbers of cells were mixed, centrifuged, and washed in the same manner as the experimental samples, but were not PEG treated (mixed controls). The following day, all dishes were fed selective medium (containing 1,000 μg of G418 per ml and 100 μg of chloramphenicol per ml). The medium was changed every 3 days for 2 weeks, after which clones were isolated by the glass cylinder method for further analysis. From three 10-cm dishes a total of 57 clones were identified; 9 of these displayed a round morphology somewhat similar to the $E11\text{-}NMT^{neo}$ parental cell and 48 had a flat morphology somewhat intermediate between those of $CREF^{cap}$ and $E11\text{-}NMT^{neo}$ cells. There were no clones in the mixed-control dishes plated at 10^4 cells per 10-cm dish. Several round and flat hybrid colonies were isolated, and chromosomal analysis revealed that approximately 80 chromosomes existed per cell. Since $CREF^{cap}$ cells contain 44 chromosomes and $E11\text{-}NMT^{neo}$ cells contain 39 chromosomes, these findings suggest that the cells were hybrids and maintained all of the chromosomes from both cell types.

Tumorigenicity, anchorage independence, and EGF receptor binding. Congenitally athymic nude BALB/c mice were obtained by breeding BALB/c *nu/nu* homozygous males with BALB/c +/*nu* females in a pathogen-free environment. Mice homozygous for the *nu/nu* allele and 4 to 6 weeks old were used for analysis of tumorigenicity. Cells in logarithmic growth were detached from the substrate with 0.5% trypsin, washed with growth medium, and centrifuged. The pellet was suspended in medium without serum at a concentration of 10^7 cells per 0.2 ml. A 20-gauge needle was used to inject 0.2 ml of the cell suspension subcutaneously into the back of a nude mouse. Inocula which produced a progressively growing tumor were scored positive for tumorigenicity. Negative animals were maintained for 6 months.

The ability of the parental and hybrid cells to grow in agar was assayed as described previously (12). After 21 days of growth in Dulbecco modified Eagle medium containing 7.5% fetal bovine serum and 0.4% Noble agar, colonies of >0.1 mm in diameter were counted with the aid of an Olympus tissue culture microscope and a calibrated grid.

Specific binding of epidermal growth factor (EGF) to cell membrane receptors was determined as described previously (11). Receptor-binding data are expressed as specific binding, i.e., the total binding obtained with ^{125}I-EGF minus the binding obtained in the presence of excess unlabeled EGF. The latter value represented <5% of the total binding. Results of replicate assays agreed to within ±15%.

Nucleic acid analysis. High-molecular-weight DNA was isolated from cells as previously described (6). Cellular DNAs (10 μg per sample) were digested with the appropriate restriction endonuclease, size fractionated through 0.6% agarose gels, transferred to nitrocellulose, and probed for the presence of Ad5 DNA sequences by using ^{32}P-labeled Ad5 DNA (10). Following hybridization, the filters were washed and exposed for autoradiography.

In vitro transcription within isolated nuclei was performed as previously described (14). Nuclei from approximately 10^8

TABLE 1. Properties of cells used in analysis of progression suppression in Ad5-transformed rat cells

Cell line	Morphology[a]	% Cloning efficiency in agar[b]	^{125}I-EGF binding (cpm/10^6 cells)[c]	Tumorigenicity in nude mice[d]
CREF	Fib/normal	<0.001	1,460 (100%)	0/3
E11	Epi/flat	2.0	181 (12.4%)	3/3 (38 days)
E11-NMT	Epi/round	39.0	141 (10.2%)	3/3 (18 days)
R1	Epi/round	72.0	63 (4.3%)	3/3 (17 days)
R2	Epi/round	57.0	80 (5.5%)	3/3 (17 days)
F1	Epi/flat	2.0	584 (40.0%)	0/3
F2	Epi/flat	1.5	818 (56.0%)	0/3

[a] Fib, Cells displaying a fibroblastic morphology; Epi, cells displaying an epithelioid morphology.

[b] CREF cells were suspended in 0.4% agar medium at 1×10^5 cells per 60-mm plate. E11 and hybrid cells were resuspended in agar medium at a density of 2×10^3 or 5×10^3 cells per plate. Colonies were scored by microscopic evaluation 14 to 21 days postplating.

[c] Cells were plated at 4×10^5 (CREF) or 1×10^6 (E11-derived and hybrid cells) cells per 35-mm dish, and binding assays were performed 72 to 96 h postplating. Values represent the average specific binding of ^{125}I-EGF binding (adjusted by subtraction of nonspecific binding) from triplicate samples, which varied plus or minus 10%. Values in parentheses represent the percentage of CREF control binding, which is set at 100.

[d] Cells were injected subcutaneously at 1×10^7 cells per animal. The first ratio is the number of animals producing tumors/total number of animals injected. The number in parentheses is the latency period needed for formation of a palpable tumor.

cells were isolated, and RNA transcripts previously initiated by RNA polymerase II were allowed to elongate in the presence of [^{32}P]UTP. Nuclear RNA was isolated and denatured by treatment with 0.1 N NaOH for 5 min on ice. For nitrocellulose dot filters, 7 μg of plasmid DNA was denatured by boiling in 0.1 N NaOH for 15 min, diluted with cold 2 M NaCl, and spotted on a 45-μm-pore-size nitrocellulose filter. The plasmid probes used included E1A (Ad5; nucleotides [nt] 0 to 1338), E1B (Ad5; nt 3328 to 3786), E2 (Ad5; nt 22179 to 22435), E4 (Ad5; nt 32264 to 35937), chicken β-actin, rat tubulin, rat ligandin, and pBR322. The steady-state levels of the Ad5 E1A and E1B mRNAs in the various cell types were determined by hybridizing total cytoplasmic RNA to uniformly labeled [^{32}P]UTP antisense RNA probes and digesting the hybrid products with RNase T_2 as previously described (14).

RESULTS

Biological and biochemical properties of Ad5-transformed, untransformed, and hybrid rat embryo cell cultures used to analyze the progression phenotype. The properties of CREF and H5ts125-transformed Sprague-Dawley secondary rat embryo cells are indicated in Table 1. The cloned E11 cell line exhibited a low efficiency of growth in agar (ca. 2%) and induced tumors in nude mice with an average latency period of tumor formation of 38 days. In contrast, E11-NMT cells, derived from a tumor induced in a nude mouse after the subcutaneous inoculation of 10^6 E11 cells, grew efficiently in agar-containing medium (39 to 45%) and displayed a reduced tumor latency period, only 18 days, when inoculated subcutaneously into nude mice. When grown in monolayer culture, however, both cell types exhibited similar population doubling times of approximately 15 h (1). To determine whether untransformed rat embryo cells contain a dominant-acting suppressor gene function which could inhibit expression of the progression phenotype in E11-NMT cells, we constructed somatic cell hybrids between E11-NMT cells and the established and phenotypically normal rat embryo cell line CREF. Microscopic observations of hybrid cells formed after PEG-induced fusion of CREF^{cap} and E11-NMT^{neo} cells and selection in medium containing 100 μg of chloramphenicol per ml and 1,000 μg of G418 per ml indicated a predominance of presumptive hybrid colonies with a flat morphology and fewer colonies with a round morphology. On the basis of these morphological differences, two flat hybrids (F1 and F2) and two round hybrids (R1 and R2) were chosen for detailed analysis (Fig. 1). The morphology of CREF^{cap} cells differs from that of E11-NMT^{neo} cells (Fig. 1), permitting easy differentiation between these two cell types in mixed cultures. The true hybrid nature of the cells selected in medium containing chloramphenicol and G418 was verified by chromosome analysis. CREF^{cap} cells contained an average of 44 chromosomes, E11-NMT^{neo} cells contained an average of 39 chromosomes, and the four hybrids contained 78 (F1), 78 (F2), 84 (R1), and 80 (R2) chromosomes.

Since a major biological difference between E11 and E11-NMT^{neo} cells is in their ability to form colonies when seeded in 0.4% agar, the anchorage independence phenotype of the hybrid cells was determined. The E11 cell line grew poorly in agar, whereas the tumor-derived E11-NMT^{neo} cell line grew with high efficiency in agar (Table 1). In contrast, CREF^{cap} cells displayed an anchorage-dependent phenotype and did not form macroscopic colonies in agar-containing medium (<0.001%), a phenotype displayed by untransformed normal early-passage rat embryo cells. Both round hybrids exhibited the progressed phenotype, as indicated by their enhanced ability to grow in agar. In fact, the cloning efficiency of R1 and R2 actually exceeded that of the parental E11-NMT^{neo} cells (Table 1). In contrast, the flat hybrids exhibited a suppression in their ability to grow in agar and displayed a cloning efficiency in agar similar to that observed in E11 cells.

To determine whether the ability of the various hybrid clones to grow in agar correlated with their tumorigenic potential in athymic nude mice, 10^7 CREF^{cap}, E11, E11-NMT^{neo}, R1, R2, F1, or F2 cells were injected subcutaneously into animals (Table 1). Tumors did not develop in CREF^{cap}-injected nude mice, whereas tumors did develop in animals injected with E11 or E11-NMT^{neo} cells. As previously observed, the latency period for tumor development was shorter when E11-NMT^{neo} cells were used than when E11 cells were used (1), even with this large inoculum of transformed cells. The round hybrids were tumorigenic and displayed the same reduced tumor latency period exhibited by the E11-NMT^{neo} parental cells. In contrast, both flat hybrids were unable to form tumors under the conditions used in this assay.

The binding of ^{125}I-EGF to cell surface receptors in parental and hybrid cells is shown in Table 1. Both E11 and E11-NMT^{neo} cells exhibited low levels of specific EGF binding (12.4 and 10.2% of CREF^{cap} binding, respectively). The R1 and R2 hybrids specifically bound even less ^{125}I-EGF (4.3 and 5.5% of the binding seen in CREF^{cap} cells, respectively). In contrast, the flat hybrids displayed an intermediate level of specific binding of ^{125}I-EGF, with F1 and F2 displaying 40 and 56%, respectively, of the binding in control CREF^{cap} cells.

Arrangement of integrated Ad5 DNA sequences in parental and hybrid cells. In previous studies we have demonstrated that progression of the transformed phenotype in E11 cells is not associated with changes in the integration pattern of Ad5 DNA sequences (1). To determine the presence and arrange-

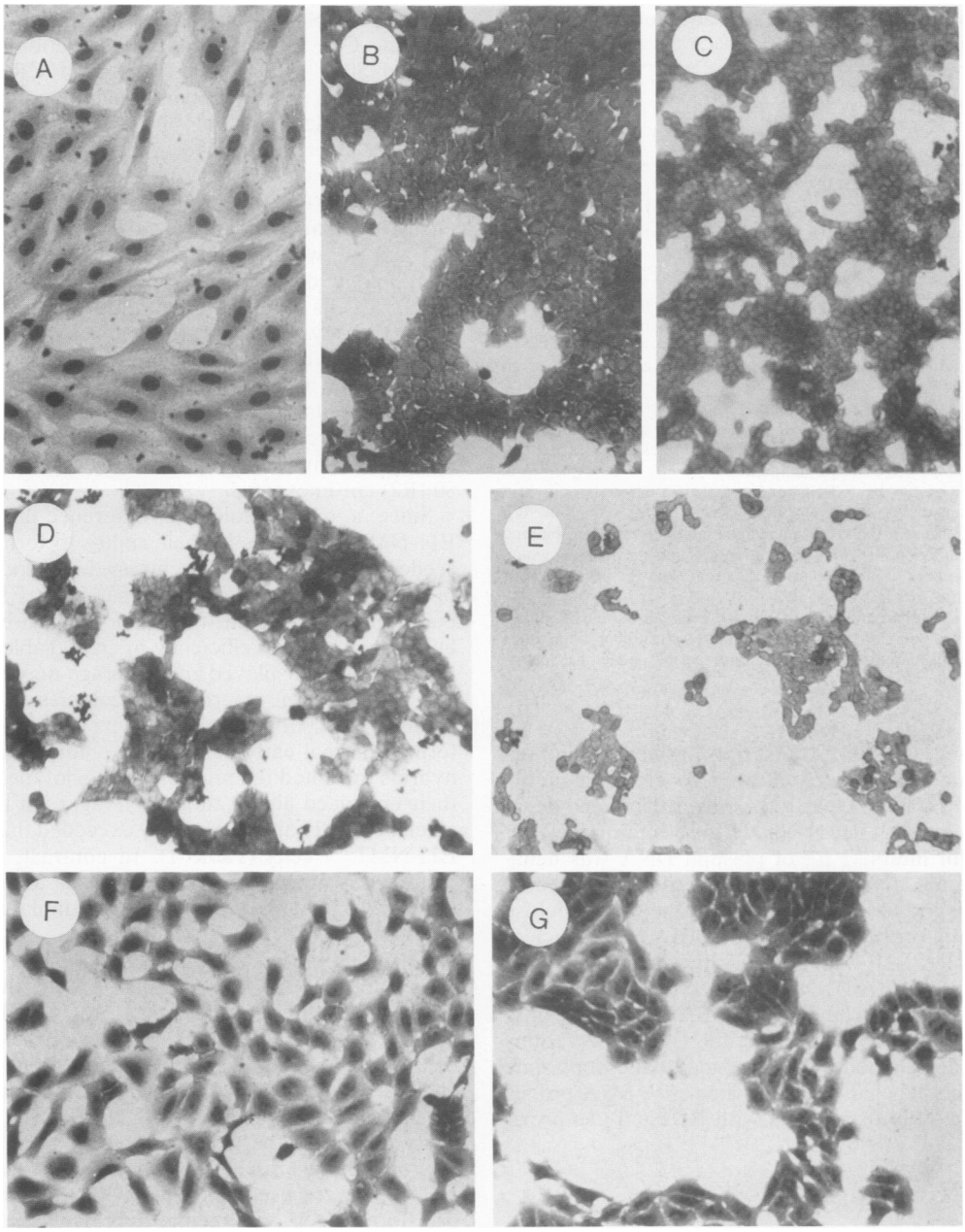

FIG. 1. Morphology of CREF^{cap} (A), E11 (B), E11-NMT^{neo} (C), and CREF^{cap}/E11-NMT^{neo} hybrid R1 (D), R2 (E), F1 (F), and F2 (G) cells. Cells were grown in 35-mm dishes, fixed with 4% formaldehyde, and stained with Giemsa stain. Magnification, ca. ×200.

ment of integrated Ad5 DNA within the cellular genomes of the parental and hybrid cells, we performed Southern transfer-hybridization analyses. These showed that the CREF^{cap} cellular genome did not contain integrated Ad5 DNA (Fig. 2). However, cellular DNAs from the E11, E11-NMT^{neo}, R1, R2, F1, and F2 cultures contained the same general arrangement of integrated Ad5 DNA, as evidenced by similar viral DNA-containing fragment patterns after digestion by either *Xba*I (Fig. 2A) or *Kpn*I (Fig. 2B). These results indicate that Ad5 DNA is retained in E11 cells that have been passed through nude mice as tumors and in E11-NMT^{neo} cells transfected with pSV2-neo, as well as in E11-NMT^{neo} cells fused with CREF^{cap} cells. More importantly, there appears to be no loss or gross rearrangement of integrated Ad5 DNA in the cellular genomes within the different cells in the E11 series displaying different stages in the progression lineage.

Nuclear RNA transcriptional rates and steady-state mRNA levels of the Ad5 E1A and E1B genes in transformed parental and hybrid cells. To determine whether the suppressed phenotype of the flat hybrids was due to changes in the transcription of the integrated Ad5 genes necessary for expression of the transformed phenotype of rat embryo cells (E1A and E1B), we performed nuclear run-on assays (Fig. 3). Using a similar amount of total labeled nuclear RNA (10^7 cpm) representing the same number of nuclei from each cell line shown, it is clear that there is no significant difference in the expression of Ad5 E1A, E1B, E2, or E4 genes among E11, E11-NMT^{neo}, R1, R2, F1, or F2 cells. The slight variations that are observed are not significant when the

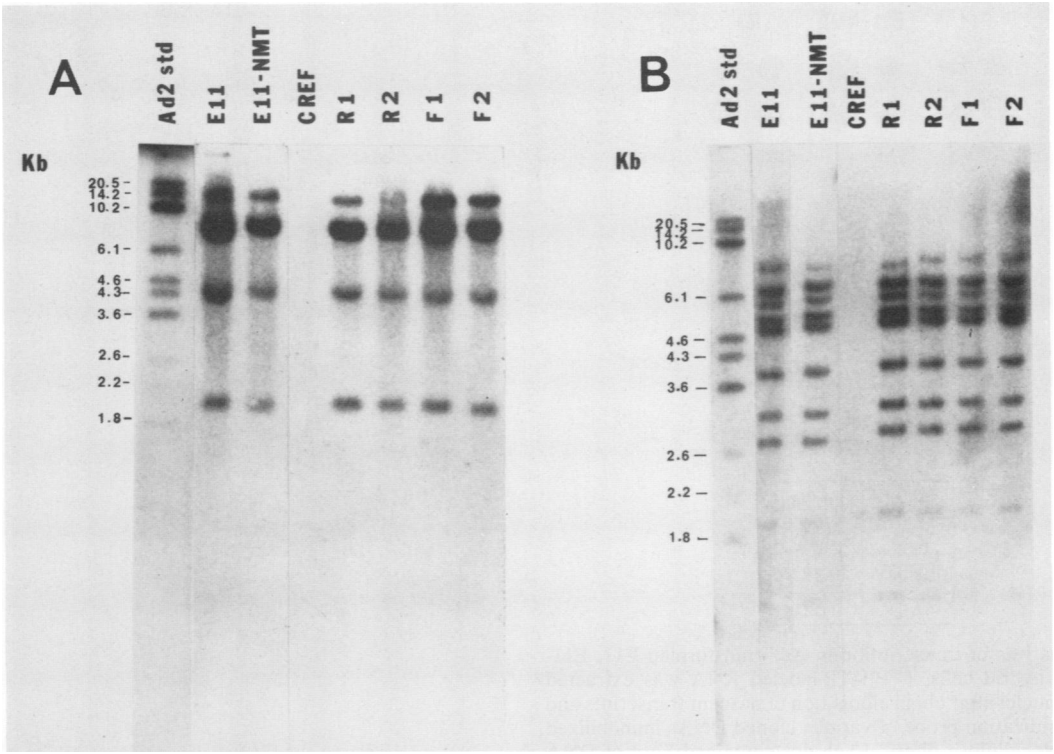

FIG. 2. Arrangement and stability of integrated Ad5 DNA sequences in CREF^{cap}, E11, E11-NMT^{neo}, and CREF^{cap}/E11-NMT^{neo} hybrid cells. Cellular DNA (10 μg) was digested with *Xba*I (A) or *Kpn*I (B) and analyzed by Southern transfer-hybridization with ^{32}P-labeled Ad5 DNA as a probe. Ad2 DNA was digested independently with *Eco*RI or *Bam*HI and mixed 1:1, and 10^{-4} μg of this mixture was included in the gel as a size standard.

comparative rates of transcription of common genes (actin and tubulin) are compared among the cell lines.

To determine whether there were any differences in the level or stability of cytoplasmic Ad5 E1A and E1B mRNAs which could account for differences in the displayed phenotype, we hybridized total cytoplasmic RNA from E11, E11-NMT^{neo}, and hybrid cells to antisense ^{32}P-labeled SP6 polymerase-generated RNA specific for E1A and E1B gene sequences (Fig. 4). After RNase T_2 digestion of hybrids, bands of 294 and 72 nt representing E1A and E1B transcripts, respectively, were observed in all cell lines. These RNase protection assays show that there are no significant differences in the levels of the protected 294-nt fragments that represent combined levels of both E1A 12S and 13S RNA species. The levels of the RNase-resistant 72-nt fragments representing E1B appear to differ slightly among R1 and R2 cells as they do among F1 and F2 cells. When using an antisense SP6 probe that scored the 3′ end of the E1B 13S and 22S mRNAs, no differences were observed in the accumulation of the two E1B mRNAs in all the cell types which expressed the Ad5 E1B gene (data not shown). These results indicate that there are no significant differences in the steady-state levels of RNA from Ad5 transforming genes in the two morphological types of hybrid cells. This was further confirmed by analyzing the steady-state levels of the E1A 289R and 243R proteins and E1B 175R protein in each of the cell lines and demonstrating no differences in the relative levels of these Ad5-encoded polypeptides (data not shown).

DISCUSSION

There is now extensive evidence indicating that genetic elements which can suppress the expression of the malignant phenotype in transformed cells exist in the eucaryotic genome (18, 20, 21, 25, 28). Transformed cells resulting from the action of specific viral or cellular oncogenes represent useful model systems for studying the process of tumor suppression, since in many instances the transforming gene products of these agents have been identified and characterized and their role in mediating the expression of the transformed phenotype can be directly evaluated (1, 4, 26, 36). Suppressed somatic cell hybrids can be formed between normal and tumor cells transformed by various oncogenes, and evidence has been presented indicating that suppression of the malignant phenotype can occur at several levels of gene regulation (22, 31–33, 35). Somatic cell hybrids created between cells transformed with either simian virus 40, c-Ha-*ras*, or v-*ras* and normal cells were suppressed in their tumorigenicity, although no changes in the levels of expression of the transforming gene products were demonstrated (15–17, 37). In contrast, Griegel et al. (17) and Willecke et al. (37) have observed a reduction in expression of the Ha-*ras* p21 transforming protein in four of five hybrids formed between diploid embryonic rat fibroblasts and Ha-*ras*-1-transformed rat-1 cells which displayed a suppression of expression of the transformed state. Similarly, Dyson et al. (8) have demonstrated a correlation between suppression of the transformed state and decreased transcription of the v-*src* oncogene in somatic cell hybrids. In the present study, we have shown that an established normal rat embryo cell line, CREF, can suppress the highly progressed tumorigenic phenotype of an Ad5-transformed rat embryo cell line and that this effect is not a consequence of a reduced rate of transcription or steady-state levels of RNA encoded by the E1A and E1B transforming genes of Ad5 or altered expres-

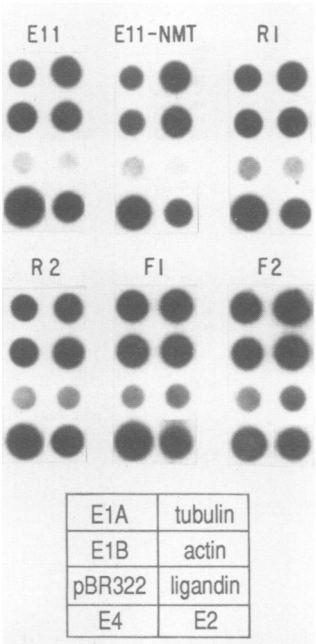

FIG. 3. Analysis of transcription in Ad5-transformed E11, E11-NMT^{neo}, and hybrid cells. [^{32}P]UTP-labeled RNA was extracted from isolated nuclei after chain elongation of nascent transcripts and used as a hybridization probe of various cloned DNAs immobilized as dots on nitrocellulose filters. Each dot contained 7 μg of DNA which was hybridized to 10^7 cpm of labeled RNA from each cell line, hybrids were digested with RNase, and filters were subjected to autoradiography. The types and locations of cloned DNAs immobilized in each dot are shown in the box under the autoradiograph. pBR322 DNA was included as a control to determine the amount of nonspecific hybridization in this experiment.

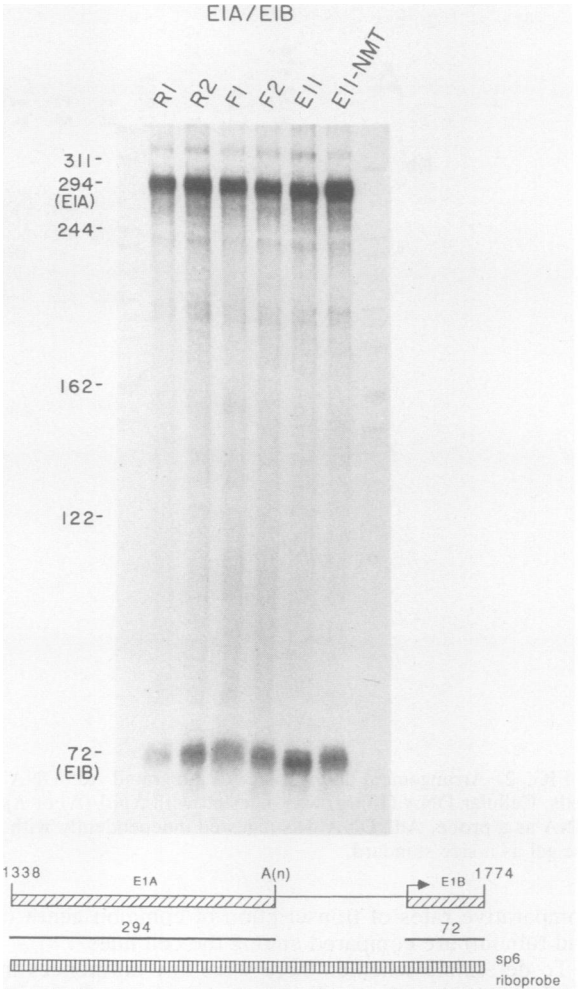

FIG. 4. Analysis of steady-state levels of Ad5 E1A and E1B RNA. Total cytoplasmic RNA (12.5 μg) extracted from each cell line was hybridized for 24 h to antisense ^{32}P-labeled RNA probes containing sequences from the E1A and E1B transcription units of Ad5. RNase T_2-resistant hybrids were size fractionated through denaturing 5% polyacrylamide gels, and the gels were autoradiographed. RNase-resistant fragments of predicted sizes of 294 nt (E1A specific) and 72 nt (E1B specific) were localized by using a labeled size standard run in lanes flanking analyzed RNAs.

sion of E1A- and E1B-encoded transforming polypeptides. These results suggest that suppression of the Ad5-transformed progressed phenotype occurs at a control level distal to oncogene expression and that cellular genes, which can modulate the ability of viral oncogenes to regulate the expression of the transformed state, exist in normal cells.

Our studies demonstrate that the CREF^{cap} genome is able to revert a highly progressed Ad5-transformed cell line, E11-NMT^{neo}, to a nontumorigenic phenotype. However, the suppressed hybrids do not revert to a completely normal state, as indicated by their retention of a low level of anchorage independence (this is most probably a result of unaltered viral E1A and E1B gene expression). In this respect, the flat hybrids formed between the fusion of E11-NMT^{neo} and CREF^{cap} cells have retained specific phenotypes associated with the unprogressed E11 transformed rat embryo cell. The inability to completely reverse the transformed phenotype of E11-NMT^{neo} cells by the CREF^{cap} genome suggests that regulation of the progressed-transformed versus the unprogressed-transformed phenotype may be under different genetic control.

Both E11 and E11-NMT^{neo} cells, as well as the round hybrids, bound low levels of ^{125}I-EGF in comparison with CREF cells, whereas the flat hybrids displayed increased ^{125}I-EGF binding (Table 1). A similar situation, i.e., increased ^{125}I-EGF binding in suppressed hybrids, was observed in Ha-*ras*-1-transformed rat-1/diploid rat embryo fibroblast hybrids (37). In this system, no correlation was found between the level of transforming growth factors secreted by hybrid cells and their tumorigenic potential. For E11-NMT cells, serum-free conditioned medium contains transforming growth factors which compete with ^{125}I-EGF for binding to the EGF receptors in CREF cells and which reversibly induce CREF cells to grow when suspended in 0.4% agar (11). Additional experiments are required to determine whether a reduction in transforming growth factor production and secretion by flat hybrids mediates their loss of the progression phenotype. However, on the basis of the presently described studies and similar studies by other investigators (8, 15–17, 37), it is clear that the effect of a specific suppressor gene from a normal cell on a tumor cell may vary from one hybrid system to another. Whether this difference is a consequence of biochemical differences in the suppressor gene products themselves or whether inherent differences exist in the cell systems analyzed remains to be determined.

Somatic cell hybridization of E11-NMT^{neo} and CREF^{cap} cells resulted predominantly in hybrid cells which had a flat morphology and which were intermediate in size between

and morphologically distinguishable from the parental E11-NMT^{neo} and CREF^{cap} cells (Fig. 1). However, colonies which had a round morphology similar to E11-NMT^{neo} cells and which survived genetic selection (with chloramphenicol and G418) were also observed (Fig. 1). These round hybrids, which contained the anticipated number of chromosomes for hybrid cells, displayed phenotypes similar to E11-NMT^{neo} cells. The lack of suppression of both the transformed and tumorigenic properties of E11-NMT^{neo} cells in these hybrids could be due to the complete or partial loss of a chromosome(s) containing a suppressor gene(s) or to the inactivation of a potential suppressor gene.

In the case of flat hybrids formed between CREF^{cap} and E11-NMT^{neo} cells, it is possible that expression of a functional suppressor gene product (from CREF) results in the loss of the progression phenotype by overriding the ability of the E1A gene to functionally inactivate both the endogenous and the additional CREF-suppressor gene product(s). In contrast, the lack of suppression of the progression phenotype in the round hybrids could reflect a loss or inactivation of the suppressor gene product (from the parental CREF^{cap} and/or E11-NMT^{neo} cell), resulting in an insufficient level of this negative-progression regulator to suppress the E11-NMT phenotype. The Ad5-transformed cell culture system, which displays a suppression of the progressed transformed phenotype, either by treatment with 5-azacytidine (1, 7) or by formation of somatic cell hybrids with normal cells, represents a valuable model with which to investigate the molecular basis of tumor cell progression and should provide a means of identifying and molecularly cloning the genes which mediate suppression of the tumorigenic phenotype.

ACKNOWLEDGMENTS

Research support for these studies was provided by Public Health Service grants CA35675 (P.B.F.), CA43208 (P.B.F.), CA48707 (L.E.B.), CA40065 (J.W.S.), and CA50195 (J.W.S.) from the National Cancer Institute and American Cancer Society grants ACS CD-374A (J.W.S.) and JFRA-235 (L.E.B.). P.B.F. is a Chernow Research Scholar. L.E.B. is a Sinsheimer Scholar.

We appreciate the assistance of Armand F. Miranda in performing the immunofluorescence studies.

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