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Induction of Neuronal and Tumor-Related Genes by Adenovirus Type 12 E1A▽

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Adenovirus type 12 (Ad12) E1A protein (E1A-12) contains a unique 20-amino-acid spacer region between the second and third conserved regions. Substitution of a single amino acid in the spacer is able to abrogate Ad12 tumorigenesis. To investigate the function of the spacer, microarray analysis was performed on cells transformed by tumorigenic and nontumorigenic Ad12s that differ only by one amino acid in the spacer. Fewer than 0.8% of approximately 8,000 genes in the microarray exhibited differential expression of threefold and higher. Of these, more than half of the known genes with higher expression in the wild-type Ad12-transformed cells have neuronal-specific functions. Some of the other differentially expressed genes are involved in the regulation of the cell cycle, transcription, cell structure, and tumor invasiveness. Northern blot analyses of a subset of the neuronal genes, including Robo1, N-MYC, and α-internexin, confirmed their strong expression in multiple Ad12 tumorigenic cell lines. In contrast, these neuronal genes displayed only minor or negligible expression in cells transformed by spacer-mutated Ad12. Significantly, stable introduction of E1A-12 into nontumorigenic Ad5-transformed cells induced neuronal gene expression. We found that the neuron-restrictive silencer factor, which serves as a master repressor of neuronal genes, was inactivated in both Ad12- and Ad5-transformed cells via cytoplasmic retention, though only Ad12-transformed cells exhibited neuronal gene induction. Mutational analyses of the α-internexin promoter demonstrated that E1A-12-mediated neuronal gene induction further required the activation of neuronal promoter E-box elements. These results indicate that the spacer is involved in mediating neuronal and tumor-related genes.

Human adenoviruses have been very useful in the study of tumorigenesis, a cancer-causing process that involves multiple biological events, including oncogenic transformation of cells, immune evasion, angiogenesis, and metastasis. Currently, 51 serotypes of adenoviruses have been identified, all of which are able to transform rodent cells in culture (7, 54, 55, 72). It is well documented that two viral immediate-early gene products, E1A and E1B, are responsible for cell transformation (7, 54, 55, 72). E1A stimulates the cell cycle by binding to the tumor repressor Rb, which leads to the release and activation of the transcription factor E2F. In coordination, E1B mediates the inhibition of cell growth arrest and apoptosis through its interaction with p53. In contrast to the transforming capacity of all adenoviruses, only a small group of serotypes, such as adenovirus type 12 (Ad12), are tumorigenic in immunocompetent adult rodents (54, 55, 72).

Ad12 tumorigenicity is determined solely by the viral E1A protein (E1A-12) (55, 72). E1A-12 is capable of mediating shutoff of major histocompatibility complex (MHC) class I at the transcriptional level (1, 16, 22, 36, 52, 59, 63–65, 76, 77). Moreover, almost every component of the antigen processing machinery, such as Tap-1 and -2, LMP-2 and -7, tapasin, MECL-1, and PA28, exhibits downregulation of expression in Ad12-transformed cells (53, 57, 58, 66). This leads to diminished expression of MHC class I antigens on the cell surface, which in turn contributes to viral tumorigenesis by providing Ad12-transformed cells with a means to escape cytotoxic T-lymphocyte (CTL)-mediated immunosurveillance (4, 54, 55, 72, 73). Conversely, E1A proteins of nontumorigenic adenoviruses, including Ad5, are unable to inhibit MHC class I expression (54, 55, 72). As a consequence, cells transformed by Ad5 can be recognized and destroyed by host CTLs (4, 16, 59, 73). It is noteworthy that the C-terminal region of E1A-5, which is involved in interacting with the transcriptional corepresssor C-terminal binding protein, may also play a role in suppressing Ad5 tumorigenesis (12).

While MHC class I shutoff is required for viral tumorigenesis, diminished MHC class I expression on the cell surface by itself is insufficient for Ad12 to cause tumors. Compared with E1A-5 and the counterparts of other nontumorigenic adenoviruses, tumorigenic E1A-12 contains a unique "spacer" region that is composed of 20 amino acids between conserved regions CR2 and CR3 (Fig. 1A). This spacer region is essential for Ad12 tumorigenesis (54, 55, 72). Deletion of the spacer or alteration of even a single amino acid (alanine to proline) in the spacer (E1A-12sm [Fig. 1A]) can abolish Ad12 tumorigenesis (55, 72). However, Ad12 spacer mutants retain the ability to repress MHC class I expression as well as to transform cells in culture (54, 55, 72). This indicates that the spacer encodes a tumorigenic function that is completely distinct from MHC class I shutoff. While the actual function of the spacer in Ad12 tumorigenesis is not known, it has been suggested that this region of E1A-12 plays a role in evasion of natural killer cell-mediated cytolysis (55, 72).

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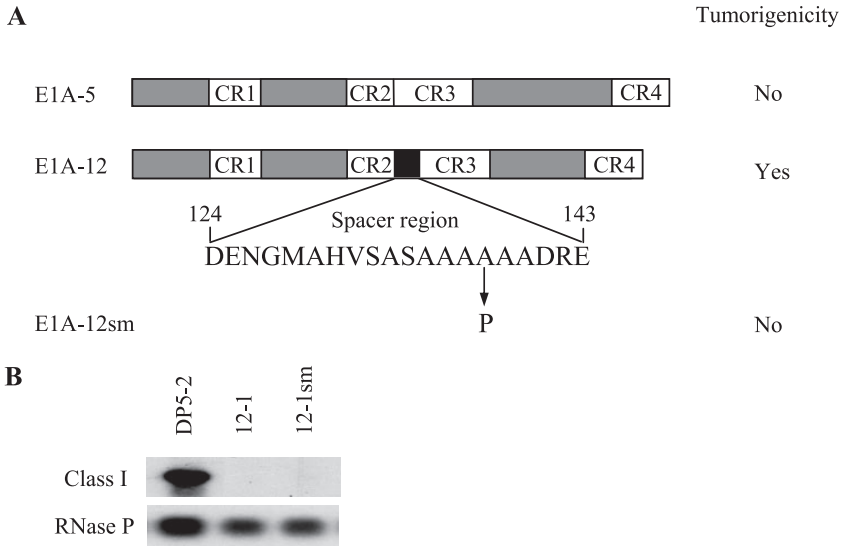

FIG. 1. The E1A-12 tumorigenic spacer does not mediate MHC class I downregulation. (A) Schematic representations of E1A-5, E1A-12, and E1A-12sm. The E1A proteins contain four conserved regions, CR1 to CR4. A salient feature of E1A-12 is the unique 20-amino-acid spacer region between CR2 and CR3 that is essential for tumorigenesis. E1A-12sm is the same as E1A-12 except for a single amino acid substitution (alanine to proline) at position 138 in the spacer region. (B) Northern blot analysis of MHC class I. Total RNAs isolated from cell lines DP5-2, 12-1, and 12-1sm, which were transformed by Ad5, Ad12, and spacer point mutant Ad12, respectively, were analyzed by Northern blotting using ^{32}P-labeled MHC class I DNA as the probe. As a quantitative control, the same blot was rehybridized with a ^{32}P-labeled RNase P probe.

cDNA microarray analyses have been successfully employed to identify genes that are differentially expressed in tumorigenic Ad12- and/or nontumorigenic Ad5-infected or -transformed cells (15, 27, 67). Using a cDNA microarray, we have recently demonstrated that several cancer-related genes, including those for growth factor receptor binding protein 10 and protease nexin 1, which are involved in mediating cell growth and extracellular matrix proteolysis, respectively, are expressed at significantly higher levels in tumorigenic Ad12-transformed cells than in nontumorigenic Ad5-transformed cells (27).

In the present study, we extended our microarray analysis to cells transformed by tumorigenic and nontumorigenic Ad12s that differ by only a single amino acid in the E1A-12 spacer region. We found that the spacer is involved in modulating the expression of numerous genes that are implicated in the cell cycle, transcriptional regulation, immune response, and tumor invasiveness. Interestingly, the E1A-12 spacer, together with other regions in E1A-12, also plays an important role in upregulating a number of neuronal genes. Significantly, the induction of neuronal gene expression by E1A-12 involves the cytoplasmic retention of the neuronal and tumor repressor neuron-restrictive silencer factor (NRSF), as well as the activation of neuronal promoter E-box elements by the basic helix-loop-helix (bHLH) factors. This neuronal phenotype induced by E1A-12 likely contributes to Ad12 tumorigenesis.

MATERIALS AND METHODS

Cell lines. Hooded Lister rat cell lines transformed by Ad5 (DP5-2), Ad12 (12-1), an Ad12 spacer point mutant (12-1sm, originally named RK715B), and an Ad12 spacer deletion mutant (12-1sd, originally named RK710A) were described previously (72). The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen)-10% fetal bovine serum–2 mM L-glutamine-100 units/ml penicillin–100 μg/ml streptomycin. Mouse cell lines transformed by Ad5 (KAd5, Wt5a, and BrAd5) or by Ad12 (F10-12, Bem12-3, and 12A1) as previously described (16, 27) were cultured in Eagle's minimal essential medium (Biowhittaker) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate.

RNA preparation and Northern blot analysis. Total RNA was prepared from cells using RNeasy kit (Qiagen) according to the manufacturer's instructions. Twenty micrograms of total RNA from each sample was used for Northern blot analysis as previously described (27).

Microarray analysis. Global mRNA expression patterns in two genetically matched Ad12-transformed cells (12-1 and 12-1sm) were analyzed using an Affymetrix rat genome U34A gene chip. This chip contains approximately 7,000 known rat genes, including about 1,200 genes with neuronal functions, as well as about 1,000 expressed sequence tags (ESTs). Microarray analysis was conducted according to the manufacturer's instructions in the Microarray Facility of the University of Pennsylvania. Briefly, total RNA was used to generate double-stranded cDNA using a primer consisting of poly(dT) sequences and the T7 promoter. The cDNA was then transcribed in vitro to produce biotinylated cRNA, which was then fragmented to oligonucleotides with a length of about 200 bases. After being heated at 99°C for 5 min, the biotinylated oligonucleotides were hybridized to the U34A microarray for 16 h at 45°C. The microarray was then washed and incubated with phycoerythrin-labeled streptavidin. After excitation at 570 nm, fluorescence signals were collected using a confocal scanner and expression levels for targeted genes were quantitated using Affymetrix Microarray Suite 5.0 software.

Statistical analysis. To analyze the statistical significance of neuronal gene expression as identified by microarray, a two-sided exact binomial test was performed using SAS version 8.02 (SAS Institute Inc., Cary, NC). A P value of <0.05 was considered statistically significant.

Cell transfection and establishment of stable cell lines. Cell transfection was performed using Lipofectamine 2000 transfection regent (Invitrogen) based on the manufacturer's protocol. To establish stable Ad5 cell lines expressing E1A-12, DP5-2 cells were transfected with plasmid pCMV-Ad12E1A, which contains E1A-12 13S cDNAs in the pRc/CMV vector (Invitrogen). Single-cell colonies resistant to Geneticin (Invitrogen) were selected. Expression of E1A-12 was confirmed by Western blot analysis. As a control, stable DP5-2 cell lines transfected with empty plasmid pRc/CMV were also generated.

Western blot analysis. Western blot analysis was carried out as described previously (26, 39). Rabbit anti-E1A-5 antibody (13 S-5) was purchased from Santa Cruz Biotechnology. Rabbit anti-E1A-12 was previously described (60). Rabbit anti-NRSF antibody (ab21635) was purchased from Abcam. Monoclonal β-actin (AC-15) antibody was acquired from Sigma.

Indirect immunofluorescence and confocal microscopy. Cells grown on glass coverslips were washed with phosphate-buffered saline (PBS), fixed with para-

formaldehyde (4% in PBS), and then permeabilized with 0.2% Triton X-100 in PBS. The coverslips were blocked with 3% bovine serum albumin in PBS for 45 min. The cells were stained with mouse anti-NRSF (ab52850) and Alexa Fluor 488-conjugated anti-mouse antibody (Invitrogen). The cells were then mounted in ProLong Gold antifade reagent with DAPI (4′,6′-diamidino-2-phenylindole) (Invitrogen) and visualized with a Nikon confocal microscope (Eclipse TE300; 60× objective lens) equipped with Radiance2000 (Bio-Rad) and the software lasersharp2000 (Bio-Rad).

EMSA. Nuclear and cytoplasmic cell extracts were prepared as described previously (30, 39). Double-stranded oligonucleotide repressor element 1 (RE1) (5′-cagATTGGGTTTCAGAACCACGGACAGCACC; bases in lowercase are overhang ends that do not anneal to the other strand) was labeled with [α-^{32}P]dCTP (Amersham Biosciences) using Klenow enzyme (New England Biolabs). This RE1 oligonucleotide contains a strong recognition sequence for NRSF and is derived from the promoter region of the type II sodium channel gene (37). Electrophoretic mobility shift assay (EMSA) was conducted as previously described (36). Briefly, nuclear or cytoplasmic extracts were incubated with ^{32}P-labeled oligonucleotides for 45 min at room temperature. Protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel. The gel was then dried and autoradiographed.

Luciferase assay. Neuronal promoter-driven firefly luciferase reporters were constructed by cloning either the mouse neuronatin alpha promoter region (nucleotides −448 to +123) or the rat α-internexin promoter region (nucleotides −1100 to +73) into the pGL3 vector (Promega). Subconfluent cells grown on 24-well plates were transfected with 100 ng of these luciferase reporter plasmids together with 10 ng of *Renilla* luciferase reporter pRL-TK (Promega), which was used as an internal control of transfection efficiency. At 24 h posttransfection, the cells were lysed and luciferase activity was measured using Promega's dual-luciferase assay kit. Each transfection was performed in duplicate, and each experiment was repeated three times.

RESULTS

Identification of differentially expressed genes in Ad12 tumorigenic and nontumorigenic cells by microarray analysis. The tumorigenicity of Ad12 correlates with the ability of E1A-12 to repress MHC class I expression (54, 55, 72). However, Ad12 tumorigenesis requires not only MHC class I shutoff but also an unknown function encoded by the unique spacer located between conserved regions CR2 and CR3 of E1A-12 (Fig. 1A). Significantly, alteration of a single amino acid (alanine to proline) in the spacer (E1A-12sm [Fig. 1A]) abolishes Ad12 tumorigenesis but has no effect on MHC class I repression by the E1A (54, 55, 72). Indeed, the Northern blot analysis shown in Fig. 1B confirmed that transcription of MHC class I remains repressed to the same extent in both 12-1sm cells containing the point mutant spacer and the parental 12-1 cells containing the wild-type spacer. This is in sharp contrast to strong expression of the class I transcripts in the nontumorigenic Ad5-transformed rat cells expressing E1A-5 (DP5-2 [Fig. 1B]). Thus, a tumorigenic function distinct from MHC class I shutoff is encoded by the E1A-12 spacer.

To investigate the function of the E1A-12 spacer in Ad12 tumorigenesis, we performed microarray analysis to compare gene expression profiles in 12-1 and 12-1sm cells. Since tumorigenic 12-1 and nontumorigenic 12-1sm cells are haplotypically matched, any difference in cellular gene expression can be attributed solely to the single point mutation in the spacer. As outlined in Fig. 2, an Affymetrix rat genome U34A gene chip, which contains sequences of approximately 7,000 known genes and 1.000 ESTs, was used for the microarray analysis. Based on a differential expression ratio of threefold and higher, only 33 known genes and 27 ESTs were identified as highly differentially expressed in 12-1 and 12-1sm cells (Fig. 2). This represents fewer than 0.8% of the genes on the microarray. Twenty

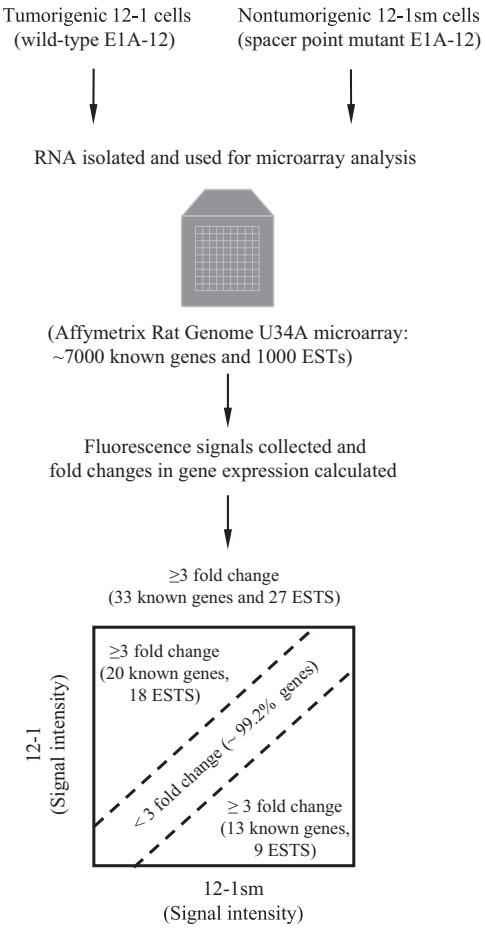

FIG. 2. Microarray analysis of differential gene expression between tumorigenic and nontumorigenic Ad12-transformed cells. Total RNAs isolated from tumorigenic 12-1 cells and nontumorigenic 12-1sm cells were used for microarray analysis on an Affymetrix rat genome U34A microarray. The number of genes displaying a threefold or greater change in expression between the two cell lines is indicated.

of the 33 known genes were expressed at higher levels in the tumorigenic 12-1 cells, with the remaining 13 known genes showing greater expression in the nontumorigenic 12-1sm cells. Of the 27 ESTs, 18 were expressed at higher levels in the 12-1 cells and 9 were expressed at higher levels in the 12-1sm cells.

Table 1 lists all of the highly differentially expressed genes and ESTs identified by the microarray analysis. These genes were further categorized into different groups according to their basic functions. Most surprisingly, more than half (11 out of 20, $P < 0.001$) of the known genes that exhibited higher expression in 12-1 cells perform neuronal functions (Table 1). For example, transcripts encoding fatty acid binding protein were dramatically elevated by more than 55-fold in 12-1 cells compared with 12-1sm cells. 12-1 cells also had greater expression levels of genes involved in neuronal development: C1-13 (10.6-fold), rS-Rex-b (9.2-fold), and neuronatin alpha (4.0-fold) (3, 33, 38, 42, 71). Other genes elevated in expression in 12-1 cells include those for the transmembrane receptor Robo1 (roundabout homolog 1, 4.9-fold) and the homeodomain transcription factor Nkx6.1 (3.5-fold), which play important roles in axon guidance and pathfinding (8, 10, 35, 50, 74);

TABLE 1. Genes differentially expressed in tumorigenic and nontumorigenic Ad12-transformed cells as determined by microarray analysis

Category[a]	Ratio[b]	GenBank accession no.	Gene product name
Neuronal	55.7	U02096	Fatty acid binding protein (brain type)
	24.3	M63656	Aldolase C (brain type)
	24.3	X73411	snRNP-associated polypeptide N (brain type)
	13.0	M29293	Neural snRNP
	10.6	X52817	Neural C1-13
	9.2	U17604	rS-Rex-b (brain type)
	4.9	AF041082	Transmembrane receptor Robo1
	4.0	U08290	Neuronatin alpha
	3.5	U09357	Neural receptor-type protein tyrosine phosphatase zeta/beta
	3.5	AF004431	Homeodomain protein Nkx6.1
	3.2	X06984	Aldolase C (brain type)
	3.2	X55812	SKR6, a CB1 cannabinoid receptor
	−3.2	AF023621	Sortilin
Cell cycle	10.6	D14014	Cyclin D1
	4.6	X75207	Cyclin D1
Transcription	6.1	Y00396	c-MYC oncogene and flanking regions
	3.0	U56242	Transcription factor Maf2
	−3.2	L03556	Hox1.3 protein (hox1.3)
	−3.2	X60769	Silencer factor B
	−6.5	L23148	Inhibitor of DNA binding, splice variant Id1.25
Tumor invasiveness	4.0	D10926	Tissue factor pathway inhibitor
	3.2	D63886	Membrane-type matrix metalloproteinase
	3.2	M23697	Tissue-type plasminogen activator
Cell structure	12.1	AJ224879	Collagen alpha 1 type II
	−3.0	AF097887	Chp mRNA
	−3.7	X81448	Keratin 18
	−6.5	S76054	Cytokeratin-8
Immune response	3.7	U77777	Gamma interferon-inducing factor isoform alpha precursor
	−7.0	U17035	Mob-1
Apoptosis	−3.0	X96437	PRG1
Miscellaneous	10.8	M81681	Biliverdin reductase
	−4.0	X06483	Ribosomal protein L32
	−4.9	L16764	HSP70
	−10.6	Z27118	HSP70
	−12.1	X62951	mRNA (pBUS19) with repetitive elements
	−21.1	AJ001044	EGP-314 protein homologue
ESTs/unknown	9.2	AI230247	
	9.2	AI179399	
	9.2	AI105137	
	8.0	AA817854	
	6.1	AA893743	
	5.7	AA945737	
	4.3	RX013713	
	4.3	AA900476	
	4.0	AA899106	
	3.7	AI232379	
	3.5	AA893082	
	3.5	AA800503	
	3.5	AA800708	
	3.2	AI231292	
	3.2	AA800908	
	3.2	AAA799328	
	3.0	AA894305	
	3.0	AA891204	
	−3.0	AI070295	
	−3.2	AI177161	
	−3.7	AA894130	
	−5.3	RX049893	
	−6.1	H31287	
	−13.9	AA818604	
	−19.7	AA892468	
	−21.1	AI072634	
	−59.7	AA848563	

[a] Genes are categorized based on their known functions.
[b] Differential gene expression ratios between tumorigenic 12-1 and nontumorigenic 12-1sm transformed cells. A positive ratio represents higher expression in 12-1 cells, whereas a negative value denotes greater expression in 12-1sm cells.

the type 1 cannabinoid receptor SKR6 (3.2-fold), which is involved in neuronal signal transmission (45); and the neuronal aldolase C (24.3- and 3.2-fold for two separate sequences), which is involved in glycolysis (20) and in the regulation of neurofilament light subunit (NF-L) expression (9). In contrast, only a single neuronal-like gene, that for sortilin, which functions as a neurotensin receptor in neuronal cells (46) or as a sorting protein in the glucose transporter Glut4 vesicles in fat and muscle cells (41), was identified with 3.2-fold greater expression in 12-1sm cells. These results indicate that neuronal genes are predominantly expressed at higher levels in the tumorigenic 12-1 cells. These data also suggest that the E1A-12 spacer plays a critical role in modulating neuronal gene expression.

In addition to the neuronal genes, other genes that are involved in the regulation of the cell cycle, transcription, tumor invasiveness, cell structure, immune response, and apoptosis were differentially expressed in the tumorigenic 12-1 and nontumorigenic 12-1sm cells (Table 1). For example, cyclin D1, whose disregulation is known to be involved in the development of several types of cancers, including neuroendocrine tumors (13, 19), was overexpressed in 12-1 cells (10.6- and 4.6-fold for two separate sequences of the gene). Significantly, two oncogenes, those for c-MYC (6.1-fold) and Maf2 (3.0-fold), were also upregulated in 12-1 cells. In contrast, three other transcription factors, including the negative regulator silencer factor B (3.2-fold), were expressed more in 12-1sm cells. In the category of tumor invasiveness, genes involved in regulating extracellular matrix proteolysis were expressed at greater levels in the tumorigenic 12-1 cells; they include genes for tissue factor pathway inhibitor (4-fold), membrane-type matrix metalloproteinase (3.2-fold), and tissue-type plasminogen activator (3.2-fold). These findings strongly indicate that the E1A-12 spacer is involved in regulating tumor-related genes.

E1A-12 induces neuronal and tumor-related gene expression. To confirm that neuronal and tumor-related genes are more highly expressed in 12-1 cells than in 12-1sm cells, we performed Northern blot analyses. In addition, two other haplotypically matched transformed cell lines were used in the Northern blot analyses: DP5-2 cells, which contain wild-type E1A-5 that naturally lacks the spacer, and 12-1sd cells, which contain a complete deletion of the 20-amino-acid spacer in E1A-12. The inclusion of DP5-2 and 12-1sd cells was instrumental in elucidating the roles of the spacer in cellular gene regulation. As revealed in the Northern blot (Fig. 3), the representative neuronal genes for C1-13, S-Rex, Robo1, and neuronatin alpha all displayed much higher expression in the tumorigenic 12-1 cells (lane 2) than in the nontumorigenic 12-1sm cells (lane 3). Of these four genes, C1-13 and S-Rex were not expressed at detectable levels in the nontumorigenic 12-1sm cells, whereas Robo1 and neuronatin were marginally expressed (Fig. 3). The 12-1sm cells also exhibited a dramatic reduction in expression of the tumor-related cell cycle regulator cyclin D1 and the extracellular matrix proteolysis regulator membrane-type matrix metalloproteinase compared with 12-1 cells (Fig. 3, lanes 2 and 3). These findings are in accordance with the microarray data described above. Significantly, the spacer deletion mutant 12-1sd cells displayed gene expression patterns similar to those found in 12-1sm cells (Fig. 3, lanes 3 and 4). In contrast, none of these neuronal and tumor-related genes were expressed at detectable levels in DP5-2 cells (Fig. 3, lane 1). It is noted that other tumor-related genes, such as those for c-MYC, tissue factor pathway inhibitor, and tissue-type plasminogen activator, were expressed at comparable or slightly higher levels in DP5-2 cells compared with 12-1 cells, though these genes exhibited lower expression in 12-1sm cells as confirmed by Northern blotting (data not shown). These results indicate a strong correlation between the presence of the E1A-12 spacer and the elevated expression of neuronal and tumor-related genes.

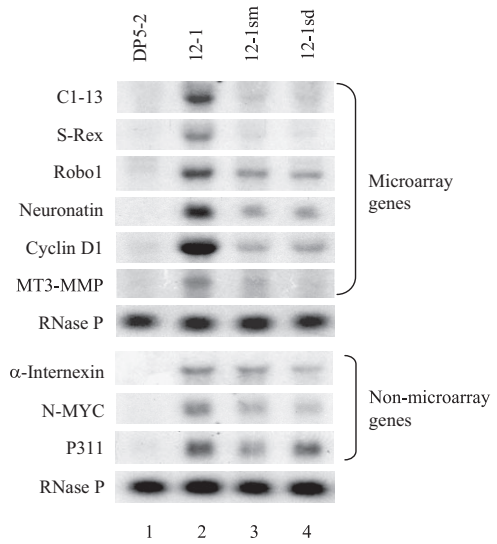

FIG. 3. Neuronal and tumor-related genes are expressed in Ad12 tumorigenic cells. Neuronal and tumor-related genes identified by microarray analyses (upper portion) or not reported in the microarray (bottom portion) were analyzed by Northern blotting. Total RNAs isolated from different E1A-expressing cell lines were hybridized with a ^{32}P-labeled DNA probe derived from the indicated gene. As a quantitative control, RNA levels of RNase P were compared in each cell line. DP5-2 is a cell line transformed by Ad5, and 12-1, 12-1sm, and 12-1sd are cell lines transformed by wild-type, spacer point mutant, and spacer deletion mutant Ad12, respectively. MT3-MMP, membrane-type matrix metalloproteinase.

To help confirm that neuronal and tumor-related gene expression is a feature of 12-1 cells, we performed Northern blot analyses of other neuronal genes that were not reported in the microarray. These genes include those for the neuronal intermediate filament α-internexin, the neuronal oncoprotein N-MYC, and P311 (also called neuronal protein 3.1). As shown in Fig. 3, these genes displayed expression patterns very similar to those of the Robo1, neuronatin, and cyclin D1 genes (as described above) in DP5-2 and 12-1 cells, with undetectable expression in DP5-2 cells (lane 1) and greater expression in 12-1 cells (lane 2). Notably, the expression of α-internexin and P311 was not dramatically reduced in 12-1sm and 12-1sd cells, suggesting that regions outside the spacer in E1A-12 also play a role in regulating expression of some neuronal and tumor-related genes. These data clearly indicate an important role of E1A-12 in inducing neuronal and tumor-related genes.

We next examined whether E1A-12, when introduced into Ad5 cells, was able to induce the expression of neuronal and tumor-related genes. To this end, plasmids expressing the 13S

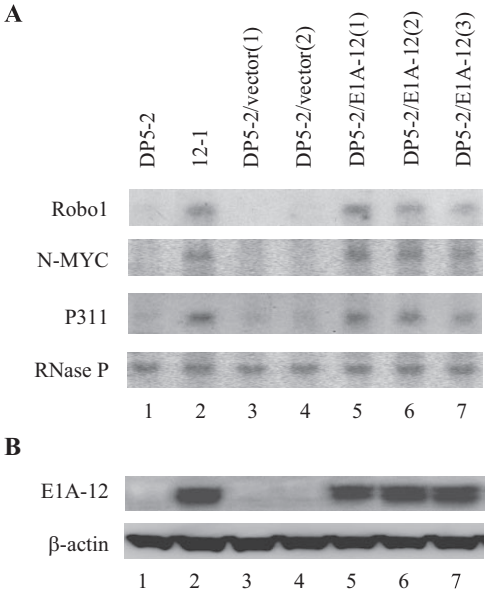

FIG. 4. Stable transfection of E1A-12 into Ad5 nontumorigenic cells induces neuronal and tumor-related gene expression. (A) Northern blot analyses of representative neuronal and tumor-related genes. Ad5-transformed cells (DP5-2) were transfected with 13S E1A-12-expressing plasmids to generate a stable cell line (DP5-2/E1A-12) that coexpresses E1A-5 and E1A-12. Total RNAs prepared from normal DP5-2 cells, Ad12-transformed cells (12-1), vector-transfected cells (DP5-2/vector), and stable DP5-2/E1A-12 cells were used for Northern blot hybridization. All stably transfected cell lines were independently derived. (B) Western blot analyses of E1A-12 expression. Whole-cell extracts from the cells shown in panel A were immunoblotted using an antibody against E1A-12. As a quantitative control, the same blot was reprobed with β-actin antibody. The number assigned to each lane corresponds with that in panel A.

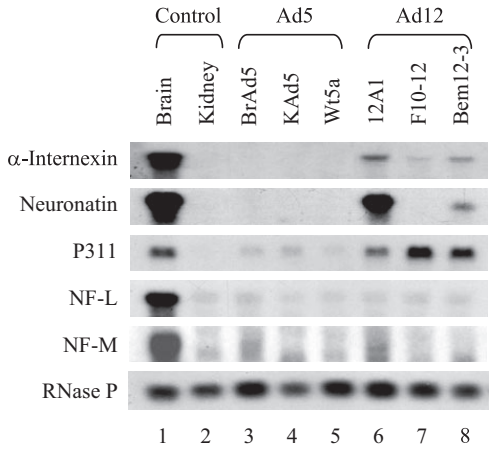

FIG. 5. Northern blot profiles of representative neuronal genes from different Ad5- and Ad12-transformed cells. Total RNAs isolated from Ad5- and Ad12-transformed cells and from normal mouse brain and kidney cells (serving as controls) were hybridized with ^{32}P-labeled DNA probes derived from neuronal genes. As a quantitative control, RNA levels of RNase P are also shown.

E1A-12 were introduced into DP5-2 cells to establish DP5-2/E1A-12 stable cell lines that express both E1A-5 and E1A-12 proteins. As a negative control, an empty vector plasmid was used to generate stable DP5-2/vector cells. Total RNAs isolated from these stable cells were then analyzed by Northern blotting to examine expression of neuronal and tumor-related genes. As shown in Fig. 4A, representative genes, including those for Robo1, N-MYC, and P311, were all expressed in three independent DP5-2/E1A-12 cell lines (lanes 5 to 7) at levels similar to those found in the tumorigenic 12-1 cells (lane 2). In contrast, no expression was observed for these genes in the parental DP5-2 cells (lane 1) and two independent vector-transfected DP5-2/vector cell lines (lanes 3 and 4). Western blot analyses confirmed that E1A-12 was expressed in 12-1 and DP5-2/E1A-12 cells (Fig. 4B). It is noted that the introduction of E1A-12 and empty vector plasmids into DP5-2 cells did not affect E1A-5 expression, as comparable levels of E1A-5 were found in all of these cells (data not shown). These results strongly demonstrate that E1A-12 is involved in upregulating neuronal and tumor-related genes.

Since E1A-12 is the key determinant of viral tumorigenesis (55, 72), it was not surprising that tumor-related genes were upregulated by the viral oncoprotein. Unexpectedly, however, neuronal genes were also induced by E1A-12 in 12-1 cells. To address this phenomenon, we first inquired if induction of neuronal genes by E1A-12 was a common feature among Ad12 tumorigenic cell lines. Northern blot analyses were performed to examine neuronal gene expression in three additional Ad12-transformed mouse cell lines (12A1, F10-12, and Bem12-3). For comparison, three Ad5-transformed mouse cell lines (Brad5, KAd5, and Wt5a), which are haplotypically matched to the Ad12-transformed mouse cells (16, 27), were also analyzed. In addition, RNAs from normal cells of BALB/c brain and kidney were included as controls. As expected, Northern blot analyses demonstrated that neuronal genes, including those for α-internexin, neuronatin, P311, and the NF-L and neurofilament medium (NF-M) subunits, were highly expressed in brain cells (Fig. 5, lane 1) but were not expressed in kidney cells (lane 2). Significantly, α-internexin, neuronatin, and P311 were also expressed to a notable extent in all three Ad12 mouse cell lines (Fig. 5, lanes 6 to 8), with the single exception that expression of neuronatin was not detected in F10-12 cells (lane 7). Interestingly, certain neuronal genes, i.e., those for NF-L and NF-M, were not expressed in the three Ad12-transformed mouse cell lines (Fig. 5, lanes 6 to 8). This is in agreement with the finding that expression of NF-L and NF-M was not detected in 12-1 rat cells (data not shown). Little or no expression was observed for any of the neuronal genes, including those for NF-L, NF-M, α-internexin, neuronatin, and P311, in the three Ad5-transformed mouse cell lines (Fig. 5, lanes 3 to 5). In summary, these results indicate that E1A-12, but not E1A-5, is able to induce a selective group of neuronal genes.

Induction of neuronal gene expression by E1A-12 results from loss of NRSF repression and stimulation of the neuronal E-box elements by bHLH transactivators. Neuronal gene expression in nonneuronal cells is repressed by the master neuronal regulator NRSF, also known as REST (repressor element 1-silencing transcription factor) (14, 43). NRSF recognizes and binds to *cis*-acting DNA sequences called RE1 of neuronal genes. NRSF is rarely or not expressed in neuronal cells but is highly expressed in nonneuronal cells (14, 43). Western blot analysis was performed to examine NRSF expres-

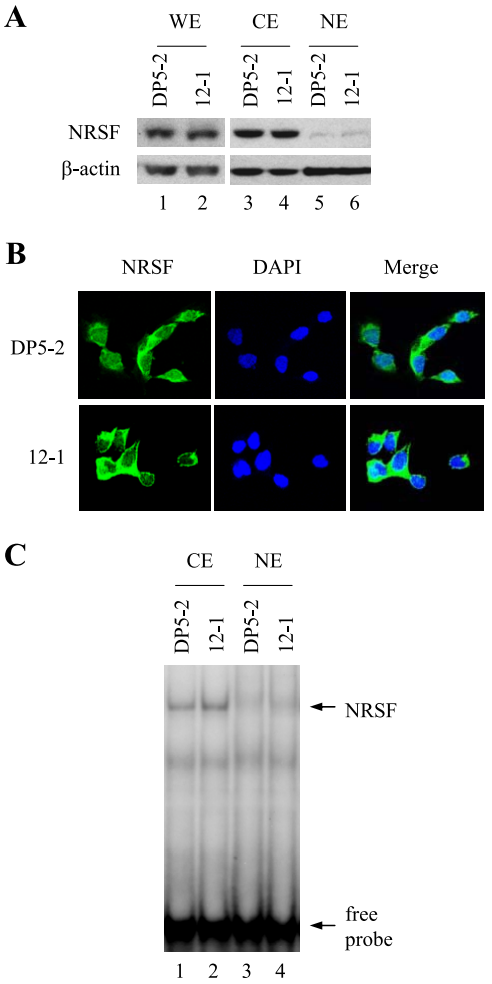

FIG. 6. NRSF is retained in the cytoplasm of Ad5- and Ad12-transformed cells. (A) Western blot analyses of NRSF expression. Protein extracts of whole cells (WE), cytoplasm (CE), and nuclei (NE) from DP5-2 (Ad5) and 12-1 (Ad12) cells were analyzed by Western blotting using an anti-NRSF antibody. As a quantitative control, the same blots were reprobed with an antibody against β-actin. (B) Confocal immunofluorescent microscopy. Indirect immunofluorescence was performed for staining NRSF (green). Nuclei were stained with DAPI (blue). (C) EMSA. Equal amounts (15 μg) of CE and NE from DP5-2 and 12-1 cells were analyzed using a ^{32}P-labeled double-stranded DNA probe that contains an RE1 sequence strongly recognized by NRSF. Positions of free and NRSF-bound RE1 probe are indicated.

sion in Ad5 (DP5-2)- and Ad12 (12-1)-transformed cells. As shown in Fig. 6A, comparable expression of NRSF was observed in DP5-2 and 12-1 whole-cell extracts (lanes 1 and 2). Since the presence of NRSF in 12-1 cells did not appear to prevent the expression of neuronal genes, including that for α-internexin, that contain an RE1 DNA recognition site, this led us to speculate that NRSF function might become impaired in Ad12-transformed cells. We first examined whether the transcription repressor NRSF was able to translocate into the nuclei of 12-1 cells. Surprisingly, NRSF was found exclusively in the cytoplasmic extract and not in the nuclear extract of 12-1 cells (Fig. 6A, lanes 4 and 6). Interestingly, NRSF was also sequestered in the cytoplasm of DP5-2 cells (Fig. 6A, compare lanes 3 and 5). These findings were confirmed by confocal fluorescence microscopy (Fig. 6B). To further demonstrate these data, we next tested DNA binding activity of NRSF in 12-1 and DP5-2 cells using EMSA. As shown in Fig. 6C, no NRSF DNA binding was found in the nuclear protein extracts of the 12-1 or DP5-2 cells (lanes 3 and 4). Instead, NRSF DNA binding activity was observed in the cytoplasmic protein extracts of both the 12-1 and DP5-2 cells (Fig. 6C, lanes 1 and 2). This DNA binding activity was abrogated when the cytoplasmic extracts were first treated with an anti-NRSF antibody (data not shown), confirming that the DNA binding activity was rendered by NRSF. These results indicate that the repression of neuronal gene expression by NRSF is nullified in both Ad12- and Ad5-transformed cells via the retention of NRSF activity in the cytoplasm.

While the cytoplasmic retention of NRSF activity in both Ad12- and Ad5-transformed cells is a precondition for neuronal gene expression, it is perplexing that neuronal gene expression occurs only in the Ad12-transformed cells and not in the Ad5-transformed cells. This suggested that the inactivation of NRSF is not the sole determinant for neuronal gene expression. Indeed, for neuronal gene expression to occur, the loss of NRSF function needs to be accompanied by activation of neuronal promoters by transcription factors (14, 43). To determine if neuronal gene promoters are activated in Ad12-transformed cells, a luciferase reporter controlled by the rat α-internexin (Intx) promoter (Fig. 7A) was transfected into 12-1 cells. For comparison, the same luciferase reporter was also transfected into DP5-2 cells. The presence of the α-internexin promoter greatly activated luciferase expression in 12-1 cells, but not in DP5-2 cells (Fig. 7B, bars 2). As expected, no luciferase activity was generated by a control reporter lacking the α-internexin promoter in 12-1 or DP5-2 cells (Fig. 7B, bars 1). Similar results were obtained when the α-internexin promoter-driven luciferase reporter was analyzed in a second pair of Ad12- and Ad5-transformed mouse cell lines and when a neuronatin promoter-driven luciferase reporter was analyzed in these cell lines (data not shown). These results suggest that, in addition to inhibition of NRSF activity (see above), the induction of neuronal genes in Ad12 tumorigenic cells is attributable to the activation of the neuronal promoters.

How are neuronal gene promoters activated in Ad12 tumorigenic cells? It is known that a class of transcription factors with bHLH motifs, which recognize the core hexamer sequence 5′-CANNTG termed the E box, is involved in stimulating neuronal gene expression (5, 56). Sequence analyses of the promoters of α-internexin and neuronatin revealed that both genes contain E boxes. In the α-internexin promoter, two E boxes reside closely upstream of the transcription initiation site (+1). As shown in Fig. 7A, E-box 1 (5′-CAGATG) is located from nucleotide −342 to −347 and E-box 2 (5′-CAG CTG) is located from nucleotide −130 to −135. Mutations were introduced into these two E boxes to determine if they are important for the activation of the α-internexin promoter in Ad12 tumorigenic cells. The α-internexin promoter, containing one or both mutated E boxes, was appended to the luciferase reporter gene (Fig. 7A) and transfected into 12-1 and DP5-2 cells. Compared with the wild-type α-internexin promoter (Intx, Fig. 7A), conversion of the two conserved bases TG to AT in E-box 1 (Intx-mt1, Fig. 7A) dramatically

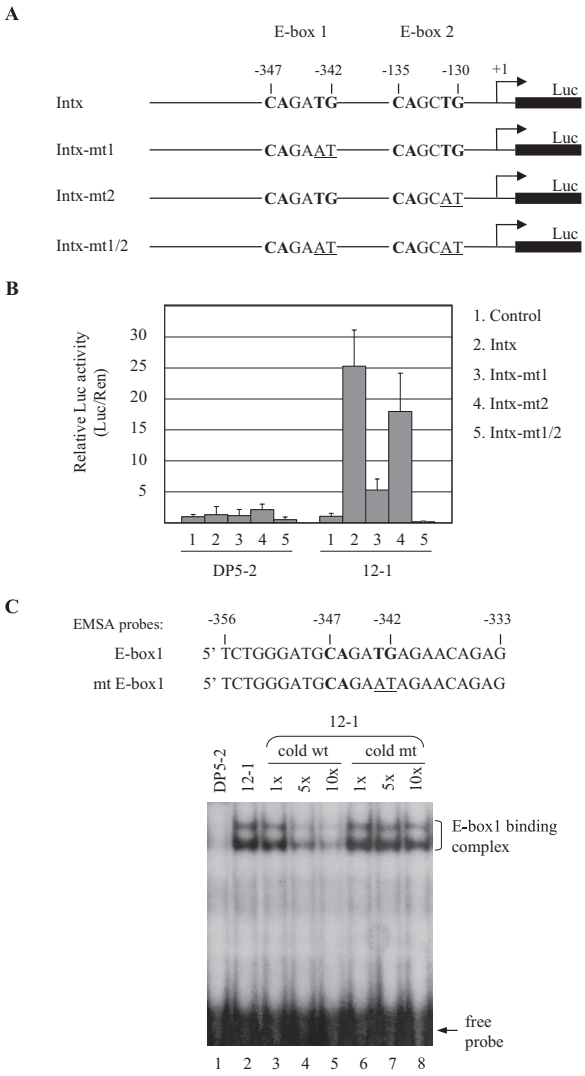

FIG. 7. Promoter activation of α-internexin in Ad12 cells requires E-box elements. (A) Schematic representations of firefly luciferase reporter driven by the rat α-internexin promoter. The α-internexin promoter (Intx, −1100 to +73) is denoted by a thin line and the luciferase gene by a black bar. The sequences and locations of two E-box elements in the promoter relative to the transcription initiation site (+1, indicated by an arrow) are given. Conserved E-box sequences are shown in bold, and mutations in the E-box elements are underlined. Names of each reporter are indicated on the left side. (B) Luciferase activity assay. DP5-2 and 12-1 cells were transfected with plasmids of firefly luciferase reporter shown in panel A together with *Renilla* luciferase reporter plasmids (internal control). At 24 h post-transfection, the cells were lysed and luciferase activities were measured. Data represent averages and standard deviations from three independent experiments. The control is firefly luciferase reporter lacking a promoter. (C) EMSA. EMSA was conducted by incubating nuclear extracts (10 μg) of DP5-2 and 12-1 cells with ^{32}P-labeled E-box1 double-stranded oligonucleotide (only sense sequences shown) derived from the α-internexin promoter. For DNA binding competition assay, the nuclear extract of 12-1 cells was preincubated with 1-, 5-, and 10-fold unradiolabeled wild-type (wt) or mutant (mt) E-box1 double-stranded oligonucleotides, followed by EMSA as described above. The E-box 1 conserved sequences are shown in bold, and mutations in E-box 1 are underlined.

reduced luciferase activity in 12-1 cells (Fig. 7B, bars 2 and 3). However, the same nucleotide alteration in E-box 2 (Intx-mt2, Fig. 7A) resulted in only a moderate decrease in luciferase activity in 12-1 cells (Fig. 7B, bar 4). Significantly, a combination of both E-box mutations (Intx-mt1/2, Fig. 7A) completely abrogated luciferase transactivation in 12-1 cells (Fig. 7B, bar 5). In contrast, none of these mutations affected the already-deficient expression of the reporter in DP5-2 cells (Fig. 7B). These data strongly suggest that recognition of the two E boxes, especially E-box 1, by a certain neuronal bHLH transactivator(s) is critical for α-internexin expression in Ad12 tumorigenic cells.

To examine whether bHLH factors can in fact bind to the α-internexin promoter E-box 1, we performed EMSA by incubating nuclear extracts of DP5-2 and 12-1 cells with ^{32}P-labeled double-stranded DNA probe E-box1 (position −356 to −333, Fig. 7C). No DNA binding activity was observed in the nuclear extract of DP5-2 cells (Fig. 7C, lane 1). In strong contrast, two major DNA binding bands were generated by the nuclear extract of 12-1 cells (lane 2). Moreover, DNA binding competition analyses revealed that unradiolabeled E-box1 oligonucleotide is able to compete for the binding activity in a dose-dependent manner (Fig. 7C, lanes 3 to 5), whereas alteration of the two conserved bases TG to AT in the E-box1 (mt E-box1) disabled the competition (lanes 6 to 8). These data demonstrate that only Ad12 tumorigenic cells, but not Ad5 nontumorigenic cells, express nuclear bHLH factors capable of binding to the promoter E-box 1 for α-internexin transactivation. Taking all the data together, induction of neuronal gene expression by tumorigenic E1A-12 involves the activation of neuronal promoter E-box elements by certain bHLH factors, as well as the relief of NRSF repression.

DISCUSSION

Ad12 tumorigenesis is determined solely by the viral multifunctional oncoprotein E1A-12. As with its nontumorigenic adenovirus counterparts, including E1A-5, E1A-12 is capable of reprogramming cellular gene expression and transforming cells. Uniquely, E1A-12 is able to mediate MHC class I shutoff, which enables Ad12 tumorgenic cells to escape from CTL-mediated cytolysis (55, 72). Importantly, Ad12 tumorigenesis also requires the unknown functions encoded by the 20-amino-acid spacer between the conserved regions CR2 and CR3. In the present study, we demonstrate that the spacer, along with other regions in E1A-12, plays a critical role in upregulating numerous neuronal and tumor-related genes.

Since the E1A-12 spacer is essential for tumorigenesis, this suggests that certain neuronal genes that it upregulates are involved in the tumorigenic process. For example, the induction of Robo1, which is involved in regulating axon path finding and neuronal migration (8, 35, 74), may have a role in directing the migration and invasiveness of Ad12 tumorgenic cells. In fact, Robo1 has been reported to participate in mediating glioma cell migration (48). Moreover, Robo1 has been found to be highly expressed in several nonneuronal tumors, including hepatocellular carcinoma (32) and colorectal cancer (23). Like Robo1, other neuronal genes, such as those for N-MYC and P311, that are upregulated by E1A-12 may also play an important role in Ad12 tumorigenesis. N-MYC, namely, the

neuronal MYC oncoprotein, possesses cell-transforming and oncogenic functions (31, 34). P311, a small protein widely expressed in neuronal cells, has been found to be implicated in the regulation of cell growth and migration (44, 47, 62). Although a direct involvement of these neuronal genes in Ad12 tumorigenesis has yet to be confirmed, it is intriguing to speculate that Ad12 may usurp the functions of these neuronal genes to induce tumorigenesis.

While our data demonstrate that E1A-12, but not E1A-5, is able to induce neuronal gene expression, Shaw et al. (61) previously suggested that human adenoviruses, including Ad12 and Ad5, preferentially transform human neuronal cells. Their claim was based on the observation that neuronal genes such as those for NF-L and NF-M were expressed in Ad12- and Ad5-transformed cells derived from human embryonic retina and kidney tissues (61). However, in agreement with our data, they found that in rodent systems neuronal genes were expressed only in Ad12-transformed cells and not in Ad5-transformed cells (61). This phenomenon can hardly be explained by the preferential transformation of neuronal cells. In fact, our data have demonstrated that stable transfection of an E1A-12 plasmid into Ad5-transformed cells, which otherwise do not express neuronal genes, is able to induce neuronal gene expression. Furthermore, we found that only a subset of neuronal genes were expressed in Ad12-transformed cells. No expression of NF-L or NF-M was observed in any of our Ad12- or Ad5-transformed cell lines, suggesting that neither Ad12- nor Ad5-transformed cells were derived from neuronal cells. These data strongly indicate that the expression of neuronal genes in Ad12-transformed cells is activated by E1A-12 and is not a consequence of preferential transformation of neuronal cells.

In accordance with our finding that E1A-12 functions to activate neuronal gene expression, intraperitoneal and subcutaneous tumors induced by Ad12 in rodents have been shown to contain neuroectodermal characteristics and to express several neuronal genes (29, 49, 51). Interestingly, it has been found that several other nonneuronal tumors, including breast, ovarian, colorectal, and pancreatic cancers, also display aberrant expression of neuronal genes (2, 11, 21, 23, 75). This indicates that the induction of neuronal gene expression is not limited to Ad12 tumorigenic cells, but is relatively common in tumor cells. While it is not clear why these tumor cells express neuronal genes, it is possible that the neuronal gene expression may provide the tumor cells with some survival advantage, e.g., immune privilege as found with neuronal cells. It is noted that like neuronal cells, Ad12 tumorigenic cells and most other tumors do not express MHC class I and are capable of evading host immune surveillance. Intriguingly, in addition to regulating neuronal differentiation, NRSF was recently identified as a tumor suppressor in epithelial cells that controls oncogenic transformation (28, 68, 69). This dual role of NRSF in repression of neuronal gene expression and tumor formation also suggests that neuronal genes likely play an important role in tumorigenesis.

Neuronal gene expression is regulated by both transcription repressors and activators. In nonneuronal cells, neuronal genes are normally repressed by NRSF. Rarely is NRSF expressed in neuronal cells (14, 43). The fact that comparable high expression of NRSF was observed in both Ad12- and Ad5-transformed cells suggests again that these cells were unlikely to be derived from neuronal cells. Yet, the cytoplasmic retention of NRSF in both Ad12- and Ad5-transformed cells should serve as a means by which viral gene products overcome repression of neuronal genes. While it remains to be elucidated how NRSF is retained in the cytoplasm of Ad12- and Ad5-transformed cells, our preliminary data indicate that the NRSF cytoplasmic retention is determined solely by E1A protein (data not shown). It is noteworthy that by interacting with the NRSF corepressor CoREST, the ICP0 protein of herpes simplex virus type 1 is able to mediate nuclear export of NRSF to the cytoplasm (24, 25).

Although the relief of NRSF repression should enable both Ad12- and Ad5-transformed cells to express neuronal genes, surprisingly, neuronal gene expression was found to occur only in Ad12 tumorigenic cells. What could account for this difference? The inhibition of NRSF repression is recognized as being required but not sufficient to promote neuronal gene expression (14, 43). It is likely that the induction of neuronal genes by E1A-12 also requires the activation of neuronal promoters. Indeed, our data demonstrate that the induction of α-internexin by E1A-12 (but not E1A-5) is dependent on the binding of certain nuclear factors to its promoter E-box elements. It is well documented that E-box elements are recognized by bHLH transcription factors. Many neuronal transcription factors, such as neurogenin and NeuroD, are bHLH factors (5, 56), which bind to E-box elements of neuronal gene promoters and, as such, play an essential role in regulating neuronal development (5, 56). It is relevant to note that several bHLH factors, including NeuroD, were found to be overexpressed in both neuronal and nonneuronal tumors (6, 17, 18, 40, 70). Interestingly, the aforementioned N-MYC oncoprotein, which is highly expressed in Ad12 tumorigenic cells, is also a bHLH transcription factor. It has yet to be determined whether N-MYC is involved in inducing neuronal gene expression in Ad12 tumorigenesis.

In summary, this study reveals that the E1A-12 spacer mediates the upregulation of certain neuronal and tumor-related genes. Our data further indicate that E1A-12-mediated induction of neuronal gene expression requires both the inhibition of NRSF and the activation of neuronal promoter E-box elements that are recognized by bHLH factors. These results may provide a greater understanding of the mechanisms by which neuronal genes become expressed in nonneuronal cells and their contribution to human cancers.

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