Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 9125–9130, August 1997
Cell Biology

Subtraction hybridization identifies a transformation progression-associated gene *PEG-3* with sequence homology to a growth arrest and DNA damage-inducible gene

(subtractive cDNA library/*gadd34*/*MyD116*/cancer progression/gene transcription/DNA transfection)

ZAO-ZHONG SU*, YIJIE SHI*, AND PAUL B. FISHER*†‡

Departments of †Pathology and *Urology, Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons of Columbia University, New York, NY 10032

Communicated by George J. Todaro, University of Washington, Seattle, WA, June 18, 1997 (receive for review March 17, 1997)

ABSTRACT Cancer is a progressive multigenic disorder characterized by defined changes in the transformed phenotype that culminates in metastatic disease. Determining the molecular basis of progression should lead to new opportunities for improved diagnostic and therapeutic modalities. Through the use of subtraction hybridization, a gene associated with transformation progression in virus- and oncogene-transformed rat embryo cells, progression elevated gene-3 (PEG-3), has been cloned. *PEG-3* shares significant nucleotide and amino acid sequence homology with the hamster growth arrest and DNA damage-inducible gene *gadd34* and a homologous murine gene, *MyD116*, that is induced during induction of terminal differentiation by interleukin-6 in murine myeloid leukemia cells. *PEG-3* expression is elevated in rodent cells displaying a progressed-transformed phenotype and in rodent cells transformed by various oncogenes, including Ha-*ras*, v-*src*, mutant type 5 adenovirus (Ad5), and human papilloma virus type 18. The *PEG-3* gene is transcriptionally activated in rodent cells, as is *gadd34* and *MyD116*, after treatment with DNA damaging agents, including methyl methanesulfonate and γ-irradiation. In contrast, only *PEG-3* is transcriptionally active in rodent cells displaying a progressed phenotype. Although transfection of *PEG-3* into normal and Ad5-transformed cells only marginally suppresses colony formation, stable overexpression of *PEG-3* in Ad5-transformed rat embryo cells elicits the progression phenotype. These results indicate that *PEG-3* is a new member of the *gadd* and *MyD* gene family with similar yet distinct properties and this gene may directly contribute to the transformation progression phenotype. Moreover, these studies support the hypothesis that constitutive expression of a DNA damage response may mediate cancer progression.

The carcinogenic process involves a series of sequential changes in the phenotype of a cell resulting in the acquisition of new properties or a further elaboration of transformation-associated traits by the evolving tumor cell (1–4). Although extensively studied, the precise genetic mechanisms underlying tumor cell progression during the development of most human cancers remain enigmas. Possible factors contributing to transformation progression include activation of cellular genes that promote the cancer cell phenotype, i.e., oncogenes; activation or modification of genes that regulate genomic stability, i.e., DNA repair genes; loss or inactivation of cellular genes that function as inhibitors of the cancer cell phenotype, i.e., tumor suppressor genes; and/or combinations of these genetic changes in the same tumor cell (1–6). A useful model system for defining the genetic and biochemical changes mediating tumor progression is the type 5 adenovirus (Ad5)/early passage rat embryo (RE) cell culture system (1, 7–14). Transformation of secondary RE cells by Ad5 is often a sequential process resulting in the acquisition and further elaboration of specific phenotypes by the transformed cell (7–10). Progression in the Ad5-transformation model is characterized by the development of enhanced anchorage independence and tumorigenic potential (as indicated by a reduced latency time for tumor formation in nude mice) by progressed cells (1, 10). The progression phenotype in Ad5-transformed RE cells can be induced by selection for growth in agar or tumor formation in nude mice (7–10), referred to as spontaneous progression, by transfection with oncogenes (13), such as Ha-*ras*, v-*src*, v-*raf*, or the E6/E7 region of human papillomavirus type 18 (HPV-18), referred to as oncogene-mediated progression, or by transfection with specific signal transducing genes (14), such as protein kinase C, referred to as growth factor-related, gene-induced progression.

Progression, induced spontaneously or after gene transfer, is a stable cellular trait that remains undiminished in Ad5-transformed RE cells even after extensive passage (>100) in monolayer culture (13). However, a single treatment with the demethylating agent 5-azacytidine (AZA) results in a stable reversion in transformation progression in >95% of cellular clones (10, 13, 14). The progression phenotype is also suppressed in somatic cell hybrids formed between normal or unprogressed-transformed cells and progressed cells (11–13). These findings suggest that progression may result from the activation of specific progression-promoting genes or the selective inhibition of progression-suppressing genes, or possibly a combination of both processes.

The final stage in tumor progression is acquisition by transformed cells of the ability to invade local tissue, survive in the circulation, and recolonize in a new area of the body, i.e., metastasis (15–17). Transfection of an Ha-*ras* oncogene into cloned rat embryo fibroblast (CREF) cells (18) results in morphological transformation, anchorage independence, and acquisition of tumorigenic and metastatic potential (19–21). Ha-*ras*-transformed CREF cells exhibit major changes in the transcription and steady-state levels of genes involved in suppression and induction of oncogenesis (21, 22). Simultaneous overexpression of the Ha-*ras* suppressor gene K*rev*-1 in Ha-*ras*-transformed CREF cells results in morphological reversion, suppression of agar growth capacity, and a delay in *in vivo* oncogenesis (21). Reversion of transformation in Ha-*ras* plus K*rev*-1-transformed CREF cells correlates with a return in the transcriptional and steady-state mRNA profile to that of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1997 by The National Academy of Sciences 0027-8424/97/949125-6$2.00/0
PNAS is available online at http://www.pnas.org.

Abbreviations: *PEG-3*, progression elevated gene-3; *gadd34*, growth arrest and DNA damage-inducible gene 34; *MyD116*, myeloid differentiation-inducible gene 116; Ad5, type 5 adenovirus; RE, rat embryo; HPV-18, human papillomavirus type 18; AZA, 5-azacytidine; CREF, cloned rat embryo fibroblast; mda, melanoma differentiation associated; GAPDH, glyceraldehyde-3-phosphate.

‡To whom reprint requests should be addressed. e-mail: pbf1@columbia.edu.

untransformed CREF cells (21, 22). Following long latency times, Ha-*ras* plus K*rev*-1-transformed CREF cells form both tumors and metastases in athymic nude mice (21). The patterns of gene expression changes observed during progression, progression suppression, and escape from progression suppression supports the concept of "transcriptional switching" as a major component of Ha-*ras*-induced transformation (21, 22).

To identify potential progression-inducing genes with elevated expression in progressed versus unprogressed Ad5-transformed cells we used subtraction hybridization (13, 23). This approach resulted in the cloning of progression elevated gene-3 (*PEG-3*), which is expressed at elevated levels in progressed cells (spontaneous, oncogene-induced, and growth factor-related, gene-induced) rather than in unprogressed cells (parental Ad5-transformed, AZA-suppressed, and suppressed hybrids). Transfection of *PEG-3* into unprogressed, parental Ad5-transformed cells induces the progression phenotype, without significantly altering colony formation in monolayer culture or affecting cell growth. *PEG-3* expression is also elevated following DNA damage and oncogenic transformation of CREF cells by various oncogenes. Sequence analysis indicates that *PEG-3* has 73 and 68% nucleotide and 59 and 72% amino acid similarities, respectively, with the *gadd34* and *MyD116* genes. However, unlike *gadd34* and *MyD116*, which encode proteins of ≈65 and ≈72 kDa, respectively, *PEG-3* encodes a protein of ≈50 kDa with only ≈28 and ≈40% amino acid similarities to *gadd34* and *MyD116*, respectively, in its carboxyl terminus. These results indicate that *PEG-3* represents a new member of the *gadd34*/*MyD116* gene family with both similar and distinct properties. Unlike *gadd34* and *MyD116*, which dramatically suppress colony formation (24), *PEG-3* only modestly alters colony formation following transfection, i.e., ≤20% reduction in colony formation in comparison with vector transfected cells. Moreover, a direct correlation only exists between expression of PEG-3, and not *gadd34* or *MyD116*, and the progression phenotype in transformed rodent cells. These findings provide evidence for a potential link between constitutive induction of a stress response, characteristic of DNA damage, and induction of cancer progression.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Anchorage-Independent Growth Assays. The isolation, properties, and growth conditions of the E11, E11-NMT, E11-NMT × CREF somatic cell hybrids, E11 × E11-NMT somatic cell hybrids, and the E11-NMT AZA clones have been described (1, 7–13). E11-*ras* R12 and E11-HPV E6/E7 clones were isolated by transfection with the Ha-*ras* or the HPV-18 E6/E7 genes, respectively. The isolation, properties, and growth conditions of CREF, CREF-H5hr1 A2, CREF-*ras*, the CREF-*ras*/K*rev*1 B1, B1 T, and B1 M, and the CREF-*ras*/K*rev*1 B2, B2 T, and B2 M clones have been described (21). CREF-*src* and CREF-HPV-18 clones were isolated by transfection with the v-*src* and HPV-18 E6/E7 genes, respectively. All cells were grown in Dulbecco's modified Eagle's minimum essential medium supplemented with 5% fetal bovine serum at 37°C in a 5% $C0_2$/95% air humidified incubator. Anchorage-independence assays were performed by seeding various cell densities in 0.4% Noble agar on a 0.8% agar base layer both of which contain growth medium (7).

Cloning and Sequencing of the *PEG-3* cDNA. The *PEG-3* gene was cloned from E11-NMT cells using subtraction hybridization as described (23). A full-length *PEG-3* cDNA was obtained using the rapid amplification of cDNA end (RACE) procedure and direct ligation (25, 26). Sequencing was performed by the dideoxy chain termination (Sanger) method (27). The coding region of *PEG-3* was cloned into a pZeoSV vector (Invitrogen) as described (25, 26).

RNA Analysis and *in Vitro* Transcription Assays. Total cellular RNA was isolated by the guanidinium/phenol extraction method and Northern blotting was performed as described (28). Fifteen micrograms of RNA were denatured with glyoxal/dimethyl sulfoxide and electrophoresed in 1% agarose gels, transferred to nylon membranes, and hybridized sequentially with ^{32}P-labeled PEG-3, Ad5 E1A, and glyceraldehyde-3-phosphate (GAPDH) probes (28, 29). Following hybridization, the filters were washed and exposed for autoradiography. The transcription rates of PEG-3, *gadd34*, *MyD116*, GAPDH, and pBR322 were determined by nuclear run-on assays (12, 21).

***In Vitro* Translation of PEG-3.** Plasmid pZeoSV containing *PEG-3* cDNA was linearized by digestion with *Xho*I and used as a template to synthesize mRNA. *In vitro* translation of *PEG-3* mRNA was performed with a rabbit reticulocyte lysate translation kit as described by Promega.

DNA Transfection Assays. To study the effect of *PEG-3* on monolayer colony formation, the vector (pZeoSV) containing no insert or a pZeoSV-PEG-3 construct containing the *PEG-3* coding region were transfected into the various cell types by the Lipofectin method (GIBCO/BRL) and Zeocin-resistant clones were isolated or the efficiency of Zeocin colony formation was determined (29, 30).

RESULTS AND DISCUSSION

Expression of the *PEG-3* Gene Correlates Directly with the Progression Phenotype in Viral- and Oncogene-Transformed Rodent Cells. A critical component of cancer development is progression, a process by which a tumor cell develops either qualitatively new properties or displays an increase in the expression of traits that enhance the aggressiveness of a tumor (1–4). Insight into this process offers the potential of providing important new targets for intervening in the neoplastic process (1–4). In the Ad5-transformed RE cell culture model system, enhanced anchorage-independent growth and *in vivo* tumorigenic aggressiveness, i.e., markers of the progression phenotype, are stable traits that can be induced spontaneously or by gene transfer (oncogenes and growth factor-related genes) (Table 1). Upon treatment of progressed cells with AZA, the progression phenotype can be stably reversed (1, 10). A reversion of progression also occurs following somatic cell hybridization of progressed cells with unprogressed Ad5-transformed cells or with normal CREF cells. A further selection of these unprogressed Ad5-transformed cells by injection into nude mice results in acquisition of the progressed phenotype following tumor formation and establishment in cell culture. These studies document that progression in this model system is a reversible process that can be stably produced by appropriate cellular manipulation. In this context, the Ad5-transformed RE model represents an important experimental tool for identifying genes that are associated with and that mediate cancer progression.

To directly isolate genes elevated during progression we employed an efficient subtraction–hybridization approach, previously used to clone the p21 gene (melanoma differentiation-associated gene-6; mda-6) (23, 25) and a novel cancer growth-suppressing gene mda-7 (26, 29). For this approach, cDNA libraries from a progressed mutant Ad5 (H5ts125)-transformed RE clone, E11-NMT (10), and its parental unprogressed cells, E11 (10, 31), were directionally cloned into the λ Uni-ZAP phage vector and subtraction hybridization was performed between the double-stranded tester (E11-NMT) and the single-stranded driver DNA (E11) by mass excision of the libraries (23). With this strategy, in combination with the RACE procedure and DNA ligation techniques, a full-length *PEG-3* cDNA displaying elevated expression in E11-NMT versus E11 cells was cloned. Northern blot analysis indicates that *PEG-3* expression is ≥10-fold higher in all progressed Ad5-transformed RE cells, including E11-NMT, specific E11-NMT × CREF somatic cell hybrid clones, R1 and R2, expressing an aggressive transformed phenotype, and specific E11 × E11-NMT somatic cell hybrid clones, such as IIa that display the progression phenotype (Fig. 1 and Table 1). *PEG-3* mRNA levels also increase following induction of progression by stable expression of the Ha-*ras* and HPV-18 E6/E7 oncogenes in E11 cells (Fig. 1). A further correlation

Table 1. Expression of *PEG-3* in Ad5-transformed RE cells directly correlates with expression of the progression phenotype

Cell type*	Agar cloning efficiency, %†	Tumorigenicity in nude mice‡	Progression phenotype
RE	<0.001	0/10	Prog$^-$
CREF	<0.001	0/18	Prog$^-$
E11	2.9 ± 0.3	8/8 (36)	Prog$^-$
E11-NMT	34.3 ± 4.1	6/6 (20)	Prog$^+$
CREF × E11-NMT F1	2.0 ± 0.3	0/6	Prog$^-$
CREF × E11-NMT F2	1.5 ± 0.1	0/6	Prog$^-$
CREF × E11-NMT R1	72.5 ± 9.4	3/3 (17)	Prog$^+$
CREF × E11-NMT R2	57.4 ± 6.9	3/3 (17)	Prog$^+$
E11 × E11-NMT IIId	5.6 ± 0.7	3/3 (56)	Prog$^-$
E11 × E11-NMT IIIdTD	41.0 ± 4.9	3/3 (19)	Prog$^+$
E11 × E11-NMT A6	0.3 ± 0.0	2/3 (44)	Prog$^-$
E11 × E11-NMT A6TD	29.3 ± 3.5	NT	Prog$^+$
E11 × E11-NMT 3b	1.5 ± 0.2	3/3 (31)	Prog$^-$
E11 × E11-NMT IIa	29.5 ± 2.8	3/3 (23)	Prog$^+$
E11-NMT AZA C1	2.8 ± 0.5	NT	Prog$^-$
E11-NMT AZA B1	1.6 ± 0.3	3/3 (41)	Prog$^-$
E11-NMT AZA C2	2.0 ± 0.1	3/3 (50)	Prog$^-$
E11-*ras* R12	36.8 ± 4.6	3/3 (18)	Prog$^+$
E11-HPV E6/E7	31.7 ± 3.1	3/3 (22)	Prog$^+$

NT, not tested; Prog$^-$, progression phenotype is not expressed; Prog$^+$, progression phenotype is expressed.

*Cell line descriptions can be found in *Materials and Methods*.

†Anchorage-independent growth was determined by seeding variable numbers of cells in 0.4% agar on a 0.8% agar base layer. Results are the average number of colonies from four replicate plates ± SD.

‡Tumorigenicity was determined by injecting nude mice with 2×10^6 or 1×10^7 RE, CREF, and CREF × E11-NMT hybrids. Identified are the number of animals with tumors per number of animals injected and the numbers in parentheses indicate average latency time in days, i.e., first appearance of a palpable tumor.

between expression of *PEG-3* and the progression phenotype is provided by E11 × E11-NMT clones, such as IIId and A6, that initially display a suppression of the progression phenotype and low *PEG-3* expression, but regain the progression phenotype and *PEG-3* expression following tumor formation in nude mice, i.e., IIIdTD and A6TD (Table 1 and Fig. 1). In contrast, unprogressed Ad5-transformed cells, including E11, E11-NMT × CREF clones F1 and F2, E11 × E11-NMT clones IIId, A6, and 3b, and AZA-treated E11-NMT clones B1, C1, and C2, have low levels of *PEG-3* RNA. These results provide evidence for a direct relationship between the progression phenotype and *PEG-3* expression in this Ad5-transformed RE cell culture system. They also demonstrate that the final cellular phenotype, i.e., enhanced anchorage independence and aggressive tumorigenic properties, is a more important determinant of *PEG-3* expression than is the agent (oncogene) or circumstance (selection for tumor formation in nude mice) inducing progression.

A second rodent model used to study the process of cancer progression employs CREF clones modified by transfection to express dominant acting oncogenes (such as Ha-*ras*, v-*src*, HPV-18, and the mutant adenovirus H5hr1) and tumor suppressor genes (such as *Krev*-1, RB, and wild-type p53) (refs. 19–22 and unpublished data). In this model system, Ha-*ras*-transformed CREF cells are morphologically transformed, anchorage independent, and induce both tumors and lung metastases in syngeneic rats and athymic nude mice (19–22). The *Krev*-1 (Ha-*ras*) suppressor gene reverses the *in vitro* and *in vivo* properties in Ha-*ras*-transformed cells (21). Although suppression is stable *in vitro*, Ha-*ras*/*Krev*-1 CREF cells induce both tumors and metastases after extended times in nude mice (21). Expression of *PEG-3* is not apparent in CREF cells, whereas tumorigenic CREF cells transformed by v-*src*, HPV-18, H5hr1, and Ha-*ras* contain high levels of *PEG-3* RNA (Fig. 2). Suppression of Ha-*ras* induced transformation by *Krev*-1 inhibits *PEG-3* expression. However, when Ha-*ras*/*Krev*-1 cells escape tumor suppression and form tumors and metastases in nude mice, *PEG-3* expression reappears, with higher expression in metastatic-derived than tumor-derived clones (Fig. 2). These findings provide further documentation of a direct relationship between induction of a progressed and oncogenic phenotype in rodent cells and *PEG-3* expression. As indicated above, it is the phenotype rather than the inducing agent that appears to be the primary determinant of *PEG-3* expression in rodent cells.

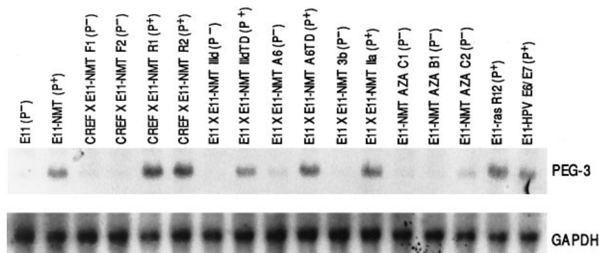

FIG. 1. PEG-3 expression in Ad5-transformed RE cells displaying different stages of transformation progression. Fifteen micrograms of cellular RNA isolated from the indicated cell types were electrophoresed, transferred to nylon membranes, and hybridized with an ≈700 bp 3′ region of the *PEG-3* gene (*Upper*) and then stripped and probed with GAPDH (*Lower*).

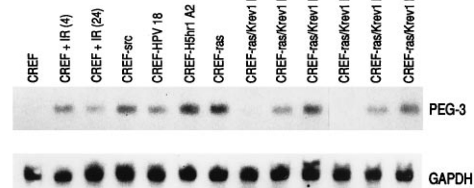

FIG. 2. PEG-3 expression in γ-irradiated and oncogene-transformed CREF cells. The experimental procedure was as described in the legend to Fig. 1. CREF cells were γ-irradiated with 10 Gy and RNA was isolated 4 and 24 hr later.

The *PEG-3* Gene Displays Sequence Homology with the Hamster *gadd34* and Mouse *MyD116* Genes and Is Inducible by DNA Damage. The cDNA sizes of PEG-3, *gadd34*, and *MyD116* are 2210, 2088, and 2275 nt, respectively. The nucleotide sequence of *PEG-3* is ≈73% and the amino acid sequence is ≈59% homologous to the *gadd34* (32) gene (Fig. 3 and data not shown). *PEG-3* also shares significant sequence homology, ≈68% nucleotide and ≈72% amino acid, with the murine homologue of *gadd34*, *MyD116* (33, 34) (Fig. 3 and data not shown). Differences are apparent in the structure of the 3′ untranslated regions of *PEG-3* versus *gadd34/MyD116*. ATTT motifs have been associated with mRNA destabilization. In this context, the presence of three ATTT sequences in *gadd34* and six tandem ATTT motifs in *MyD116* would predict short half-lives for these messages. In contrast *PEG-3* contains only one ATTT motif, suggesting that this mRNA may be more stable. The sequence homologies between *PEG-3* and *gadd34/MyD116* are highest in the amino-terminal region of their encoded proteins, i.e., ≈69 and ≈76% homology with *gadd34* and *MyD116*, respectively, in the first 279 aa. In contrast, the sequence of the carboxyl terminus of *PEG-3* significantly diverges from *gadd34/MyD116*, i.e., only ≈28 and ≈40% homology in the carboxyl-terminal 88 aa. In *gadd34* and *MyD116* a series of similar 39 aa are repeated in the protein, including 3.5 repeats in *gadd34* and 4.5 repeats in *MyD116*. In contrast, *PEG-3* contains only 1 of these 39 aa regions, with ≈64% and ≈85% homology to *gadd34* and *MyD116*, respectively. On the basis of sequence analysis, the *PEG-3* gene should encode a protein of 457 aa with a predicted M_r of ≈50 kDa. To confirm this prediction, *in vito* translation analyses of proteins encoded by the *PEG-3* cDNA were determined (Fig. 4). A predominant protein after *in vitro* translation of *PEG-3* has a molecular mass of ≈50 kDa (Fig. 4). In contrast, *gadd34* encodes a predicted protein of 589 aa with an M_r of ≈65 kDa and *MyD116* encodes a predicted protein of 657 aa with an M_r of ≈72 kDa. The profound similarities in the structure of *PEG-3* versus *gadd34/MyD116* cDNA and their encoded proteins suggest that *PEG-3* is a new member of this gene family. Moreover, the alterations in the carboxyl terminus of *PEG-3* may provide a functional basis for the different properties of this gene versus *gadd34/MyD116*.

The specific role of the *gadd34/MyD116* gene in cellular physiology is not known. Like hamster *gadd34* and its murine homologue *MyD116*, *PEG-3* steady-state mRNA and RNA transcriptional levels are increased following DNA damage by methyl methanesulfonate and γ-irradiation (Figs. 2 and 5 and data not shown). In contrast, nuclear run-on assays indicate that only the *PEG-3* gene is transcriptionally active (transcribed) as a function of transformation progression (Fig. 5). This is apparent in CREF cells transformed by Ha-*ras* and in E11-NMT and various E11-NMT subclones either expressing or not expressing the progression phenotype (Fig. 5). The *gadd34/MyD116* gene, as well as the *gadd45*, *MyD118*, and *gadd153* genes, encode acidic proteins with very similar and unusual charge characteristics (24). *PEG-3* also encodes a putative protein with acidic properties similar to the

```
        1                                                 50
PEG-3   MAPSPRPQHV LHWKEAHSFY LLSPLMGFLS RAWSRLRGPE VSEAWLAETV
GADD34  MAPSPRPQHI LLWRDAHSFH LLSPLMGFLS RAWSRLRVPE APEPWPAETV
MYD116  MAPSPRPQHV LHWRDAHNFY LLSPLMGLLS RAWSRLRGPE VPEAWLAKTV
        51                                               100
PEG-3   AGANQIEADA LLTPPPVSEN HLPLRETEGN GTPEWSKAAQ RLCLDVEAQS
GADD34  TGADQIEADA HPAPPLVPEN HPPQGEAEES GTPEEGKAAQ GPCLDVQANS
MYD116  TGADQIEAAA LLTPTPVSGN LLPHGETEES GSPEQSQAAQ RLCL.VEAES
        101                                              150
PEG-3   SPPKTWGLSD IDEHNGKPGQ DGLREQEVEH TAGLPTLQPL HLQGADKKVG
GADD34  SPPETLGLSD DD....KQGQ DGPREQGRAH TAGLPILLSP GLQSADKSLG
MYD116  SPPETWGLSN VDEYNAKPGQ DDLREKEMER TAGKATLQPA GLQGADKRLG
        151                                              200
PEG-3   EVVAREEGVS ELAYPTSHWE GGPAEDEEDT ETVKKAHQAS AASIAPGYKP
GADD34  EVVAGEEGVT ELAYPTSHWE GCPSEEEEDG ETVKKAFRAS ADS..PGHKS
MYD116  EVVAREEGVA EPAYPTSQLE GGPAENEEDG ETV.KTYQAS AASIAPGYKP
        201                                              250
PEG-3   STSVYCPGEA EHRATEEKGT DNKAEP.... .SGSHSRVWE YHTRERPKQE
GADD34  STSVYCPGEA EHQATEEKQT ENKADPPSSP .SGSHSRAWE YCS....KQE
MYD116  STPVPFLGEA EHQATEEKGT ENKADPSNSP SSGSHSRAWE YYSREKPKQE
        251                                              300
PEG-3   GETKPEQHRA GQSHPCQNAE AEEGGPETS. ...VCSGSAF LKAWVYRPGE
GADD34  GEADPEPHRA GKYQLCQNAE AEEEEEAKVS SLSVSSGNAF LKAWVYRPGE
MYD116  GEAKVEAHRA GQGHPCRNAE AEEGGPETT. ..FVCTGNAF LKAWVYRPGE
        301                                              350
PEG-3   DTEEEEDSDL DSAEEDT.AH TCTTPHTSAF LKAWVYRPGE DTEEED....
GADD34  DTEDDDDSDW GSAEEEGKAL SSPTSPEHDF LKAWVYRPGE DTEDDDDSDW
MYD116  DTEEEDNSDS DSAEEDT.AQ TGATPHTSAF LKAWVYRPGE DTEEEDSD..
        351                                              400
PEG-3   .......... .......... .......... ......DGDW DSAEEDA.SQ
GADD34  GSAEEEGKAL SSPTSPEHDF LKAWVYRPGE DTEDDQDSDW GSAEKDGLAQ
MYD116  SDSAEEDTAQ TGATPHTSAF LKAWVYRPGE DTEEE.NSDL DSAEEDT.AQ
        401                                              450
PEG-3   SCTTPHTSAF LKAWVYRPGE .......... .......... ..........
GADD34  TFATPHTSAF LKTWVCCPGE DT........ .......... ..........
MYD116  TGATPHTSAF LKAWVYRPGE DTEEENSDLD SAEEDTAQTG ATPHTSPFLK
        451                                              500
PEG-3   .......... ..DTEEEDDS ENVAPVDSET VDSCQS.... TQHCLPVEKT
GADD34  .......... .....EDDDC EVVVPEDSEA ADPDKSPSHE AQGCLPGEQT
MYD116  AWVYRPGEDT EDDTEEEEDS ENVAPGDSET ADSSQSPCLQ PQRCLPGEKT
        501                                              550
PEG-3   KGCGEAEPPP FQWPSIYL.. .....DRSQH HLGLPLSCP. ..FDCRSGSD
GADD34  EGLVEAEHSL FQ.VAFYLPG EKPAPPWTAP KLPLRLQRRL TLLRTPTQDQ
MYD116  KGRGE.EPPL FQ.VAFYLPG EKPESPWAAP KLPLRLQRRL RLFKAPTRDQ
        551                                              600
PEG-3   LSKPPPGIRA LRFL...... .......... .......... ..........
GADD34  DPETPLRARK VHFSENVTVH FLAVWAGPAQ AARRGPWEQL ARDRSRFARR
MYD116  DPEIPLKARK VHFAEKVTVH FLAVWAGPAQ AARRGPWEQF ARDRSRFARR
        601                                              650
PEG-3   .......... .......... .......... .......... ..........
GADD34  IAQAEEKLGP YLTPAFRARA WARLGNPSLP LALEPICDHT FFPSQ.....
MYD116  IAQAEEKLGP YLTPDSRARA WARLRNPSLP QSEPRSSSEA TPLTQDVTTP
        651               669
PEG-3   .......... .........
GADD34  .......... .........
MYD116  SPLPSETPSP SLYLGGRRG
```

FIG. 3. Predicted amino acid sequences of the PEG-3, gadd34, and MyD116 proteins. Sequences shared by the three genes are shaded. *PEG-3* encodes a putative protein of 457 aa (M_r of ≈50 kDa), the *gadd34* gene encodes a putative protein of 589 aa (M_r of ≈65 kDa), and the *MyD116* gene encodes a putative protein of 657 aa (M_r of ≈72 kDa).

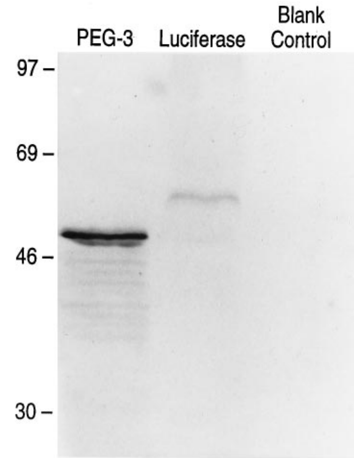

FIG. 4. *In vitro* translation of the *PEG-3* gene. Lanes: Luciferase, *in vitro* translation of the luciferase gene (≈61 kDa) (positive control); Blank Control, contains the same reaction mixture without mRNA (negative control); *PEG-3*, contains the translated products of this cDNA. Rainbow protein standards (Amersham) were used to determine the sizes of the *in vitro* translated products.

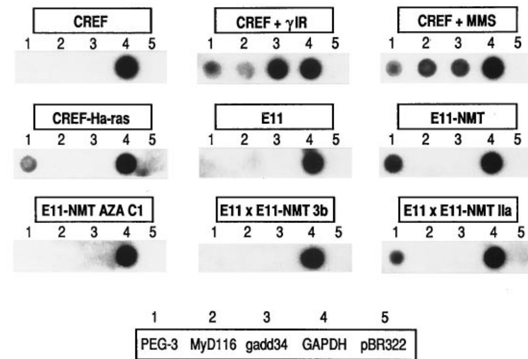

FIG. 5. Transcription of the *PEG-3*, *gadd34*, and *MyD116* genes as a function of DNA damage and transformation progression. Nuclear run-on assays were performed to determine comparative rates of transcription. Nuclei were isolated from CREF cells treated with methyl methanesulfonate (MMS) (100 μg/ml for 2 hr followed by growth for 4 hr in complete medium) or γ-irradiation (10 Gy followed by 2 hr growth in complete medium). DNA probes include *PEG-3*, *MyD116*, *gadd34*, GAPDH, and pBR322.

gadd and *MyD* genes (Fig. 3). The carboxyl-terminal domain of the murine MyD116 protein is homologous to the corresponding domain of the herpes simplex virus 1 $\gamma_1$34.5 protein, which prevents the premature shutoff of total protein synthesis in infected human cells (35, 36). Replacement of the carboxyl-terminal domain of $\gamma_1$34.5 with the homologous region from *MyD116* results in a restoration of function to the herpes viral genome, i.e., prevention of early host shutoff of protein synthesis (36). Although further studies are required, preliminary results indicate that expression of a carboxyl-terminus region of *MyD116* results in nuclear localization (36). Similarly, *gadd45*, *gadd153*, and *MyD118* gene products are nuclear proteins (24, 37). Moreover, both *gadd45* and *MyD118* interact with the DNA replication and repair protein proliferating cell nuclear antigen and the cyclin-dependent kinase inhibitor p21 (37). *MyD118* and *gadd45* also modestly stimulate DNA repair *in vitro* (37). The carboxyl terminus of *PEG-3* is significantly different than that of *MyD116* (Fig. 3). Moreover, the carboxyl-terminal domain region of homology between *MyD116* and the $\gamma_1$34.5 protein is not present in PEG-3. In this context, the localization, protein interactions, and properties of *PEG-3* may be distinct from *gadd* and *MyD* genes. Once antibodies with the appropriate specificity are produced it will be possible to define *PEG-3* location within cells and identify potentially important protein interactions mediating biological activity. This information will prove useful in elucidating the function of the *PEG-3* gene in DNA damage response and cancer progression.

PEG-3 Lacks Potent Growth-Suppressing Properties Characteristic of the *gadd* and *MyD* Genes. An attribute shared by the *gadd* and *MyD* genes is their ability to markedly suppress growth when expressed in human and murine cells (24, 37). When transiently expressed in various human tumor cell lines, *gadd34/MyD116* is growth inhibitory and this gene can synergize with *gadd45* or *gadd153* in suppressing cell growth (24). These results and those discussed above suggest that *gadd34/MyD116*, *gadd45*, *gadd153*, and *MyD118* represent a novel class of mammalian genes encoding acidic proteins that are regulated during DNA damage and stress and involved in controlling cell growth (24, 37). In this context, *PEG-3* would appear to represent a paradox, since its expression is elevated in cells displaying an *in vivo* proliferative advantage and a progressed-transformed and tumorigenic phenotype.

To determine the effect of *PEG-3* on growth, E11 and E11-NMT cells were transfected with the protein coding region of the *PEG-3* gene cloned into a Zeocin expression vector, pZeoSV (Fig. 6). This construct permits an evaluation of growth in Zeocin in the presence and absence of *PEG-3* expression. E11 and E11-NMT cells were also transfected with the p21 (mda-6) and mda-7 genes, previously shown to display growth inhibitory properties (25, 26, 29). Colony formation in both E11 and E11-NMT cells is suppressed 10–20%, whereas the relative colony formation following p21 (mda-6) and mda-7 transfection is decreased by 40–58% (Fig. 6 and data not shown). Colony formation is also reduced by 10–20% when *PEG-3* is transfected into CREF, normal human breast (HBL-100), and human breast carcinoma (MCF-7 and T47D) cell lines (data not shown). Although the *gadd* and *MyD* genes were not tested for growth inhibition in E11 or E11-NMT cells, previous studies indicate colony formation reductions of 50–75% in several cell types transfected with *gadd34*, *gadd45*, *gadd153*, *MyD116*, or *MyD118* (24, 37). The lack of dramatic growth-suppressing effects of *PEG-3* and its direct association with the progression state suggest that this gene may represent a unique member of this acidic protein gene family that directly functions in regulating progression. This may occur by constitutively inducing signals that would normally only be generated during genomic stress. In this context, *PEG-3* might function to alter genomic stability and facilitate tumor progression. This hypothesis is amenable to experimental confirmation.

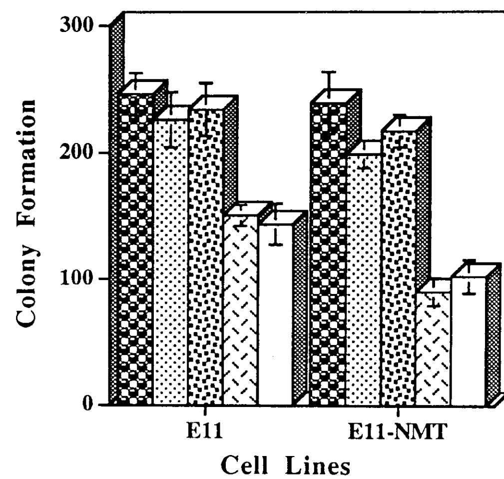

FIG. 6. Effect of transfection with *PEG-3*, mda-7, and p21 (mda-6) on colony formation of E11 and E11-NMT cells in monolayer culture. Target cells were transfected with 10 μg of a Zeocin vector (pZeoSV), the *PEG-3* gene cloned in pZeoSV (PEG-3), the pREP4 vector, the *mda-7* gene cloned in pREP4 (mda-7), and the *mda-6* (p21) gene cloned in pREP4 (p21 (mda-6). Data represent the average number of Zeocin- or hygromycin (pREP4 transfection)-resistant colonies ± SD for four plates seeded at 1×10^5 cells per 6-cm plate. ▩, Zeocin vector; ▤, PEG-3; ▤, pREP4 vector; ▧, mda-7; □, p21 (mda-6).

PEG-3 Induces a Progression Phenotype in Ad5-Transformed RE Cells. An important question is whether *PEG-3* expression simply correlates with transformation progression or whether it can directly contribute to this process. To distinguish between these two possibilities we have determined the effect of stable elevated expression of *PEG-3* on expression of the progression phenotype in E11 cells. E11 cells were transfected with a Zeocin expression vector either containing or lacking the *PEG-3* gene, and random colonies were isolated and evaluated for anchorage-independent growth (Fig. 7). A number of clones were identified that displayed a 5- to 9-fold increase in agar cloning efficiency in comparison with E11 and E11-Zeocin vector-transformed clones. To confirm that this effect was indeed the result of elevated

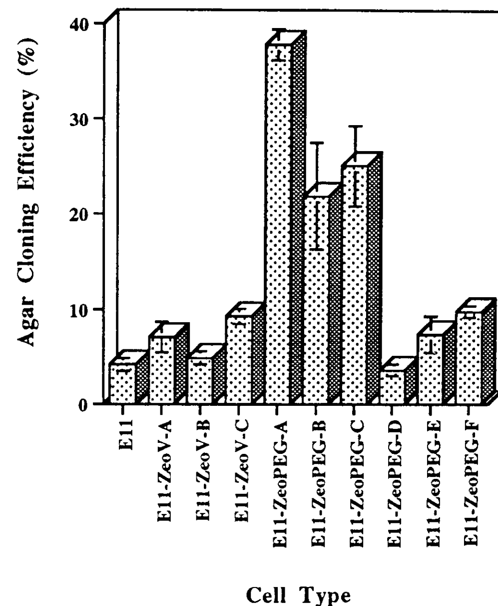

FIG. 7. Effect of stable *PEG-3* expression on anchorage-independent growth of E11 cells. Agar cloning efficiency of E11, Zeocin-resistant pZeoV (vector)-transfected E11, and Zeocin-resistant pZeoPEG-transfected E11 cells. Average number of colonies developing in four replicate plates ± SD.

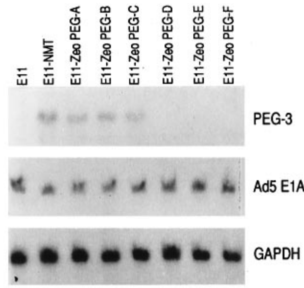

FIG. 8. Expression of *PEG-3*, Ad5 E1A, and GAPDH RNA in pZeoPEG-transfected E11 cells. The experimental procedure was as described in the legend to Fig. 1. Blots were probed sequentially with *PEG-3*, Ad5 E1A, and GAPDH. The E11-ZeoPEG clones are the same clones analyzed for anchorage independence in Fig. 7.

PEG-3 expression, independent Zeocin-resistant E11 clones either expressing or not expressing the progression phenotype were analyzed for *PEG-3* mRNA expression (Fig. 8). This analysis indicates that elevated anchorage independence in the E11 clones correlates directly with increased *PEG-3* expression. In contrast, no change in Ad5 E1A or GAPDH mRNA expression is detected in the different clones. These findings demonstrate that *PEG-3* can directly induce a progression phenotype without altering expression of the Ad5 E1A transforming gene. Further studies are required to define the precise mechanism by which *PEG-3* elicits this effect.

Cancer is a progressive disease characterized by the accumulation of genetic alterations in an evolving tumor (1–6). Recent studies provide compelling evidence that mutations in genes involved in maintaining genomic stability, including DNA repair, mismatch repair, DNA replication, microsatellite stability, and chromosomal segregation, may mediate the development of a mutator phenotype by cancer cells, predisposing them to further mutations resulting in tumor progression (38). Identification and characterization of genes that can directly modify genomic stability and induce tumor progression will provide significant insights into cancer development and evolution. This information would be of particular benefit in defining potentially novel targets for intervening in the cancer process. Although the role of *PEG-3* in promoting the cancer phenotype remains to be defined, the current studies suggest a potential causal link between constitutive induction of DNA damage response pathways that may facilitate genomic instability and cancer progression. In this context, constitutive expression of *PEG-3* in progressing tumors may directly induce genomic instability or it may induce or amplify the expression of downstream genes involved in this process. Further studies are clearly warranted and will help delineate the role of an important gene, PEG-3, in cancer.

CONCLUSIONS

Subtraction hybridization results in the identification and cloning of a gene *PEG-3* with sequence homology and DNA damage-inducible properties similar to gadd34 and MyD116. However, *PEG-3* expression is uniquely elevated in all cases of rodent progression analyzed to date, including spontaneous and oncogene-mediated, and overexpression of *PEG-3* can induce a progression phenotype in Ad5-transformed cells. Our studies suggest that *PEG-3* may represent an important gene that is both associated with (diagnostic) and causally related to cancer progression. They also provide a potential link between constitutive expression of a DNA damage response pathway and progression of the transformed phenotype.

We thank Drs. Albert Fornace, Jr., Barbara Hoffman, and Dan Liebermann for supplying the *gadd34* and *MyD116* cDNAs. We thank Dr. John Smith (Corixa Corp., Seattle) for confirmation of the DNA sequence of the *PEG-3* cDNA. This research was supported in part by National Institutes of Health Grant CA35675 and the Chernow Endowment. P.B.F. is a Chernow Research Scientist.

1. Fisher, P. B. (1984) in *Tumor Promotion and Cocarcinogenesis in Vitro: Mechanisms of Tumor Promotion*, ed. Slaga, T. J. (CRC, Boca Raton, FL), pp. 57–123.
2. Bishop, J. M. (1991) *Cell* **64,** 235–248.
3. Vogelstein, B. & Kinzler, K. W. (1991) *Trends Genet.* **9,** 138–141.
4. Knudson, A. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10914–10921.
5. Levine, A. J. (1993) *Annu. Rev. Biochem.* **62,** 623–651.
6. Hartwell, L. H. & Kastan, M. B. (1994) *Science* **266,** 1821–1828.
7. Fisher, P. B., Goldstein, N. I. & Weinstein, I. B. (1979) *Cancer Res.* **39,** 3051–3057.
8. Fisher, P. B., Dorsch-Hasler, K., Weinstein, I. B. & Ginsberg, H. S. (1979) *Nature (London)* **281,** 591–594.
9. Fisher, P. B., Bozzone, J. H. & Weinstein, I. B. (1979) *Cell* **18,** 695–705.
10. Babiss, P. B., Zimmer, S. G. & Fisher, P. B. (1985) *Science* **228,** 1099–1101.
11. Duigou, G. J., Babiss, L. E., Iman, D. S., Shay, J. W. & Fisher, P. B. (1990) *Mol. Cell. Biol.* **10,** 2027–2034.
12. Duigou, G. J., Su, Z.-z., Babiss, L. E., Driscoll, B., Fung, Y.-K. T. & Fisher, P. B. (1991) *Oncogene* **6,** 1813–1824.
13. Reddy, P. G., Su, Z.-z. & Fisher, P. B. (1993) in *Chromosome and Genetic Analysis: Methods in Molecular Genetics*, ed. Adolph, K. W. (Academic, Orlando, FL), Vol. 1, pp. 68–102.
14. Su, Z.-z., Shen, R., O'Brian, C. A. & Fisher, P. B. (1994) *Oncogene* **9,** 1123–1132.
15. Fidler, I. J. (1990) *Cancer Res.* **50,** 6130–6138.
16. Liotta, L. A., Steeg, P. G. & Stetler-Stevenson, W. G. (1991) *Cell* **64,** 327–336.
17. Fidler, I. J. (1995) *J. Natl. Cancer Inst.* **87,** 1588–1592.
18. Fisher, P. B., Babiss, L. E., Weinstein, I. B. & Ginsberg, H. S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3527–3531.
19. Boylon, J. F., Jackson, J., Steiner, M., Shih, T. Y., Duigou, G. J., Roszman, T., Fisher, P. B. & Zimmer, S. G. (1990) *Anticancer Res.* **10,** 717–724.
20. Boylon, J. F., Shih, T. Y., Fisher, P. B. & Zimmer, S. G. (1992) *Mol. Carcinog.* **3,** 118–128.
21. Su, Z.-z., Austin, V. N., Zimmer, S. G. & Fisher, P. B. (1993) *Oncogene* **8,** 1211–1219.
22. Su, Z.-z., Yemul, S., Estabrook, A., Friedman, R. M., Zimmer, S. G. & Fisher, P. B. (1995) *Int. J. Oncol.* **7,** 1279–1284.
23. Jiang, H. & Fisher, P. B. (1993) *Mol. Cell. Differ.* **1,** 285–299.
24. Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A. & Fornace, A. J., Jr. (1994) *Mol. Cell. Biol.* **14,** 2361–2371.
25. Jiang, H., Lin, J., Su, Z.-z., Kerbel, R. S., Herlyn, M., Weissman, R. B., Welch, D. R. & Fisher, P. B. (1995) *Oncogene* **10,** 1855–1864.
26. Jiang, H., Lin, J. J., Su, Z.-z., Goldstein, N. I. & Fisher, P. B. (1995) *Oncogene* **11,** 2477–2486.
27. Su, Z.-z., Leon, J. A., Jiang, H., Austin, V. A., Zimmer, S. G. & Fisher, P. B. (1993) *Cancer Res.* **53,** 1929–1938.
28. Jiang, H., Su, Z.-z., Datta, S., Guarini, L., Waxman, S. & Fisher, P. B. (1992) *Int. J. Oncol.* **1,** 227–239.
29. Jiang, H., Su, Z.-z., Lin, J. J., Goldstein, N. I., Young, C. S. H. & Fisher, P. B. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9160–9165.
30. Su, Z.-z., Grunberger, D. & Fisher, P. B. (1991) *Mol. Carcinog.* **4,** 231–242.
31. Fisher, P. B., Weinstein, I. B., Eisenberg, D. & Ginsberg, H. S. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2311–2314.
32. Fornace, A. J., Jr., Alamo, I., Jr., & Hollander, M. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8800–8804.
33. Lord, K. A., Hoffman-Liebermann, B. & Liebermann, D. A. (1990) *Oncogene* **5,** 387–396.
34. Lord, K. A., Hoffman-Liebermann, B. & Liebermann, D. A. (1990) *Nucleic Acids Res.* **18,** 2823.
35. Chou, J. & Roizman, B. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5247–5251.
36. He, B., Chou, J., Liebermann, D. A., Hoffman, B. & Roizman, B. (1996) *J. Virol.* **70,** 84–90.
37. Vairapandi, M., Balliet, A. G., Fornace, A. J., Jr., Hoffman, B. & Liebermann, D. A. (1996) *Oncogene* **11,** 2579–2594.
38. Loeb, L. A. (1994) *Cancer Res.* **54,** 5059–5063.