

The p66Shc Longevity Gene Is Silenced through Epigenetic Modifications of an Alternative Promoter*

Received for publication, January 10, 2002, and in revised form, March 5, 2002
Published, JBC Papers in Press, April 10, 2002, DOI 10.1074/jbc.M200280200

Andrea Ventura^{‡§}, Lucilla Luzi^{‡¶}, Sonia Pacini^{§||}, Cosima T. Baldari^{||},
and Pier Giuseppe Pelicci^{‡¶**}

From the [‡]Department of Experimental Oncology, European Institute of Oncology, Milan 20141, the [¶]Fondazione Italiana per la Ricerca sul Cancro Institute for Molecular Oncology, Milan 20139, and the ^{||}Department of Evolutionary Biology, University of Siena, Siena 53100, Italy

The mammal Shc locus encodes three overlapping isoforms (46, 52, and 66 kDa) that differ in the length of their N-terminal regions. p46/p52Shc and p66Shc have been implicated, respectively, in the cytoplasmic propagation of growth and apoptogenic signals. Levels of p66Shc expression correlate with life span duration in mice. p46Shc and p52Shc are ubiquitously expressed, whereas p66Shc is expressed in a cell lineage-specific fashion. However, the mechanisms underlying the regulation of Shc protein expression are unknown. Here we report the identification of two alternative promoters, driving the transcription of two mRNAs coding for p46/p52Shc and p66Shc. We show that treatment with an inhibitor of histone deacetylases or with a demethylating agent results in induction of p66Shc expression in cells that normally do not express this isoform but leaves the levels of the two other isoforms unchanged. Moreover, analysis of the methylation pattern of the p66Shc promoter in a panel of primary and immortalized human cells showed inverse correlation between p66Shc expression and methylation density of its promoter. These results identify histone deacetylation and cytosine methylation as the mechanisms underlying p66Shc silencing in nonexpressing cells.

Three isoforms of 66, 52, and 46 kDa are encoded by the human or mouse Shc locus. They share a common modular architecture, with an N-terminal phosphotyrosine binding domain and a C-terminal Src homology-2 domain, separated by a proline-rich region (CH1). The presence of overlapping N-terminal sequences of 46 and 110 amino acids, respectively, distinguishes the p52 and p66Shc isoforms from p46 (for review, see Ref. 1).

Despite their high structural similarity, a growing body of experimental evidence suggests that the Shc isoforms are functionally nonredundant. In response to a variety of growth factors, p46/p52Shc bind to phosphorylated receptors through their phosphotyrosine binding and/or SH2 domains and are in turn phosphorylated on three tyrosine residues within their CH1 regions. These phosphotyrosine residues then act as dock-

ing sites for the Grb2·SOS complex, through direct interaction with the Grb2 SH2 domain, allowing its juxtaposition to, and activation of Ras proteins (2–4). p46/52 Shc isoforms couple, therefore, activated receptor kinases to Ras and are implicated in the cytoplasmic propagation of mitogenic signals. It is as yet not known, however, whether functional differences exist between p46 and p52Shc.

p66Shc is also tyrosine phosphorylated after growth factor receptor activation and binds Grb2; however, it does not mediate Ras activation (5, 6). We have shown recently that p66Shc is, instead, involved in signal transduction pathways that regulate the cellular response to oxidative stress and life span in mice (7). Indeed, p66Shc^{−/−} cells are more resistant to oxidative stress-induced apoptosis, and knockout mice for p66Shc live about 30% longer than littermate controls (7). p66Shc is phosphorylated on serine 36 after either treatment with growth factors (epidermal growth factor and Insulin) or oxidative stress (7, 8). Serine 36 phosphorylation is believed to be required for p66Shc function because the expression of p66Shc carrying a serine 36 to alanine mutation is unable to rescue the oxidative response defect observed in p66^{−/−} mouse embryo fibroblasts (MEFs)¹ (7).

Recent work suggests that in addition to post-translational modifications of Shc proteins, transcriptional regulation could also play a role in regulating their biological functions. Shc proteins are in fact down-regulated during neuron differentiation, whereas the levels of a neuron-specific Shc family member (N-Shc/Rai/ShcC) increase progressively (9, 10), a process that is required for proper neuron maturation (11). Furthermore, the absolute levels of p66Shc correlate with life span in mice because p66Shc^{+/−} animals display an intermediate life span compared with wild type and p66Shc^{−/−} littermates, indicating that even subtle differences in expression levels of this isoform can have a significant effect (7). Finally, p66Shc expression is restricted to certain tissues and cell lines, being absent in brain, in most hematopoietic cell lines, in peripheral blood lymphocytes (PBL) and in a subset of breast cancer cell lines (4, 12–14). In breast cancer cell lines and in primary breast cancers, variability in the expression levels of p66Shc has been reported (12–14), and p66 down-regulation has been shown to correlate with high expression levels of erbB-2 (12, 13).

Nothing is known regarding the molecular mechanisms that regulate the differential expression of the various Shc isoforms. In particular, it is not known whether regulation occurs at the transcriptional or post-transcriptional levels and whether the three isoforms are produced as the result of alternative splicing

* This work was supported in part by grants from the Associazione Italiana per la Ricerca sul Cancro (to P. G. P. and C. T. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by fellowships from the Fondazione Italiana per la Ricerca sul Cancro.

** To whom correspondence should be addressed: Dept. of Experimental Oncology, European Institute of Oncology, Via Ripamonti 435, Milan 20141, Italy. Tel.: 39-2-5748-9831; Fax: 39-2-5748-9851; E-mail: gpellicci@ieo.it.

¹ The abbreviations used are: MEF(s), mouse embryo fibroblast(s); 5-aza-dC, 5-aza-2'-deoxycytidine; PBL, peripheral blood lymphocytes; PIPES, 1,4-piperazinediethanesulfonic acid; RACE, rapid amplification of cDNA ends; TSA, trichostatin A.

or alternative promoter usage. Considering the heterogeneity of p66Shc expression and the finding that its absolute expression levels correlate directly with life span in mammals (7), deciphering the mechanisms that regulate Shc expression could be crucial to understanding further the functions of Shc proteins and to design strategies for the manipulation of their expression levels *in vivo*.

We report here that two distinct transcripts, originating from alternative promoters, encode for p52/46Shc and p66Shc and that expression of p66Shc is regulated by epigenetic modifications of its promoter region.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—U2OS, WI38, IMR90, MDA-MB-453, MDA-MB-361, BT20, NIH3T3, and MEFs (wild type and Shc^{-/-}) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine calf serum, 100 μ g/ml streptomycin, and 100 μ g/ml penicillin. U937, Jurkat, 32D, and HL60 were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine calf serum, 100 μ g/ml streptomycin, and 100 μ g/ml penicillin. Wild type MEFs were prepared according to standard procedures. Shc^{-/-} MEFs were a kind gift from Dr. T. Pawson. Peripheral blood mononuclear cells were isolated from whole blood by density centrifugation on Ficoll-Paque (Amersham Biosciences) and subsequently depleted of macrophages by adherence. Shc antibody was obtained from Transduction Laboratories (anti-Shc SH2 monoclonal).

RNAse Protection Assay—Riboprobes were produced from PCR fragments that had been cloned in pGEM3 or in PCR2.1 vectors and verified by sequencing. Plasmid DNA was cleaved with appropriate restriction enzymes and transcribed *in vitro* using T3 or T7 RNA polymerases in the presence of [α -³²P]UTP. The full-length riboprobe was purified from polyacrylamide gel, and 5×10^5 cpm were hybridized with 15–30 μ g of total RNA in 30 μ l of hybridization buffer (40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide). After overnight hybridization at 50 °C, the samples were treated with 0.5 unit of RNase ONE (Promega) for 1 h at 37 °C. RNA was ethanol precipitated, loaded on a 6% polyacrylamide/urea gel, and subjected to autoradiography.

5'-RACE—The 5'-RACE was performed on 200 ng of WI38 poly(A) RNA using the SMART RACE cDNA Amplification Kit (CLONTECH, Palo Alto, CA) following the manufacturer's instructions. RACE products were cloned in the pCR2.1 TOPO vector (Invitrogen). Individual colonies were grown, and plasmid DNA was recovered and subjected to automated sequencing. The sequence of the gene-specific primer used to obtain the RACE product is 5'-GAAGTCCAGGGCAGCATTGA-3'.

Trichostatin A (TSA) and 5-Aza-dC Treatment—Cells were split at low density the day before treatment and treated with TSA (Sigma), 5-aza-dC (Sigma), at the indicated concentrations, ethanol (as control for TSA), or 1 \times phosphate-buffered saline (as control for 5-aza-dC). For the combined treatments, cells were first incubated with 1 μ M 5-aza-dC for 24 h, and then 20 nM TSA was added for an additional 20 h. Alternatively, 5-aza-dC and TSA were administered simultaneously, and cells were cultured for 20 h.

RNA and Reverse Transcription-PCR—Total RNA was extracted from the various cell lines using the RNeasy kit (Qiagen) following the manufacturer's instructions. Reverse transcription reactions were performed with 2 μ g of total RNA using the SuperScript II Reverse Transcriptase (Invitrogen), following the manufacturer's instructions. Random hexamers were used for the first strand synthesis. PCRs were performed using 2 μ l of cDNA in a 50- μ l volume using 200 μ M dNTPs, 1.5 mM MgCl₂, 1 pmol of each oligonucleotide, and 1 unit of Taq polymerase (Roche Molecular Biochemicals). The following sets of primers were used: for amplification of the p66Shc transcript: forward, 5'-CGGTGCGGAGACTCCATGAG-3'; reverse, 5'-GCCATGAGGTTAAG-GCTGCTG-3' (this primer maps to a region common to both p66Shc and p52/46Shc transcripts and was also used as the reverse primer for the amplification of the p52/46Shc transcript); for amplification of the p52/46Shc transcript: forward, 5'-CAACCTGAAGCTGGCAATCC-3'; for the β -actin amplifications: forward, 5'-TTCTACAATGAGCTGCGT-GTG-3'; reverse, 5'-CAGGAAGGAAGGCTGGAAGA-3'.

Bisulfite Genomic Sequencing—Genomic DNA was extracted from the indicated cell lines according to standard procedures and treated with sodium bisulfite essentially as described by Clark *et al.* (15). Briefly, 2 μ g of genomic DNA was digested with EcoRI, resuspended in 100 μ l of dH₂O, and 11 μ l of 3 N NaOH was added, and DNA was incubated at 37 °C for 20 min. After denaturation, 1.1 ml of 3.5 M NaHSO₃ (Sigma), and 1 mM hydroquinone, pH 5.0 (Sigma), were added,

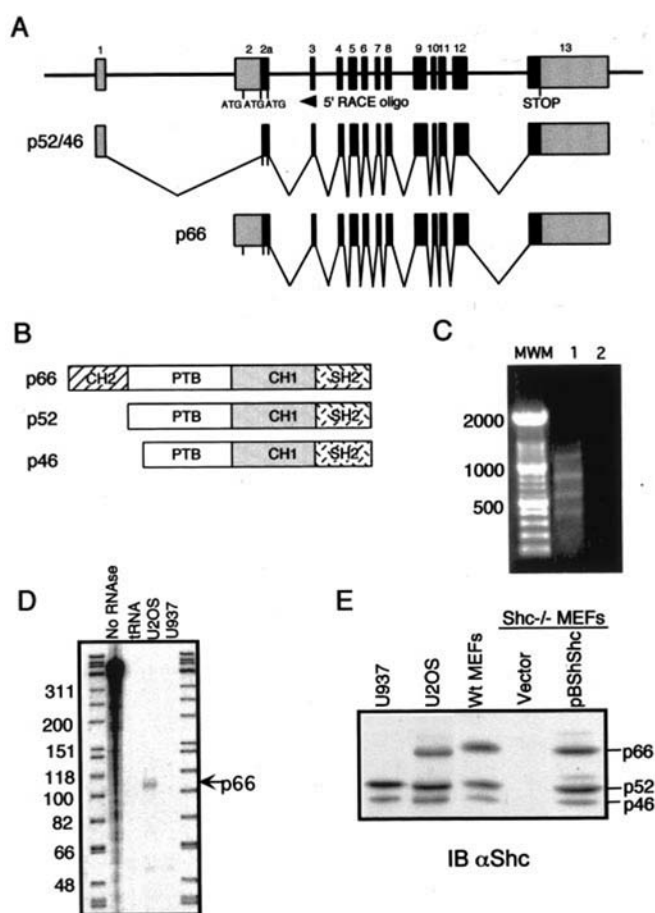


FIG. 1. Mapping of Shc transcripts cap sites. A, schematic representation of the human Shc locus and splicing patterns. The arrowhead indicates the position of the 5'-RACE primers. B, modular organization of the Shc isoforms. C, 5'-RACE products for Shc mRNAs. Poly(A) RNA from WI38 cells was subjected to 5'-RACE to identify the transcription start sites of Shc mRNAs (lane 1). As a control, an identical reaction was set up in the absence of RNA (lane 2). MWM is the 100-bp ladder molecular weight marker. D, RNase protection using a riboprobe spanning positions -199 to +101. 20 μ g of total RNA extracted from U2OS and U937 cells was used, as indicated. Yeast tRNA was used as a control for RNase digestion. E, immunoblotting analysis using an anti-Shc antibody of Shc^{-/-} MEFs transfected with a construct containing the entire human Shc locus (pBSHShc). Shc^{-/-} MEFs transfected with the empty vector as well as 32D and NIH3T3 cells are shown as controls.

and the solution incubated 16 h at 55 °C. Treated DNA was extracted from the solution using 20 μ l of glass milk (GeneClean II kit; Stratech Scientific Ltd., London) and resuspended in 100 μ l of deionized water. Desulfonation was performed by adding 11 μ l of 3 N NaOH and incubating at 37 °C for 15 min. DNA was finally precipitated and resuspended in 100 μ l of 1 \times Tris-EDTA, pH 8.0. 2–4 μ l of resuspended DNA was used in a PCR using primers designed to amplify the p66Shc promoter region. Cycling conditions were as follows: five cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min followed by 25 cycles at 94 °C for 30 s, 50 °C for 2 min, 72 °C for 90 s. In some cases, 1 μ l of PCR was subjected to 20 cycles of seminested PCR. The PCR products were cloned in the pCR2.1-TOPO vector. Individual colonies were grown; plasmid DNA was recovered and subjected to automated sequencing at the DNA Sequencing Facility of the FIRC Institute of Molecular Oncology. Additional information and primer sequences are available upon request. Primers used in the first round of PCR were: hMetp66F, 5'-T-TAGATTATTAGTTGTTTGTATAGGGTAG-3'; hMetp66B, 5'-AAAAA-CCAAACAAAAAATATCCCCAAACCC-3'. For the seminested PCR hMetp662B 5'-CTAATTAACCTCTATAACCCAAAAATCAC-3' was used instead of p66Metp66B.

Promoter Sequence Analysis—A mouse genomic clone containing the first and second exons of Shc as well as the 5'-flanking region and the first exon of Cks1 (5) was sequenced at the DNA Sequencing Facility of FIRC Institute of Molecular Oncology; the sequence obtained was aligned and

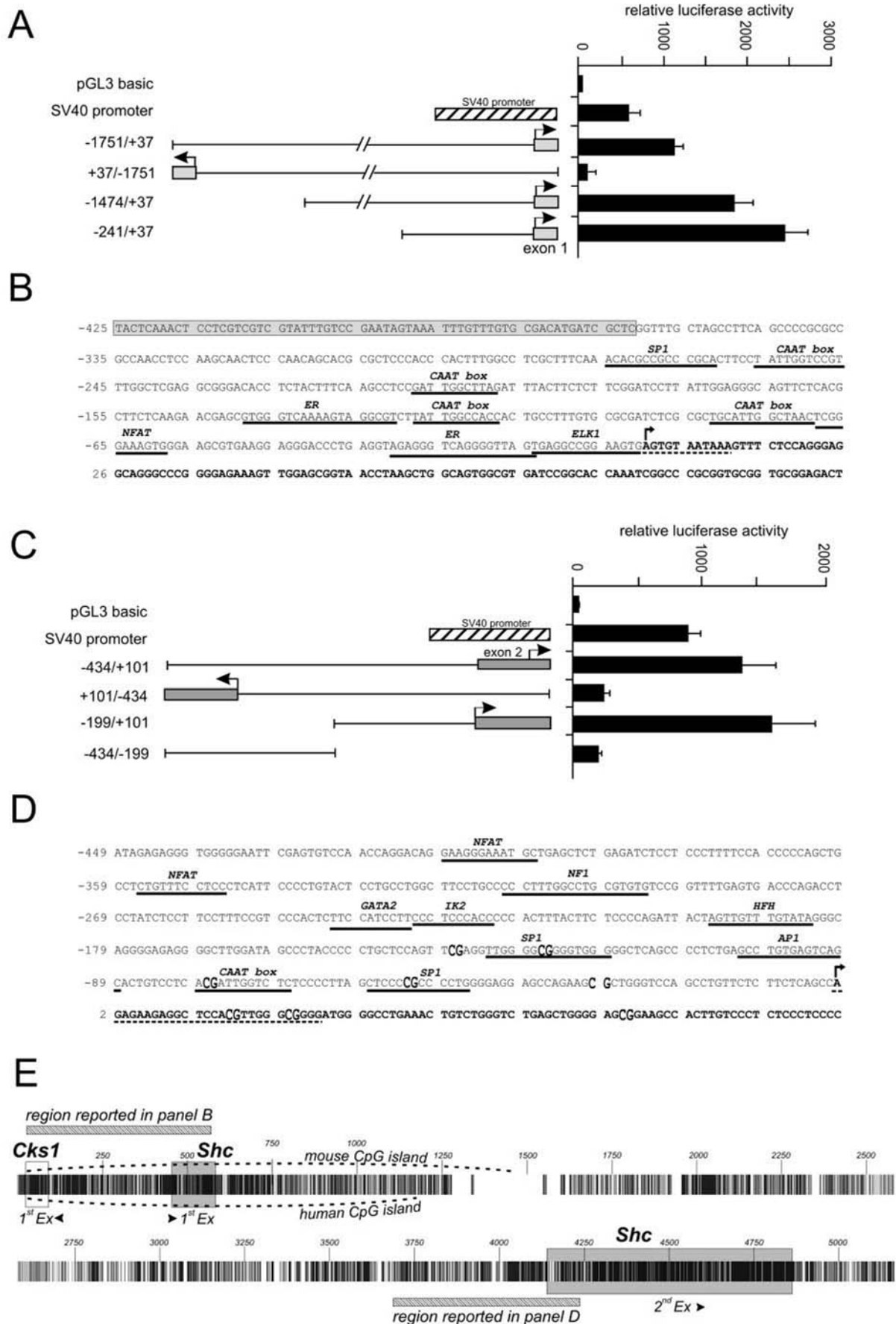


FIG. 2. Identification of two promoter activities in the Shc locus. A and C, promoter activity of various Shc genomic fragments containing the p52/46Shc (A) and p66Shc (C) cap sites. The indicated fragments were cloned in pGL3-Basic in the described orientation, and their ability to drive the transcription of the luciferase gene was assayed after transient transfection in U2OS cells. Transfections were performed in triplicate.

compared with mouse genomic traces at the Ensembl trace repository (trace.ensembl.org/). 50 traces were found to overlap with the sequence. The 5.1-kbp contig, spanning the genomic sequence from the first exon to the end of the second exon of Shc gene and its human corresponding region have been submitted to GenBank under Accession numbers AF455140 and AF455141, respectively. These regions include the p46/p52 and p66Shc putative promoters, extending from the first exon of Cks1 to the second exon of Shc. Match™. Public at BIOBASE portal (www.gene-regulation.com/) was used to search for transcription factor binding sites. The program searches for transcription factor binding sites using the mononucleotide weight matrices TRANSFAC® 5.0 library. "CpG islands revealing" at WebGene (www.itba.mi.cnr.it/webgene/) was used to predict the presence of CpG islands. The program locates CpG islands as defined by Gardiner-Garden and Frommer (16).

Plasmids, Transfections, and Luciferase Assays—The various inserts were obtained by PCR or digesting with appropriate restriction enzymes the genomic clone containing the Shc locus. They were cloned into pGL3 basic (Promega) and sequenced to assure fidelity. Transfections of adherent cells were performed using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. 1 µg of plasmid DNA and 250 ng of CMV/gal (in which the β-galactosidase gene expression is driven by the cytomegalovirus promoter) were transfected in triplicate in six wells/plates. Luciferase activity was assayed 36 h after transfection using the luciferase assay system (Promega) according to the manufacturer's instructions and normalized to the levels of β-galactosidase activity. U937 and Jurkat cells were cotransfected with 1 µg of p66/p52 promoter reporter DNA and 0.5 µg of CMV/gal control as described (17). Luciferase activity was quantitated after 24 h and normalized to the levels of β-galactosidase activity. For transfections in the Shc^{-/-} MEFs, cells were plated at 60% confluence on 10-cm dishes, and 5 µg of plasmid DNA was transfected using LipofectAMINE Plus.

In Vitro Methylation—20 µg of plasmid DNA was incubated with 20 units of SssI methylase (New England Biolabs) for 2 h at 37 °C in a buffer containing 50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 1 mM dithiothreitol, and 160 µM S-adenosylmethionine. As a control the same amount of plasmid DNA was incubated in absence of enzyme. DNA was precipitated and resuspended at a concentration of 0.5 µg/µl. An aliquot was loaded on a 1.2% agarose gel to evaluate DNA integrity and recovery.

RESULTS

p66Shc and p46/p52Shc are encoded by two distinct transcripts (Ref. 5 and Fig. 1, A and B). It is not clear, however, whether they result from alternative splicing or alternative promoter usage. To discriminate between these two possibilities, we first mapped their transcription start sites by 5'-RACE experiments, using, as template, mRNAs from WI38 cells, which express all three isoforms, and a primer derived from a region (exon 3) common to the two transcripts (indicated in Fig. 1A).

Four RACE products were obtained of ~350, 600, 800, and 1,200 bp (Fig. 1C), which were cloned and sequenced. Sequencing of several clones obtained from the 600- and 1,200-bp RACE products revealed that they originated from nonspecific primer annealing. In contrast, five of five clones sequenced from the 350-bp product corresponded to the p46/p52 transcript and contained exon 1 sequences spliced to exon 2A. Four of these clones showed an identical start site, which maps to the 5'-extremity of exon 1 and extends the known human p46/p52 transcript of 11 bases (dashed underline in Fig. 2B). The remaining clone started 23 bp downstream. Eight of eight clones obtained from the 800-bp RACE product contained sequences specific for the p66Shc transcript, including the p66Shc start codon. Four of eight showed an identical start site, which maps to the 5'-extremity of exon 2 and extends the known p66Shc transcript of 27 bases (dashed underline in Fig. 2D). The other

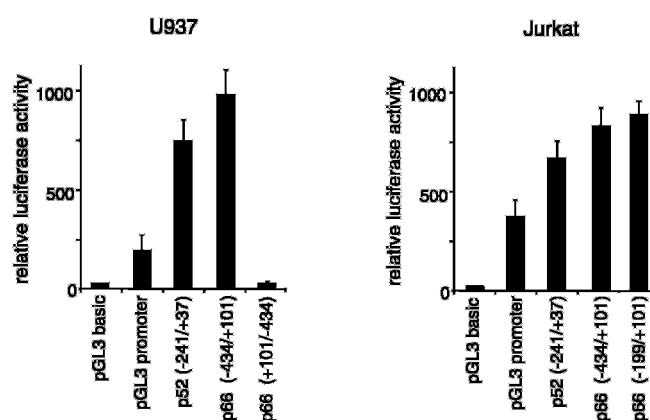


FIG. 3. The p66Shc promoter is active when transiently transfected in cell lacking endogenous p66Shc. Activity of the p46/p52Shc and p66Shc promoter fragments assayed by transient transfection in U937 (left panel) or Jurkat (right panel) cells is shown. Experiments were performed in triplicate. The error bar is ± 1 S.D.

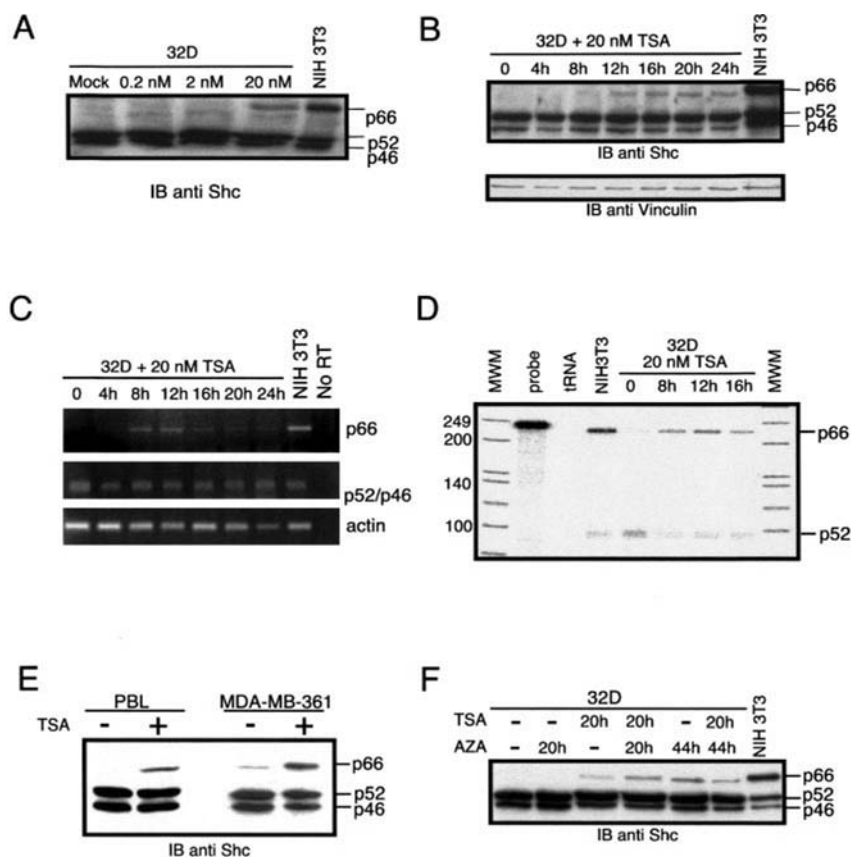
four clones had start sites located 4, 14, 20, and 65 bp downstream, respectively. To confirm that the extended sequences are, indeed, contained within the p66 transcript, we performed RNase protection experiments. A p66Shc-specific riboprobe was synthesized from a Shc genomic fragment containing bases -199 to +101 (wherein +1 is the major transcription start site identified in the RACE experiment). As shown in Fig. 1D, and in agreement with the 5'-RACE results, a single protected fragment, 100–118 nucleotides in size, was detected in RNA from U2OS cells (which express p66Shc; Fig. 1E), but not from U937 cells (which lack p66Shc expression; Fig. 1E).

In summary, the positions of the p66Shc and p52/46Shc transcription start sites define the intron-exon structure of the 5'-region of the Shc locus and suggest that two promoters are responsible for the transcription of the two Shc mRNAs. To confirm this hypothesis, we first evaluated the potential of a genomic clone containing the Shc locus (up to the identified 5'-extremity) to encode the three Shc isoforms upon transient transfection into MEFs derived from Shc-null mice (18). A clone of about 23 kbp, containing all of the mapped Shc exons (1–13) and extending for about 2 kbp upstream the first exon, previously isolated from a human genomic DNA library (5), was cloned into pBlueScript (pBShShc). As shown in Fig. 1E, transient transfection of this clone into Shc^{-/-} MEFs led to expression of the three Shc isoforms, with a relative expression pattern comparable with that observed in wild type MEFs, indicating that it contains the Shc promoter region(s).

Then, to map the positions of the two putative Shc promoters, DNA fragments located immediately upstream of the identified transcription start sites were subcloned into the pGL3-Basic plasmid upstream of the luciferase reporter gene and transfected into U2OS cells. A 1.8-kbp genomic fragment spanning position -1751 to +37 and containing the putative p46/p52 promoter showed, in the 5' → 3' orientation, a strong promoter activity compared with the SV40 promoter. 5'-Deletions of this region allowed narrowing down of the promoter region to a 278-bp fragment spanning positions -241 to +37 (Fig. 2, A and B). A similar approach was used to identify the p66Shc promoter. A 535-bp genomic fragment spanning posi-

Representative results of at least three experiments are shown. The error bar indicates ± 1 S.D. B and D, sequence of the p46/p52Shc (B) and p66Shc (D) promoter regions. The arrow indicates the major transcription start site mapped by 5'-RACE. Transcribed sequences are in bold. Putative transcription factor binding sites conserved at similar positions in human and mouse species are underlined. In the p66 promoter, CpG residues analyzed with the sodium bisulfite genomic sequencing technique (see below) are stretched and shown in bold. E, graphical representation of the alignment of the human and mouse genomic regions containing the putative p46/p52 and p66 promoters. Each vertical bar represents an identical base in the mouse-human alignment. Gray boxes indicate exons. Arrows specify the transcribed strand. Human and mouse CpG islands are indicated by dotted curves.

FIG. 4. p66Shc expression can be induced by deacetylase inhibitors and demethylating agents. A, 32D cells were treated for 20 h with TSA at the indicated concentrations. Lysates were resolved by SDS-PAGE and analyzed by Western blotting using an anti-Shc monoclonal antibody. B, 32D cells were treated with 20 nM TSA and analyzed, at the indicated time points, by Western blotting using anti-Shc (upper panel) or anti-vinculin (lower panel) antibodies. C, reverse transcription-PCR using primers specific for p66Shc (top panel), p46/p52Shc (middle panel), and β -actin (bottom panel) on total RNA from 32D cells treated with 20 nM TSA, as indicated. D, RNase protection assay on total RNA from TSA-treated 32D cells using a riboprobe that distinguishes between p66Shc and p46/p52Shc transcripts. E, human PBL or MDA-MB-361 cells were incubated for 20 h (MDA-MB-361) or 24 h (PBL) with 20 nM TSA or mock treated with carrier (ethanol), lysed, and analyzed by Western blotting using an anti-Shc antibody. F, 32D cells were treated with 20 nM TSA and/or 1 μ M 5-aza-dC, as indicated, lysed at the indicated time points, and analyzed by Western blotting using anti-Shc antibody.



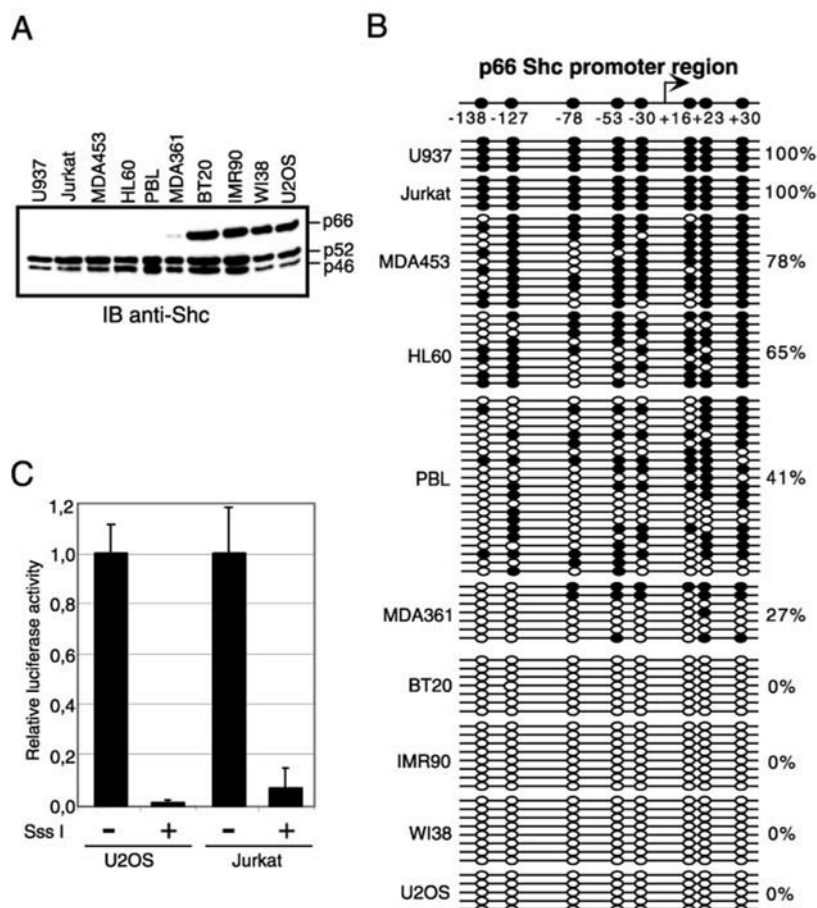
tions -434 to +101 showed a strong promoter activity, which, again, was orientation-dependent (Fig. 2, C and D). To restrict the promoter region further, we tested two shorter fragments: -199/+101 and -434/-199. As shown in Fig. 2C, only the -199/+101 fragment, which retains the p66Shc transcription start site, retained promoter activity. These results demonstrate the existence of an alternative promoter positioned in the first intron of the Shc locus.

Alignment of human and mouse sequences revealed a high degree of conservation around the p46/p52 and p66Shc promoters (74% identity for -241/+37 and 73.7% for -199/+101). In both species, the p46/p52 promoter lies within a CpG island, in good agreement with its constitutive activity (Fig. 2E). Additionally, the p66Shc promoter, although not contained within a CpG island, is located in a region with a high GC content (about 60%). Computational searches for regulatory sequences revealed that neither the p46/p52 nor the p66Shc promoter contains an identifiable TATA box, placing them in the TATA⁻/Inr⁺ class of promoters (19). Several putative binding sites for transcription factors were identified. The p46/p52 promoter contains four CAAT boxes, one GC box/Sp1, two estrogen receptors, and one ELK1. In the p66Shc promoter region there are two GC box/Sp1 sites, one CAAT box, one hepatocyte nuclear factor-3/forkhead homolog, and one AP1 site (Fig. 2, B and D). Finally, sequence similarity searches of the human and mouse Shc sequences revealed the presence of the first exon of the CDC kinase subunit 1 (Cks1) gene, as close as 362 bp 5' to Shc exon 1. The Cks1 gene is located in the opposite orientation, therefore suggesting that the Cks1 and Shc p46/p52 promoters are located head to head within 362 bp of genomic sequences.

p66Shc cell type-specific expression is likely to be regulated at the transcriptional level, as suggested by the lack of p66Shc transcripts in cells that do not express the protein (Ref. 5; see also Fig. 1, D and E, and data not shown). To test this hypothesis, we transiently transfected the p66 and p46/p52 promoters

into U937 and Jurkat cells, which express p46/p52 and lack detectable p66Shc transcripts and polypeptides (Fig. 1E and not shown). Results revealed that both promoter constructs are active in these cell types (Fig. 3). Although this is expected for the p52/46 promoter, the activity of the p66Shc promoter constructs in cells lacking endogenous p66 expression suggests that either it does not contain relevant regulatory sequences or that it is physiologically regulated by epigenetic changes. To test the latter hypothesis, we investigated the effects of histone deacetylase inhibitors or demethylating agents on the expression of p66Shc. Histone deacetylation has been shown to correlate with transcriptional repression, and a body of experimental evidence indicates that histone deacetylases are recruited to methylated promoters by methylcytosine-binding proteins (20, 21). 32D cells, which are immortal hematopoietic precursors lacking p66Shc expression, were treated with the histone deacetylase inhibitor TSA and analyzed for p66Shc expression at the protein and RNA levels. Dose response experiments showed that a 24-h treatment with 20 nM TSA results in induction of p66Shc protein expression (Fig. 4A). Time course experiments (Fig. 4B) showed that the p66Shc protein becomes detectable after 12 h, and its levels peak after 16–20 h of treatment with 20 nM TSA. Semiquantitative reverse transcription-PCR revealed that the p66Shc mRNA is not detectable in untreated 32D cells, although it becomes readily detectable after 8 h of TSA treatment and peaks after 12 h, with a kinetics that is consistent with that of the protein (compare Fig. 4, B and C). Similar results were obtained by RNase protection, using a riboprobe designed to discriminate the p66 and p52/46Shc mRNAs (Fig. 4D). Treatment with TSA was also shown to induce the expression of p66Shc in PBL and in the human breast cancer cell line MDA-MB-361 (Fig. 4E), thus indicating that histone deacetylases are involved in p66Shc silencing in primary (PBL), immortalized (32D) and transformed (MDA-MB-361) cells. TSA treatment caused also a slight, yet consist-

FIG. 5. p66Shc expression and promoter methylation. *A*, immunoblot analysis of Shc expression in a panel of human cell lines and primary cells. Lysates prepared from the indicated cell lines were immunoblotted with an anti-Shc antibody. *B*, genomic DNA was extracted from the indicated cells and subjected to bisulfite genomic sequencing. Numbers indicate the position of the CpG residues analyzed relative to the p66Shc mRNA transcription start site (arrow). Horizontal lines represent individual sequenced alleles. Filled and empty circles indicate methylated and nonmethylated cytosines, respectively. Numbers in parentheses indicate the fraction of methylated cytosines found in each cell type. *C*, the p66Shc promoter (−199/+101) was methylated *in vitro* using the CpG methylase SssI, and its activity was assayed 36 h after transfection in U2OS or Jurkat cells, as indicated. For comparison, the activity of the mock treated DNA was set to 1. Experiments were performed in triplicate, and error bars indicate ± 1 S.D.



ent, reduction of the p46/p52 mRNA (Fig. 4, *C*, middle panel, and *D*). Although the underlying mechanism(s) is presently unknown, this finding suggests that the p52/46 promoter is negatively regulated by an histone deacetylase-sensitive factor. Alternatively, derepression of the downstream p66Shc promoter and assembly of the transcription machinery might interfere with the elongation of the p46/p52 transcript.

To investigate the role of cytosine methylation, 32D cells were then treated with the demethylating agent 5-aza-dC. Fig. 4*F* shows that a 44-h treatment with 1 μ M 5-aza-dC was sufficient to induce p66Shc expression. A shorter (20-h) treatment was ineffective, in agreement with the requirement of DNA replication for 5-aza-dC to be effective. 5-Aza-dC does not seem to synergize with TSA on the p66Shc promoter because no further increase in the expression of p66Shc was observed when the two drugs were combined (Fig. 4*F*). It appears, therefore, that p66Shc expression can be induced by either histone deacetylase inhibitors or demethylating agents. Considering that histone deacetylases are involved in transcriptional repression of methylated DNA, these results suggest that the p66Shc promoter is regulated through methylation.

The p66Shc promoter contains a relatively high frequency of CpG dinucleotides (about 4% in a 200-bp scanning window), although not to the extent of being recognized as a CpG island by current methods (16). We examined the methylation status of eight CpG residues in the region comprising position −139 and +66 (indicated in Fig. 2*D*). Nine different cell lines and PBL from a healthy donor, expressing different amounts of p66Shc (Fig. 5*A*), were studied with the sodium bisulfite genomic sequencing method (15) (Fig. 5*B*). In the cell lines expressing high levels of p66Shc (WI38, U2OS, IMR90, and BT-20), all the CpG examined were unmethylated (Fig. 5*B*). Strikingly, among the cell lines not expressing detectable

amounts of p66Shc (U937, HL60, Jurkat, MDA-MB-453, and PBL), the fraction of methylated cytosines ranged between 41 and 100% (Fig. 5*B*). The MDA-MB-361 breast cancer cell line, which expresses low, but detectable, levels of p66Shc (Fig. 5*A*), showed an intermediate degree of cytosine methylation (27%) in the p66Shc promoter region. Finally, to provide direct evidence for the role of CpG methylation in transcriptional silencing of p66Shc, we evaluated the effect of *in vitro* methylation on p66Shc promoter activity. The −199/+101 construct was incubated with the SssI methylase, which selectively methylates CpG residues, and transfected in cells expressing (U2OS) or not expressing (Jurkat) p66Shc. In both cases, the activity of the methylated promoter was markedly lower than that of its unmethylated counterpart (Fig. 5*C*).

DISCUSSION

Here we describe the identification and initial characterization of two promoters responsible for the regulated expression of the three Shc isoforms. For the p66Shc promoter, we have identified epigenetic modifications, namely histone deacetylation and cytosine methylation, to be the mechanisms underlying transcriptional silencing of p66Shc in specific cell types. Notably, we show that the histone deacetylase inhibitors or demethylating agents are capable of restoring p66Shc expression in primary, immortal, and transformed cells. This is of particular relevance because similar results have been reported previously for the expression of other genes in transformed cell lines, in which the global pattern of DNA methylation and histone acetylation is consistently abnormal (22, 23).

Alternative promoters are a frequent feature of eukaryotic genes (24) and represent a mechanism to generate protein isoform diversity and to regulate their differential expression tightly (25–31). In the case of the human porphobilinogen

deaminase gene, for example, two distinct promoters are responsible for the generation of two isoforms, which differ at their N terminus and differentially are expressed (ubiquitously and in erythroid cells, respectively) (32).

Although the role of promoter methylation and histone deacetylation in gene silencing is well established (21), there are few reports showing that such mechanisms work on alternative promoters. Archey *et al.* (33) reported that the methylation status of an alternative promoter for the human transforming growth factor- β 3 gene correlates with its activity. Notably, similarly to our findings, the correlation between CpG methylation and promoter activity was evident in a region that is not part of a CpG island. A causal link between alternative promoter methylation and silencing of the respective isoform has been demonstrated recently for transcript A of the human RAS effector homolog (RASSF1) (34).

In the case of Shc, the existence of two promoters explains the different expression patterns of p52/46 and p66Shc, with ubiquitous expression of p52/46 and tissue-specific expression of p66Shc. The requirement for independently controlled p66Shc expression is even more compelling because this isoform is involved in a different signal transduction pathway with respect to p52Shc: p52Shc is implicated in coupling activated tyrosine kinases to Ras, thereby ensuring the transduction of growth and survival signals, and p66Shc is indispensable for the execution of oxidative stress-induced apoptosis (7).² Stable chromatin changes, such as those imposed by DNA hypermethylation, might therefore represent a permanent mechanism to silence p66Shc expression in tissues where apoptosis might be particularly harmful (such as the adult brain). More recently we have demonstrated that p66Shc is also involved in apoptosis of lymphoid cells, an event that is accompanied by marked up-regulation of p66Shc expression.³ Lymphocytes carry hypermethylated p66Shc promoter and do not express p66Shc. It is therefore possible that modifications of p66Shc promoter methylation might also occur in adult cells as an additional mechanism of p66Shc regulation.

It is also noteworthy that p66Shc promoter methylation correlates with p66Shc expression in a subset of breast cancer cell lines (Fig. 4). Promoter methylation has been frequently observed for tumor suppressor genes in cancer cells, where it represents an effective alternative to mutational inactivation (23). Loss of p66Shc expression in these cells could contribute to their transformed phenotype. First, because p66Shc has been shown to be required for oxidative stress-mediated apoptosis (7),² its loss could confer a growth advantage on tumoral cells. Second, loss of p66Shc could allow more effective mitogenic signaling by ErbB2. In fact, p66Shc negatively regulates tyrosine kinase signaling (5, 6), and an inverse correlation between p66Shc and ErbB2 expression has been reported in breast cancer cell lines (12, 13).

Evidence is accumulating that some of the methylation changes observed in cancer may initiate in subpopulations of normal cells as a function of age and increase progressively during carcinogenesis, suggesting that age-related methylation may be a fundamental marker of the field defect in patients with neoplasia (for review, see Refs. 23 and 35). In colon cancer, for example, a pattern of age-related methylation has been shown for several genes, including those for estrogen receptor, insulin-like growth factor II, N33, and MyoD, which progresses to full methylation in adenomas and neoplasms (36, 37). Con-

sidering the function of p66Shc in determining life span, variation of p66Shc promoter methylation may contribute to both aging and tumor development in specific tissues. Manipulation of p66Shc expression by chromatin modifiers, such as histone deacetylase inhibitors and demethylating agents, might therefore provide a mechanism to interfere with processes such as aging or tumorigenesis of given tissues.

Acknowledgments—We are grateful to Doreen Cantrell, Emanuela Colombo, Enrica Migliaccio, Mark Pearson, Giuliana Pelicci, and Veronica Raker for helpful discussion and to Sara Volorio, Mirko Riboni, and Lorin Bernard for sequencing of various DNA clones.

REFERENCES

- Luzi, L., Confalonieri, S., Di Fiore, P. P., and Pelicci, P. G. (2000) *Curr. Opin. Genet. Dev.* **10**, 668–674
- Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G., Schlessinger, J., and Pawson, T. (1992) *Nature* **360**, 689–692
- Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993) *Nature* **363**, 83–85
- Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Pawson, T., and Pelicci, P. G. (1992) *Cell* **70**, 93–104
- Migliaccio, E., Mele, S., Salcini, A. E., Pelicci, G., Lai, K. M., Superti-Furga, G., Pawson, T., Di Fiore, P. P., Lanfrancone, L., and Pelicci, P. G. (1997) *EMBO J.* **16**, 706–716
- Okada, S., Kao, A. W., Ceresa, B. P., Blaikie, P., Margolis, B., and Pessin, J. E. (1997) *J. Biol. Chem.* **272**, 28042–28049
- Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L., and Pelicci, P. G. (1999) *Nature* **402**, 309–313
- Kao, A. W., Waters, S. B., Okada, S., and Pessin, J. E. (1997) *Endocrinology* **138**, 2474–2480
- Cattaneo, E., and Pelicci, P. G. (1998) *Trends Neurosci.* **21**, 476–481
- Conti, L., De Fraja, C., Gulisano, M., Migliaccio, E., Govoni, S., and Cattaneo, E. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 8185–8190
- Conti, L., Sipione, S., Magrassi, L., Bonfanti, L., Rigamonti, D., Pettrrossi, V., Peschanski, M., Haddad, B., Pelicci, P., Milanese, G., Pelicci, G., and Cattaneo, E. (2001) *Nat. Neurosci.* **4**, 579–586
- Xie, Y., and Hung, M. C. (1996) *Biochem. Biophys. Res. Commun.* **221**, 140–145
- Stevenson, L. E., and Frackelton, A. R., Jr. (1998) *Breast Cancer Res. Treat.* **49**, 119–128
- Jackson, J. G., Yoneda, T., Clark, G. M., and Yee, D. (2000) *Clin. Cancer Res.* **6**, 1135–1139
- Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994) *Nucleic Acids Res.* **22**, 2990–2997
- Gardiner-Garden, M., and Frommer, M. (1987) *J. Mol. Biol.* **196**, 261–282
- Pacini, S., Olivieri, C., Di Somma, M. M., Isacchi, A., Lanfrancone, L., Pelicci, P. G., Telford, J. L., and Baldari, C. T. (1998) *J. Biol. Chem.* **273**, 20487–20493
- Lai, K. M., and Pawson, T. (2000) *Genes Dev.* **14**, 1132–1145
- Suzuki, Y., Tsunoda, T., Sese, J., Taira, H., Mizushima-Sugano, J., Hata, H., Ota, T., Isogai, T., Tanaka, T., Nakamura, Y., Suyama, A., Sakaki, Y., Morishita, S., Okubo, K., and Sugano, S. (2001) *Genome Res.* **11**, 677–684
- Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998) *Nature* **393**, 386–389
- Ng, H. H., and Bird, A. (1999) *Curr. Opin. Genet. Dev.* **9**, 158–163
- Antequera, F., Boyes, J., and Bird, A. (1990) *Cell* **62**, 503–514
- Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J. P. (1998) *Adv. Cancer Res.* **72**, 141–196
- Ayoubi, T. A., and Van De Ven, W. J. (1996) *FASEB J.* **10**, 453–460
- Nolan, K., Lacoste, J., and Parsons, J. T. (1999) *Mol. Cell. Biol.* **19**, 6120–6129
- Colbert, M. C., and Ciejek-Baez, E. (1988) *Dev. Biol.* **130**, 392–396
- Dhulipala, P. D., Lee, L., Rao, V. N., and Reddy, E. S. (1998) *Oncogene* **17**, 1149–1157
- Flouriout, G., Griffin, C., Kenealy, M., Sonntag-Buck, V., and Gannon, F. (1998) *Mol. Endocrinol.* **12**, 1939–1954
- George, C. X., and Samuel, C. E. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 4621–4626
- Kraus, J., Woltje, M., Schonwetter, N., and Holtt, V. (1998) *FEBS Lett.* **428**, 165–170
- Ishikawa, Y., Bianchi, C., Nadal-Ginard, B., and Homey, C. J. (1990) *J. Biol. Chem.* **265**, 8458–8462
- Chretien, S., Dubart, A., Beaupain, D., Raich, N., Grandchamp, B., Rosa, J., Goossens, M., and Romeo, P. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 6–10
- Archey, W. B., Sweet, M. P., Alig, G. C., and Arrick, B. A. (1999) *Cancer Res.* **59**, 2292–2296
- Dammann, R., Li, C., Yoon, J. H., Chin, P. L., Bates, S., and Pfeifer, G. P. (2000) *Nat. Genet.* **25**, 315–319
- Ahuja, N., and Issa, J. P. (2000) *Histol. Histopathol.* **15**, 835–842
- Ahuja, N., Li, Q., Mohan, A. L., Baylin, S. B., and Issa, J. P. (1998) *Cancer Res.* **58**, 5489–5494
- Issa, J. P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., and Baylin, S. B. (1994) *Nat. Genet.* **7**, 536–540

² M. Trinei, M. Giorgio, L. Lafrancone, and P. G. Pelicci, unpublished results.

³ C. T. Baldari, unpublished results.