Supplementary Data.

Experimental Procedures

Cell culture and infections conditions.

Briefly, primary human mesothelial cells (HM) from patients that developed pleural fluid accumulation because of congestive heart failure were grown in tissue culture and infected with SV40 (S-HM). "Flat foci" developed 2-3 weeks from infection and tridimensional foci were observed 4-6 weeks from infection. Tridimensional foci were hand picked with a Pasteur glass pipette (one focus only from each 5 cm dish), transferred to a 96 well plate, expanded, and established in tissue culture. Each of the 7 cell lines (S-HML) described in this paper was derived from a different patient. These 7 S-HML appear immortal because they grew for > 100 tissue culture passages, and express telomerase (35). S-HML have a transformed phenotype in that these cells show loss of contact inhibition, grow in soft agar and in no serum and invade a matrix gel. The studies were performed on S-HML1. In addition cell lines S-HML 4,5,6,7,10,12 were used to verify the reproducibility of different results as indicated in figure legends. All cell lines were fingerprinted using the GenePrint fluorescent STR system (Promega, Madison, WI).

Antibodies. We used AB-597 mouse monoclonal specific for the SV40 VP1 (a generous gift from F.J. O'Neill; Salt Lake City, UT), mouse monoclonal PAb 419, specific for Tag (Calbiochem), rabbit polyclonal D-20 specific for RXR, mouse monoclonal C4 specific for TRα1 and mouse monoclonal 27 specific for histone H1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal

MAB374 specific for glyceraldehyde-3-phosphate dehydrogenase (Chemicon) was used as loading control.

Transfection, Northern blotting and Southern blotting experiments. Before transfection cells were handled as described (12). 5 x 10^6 cells in 0.8 ml of RPMI were transfected with 10 μg of DNA by electroporation using a Gene Pulser II apparatus (BioRad, Hercules, CA). Efficiency of transfection of different S-HML was >90% and 20-40% in HM (as determined by transfecting cells with pGreenLantern2, Life Technologies, Gaithersburg, MD, and scoring green fluorescent cells 24 hr after electroporation by FACS). Total cellular RNAs were obtained using the RNeasy kit (Qiagen, Valencia, CA). 40 μg of total RNA from each sample were run on 0.8% formaldehyde-MOPS agarose gels, capillary-transferred onto nylon membranes (Amersham, Piscataway, NJ), hybridized with ^{32}P-labelled SV40 probes following standard protocols. The probe for the early mRNAs (early-probe) consisted of a 2,473 bp PCR amplification of the SV40 early genes (positions 5163-2690 of the SV40 genome). The probe for the late mRNA (late-probe) consisted of a 2,258 bp PCR amplification of the SV40 late genes (positions 335-2593). Southern blot analyses were performed according to standard protocols using a ^{32}P-labeled, *Hind* III-digested SV40 DNA as probe.

In situ hybridization. Briefly, cells grown on glass histochemistry slides were fixed in 10% buffered formalin for 24 hr, extensively rinsed with PBS, then incubated with PBS containing 10 μg/ml RNAse A for 1 hr at R.T. Protease digestion was performed using 2 mg/ml pepsin in PBS at R.T. for 5'. After denaturation at 95°C for 2', hybridization was carried out in 100 μl of buffer (10%

formamide, 7.5% dextran sulfate, 2X saline sodium citrate, 4 nmol/ml of biotinylated oligos) for 2hr at 40°C in a humidified chamber. The probe was an equimolar mixture of two 5'-biotinylated oligos (5'-TCCTCCTTTTATGACGAGCTTTGGCACTTGCACTGG-3' and 5'-CACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATG-3') complementary, respectively, to positions 1565-1600 and to positions 2554-2592 of the SV40 late region. A 36-mer, 5'-biotinylated oligonucleotide containing random bases was used in parallel experiments as the negative control. After hybridization, slides were washed in PBS containing 150 mM NaCl and 2.5% Bovine Serum Albumin (BSA) pre-equilibrated at 45°C. Slides were then incubated for 30' at R.T. with HRP-conjugated Streptavidin in PBS containing 2.5% BSA. Color was developed using the DAB peroxidase substrate kit (Vector Laboratories, Southfield, MI). Slides were then inspected by bright field microscopy and photographed.

Fluorescence In situ Hybridization (FISH). Preparation of metaphase spreads from Focus 1 cells was performed using standard cytogenetic procedures. A plasmid containing the early region of the SV40 genome was biotinylated by random priming in a reaction containing 600 ng DNA, in a total volume of 100 μl using the Bioprime DNA-labeling system (Invitrogen). Hybridization of probe to metaphase spreads and detection of FISH signals were carried out as described (Supplementary reference 1). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole and observed with a Zeiss Axioplan epifluorescence microscope equipped with a cooled charge-coupled device camera operated by a Metasystems Workstation.

Plasmid construction, luciferase and RNAi assays. We constructed plasmids +1Luc and +55Luc in which a firefly luciferase gene was either under the control of the entire SV40 regulatory region (late orientation, plasmid +1Luc), or replaced the VP-2 gene (plasmid +55Luc). The entire SV40 regulatory region was PCR-amplified using primers 5'- GGGCTCGAGCTTTGCAAAGCTTTTTGC- 3' (primer A, underlined sequences are homologous to SV40) and 5'- GGGTCATGATGGCCTGAAATAACCTCTG- 3' (underlined sequences are homologous to SV40). The resulting PCR product was cloned into the *Xho* I and *Nco* I sites of pGL2 (Promega, Madison, WI) to give rise to plasmid +1Luc. A 640 bp PCR fragment containing the entire SV40 regulatory region, the agnoprotein gene, and the late intron including position 561 (one nucleotide upstream from the VP-2 ATG) was amplified using primer A (see above) and 5'- GGGTCATGAGGACCTGAAATAAAAGAC- 3' (underlined sequences are homologous to SV40) and cloned into the *Xho* I and *Nco* I sites of pGL2 (plasmid +55Luc). These two plasmids were independently transfected into different HM and S-HML (renilla luciferase was the internal control) and the results were evaluated using a Veritas (Turner Biosystems, Sunnyvale, CA) luminometer.

silencing RNA (siRNA) used for downregulation of Drosha was performed by electroporating 1 nmol/10^6 cells of the Hs_RNASE3L_4 HP siRNA (Qiagen). Control siRNA was also from Qiagen. Quantification of the Drosha mRNA was done using real time RT-PCR using SYBR green incorporation (see above) and the Hs_RNASEN_1_SG QuantiTect Primer Assay (Qiagen).

5-Aza-2'-deoxycytidine, Trichostatin A, 9-cis-retinoic acid and T_3 treatments. Different S-HML were cultured in the presence of a range of concentrations of either the demetylating agent 5-Aza-2'-deoxycytidine (0 to 50μM) or the Histone Deacetylase inhibitor Trichostatin A (o to 1 mg/ml) for three days. Alternatively, S-HML cells were treated with different concentrations (from 0 to 0.1 μg/ml) 3,3',5-Triiodo-L-thyronine (T_3) or 9-cis-retinoic acid (from 0 to 1 ng/ml) for 48 hr. All chemicals were from Sigma. Subsequently, the tissue culture media and the treated cells were handled as described (12). Briefly, tissue culture media were centrifuged at 100,000 g for 3 hr then the supernatant was discarded. Pellets were suspended in 100 μl of buffer A (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% SDS, 50 mM 2-mercapto-ethanol). After normalizing the volumes for number of cells, this suspension was loaded on SDS-PAGE and blotted. Cells were dissociated with trypsin/EDTA, washed three times in tissue culture medium containing 10% FBS and pelletted. Pellets were lysed in buffer A, and after protein quantification by Bradford assay the lysates were loaded on SDS-PAGE. VP-1 expression in the tissue culture media and in treated cells was measured by Western blot analysis.

Antisense TRα1 and shRXR. The complete cDNA for TRα1 was obtained by PCR using a liver cDNA library as the template and oligos 5'-ATGGAACAGAAGCCAAGC-3' (corresponding to the first 18 bases of the TRα1 mRNA starting from the initiation codon) and 5'-TTAGACTTCCTGATCCTC-3' (complementary to the last 18 positions of the TRα1 mRNA). The 1233 bp amplification product was purified from the gel, its ends were blunted using T4

DNA polymerase, and cloned into the *Hind* III site (after blunting the *Hind* III 5' protruding ends using T4 DNA polymerase) of pGreenlantern 2 (and therefore downstream from a Green Fluorescent protein (GFP). Recombinant plasmids-bearing colonies were screened by PCR to check for orientation of the inserts, and one plasmid carrying the TRα1 cDNA cloned in antisense orientation was amplified to give rise to plasmid pANTITR (which includes a GFP gene under the control of a CMV promoter and, downstream from it, an antisense cDNA for TRα1).

The oligonucleotide 5'-GATCCCGACATGGCTTCCTTCACCAAGCACATCTGCGAAGCTTGGCAGATGTGCTTGGTGAAGGAAGCCATGTTTTTTT- 3' was annealed to oligonucleotide 5'-CTAGAAAAAAACATGGCTTCCTTCACCAAGCACATCTGCCAAGCTTCGCAGATGTGCTTGGTGAAGGAAGCCATGTCGG-3' and ligated into the *Bam* HI and *Xba* I sites of pGE1 plasmid of the GeneEraser system (Stratagene). This plasmid (named shRXR) expressed a small hairpin RNA targeting positions 376-405 of the RXRα mRNA (numbering starts at the mRNA initiation codon).

Bisulfite sequencing experiments. Methylation-specific PCR conducted on Mbo I-digested, S-HML-extracted SV40 DNA was performed as described (Supplementary ref. 2). PCR products were then used for DNA sequencing.

RNA polymerase II quantitative chromatin immunoprecipitation. 80% confluent S-HML1 cells on three 15-cm plates were cross-linked by incubation with 20 ml of 1% formaldehyde in cell medium (5% FBS in DMEM) for 10 min at

RT. The cross-linking reaction was stopped by addition of glycine to a final concentration of 0.125 M. Cells were then washed with phosphate-buffered saline, resuspended in 2 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.0]) and subjected to sonication with a Branson Sonifier 250 (Danbury, CT) to obtain DNA fragments ≤ 600 bp in length. After sonication, cellular debris was removed by centrifugation and the clear lisate was diluted 1:10 with immunoprecipitation buffer: (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.0], 167 mM NaCl). Subsequently, the chromatin was precleared with protein A agarose beads. For the evaluation of the chromatin input, 50 µl of the cleared chromatin was subjected to crosslinking reversal and DNA was extracted for agarose gel analysis and quantitative PCR. For the Pol II chromatin IP, 10 µg of rabbit polyclonal antibody H-224 (Santa Cruz Biotechnology, Santa Cruz, CA) was added to 5 ml of the chromatin mixture, and incubated at 4°C O/N on a rocking platform. As a negative control, a non-immune rabbit antibody was used as the primary antibody.

Protein A agarose beads (Upstate, Billerica, MA) pre-coated with Salmon Sperm DNA were added to the Pol II IP solution and control. After a 2 hours incubation at 4°C, the beads were collected by centrifugation and washed four times with 1 ml of Low Salt washing solution (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.0, 150 mM NaCl), once with a High Salt washing solution (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.0, 500 mM NaCl) , once with a LiCl solution (0.25 M LiCl, 1% NP-40, 1% SDC, 1 mM EDTA, 10 mM Tris-HCl pH8.0) and twice with a TE solution (10 mM Tris-H, pH 8, 1 mM

EDTA, pH 8). Bound chromatin was eluted from the agarose beads by incubation with a 1% SDS, 0.1 M NaHCO3 solution at room temperature for 30 minutes on a rotating platform. The eluates from the Pol II immunoprecipoitation and control were incubated at 65 °C O/N for crosslinking reversal. DNA was isolated through a Qiaquick PCR purification Kit (Qiagen). For the quantitative PCR, 12 couples of primers (L1F and L1R, L2F and L2R, L3F and L3R, L4F and L4R, L5F and L5R, L6F and L6R, E7F and E7R, E8F and E8R, E9F and E9R, E10F and E10R, E11F and E11R, E12F and E12R, see Supplementary Table S1) were used to amplify SV40 DNA from the Pol II immunoprecipitation and input chromatin samples. The relative Pol II occupancy along the SV40 genome was determined calculating the ratio of Pol II antibody-precipitated DNA to input chromatin DNA (we measured the 18S ribosomal DNA; sequences of primers are listed in Supplementary Table S1). DNA obtained from the pre-immune antibody control was routinely less than 5% of the equivalent Pol II-immunoprecipitated DNA (18S sequences of the input DNA were used as a normalization factor, while pre immune serum was used as the negative control).

SV40 late mRNAs: half-life measurements. Confluent cell layers were expanded 1:2 in T75 flasks. 18 hours later, cells received medium containing α-amanitin and were pre-incubated at 37°C for 15', dissociated with trypsin/EDTA, harvested, and snap frozen (time point 0, and subsequent time points). Total cellular mRNA was obtained using the RNeasy kit (Qiagen) in the presence of RNAse-free DNAse I. The concentration of RNA in each sample was measured by using a spectrophotometer (GE Healthcare, Uppsala, Sweden), and the

quality of the mRNA was assayed in 1% formaldehyde agarose gels. 2 μg of total RNA from each sample were reverse transcribed using a first strand synthesis kit (MBI-Fermentas, Hanover, MD), in the presence of 10 pmol of each of the following primers: 5'- CGCCACAAGCCAGTTATCCC-3' (complementary to the human 28S rRNA gene), and 5'- CTGCATTCTAGTTGTGG (complementary to the last 17 bases of the VP-1 mRNA upstream from the VP-1 termination codon). The amount of VP-1 mRNA in each sample was then determined by Real Time PCR using a Gene Amp 5700 (PE-Applied Biosystems, Wellesley, MA). Real Time PCR was performed as follows: 1/5 of the reverse transcription reaction from each sample was diluted serially in H_2O to determine the optimal range of dilution for the samples (C_T between 15 and 25). We used the following combination of primers and probes (Applied Biosystems): VP-1 mRNA was quantitated using primers 5'-ACAGGACCAAATATCCTGCTCAAA-3' and 5'-CAGCCTTGTGGTCAGTGTTCA-3'; the probe for this pair of oligos was 6FAM-AACCCCAAAAAATGCTACAGTTGACAGTCAGC-TAMRA. 28S rRNA was quantitated using primers 5'-GAATCCGCTAAGGAGTGTGTAACA-3' and 5'-CTCCAGCGCCATCCATTT-3'; the probe for this pair of oligos was 6FAM-CTCACCTGCCGAATCAACTAGCCCTG-TAMRA. Oligos-probes combinations were chosen using the Primer Express 1.0 software (Applied Biosystems). After estimating the sample with higher levels of both VP-1 and 28S RNAs, 1:2 serial dilutions were made in H_2O (range 1 to 128) to construct a calibration curve for both VP-1 and 28S RNAs. Similar calibration curves were run with each experiment, as described (39).

Name	Sequence (5’-3’)	Position on SV40 DNA
L1F	GGTTCGTAGGTCATGGACTGAA	373…394
L1R	CACAAAATTGCAGCAAAAGCTC	434…455

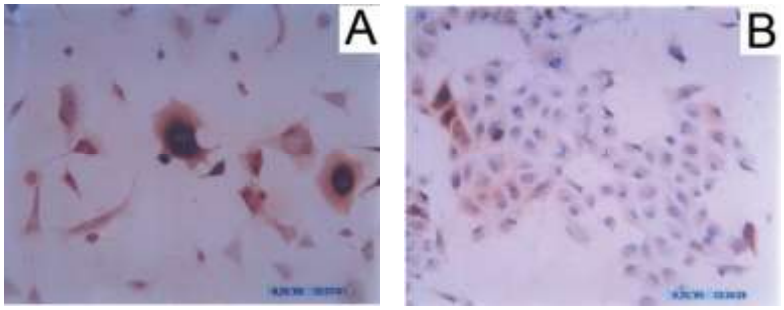
L2F	GCCTAACAACCTCTGAGGCAAT	710…731
L2R	TCACACCAGTCACAGTTTGCAG	811…832
L3F	TAACTGAGAGGTGGGAAGCTCAA	1334…1356
L3R	ATCCACTGAGGAGCAGTTCTTTG	1423…1445
L4F	TAAATCCTCAAATGGGCAATCC	1659…1680
L4R	GGCAAAGGAATTCTAGCCACAC	1773…1794
L5F	GCAACCACAGTGCTTCTTGATG	2225…2246
L5R	GGTAAACAGCCCACAAATGTCA	2299…2320
L6F	ACCCTTAGAAAGCGGTCTGTGA	2372…2393
L6R	CACCCTCTGTGTCCTCCTGTTA	2434…2455
E7F	AGCCTTGGGACTGTGAATCAAT	2810…2831
E7R	ATGATGATGAAGACAGCCAGGA	2896…2917
E8F	TACAAATCTGGCCTGCAGTGTT	3192…3213
E8R	GGGATTATTTGGATGGCAGTGT	3304…3325
E9F	TGAATCCATTTTGGGCAACAA	3609…3629
E9R	GGTTCTACAGGCTCTGCTGACA	3668…3689
E10F	TCATCACATTTTGTTTCCATTGC	3967…3989
E10R	AAAGTTTGCCAGGTGGGTTAAA	4060…4081
E11F	AAGGAAAGTCCTTGGGGTCTTC	4400…4421
E11R	GGTGGAATGCCTTTAATGAGGA	4516…4537
E12F	ACTCAGGCCATTGTTTGCAGTA	4857…4878
E12R	AACCTGACTTTGGAGGCTTCTG	4931…4952
18SF	TGATTAAGTCCCTGCCCTT	N.A.
18SR	TCAAGTTCGACCGTCTTCTCAG	N.A.
cdc2F	AGAAATACTTGGATTCTATC	N.A.
cdc2R	GAGTGTTACTACCTCATGTG	N.A.
GAPDHF	ACCATGGAGAAGGCTGGGGC	N.A.
GAPDHR	GATGGCATGGACTGTGGTC	N.A.

Supplementary Table S1. List of primers used for ChIP and for strand-specific quantitative RT-PCR (see text Fig. 3 and 6, and supplementary Fig. S5). Primers L1R, L2R, L3R, L4R, L5R and L6R were used to prime late “sense” RNA; primers L1F, L2F, L3F, L4F, L5F and L6F were used to prime late “antisense” RNA. Primers E7F, E9F and E11F were used to prime early “sense” RNA, and

primers E7R, E9R and E11R to prime early “antisense” RNA. As a negative control, the same reactions without reverse transcriptase were set up to detect any residual genomic DNA contamination in the RNA template. Additionally, putative mispriming was analyzed by PCR amplifying 18S rRNA, glyceraldehyde 3-phosphate dehydrogenase and β-actin. In these conditions we PCR amplified SV40 sequences only. After inactivation of the reverse transcriptase at 70 °C for 15 minutes, one fifth of each reaction was subsequently used for Sybr green-based quantitative PCR (kit “Power SYBR® Green PCR Master Mix”; Perkin Elmer, Waltham, MA) in a ABI 7300 thermocycler (Applied Biosystems). The same primers listed above were used for the primer couples in the quantitative PCR (qPCR) reactions. Each sample from the RT reaction was primed in the qPCR reaction with the same primer used for RT plus the corresponding “opposite orientation” primer (see Supplementary data, Fig. S5).

Additional Results.

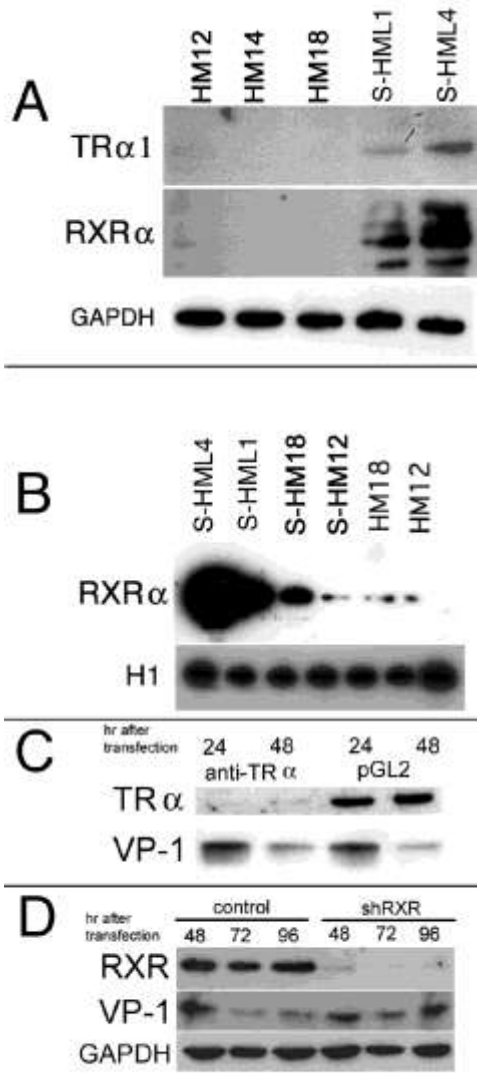
Immunohistochemistry consistently showed that two weeks after SV40 infection nearly 100% of S-HM expressed VP-1 (Fig. S1). At the same time small clusters of nearby morphologically transformed cells became microscopically detectable (cells appeared smaller, with increased nuclear/cytoplasmic ratio and grew densely packed; Fig. S1). These clusters of cells (usually referred as flat foci) generally develop into a tridimensional focus of transformed cells that becomes visible by the naked eye about 4 to 6 weeks after SV40 infection. “Early” SV40-transformed HM stained negative for VP-1 at the stage of flat foci (Fig. S1).



Supplementary Fig. S1. SV40 late gene silencing is established early during the process of HM transformation. VP-1 immunostaining of representative primary S-HM cells 2 weeks after SV40 infection. (*A*), infected HM. (*B*), flat focus of transformed S-HML. Note that the two panels represent two fields of the same slide. Original magnification 400 X. Nuclei were lightly counterstained with hematoxylin.

Supplementary Fig. S2. Titration of the expression levels of SV40 DNA in S-HML1. Lanes: 1 through 8, dilutions (from 64 to 0.5 ng) of purified SV40 DNA linearized with *Bam* HI; 9 and 10, two independent SDS/alkali extractions of SV40 DNA (then linearized with *Bam* HI) from 5 x 10^6 S-HML1 cells. Intensity of bands was measured using a phosphoimager. This experiment indicated that S-HML1 contains an average of 56 copies of SV40 DNA/cell. Other S-HML contained up to 1,000 copies of SV40 DNA/cell.

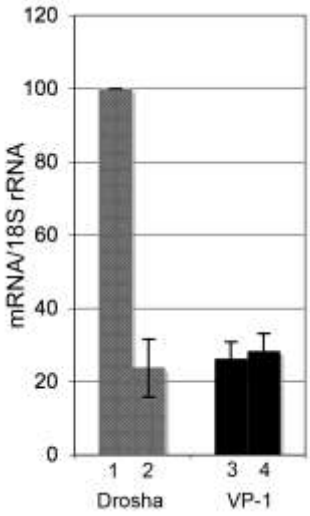
Is transcription of the late gene inhibited in S-HML by DNA methylation and/or by known transcriptional repressors? We treated S-HML with demethylating agents and with Histone Acetyl Transferase inhibitors. We artificially down regulated the two major transcriptional repressors of the late promoter TRα and RXRα (Supplementary Fig. S3). We also treated S-HML with the cognate hormones of TRα and RXRα. We found that none of these strategies influenced the VP-1 expression levels in S-HML.



Supplementary Fig. S3. Late genes silencing in S-HML is not due to known transcriptional repressors of the SV40 late promoter. (*A* and *B*), S-HML cells overexpress SV40 late promoter inhibitors. (*A*) Western blot analysis of 50 μg of total protein extracts obtained from three independent HM cultures and from S-HML1 and S-HML4. GAPDH: Glyceraldehyde-3-phosphate-dehydrogenase. (*B*) RXRα expression levels in two S-HML**,** two S-HM (72 hr after SV40 infection) and in two HM. Western blot analysis was performed on 10 μg of nuclear

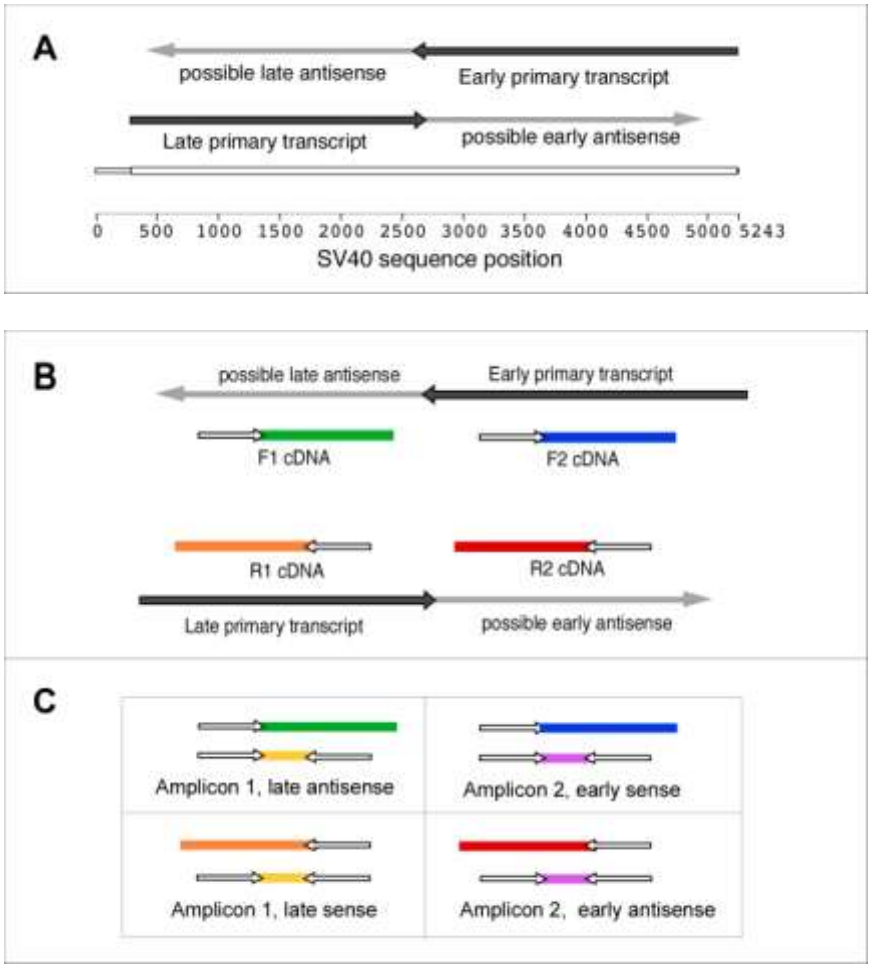
extracts. H1: histone H1. (*C and D*), neither TRα1 nor RXRα are responsible for SV40 late gene silencing in S-HML. (C), we constructed a recombinant plasmid expressing a Green Fluorescent Protein (GFP) mRNA that included, downstream of the GFP termination codon, the entire TRα1 cDNA sequence positioned in antisense orientation with respect to the TRα1 mRNA. Transfectants were sorted for Green fluorescence; the control plasmid was pGreenLantern 2 (the vector used to develop the antisense TRα1 plasmid). S-HML transfected with the anti-TRα1 plasmid displayed more than 10 fold reduction of the TRα1 expression levels both 24 and 48 hr after transfection compared to controls, but no differences in VP-1 expression were observed between the anti-TRα1 transfected S-HML1 cells and the same cells transfected with the control plasmid (note, the gel was over-expressed overnight to visualize the bands) (*D*), RNA interference targeting of RXRα using the GeneEraser system (Stratagene). We cloned in pGE-1 a 29 nucleotide long hairpin corresponding to a portion of the RXRα mRNA (shRXR). The manufacturer provides a pGE-control plasmid containing a hairpin with no homology to human sequences (control plasmid), which was used as the negative control. Transfectants were selected using G418. As shown in the Western Blot analysis, we attained a more than 10-fold reduction of RXRα expression 48 hr after transfection with the shRXRα compared to the control, and the RXR expression levels become undetectable 72 and 96 hr after transfection with the shRXRα. Nonetheless, no differences in VP-1 expression where observed between shRXRα and control, suggesting that neither TRα nor RXRα influenced VP-1 production.

Can Drosha downregulation restore VP-1 mRNA expression?



Supplementary Fig. S4. Artificial downregulation of the Drosha mRNA using RNAi does not rescue VP-1 mRNA expression. The Drosha mRNA was downregulated using a commercially available, certified miRNA (Qiagen, Valencia, CA). Cells were transfected (by electroporation) with both control and miRNA targeting Drosha (columns 1 and 2, respectively). 48 hr after transfections, cells were collected, total RNA was extracted from different transfectants in parallel. mRNA expression levels are expressed in arbitrary units normalized for amounts of 18S rRNA. Drosha mRNA levels of the control transfectant were set at 100, while the other values represent percentages of 100 (the histogram is the average of 3 independent experiments). Oligos to detect the VP-1 mRNA were LA1 and LA2 using standard Sybr-green quantitative RT-PCR reactions (see Materials and Methods). Colums 3 and 4: VP-1 mRNA expression in control miRNA and in anti-Drosha miRNA transfected cells, respectively.

Experimental strategy used to generate the Transcriptional map of the SV40 genome in S-HML, see text Fig. S5.

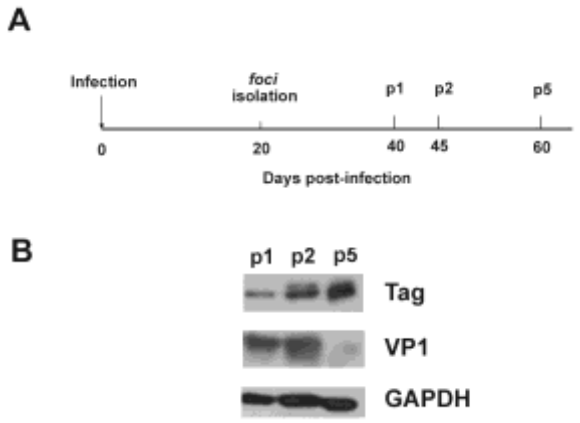


Supplementary Fig. S5. (*A*), scheme of the SV40 genome (represented in linearized form) with primary transcripts. (*B*), RNA from S-HML is reverse transcribed. Four independent reverse-transcriptase reactions are represented in the figure, with primers F1, F2, R1 and R2. (*C*), the amount of the specific cDNAs generated by RT is measured by quantitative PCR (qPCR). Four qPCRs are indicated with templates F1, F2, R1 and R2. qPCR uses the same primers used to reverse transcribe the sense and the antisense RNA. For example, F1cDNA (from the antisense) and R1 cDNA (from the

sense) (*B*) will be qPCR (*C*) using the same primers F1 and R1 to establish a ratio between sense and antisense in parallel reactions. Because the two PCR reactions are identical, the ratio can be accurately established and depends only on the amount of template.

Is late gene silencing observed in other human cell types?

To begin testing this hypothesis, we obtained primary human astrocytes from cadavers, established them in culture and infected these cells with SV40 using the same procedure described to infect HM (See above, page 1). Brain cells were chosen, because these cells, together with mesothelial cells, appear to be most susceptible to SV40 carcinogenesis (1). As described in Fig 1 A, 20 days post infection, morphologically transformed tridimensional foci were established in tissue culture and as soon as a 25 cm^2 flask was semi-confluent (40 days post infection, P1) the levels of Tag and VP-1 were measured by Western blot at day 40, 45 and 60. A total of 6 foci were established in tissue culture and in all of them we observed a progressive reduction in VP-1 expression and a parallel increase in Tag expression (Fig 6). These preliminary results, mirror those observed in HM, and suggest that late gene silencing may be a general phenomenon, at least in those types of human cells that are most susceptible to SV40 infection and transformation.



Supplementary Fig. S6. Tag and VP1 protein expression is progressively reduced in human astrocytes transformed by SV40 infection. (A) Scheme of post-infection time-course analysis of astrocytes transformed by SV40. (B) Immunoblotting of Tag and VP1 proteins at the indicated time points, using GAPDH as loading control.

Supplementary References.

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2. Paulin R, Grigg GW, Davey MW, Piper AA. Urea improves efficiency of bisulphite-mediated sequencing of 5'-methylcytosine in genomic DNA. Nucleic Acids Res 1998;26:5009-10.