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Polyomavirus tumorantigens have a profound effect on gene expression in mouse fibroblasts

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Polyomavirus (Py) large and small tumorantigens together are competent to induce S phase in growth-arrested mouse fibroblasts. The capacity of the large tumorantigen to bind the pocket proteins, pRB, p130 and p107, is important for the transactivation of DNA synthesis enzymes and the cyclins E and A, while the interference of small tumorantigen with protein phosphatase PP2A causes a destabilization of the cdk2 inhibitor p27, and thus leads to strong cyclin E- and cyclin A-dependent cdk2 activity. Py small tumorantigen, in addition, is able to transactivate cyclin A. Hence, this protein might have a much wider effect on gene expression in arrested mouse fibroblasts than hitherto suspected. This may have a profound part in the known capacity of Py to form tumors in mice. Therefore, it was interesting to gain an insight into the spectrum of transcriptional deregulation by Py tumorantigens. Accordingly, we performed microarray analysis of quiescent mouse fibroblasts in the absence and presence of small or large tumorantigen. We found that the viral proteins can induce or repress a great variety of genes beyond those involved in the S phase induction and DNA synthesis. The results of the microarray analysis were confirmed for selected genes by several methods, including real-time PCR. Interestingly, a mutation of the binding site for pocket proteins in case of LT and for PP2A in case of ST has a variable effect on the deregulation of genes by the viral proteins depending on the gene in question. In fact, some genes are transactivated by LT as well as ST completely independent of an interaction with their major cellular targets, pocket proteins and PP2A, respectively.
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Introduction

Polyomavirus (Py) can form solid tumors in many different organs of newborn mice and thus must be able to infect and replicate in a great variety of cell types (Benjamin, 2001). Such cells are in diverse stages of differentiation and are most frequently growth arrested. As the virus requires cells in the S phase for the replication of its own DNA, it must be able to drive the infected cells out of the quiescent state into the S phase. This is accomplished by the activity of three early viral proteins, the large, middle and small tumor antigens (henceforth abbreviated LT, MT and ST, respectively). LT is a pleiotropic protein that specifically binds to the viral origin of replication and carries helicase activity. In its replication capacity, it combines functions of the ORCs and the MCMs (Bell and Dutta, 2002) of the cellular replication machinery. In addition, it is the major protein involved in creating optimal conditions for virus replication in the host cell by interfering with the cellular mechanisms of growth control (Moran, 1993). It binds to the underphosphorylated form of the 'pocket proteins', p130, p107 and p110 the retinoblastoma protein (pRB), thereby obstructing the function of these proteins as negative regulators of members of the transcription factor family E2F. This leads to the transcription of a variety of genes whose products play a role in DNA replication (DNA synthesis- and precursor-producing enzymes (Ogris *et al*., 1993b; Mudrak *et al*., 1994) and in the initiation and propagation of the S phase (cyclins E and A, Schüchner *et al*., 2001). MT antigen is the major transforming protein of Py. It interacts with protein phosphatase 2A (PP2A) and several proteins at the inner surface of the cell membrane, thereby initiating signal transduction pathways, including antiapoptotic ones (Gottlieb and Villarreal, 2001). Therefore, it has been suggested that MT might serve to prevent apoptosis. In case of SV40, this is mediated by the LT antigen that binds and inactivates p53, a capacity lacking in Py LT protein (Pipas, 1992). In conjunction with LT antigen, ST of polyoma virus and of SV40 are essential for the initiation of the S phase in serum-starved Swiss 3T3 cells (Ogris *et al*., 1992) and human diploid fibroblasts (Porras *et al*., 1999), respectively. ST protein, like MT, interacts with the cellular protein phosphatase PP2A (Campbell *et al*., 1995), replacing the B subunit of the

trimeric enzyme with consequences for its activity and specificity. This function plays an important role in the activation of the cyclin E and cyclin A-specific protein kinase, cdk2. ST accomplishes this by causing a shift of the cdk inhibitor p27 to its phosphorylated form, which is followed by a rapid destruction of p27 by the ubiquitin–proteasome system (Schüchner and Wintersberger, 1999). Therefore, it is not surprising that ST is also mandatory for the tumorigenic transformation of human diploid cells (Hahn *et al*., 1999) together with LT protein, mutated Ras and telomerase (Yu *et al*., 2001; Hahn *et al*., 2002). In this context, it is however important to remember that ST antigens from Py and from SV40 were also found to contribute to the transactivation of cyclin A (Porras *et al*., 1996; Schüchner *et al*., 2001). ST protein from SV40 had previously been found to be capable of transactivation, but the earlier work concerned viral promoters (Loeken *et al*., 1988; Bikel and Loeken, 1992; Loeken, 1992). It was therefore of interest to find out if ST can more widely influence the transcriptional potential of cells.

It is noteworthy that both, LT and ST, evolved to interact with widely used regulators of cell growth, the pocket proteins and PP2A, respectively. These interactions assure that the requirements for virus multiplication can be provided but at the same time makes it likely that a variety of reactions, not necessarily linked to S phase induction, will also be deregulated by the viral proteins with unforeseen consequences for the cell. In order to apprehend the potential of PyT antigens to affect the transcriptional programme of mouse fibroblasts, we carried out a microarray analysis on gene expression in quiescent cells in the absence and in presence of the viral protein. We discovered that T antigens can induce or inhibit the expression of a surprisingly large number of genes. Examples of such genes were studied in more detail and the effect of ST on their expression was compared to that of the LT protein.

Results and discussion

Microarray analysis

We have previously produced cell lines (on the basis of murine Swiss 3T3 cells) that contain the information for Py LT or ST antigen under the control of the dexamethasone-responsive MMTV promoter (Ogris *et al*., 1992). These cells do not produce the viral protein if grown in the absence of the hormone. After addition of dexamethasone to a final concentration of 1 μmol/l, viral proteins become detectable after 4 h and reach high levels after about 8 h; levels of T antigen remain high up to 48 h after hormone addition. The level of viral protein reached under these conditions appears to correspond approximately to that produced in infected cells. Lower concentrations of dexamethasone lead to less viral protein, which in case of LT suffices for transactivation but is insufficient to allow the replication of a plasmid carrying a viral origin of replication (Ogris *et al*., 1993a). From this result we can exclude that our cells produce levels of viral protein that are far beyond those achieved in virus infection. This cell system enabled us to study gene expression within the same serum-starved, quiescent cells in the absence or in presence of T antigen. We used a mouse cDNA microarray containing about 17 000 cDNAs to search for genes whose expression is altered by PyST and PyLT. Cells were made quiescent by serum starvation for 72 h and the culture was then divided into two equal parts. One was used to isolate RNA from normal quiescent cells kept in the absence of T antigen, the second one received dexamethasone to 1 μmol/l to induce the viral protein and RNA was extracted 28 h later. This time point was chosen because earlier work had shown that it corresponds to the onset of the S phase in cells expressing ST and LT proteins together (Ogris *et al*., 1992). T antigen expression was determined prior to and after hormone addition in order to assure the expected absence (before hormone treatment) and presence (after hormone treatment) of viral protein (Figure 1). Swiss 3T3 cells containing no viral information served as a control for effects of dexamethasone that were not due to the activity of T antigen. Total RNA was used for the preparation of cDNA that was thereby labelled with Cy3-dUTP or Cy5-dUTP, respectively. The labelled cDNAs were used for hybridization in the microarray analysis. The experiment was

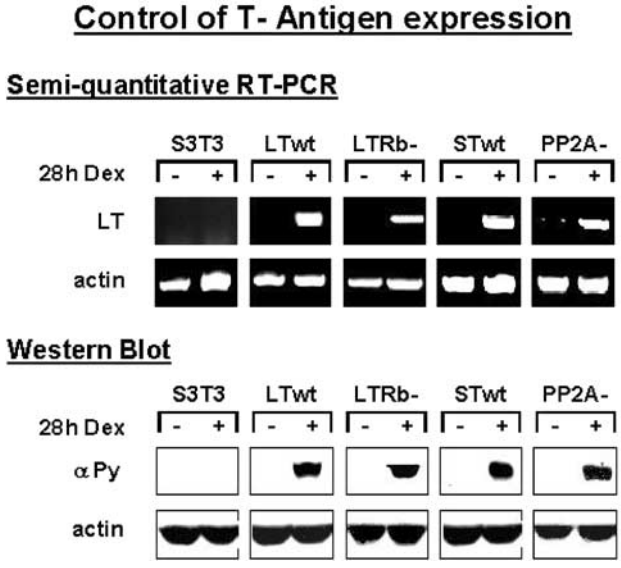

Figure 1 Control of T-antigen expression. Cells were arrested for 72 h and then incubated in the presence (+Dex) or in the absence (−Dex) of dexamethasone (1 μmol/l final concentration). RNA or protein was extracted from these cell cultures 28 h after induction of T antigen. Serum-starved Swiss 3T3 cells (plus or minus dexamethasone) were used as a negative control and β-actin served as a loading control. RT–PCR (upper part) was performed with total RNA from cells expressing (+Dex) or not expressing (−Dex) LT or ST antigen. Western blot analysis (lower part) of protein extracts using an antibody against the N-terminus of Py T antigens. 3T3 are Swiss 3T3 cells, LT are Swiss 3T3 cells expressing LT under the control of the MMTV promoter, ST are 3T3 cells expressing ST under the control of the MMTV promoter. LTRb-cells express a mutant of LT antigen (E146N) defective in binding pocket proteins; PP2A-cells express a mutant of ST protein (Ins107AL) defective in binding PP2A

carried out twice whereby color inversion was performed. Genes, whose expression was significantly changed solely by addition of dexamethasone to 3T3 cells in the absence of viral protein, were eliminated from our selection. Genes were considered as regulated when the presence of the viral protein resulted in a two-fold or higher up- or downregulation. Under these conditions, PyST was observed to upregulate 281 and to repress 242 of the genes represented by the microarray. The upregulated genes are listed in groups according to function in Table 1; the downregulated genes are shown in Table 2. The results of an identical microarray analysis carried out with cells expressing LT antigen are shown in Tables 3 and 4. LT expression resulted in an upregulation of 222 genes and a downregulation of 157 of the genes on our array. Microarray data on cells stimulated by addition of serum were carried out for comparison. Here, the time point chosen was 18 h, which according to FACS analysis corresponds to the onset of the S phase in serum stimulated cells, and hence approximates the 28 h point in quiescent cells expressing T antigens. At this time, serum addition induced the expression of 643 genes and repressed 537 genes on our array. (The data are available on request). Several genes induced or repressed by ST and LT were selected for confirmation and extension of the microarray data by detailed analysis. We first used semiquantitative reverse transcription–polymerase chain reactions (RT–PCR) or immunoblotting if antibody against the protein was available to confirm the effects of the viral proteins using the 28 h time point. Considering that this time point may not always reveal the optimal induction or repression accomplished by the viral protein, we then carried out real-time PCR reactions on RNA isolated 8, 18 and 28 h after induction of the viral protein. These series of reactions were carried out with cells expressing wild-type LT or ST protein as well as the LT mutant lacking an interaction with pocket proteins and an ST mutant defective in binding PP2A. Furthermore, the effect of dexamethasone on arrested Swiss 3T3 cells not carrying information for viral protein was also determined 8, 18 and 28 h after addition of hormone. For reasons of clarity, we show only those data for the mutants that correspond to the time points where optimal effects were attained with the wild-type proteins and we show only the longest, that is, the 28 h time point for 3T3 cells treated with dexamethasone. Moreover, for two upregulated genes, namely those coding for MCM6 and MCM7, promoter-luciferase constructs were produced and promoter activation was studied after transient co-transfection with T antigen.

Confirmation of microarray data for selected genes

Genes upregulated by viral proteins From the groups depicted in Tables 1 and 2, we first selected examples of genes whose products are involved in the progression of the G1 phase of the cell cycle and those that have a function during the S phase for further analysis. This selection included genes whose expression was deregulated to different degrees by the viral protein. Using in addition to the wild type, a mutant of ST unable to interact with PP2A, it was observed that this mutation affects the activity of ST on all of the genes examined, albeit to a variable degree, depending on the gene examined. For instance, while the induction of cyclin D1 and that of c-myc is reduced by 80–100% if the interaction site for PP2A was mutated, the transactivation of mcm6 and mcm7 is much less dependent on an interaction with PP2A. In this case, the PP2A-mutant exhibited 40% or more of the activity of the wild-type ST. It is likely that at least that part of the capacities of ST that does depend on an interaction with PP2A influences promoter activity indirectly, by deregulating signal transduction pathways (Sonntag *et al*., 1993; Frost *et al*., 1994) or by causing a more direct change in the phosphorylation status of transcriptional regulators. The situation is quite similar in the case of the dependence of LT functions on an intact binding site for pocket proteins except that in this case some of the genes chosen were deregulated completely independent of an interaction of LT with pocket proteins. It should be pointed out that in all experiments we used one and the same cell lines harboring the mutated versions of either ST or LT. The variability of the effect of the mutations therefore cannot be attributed to differences in the cell lines used. Rather, it indicates that ST as well as LT can transactivate or repress certain genes by a mechanism independent of an interaction with PP2A or pocket proteins, respectively. These observations on individual genes agree well with the data obtained on microarray analysis carried out with the PP2A mutant of ST and the pocket protein mutant of LT, respectively (available on request). These data revealed not only a reduced effect on divers genes that are up- or down-regulated by the viral proteins, but they in addition disclosed genes that are deregulated only if the major interaction of LT or ST was eliminated by mutation. While nothing is known of potential interactions of ST with cellular partners other than PP2A and chaperones of the dnaK type, interactions in addition to those involving pocket proteins and chaperones are known for LT. For instance, SV40 LT was reported to interact with components of the general transcription machinery (Damania *et al*., 1998). Although such an interaction was not so far shown or looked for in Py LT antigen, the functional similarity between SV40 and polyoma LT suggests that it may well exist. If so, it is likely to contribute to the transactivating capacity of this viral protein. Furthermore, LT proteins of both SV40 and polyoma virus were previously shown to bind the co-activator protein CPB/p300 (Eckner *et al*., 1996; Nemethova and Wintersberger, 1999), which again may influence gene expression independent of an interaction with pocket proteins.

While the cyclins E and A were found earlier to be transactivated by LT and ST, this study includes cyclin D1 as a further target for ST antigen. RT–PCR and real-time PCR (Figure 2) support the data obtained in the microarray analysis. This result is in accord with a report on SV40 ST protein, which was also observed to induce cyclin D1 expression in a manner dependent on

Table 1 Genes induced two-fold or higher by Py small T-antigen in cDNA microarray

GenBank	*Clone description*	*Fold induction*
Cell cycle regulators and DNA synthesis genes		
AI850048	Cyclin D1	2.53
AI323675	PCTAIRE-motif protein kinase 3	2.02
AI840227	Mini chromosome maintenance deficient 7 (*S. cerevisiae*)	2.01
AI854509	Mini chromosome maintenance deficient 6 (*S. cerevisiae*)	2.02
AI849847	Proliferating cell nuclear antigen	2.06
AI840542	RAD21 homolog (*S. pombe*)	2.30
Oncogenes, tumor suppressors, stress and apoptosis genes		
AI849916	Polymyositis/scleroderma autoantigen 1	4.54
AI841358	Heat shock 70 kDa protein 8	4.40
AI850962	Transducer of ERBB2, 2	4.39
AI845078	Small protein effector 1 of Cdc42	4.04
AI528587	c-mer proto-oncogene	3.88
AI841755	Aplysia ras-related homolog B (RhoB)	3.86
AI842320	Neurofibromatosis 2	3.37
AI844365	Bcl2-like	2.90
AI836493	Myelocytomatosis oncogene (c-myc)	2.88
AI848789	B-cell CLL/lymphoma 7C	2.70
AI838486	Heat shock protein, 105 kDa	2.67
AI852670	Cerebral cavernous malformations 1	2.39
AI836313	Heat shock protein, 86 kDa 1	2.18
AI844691	Tumor differentially expressed 1	2.16
AI845042	Annexin A5	2.13
AI842572	Rho, GDP dissociation inhibitor (GDI) beta	2.12
AI528760	Cold shock domain protein A	2.09
Genes involved in transcription, translation, immune response and degradation pathway		
AI841501	Ribosomal protein S3a	3.86
AI845964	Ribosomal protein S17	3.36
AI853670	Leucine zipper protein 1	3.24
AI464459	Cytokine inducible SH2-containing protein 2	3.19
AI835282	Eucaryotic translation elongation factor 1 beta 2	3.14
AI662267	Homeodomain interacting protein kinase 1	3.13
AI845514	ATP-binding cassette, subfamily A (ABC1), member 1	2.99
AI843614	Retinol binding protein 1, cellular	2.90
AI840032	Translationally regulated transcript (21 kDa)	2.86
AI836784	Splicing factor, arginine/serine-rich 5 (SRp40, HRS)	2.82
AI836484	RNA binding motif protein, X chromosome	2.78
AI835939	Eucaryotic translation elongation factor 1 alpha 1	2.57
AI852094	Polybinding protein, cytoplasmic 1	2.47
AI323595	B-cell receptor-associated protein 31	2.39
AI849117	Ribosomal protein L10A	2.37
AI413443	Ubiquitin-conjugating enzyme E2G 2	2.36
AI850137	Nucleolin	2.29
AI841821	Ia-associated invariant chain	2.26
AI844203	Eucaryotic elongation factor-2 kinase	2.21
AI851284	Interferon-related developmental regulator 1	2.19
AI850329	High mobility group box 1	2.18
AI839283	Inhibitor of DNA binding 3	2.10
AI852144	Pre-B-cell colony-enhancing factor	2.08
AI852911	Kruppel-like factor 15	2.07
AI847690	Ribosomal protein S4, X-linked	2.06
AI850396	Nuclear factor, erythroid derived 2,-like 1	2.04
AI323569	Zinc-finger homeobox 1a	2.03
AI836652	Ubiquitin B	2.01
Cell surface, signal transduction, cytoskeleton and metabolism proteins continuation		
AI853846	Fatty acid binding protein 5, epidermal	4.47
AI847285	Mitogen-activated protein kinase kinase kinase 6	4.41
AI845630	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	4.36
AI836976	Glutamate dehydrogenase	4.06
AI848830	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	3.97
AI835559	Thymopoietin	3.65
AI847745	Phosphatase and tensin homolog	3.51
AI839581	Porcupine homolog (Drosophila)	3.46
AI427514	Metallothionein 1	3.33
AI841644	Guanine nucleotide binding protein, beta 2, related sequence 1	3.24
AI838964	Syntrophin, acidic 1	3.16
AI845452	Procollagen, type XI, alpha 2	3.10

Table 1 (*continued*)

GenBank	*Clone description*	*Fold induction*
Cell surface, signal transduction, cytoskeleton and metabolism proteins continuation		
AI849502	Amyloid beta (A4) precursor-like protein 2	3.08
AI836893	Laminin receptor 1 (67 kDa, ribosomal protein SA)	3.08
AI853686	Glutamate receptor, ionotropic, AMPA3 (alpha 3)	3.01
AI851272	Dual specificity phosphatase 14	3.00
AI839368	Carbonyl reductase 1	2.97
AI845051	Beta-spectrin 3	2.93
AI845046	Trans-Golgi network protein 1	2.93
AI851776	Myosin Ic	2.90
AI840685	Lysosomal membrane glycoprotein 1	2.90
AI847962	Transmembrane 4 superfamily member 2	2.86
AI844634	Actin, alpha 1, skeletal muscle	2.82
AI849308	Glutaredoxin 1 (thioltransferase)	2.80
AI840364	Neural precursor cell expressed, developmentally downregulated gene 4b	2.77
AI840657	Silica-induced gene 81	2.75
AI844844	Receptor (calcitonin) activity modifying protein 2	2.75
AI385536	Cadherin 2	2.71
AI841041	Huntingtin-associated protein 1	2.64
AI854038	SH3 domain protein 5	2.61
AI836919	Crystallin, alpha B	2.60
AI845273	Aspartate-beta-hydroxylase	2.59
AI846934	Lipin 1	2.59
AI849894	Guanine nucleotide binding protein, beta 2	2.59
AI847216	Cytochrome *c* oxidase, subunit VI a, polypeptide 1	2.57
AI847216	Nucleophosmin 1	2.57
AI844017	Aldo-keto reductase family 1, member B3 (aldose reductase)	2.53
AI847207	Microspherule protein 1	2.52
AI843247	Dopamine receptor 4	2.48
AI450702	ATPase, Na+/K+ transporting, beta 3 polypeptide	2.47
AI850974	Phosphatidylinositol 3-kinase, reg. subunit, polypeptide 1 (p85 alpha)	2.46
AI854176	Muscleblind-like (Drosophila)	2.42
AI841957	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 6	2.39
AI838730	Popeye 3	2.37
AI851805	Platelet-derived growth factor, C polypeptide	2.31
AI325068	Solute carrier family 25 (mitoch. carrier; adenine nt translocator), member 5	2.29
AI839744	Growth arrest specific 1	2.28
AI851616	Pyruvate dehydrogenase E1alpha subunit	2.27
AI851427	Protein kinase, cAMP-dependent regulatory, type II beta	2.26
AI843104	Fibroblast growth factor inducible 14	2.26
AI854743	Adenylate kinase 3 alpha like	2.25
AI853659	Cut (Drosophila)-like 1	2.25
AI851564	Acetyl-Coenzyme A dehydrogenase, long-chain	2.24
AI843768	Amyloid beta (A4) precursor protein	2.23
AI427644	Epidermal growth factor receptor	2.23
AI836347	Tubulin alpha 2	2.22
AI852430	Peripheral myelin protein, 22 kDa	2.21
AI841311	Microtubule-associated protein 1 light chain 3	2.21
AI414231	Villin	2.19
AI842086	Basigin	2.18
AI840757	Tubulin alpha 1	2.18
AI840676	Beta-spectrin 2, nonerythrocytic	2.18
AI853695	Integrin-associated protein	2.18
AI852827	Catechol-*O*-methyltransferase	2.14
AI847067	Neural precursor cell expressed, developmentally downregulated gene 4a	2.14
AI835817	Thymus expressed acidic protein	2.13
AI849996	Latexin	2.12
AI844775	Thioredoxin	2.10
AI848212	Fumarylacetoacetate hydrolase	2.10
AI844421	Tissue inhibitor of metalloproteinase 2	2.10
AI841941	Tetratricopeptide repeat domain	2.10
AI845316	Stearoyl-coenzyme A desaturase 2	2.07
AI834834	G protein-coupled receptor, family C, group 5, member B	2.03
AI843656	Protein kinase C, delta	2.02
AI853012	Cystatin B	2.02
AI850110	WD repeat domain 1	2.00
AI839138	Upregulated by 1,25-dihydroxyvitamin D-3	2.00
152 ESTs with unknown function		

Table 2 Genes repressed two-fold or higher by Py small T-antigen in cDNA microarray

GenBank	*Clone description*	*Fold repression*
Cell cycle regulators and DNA synthesis genes		
AI323295	DNA-damage inducible transcript 3	2.17
Oncogenes, tumor suppressors, stress and apoptosis genes		
AI838312	Decorin	9.13
AI851574	Melanoma antigen, family D, 2	4.70
AI450810	Promyelocytic leukemia	3.81
AI842716	Cytochrome *P*450, 51	2.58
AI842612	Melanocyte proliferating gene 1	2.04
Genes involved in transcription, translation, immune response and degradation pathway		
AI854552	Interferon-induced protein with tetratricopeptide repeats 3	9.28
AI854626	Lymphocyte antigen 6 complex	6.19
AI838634	Ubiquitin-specific protease 25	5.10
AI449282	Interferon-inducible GTPase	4.22
AI848402	Histocompatibility 2, T region locus 10	3.34
AI836367	TAP binding protein	2.93
AI836233	Proteasome (prosome, macropain) subunit, beta type 10	2.86
AI838322	Small inducible cytokine A21a (leucine)	2.81
AI451137	Interferon activated gene 203	2.77
AI528519	Complement component 3	2.70
AI528713	Interferon gamma inducible protein, 47 kDa	2.64
AI428543	Zinc-finger protein 261	2.63
AI447752	Nuclear receptor subfamily 3, group C, member 1	2.60
AI853915	Guanine nucleotide binding protein, beta 4	2.58
AI851597	Eucaryotic translation elongation factor 1 alpha 2	2.48
AI429495	ATP-dependent interferon responsive	2.43
AI841665	Matrix metalloproteinase 14 (membrane-inserted)	2.41
AI852236	Histocompatibility 2, D region locus 1	2.36
AI853714	Cathepsin B	2.30
AI853077	Calpastatin	2.27
AI852036	Nuclear receptor-binding SET-domain protein 1	2.20
AI326933	Ubiquitin-conjugating enzyme 8	2.17
AI448932	T-box 2	2.14
AI840449	Small nuclear ribonucleoprotein D1	2.14
AI852351	Mitochondrial ribosomal protein L11	2.14
AI528521	Special AT-rich sequence binding protein 1	2.05
AI852357	Eucaryotic translation initiation factor 4A2	2.01
Cell surface, signal transduction, cytoskeleton and metabolism proteins		
GenBank	Clone description	Fold Repression
AI842984	Tenascin C	9.66
AI661287	Transforming growth factor, beta induced, 68 kDa	9.04
AI854636	Early growth response 1	6.81
AI326106	Guanylate nucleotide binding protein 2	6.53
AI449282	Frizzled homolog 9 (Drosophila)	5.16
AI835595	Secreted phosphoprotein 1	4.64
AI836468	Myristoylated alanine-rich protein kinase C substrate	4.57
AI844874	Procollagen, type I, alpha 1	4.17
AI850915	UDP-glucose ceramide glucosyltransferase-like	3.90
AI836323	Sortilin 1	3.85
AI848233	sprouty homolog 2 (Drosophila)	3.84
AI448916	Slit homolog 2 (Drosophila)	3.79
AI842053	Peptidylprolyl isomerase C-associated protein	3.64
AI847324	Insulin receptor substrate 1	3.61
AI449540	Signal transducer and activator of transcription 1	3.53
AI853346	Thymidylate kinase family LPS-inducible member	3.52
AI528734	Stromal cell derived factor 1	3.50
AI450263	Lectin, galactose binding, soluble 6	3.27
AI836324	Carboxypeptidase E	3.20
AI465143	Lectin, galactose binding, soluble 1	3.13
AI846654	Heparan sulfate 6-*O*-sulfotransferase 2	3.08
AI413932	Emerin	3.03
AI840949	Argininosuccinate synthetase 1	2.98
AI325230	Dopachrome tautomerase	2.79
AI837432	Solute carrier family 7 (cationic, transporter), member 10	2.74
AI838652	Procollagen, type I, alpha 2	2.71
AI839177	Procollagen, type XII, alpha 1	2.66
AI844075	LIM only 4	2.64
AI385730	Procollagen, type XI, alpha 1	2.40

Table 2 (*continued*)

GenBank	*Clone description*	*Fold repression*
AI853169	Stearoyl-Coenzyme A desaturase 1	2.37
Cell surface, signal transduction, cytoskeleton and metabolism proteins continuation		
AI835931	Brain protein I3	2.36
AI427643	Glia maturation factor, gamma	2.35
NA	VpreB	2.34
AI838326	Serine (or cys) proteinase inhibitor, clade H (hsp 47), member 1	2.26
AI836826	Glycoprotein 38	2.26
AI450121	Peroxisomal biogenesis factor 13	2.25
AI836381	Actin-like	2.25
AI840949	Argininosuccinate synthetase 1	2.22
AI847852	Protein tyrosine phosphatase, receptor-type, M	2.21
AI448959	Fibulin 2	2.21
AI853088	Follistatin-like	2.18
AI853451	Protein kinase, cAMP dependent, catalytic, alpha	2.15
AI844939	Open reading frame 12	2.15
AI843816	Exostoses (multiple) 1	2.13
AI847131	Ankyrin repeat hooked to zinc-finger motif	2.11
AI449353	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	2.10
AI450719	Single-strand selective monofunctional uracil DNA glycosylase	2.09
AI450103	Serine protease inhibitor 6	2.09
AI854719	Prostaglandin E synthase	2.07
AI854572	Coatomer protein complex, subunit gamma 1	2.07
AI852039	Elongation of v.l. chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1	2.07
AI893625	Syndecan 2	2.07
AI844799	Phosphatidylinositol membrane-associated	2.04
AI847778	*N*-acetyl galactosaminidase, alpha	2.04
AI846240	Phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 4, p150	2.03
AI893646	Protein tyrosine phosphatase, receptor type, K	2.03
AI840537	Cadherin 11	2.02
AI843939	Adenylate kinase 1	2.02
AI323769	Ceruloplasmin	2.02
AI853322	Protein tyrosine phosphatase, receptor type, S	2.01
AI842633	Human immunodeficiency virus type I enhancer-binding protein 1	2.00
148 ESTs with unknown function		

an interaction with PP2A (Watanabe *et al*., 1996). Immunoblotting (not shown) indicates that cyclin D1 protein was high in arrested cells and did not change significantly after T antigen induction or serum addition, suggesting that the protein is rather stable in Swiss 3T3 cells. Py LT antigen transactivated cyclin D1 about six-fold, while the corresponding protein from SV40 was reported to inhibit cyclin D1 expression (Lukas *et al*., 1994).

C-myc is strongly transactivated by ST (Figure 2), while LT had only a minor effect. The strong transactivation of c-myc by ST may be particularly relevant as c-myc modulates the expression of a great variety of genes (Coller *et al*., 2000; Menssen and Hermeking, 2002; Fernandez *et al*., 2003). This raises the possibility that ST changes the expression of some genes through its effect on c-myc. In fact, among the genes observed as regulated by c-myc in all three studies referred to above are those coding for ribosomal proteins, heat shock proteins and nucleolin, which are also found to be upregulated by ST in our analysis.

Many proteins involved in DNA synthesis and precursor production were previously shown to be transactivated by PyLT (Ogris *et al*., 1993b; Mudrak *et al*., 1994). This includes PCNA, which in this study was also found to be induced by ST protein. The list also contains two important proteins of cellular prereplication complexes, MCM6 and MCM7. Both of these proteins were transactivated by LT and ST antigens (Figure 3). Genes coding for MCM proteins are regulated by E2F (Tsuruga *et al*., 1997; Ohtani *et al*., 1999); their induction by LT and ST may therefore resemble that of the other E2F-controlled genes reported previously. We have isolated the murine promoters for the mcm6 and the mcm7 gene and created promoter-luciferase constructs for transient transfections together with LT or ST or their mutants (Figure 4). The results of these assays confirmed those obtained with real-time PCR and RT–PCR. In agreement with the more direct function of LT protein on E2F regulated promoters, transactivation of these promoters in transient transfection assays is consistently stronger by LT than by ST antigen. Considering the coordinated regulation of all six MCM proteins (MCM 2–7) during serum stimulation of REF 52 cells (Ohtani *et al*., 1999), it is likely that also the other MCM proteins are transactivated by both T antigens.

Several oncogenes and tumor suppressors exhibit altered expression in the presence of LT or ST. An example is the tumor suppressor protein

Table 3 Genes induced two-fold or higher by Py large T-antigen in cDNA microarray

GenBank	*Clone description*	*Fold induction*
Cell cycle regulators and DNA synthesis genes		
AI840227	Mini chromosome maintenance deficient 7 (*S. cerevisiae*)	4.00
AI854509	Mini chromosome maintenance deficient 6 (*S. cerevisiae*)	3.56
AI894115	Cyclin D1	3.48
AI849847	Proliferating cell nuclear antigen	3.03
AI837745	Telomerase binding protein, p23	2.72
AI849171	Polymerase (DNA directed), delta 2, regulatory subunit (50 kDa)	2.58
AI840088	Replication protein A2	2.44
AI846943	Cyclin-dependent kinase 4	2.32
AI844106	Thymidylate kinase	2.32
AI838819	MYB binding protein (P160) 1a	2.08
Oncogenes, tumor suppressors, stress and apoptosis genes		
AI846348	Hypoxia induced gene 1	2.93
AI854800	Cytochrome *P*450, 51	2.87
AI844365	Bcl2-like	2.66
AI836493	Myelocytomatosis oncogene (c-myc)	2.61
AI840074	Ras-GTPase-activating protein (GAP<120>) SH3-domain-binding protein 2	2.57
AI528760	Cold shock domain protein A	2.53
AI838255	Chaperonin subunit 6a (zeta)	2.43
AI429136	Transforming, acidic coiled-coil containing protein 3	2.36
AI837834	t-complex protein 1	2.25
AI841137	Differentially expressed in brain tumors	2.09
AI841116	Peroxiredoxin 2	2.07
AI841173	Rho interacting protein 3	2.06
AI842297	RAS-related C3 botulinum substrate 1	2.04
AI854010	RAS-like, family 2, locus 9	2.04
AI842320	neurofibromatosis 2	2.01
Genes involved in transcription, translation, immune response and degradation pathway		
AI845040	Translationally regulated transcript (21 kDa)	3.00
AI836520	Histone gene complex 2	2.75
AI840449	Small nuclear ribonucleoprotein D1	2.74
AI836982	Proteaseome (prosome, macropain) 28 subunit, 3	2.56
AI849860	Stem-loop binding protein	2.53
AI838842	RNA binding motif protein 3	2.40
AI839283	Inhibitor of DNA binding 3	2.29
AI843304	CCAAT/enhancer binding protein (C/EBP), alpha	2.20
AI835853	H2A histone family, member Z	2.12
AI326849	Transcriptional regulator protein	2.11
AI464523	T-box 5	2.07
AI835911	Heterogeneous nuclear ribonucleoprotein K	2.05
AI853852	Ribosomal protein S18	2.02
AI847332	Interferon alpha responsive gene, 15 kDa	2.01
Cell surface, signal transduction, and cytoskeleton and metabolism proteins		
AI834834	G protein-coupled receptor, family C, group 5, member B	5.82
AI839335	Adducin 3 (gamma)	4.90
AI852527	Importin beta	3.64
AI841020	Phosphoglycerate kinase 1	3.56
AI849502	Amyloid beta (A4) precursor-like protein 2	3.54
AI851778	Connective tissue growth factor	3.44
AI849308	Glutaredoxin 1 (thioltransferase)	3.43
AI840757	Tubulin alpha 1	3.31
AI326287	Tubulin alpha 8	3.26
AI528531	Pyruvate dehydrogenase E1alpha subunit	3.16
AI836347	Tubulin alpha 2	3.11
AI840025	Secreted acidic cysteine-rich glycoprotein	2.90
AI850162	Laminin, gamma 1	2.85
AI427514	Metallothionein 1	2.83
AI836976	Glutamate dehydrogenase	2.83
AI840604	Tubulin alpha 4	2.83
AI854035	Kelch-like ECH-associated protein 1	2.82
AI847285	Mitogen-activated protein kinase Kinase kinase 6	2.75
AI835559	Thymopoietin	2.75
AI841394	Phosphoglycerate mutase 1	2.70
AI841966	Protein kinase, AMP-activated, gamma 1 noncatalytic subunit	2.61
AI837206	Prothymosin alpha	2.57

Table 3 (*continued*)

GenBank	*Clone description*	*Fold induction*
Cell surface, signal transduction, and cytoskeleton and metabolism proteins continuation		
AI850901	Parvin, alpha	2.56
AI851776	Myosin Ic	2.50
AI839899	Regulatory factor X-associated ankyrin-containing protein	2.46
AI836844	Calmodulin 1	2.46
AI450702	ATPase, Na+/K+ transporting, beta 3 polypeptide	2.45
AI894247	Tubulin, beta 5	2.44
AI849335	Neighbor of Punc E11	2.43
AI844634	Actin, alpha 1, skeletal muscle	2.35
AI448908	Chloride intracellular channel 4 (mitochondrial)	2.31
AI851107	Peripheral myelin protein, 22 kDa	2.30
AI845765	Sterol-C5-desaturase homolog (*S. cerevisae*)	2.29
AI843707	Treacher Collins Franceschetti syndrome 1, homolog	2.27
AI836137	Pyruvate kinase 3	2.27
AI842299	Laminin B1 subunit 1	2.23
AI839138	Upregulated by 1,25-dihydroxyvitamin D-3	2.21
AI414231	Villin	2.21
AI850309	Coagulation factor II (thrombin) receptor	2.20
AI842934	Dystroglycan 1	2.20
AI849775	Osteoblast specific factor 2 (fasciclin I-like)	2.17
AI850206	Paralemmin	2.15
AI447993	Butyrophilin-like 2	2.14
AI854752	Radixin	2.14
AI849985	Solute carrier family 12, member 2	2.12
AI842656	Lurcher transcript 1	2.11
AI836968	Alpha actinin 4	2.11
AI847067	Neural precursor cell expressed, developmentally downregulated gene 4a	2.09
AI853464	Pantophysin	2.09
AI845824	sorbitol dehydrogenase 1	2.08
AI838504	Enhancer of rudimentary homolog (Drosophila)	2.08
AI847962	Transmembrane 4 superfamily member 2	2.08
AI842586	Troponin T2, cardiac	2.07
AI844511	STIP1 homology and U-Box containing protein 1	2.06
AI841014	Guanine nucleotide binding protein, alpha stimulating	2.06
AI836349	Junctophilin 3	2.06
AI841283	Novel nuclear protein 1	2.05
AI662267	Homeodomain interacting protein kinase 1	2.04
AI385637	Frizzled homolog 8 (Drosophila)	2.04
AI836694	ATP synthase, H+ transporting, mitroch. F1 complex, gamma polypept. 1	2.03
AI843739	FK506 binding protein 12-rapamycin associated protein 1	2.02
AI841010	Adenine phosphoribosyl transferase	2.02
AI853846	Fatty acid binding protein 5, epidermal	2.02
AI838959	Actin, alpha 2, smooth muscle, aorta	2.01
119 ESTs with unknown function		

neurofibromatosis 2 (NF2) (Lutchman and Rouleau, 1995). Figure 5 shows the induction of NF2 by LT as well as ST, which is dependent on the interactions with pocket proteins and PP2A, respectively. The NF2 promoter has some similarity to the cyclin A promoter with which it shares binding sites for transcription factors SP1, E2F and NF-Y (Kino *et al*., 2001). This might suggest that its transactivation by the two T antigens may follow similar mechanisms. Surprisingly, however, and in vast contrast to cyclin A, serum addition dramatically reduced NF2 expression, confirming the results of the microarray analysis of serum-stimulated cells. The same was observed with the gene coding for the G protein coupled receptor (GPCR) (Figure 6). This gene, together with the one coding for myosin, belongs to the large group of genes in the tables listed under genes coding for signal transducers, cell surface proteins and cytoskeletal proteins. Both GPCR and myosin are also targets of the LT protein (Figure 6).

Genes downregulated by viral proteins Among the genes that are strongly repressed by ST are those coding for decorin and cadherin 11 (Figure 7). Both of these proteins are located in the cell surface and exhibit various cell-type-specific effects during differentiation and cell growth. For instance, cadherin 11 is upregulated during myoblastic or osteogenic differentiation of C3H10T1/2 and C2C12. In contrast, adipogenic differentiation of these cells as well as of 3T3-L1 cells causes a decrease of cadherin expression (Shin *et al*., 2000). In human osteoblasts, dexamethasone was reported to inhibit cadherin 11 expression (Lecanda *et al*., 2000). We found that also in Swiss 3T3 cells dexamethasone

Table 4 Genes repressed two-fold or higher by Py large T-antigen in cDNA microarray

GenBank	*Clone description*	*Fold repression*
Cell cycle regulators and DNA synthesis genes		
AI840919	DNA-damage inducible transcript 3	2.45
AI848752	topoisomerase (DNA) II beta	2.27
Oncogenes, tumor suppressors, stress and apoptosis genes		
AI846778	Decorin	3.86
AI852541	Tumor rejection antigen gp96	2.46
AI450853	Cerebral cavernous malformations 1	2.45
AI843677	Erbb2 interacting protein	2.40
AI528704	Rho-associated coiled-coil forming kinase 1	2.26
AI853115	Rho GTPase activating protein 5	2.21
AI834803	DnaJ (Hsp40) homolog, subfamily B, member 12	2.21
AI846385	RAB23, member RAS oncogene family	2.06
AI848314	Natural killer tumor recognition sequence	2.05
Genes involved in transcription, translation, immune response and degradation pathway		
AI854552	Interferon-induced protein with tetratricopeptide repeats 3	6.57
AI852028	Ubiquitin specific protease 9, X chromosome	3.25
AI850966	Transcription elongation factor A (SII) 1	2.62
AI447752	Nuclear receptor subfamily 3, group C, member 1	2.48
AI836309	Eucaryotic translation initiation factor 3	2.47
AI528713	Interferon gamma inducible protein, 47 kDa	2.46
AI846176	Histocompatibility 2, T region locus 10	2.28
AI448195	Trans-acting transcription factor 3	2.26
AI449282	Interferon-inducible GTPase	2.24
AI853077	Calpastatin	2.20
AI841432	Heterogeneous nuclear ribonucleoprotein H1	2.05
AI449004	Heterogeneous nuclear ribonucleoprotein A2/B1	2.00
AI845529	Ribonuclease P1	2.00
Cell surface, signal transduction, and cytoskeleton and metabolism proteins		
AI847805	Secreted phosphoprotein 1	5.29
AI661287	Transforming growth factor, beta induced, 68 kDa	4.94
AI450263	Lectin, galactose binding, soluble 6	3.28
AI528637	Beta-2 microglobulin	3.22
AI326106	Guanylate nucleotide binding protein 2	3.19
AI842984	Tenascin C	3.16
AI893625	Syndecan 2	3.04
AI840134	Myristoylated alanine-rich protein kinase C substrate	2.99
AI842819	Branched chain aminotransferase 1, cytosolic	2.93
AI850620	Matrin 3	2.81
AI528734	Stromal cell derived factor 1	2.73
AI838226	Glycine transporter 1	2.45
AI843323	Latent transforming growth factor beta binding protein 2	2.42
AI854587	Proline dehydrogenase	2.41
AI426812	Imprinted and ancient	2.39
AI839302	Clusterin	2.34
AI327322	Chondroitin sulfate proteoglycan 6	2.31
AI850263	Lysosomal membrane glycoprotein 2	2.31
AI323554	Crystallin, zeta	2.30
AI427643	Glia maturation factor, gamma	2.29
AI851370	Tetratricopeptide repeat domain	2.27
AI848700	Kinesin family member 5B	2.26
AI662153	Kidney androgen regulated protein	2.21
AI854636	Early growth response 1	2.21
AI528639	Adenylate cyclase 7	2.18
AI852712	Gap junction membrane channel protein alpha 1	2.15
AI846016	Development and differentiation enhancing	2.14
AI838700	Lymphoid nuclear protein related to AF4-like	2.13
AI839465	Neuropilin	2.11
AI323769	Ceruloplasmin	2.10
AI844503	GTP cyclohydrolase 1	2.09
AI835169	Potassium voltage-gated channel, subfamily Q, member 2	2.09
AI853162	Short stature homeobox 2	2.08
AI450790	Sideroflexin 1	2.08
Cell surface, signal transduction, and cytoskeleton and metabolism proteins continuation		
AI840537	Cadherin 11	2.01
AI326787	Lipoprotein lipase	2.01
AI838607	Thrombospondin 1	2.01
96 ESTs with unknown function		

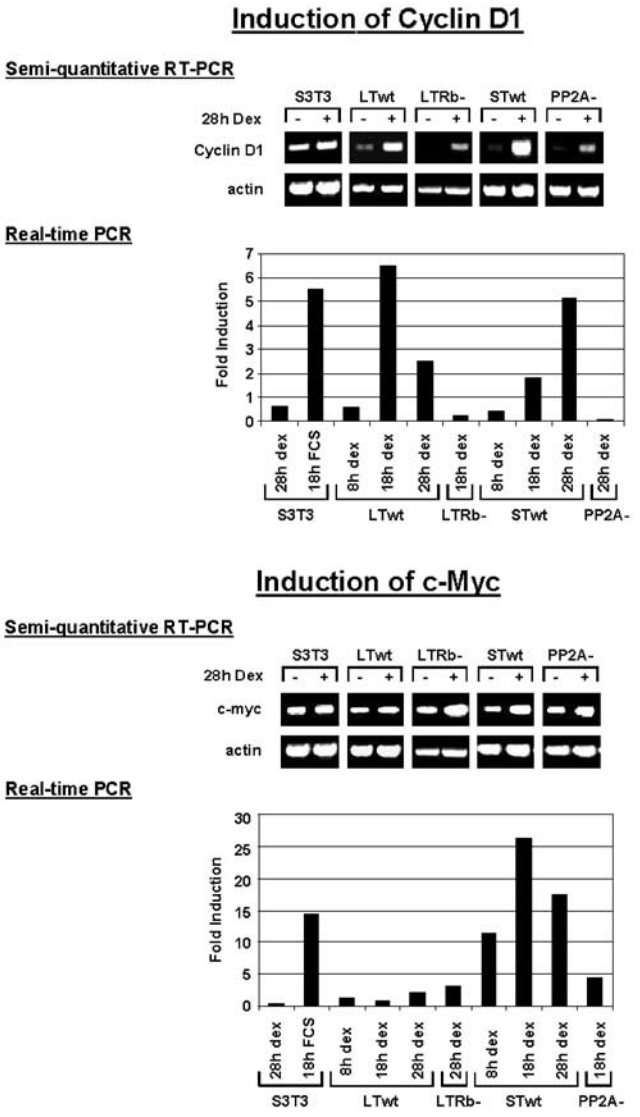

Figure 2 Induction of cyclin D1 and c-myc. In this and in all further figures, with the exception of Figure 4, cells were treated as in Figure 1. Real-time PCR and semiquantitative RT–PCR were performed with specific primers as described in Materials and methods

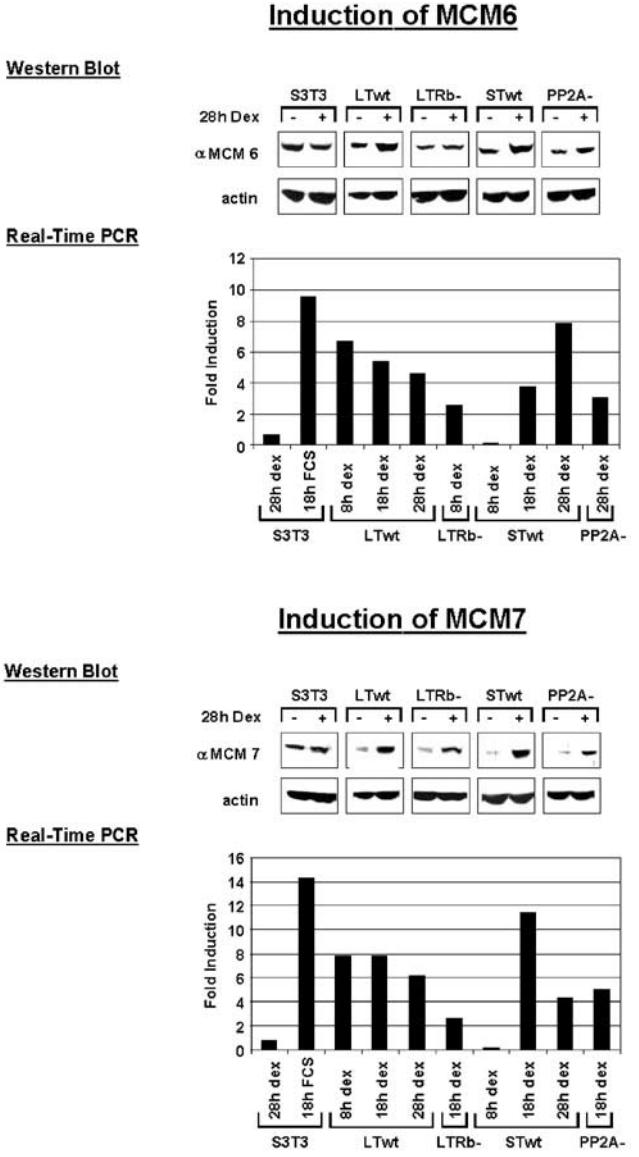

Figure 3 Induction of MCM6 and MCM7. The primers and antibodies used are described in Materials and methods

caused some decrease of cadherin 11 expression, but the effect of ST protein was by far stronger (Figure 7), excluding that this effect is solely due to the addition of dexamethasone to our cells. Decorin was found to be downregulated in some tumors (for reviews, see for instance Ständer *et al*., 1999; Kresse and Schönherr, 2001). It binds to TGF-β and inhibits its activity. On the other hand, it was found to upregulate p21 expression in some tumor cells. Its strong downregulation in our ST expressing cells could thus contribute to the growth-inducing potential of the viral protein. Interestingly, downregulation of both of these genes by LT was completely independent of an interaction with pocket proteins.

Finally, one group of genes whose expression not unexpectedly is affected by LT and ST involves genes for transcription factors and translational regulators, immune response genes and genes whose products function in degradation pathways. While components of the transcription machinery are frequently upregulated by T antigens (see for instance Felton-Edkins and White, 2002), many genes of the immune response here were found to be downregulated, thus creating conditions that would allow the virus to escape the cellular immune response.

In summary, this microarray analysis disclosed an astonishingly large number of genes that are up- or downregulated after the expression of PyT antigens in quiescent cells. All selected genes chosen for documentation of their deregulation confirmed the data obtained by the microarray analysis. Considering that the list in Tables 1–4 still misses genes known to code for S phase-

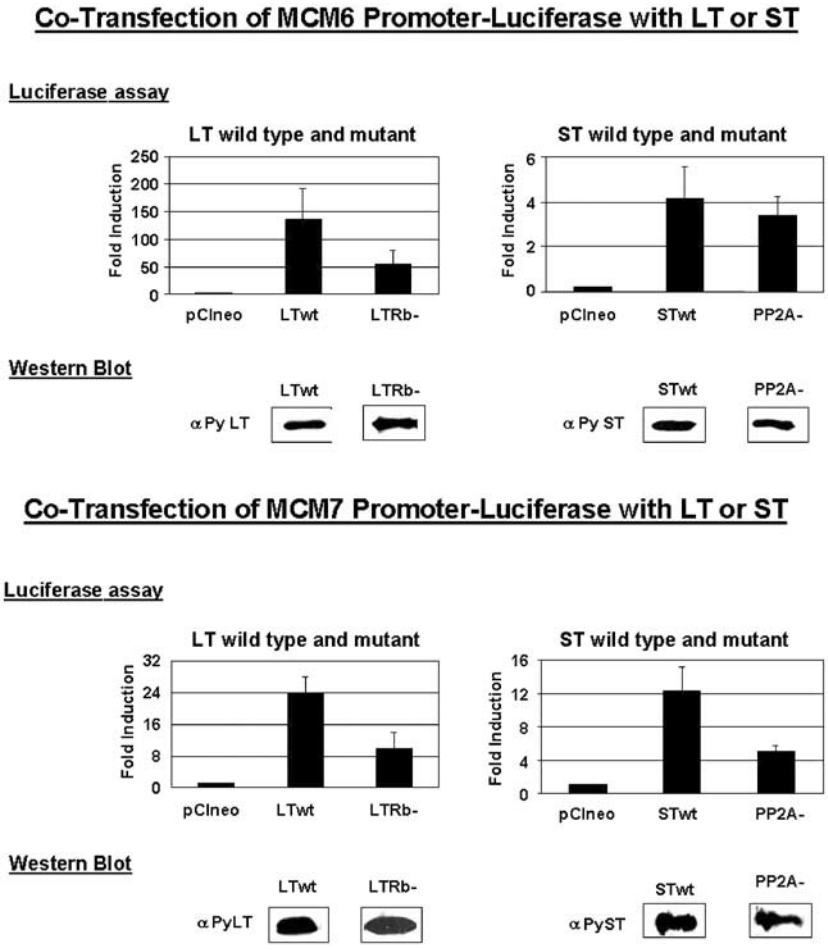

Figure 4 Effect of LT and ST on the mcm6 and mcm7 promoters. Transient co-transfection assays were performed using mcm6 and mcm7 promoter-luciferase constructs and LT or ST expressing plasmids. For details see Materials and methods. The equal expression of the wild type and mutated viral proteins was verified by immunoblotting using a polyclonal antibody directed towards the LT/ST common region at the N-termini of the viral proteins

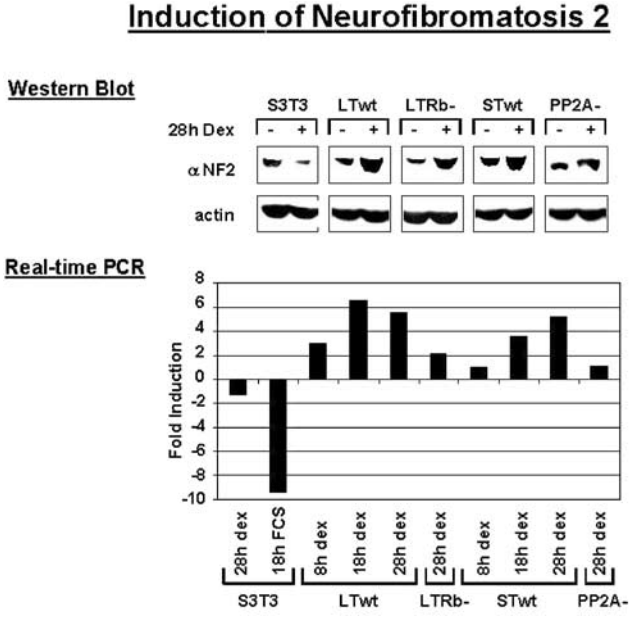

Figure 5 Induction of the neurofibromatosis gene 2. The antibody used for immunoblotting was NF2 (sc-331) from Santa Cruz

specific enzymes and proteins that were already shown or are expected to be transactivated by viral proteins, the total number of genes influenced in their expression by Py T antigens may be even larger. Obviously, the selection pressure on the evolution of Py was to create optimal conditions for virus replication in many different cell types, that is, driving these different cells from the quiescent state into the S phase. This may require interference with different mechanisms of growth regulation and hence with a broad spectrum of transcriptional control processes. One may ask why it needs both LT and ST to drive efficiently Swiss 3T3 cells out of the quiescent state into the S phase (Ogris *et al*., 1992), when ST alone is such an efficient deregulator of gene expression. There are several explanations for this: (i) compared with LT, ST is weak in inducing some DNA synthesis- and precursor-producing enzymes such as thymidine kinase (unpublished). (ii) We found earlier that both LT and ST are required to induce sufficiently high levels of cyclin E/cdk2 activity (Schüchner and Wintersberger, 1999). Cyclin E/cdk2 is essential for S phase induction in most vertebrate cells. (iii) By targeting PP2A, an enzyme that is involved in many

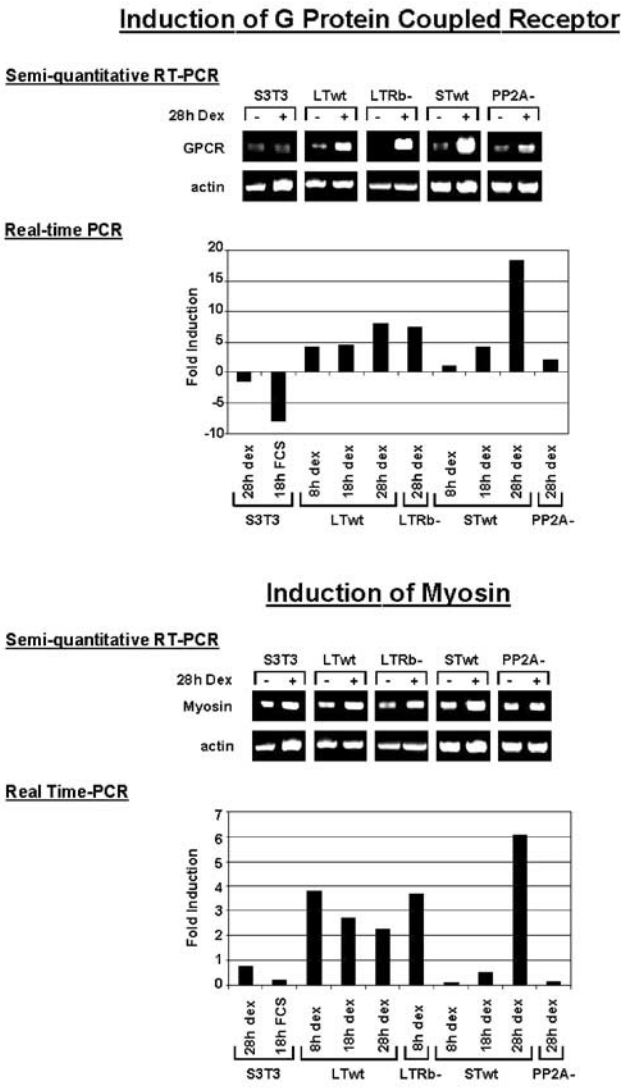

Figure 6 Upregulation of the genes for the GPCR and for myosin. The oligonucleotides used for the PCR reactions are described in Materials and methods

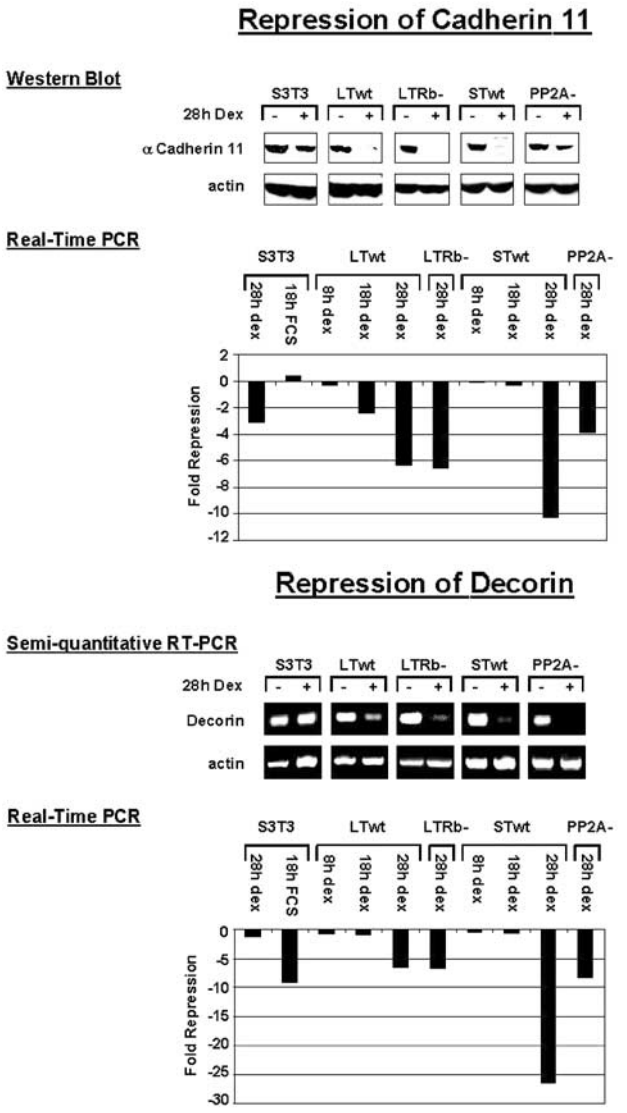

Figure 7 Strong repression of the tumor suppressor proteins cadherin 11 and decorin by ST and LT antigens. The antibody used in the immunoblot of cadherin 11 is described in Materials and methods

cellular regulatory processes, ST may not only affect reactions that favor S phase induction but also some that hinder this process and it may require LT to neutralize these effects.

We do not want to imply that all of the genes deregulated by the viral T antigens are direct targets of these proteins; the expression of some of the genes may be affected by secondary reactions to gene products up- or down-regulated by the T antigens. Still, their deregulation in quiescent cells does depend on the activity of the viral proteins. An example is c-myc, which we found to be strongly upregulated by ST but which itself is an important transactivator. Moreover, the products of some of the genes deregulated by LT or ST may have no role in S phase induction but they may nonetheless contribute to tumorigenesis. Tumor formation was often considered to be an accidental reaction following virus infection, initiated by the competence of the viral proteins to interfere with growth control and apoptosis. However, as Py is replicating rather slowly, the formation of tumors may have even been of evolutionary advantage for the virus, if tumor cells create longer-lasting conditions favorable for virus replication.

Materials and methods

Cell culture and stable transfections

Swiss 3T3 fibroblasts and derived cell lines conditionally expressing the Py LT or the Py ST antigen under the control of the dexamethasone-inducible MMTV promoter (Ogris *et al*., 1992) were grown in Dulbecco's modified Eagles medium containing 10% fetal calf serum, penicillin (60 μg/ml) and streptomycin (100 μg/ml) in a 7.5% CO_2 atmosphere. When the cells reached 80% confluency, they were arrested by serum

deprivation (final concentration of FCS 0.2%) for 72 h. The cells were then induced with dexamethasone at a final concentration of $10^{-}6$ mol/l for 28 h.

RNA extraction

Total RNA was isolated using TRIzol reagent (Life Technologies/GIBCO-BRL) as recommended by the supplier.

Microarray preparation

The mouse microarrays used contained a set of 17 000 sequence verified mouse cDNA clones printed onto polylysine-coated slides. Prior to use, the arrays were UV-cross-linked, rehydrated in water steam and chemically blocked using 1.37 g of succine anhydride, 90 ml *N*-methyl-pyrrolidone and 10 ml of 0.2 M boric acid pH 8.0 for 15 min. After blocking, the slides were rinsed in 0.1% SDS and prehybridized with prehybridization buffer containing 210 μl formamide, 120 μl 20 × SSPE, 60 μl 50 × Denhardts, 6 μl salmon sperm DNA (10 μg/μl), 190 μl water and 15 μl 20% SDS at 50°C for 1 h.

Fluorescent probe preparation

In total, 100 μg RNA in 17 μl water was primed with 2 μl oligo-dT_{12-18}-primer (500 μg/ml) at 65°C for 5 min. Reverse transcription was performed in a mixture containing 8 μl 5 × first-strand reaction buffer provided with SuperScript™ II RNase H^- Reverse transcriptase (Invitrogen Life Technologies), 4 μl 100 mM dithiothreitol, 4 μl of 10 × dNTP (5 mM dATP, dCTP, dGTP and 2 mM dTTP), 1 μl RNasin (40 U/μl Promega), 2 μl SuperScript™ II RNase H^- Reverse transcriptase (200 U/μl Invitrogen Life Technologies), 4 μl of Cy3-dUTP or Cy5-dUTP (1 nM/μl Amersham Pharmacia) and incubated at 42°C for 60 min, followed by the addition of another 1 μl SuperScript™ II RNase H^- Reverse transcriptase for an additional 1 h incubation. The reaction was stopped by incubation at 94°C for 3 min. The remaining RNA was eliminated by adding 2 μl RNase ONE™ (6 U/μl, Promega), 5 μl 10 × RNaseONE™ reaction buffer and incubating at 37°C for 10 min. The Cy3-dUTP and Cy5-dUTP labelled probes were pooled and precipitated with blocking solution containing 3 μg poly$(dA)_{40-60}$, 6 μg tRNA, 15 μg mouse Cot 1 DNA, 33 μl 10 mol/l ammonium acetate and 330 μl 96% ethanol at −80°C for 20 min. The pellet was washed with 400 μl 80% ethanol, dissolved in 10.5 μl H_2O and then mixed with 6 μl 20 × SSPE, 1.5 μl 50 × Denhardts solution, 10.5 μl formamide and 0.75 μl 20% SDS. The probe was denatured at 94°C for 1 min followed by prehybridization at 50°C for 1 h. It was then added to the microarray slides and incubated at 50°C overnight.

Data analysis

After the hybridization, the slides were washed in 1 × SSC, 0.1% SDS, followed by washing with 0.2 × SSC, 0.1% SDS and finally 0.2 × SSC. After drying by centrifugation the microarrays were scanned with a Gene Pix 4060 scanner. Analysis was performed using Gene Pix Pro 4.1 microarray information management system (JMIMS) developed by Volker Leidl and Gerald Löffler (Research Institut of Molecular Pathology, Vienna).

RT–PCR

In total, 10 μg RNA was reverse transcribed to first-strand cDNA using oligo-dT-primer and RevertAid™M-MuLV Reverse Transcriptase (MBI), either 1/40 or 1/80 was used for PCR. The following primers were used: cyclin D1: forward 5′-CTGTGCGCCCTCCGTATCTTA-3′, reverse 5′-GGCG GCCAGGTTCCACTTGAG-3′; mcm6: forward 5′-TGCAC GAGCCT CTTCCCTACT-3′, reverse 5′-TCCCGCATGTC CATCTTATCA-3′; mcm7: forward 5′-AGGCGCTGCTGC TGCTTCTG-3′, reverse 5′-GCCGGCTGTGCTGGTGGAC-3′;

LT forward 5′-GAGAGCGGCCACAGTCCAC-3′, reverse 5′-ATCGGGCTCAGCA ACACAAG-3′; ST: forward 5′-CA GGCATATAAGCAGCAGTC-3′, reverse 5′-CATCTC GG GTTGGTGTTC-3′; Neurofibromatosis 2: forward 5′-AAG CAGCCCAAGACATTCA-3′, reverse 5′-CTCGGGAGGG CAGTAGAC-3′; G protein-coupled receptor: forward 5′-TGCCCCTGCTCCTGGTGATTG-3′, reverse 5′-TAGG CGCAGGCTGGCTTCGTG-3′;

Myosin forward 5′-TGGAAGTCCGGCGGCAGAGTC-3′, reverse 5′-CCGGGGGCGA GGCTTGTAAC-3′; cadherin 11: forward 5′-ACCCCAAGGCACTCTCCAACC-3′; reverse 5′-TCACCACCCCCTTCATCATCATAG-3′; c-Myc: forward 5′-GCCCGCGCCCA GTGAGGATA-3′, reverse 5′-GCGGC GGCGGTGAGGTC-3′; decorin: forward 5′-CCCCT AC CGATGCCAGTGT-3′, reverse 5′-TTGCCGCCCAGTTC TATGAC-3′; β-actin forward 5′- TGGCACCACACC TTCTACAATGAG -3′, reverse 5′-CAAGAAGGAGGCTG GAAAAGAG-3′.

PCR cycle conditions were the following: initial denaturation was for 2 min at 95°C this was followed by 20–25 cycles consisting of 1.5 min at 95°C, 1.5 min for annealing (annealing temperatures were adjusted for each primer pair) and 2 min at 72°C for synthesis. Dilutions of cDNA were performed to determine the linear range for each primer pair. PCR products were resolved on a 1.5% agarose gel containing ethidium bromide and were visualized with UV light.

Real-time PCR

Quantitative PCR was performed using Light Cycler Fast Start DNA Master SYBR Green I Kit as recommended by the supplier (Roche). The same primers as described in RT–PCR were used to verify the expression of the selected genes. The β-actin gene was chosen for normalization of the data.

Immunoblotting

After 72 h serum starvation and 28 h induction with dexamethasone, cells were harvested and lysed in buffer (20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing complete protease inhibitor cocktail (Boehringer Mannheim) as described elsewhere (Adamczewiski *et al*., 1993). Protein concentration was measured with the BioRad protein assay (BioRad) following the manufacturer's instructions. Equal amounts of protein were separated on an 11% SDS–PAGE, transferred onto a nitrocellulose membrane and probed with the indicated antibodies. Antibodies against MCM6 (sc-9843), NF2 (sc-331), cyclin D1 (sc-246) and cadherin-11 (sc-6463) were purchased from Santa Cruz Biotechnology. MCM7 (Ab-1) antibody was purchased from Oncogene. β-Actin (AC-74) antibody was purchased from SIGMA. LT and ST antigens were detected by a rabbit polyclonal antibody directed against the common N-terminal region of these proteins.

Antibody binding was revealed using appropriate peroxidase-conjugated secondary antibodies (Jackson) and ECL reagents (Amersham) following the manufacturer's instruction.

Transient transfection and luciferase reporter assays

Mouse mcm6 and mcm7 promoters were amplified by PCR using the following primers: mcm6: forward 5′-CGGGGT ACCGCTTCATGGCGTATGCTT-3′; reverse 5′CCGCTCGA GGAGGTCCATGGCGGCGCC-3′; mcm7: forward 5′-CGGGGTACCAGGATAC AGTCTTTATAGATGAG-3′; reverse 5′-CCGCTCGAGCGCTGCCGGGGACGGTGT-3′. The amplified fragments were inserted into the pGL3 Basic Vector (Promega).

Asynchronously growing REF52 cells were seeded at a density of 5×10^4 cells per six-well plate. The next day, the cells were transfected with polyethylenimine (PEI25000) together with plasmid carrying the information for LT or ST (wild type or mutants) under the CMV promoter and a plasmid expressing β-galactosidase as described previously (Schüchner *et al*., 2001). Subsequently, the cells were arrested by serum deprivation (0.2% serum) for 48 h. Cells were then harvested and luciferase activity was measured in the assay buffer (25 mM Tricine pH 7.8, 0.5 mM EDTA, 0.54 mM Na-Tripolyphosphate, 16.3 mM $MgSO_4 \cdot 7H_2O$, 0.1% Triton X-100, 4.6 μM Luciferin, 1.2 mM ATP and 5.6 mM DTT) using an Auto-Luminat. To normalize the transfection efficiency, β-galactosidase activity was measured as described in Sambrook *et al*. (1989). The protein concentration was determined and likewise used for normalization. An aliquot of each extract was used for determination of the level of T antigen by immunoblotting.

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