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# Review

# Oncogenic potentials of the human polyomavirus regulatory proteins

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**Abstract.** The polyomaviruses BK, JC and SV40 are common in the human population. Their DNA genomes encode large T-antigen, small t-antigen, agnoprotein, and the capsid proteins VP1 – 3. Studies with these viruses have contributed extensively to the understanding of processes such as replication, transcriptional and posttranscriptional regulation, and cell cycle control. All three viruses can transform human cells *in vitro*, can induce tumours in animal models, and are strongly association with certain human cancers. It is generally assumed that large T-antigen is the major protein involved in neoplastic processes and that large T-antigen predominantly exerts its effect through deregulation of the tumour suppressors p53 and the retinoblastoma family members. However, additional properties of large T-antigen as well as the other viral proteins contribute to oncogenic processes. This review presents the different mechanisms by which the polyomavirus proteins can induce transformation and discusses which mechanisms may be operational in polyomavirus-positive cancers.

**Keywords.** Large T-antigen, small t-antigen, agnoprotein, cancer, SV40, BKV, JCV.

## 1. Introduction

In 1953, Ludwig Gross discovered the first polyomavirus while he was studying murine leukaemia virus. He observed that some animals inoculated with this retrovirus developed not only leukaemia but also adenocarcinomas of the parotid gland. Extracts of the tumour contained a virus that induced the formation of a variety of solid tumours in newborn mice, hence its name polyomavirus. Simian vacuolating virus 40 (SV40), a natural infectious agent in rhesus macaque (*Macaca mulatta*), was the second member of this family to be isolated. It was discovered as a contaminant agent in the Salk poliovirus vaccines [1]. In 1971, two new polyomaviruses solely infecting humans were described. BK virus (BKV) was detected in the urine of a renal transplant patient with the initials, B.K., while JC virus (JCV) was characterized in the brain of a Hodgkin lymphoma patient with initials, J.C. who suffered from progressive multifocal leukoencephalopathy. While serological studies and polymerase chain reaction (PCR) analyses have shown that almost everybody is latently infected with BKV and/or JCV, SV40 infections in humans were considered rare. Occasional SV40 infections occurred through transmission of the virus from monkeys to people living in close contact with these animals or through vaccina-

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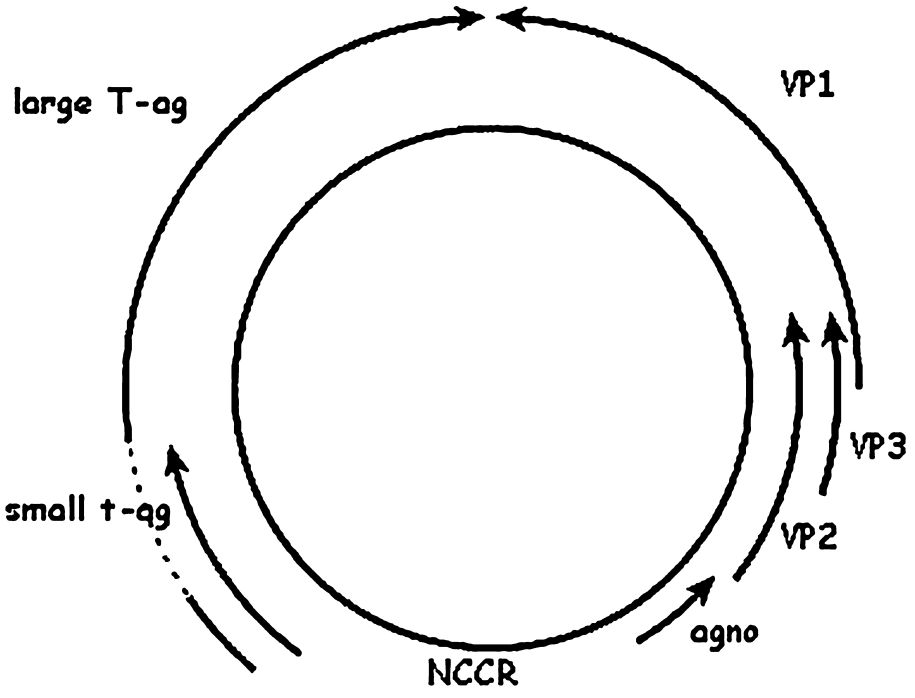


**Figure 1.** Genetic organization of the circular double-stranded DNA genome of human polyomaviruses (HPyVs). The gene products encoded by the early region (LT-ag and st-ag) and the region (agnoprotein, capsid proteins VP1, VP2, and VP3) are indicated. The non-coding control region (NCCR) consisting of the origin of replication and the transcription control region is interspersed between the early and late region. The sequences of the NCCR control viral DNA replication and transcription of the early and late genes. The alternative early proteins are not indicated. See text for details.

tion with the contaminated poliovirus or adenovirus vaccines in the 1950s and 1960s. However, recent observations support the possibility that SV40 can spread in humans by horizontal infection and even vertical transmission. PCR data revealed that individuals who were never exposed to contaminated poliovirus vaccines could be infected with SV40 [2–6]. Therefore, SV40 is considered as the third member of the human polyomaviruses (HPyV) in this review.

All three HPyVs can transform human cells *in vitro* and can induce different types of tumours in several rodent species. Moreover, injection of HPyV-transformed cells in animal models or ectopic expression of viral regulatory proteins in transgenic animals can also provoke tumours. The results of such studies have been excellently reviewed by others and are beyond the scope of this review [2, 7–12]. These findings clearly illustrate the oncogenic potential of HPyV. The molecular mechanisms by which the different viral proteins may contribute to transformation will be elaborated here, and a review of these mechanisms that seem to be operational in HPyV-positive cancers will be provided.

## 2. The HPyV regulatory proteins

### 2.1. The HPyV genome

HPyVs are non-enveloped viruses with an icosahedral capsid that consists of the capsid proteins VP1, VP2, and VP3. Their genome consists of a circular double-stranded DNA molecule of around 5000 base pairs packed with cellular histone proteins. Both BKV and JCV share approximately 72% nucleotide homology with each other and 70% nucleotide homology with SV40. The viral genome can be divided into three functional regions: the non-coding control region (NCCR), which is interspersed between the early and the late regions (Fig. 1). The early region, encoding the regulatory proteins large tumour antigen (LT-ag) and small tumour antigen (st-ag), is predominantly expressed early during the infection cycle, but can also be expressed during the late phase after the onset of viral replication [13,14]. The early region of HPyV encodes additional proteins due to translation of alternative spliced transcripts (see 2.4). The late region encompasses the genetic information for the capsid proteins and the regulatory agnoprotein and is per definition transcribed only after viral DNA replication begins, although low levels of transcription may occur early after infection as well. The NCCR consists of the origin of replication and the transcription control region (TCR). The TCR regulates transcription of both the early and the late genes [13,14]. HPyV isolates that have not been passaged in cell culture display more sequence variability in their TCR than in other regions of the genome [15–17].

### 2.2. Large T-antigen (LT-ag)

The HPyV LT-ag is a multifunctional protein whose activities are engaged in several processes. LT-ag participates in viral DNA replication and transcrip-

tion, and contributes to create an optimal cellular environment by e.g. driving the cell into the S phase of the cell cycle. Distinct regions of the protein display different activities required for DNA replication, including ATPase, DNA helicase, specific DNA binding, and recruitment of cellular DNA replication proteins such as DNA polymerase α, replication protein A, nucleolin, and topoisomerase I (Table 1; [11, 14, 18]).

### 2.3. Small t-antigen (st-ag)

Alternative splicing of the early transcript generates a 17-kDa protein, referred to as st-ag. This protein shares the first 80 (JCV and BKV), respectively 82 (SV40) amino-terminal residues with LT-ag, while the remaining carboxy-terminal 92 residues are unique. For SV40, it was shown that the amount of small t-ag versus large T-ag messenger RNA (mRNA) in infected cells seems to be cell-specific and a specific protein called ASF mediated the production of the two splice variants. Five to 10 times more large T-ag mRNA compared to small t-ag transcripts were detected in SV40-infected human 293 cells, while a 100-fold excess of large T-ag mRNA was measured in cell extracts of HeLa cells [55]. The mechanism for alternative splice utilization of the BKV or JCV early pre-mRNA transcript has not been studied so far.

Considerably fewer studies have been aimed at characterizing the role of st-ag in virus multiplication compared to LT-ag. St-ag is located both in the nucleus and the cytoplasm. This Cys-rich protein seems to be dispensable for the viral life cycle, but it provides a helper function for LT-ag by augmenting viral replication and trans-activation of the viral promoter, resulting in increased virus yield in permissive cells [14, 56]. SV40 st-ag seems to exert its effect by inhibition of the protein phosphates 2A (PP2A), a family of abundantly expressed serine-threonine phosphatases implicated in the regulation of many cellular processes, including regulation of different signal transduction pathways and cell cycle progression. The heterotrimeric holoenzyme consists of the catalytic subunit C, the scaffold protein A, and the regulatory B subunit, of which several members exist. St-ag was originally believed to compete and replace the B subunits in their binding to the A subunit, thereby inhibiting the catalytic activity of PP2A [46]. Another possibility is that st-ag binds to newly synthesized AC dimers, thereby bypassing the need to compete with the B subunits [57]. The unique carboxy-terminal region of st-ag is involved in PP2A interaction, as point mutation of residues Cys-97, Pro101, and Cys-103, as well as deletion of residues 110–119 block st-ag association with A subunit [46]. All three sites are conserved in BKV and JCV st-ag, and the region encompassing residues 110–119 shows 50% (JCV) to 70% (BKV) homology with SV40 st-ag. This may indicate that BKV and JCV st-ag also bind PP2A, but this remains to be demonstrated.

### 2.4. Alternative early proteins

The early region of HPyV encodes additional proteins due to translation of alternative spliced transcripts. Both SV40-infected and SV40-transformed cells express an additional early protein, referred to as 17kT. This protein shares the 131 amino-terminal residues with LT-ag, and has an additional 4 unique amino acids (ALLT). The exact role of 17kT in virus propagation remains unknown, but 17kT is more abundantly expressed in transformed cells than during lytic infection. SV40 17kT could in cooperation with activated H-Ras immortalize rat embryo fibroblasts and induce dense focus formation [58]. The amino-terminal region of LT-ag possesses multiple functions, and separate expression of 17kT can allow different regulation of its various activities e.g. by differential phosphorylation, which is known to influence the functions of LT-ag. In this respect, LT-ag and 17kT display distinct phosphorylation patterns [59]. In addition to LT-ag and st-ag, JCV expresses the proteins $T'_{135}$ (first 132 amino acids of LT-ag and 3 unique carboxy-terminal residues), $T'_{136}$ (first 132 amino acids of LT-ag and 4 unique carboxy-terminal residues), and $T'_{165}$ (first 132 and last carboxy-terminal amino acids of LT-ag) [29]. Stable Rat-2 cell lines could be readily established expressing the complete early region of JCV expressing all five proteins (LT-ag, st-ag, $T'_{135}$, $T'_{136}$ and $T'_{165}$), LT-ag alone, or only a single T' protein. All cell lines had doubling time and saturation densities that were comparable to control Rat-2 cells. Cells expressing all five early proteins exhibited anchorage-independent growth, and so did cells that expressed only LT-ag or $T'_{135}$. Neither oncogenic H-Ras alone, nor any of the T′ proteins separately could immortalize rat embryo fibroblasts, while co-expression of individual T' proteins plus mutated H-Ras led to immortalization, suggesting that the T′ proteins are required but not sufficient for immortalization [58]. While 17kT is expressed at very low levels, the JCV T′ proteins are expressed at high levels in a lytic infection and the half-life of $T'_{136}$ is similar to that of JCV LT-ag. The splicing is regulated differentially in transformed versus lytic-infected cells and temporally during the course of a productive infection [29]. The T′ proteins are required for efficient JCV replication, as mutated JCV lacking the expression of all three T' proteins replicated 10- to 20-fold slower in human foetal glial cells compared to wild-type JCV. The unique carboxy-termini of the T′ proteins have distinct phosphorylation patterns, which

may cause altered biological properties [60]. Alternative small open reading frames that may encode peptides are found in the BKV genome. BELP, a putative 39-amino acid hydrophobic peptide encoded by the early region corresponds to the previously described SV40 early leader peptide, SELP. SV40 mutants carrying a 23-amino acids deletion in SELP behaved like wild-type virus [61]. Another open reading frame, located at the 3′ end of the BKV early region, may theoretically encode a 75-amino acid peptide [62]. Although the biological relevance of these proteins has not been unravelled, it was proposed that they might influence tissue tropism, and the pathogenic and oncogenic potentials of BKV *in vivo* [29, 62, 63]. The expression levels of these alternative HPyV early proteins have not been examined in human tumours.

### 2.5. Agnoprotein

The leader sequence of the late transcript of all three HPyV encodes an ~70-amino acids long polypeptide, known as the agnoprotein. Viral cell propagation with agnoprotein-deficient mutants or depletion of agnoprotein through RNA interference, and the identification of interaction partners (see Table 1) have helped to elucidate the function of this viral protein. The agnoprotein of all three HPyVs is phosphorylated *in vivo* [42, 64]. Phosphorylation mutants of JC virus agnoprotein were unable to sustain the viral infection cycle, because virus particles deficient in DNA content were produced [64]. The agnoprotein predominantly resides in the cytosol and in the perinuclear region in association with the outer nuclear membrane, but a minor fraction of the protein can also be detected in the nucleus [65–67; our unpublished results]. The SV40 agnoprotein has been suggested to play a role in virion assembly, as viral propagation with SV40 mutants lacking functional agnoprotein was reduced compared to wild-type virus [68–72]. SV40 agnoprotein facilitates perinuclear-nuclear localization of VP1 [73, 74]. BKV strain AS, encoding a N-terminal modified agnoprotein, can be successfully propagated in cell culture, while BKV variants with deleted agnogene were detected in the urine of renal transplant patients [75, 76]. A JCV variant with a 21-bp deletion near the 3' end of the agnogene was isolated from the urine in a population from Papua New Guinea, while a JCV mutant deficient in agnoprotein expression was viable but replicated less efficiently than the wild type [64, 77]. Specific depletion of JCV agnoprotein expression by small interfering RNA (siRNA) reduced the expression of both early and late gene expression and virus production [78, 79]. These findings suggest that the BKV and JCV agnoproteins might be involved in virion assembly, but they are not absolutely required for viral multiplication. The interactions of agnoprotein with the cellular proteins HP1α and fasciculation and elongation protein zeta 1 (FEZ1) have been proposed to promote the translocation of virions out of the nucleus [25, 28].

JCV agnoprotein can bind LT-ag, an interaction that requires the amino-terminal part of agnoprotein and the central part of LT-ag. The biological relevance of the interaction is poorly understood, but agnoprotein repressed both basal and LT-ag-mediated late transcription and viral DNA replication [54]. Whether agnoprotein influences the transforming ability of LT-ag is not known.

## 3. Molecular mechanisms for HPyV-induced transformation

The oncogenic potential of all three HPyV *in vitro* and in animal models, and their association with human cancers have been excellently reviewed by others and is beyond the scope of this review [2, 7, 8, 10–12, 33, 80]. Here, we provide an overview of the putative mechanisms by which HPyV proteins may induce transformation (summarized in Fig. 2). The combination of mechanisms that is required for HPyV-induced cancer in humans is difficult to disclose, and probably depends on the cellular context and other factors.

### 3.1. Effect on the cell cycle

HPyV and other small DNA viruses have evolved a common strategy to invoke unscheduled S phase entry, thereby replicating the viral genome together with the host cellular DNA. While progression into S phase is vital to the propagation of the virus in permissive cells, such an event may contribute to oncogenic transformation in nonpermissive cells. This virus-induced S phase entry is predominantly achieved by inactivation of the tumour suppressor proteins pRb and p53. LT-ag is the major actor in this process, but st-ag and agnoprotein may play a supporting role.

#### 3.1.1. Targeting the retinoblastoma family

The retinoblastoma protein (pRb), originally described in a rare childhood eye tumour called retinoblastoma in which this protein was missing or defective, controls cell cycle progression from the G1 to S phase. pRb is a member of the retinoblastoma family that also includes p107 and p130 (or pRb2). The pRb proteins undergo cell cycle-dependent phosphorylation. During the G1 to S phase transition, pRb is converted from its hypo- to hyperphosphorylated form, while p107 and p130 become hyperphosphory-

**Table 1.** Cellular proteins associated with the regulatory proteins of the human polyomaviruses.

| Cellular protein | Function | Viral protein | Interaction | Biological implication | References |
|---|---|---|---|---|---|
| AP-1 (c-Jun and c-Fos) | transcription factor | LT (SV40, JCV) | *in vivo* | altered gene expression | 19, 20 |
| AP-2 | transcription factor | LT (SV40) | *in vivo* | altered gene expression | 19 |
| Brn1 | transcription factor | LT (JCV) | *in vivo* | unknown | 21 |
| Bub1 | mitotic spindle checkpoint protein | LT (SV40) | *in vivo* | override spindle checkpoint | 22 |
| Bub3 | mitotic spindle checkpoint protein | LT (SV40) | *in vivo* | override spindle checkpoint | 22 |
| β-catenin | transcription factor | LT (JCV) | *in vivo* | altered gene expression | 20 |
| Cul7 (p185) | proteosomal-induced degradation | LT (SV40) | *in vivo* | unknown | 11, 23 |
| DNA polymerase α | DNA replication | LT (SV40) | *in vivo* | viral DNA replication | 20 |
| Fbw7 | proteosomal-induced degradation | LT (SV40) | *in vivo* | unknown | 11, 24 |
| FEZ1 | microtubules interaction protein | agnoprotein (JCV) | *in vivo* | unknown, but overexpression of FEZ1 inhibits viral propagation | 25 |
| Gelsolin | orginization of actin filaments | LT (SV40) | *in vitro* and Y2H | unknown | 26 |
| Histone H1 | chromatin structure | LT (SV40) | *in vivo* | DNA replication | 27 |
| Histone H3 | chromatin structure | LT (SV40) | *in vivo* | DNA replication | 27 |
| HPα-1 | chromatin-interaction protein | agnoprotein (JCV) | *in vivo* | nuclear egress of viral particles | 28 |
| Hsc70 | DnaK | LT (SV40, JCV)<br>$T'_{135}$, (JCV) | *in vivo*<br>*in vivo* | disruption of pRb-E2F complex | 11<br>29 |
| IRF-1 (interferon-regulatory factor-1) | transcription factor and tumor suppressor | LT (SV40) | *in vivo* | LT-ag induced degradation? | 30 |
| IRS-1 (insulin receptor substrate-1) | activator of PI3K | LT (SV40, JCV) | *in vivo* | signal transduction | 20, 31–35 |
| Ku70 | part of DNA-PK | agnoprotein (JCV) | *in vitro* (?) | influences DNA repair | 36 |
| Lamin C | component nuclear lamina and matrix | LT (SV40) | *in vitro* and Y2H | unknown | 26 |
| Laminin γ | component of basal membrane and extracellular matrix | LT (SV40) | *in vitro* and Y2H | unknown | 26 |
| MDM2 | oncogene | LT (SV40) | *in vitro* | unknown | 37 |
| Nbs1 | DNA repair and replication | LT (SV40) | *in vivo* | genomic instability | 11, 38, 39 |
| NF2 | tumor suppressor protein | LT (JCV) | *in vivo* | override G1/S checkpoint | 40 |
| Nucleolin | DNA replication | LT (SV40) | *in vivo* | DNA replication | 41 |
| p193 | pro-apoptotic protein | LT (SV40) | *in vivo* | preventing apoptosis | 23 |
| p50 | unknown | agnoprotein (BKV) | *in vivo* | unknown | 42 |
| p52 | unknown | agnoprotein (JCV) | *in vivo* | unknown | 43 |
| p53 | tumor suppressor | LT (SV40, BKV, JCV)<br>agnoprotein (JCV) | *in vivo*<br>*in vivo* | promotes G1/S transition<br>transactivation of p21promoter | 20<br>44 |
| p75 | unknown | agnoprotein (BKV) | *in vivo* | unknown | 42 |
| p100 | unknown | agnoprotein (BKV) | *in vivo* | unknown | 42 |
| p103 | unknown | agnoprotein (JCV) | *in vivo* | unknown | 43 |

**Table 1** *(Continued)*

| Cellular protein | Function | Viral protein | Interaction | Biological implication | References |
|---|---|---|---|---|---|
| p112 | unknown | agnoprotein (JCV) | *in vivo* | unknown | 43 |
| p158 | unknown | agnoprotein (JCV) | *in vivo* | unknown | 43 |
| p300/CBP | transcriptional coactivators | LT (SV40) | *in vivo* (indirect via p53) | stimulation of E2F-mediated transcription | 11 |
| p400 | DNA damage response | LT (SV40) | *in vivo* (indirect via p53) | unknown | 45 |
| PP2A | ser/thr protein phosphatase | st (SV40) | *in vivo* | abnormal protein dephosphorylation | 46 |
| pRb/p107/p130 | tumor suppressor | LT (SV40, BKV, JCV) T' (JCV) | *in vivo* | promotes G1/S transition | 47<br>29 |
| Pur-α | transcription factor | LT (JCV) | *in vivo* | altered gene expression | 20 |
| Ran | GTPase | LT (SV40) | in vitro | centrosome amplification resulting in genomic instability | 48 |
| Replication protein A | DNA replication | LT (SV40) | in vivo | viral DNA replication | 49 |
| RNA polymerase II (140kDa subunit) | transcription factor | LT (SV40) | *in vivo* | altered gene expression | 19 |
| Sp1 | transcription factor | LT (SV40) | *in vivo* | altered gene expression | 19 |
| TAF1 ($TAF_{II}250$) | transcription factor | LT (SV40) | *in vivo* | altered gene expression | 19 |
| TAF4 ($TAF_{II}135$) | transcription factor | LT (SV40) | *in vivo* | altered gene expression | 19 |
| TAF5 ($TAF_{II}100$) | transcription factor | LT (SV40) | *in vivo* | altered gene expression | 19 |
| TBP | transcription factor | LT (SV40) | *in vivo* | altered gene expression | 19 |
| TEF-1 | transcription factor | LT (SV40) | *in vivo* | altered gene expression | 19 |
| Thymosin β4 | organization of actin filaments | LT (SV40) | *in vitro* and Y2H | unknown | 26 |
| Topoisomerase I | DNA replication protein | LT (SV40)<br>st (SV40) | *in vivo* | viral DNA replication | 50 |
| Tst-1/Oct6/SCIP | transcription factor | LT (JCV; SV40) | *in vivo* | synergistic activation of JCV promoter | 20, 21 |
| Tubulin | intracellular protein trafficking | LT (SV40)<br>st (SV40)<br>agnoprotein (JCV) | *in vivo*<br>*in vitro*<br>*in vivo* | unknown<br>unknown<br>unknown | 51<br>52<br>25, 43 |
| YB-1 | transcription factor | LT (JCV)<br>agnoprotein (JCV) | *in vivo*<br>*in vivo* | altered gene expression | 20, 53<br>54 |

lated during the late G1 to S phase. The phosphorylation is most notably mediated by cyclinD1/cdk4 and cyclin E/cdk2 [81]. The retinoblastoma proteins control S phase cell cycle progression by modulating the transcription of E2F-responsive genes. E2F is a family of transcription factors that consists of at least eight known members, some of which have multiple isoforms. The expression of some of the E2Fs is cell cycle-dependent and cell-specific [82]. E2F1, -2, -3a, and -3b are transcriptional activators and interact with pRb, while E2F4 and E2F5 are transcriptional repressors that preferentially bind p107 and p130. E2F6, E2F7, and E2F8 lack a pocket protein interaction domain, and function as repressors. E2F1 to E2F6 bind DNA through a heterodimerization with a dimerization partner (DP1- or DP-2), while E2F7 and E2F8 can bind DNA directly. Hypophosphorylated pRb family members regulate the activity of E2Fs predominantly by recruiting histone deacetylases (HDACs), chromatin remodelling factors and histone methyltransferases. Moreover, pRb can repress transcription by physically blocking the tran-

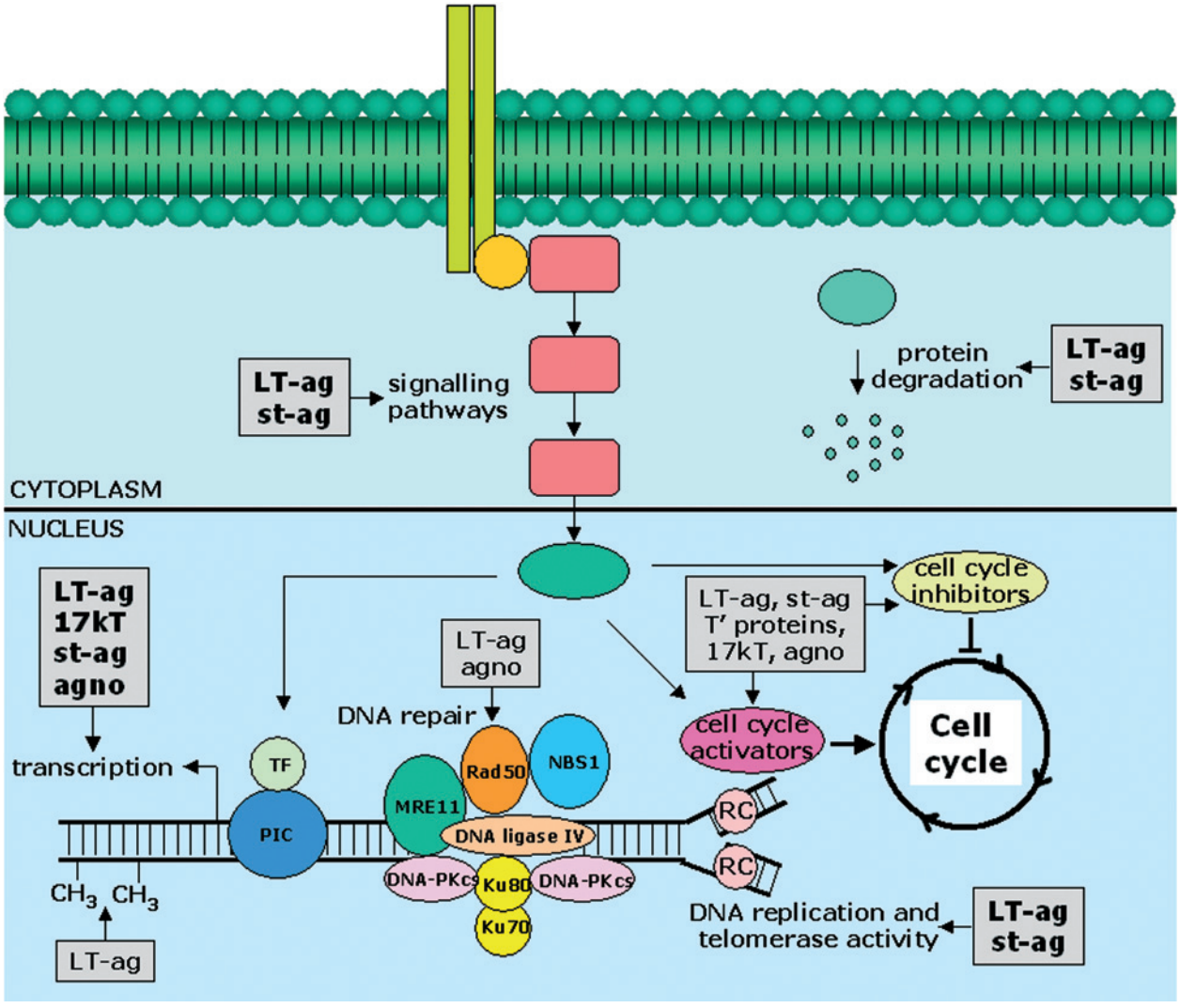


**Figure 2.** Mechanisms by which the different HPyV proteins can contribute to cellular transformation. Signalling cascades regulate several cellular processes, such as gene transcription, DNA replication, and cell cycle progression. The HPyV regulatory proteins can interfere with signalling pathways or with their targets, such as transcription factors (TF), general transcription factors (PIC, preinitiation complex), or cell cycle activators and inhibitors. Some of the HPyV regulatory proteins also influence protein degradation, DNA repair, DNA replication, and telomerase activity. LT-ag has also been shown to enhance DNA methylation, thereby repressing transcription. The concerted action of the HPyV proteins disturbs crucial cellular processes, which may lead to cellular transformation. See text for details.

scription activation domain of E2F and by preventing the assembly of the preinitiation complex. E2Fs stimulate transcription of genes that encode proteins controlling cell cycle progression and DNA replication such as cyclins A, D1 and E, dihydrofolate reductase, thymidine kinase, Cdk2, cdc2, cdc6, DNA polymerase α, c-Myc, c-Myb, but also genes whose products are involved in DNA repair, differentiation and apoptosis. On the other hand, E2Fs represses the transcription of tumour suppressor genes such as $p19^{ARF}$ [82–86]. In quiescent cells, E2F-regulated genes are not expressed because their promoters are occupied primarily with p130/E2F4 complexes, which repress transcription. Inactivation of Rb protein by phosphorylation results in the replacement of these complexes by activating E2F1–3. While repression of E2F-mediated transcription seems to be the major mechanism by which retinoblastoma proteins retain cells in the G1 phase, other mechanism may apply. Indeed, p107 and p130 can also prevent cell cycle progression by acting as Cdk2 inhibitors [87].

Shortly after pRb was discovered, SV40 LT-ag was identified as a binding partner for pRb and later also for the other retinoblastoma family members. In fact, the LT-ags of all three HPyVs have been shown to interact with the Rb family members, although with different affinity. The LT-ag LxCxE motif (residues 103–107) is absolutely required for this interaction [47, 60, 88, 89]. LT-ag preferentially usurps hypophosphorylated pRb, resulting in disruption of pRb/E2F

complexes. Disruption of pRb/E2F complexes involves the N-terminal J domain of LT-ag. This region binds Hsc70, a chaperone with weak intrinsic ATPase activity. In the presence of co-chaperones (e.g. the DnaK/DnaJ families of co-chaperones) and the J domain-containing protein (i.e. LT-ag), the ATPase activity of Hsc70 increases dramatically. The energy generated by hydrolysis of ATP is used to split the pRb/E2F complex [90, 91]. Disruption of pRb family members/E2F complexes by LT-ag may be cell type-specific as SV40 LT-ag abrogated p107/E2F and p130/E2F, but not pRb/E2F complexes, in NIH3T3 cells and in cultured monkey kidney epithelial cells, while pRb/E2F and p107/E2F complexes remained intact in LT-ag expressing BSC40 cells. How LT-ag discriminates between different pRb family members/E2F complexes, as well as the biological significance of this differential action is unclear, but cell-specific disruption of pRb/E2F complexes by LT-ag may account for the cell type-specific transformation by HPyV [11, 18, 87, 92]. A second mode of LT-ag to target the pRb pathway is by influencing the expression levels of the retinoblastoma family members. Transformation of mouse embryonic fibroblasts (MEFs) by SV40 LT-ag led to proteasome-dependent degradation of p130, but did not alter the levels of pRb and even resulted in increased levels of p107. BKV LT-ag, on the other hand, downregulated the expression and phosphorylation of all three Rb family members [47, 60, 89]. It is not known whether JCV LT-ag influences the expression of all Rb proteins, but it was shown to reduce the protein levels of p130. Overexpression of p130 overcame cellular transformation mediated by JCV LT-ag and resulted in suppression of tumour formation both *in vitro* and *in vivo* [83]. This suggests that targeting of Rb proteins by JCV LT-ag is important in JCV-induced transformation. Finally, a third mechanism by which LT-ag may modulate the biological action of the Rb members is by affecting their phosphorylation. Incubation of purified LT-ag with p130 in a cell-free system reduced the phosphorylation of p130. LT-ag-induced dephosphorylation required an intact J domain and was inhibited by okadaic acid, suggesting that the J domain recruits a phosphatase that acts on p130. The rationale behind LT-ag-mediated dephosphorylation of p130 is not completely understood because hyperphosphorylation of p130 during the late G1 phase seems to be required for progression to the S phase [83–86].

The alternative early proteins may also contribute to transformation by targeting the Rb family members. The 17kT protein was shown to reduce the levels of p130, to stimulate cell cycle progression of quiescent fibroblasts, and to transactivate the E2F-responsive cyclin A promoter. All these functions seem to require the J domain of the protein [93, 94]. The exact role of 17kT in cellular transformation remains elusive, but it can transform rat F111 fibroblasts [59]. All three JCV T′ proteins retain the ability to bind the retinoblastoma family members, although with different efficiencies. The binding is preferentially to the hyperphosphorylated forms of the Rb family members. While LT-ag had the highest affinity for pRb, $T'_{135}$ and $T'_{136}$ interacted more efficiently with p107 than LT-ag or $T'_{165}$. $T'_{165}$ bound less efficiently to p130 than the three other LT-ag forms. JCV-transformed PHFG cells displayed reduced p130 levels and only un(der)phosphorylated protein, while the concentrations of p107 and pRb proteins were substantially increased compared to non-transformed cells. Also, in Rat-2 cell lines stably expressing individual T' proteins, reduced levels of hyperphosphorylated p107 and p130 were detected compared to parental Rat-2 cells [58, 60, 87]. These findings indicate that the T' proteins may contribute to cellular transformation by the combined action of reducing the levels of pRb, interacting with and altering the phosphorylation pattern of the pRb family members, thereby generating active E2F. JCV $T'_{135}$ also binds Hsc70, but the biological relevance for oncogenesis is not known [results not shown in 60].

3.1.2. Targeting p53 family

The multifunctional p53 is the best-studied member of a family that also includes p63 and p73 [95]. The p53 protein represses cell cycle proliferation and angiogenesis, and stimulates apoptosis mainly by acting as a transcription factor that controls the expression of genes whose gene products are involved in these processes. p53 arrests cell cycle progression in the G1/S and G2 phases. G1/S phase arrest is obtained through p53-mediated activation of the gene encoding cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$, a negative regulator of cyclin E/cdk2 and cyclin D/cdk4–6. These kinase complexes phosphorylate pRb, p107, and p130 during G1. G2 arrest by p53 is less clear, but it may involve the effectors $p21^{Waf1/Cip1}$and 14–3-3σ [96].

Thus one mechanism by which HPyV may induce transformation is sequestering the cellular regulator p53. LT-ag therefore negatively interferes with the transcription of p53 target genes, such as $p21^{Waf1/Cip1}$, and this may drive the cell into proliferation and contribute to transformation [97–99]. At present it is not completely clear whether LT-ag inhibits some or all of p53 functions or even whether LT-ag may resuscitate functions [11, 18].

The JCV agnoprotein also interacts with p53, and constitutive expression of agnoprotein in NIH3T3 cells increased $p21^{Waf1/Cip1}$ promoter activity and $p21^{Waf1/Cip1}$ protein levels, accounting for the slower

growth rate and accumulation in the G2/M phase of these cells [44]. Thus, while LT-ag negatively interferes with p53-mediated expression of $p21^{Waf1/Cip1}$, agnoprotein seems to exert an opposite effect. Since agnoprotein has been detected in LT-ag positive cancer cells [100], it seems unlikely that agnoprotein counteracts the action of LT-ag on p53.

SV40 LT-ag does not bind the p63 and p73 members of the p53 family, suggesting that they are not important for LT-ag-induced transformation [90]. The interaction between JCV and BKV LT-ag and p63 and p73 has not been addressed yet, nor is it known whether other HPyV proteins can interact with p63 and/or p73.

3.1.3. Targeting the CBP family

The co-activator proteins p300 and CBP (CREB-binding protein) are recruited by many different transcription factors, such as CREB, nuclear receptors, STAT, NF-κB, and AP-1. CBP/p300 can stimulate transcription of genes through their intrinsic acetylation activity [101]. Moreover, CBP and p300 are involved in cell growth, cell cycle progression and development, and are implicated in cancer [102, 103]. Neither CBP nor p300 interacts directly with SV40 LT-ag, but rather p53 serves as a scaffold to bridge the interaction between SV40 LT-ag and p300/CBP. LT-ag can enhance the histone acetylase activity of p300/CBP, and the presence of LT-ag increases phosphorylation of p53 at Ser-15. This phosphorylation event stabilizes the interaction between p53 and p300/CBP. CBP in turn acetylates LT-ag on K697, a residue that is conserved in JCV and BKV [11, 18]. The biological consequence of LT-ag acetylation is not completely understood, but it did not affect the interaction of SV40 LT-ag with p53, pRb, or Fbw7 (see section 3.6.), nor did it alter the subcellular localization of LT-ag. However, acetylation strongly reduced the half-life of LT-ag from >36 h for unacetylated to ~10 h for the acetylated form. The mechanism involved in degradation of acetylated LT-ag has not been solved, but seems to be independent of the ubiquitin-proteasome pathway, since the proteasome inhibitor MG-132 did not inhibit accelerated degradation of acetylated LT-ag [104]. Another puzzling question is: Why would LT-ag recruit CBP to induce its own destabilization? Transient downregulation of LT-ag is required to allow a switch from the early to the late phase during viral propagation. One way to achieve this is by LT-ag-induced acetylation mediated by CBP/p300. However, when excessive amounts of LT-ag compared to CBP/p300 start to amass in the cell, the majority of LT-ag will not be acetylated and will have a prolonged life span. On the other hand, it is feasible that accumulation of abnormally high LT-ag concentrations during neoplastic processes allows LT-ag to usurp most of CBP/p300, thereby affecting p53-mediated transcription, but also transcription mediated by other transcription factors that interact with p300/CBP [101]. Sequestering of p300/CBP by LT-ag may negatively interfere with transcription of genes whose products are involved in cell cycle progression, thereby contributing in the transformation process. Indeed, diminished CBP protein levels in heterozygous $CBP^{+/-}$ mice or in patients with the haploinsufficient disorder Rubinstein-Taybi syndrome were associated with increased incidence of tumours [105]. It should be noted that LT-ag might help to deliver p300/CBP to E2F, which may enhance transcription of E2F target genes and G1 to S cell cycle progression (section 3.1.1) [11, 18]. Another example that underscores a role for the LT-ag/CBP/p300 link in transformation comes from the observation that stable transfection of NIH3T3 cells with the acetylation-mimicking mutant L697Q had reduced anchorage-independent growth compared with cells expressing wild-type or non-acetylatable K697R LT-ag [104].

p400, a chromatin-remodelling enzyme closely related to p300 and CBP, plays a role in the regulation of p53-mediated expression of $p21^{Waf1/Cip1}$, as depletion of p400 by RNA interference resulted in G1 arrest and $p21^{Waf1/Cip1}$ induction [106]. Moreover, p400 can be recruited by E2F1 and may be involved in E2F-dependent acetylation, transcription, and S phase entry [107]. p400 was shown to interact with p53 and can also bind LT-ag [45]. The biological relevance of this interaction, as well as the putative role in transformation remains unsolved, but an adenovirus E1A mutant defective in p400 binding was also defective in transformation. Because E1A and large T-ag share functional similarities, the interaction of LT-ag with p400 may also be involved in transformation [108].

### 3.2. Effect on telomerase

To circumvent loss of terminal DNA after each round of eukaryotic chromosome replication, eukaryotic cells utilize telomerase ribonucleoprotein. This RNA-protein complex carries the reverse transcriptase telomerase enzyme and an internal template for telomeric repeat synthesis that specifically recognizes chromosome ends as substrates. Surprisingly, telomerase does not behave as a constitutive enzyme, but its activity is extensively regulated in a cell type- and stimulus-specific manner [109, 110]. Constitutive telomerase activity has been associated with immortalization, and cancer cells strongly upregulate telomerase activity and remain dependent on telomerase function for viability [111]. Thus, inducing the telomerase activity could be one strategy by which oncogenic virus can immortalize cells. Indeed, SV40 as well as BKV infection induces telomerase activity in

primary human mesothelial cells, but not in primary fibroblasts [112]. As immortalized human cells are much more amenable to appropriate transforming stimuli compared to non-immortalized cells, HPyV may render host cells more vulnerable to transformation by immortalizing them through enhancing the telomerase activity. Telomerase activity was undetectable or minimal in mesothelial cells infected with or transformed by SV40 st-ag mutants, while human epithelial cells expressing st-ag had higher telomerase activity compared to control cells. These results suggest that st-ag is required for activating this enzyme [113, 114]. The mechanism by which st-ag activates telomerase has not been solved, but hTERT, a component of the telomerase complex, contains two consensus Akt kinase phosphorylation sites and can be activated by Akt *in vitro* [115]. Thus st-ag may inhibit PP2A-mediated dephosphorylation of the protein kinase Akt/PKB, resulting in more active Akt, which can phosphorylate the telomerase reverse transcriptase subunit, thereby increasing telomerase activity [113].

### 3.3. Effect on apoptosis/cell survival

Evasion of apoptosis is one of the hallmarks of cancer, and viruses, including HPyV, have developed several mechanisms to prevent apoptosis of the infected cell. An intrinsic p53-independent anti-apoptotic activity has been mapped in residues 525–541 of SV40 LT-ag [11]. This region bears homology with the BH-1 domain, a region present in anti-apoptotic proteins such as Bcl-2 and Bcl-$x_L$ [116]. In addition, the amino-terminal domain of SV40 LT-ag binds the pro-apoptotic protein p193 and antagonizes its apoptotic activity. Loss of p193 activity is associated with marked growth enhancement in NIH3T3 cells, suggesting that the anti-apoptotic activity of LT-ag towards p193 may contribute to LT-ag-mediated neoplastic events [23].

SV40 st-ag exerted a p53-independent anti-apoptotic effect in rat embryonic fibroblast cells, while *in vivo* CD95-mediated apoptosis in transgenic mice with liver-specific expression of st-ag was blocked [117, 118]. The exact mechanism by which st-ag prevents apoptosis is not known, but may involve the prevention of PP2A-mediated dephosphorylation of Akt or its substrate Bax. Phosphorylation of Akt, a crucial event in PI3K/Akt-mediated cell survival, was increased in the livers of st-ag transgenic mice and in primary human keratinocytes expressing st-ag [114, 119, 120]. Phosphorylation levels of Bax, which is a pro-apoptotic protein, were also increased in SV40 st-ag-expressing cells [121]. On the other hand, SV40 st-ag is capable of inducing apoptosis in U2OS, retinoic acid-stimulated HL-60, and p53-deficient H1299 cells [122, 123]. Remarkably, the ability of st-ag to induce apoptosis in U2OS and H1299 cells was also PP2A-dependent [122]. How st-ag can induce or evade apoptosis by inhibiting the same target protein PP2A is not understood, but the cell-specific composition of PP2A and hence cell-specific substrate specificity of this protein phosphatase may be responsible.

### 3.4. Effect on signalling pathways

Several studies suggest that the oncogenic behaviour of HPyV may involve deregulation of different cell-signalling pathways by LT-ag and st-ag (Fig. 2).

#### 3.4.1. The phosphoinositide-3 kinase-Akt/protein kinase B (PI3K/Akt) pathway

The PI3K/Akt pathway is a critical determinant of cellular processes such as metabolism, cell cycle, and apoptosis. Each of these processes is also of great significance to a productive SV40 infection [124]. In addition, aberrant activation of this pathway has been associated with cancer [125, 126]. The cytoplasmic insulin receptor substrate-1 (IRS-1) is a potent activator of the PI3K/PKB signalling pathway. JCV LT-ag-positive cells contained elevated levels of IRS-1 and its receptor, insulin-like growth factor I receptor (IGF-IR). Moreover, IRS-1 resided predominantly in the nucleus, where it was associated with Rad51 (see section 3.7). IRS-1 could also physically interact with SV40 and JCV LT-ag. Cells derived from IGF-IR-deficient mice, but which expressed wild-type p53 and Rb proteins, were refractory to JCV-induced transformation. A dominant negative IRS-1 mutant inhibited growth and survival of JCV LT-ag transformed cells in anchorage-independent culture conditions, while SV40 LT-ag could not transform cells that lacked IRS-1 or cells that contained pSer-IRS-1 or inactive IRS-1. These observations emphasize the importance of the functional interaction between LT-ag and the IGF-IR/IRS-1 signalling pathway in cellular transformation by this viral protein [20, 33, 127, 128]. So far, whether BKV LT-ag binds IRS-1 has not been examined, nor has the activity of the IRS-1/PI3K/PKB pathway in HPyV positive tumours been systematically investigated.

SV40 st-ag has been shown to modulate the activity of different signalling pathways. It was found that st-ag caused PKCζ-dependent activation of the MEK/ERK MAP kinase cascade (see 3.4.3) and of NF-κB, with concomitant stimulation of cell growth. Activation of the atypical PKCζ by st-ag was dependent on st-ag's property to bind PP2A and was mediated through PI3K [129]. Others have found a role for Akt and Rac, other PI3K downstream targets, in st-ag-mediated transformation of immortalized human mammary epithelial cells [130]. However, a recent report showed that SV40 st-ag activated Akt, but not ERK

in the rat fibroblast cell line REF52 and the human fibroblast cell line BJ. Activation was dependent on PP2A, as mutation of the PP2A-binding domain abrogated st-ag-induced Akt phosphorylation [131]. Cell-type-specific activation of signalling pathways by st-ag may explain the discrepancy between the different studies. Although both mouse polyomavirus st-ag and SV40 st-ag can interact with PP2A, Rodriguez-Viciana et al. found that mouse polyomavirus preferentially activated the MEK/ERK MAP kinase cascade, while SV40 st-ag promoted phosphorylation of Akt. Thus, the effects of polyomavirus st-ag proteins on PP2A may be more complicated than originally envisioned [131].

3.4.2. The Wnt signalling pathway

The Wnt pathway is implicated in cell proliferation, cell survival, and transcription, and is also important for regulating pattern formation during development. This pathway is constitutively activated in several particular cancers due to mutations in proteins that constitute this pathway [132, 133]. JCV LT-ag binds directly to β-catenin, one of the components of the Wnt pathway, resulting in increased stability and nuclear translocation of β-catenin, and enhanced expression of β-catenin target genes such as c-myc and cyclin D1. Interestingly, JCV is associated with medulloblastomas and tumours of the colon and prostate, cancers in which the Wnt pathway frequently is aberrantly activated [20, 127]. Whether constitutive activation of the Wnt signalling pathway by LT-ag is adequate to induce cancer or also requires mutations in the components of this pathway remains to be established.

3.4.3. The Ras/Raf/MAP kinase signalling pathway

The Ras/Raf/MAP kinase signalling cascade controls biological processes such as proliferation, cell survival/cell death, mobility, differentiation, and gene expression. Not unexpectedly, mutations in genes encoding Ras and Raf are common in cancer cells [134, 135]. SV40 LT-ag could increase the activity of the small GTPase Ras, as well as of c-Raf, which is a direct downstream target of Ras [136, 137]. The precise mechanism by which SV40 LT-ag activates c-Ras and c-Raf remains elusive, but full c-Raf activation required an intact J domain. Hsp27, which interacts with the J domain of SV40 LT-ag, has been shown to inactivate c-Raf. Thus SV40 LT-ag may sequester Hsp27, resulting in derepression of Raf-1 [137]. The activity of Ras and c-Raf in HPyV-positive cancers has not been scrupulously examined.

3.4.4. The STAT3 pathway

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway consists of the tyrosine kinases JAK and the transcription factors STAT. Phosphorylation of STAT by JAK permits the dimerization of STATs and nuclear entrance where they activate transcription of their target genes. As such, the JAK/STAT signalling pathway can modulate cell proliferation, differentiation, cell migration, and apoptosis [138]. STAT3 is a potent apoptosis inhibitor, probably through its induction of the anti-apoptotic *bcl-x*$_L$ gene. SV40 LT-ag may prevent apoptosis in a STAT3-dependent manner, because depletion of STAT3 in LT-ag transformed MEF cells resulted in apoptosis, while reintroduction of STAT3 increased growth rate and restored the ability for anchorage-independent growth. By sequestering pRb, LT-ag increased expression of the E2F-responsive genes encoding the tyrosine kinases Src and IGF-IR. Both these kinases are able to activate STAT3 [139]. The activity state of STAT3 in HPyV-positive cancers has not been monitored.

3.4.5. The Notch signalling pathway

The transmembrane Notch receptors undergo proteolytic cleavage upon ligand binding. The activated intracellular fragment of Notch can regulate different signalling pathways, including PI3K/Akt, the MAP kinase JNK, and the NF-κB pathway. Moreover, this proteolytic fragment binds the transcription factor CSL (acronym for CBF1, Suppressor of Hairless, Lag-1), resulting in the release of transcriptional co-repressor proteins with histone deacetylase activity and subsequent activation of transcription. The oncogenic potential of Notch proteins resides in their ability to inhibit apoptosis and to promote cell proliferation and angiogenesis. However, Notch has also been associated with tumour-suppressive roles. The opposed function of Notch seems to depend on the cellular context [140]. Recently, it was reported that Notch-1 expression is enhanced in SV40-positive mesothelioma biopsies, in SV40-transformed mesothelial cells, and in cell lines derived from SV40-positive mesotheliomas compared to normal mesothelial cells. Infection of primary human mesothelial cells with SV40 resulted in upregulation of Notch-1 transcript levels and promoted cell cycle progression. These findings indicate that SV40-induced activation of the Notch-1 signalling pathway is involved in malignant transformation of mesothelial cells by this virus [141].

3.4.6. The hepatocyte growth factor receptor (Met) pathway

Signalling through the hepatocyte growth factor receptor Met leads to cell growth, motility, and morphogenesis. Perturbed hepatocyte growth factor receptor signalling contributes to oncogenesis and

tumour progression in several human cancers and promotes aggressive cellular invasiveness that is strongly linked to tumour metastasis [142]. SV40 LT-ag-positive, but not SV40-negative malignant mesothelioma cells expressed constitutive active Met. Transfection of LT-ag complementary DNA (cDNA) or SV40 DNA in human mesothelial cells resulted in enhanced levels and phosphorylation of Met, and a higher rate of S phase entry compared to non-transfected cells. These findings indicate that SV40 may direct malignant transformation of human mesothelial cells by activating the Met receptor. Increased Met expression occurred in the presence of LT-ag mutants that failed to bind pRb, but no phosphorylated Met was detected with such mutants. These results suggest that LT-ag may increase Met expression in a pRb-independent manner, but activation of the receptor happens in a pRb-dependent manner. The exact mechanism for LT-ag-provoked Met phosphorylation remains to be elucidated [143]. BKV infection of human primary mesothelial cells also triggered Met phosphorylation, but BKV failed to transform these cells, but rather induced lysis. The difference in outcome of an SV40 or BKV infection can be explained by the fact that BKV replicated faster than SV40 in primary mesothelial cells. Accordingly, artificial acceleration of SV40 replication by down-regulation of p53 resulted in cells lysis [112]. Thus, HPyV replication efficiency influences whether the infected cells are more likely to be lysed or transformed [144].

3.4.7. The cGMP-dependent protein kinase II pathway

One of the targets of the second messenger cyclicGMP (cGMP) is the cGMP-dependent protein kinase II. This kinase plays an important role in the induction of apoptosis, and can affect cell proliferation, differentiation, gene regulation, and apoptosis [145, 146]. SV40 LT-ag-immortalized human fibroblasts displayed strongly diminished expression of cGMP-dependent protein kinase II [147], while the LT-ag-expressing hybrid cell line IOSE-Ov29 lacked cGMP-dependent protein kinase II expression and gained enhanced malignancy-associated phenotype compared to the fusion cell lines from which it was derived [148]. These observations indicate that LT-ag may induce loss of cGMP-dependent protein kinase II expression to promote immortalization and favour development into a malignant cell.

3.4.8. The integrin signalling pathway

St-ag may influence the integrin signalling pathway, as transcript levels of *osteopontin*, *paxillin*, *f-spondin*, *gelsolin, and matrix metalloprotease-1* genes were changed in directions consistent with integrin activation, while genes involved in cell-cell adhesion were downregulated. In addition, inhibition of integrin reduced the anchorage-independent growth capacity of cells expressing SV40 st-ag [149]. Whether modulation of signalling pathways by st-ag is essential for transformation is not known, nor has the activity state of these signalling pathways been investigated in st-ag-expressing human cancers.

### 3.5. Effect on gene expression

3.5.1. Effect on transcription regulatory proteins

Earlier studies have shown that as much as 3% of the mRNA species in SV40-transformed cells are novel compared with non-transformed cells and that infection with HPyV induces major changes in the gene expression profile of the host cell [reviewed in 19]. Differential display, microarray analysis, and proteomic studies allowed identification of genes or their gene products that are differentially expressed in HPyV-positive samples. Not surprisingly, genes with changed expression pattern encode proteins involved in proliferation (many of them E2F-responsive genes), transcription, translation, DNA repair, apoptosis, angiogenesis, and DNA methylation [see e.g. 149–156]. The mechanisms by which HPyV proteins affect cellular transcription are briefly discussed here. For a more extensive overview, the reader is referred to other reviews on the matter [19, 20, 130].

Both LT-ag and st-ag can alter the amounts of cellular transcription factors, which in turn will affect the activity of promoters that contain binding motifs for such transcription factors. SV40 LT-ag can, for example, influence the mRNA levels of Sp1, AP-1 (c-Fos and c-Jun), c-Myc, and TFIIC, while st-ag transactivates or represses the c-*fos* promoter in a cell-dependent fashion [157–159]. SV40 st-ag was shown to induce expression of the c-*myc* gene, which encodes the c-Myc transcription factor that plays an import role in cell cycle regulation [149, 156].

The viral proteins can also alter the transcriptional activity of particular transcription factors. SV40 LT-ag not only modified the relative proportions of splice variants of TEF1, a transcription factor that influences SV40 promoter activity, but by binding it also modulated TEF-1's activity, a property that seems to be involved in transformation. The LT-ag mutant (S189N) that could not bind TEF-1 was eightfold less efficient in focus formation assays, but the effect of this mutation on other LT-ag functions was not addressed, hence the importance of LT-ag-TEF-1 interaction in the neoplastic process remains elusive [160]. Numerous studies have shown that SV40 st-ag-mediated inhibition of PP2A resulted in activation of

different signal transduction pathways, which in turn stimulated the activity of the transcription factors Sp1, NF-κB, AP-1, Elk-1, c-Myc, FHL2, and CREB [19, 129, 159, 161, 162]. Aberrant activity of these transcription factors has been firmly associated with several different human tumours [163–169].

HPyV LT-ag and st-ag can interact with transcription regulatory proteins, including TATA-binding protein (TBP), TBP-associated factors (TAF1, 4, 5, and 9), TFIIB, the 140-kDa subunit of RNA polymerase II, Sp1, ATF, c-Jun (AP-1), AP-2, TEF-1, Tst-1, Pur-α, YB-1, CBP and p300 [19]. The agnoprotein of JCV can interact with YB-1, and this negatively influenced YB-1-induced expression of both early and late viral genes [170]. Whether agnoprotein influences the expression of YB-1-responsive cellular genes has not been investigated. The biological consequences and the relevance in tumour formation of the interactions between HPyV regulatory proteins and transcription factors remain poorly understood.

Finally, it was demonstrated that SV40 LT-ag could functionally replace the general transcription factor TAF1 [171]. TAF1 possesses an intrinsic protein kinase activity able to phosphorylate p53 at Thr-55, resulting in degradation of p53 and G1 progression [172]. pRb binds to TAF1 and inhibits its kinase activity, suggesting that LT-ag may restore the kinase activity of TAF1 by levying a distress upon pRb [173]. Moreover, TAF1 facilitates expression of genes like cyclin A and D1, thereby stimulating G1 to S phase progression [174]. All these observations sustain a model in which LT-ag can override cell cycle control through adopting the functions of TAF-1.

In conclusion, HPyV regulatory proteins can affect the expression of cellular genes, but it remains to be verified whether this mechanism is implicated in HPyV-associated tumorigenesis.

3.5.2. Effect on DNA methylation

Anomalous epigenetic modifications such as DNA and histone methylation and histone acetylation or deacetylation have been linked to human cancer [175]. HPyV-induced transcriptional silencing by DNA hypermethylation may form a mechanism by which these oncoviruses transform cells. Indeed, aberrant hypermethylation of genes encoding components of the apoptotic pathway and tumour suppressor genes was detected in SV40-infected human mesothelial and peripheral blood mononuclear cells, in SV40-positive tumours, and in JCV LT-ag positive sporadic human colorectal cancers [176–179]. Moreover, HPyV LT-ag expression in cells or in transgenic mice coincided with enhanced mRNA and protein levels of the DNA methyltransferases DNMT-1 and DNMT3b3, and correlated with increased overall DNA methylation [180–183]. This suggests that DNA hypermethylation in HPyV-positive tumours is caused by increased expression and activity of DNA methylating enzymes. The biological significance of LT-ag-triggered DNA methyltransferase activity in the oncogenic program of HPyV is illustrated by the following observations. First, SV40 LT-ag could transform Balb/c 3T3 cells, but not cells treated with DNA methyltransferase antisense oligonucleotides [180]. Second, 7 out of the 13 transgenic mice with prostate-specific expression of the SV40 early proteins developed poorly differentiated prostate cancer at 24 weeks of age, while none of the 14 transgenic mice that were treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine showed any signs of malignant disease [182, 183]. Finally, elevated DNMT-1 levels have been reported in tumours associated with HPyV [182]. The mechanism by which LT-ag influences DNA methyltransferase expression has not been completely unveiled, but a non-pRb-binding LT-ag mutant failed to increase the expression of DNA methyltransferase, indicating that LT-ag-induced DNMT-1 expression may depend upon pRb inactivation/active E2F-mediated transcription. Another unsolved question is how gene-specific hypermethylation is obtained in HPyV-positive tumour cells.

### 3.6. Effect on protein turnover

The presence of many proteins is required only during certain developmental stages or phases of the cell cycle, and therefore they have a limited half-life. Proteases alone or as part of large protein degradation complexes play a crucial role in controlling the temporal existence of target proteins. As aberrant protein degradation has been linked to several diseases, including cancer [184], deregulation of protein turnover by HPyV may be one strategy to induce tumorigenesis. Both LT-ag and st-ag can modulate the stability of cellular proteins, a property that may contribute to the transformation of cells by these viral proteins. LT-ag interacts with cullin 7 (CUL7) and with the α, β, and γ isoforms of the F-box and WD40 domain protein 7 (Fbw7). CUL7 is a scaffold protein that forms the core subunit of E3 ubiquitin ligase complex involved in degradation of target proteins, while Fbw7 is the substrate recognition component of the $SCF^{Fbw7}$ ubiquitin ligase. Stable transfection studies with either wild-type LT-ag or non-CUL7-binding LT-ag mutants revealed that wild-type LT-ag promoted proliferation and growth to high densities in both wild-type and $CUL7^{-/-}$ MEF cells, while non-CUL7-binding LT-ag mutants induced cell division and growth to high densities only in CUL7-deficient MEF cells. These results suggest that the interaction between LT-ag and CUL7 is implicated in

transformation, but the mechanism is not known [11, 185].

The association between LT-ag and Fbw7 required phosphorylation of LT-ag at Thr-701, and prevented LT-ag acetylation, but acetylation of LT-ag did not interfere with Fbw7 binding. The implication of the LT-ag/Fbw7 aggregation in transformation awaits proof, but LT-ag may act as an inhibitor of the $SCF^{Fbw7}$ ubiquitin ligase-mediated ubiquitination by sequestering it from its physiological substrates. Actually, Fwb7 seems to be an ideal prey for LT-ag in the transformation process because almost every Fwb7 substrate is an oncoprotein (e.g. cyclin E, c-Myc, c-Jun, and Notch) and Fbw7 inactivation would stabilize these substrates. The ability of LT-ag to interfere with Fbw7-mediated protein degradation is probably not absolutely required for transformation, because the N-terminus of LT-ag, a region not involved in Fbw7 binding, is sufficient to transform cells [24].

The c-Myc protein plays a central role in cellular proliferation, differentiation, apoptosis and tumorigenesis, and its expression is regulated at several levels, including phosphorylation-dependent control of protein stability. The amino acids Thr58 and Ser62 are crucial in the phosphorylation-dependent degradation of c-Myc. Proteasomal degradation of dual phosphorylated c-Myc at these residues requires dephosphorylation of Ser62 by PP2A [186]. St-ag prolonged the half-life of c-Myc in REF52 and HEK293 cells by preventing PP2A-mediated dephosphorylation of phospho-Ser62. St-ag was able to transform HEK293 cells, but whether stabilization of c-Myc was involved in the transformation process was not investigated [162, 186]. SV40 st-ag was also shown to increase the stability of p53 in cells expressing SV40 LT-ag [187, 188]. The mechanism by which st-ag stabilizes p53 has not been solved, but may involve st-ag-mediated changes in the phosphorylation pattern of LT-ag or/and p53 [188–190]. The cellular response of the st-ag-induced increased stability of p53 is elusive, but may involve changes in p53 target genes, as SV40 st-ag could induce expression of a p53 reporter [191].

### 3.7. Effect on chromosome fidelity

The progression of a normal cell into a tumour cell is accompanied by accumulation of mutations and instabilities in the cellular genome. HPyV-induced chromosomal abnormalities may therefore be implicated in tumorigenesis by these viruses. In fact, cell culture studies have proven that HPyV may induce anomalies in the host chromosomes [6, 17, 192]. Moreover, examination of HPyV-positive tumour cells revealed a statistically significant correlation between HPyV expression and chromosomal instability in human cancers. 'Rogue' lymphocytes are aneuploid, and patients with rogue lymphocytes had significantly higher JCV, but not BKV, antibody titres compared to individuals who did not have rogue lymphocytes. This observation suggests that JCV can provoke chromosomal instability in its natural host, which is further confirmed by studies of JCV LT-ag expression in colorectal cancers. Also, there JCV LT-ag expression was strongly associated with chromosomal instability ($p=0.017$). Moreover, chromosomal abnormalities could be detected 7 days after JCV infection of the diploid colon cancer cell line RKO [179,193]. The mechanism by which HPyV may provoke mutation in the host cell genome remains poorly characterized. Most studies have addressed the implication of LT-ag, but recent studies provide a hint that st-ag and agnoprotein may also be involved.

LT-ag-induced chromosomal anomalies may be exerted by multiple mechanisms because microarray analysis of prostate cDNA prepared from wild-type mice or transgenic mice with prostate-specific expression of the SV40 early region revealed the $\geq$10-fold upregulation of genes encoding proteins related to chromosome instability, DNA damage, replication, and mitosis checkpoint [194]. LT-ag-provoked genomic instability correlates with its aptitude to deregulate normal mitotic checkpoints through physical interaction with and perturbation of the mitotic spindle checkpoint proteins Bub1 and Bub3. LT-ag mutants W94A defective in Bub1 binding failed to transform Rat-1 cells, but could still immortalize rat embryo fibroblasts [22]. Interestingly, inhibition of Bub1 expression in human fibroblasts resulted in genomic instability and anchorage-independent growth, underscoring the assumption that LT-ag-induced inactivation of Bub1 may contribute to a neoplastic phenotype of the cell [195]. On the other hand, studies with transgenic mice expressing the SV40 early region in a prostate-specific manner suggested that the viral proteins might promote malignant transformation by upregulation of Bub1 expression and hyperphosphorylation of Bub1. Hyperphosphorylation of Bub1 may account for aneuploidy, which contributes or even drives tumour development. Phosphorylation of Bub1 is mediated by the MEK/ERK pathway [194]. It might be assumed that st-ag contributes to hyperphosphorylation of Bub1 through activation of the MEK/ERK pathway by inhibiting PP2A. Interestingly, upregulation and hyperphosphorylation of Bub1 was also observed in human prostate cancer cell lines and in gastric and colorectal malignancies [194 and references therein], and HPyV sequences have been detected in these three different types of tumours [6]. Furthermore, LT-ag may affect chromosome fidelity by targeting DNA

repair enzymes. Nijmegen breakage syndrome protein 1 (Nbs1), a component of the Mre11/Rad50/Nbs1 complex that functions in DNA double-strand-break repair, was shown to bind LT-ag, and SV40 LT-ag disturbed the accumulation of MRE11 at sites of DNA damage [11, 38]. Nbs1 also guarantees a single round of replication during the S phase, which ensures genome stability. However, viruses like SV40 depend on multiple rounds of replication to enhance the yield of new viral genomes during propagation. SV40 LT-ag can inhibit Nbs1's suppression function to reinitiate replication in S phase, thereby inducing multiple rounds of viral, as well as cellular DNA replication in a given cell cycle. Reinitiation of nonviral chromosomal DNA synthesis results in the production of cells with abnormal DNA content, which may contribute to the development of the neoplastic process [39]. Another DNA repair component that can be targeted by LT-ag is Rad51, an enzyme involved in error-free homologous recombination-directed DNA repair (HRR). HRR is strongly inhibited in JCV LT-ag expression cells. The mechanism involves LT-ag-induced nuclear translocation of IRS-1. In the nucleus, IRS-1 interacts with Rad51 and negatively interferes with HRR. The molecular basis for LT-ag-induced nuclear translocation of IRS-1 and IRS-1-mediated inhibition of HRR remains to be solved [34, 35]. Finally, LT-ag may induce genomic instability through elimination of the small GTPase Ran . The adenovirus oncoprotein E1A was shown to interact with Ran *in vivo.* Ran controls a variety of cellular processes, including nucleocytoplasmic transport of RNA and proteins, cell cycle progression, and mitotic spindle organization. Expression of E1A, but not of a non-Ran-binding mutant elicited centromere amplification. These findings indicate that E1A may contribute to genomic instability through modifying the activity of Ran. A similar mechanism may apply for HPyV, as SV40 LT-ag can interact with Ran *in vitro*, but aimed studies are required to confirm this assumption [48].

A role of st-ag in inducing chromosome instability is supported by the observation that ectopic expression of st-ag in human diploid fibroblasts resulted in cell cycle block prior to mitotic metaphase due to inability to form organized centrosomes. The st-ag-mediated inhibition of centrosome function was PP2A-dependent, but the exact mechanism by which st-ag prevents centrosome formation has not been solved [196].

The agnoprotein may also disturb DNA repair, because JCV agnoprotein has been found to interact with Ku70, which is a part of DNA-dependent protein kinase (DNA-PK) involved in error-prone nonhomologous end joining (NHEJ) DNA repair *in vivo.* The mechanism of agnoprotein-mediated inhibition of DNA repair has not been completely solved, but may involve sequestering Ku70 into the cytoplasm. The involvement of agnoprotein in DNA repair was further confirmed by treating cells with the anti-tumour drug cisplatin. Cisplatin-treated NIH3T3 cells accumulated in S phase and G2/M, while NIH3T3 cells expressing agnoprotein did not. Instead, a larger fraction of the agnoprotein-expressing cells became aneuploid [36]. The impaired DNA damage-induced cell cycle arrest in the agnoprotein-expressing cells may possibly be a contributor to the genomic instability conferred on cells when they undergo polyomavirus infection as well as in malignancy [53].

### 3.8. Angiogenesis

Vascular endothelial growth factor (VEGF) plays a key role in the induction of the formation of new blood vessels during tumour development and progression. Cataloni and colleagues found that transfection of normal mesothelioma cells as well as lung carcinoma A549 cells with an SV40 LT-ag expression plasmid resulted in increased VEGF levels. SV40 st-ag alone had no effect, but potentiated LT-ag-induced expression of VEGF [197]. However, studies by another group showed that transfection of primary normal mesothelioma cells with SV40 DNA augmented VEGF expression, while transfection with an LT-ag expression plasmid alone induced VEGF production [198]. The use of different cells, different expression plasmids, or/and different transfection methods may account for the discrepancy between these investigations. The exact mechanism for SV40/LT-ag-induced expression of VEGF has not been solved, but seems to involve p53, because LT-ag could not provoke increased VEGF levels in cells with non-functional p53 [197]. p53 has been shown to downregulate VEGF promoter activity by sequestering the transcription factor Sp1 [199]. By binding p53, LT-ag may prevent p53 to usurp Sp1, thus allowing Sp1 to stimulate VEGF promoter activity. Recent findings suggested a crucial role for Notch signalling in tumour angiogenesis [140]. As outlined above, LT-ag can activate the Notch pathway, and this may also promote angiogenesis.

### 3.9. Integration

The genomes of the HPyV are often found integrated in cancer cells, but the integration site of the viral genome has been located in relatively few cases [1, 200, 201]. Although certainly not the major cause of HPyV-induced transformation, it is plausible that integration may disturb the expression of crucial genes such as proto-oncogenes or tumour-suppressor genes. SV40 DNA integration was detected at 12q23 in four SV40-immortalized human bronchial epithelial cell

lines [202]. This chromosomal region encompasses genes encoding insulin-like growth factor 1 (IGF-1), damage-regulated autophagy modulator (DRAM), SLC5A8, APAF-1, and PFM1 monocarboxylate co-transporter. The role of IGF-1 has been discussed previously in this review, and enhanced expression may provide the cell with proliferative advantages [202]. DRAM is a p53 target gene that encodes a lysosomal protein required for the induction of autophagy and essential for p53-mediated apoptosis [203]. Inhibition of the autophagy pathway is an important strategy for viral survival, and this cellular process is associated with various tumours [204]. SLC5A8 encodes a sodium monocarboxylate co-transporter that functions as a growth suppressor [205]. APAF-1 is a key factor in the mitochondrial apoptotic pathway downstream of p53 and is a potential tumour-suppressor gene [206], while PFM1 encodes a transcription factor suggested to play a role in cell differentiation and tumour suppression [207]. SV40 integration in this region may thus disconcert expression of these genes, thereby enforcing oncogenic processes.

Based on published sequences, Shera and her colleagues found a statistically significant correlation between SV40 DNA integration and nuclear matrix attachment regions. Nuclear matrix attachment domains anchor the DNA to the fibres of the chromosomal scaffold and serve as origins of replication, but can also be associated with chromosomal segments densely populated with transcription factor binding sites. This may facilitate transcription that is initiated within the region. As SV40 DNA integration retains the early coding region intact, the nuclear matrix attachment regions may stimulate transcription of the early viral proteins, thereby contributing to the malignant process. Interestingly, this site-specific preference of integration is not restricted to SV40, but was also observed for other small tumour viruses such as high-risk human papillomavirus, hepatitis B virus, and human T-cell leukaemia virus type 1 [208]. Fragile sites are specific chromosomal loci prone to breakage and rearrangements. It has been suggested that these sites represent target sites for viral integration and that integrated viral DNA may lead to silencing or activating adjacent genes or cause chromosomal rearrangements. Integrated SV40 DNA has been detected in fragile site FRA7H in the chromosomal region 7q32 of SV40-transformed human fibroblasts and near a fragile site at 9q12–21.1 in SV40-immortalized human uroepithelial cells [209, 210]. Treatment of SV40-immortalized, but not normal uroepithelial cells, induced breaks and deletions at 9q12–21.1, suggesting that chemical carcinogens in synergy with a DNA tumour virus could initiate a cascade of events that contribute to the genomic instability associated with tumorigenesis [209].

### 3.10. The effect on fatty acid synthesis and lipid membrane metabolism

Stearoyl-CoA desaturase is a regulatory enzyme in lipogenesis, catalyzing the rate-limiting step in the overall *de novo* synthesis of monounsaturated fatty acids. Enhanced expression levels of this enzyme were measured in SV40-transformed human lung fibroblasts WI38, while depletion of stearoyl-CoA desaturase in these cells by stable antisense cDNA transfection strongly reduced the proliferation rate, ablated anchorage-independent growth, and induced apoptosis [211]. The mechanism by which LT-ag affects stearoyl-CoA desaturase expression has not been corroborated, nor have stearoyl-CoA desaturase levels in HPyV-associated tumours been monitored. Moreover, the functional relevance of LT-ag-induced expression of stearoyl-CoA desaturase remains elusive, but it may be required to maintain the neoplastic phenotype of SV40-transformed cells, as elevated stearoyl-CoA desaturase activity results in increased synthesis of membrane lipids, which is important to sustain high rates of proliferation and invasion and to avoid apoptosis of neoplastic cells.

### 3.11. Other mechanisms for HPyV-induced transformation

Sustained increased levels of LT-ag and/or mutant forms of LT-ag may also disturb the otherwise harmless residence of HPyV in latently infected cells. Few studies have addressed the expression levels or sequenced LT-ag in HPyV-positive cancers. In one study it was noticed that SV40 isolates from human cancer cells display mutations in the C-terminus of LT-ag, but the biological consequences of these amino acid substitutions remain to be established [5]. Another group isolated a Q171L BKV LT-ag mutant from the kidney of an AIDS patient with severe tubulointestitial nephritis. The same mutation was described in BKV DNA extracted from the urine of a renal transplant patient. This residue lies within the α-helical DNA binding domain. Because Leu, Glu, Met, and Ala are known to be strong helix formers, this mutation could change the biochemical properties of the protein [212]. Corresponding mutations in BKV-positive cancers have not been reported so far, nor have the biological properties of this mutant BKV LT-ag been compared with wild-type LT-ag.

Cell culture assays have demonstrated that rearrangements in the NCCR affect HPyV DNA replication and increase transformation potential [13, 213]. The lack of extensive identification of the NCCR in HPyV-

positive cancers and the presence of both archetypal and rearranged HPyV NCCR in sporadically examined tumour samples makes it hard to conclude whether specific NCCR structures are correlated with particular tumours. BKV variants with rearranged NCCR were isolated from human insulinoma (BKV-IR strain), Kaposi's sarcoma (BKV-IR-like), prostate hyperplasias, kidney adenocarcinomas, ureter carcinoma, and bladder urothelial carcinoma (all URO1 strain; PQPQRS anatomy) [214–216]. The BKV IR strain contains an insertion sequence-like structure able to integrate and excise from the host genome. This insertion sequence-like element may promote cell transformation by integrational activation or inactivation of proto-oncogenes and tumour-suppressor genes, respectively [217]. It remains to be confirmed whether these IR and URO1 strains display increased replication and transformation potential. The URO1 strain was also present in normal tissues, undermining the importance of the NCCR as a contributing property of the virus in the neoplastic process [17, 216].

Retrotransposon expression and retrotransposition in cancer cells might cause transcriptional deregulation, insertional mutations, DNA breaks, and an increased frequency of recombinations, contributing to genome instability. Although there is little evidence on the impact of retrotransposons on cancer cell growth and proliferation, virus-induced increased activity of retrotransposons might form a process that contributes to genetic instability and transformation [218]. Recently, it was shown that SV40 LT-ag enhanced transposition and expression of murine retrotransposable elements [219]. Whether LT-ag of HPyV can enhance retrotransposition of human retrotransposons, thereby contributing to cellular transformation, has not been established, nor has the activity of retrotransposons in HPyV-positive cancers been compared to HPyV-negative tumour cells or normal cells.

The actin cytoskeleton contributes to growth control in cells and plays a critical role in the regulation of various cellular processes linked to transformation, including proliferation, contact inhibition, anchorage-independent cell growth, and apoptosis [220]. Viral-induced perturbed expression of cytoskeletal compounds may thus contribute to transformation. Indeed, expression of SV40 st-ag resulted in loss of actin cable network in the rat embryo fibroblast line Rat1 cells, but not in mouse C3H 10T1/2 and rat F111 cells [221–223]. Madin-Darby canine kidney cells stably transfected with st-ag showed perturbed morphology with obvious disorganization of F-actin [224]. Actin dynamics are regulated by interplay of the Rho family of small GTPase proteins [225]. The st-ag-expressing cells showed both deregulation of the activity and cellular levels of Rho GTPases Rac1, RhoA, and Cdc42 as well as disruption of tight junction functions [224]. The presence of st-ag also influenced the phosphorylaton pattern of the microtubule-associated protein Tau. St-ag-mediated hyperphoshorylation of Tau resulted in dissociation of Tau from microtubules, causing destabilization of microtubules [226]. The implication of st-ag-mediated destabilization of microtubules in malignancy has not been examined. SV40 LT-ag forms *in vitro* complexes with different components of the cytoskeleton, including lamin C, laminin γ1, thymosin β4, and gelsolin [26]. The interactions have not been verified *in vivo*, nor have the biological consequences been solved, but these interactions might influence activities such as intracellular transport, signal transduction, adhesion, and migration, thereby contributing to the transformation process.

LT-ag carries several posttranslational modifications, such as phosphorylation, O-glycosylation, acylation, palmitylation, adenylation, poly(ADP)-ribosylation, and N-terminal acetylation [227]. Little is known about the function of any of these modifications, except for phosphorylation, which is known to play a major role in controlling the activity and function of the protein [228]. The posttranslational modifications of LT-ag in HPyV-positive cancers have not been examined, nor has their effect on LT-ag-induced transformation been tested.

## 4. Conclusion

Do human polyomaviruses cause cancer? This is a question asked by many researchers and clinicians. A confirmative answer is supported by the observations that these viruses are oncogenic for laboratory animals, that they are capable of transforming human cells, and that viral nucleotide sequences or proteins can be detected in human tumours [2, 6, 20, 80]. As outlined in this review, there is overwhelming evidence from studies in human and non-human cell lines and animal models that gene products of HPyV can interfere with several cellular processes that, when disturbed, may lead to transformation and tumorigenesis. However, whether these mechanisms by which HPyV proteins induce transformation in cell cultures are operational in tumour cells has been sparsely investigated. Systematic studies addressing the different concepts by which HPyVs may cause malignancies should be considered. Only a few studies like these have been performed, but encouragingly, they have indicated that specific mechanisms by which HPyVs induce transformation may be operational in particular tumours. For example, a significant associ-

ation between chromosomal instability and JCV-positive colorectal cancers was found [179,193]. Likewise, activation of the Met signalling pathway was characteristic for SV40-positive mesotheliomas [112, 143]. These results suggest that specific mechanisms for HPyV-induced transformation may be operational in certain tumours and may therefore form the basis for the design of new therapeutic strategies in the treatment of specific HPyV-positive cancers.

*References.* We apologize to those authors whose work was not cited due to space limitations.


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