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Noncoding Control Region of Naturally Occurring BK Virus Variants: Sequence Comparison and Functional Analysis

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Abstract. The human polyomavirus BK (BKV) has a proven oncogenic potential, but its contribution to tumorigenesis under natural conditions remains undetermined. As for other primate polyomaviruses, the approximately 5.2 kbp double-stranded circular genome of BKV has three functional regions: the coding regions for the two early (T, t antigens) and four late (agno, capsid proteins; VP1-3) genes separated by a noncoding control region (NCCR). The NCCR contains the origin of replication as well as a promoter/enhancer with a mosaic of *cis*-acting elements involved in the regulation of both early and late transcription. Since the original isolation of BKV in 1971, a number of other strains have been identified. Most strains reveal a strong sequence conservation in the protein coding regions of the genome, while the NCCR exhibits considerable variation between different BKV isolates. This variation is due to deletions, duplications, and rearrangements of a basic set of sequence blocks. Comparative studies have proven that the anatomy of the NCCR may determine the transcriptional activities governed by the promoter/enhancer, the host cell tropism and permissivity, as well as the oncogenic potential of a given BKV strain. In most cases, however, the NCCR sequence of new isolates was determined after the virus had been passaged several times in more or less arbitrarily chosen cell cultures, a process known to predispose for NCCR rearrangements. Following the development of the polymerase chain reaction (PCR), it has become feasible to obtain naturally occurring BKV NCCRs, and their sequences, in samples taken directly from infected human individuals. Hence, the biological significance of BKV NCCR variation may be studied without prior propagation of the virus in cell culture. Such variation has general interest, because the BKV NCCRs represent typical mammalian promoter/enhancers, with a large number of binding motifs for cellular transacting factors, which can be conveniently handled for experimental purposes. This communication reviews the naturally occurring BKV NCCR variants, isolated and sequenced directly from human samples, that have been reported so far. The sequences of the different NCCRs are compared and analyzed for the presence of proven and putative cellular transcription factor binding sites. Differences in biological properties between BKV variants are discussed in light of their aberrant NCCR anatomies and the potentially modifying influence of transacting factors.

Key Words: polyomavirus, BKV, noncoding control region, review

Introduction

Members of the polyomavirus genus within the *Papovaviridae* family are useful models for understanding molecular events involved in the regulation of eukaryotic gene expression and replication, as well as mechanisms leading to malignant cell transformation (reviewed in 1).

The public and scientific interest in primate polyomaviruses have been raised following the recent report of polyomavirus DNA and proteins in 60% of human pleural mesotheliomas (2). The polyomavirus, which is still not finally identified, appears to act as a cofactor with asbestos in the development of this invariably fatal form of lung cancer, estimated to kill 80,000 people before the year 2015 in the United States alone (2).

The overall genetic organization and the protein coding sequences are well conserved among polyomaviruses (reviewed in 3–5), while a remarkable DNA sequence variability may be observed in the noncoding control regions (NCCR), both within and between the viral species. The NCCR contains the promoter/enhancer for both early and late transcription, as well as the origin of replication. For the human polyomavirus BK (BKV), biochemical and genetic studies have identified an assortment of individual *cis*-acting sequence elements that contribute to the biological activity of the region (6–15). On this basis, the NCCR of the proposed archetypic BKV strain (WW) has been arbitrarily divided into three transcription factor binding sequence blocks, called P (68 bp), Q (39 bp), and R (63 bp), as an aid to visualize the rearrangements found in different NCCR variants (10). The rearrangements may arise as a result of unequal crossing over between daughter molecules during replication, and include partial or total duplications as well as deletions of sequence blocks (reviewed in 1).

The NCCR anatomy of a given BKV strain may have important implications for its replicational and transcriptional success in accessible host cells, as well as for the transforming potential of the virus. The transacting factor binding motifs present in the NCCRs are probably an adaptation to the menu of transcription factors offered by host cells. Therefore, the heterogenicity of the NCCR may reflect a natúral selection for optimal NCCRs, allowing viral multiplication in a broad spectrum of host cells. Experiments with the NCCRs of naturally occurring BKV strains may provide clues to better conceive *in vivo* interactions between BKV and its human host, and to determine the role of this virus in human pathology. But studies of the BKV NCCR may obviously also contribute considerably to the unraveling of the specificity, differentiation, and fidelity of gene expression control in various human cell types and tissues.

Analysis of the NCCR Anatomy of Naturally Occurring BKV Variants

A list of all the naturally occurring BKV variants described thus far is shown in Table 1. The NCCRs of these strains have been sequenced directly from human cells, tissues, and secretions without propagation of the virus in cell culture. The anatomy of the NCCR, based on the P, Q, R nomenclature proposed by Markowitz and Dynan (10), is shown in Fig. 1. We have expanded this nomenclature with the O- and S-block. The O-block represents the sequences between the translational start codon for the early genes and the P-block. It contains the basic origin of replication and the TATA box of the early promoter

Table 1. Sources of the naturally occurring BKV noncoding control region variants that have been sequenced directly without passage in cell culture

Strain	Source	Ref.
cl 6, 7, 8, 9, 32, 44, 51, 97, 104, 108, MT-1	Urine of a 17-year-old male with systemic lupus erythematosus undergoing immunosuppressive therapy	16
Dunlop	Peripheral blood leukocytes of immunocompetent individuals	17
NP132	Nasopharyngeal aspirate of a child	18
proto-2	Urine of HIV-infected patients Nasopharyngeal aspirate of a child	18, 19
TU, WWT1	Urine, mostly from children	20
WWT1, WWT2 WWT3	Urine from immunocompromised patients, bone marrow transplantants, and AIDS patients	21
WW	Urine of a renal transplant patient	22
WW (#2, 7, 8, 9, 10, 12, 13, 14,15, 16, 17, 18, 19)	Urine of bone marrow transplant recipients	23
WW (101, 125, 143, 147, 150, 152, 155, 157, 164, 209)	Urine of pregnant women	24
W1-L	Peripheral blood leukocytes of immunocompetent individuals	17

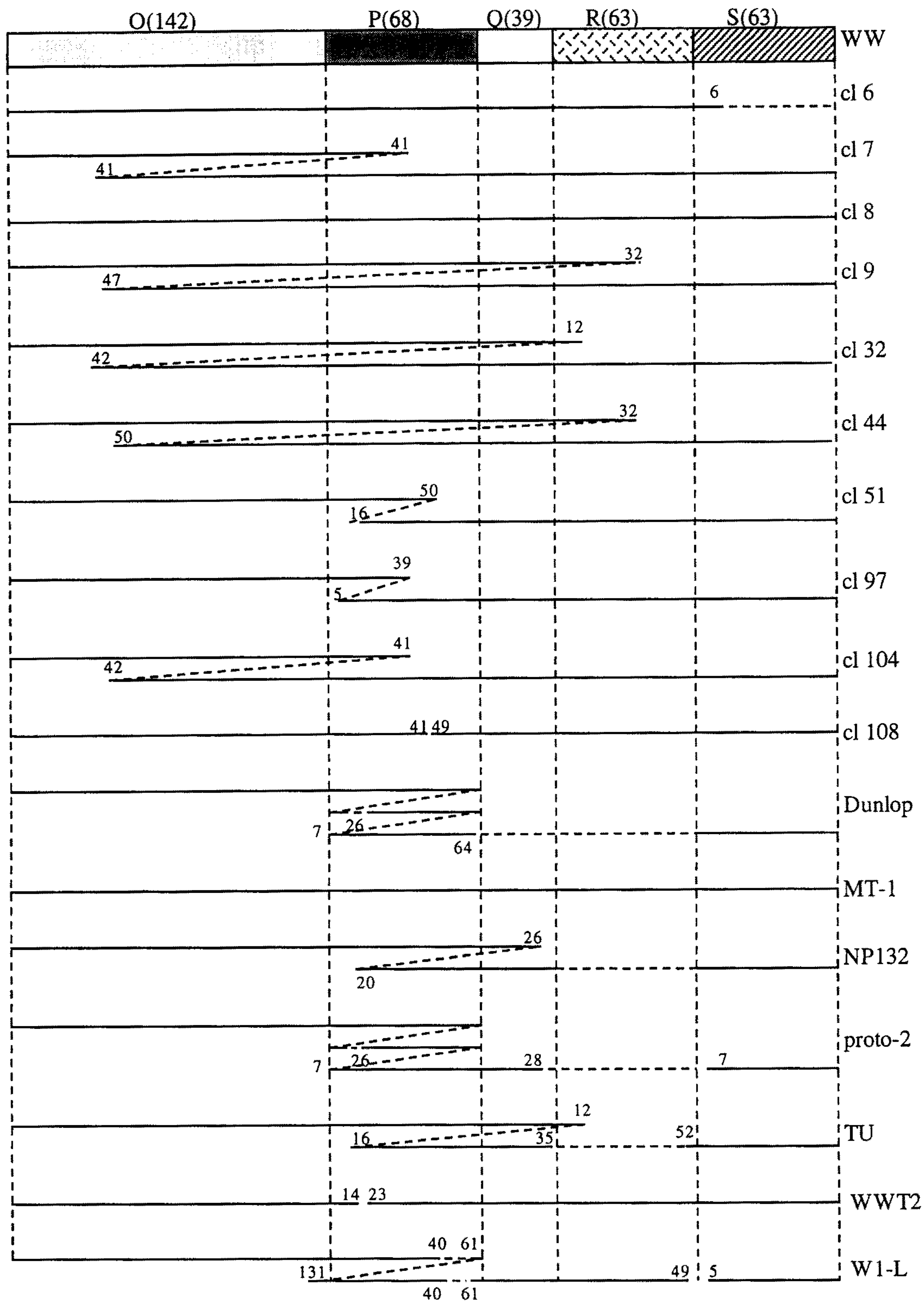

Fig. 1. Outline of the noncoding control regions (NCCRs) for naturally occurring BKV strains. The comparison is based on the linear O-P-Q-R-S-block anatomy (10) of the archetypic BKV (WW) and related strains (21,23,24) with number of base pairs given in parentheses for each block. The anatomy of other NCCR variants is illustrated by lines; parallel lines indicate a repeated sequence, and horizontal dashed lines indicate deletions relative to the archetype sequence. The start point or end point of each repeat element is shown as nucleotide numbers at the end of the solid bar. The vertical dashed lines indicate the boundaries of each block in the variant sequence. The dashed connection line between the solid parallel lines indicates the continuity of the sequences.

as well as some putative transcription factor binding sites. The S-block represents the sequences in the late leader, from the end of the R-block to the AUG start codon of the agnogene. These comparisons reveal that the majority of the NCCR variants have the linear O-P-Q-R-S anatomy (cl 6, cl 8, cl 108; MT-1; WW, WW#2, 7, 8, 9, 10, 12, 13, 15, 16, 17, 18, and 19; WWT1, WWT2, WWT3, and WW209) and also that all strains, except Dunlop, NP132, and proto-2, contain R-block sequences. This is in strong contrast to the NCCRs of cell-propagated viruses, among which only the Dik strain (25), the AS strain (26), and the RF-R2 strain (27) have retained a linear, unrearranged NCCR anatomy. The Dunlop, NP132, and proto-2 variants have a NCCR that is typical for cell-propagated virus, that is, duplication of P-block sequences and deletion of the R-block. Since similar or identical BKV strains have repeatedly been found in cell culture–passaged stocks, the possibility of PCR contamination should not be overlooked, despite strong precautions. Due to deletions and duplications in the NCCR, some of the naturally occurring variants contain aberrant block junctions (Table 2). Aberrant P-P (cl 51, cl 97, cl 108, Dunlop, proto-2, WW[#14], WWT2, and W1-L), Q-P (NP132), Q-R (TU), Q-S (NP132, proto-2), R-S (W1-L), and also aberrant P-O (cl 7, cl 104, W1-L), R-O (cl 9, cl 32, cl 44), R-P (TU), and P-S (Dunlop), junctions are found. It is striking that none of the naturally occurring NCCRs described so far have duplication of S-block sequences. The implication of these illegitimate junctions on putative binding motifs for known transcription factors are discussed later.

Table 2. Aberrant block junctions found in naturally occurring BKV NCCR variants and putative transacting factor binding sites created by them

Variants	Position	Putative binding site for
cl 7	$P_{1\text{-}41}$-$O_{41\text{-}142}$	c-*mos* promoter binding factor
cl 9	$R_{1\text{-}32}$-$O_{47\text{-}143}$	PEA-3, Pu-box, EF-1A, Ets-1, TCF-2α, T-antigen
cl 32	$R_{1\text{-}12}$-$O_{42\text{-}143}$	None
cl 44	$R_{1\text{-}32}$-$O_{50\text{-}143}$	PEA-3, PU-box, EF-1A, Ets-1, TCF-2α, T-antigen
cl 51	$P_{1\text{-}50}$-$P_{16\text{-}68}$	None
cl 97	$P_{1\text{-}39}$-$P_{5\text{-}68}$	Ets family
cl 104	$P_{1\text{-}41}$-$O_{42\text{-}143}$	c-*mos* promoter binding factor
cl 108	$P_{1\text{-}41}$-$P_{49\text{-}68}$	PEA-3, c-*mos* promoter binding factor
Dunlop	$P_{1\text{-}7}$-$P_{26\text{-}68}$	None
	$P_{1\text{-}64}$-S_{63}	None
NP132	$Q_{1\text{-}26}$-$P_{20\text{-}68}$	None
	Q_{39}-S_{63}	None
proto-2	$P_{1\text{-}7}$-$P_{26\text{-}68}$	None
	$Q_{1\text{-}28}$-2-$S_{7\text{-}63}$	None
TU	$R_{1\text{-}12}$1-$P_{16\text{-}68}$	c-*Myc*, GAGA-E74A.1
	$Q_{1\text{-}35}$-$R_{52\text{-}63}$	Sp1, hsp70, LSF(SV40), GC-box, JCV repeated sequence binding factor
WW(#14)	$P_{1\text{-}31}$-$P_{8\text{-}68}$	None
WWT2	P_{1-14}-$P_{23\text{-}68}$	None
W1-L	$P_{1\text{-}40}$-$P_{61\text{-}68}$	None
	$P_{61\text{-}68}$-4-$O_{131\text{-}142}$	None
	$R_{1\text{-}49}$-$S_{5\text{-}63}$	Glucocorticoid response element in the promoter of the metallothionin-IIA gene, p53

For references see Table 5.

Sequence Comparison of the NCCRs of Naturally Occurring BKV Strains

The sequences of the NCCR of the different strains were compared, and the deduced consensus sequence of each block is given in Fig. 2. A list of all the mutations found in the different blocks is presented in Table 3. The most obvious mutation found in the O block is the insertion of a T between position 11 and 12. This insertion is only found in the strains (the cl series) described by Sugimoto and coworkers (16). However, an insertion of an A in this position is also present in the cell-passaged strains, AS, DeBruin(DB), and DBdl (26,28), suggesting that this mutation is authentic rather than a sequencing artifact. The mutations G41→T and C101→A have so far only been described for WW (22) and cl 6 (16), respectively. The mutation A102→C has not only been detected in strains isolated by Sugimoto and coworkers (16), but also in the cell-passaged strain GS (27), again indicating the authenticity of this transversion. In the P block sequence six different point mutations are found between the NCCRs of the different BKV variants. Some of these have also been reported in cell-propagated strains (see Table 3). Both A19 and A53 are deleted in strain E, which was rescued from a human ependymoma (32). The point mutation T29→G in NP132 is found in the first P-block,

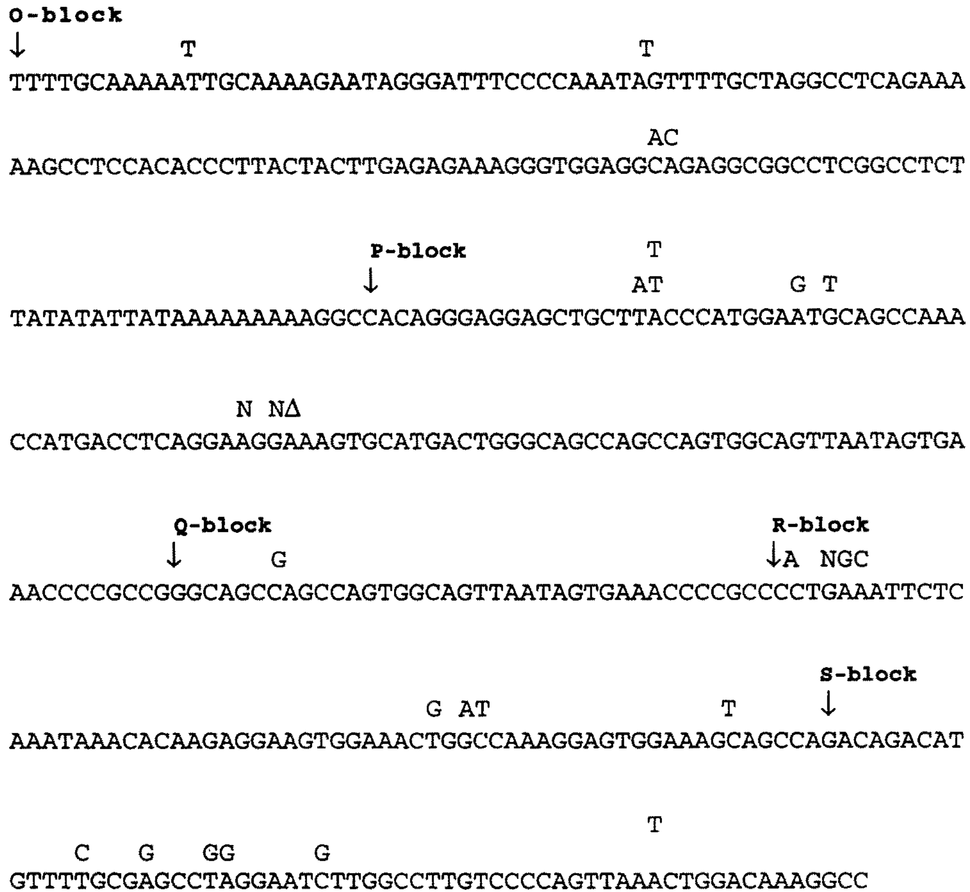

Fig. 2. Consensus sequence of the O-, P-, Q-, R-, and S-blocks of the NCCRs of the naturally occurring BKV variants. Mutations reported in the naturally occurring variants are indicated above the consensus sequence.

but not in the second partially duplicated P-block (18). The Q-block sequences appear to be well conserved. Only one point mutation (C7→G) is found. This mutation has not been reported for any of the cell culture–passaged virus strains. Several point mutations are found in the R-block. Some of them are also present in the NCCR of cell-propagated BKV strains. In the S-block, the mutation A18→G seems to be most common and has been reported in other cell-passaged BKV strains (see Table 3).

Proven and Putative Binding Motifs for Transcription Factors in the Different Blocks of the NCCR

Since biological properties of the distinct BKV strains are reflected by the structure of the NCCR, we analyzed this region for transcription factor–binding motifs. Previous studies have confirmed binding sites for the transcription factors Sp1 (Q-block; 10), NF-I (P, Q, R and S-block; 9, 10, 12, 13, 14, 33), AP-1 (P-P junctions; 10,13), the glucocorticoid/progesterone receptor (34), and the estrogen receptor (34). A list of the deduced number of binding sites for proven transcription factors in the different NCCRs of naturally occurring variants is shown in Table 4.

The O-, P-, Q-, and S-block sequences were screened for the presence of putative binding motifs for mammalian DNA binding proteins with the transcription factor recognition site file from the Transcription Factor Database (35) of the GCG Sequence Analysis Software Package, version 7.3, allowing no mismatches. The results are presented in Table 5. These results include the previously confirmed binding sites. We also

Table 3. Mutations found in the different sequence blocks of naturally occuring BKV control region variants

Block	Mutation	Cell-proposed strain[b]	NCCR variant
O	11-T-12[a]	AS[26], DB[28], DBdl[28]	cl 6, 7, 8, 9, 32, 44, 51, 97, 104, 108, MT-1
O	G41→T		WW
O	C101→A		cl 6
O	A102→C	GS[27]	cl 8, 9, 97, 104, 108, W1-L
P	T18→A	GS[27]	WW, WW101, WW(#12), WW(#15), WW(#16), WW(#19)
P	18-T-19[a]		WW209
P	A19→T		WW(#2), WW(#13), WW(#14)
P	T29→G		NP132
P	C31→T	GS[27]	WW, WW101
P	A53→N		WW(#2)
P	G55→C		WW(#7), WW(#14)
P	G55→N		WW(#2)
P	Δ56		WW(#7), WW(#14)
Q	C7→G		WWT3
R	C2→A		cl 7, 8, 9, 32, 44, 51, 97, 104, 108, MT-1
R	G4→A	AS[26], BKT-1B[29], DB[28], Dik[25], MM[30]	TU, WW, WW (#7, #8, #13, #14, #15, #16, #18, #19), WWT3, W1-L
R	G4→N		WW (#10)
R	A5→G	DB[28]	WW(#13), W1-L
R	A6→C	AS[26]	WW(#7, #14)
R	C38→G	DB[28]	WW(#13), W1-L
R	G40→A		WW(#13), W1-L
R	G41→T	AS[26]	WW(#14)
R	C56→T		cl 7, 8, 9, 32, 44, 51, 97, 104, 108, MT-1
S	T14→C		NP132
S	A18→G	tr530, 531, 532, pm522, 526, 527[31], JL[25], RF[27]	WWT1, WWT2
S	T22→G	DB[28], DBdl[28]	W1-L
S	A23→G		cl 6, 7, 8, 9, 32, 44, 51, 97, 104, 108, MT-1
S	C29→G		cl 6, 7, 8, 9, 32, 44, 51, 97, 104, 108, MT-1
S	40-T-50[a]	DB[28], Dbdl[28]	W1-L

The numbers indicate the position of the mutations in the respective sequence blocks. Cell-passaged BK virus with corresponding mutations are given.
[a] Insertion.
[b] Number refers to number in reference list.

examined whether binding motifs were created at the O-P, P-Q, Q-R, R-S, and aberrant junctions. For the legitimate junctions, new motifs were created at the P-Q and Q-R junction (see Table 5), while the results for the illegitimate junctions are presented in Table 2.

From the mutations found in the O-block, only mutations G41→T, C101→A, and A102→C affect binding motifs for transcription factors (see Table 5). The former has the potential to disturb the binding of a factor that was shown to bind the promoter of the granulocyte/macrophage colony stimulating factor gene (38). The latter two reside in binding sites for murine DNA binding proteins (39,41,46), and hence, their effect may have no biological relevance for the human BK virus. In the P-block, the mutations T29→G and C31→T affect the binding site for transcription factor NF-I, while mutation A53→N lies in a putative PEA3 binding motif. P-block mutations G55→C or N and Δ56 disturb the possible binding site for a transcription factor shown to bind this sequence in the promoter of the cytomegalovirus (CMV) *ie*-1 gene. The Q-block mutation affects the putative AP-2 and NF-I binding sites (see Table 5). Mutations in the distal part of the R-block hit putative binding motifs for the transcription factors NF-I, and ICFbf, and for factors shown to bind the SV40 enhancer core, the insulin gene promoter, and

Table 4. Deduced number of binding sites for proven transcription factors in the NCCRs of the different naturally occurring BKV strains

NCCR	Spl	NF-I	AP-1	GR	PR	ER
cl 6	1	6	0	1	1	1
cl 7	1	7	0	1	1	1
cl 8	1	6	0	1	1	1
cl 9	2	8	0	1	1	1
cl 32	2	8	0	1	1	1
cl 44	2	8	0	1	1	1
cl 51	1	7	0	1	1	1
cl 97	1	7	0	1	1	1
cl 104	1	7	0	1	1	1
cl 108	1	6	0	1	1	1
Dunlop	0	5	2	1	1	1
MT-1	1	6	0	1	1	1
NP132	1	6	1	1	1	1
proto-2	0	6	2	1	1	1
TU	1	6	0	1	1	1
WW	1	6	0	1	1	1
W1-L	1	5	0	1[a]	1[a]	1

GR = glucocorticoid receptor; PR = progesterone receptor; ER = estrogen receptor.

[a]Deletion in the S-block removes the spacer sequence between the two halves of the glucocorticoid/progesterone response element.

the promoter of the CMV *ie*-1 gene. None of the mutations in the S-block are located in transcription factor–binding motifs.

In an effort to define the biological significance of transcription factor binding motifs, elegant linker scan mutation studies were performed by the group of Subramani (7,11,15). A simplified NCCR variant with a O-P-Q-S anatomy was constructed, since the presence of repeated sequences would permit reiterated elements to compensate for one another. The mutations are summarized in Table 6, and the putative binding motifs in these segments are shown. Most of the linker exchanges had a profound influence on the promoter strength of the BKV NCCR, as determined by transient gene expression studies with the CAT reporter gene in CV-1, HeLa, or Vero cells. Some mutations had paradoxical and conflicting effects on the NCCR promoter, resulting in increased activity in some cells and decreased in others. Mutations of the NF-I motif in the P-block ($P_{24\text{-}36}$), for instance, resulted in decreased CAT activity in CV-1 and HeLa cells, but led to an increase in Vero cells. This suggests that protein(s) that bind to this motif act as activators in CV-1 and HeLa, while repressor(s) may bind the same motif in Vero cells.

The mutation in TGGGCAGCCAGCCA ($P_{68\text{-}}$–$Q_{1\text{-}13}$) had almost no effect on the transcriptional activity of the NCCR in CV-1 cells, while the promoter strength was 50% reduced in HeLa cells. On the other hand, in Vero cells a threefold increase in promoter strength was measured compared with the nonmutated promoter. This element contains putative motifs for NF-I, AP-2, Sp-1, LF-A1, and polyomavirus T-antigen. NF-I is a family of highly conserved proteins and is found in many tissue types (15 and references therein), while AP-2, originally isolated from HeLa cells, is a cell-type specific factor (69). The mutation results, however, warrant caution due to the characteristics of the cell lines used. CV-1 and Vero cells are African Green Monkey kidney cell lines and thus are hardly *in vivo* host cells for the human BKV, while HeLa cells are nonpermissive for the BKV strains tested thus far. Even though CV-1 and Vero originate in the same organ of the same species, they offered significantly different conditions to the BKV NCCR. We feel that the cited observations, as well as general considerations, underscore the difficulties with drawing biologically sound conclusions about promoter regulation on the basis of experiments in arbitrarily selected cell lines.

For the sake of simplicity, we limited our binding motif searches to complete matches to known motifs for mammalian transcription factors. Clearly, the situation is more complex: nonconsensus motifs might also be functional, as exemplified by the nonconsensus NF-I sites (9,10,12,13,14,33) and the glucocorticoid and progesterone response elements found in the S-block of the NCCR (34) and the CRE (cAMP response element)-like motif in the P-block. Early gene expression in the BKV-transformed cell line BKT-1B, as well as in BKV-infected Vero cells, is induced by cAMP analogs (29; U.M. and T.T., unpublished results). Transient gene expression studies with BKV promoter/enhancer sequences linked to the CAT reporter gene showed that these sequences could mediate cAMP-induced transcription. Cotransfection with consensus CRE oligonucleotides abolished

Table 5. Potential mammalian transacting factor binding sites in the sequence blocks of the BKV(WW) NCCR

Transcription factor or binding motif	Consensus motif[a]	BKV motif	Location	Ref.
H4TF-1	GATTTC	GATTTC	O_{27-32}	36
Promoter IL-6 gene	TTCC	TTCC	O_{30-33}	37
Promoter granulocyte/macrophage stimulating factor gene	YATTW	TATTT	O_{39-43}	38
Mouse Thy-1 gene promoter	AGGC	AGGC	O_{49-52}	39
			O_{98-101}	
			$O_{104-107}$	
			$O_{139-142}$	
T-antigen	GCCYC	GCCTC	O_{51-55}	40
			O_{63-67}	
			$O_{109-113}$	
			$O_{115-119}$	
	GDGGC	GAGGC	$O_{103-107}$	
Mouse α2(I) collagen promoter	CAGA	CAGA	O_{55-58}	41
			$O_{101-104}$	
NF-I	KCCA	TCCA	O_{66-69}	42
GATA-1	CACACCCC	CACACCCC	O_{68-74}	43
Gamma CAC1/2 CACCC-binding factor	CACCC	CACCC	O_{70-74}	44, 45
Polyomavirus B enhancer	AGAGG	AGAGG	$O_{102-106}$	46
Spl	AGGCGG	AGGCGG	$O_{104-109}$	47
Glucocorticoid receptor	CAGAG	CAGAG	$O_{101-105}$	48
Mitogen-responsive regulatory region of IL-2 gene MMTV promoter	TATA	TATA	$O_{121-124}$	49–51
			$O_{123-126}$	
			$O_{128-131}$	
Promoter rat osteocalcin gene				
TFILA-F/TBP/TRF/Dr1/En	TATAAA	TATAAA	$O_{128-133}$	52–55
Promoter rat glucagon gene	TATAT	TATAT	$O_{121-125}$	56
DBF4/NC1/NC2/UBP-1 arginosuccinate synthetase gene promoter	TATAA	TATAA	$O_{121-125}$	57–59
Unknown factor binding JCV repeated sequence	GGGNGGRR	GGGAGGAG	P_{5-12}	60
NF-I	TGGMNNNNNGCCAA	TGGATGCAGCCAA	P_{24-37}	10, 61
c-*mos* upstream enhancer	CAAACCA	CAAACCA	P_{35-41}	62
Phorbol-inducible element in plasminogen activator inhibitor type 2 promoter	TGACCTCA	TGACCTCA	P_{42-49}	63
PEA3	AGGAAG	AGGAAG	P_{49-54}	64
CMV *ie*-1 promoter	GGAAAG	GGAAAG	P_{54-59}	65
LF-A1	GGGCA	GGGCA	Q_{1-5}	66
Spl	GGGCAG	GGGCAG	Q_{1-6}	67
T-antigen	TGGGC	TGGGC	P_{68}-Q_{1-4}	68
AP-2	GGSCWSSC	GGGCAGCC	Q_{1-8}	69
NF-I	TGGNNNNNCCA	TGGGCAGCCA	P_{68}-Q_{1-9}	10, 61
NF-I–like factor binding promoter	TGGCA	TGGCA	Q_{15-19}	70
Human albumin and retinol binding protein genes				
Lymphokine	RTGRAAYCYC	GTGAAACCCC	Q_{27-36}	71
NFκB	GDRRADYCCC	GTGAAACCCC	Q_{27-36}	72
Polyomavirus T-ag	GCCYC	GCCCC	Q_{37-39}-R_{1-2}	73
NF-GMA	GRGRTTKCAY	GGGGTTTCAC	Q_{36-27}	74
hsp70	CCCGCC	CCCGCC	Q_{34-39}	75
LSF (SV40)	CCCGCC	CCCGCC	Q_{34-39}	76
Spl	CCCGCC	CCCGCC	Q_{34-39}	77

Table 5. (Continued)

Transcription factor or binding motif	Consensus motif[a]	BKV motif	Location	Ref.
VITF	TTCTCAAAT	TTCTCAAAT	R_{8-16}	78
60 kD protein binding to *cis*-acting negative element in rat prolactin gene	AAATAAA	AAATAAA	R_{13-19}	79
Pit-1	TAAACAC	TAAACAC	R_{16-22}	80
HiNF-A	AAACACA	AAACACA	R_{17-23}	81
Binding site in polyomavirus B-enhancer	AGAGG	AGAGG	R_{24-28}	46
Spi-1/PU.1	GAGGAA	GAGGAA	R_{25-30}	82
E1A-F	AGGAAGT	AGGAAGT	R_{26-32}	83
Ets-1/TCF-2	SMGGAWGY	GAGGAAGT	R_{25-32}	84
Insulin gene enhancer	GTGGAAA	GTGGAAA	R_{30-36} R_{50-56}	85
PEA3	AGGAAG	AGGAAG	R_{26-31}	64
ICFbf	RRARNNGAAACT	GAAAGTGGAAACT	R_{28-39}	86
NFI	TGGMNNNNNGCCAA	TGGAAACTGGCCAA	R_{32-45}	10, 61
SV40 enhancer-core	TGGAAAG	TGGAAAG	R_{51-57}	87
CMV *ie*-1 promoter	GGAAAG	GGAAAG	R_{52-57}	65
Promoter mouse α2(I) collagen gene	CAGA	CAGA	S_{3-6}	41
p53	RRRCWWGYYY	AGACATGTTT	S_{4-13}	88
NF-I	TGGCCTTGTCCCCAG	TGGCCTTGTCCCCAG	S_{31-45}	10
Glucocorticoid receptor	TGTCCC	TGTCCC	S_{37-42}	89
SV40 enhancer	CTGGG	CTGGG	S_{41-45}	90
Promoter murine Thy-1 gene	AGGC	AGGC	S_{59-62}	39

Computer analysis using GCG Sequence Analysis Software Package, version 7.3, with the TF Sites file from the Transcription Factor Database (35), allowing no mismatches, was performed to deduce the putative binding sites. The position of the binding motif in each block is indicated by numbers.
[a]D = A or G or T; K = G or T; M = A or C; N = A or C or G or T; R = A or G; S = C or G; W = A or T; Y = C or T.

Table 6. Early promoter/enhancer activity of the BKV PQ[a] NCCR following linker scan mutations in transacting factor binding motifs.

			BKV early promoter activity		
Target motif	Position	Putative/proven binding site	CV-1	HeLa	Vero
AGGGAGGAGC	P_{4-13}	None	49	40	53
TGGAATGCAGCC	P_{24-36}	NF-I	40–62[b]	21–45[b]	222
TTACCCAT	P_{17-24}	None	66	79	ND[c]
AAACCATGAC	P_{36-46}	c-*mos* promoter	28–80[b]	46–78[b]	ND
CTCAGGAA	P_{46-53}	PEA3	62	24	ND
GGAAGGAAAGTG	P_{50-61}	CMV *ie*-1 promoter, PEA3	93–94[b]	49–72[b]	ND
ATGACT	P_{63-68}	None	58	57	ND
TGGGCAGCCAGCCA	P_{68}-Q_{1-13}	NF-I; AP-2, LF-A1, Sp1, T-ag	61	95	304–337[b]
CCCGCC	Q_{34-39}	Sp1, LSF, hsp70, NF-κB, T-ag, NF-GNA	21	41	57
TGGACAAAGGCCA	S_{52-63}	NF-I, promoter murine Thy-1 gene	ND	ND	57

Transient transfection assays were performed in the indicated cell lines, and the expression levels of CAT reporter gene products for the mutants are given as percentages of the level achieved with the unmutated NCCR.
The data were compiled from references 7, 10, and 14.
[a]The PQ NCCR variant contains a linear arrangement of the sequence blocks O, P, Q, and S.
[b]Several different mutations were tested. The activity of the BKV promoter was determined by the particular mutation.
[c]Not done.

this cAMP-induced expression (U.M. and T.T., unpublished results). Furthermore, a linker scan mutant, in which the BKV CRE-like sequence TGACCTCA was replaced by an arbitrary sequence, strongly reduced the promoter activity as measured by reporter gene expression studies (7,11,15). Finally, studies have shown the importance of the TGACCTCA motif in viral multiplication in cell culture (91), indicating that this CRE-like motif may play an important biological role.

There are very few experimental data to support a discussion concerning the absolute or relative biological importance of the various trans-acting factor binding motifs in the BKV NCCR. The consensus motifs for PEA3 (P_{49-54} and R_{26-31}) and Spi-1/PU.1 (R_{25-30}) may be good candidates. The proteins corresponding to both motifs belong to the Ets transcription factor family. Ets members have been shown to be involved in differentiation, development, and inhibition of Ras-mediated transformation and are important for growth regulation during hematopoiesis (82,92,93). Expression of Spi-1/PU.1 is restricted to B-lymphocytes, monocytes, and macrophages. The detection of BKV in peripheral leukocytes (17) and our previous finding that BKV can infect primary human monocytes and macrophage-like cell lines (94) support the assumption that the Ets protein family may be involved in BKVs life cycle. New family members, some of which have an ubiquitous expression pattern, have recently been described (95).

The transcription factor AP-2 is another candidate. This protein may mediate both cyclic AMP and phorbol ester–induced transcription (96), and our studies (29), as well as those of others (13), have shown that BKV early expression is induced by both the protein kinase A and protein kinase C pathways. Part of this activation may be mediated through AP-2 binding. We cannot exclude, however, that the putative CRE and AP-1 binding motifs are responsible for this enhanced viral expression. Interestingly, protein–protein interaction between the SV40 large T-antigen and AP-2 was shown to block AP-2 binding to DNA (69).

Finally, one or several fully consensus NF-κB binding motifs are present in all NCCRs (Q_{27-36}). This transcription factor is activated in many different cell types following a challenge with primary (viruses, bacteria, stress factors, phorbol esters) or secondary pathogenic stimuli (inflammatory cytokines), some of which have been shown to induce BKV early expression (13,29). NF-κB functions as an immediate early mediator of immune and inflammatory responses, and seems also to be involved in growth control (reviewed in 97).

Biological Properties of BKV Strains with Divergent NCCR Sequences

Several naturally occurring variants have been tested for their ability to multiply in cell culture, and for the promoter/enhancer activity and transforming capacity. These results are summarized in Table 7, but are not directly comparable since different host cells have been used.

Table 7. Biological properties of naturally occurring BKV variants

NCCR	Multiplication in vitro	Promoter activity[a]	Transforming activity in vitro
cl 6	Low[b] (16)	Not tested	Not tested
cl 7	No	Not tested	Not tested
cl 8	Low	Not tested	Not tested
cl 9	No	Not tested	Not tested
cl 32	Not tested	Not tested	Not tested
cl 44	No	Not tested	Not tested
cl 51	Moderate	Not tested	Not tested
cl 97	No	Not tested	Not tested
cl 104	No	Not tested	Not tested
cl 108	No	Not tested	Not tested
Dunlop	Yes (100)	<TU (103)	Yes (28)
MT-1	Low[c] (99)	Not tested	Not tested
NP132	No (18)	Not tested	Not tested
proto-2	Yes	Twice WW, comparable with Dunlop (28)	Not tested
TU	Yes	>Dun (103)	Yes
WW	Poorly	1/2 proto-2 (22) or silent (24, 101)	Not tested
WWT1	Yes	Not tested	Not tested

See text for details.
[a]Transient transfection studies with the CAT reporter gene.
[b]Tested in HEK cells after transfection; compared with Gardner.
[c]Human embryo lung cell line HEL-R66; Compared with Gardner.

The BKV variants isolated by Sugimoto and coworkers (16), that is, the cl series, were only tested for viral production after transfection of viral genomes into human embryonal kidney (HEK) cells. Although cl 51 was the most efficient among these variants, the virus yield was four times lower than for BKV(Gardner). cl 51 has a duplication of $P_{16\text{-}50}$, encompassing putative/proven binding motifs for NF-I, for a protein that binds the promoter of the c-*mos* gene (see Table 5), and for the putative nonconsensus CRE discussed earlier. Linker scan mutation of this region strongly reduced the promoter strength (see Table 6). All strains with the archetypal linear O-P-Q-R-S NCCR anatomy (cl 6, cl 8, cl 108, and MT-1) multiplied poorly, or not at all in the case of cl 108. The latter BKV strain has a 7 bp deletion in the P-block ($P_{42\text{-}48}$) that removes the putative CRE-like sequence TGAC CTCA. It has been reported earlier that the archetypal strain BKV WW fails to propagate in HEK cells (98). But the WW-like strain MT-1, isolated from the urine of a patient with systemic lupus erythematosus, could be propagated, though less efficiently than BKV(Gardner), on the human embryonic lung cell line HEL-R66 (99).

Among the variants that could not be propagated in HEK cells, cl 7, cl 9, cl 44, and cl 104 contained duplications of the O-block, translocated to the P-Q-R area. No experimental studies aimed at explaining this nonpermissivity have been published, nor is it clear whether this is specific for HEK cells. It has, however, been shown (7) that the late promoter is encompassed in the early promoter/enhancer region. Thus, insertion of additional sequences in the P-block may disrupt the late promoter and therefore interfere with late transcription. Alternatively, the O-block contains binding motifs for the T-antigen, a protein known to repress its own (early) transcription. The binding of T-antigen in the proximity of the late promoter may hinder the RNA polymerase complex from transcribing the late genes. Thirdly, since the origin of replication resides in the O-block, two origins of replication could disturb normal replication. But this cannot be a general phenomenon, since significant amounts of these viral DNAs were present in the patient urine.

Strain cl 97 has no duplication of O-block sequences, but contains a duplication of $P_{5\text{-}39}$. These sequences contain putative/proven motifs for proteins that bind the JCV repeated sequence, the promoter of the gene for histone-H4 and for NF-I. Linker scan mutations have shown the importance of these sequences for the activity of the BKV promoter (see Table 6). Nevertheless, strain cl 97 cannot be propagated in HEK cells. This BKV strain resembles the multiplication-competent strain cl 51, but it lacks the duplication of the CRE-like motif found in the latter. Furthermore, BKV strains, such as Gardner, MM, Dunlop, and TU, which multiply in various cell cultures, have multiple CRE-like motifs. Finally, the reiteration of a 13 bp sequence, which contains the CRE motif, was required for the efficient replication of BKV in HEK cells (91). Thus, circumstantial evidence suggests a crucial role for the CRE-like element at some stage(s) of the viral multiplication cycle in some cell cultures.

Taken together, the cl series of BKV strains illustrate an important point when it comes to viral promoter/enhancer activity and host cell permissivity. Viral strains that exist in nature, and consequently do multiply somewhere in the host organism, are not able to propagate in commonly used host cell lines. It is hence not advisable to draw general conclusions from cell culture experiments unless the biological relevance of these cells can be tested.

The BKV Dunlop strain grows well in HEK and Vero cells, and in the human endothelial cell line HUV-EC-C (100 and our unpublished results). Its NCCR has duplicated P-block sequences, while Q- and R-block sequences are completely deleted. AP-1 binding sites are created at the P-P junctions (10). Increasing the number of AP-1 sites created a stronger promoter in WI-38 human embryonic lung fibroblasts, but an increased number of NF-I sites did not have the same effect (101). The Dunlop early promoter was 100 times stronger than the WW early promoter in WI-38 cells, as tested by transient gene expression studies with the CAT reporter gene. Transient transfection studies in HeLa (human), CV-1 (monkey), and L (mouse) cells did not demonstrate any pronounced host species preference for the early nor for the late

promoter/enhancer (102). Tavis and coworkers have shown that BKV(Dun) sequences can be detected in transformed Rat2 cells, indicating the transforming potentials of this strain (28).

Aliquots of nasopharyngeal aspirate containing BKV with the NCCR NP132 were inoculated on Vero cells and the human endothelial cell line HUV-EC-C, and were analyzed for large T-antigen expression by immunoperoxidase staining 4 days postinfection. Both cell cultures scored negatively (18). The transcriptional and transforming activities of NP132 have not been tested so far.

Proto-2 propagates well in Vero and HUV-EC cell cultures (our unpublished results). It was cloned from a BKV(Gardner) stock (22), and also found in urine from human immunodeficiency virus type 1–infected persons (19). The transcriptional activity of the NCCR was compared with that of WW and Dunlop by transient gene expression studies in CV-1 cells. The proto-2 late promoter/enhancer stimulated CAT activity two times more than the WW sequences and gave similar CAT values as those with Dunlop (22). Sequencing revealed a 17 bp deletion at the Q-S junction. This deletion removes the Sp1 binding site. This site was shown by linker scan studies to be an important element of the BKV promoter/enhancer in CV-1 and HeLa cells (see Table 6).

HEK, Vero, MCF-7 (human breast adenocarcinoma), and the human endothelial cell line HUV-EC-C are permissive for the BKV TU (20 and our unpublished results). Moreover, the TU NCCR is stable in cell culture since no mutations or rearrangements occur after many cell passages (our unpublished results). The TU variant resembles the NCCR variant cl 32 but lacks the partial O-block duplication and the complete R-block present in the latter (see Fig. 1). Unfortunately, cl 32 has not been tested in cell culture, but we speculate that due to the presence of additional O-block sequences, it will not multiply in HEK cells. The early promoter/enhancer activity of the TU NCCR was twice as strong as that of BKV Gardner, 2.5 times stronger than Dunlop, and at the same level as the BKV MM NCCR when measured by transient CAT reporter gene expression studies in CV-1 cells. The late promoter/enhancer activities of these NCCRs were at the same levels, except for Dunlop, which was three times weaker (103). Dunlop has a P_{68}-$P_{1\text{-}7}$-$P_{26\text{-}68}$-$P_{1\text{-}64}$-S_{63} anatomy and does not contain Q-block nor R-block sequences. The R-block includes proven binding site motifs for NF-I, while the Q-block contains a Sp1 motif (see Table 5). This may explain why the TU NCCR promoter is stronger than Dunlop.

Sp1 may be particularly important since the BKV late promoter does not contain a TATA box. Such promoters are GC rich and often contain the CCCGCC Sp1 motif. Our studies have also shown that TU can transform primary rat pancreatic islet cells, while BKV(Gardner) was unable to transform these cells (104). This is in accordance with the observation that deletion of the P-block repeats enhanced the transforming capacity of BKV of hamster kidney cells and rat 3YI cells (105–107). Studies by Watanabe and Yoshiike (107) have revealed a 29 bp segment located in the P-block ($P_{21\text{-}49}$) responsible for suppressed transforming capacity of BKV. This sequence contains a binding site for the transcription factor NF-I, a binding motif in the *c-mos* promoter shown to bind a murine specific nuclear protein, and a plausible CRE. The R-block sequences present in TU but absent in Gardner are probably not responsible for differences in transforming capacity. This presumption is based on the observation that BKV DeBruin(DB)d182 demonstrated a higher transforming activity of Rat2 cells than BKV(DB). BKV(DB)d182 is identical to BKV(DB), except for a 82 bp deletion that completely removes the R-block (28). On the other hand, BKV(AS) with an archetypal P-Q-R anatomy transforms Rat2 cells with an efficiency comparable to that of BKV(Gardner), which lacks R sequences (26,28).

Among the WW-like strains (WW, WWT, WW#, WW209) only WW was tested in cell culture. WW DNA was transfected in HEK or adult human skin fibroblast cells. A cytopathogenic effect was not observed until day 33 postinfection (101). MT-I with the archetypal O-P-Q-R-S NCCR anatomy grew slowly in the human embryo lung cell line HEL-R66 (99), while BKV(AS) with a linear P-Q-R anatomy, but with a deletion in the S-block, was successfully propagated in human fetal glial cells, HEK cells, hu-

man embryonic lung cells, and WI-38 cells (26). The promoter activity of WW was independently tested by two different groups. Rubinstein et al. (108) found a late promoter activity in CV-1 cells that was about one half that of Gardner, while Markowitz et al. (101) found that the early promoter was transcriptionally silent in WI-38 cells. Transforming properties of BKV(WW) strains have not been tested, but Tavis and coworkers (26,28) reported that BKV(AS) transformed Rat2 cells with an efficiency comparable to that of BKV(Gardner). The NCCR of BKV(AS) has the archetypal linear P-Q-R anatomy, but lacks almost the complete S-block.

W1-L has not been tested for its replicational or transcriptional activities, nor for its transforming potentials (17).

General Conclusions

Information concerning the relationship between BKV and its human host is still scanty and fragmented. The comtemporary comprehension of virus shedding; transmission between individuals; spread within the individual; location and host cells for permissive, persistent, or latent infections; the mechanisms of viral reactivation; the potential for functional disturbance; and disease is at best fragmentary, more probably in some respects wrong. Whether and to what extent the NCCR variation is influencing this complex virus–host interplay is totally unknown. The lack of focused and systematic approaches in this area is quite evident. Except for NP132 and W1-L, all the naturally occurring NCCR variants reported so far have been isolated from urine samples. Consequently, the range of authentic host cells for BKV is unknown. One recent communication described the presence of BKV DNA in various cell types from the kidneys, lungs, and central nervous system of a patient with hemophilia type A and acquired immunodeficiency syndrome (109). Another PCR-based investigation indicated BKV latency in brain, bone tissue, and peripheral blood cells from healthy blood donors, as well as in various tumors originating from the two former tissues (110). None of these reports, however, gave information concerning NCCR sequences. The notion that NCCR variation may influence the predeliction, organ/host cell tropism, gene expression levels, the choice of permissivity versus persistence, or latency a.o. during natural infection of the human host organism is hence based on extrapolation from experiments with cell culture–adapted BKV strains in arbitrarily chosen host cells. Since one single cell culture passage may lead to molecular rearrangements in BKV NCCR, analogies are highly questionable. Table 7 illustrates the lack of basic knowledge.

In order to conceive the natural history and biology of BKV, to design more relevant model systems for the contribution of the virus to cancer as well as other diseases, and to gain basic insight into eukaryotic gene expression control, some of the immediate challenges are a) systematic identification of *in vivo* host cells within the human organism and the determination of NCCRs present in such cells, b) comprehension of circumstances and conditions that may interfere with the transcriptional/replicative state of BKV in these cells , and c) identification of cellular transcription factors that interact with the different *cis*-acting elements in the NCCRs of naturally occurring variants. *In situ* PCR may be valuable in getting a handle on NCCR variations within different organs and cell populations in the human body. Comparative studies in authentic human host cells with natural NCCR variants and mutants generated by site-directed mutagenesis may offer an indispensable tool to resolve these problems. Finally, since the coding sequences of polyomaviruses may contribute to phenotypic variation (5,111), the newly published cassette method for cloning PCR-amplified naturally occurring BKV NCCRs into a stable genomic background allows direct comparison of biological characteristics (112).

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