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Restriction of human polyomavirus BKV DNA replication in murine cells and extracts

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Abstract

BKV causes persistent and asymptomatic infections in most humans and is the etiologic agent of polyomavirus-associated nephropathy (PVAN) and other pathologies. Unfortunately, there are no animal models with which to study activation of BKV replication in the human kidney and the accompanying PVAN. Here are reported studies of the restriction of BKV replication in murine cells and extracts and the cause(s) of this restriction. Upon infection of murine cells, BKV expressed large T antigen (TAg) but viral DNA replication and progeny were not detected. Transfection of murine cells with BKV TAg expression vectors also caused TAg expression without accompanying DNA replication. Analysis of the replication of DNAs containing chimeric BKV and murine polyomavirus origins revealed the importance of BKV core origin sequences and TAg for DNA replication. A sensitive assay was developed with purified BKV TAg that supported TAg-dependent BKV DNA replication with human but not with murine cell extracts. Addition of human replication proteins, DNA polymerase α -primase, replication protein A, or topoisomerase I to the murine extracts with BKV TAg did not rescue viral DNA replication. Notably, addition of murine extracts to human extracts inhibited BKV TAg-dependent DNA replication at a step prior to or during unwinding of the viral origin. These, and differences in replication specificity between BKV TAg and the TAg of simian virus 40 (SV40) and JC virus (JCV) and their respective origins implicate features of the BKV TAg and origin distinct from SV40 and JCV that restrict BKV replication in murine cells.

Introduction

Persistent and asymptomatic infections by the human polyomavirus BKV occur in most humans (50) and have been implicated in pulmonary, ophthalmologic, hepatic, autoimmune, neurological and renal disease (81). Free and integrated BKV genomes also have been detected in human tumor cells and tissues (12, 20), however, their significance in human cancer has not been established (52). The most significant and frequently noted consequence of BKV infection is polyomavirus-associated nephropathy (PVAN) with resulting risk of allograft loss (40). Activation of latent BKV replication in kidney allografts leading to PVAN has been suggested to be caused by inhibition of IFN- γ (1, 7), inflammation or stress, and ischemia/reperfusion (29, 39).

Animal models of BKV-associated diseases would be a great help in dissecting the cause(s) of PVAN and other BKV related pathologies. Some rodent cells have been reported to be semi-permissive for BKV infection (19, 87, 110). BKV infection or expression of the viral early region can cause malignant transformation in cultured cells (59, 77, 110) and hepatocellular carcinomas, renal and other tumors in rodents (23, 43, 93). As in human cells, BKV DNAs are maintained in some rodent cells at low levels, perhaps as episomes (12, 68); however, the processes limiting viral replication have not been defined. Murine polyomavirus (mPyV) DNA replication in mouse kidneys following injury (3, 4) or renal transplant (34) has been suggested to provide a useful model for study of PVAN. However, replication of BKV in human and murine kidney cells must be better understood to judge the relevance of such models.

Previous studies of SV40, mPyV and JCV have provided numerous insights into the processes of viral DNA replication. The viral large T-antigen (TAg) helps initiate replication by binding to multiple [G(A/G)GGC] motifs within the core origin, forming a dodecameric structure that distorts duplex DNA, opening DNA on one side of the origin and untwisting the other side (9, 25, 70). In an ATP and phosphorylation-dependent process, the TAg helicase activity unwinds DNA in a bi-directional manner (86, 100, 116). Single-stranded DNA is coated with replication protein A (RPA), and topoisomerase I relieves torsional stress ahead of the replication fork (8, 42, 91). DNA polymerase α -primase synthesizes short RNA primers that are elongated by DNA

polymerase α (30, 71), and leading strand synthesis is completed by DNA polymerase δ , RPA, proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C; (51, 63, 119). Replication of the lagging strand is mediated by DNA polymerase α -primase, DNA polymerase δ and accessory proteins (75, 111).

In addition to these shared properties, differences in replication between viruses have been reported to occur because of structural variation between viral core origins and TAg proteins (5, 6, 53, 57, 58); differences in TAg acetylation that either stimulate replication (118), or regulate TAg stability (78, 89); selective interactions with the host p180 DNA polymerase α and p48 primase subunits (10, 99) and with RPA (112); steps in replication beyond initiation (94); and modulation of replication by 5' and 3' *cis*-acting origin proximal sequences that alter activities of replication proteins, determine chromatin structure and intranuclear DNA localization (16, 32, 69, 96, 108, 113, 115, 118). Such viral and host-specific features may affect the outcomes of viral infection.

Despite its importance in human disease, BKV DNA replication in human kidney epithelial cells and its role in PVAN are not well understood. Early studies documented that archetype BKV isolated from human tissues does not replicate in cultured human cells, and consequently, most analyses have utilised naturally occurring BKV variants with genomic alterations that promote growth in cell culture (64, 103). The consequences of such alterations upon BKV infection of humans and possible viral pathogenesis is unknown. Robust BKV DNA replication in cell culture requires a 76-base-pair "core origin" very similar to that of SV40, but which does not by itself suffice for DNA replication (when mediated by SV40 TAg (24, 26). Binding sites for cellular factors that might activate or modulate replication during PVAN are located in the core origin flanking sequences, termed the enhancer (27, 60, 65, 66). Also, although BKV TAg resembles SV40 TAg in its J domain, DNA binding domain (47, 90), helicase domain, and interactions with p53 and pRb (36), physical or functional BKV TAg interactions with cellular replication factors such as DNA polymerase α -primase subunits p180 and p48, RPA, and topoisomerase I have not been characterized.

Here, we report sensitive assays of BKV replication and comparative studies of BKV replication in human and murine cells and extracts that point to an early, TAg-dependent step as being a likely cause of the replication block in murine cells and extracts, and we speculate as to possible mechanisms.

Materials and Methods

Viral infections

Primary human renal proximal tubule epithelial (RPTE) cells (Lonza) were maintained in renal epithelial cell growth media as previously described (1). Murine embryonic fibroblasts (45) were immortalized using the 3T3 protocol (107) as previously described (18), and maintained in DMEM (Gibco/BRL) containing 10% fetal bovine serum (FBS; Hyclone) and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza). Both RPTE and 3T3 cells were grown at 37°C with 5% CO₂ in a humidified incubator. BKV strains TU and Proto-2 were propagated as previously described (1). Purified stocks of BKV TU strain were produced by infecting Vero cells (ATCC CCL-81) for four weeks and harvesting progeny virus by centrifugation through a 20% sucrose cushion followed by a centrifugation in a 1.2 to 1.4 g/cm³ cesium chloride gradient(55).

BKV Proto-2 and BKV TU were used to infect 70% confluent RPTE or 3T3 cells at a multiplicity of infection (MOI) of 5 infectious units per cell (IU/cell) for 1 h at 37°C. 3T3 cells were infected and maintained in DMEM containing 2% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Total cell lysates were collected at 4 and 7 days post-infection (dpi) using E1A lysis buffer (35) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and western blot, as previously described (1). Low molecular weight DNA was isolated at 0, 4, and 7 dpi using the Hirt protocol (41). Each sample was spiked with unrelated plasmid (pRL-Null, Promega) as a control for DNA isolation.

Real-time PCR

The following primers were designed using Primer3 software (83) to amplify 125- and 84-base-pair fragments of the TU and Proto-2 non-coding control regions, respectively: TUNCCRFor (5'CGCCCCTAAAATTCTCTCTT3'), TUNCCRRev (5'ATGTCTGTCTGGCTGCTTTC3'), ProtoNCCRFor (5'CCAGCCAGTGGCAGTTAATA3'), ProtoNCCRRev (5'CATGGCCTTTGTCCAGTTTA3').

In addition, primers were used to amplify a 129-base-pair fragment of the β -lactamase coding region of the pRL-Null plasmid for normalization of the samples:

RTAmpFor (5'TCGCCGCATACACTATTCTC3'),

RTAmpRev (5'GCCGCAGTGTTATCACTCAT3'). All primers were synthesized by Invitrogen. Reactions were performed in a total volume of 25 μ l using 2x Power SYBR Green PCR master mix (Applied Biosystems), 2.5 μ l template diluted 1:10,000, and 300 nM of each primer. Amplification was performed in 96-well PCR plates (Bio-Rad) using the iCycler iQ5 real time detection system (Bio-Rad) with the following conditions: 2 min at 50°C; 10 min at 95°C; 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 56°C (Proto-2) or 58°C (TU) for 1 min. Samples were analyzed in triplicate and normalized by amplification of the β -lactamase coding region fragment using the $2^{-\Delta\Delta C(T)}$ method (54).

Plasmids

pOriBKV (termed B-B-B in Figs. 2-4) was generated by inserting the *HindIII* - *SphI* fragment (5031-282) of archetype BKV Dik strain (kindly provided by Dr. J. Lednicky) into the polylinker region of pUC18. Other similar pUC18-based plasmid DNAs with complete viral origins included: pOriJCV (Mad-1 strain; (74)), pOriSV40 (SV-S strain; ref (91)), and pOrimPyV (P-P-P, A3 strain; (85)). The pUC18 plasmid without insert served as negative control (pOri-) for cell-free DNA replication as well as a vector for cloning all viral origins. DNAs for replication assays were verified by sequencing and purified by Qiagen midi-prep kits.

In vivo DNA replication assays

Murine TCMK-1 cells were grown in DMEM medium with 10% FBS, seeded in 8-well plates (1×10^5 cells/well) and incubated overnight at 37°C. Cells were transfected by Lipofectamine and PLUS reagent (Invitrogen, Carlsbad, CA) with expression vectors for TAg (0.6 μ g of DNA) and template plasmid (0.4 μ g). After incubating cells with DNA:Lipofectamine and PLUS reagent mixture for 4 h in 500 μ l serum-free DMEM, the transfection solution was replaced with 2 ml of DMEM containing 20% FBS. Similarly, human HEK 293 cells were grown in DMEM with 10% FBS, seeded in 12-

well plates (4×10^5 cells/well) and incubated overnight at 37°C. Cells were transfected with expression vector for TAg (5 ng), template plasmid (50 ng) and pUC18 empty vector (0.65 µg) as carrier DNA by Lipofectamine and PLUS reagent. DNA:Lipofectamine and PLUS reagent mixtures were incubated with HEK 293 cells as described above. Cells were harvested 48 h after transfection and low molecular weight DNAs were isolated by the Hirt protocol and Promega Miniprep columns, digested with *EcoRI* to linearize plasmid and digested with *DpnI* to distinguish input from replicated DNA (41). The *DpnI*-resistant DNA was resolved from digested DNA by agarose gel electrophoresis (1%). After transferring the DNA to a nylon membrane, *DpnI*-resistant DNA was detected by Southern blot with a biotinylated probe of the LacZ gene (~400 nt) of the pUC18 vector and visualized by chemiluminescent nucleic acid detection (Pierce).

Expression of TAg in mammalian cells

Murine TCMK-1 cells (4×10^6 cells/60 mm plate) were transfected with 28 µg of TAg expression vectors as indicated using Lipofectamine and Plus transfection reagents as described above. 48 h after transfection, cells were harvested and lysed in lysis buffer (150 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF and protease inhibitor cocktail (Roche)) by rolling at 4°C for 30 min. The cell lysates were centrifuged for 30 min at 10,000xg. Supernatants were collected and incubated with anti-Flag beads (Sigma) for 2 h. The beads were washed 3 times in ice-cold lysis buffer and proteins bound to the beads were eluted with 50 µl of SDS loading buffer, then analysed by SDS-PAGE and western blotting using an anti-Flag antibody to detect TAg expression.

Expression of TAg in insect cells and purification

The BKV TAg cDNA was subcloned from pGEM 3Zf(-) vectors into pFastBac vector (Invitrogen, UK) with *EcoRI* and *NotI* sites. Recombinant baculoviruses containing BKV TAg were generated using Bac-to-Bac baculovirus expression system (Invitrogen) and amplified in insect SF-9 cells in TC-100 medium plus 10% FBS (both Lonza). For high yields of protein expression High Five™ insect cells (Invitrogen) were infected with the amplified virus and expression of TAg was confirmed by western blot analysis

with polyclonal antibodies against SV40 TAg that cross-react with BKV TAg (kindly provided by Dr. W. Deppert (Hamburg)). It is noteworthy that baculovirus vectors expressed BKV TAg at significantly lower levels than similar vectors coding for SV40 TAg and JCV TAg (74), in part, due to the presence of inhibitory 5' leader sequences upstream of BKV TAg cDNA and alternative splicing of BKV TAg mRNAs (data not shown). Infected cells were harvested at 48 h, homogenized (dounce pestle A, 20 strokes) in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM KCl, 0.5% MgCl₂, 0.5% IGEPAL CA630 (Sigma), 10% glycerol, 1x phosphatase and protease inhibitors (Sigma)) and clarified by centrifugation at 18,000xg for 30 min. The resulting lysate was subjected to immobilized metal affinity chromatography (IMAC) using TALON[®] resins (Clontech). After binding, the resin was washed with 20 column volumes (CV) with buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM KCl, 0.5% MgCl₂, 0.01% IGEPAL CA630) and then with 20 CV of buffer A containing 5 mM imidazole (pH 7.5). BKV TAg was eluted in buffer A containing 500 mM imidazole (pH 7.5), and fractions containing TAg were pooled and dialyzed against 500 ml of buffer containing 20 mM HEPES pH 7.5, 5 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 20% glycerol, and stored at -80°C. The purity and amount BKV TAg was determined by SDS-PAGE using Roti-mark standard (150 to 10 kDa; Carl Roth GmbH & Co.) or prestained molecular weight marker proteins (New England Bioscience). Proteins were detected either by staining with Roti-Blue colloidal coomassie staining reagent (Carl Roth), or western blot analysis (74). The purity of BKV TAg was estimated at ~90% by Coomassie blue staining (Fig. 3A). SV40 TAg, JCV TAg and mPyV TAg proteins were purified from extracts of insect High Five[™] cells infected with recombinant baculoviruses by immunoaffinity chromatography using monoclonal antibodies PAb101 and 5F (SV40 and mPyV TAg, respectively) coupled to protein A Sepharose or by immobilized metal chelate chromatography (JCV TAg) using TALON[®] resin as described previously (10, 74).

Cellular replication proteins and extracts.

DNA polymerase α -primase (98), topoisomerase I (97), and replication protein A (RPA) (73) were expressed, purified and their concentrations and activities determined as previously described (72). Logarithmically growing adherent HeLa S3 cells in

DMEM and suspension FM3A cells in RPMI supplemented with 10% and 5% FBS, respectively, were collected by centrifugation, washed once with phosphate buffered saline, and then washed extensively with hypotonic buffer (20 mM HEPES-KOH pH 7.8, 5 mM potassium acetate (KAc), 0.5 mM DTT and 1x phosphatase and protease inhibitors). Cells were homogenized (dounce homogenizer B pestle, 20 strokes), adjusted to 50 mM NaCl and incubated on ice for 30 min. Extracts were clarified by centrifugation twice at 20,000xg for 30 min and stored at -80°C. Extracts of High Five™ insect cells containing BKV TAG were prepared as above, except that subsequent to centrifugation, extracts were dialyzed into 20 mM HEPES pH 7.8, 5 mM KCl, 1 mM DTT with 1x protease inhibitors, and stored at -80°C.

In vitro DNA replication assays

Replication of DNAs *in vitro* was assayed according to Stadlbauer and co-workers with slight modifications (99). Briefly, the reactions (30 µl) contained 20 mM HEPES pH 7.8, 7 mM magnesium acetate (MgAc), 1 mM DTT, 4 mM ATP, 200 µM each CTP, UTP, and GTP, 50 µM dCTP, 100 µM each dATP, dTTP, and dGTP, 40 mM creatine phosphate di-Tris (pH 7.8), 40 µg/ml creatine kinase plus 5 µCi of [α^{32} P]-dCTP (3000 Ci/mmol), 0.25 µg test plasmid DNA, FM3A or HeLa cell extract (25 to 75 µg of protein) and purified TAGs at the indicated concentrations. After incubation for 60 min at 37°C, reaction products were precipitated with cold 10% (w/vol) trichloroacetic acid (TCA) containing 2% (w/v) sodium pyrophosphate and spotted on glass fiber filters (GF/C; Whatman), washed with 1 M HCl and analyzed by scintillation counting.

The monopolymerase replication assay (94) was assembled on ice with 0.5 µg of pOriBKV DNA or 0.5 µg of pUC-HS BNA (containing the SV40 replication origin) (85), 50 ng topoisomerase I, 100 ng DNA polymerase α -primase and 1 µg RPA, in 30 mM HEPES-KOH pH 7.8, 7 mM MgAc, 0.1 mM EGTA, 0.5 mM DTT, 200 µM each UTP, GTP, and CTP, 4 mM ATP, 100 µM each dATP, dGTP, and dTTP, and 10 µM dCTP, 40 mM creatine phosphate, 1 µg creatine kinase, 0.1 mg/ml heat-treated BSA, and 5 µCi [α - 32 P]dCTP (3000 Ci/mmol, Perkin-Elmer) in 40 µl. Purified BKV or SV40

TAg (0.2 μg) was added to start the reaction, and after incubation for 60 min at 37°C, the reaction products were precipitated with cold 10% (w/v) TCA containing 2% (w/v) sodium pyrophosphate and spotted on glass fiber filters (GF/C; Whatman), washed with 1 M HCl and analyzed by scintillation counting.

Results

Comparison of BKV infection of human and murine cells.

Human RPTE and mouse 3T3 cells were infected with the TU and Proto-2 BKV strains, at 4 and 7 dpi. Proteins and low molecular weight DNA were extracted and analyzed by western blots and quantitative real-time PCR, respectively. Although BKV TAg was expressed in both the human and murine cells, viral DNA replication was observed only in the human RPTE cells, with levels increasing between 4 and 7 dpi (Fig. 1). Incubating the infected murine cells for an additional week did not increase viral DNA, and a fluorescent focus assay revealed progeny were produced by infected RPTE cells but not by murine cells (data not shown).

Importance of core origin and flanking sequences for species-specific BKV DNA replication.

To assess whether the lack of BKV replication in murine cells is dependent upon core origin or origin flanking sequences, chimeric DNAs with the BKV core origin flanked by BKV (B-B-B) or mPyV sequences (P-B-B, B-B-P, P-B-P) were constructed (Fig 2A) and their replication analyzed following DNA transfection into cells. Exchanging the BKV early and late flanking region with mPyV sequences reduced the efficiency of chimeric DNA replication in human cells (Fig. 2A, compare lane 1 with lanes 2-4). When transfected into mouse TCMK-1 cells that support mPyV DNA (P-P-P) replication in the presence of mPyV TAg (Fig. 2A, lane 9), no replication of DNAs containing the BKV origin was detected (Fig. 2A, lane 5), consistent with the results of the viral infection assays described in Fig. 1. Furthermore, none of the BKV-mPyV chimeric templates were replicated (Fig. 2A, lanes 6-8). Although BKV and mPyV TAg were expressed at similar levels in TCMK-1 cells (Fig. 2B), BKV TAg did not support replication of any BKV origin tested in murine cells, whereas in these cells, mPyV TAg supported replication of its cognate origin (Fig. 2A) and of chimeric DNAs with the mPyV core origin (data not shown). These data indicate that the BKV core origin and its cognate TAg are primary determinants of the lack of BKV replication in murine cells.

BKV TAg-dependent replication in human cell extracts.

To define the cause of the lack of BKV replication in murine cells, an *in vitro* BKV TAg-dependent DNA replication system was established. Since insect cells efficiently express polyomavirus TAg proteins capable of supporting cell-free DNA replication (10, 74, 85, 94, 98, 99, 112) BKV TAg was expressed from baculovirus vectors in High FiveTM insect cells (Fig. 3A, lane 1 in the left and right panels). Archetype BKV, derived from human patients but which does not replicate in cultured cells was used as the source of DNA for these experiments. The insect cell extracts containing BKV TAg promoted BKV DNA replication *in vitro* in a dose-dependent manner when mixed with HeLa cell extracts (Fig. 3B, columns 1-4). Replication also depended on the presence of the BKV core origin (*ori+*) (Fig. 3B, columns 5-8). Similar results were obtained with purified BKV TAg (Fig. 3A, lane 5 in the left and right panels; data not shown), whose activity was comparable to that of purified SV40 TAg with DNAs containing the SV40 origin (Fig. 3C, columns 3 and 6).

Restriction of replication of BKV origin-containing DNA.

Reflecting the lack of viral replication observed in cultured cells, murine cell extracts did not support BKV TAg-dependent replication of BKV DNAs, although these extracts supported the mPyV TAg-dependent replication of the mPyV origin (Fig. 4, compare column 2 with 1), whereas resembled reactions carried out in parallel with SV40 TAg (Fig 4, column 3), and assays containing mPyV DNA but lacking mPyV TAg (Fig. 4, column 4). To ascertain the biochemical basis of the lack of BKV DNA replication, the murine extracts were supplemented with human DNA polymerase α -primase, RPA, or topoisomerase I. No incorporation of dNMPs into BKV origin-containing DNA was observed when human DNA polymerase α -primase and BKV TAg were added to murine extracts (Fig. 4, column 6). In contrast, the addition of human DNA polymerase α -primase resulted in SV40 TAg-dependent replication of SV40 origin-containing DNAs to about 60% of the level obtained with mPyV TAg and its cognate origin (Fig. 4, compare columns 7 and 1), consistent with published data (95). Similarly, purified human recombinant RPA (hRPA) added to murine extracts did not support replication BKV DNA (Fig. 4, column 9); however, addition of hRPA to the murine extracts stimulated mPyV DNA replication with mPyV TAg (Fig. 4,

compare columns 1 and 8), suggesting that RPA levels are limiting in these extracts and that the purified hRPA does not inhibit DNA replication *per se*. This is consistent with previous data indicating that addition of RPA or *E. coli* ssB stimulated SV40 and mPyV DNA replication, respectively, in mouse cell extracts (28, 99). Addition of purified human recombinant topoisomerase I also did not support incorporation of dNMPs into BKV DNA (Fig. 4, column 11), but stimulated mPyV TAg-dependent DNA replication (Fig. 4, compare columns 1 and 10) nearly twofold, suggesting that topoisomerase I is also limiting in murine extracts, as has been previously observed in human extracts (92).

Murine proteins inhibit BKV TAg-dependent DNA replication.

The lack of BKV TAg-dependent DNA replication in murine extracts was studied with a monopolymerase replication system comprised of purified human DNA polymerase α -primase, RPA, and topoisomerase I (94). Addition of each of these purified human proteins, individually, did not suffice to support BKV DNA replication in mouse extracts (Fig. 4). However, the three human proteins mixed together, without murine cell extracts, were capable of BKV TAg-dependent DNA replication *in vitro* with efficiencies resembling the SV40 system (Fig. 5A). Replication in the BKV monopolymerase system was not affected by human cell extracts added immediately prior to BKV TAg (Fig. 5B, compare columns 1 and 2) whereas addition of murine extracts reduced the incorporation of dNMPs by almost 50% (compare column 3 with columns 1 and 2). By comparison, the addition of murine extracts reduced the incorporation of dNMPs in the SV40 monopolymerase system by only 10% (Fig. 5C, compare column 3 with column 1), and the addition of human extracts to the SV40 system did not influence replication (compare columns 1 and 2). To determine at what step the inhibition occurs, murine extracts were introduced into the monopolymerase system at different stages of replication (Fig 6A). When added prior to the addition of TAg and human replication factors, murine extracts inhibited BKV TAg-dependent replication by more than 75% (Fig 6B, compare columns 2 and 3). In contrast, addition of murine extracts at a later stage (after DNA unwinding but prior to the initiation or the elongation reactions) had lesser inhibitory effect (Fig. 6B, compare columns 4 and 5 with columns 2 and 3). These findings contrast with the SV40 system, where little

inhibition by murine extracts is observed, regardless of the time of addition (Fig. 6C). However, these results resemble recently reported with the SV40 monopolymerase system using polypeptides that interfere with the assembly of the initiation complex (105).

Origin selectivity of BKV TAg-dependent DNA replication.

As the inhibition of BKV TAg-dependent DNA replication by murine extracts occurs predominantly before/during the origin binding and initiation, the origin selectivity of BKV TAg and other polyomavirus TAg proteins (JCV, SV40, mPyV) was assessed. In human extracts, BKV TAg efficiently promoted replication of DNAs containing either a BKV origin or a JCV origin, and less well, DNAs containing an SV40 origin (Fig. 7A). JCV TAg promoted replication of DNA containing its cognate origin, and less well, replication of DNAs with BKV or SV40 origins (Fig. 7B). In turn, SV40 TAg supported replication of DNAs with its cognate origin or with a JCV origin (74), but less well, DNAs with a BKV origin (Fig. 7C). As reported earlier, DNAs with the BKV, SV40 or JCV origins were not replicated by mPyV TAg and murine extracts (Fig 7D). These observations point to subtle but perhaps significant differences between these TAg and their interactions with cognate and non-cognate origins that might explain the restriction of replication of these viruses in cells. Also, these extend reports of BKV TAg-dependent DNA replication of the JCV origin in human extracts and cells (56, 58).

Discussion

The regulation of BKV replication in human kidney tissues and the cause(s) of BKV reactivation in allografts leading to PVAN are not understood, and combined with the lack of suitable animal models, make the development of effective preventions or interventions difficult. A murine model of BKV infection would be of great benefit; however, BKV DNA is not replicated in murine cells. While only low levels of BKV TAg are expressed following infection by virions (Fig. 1), levels of BKV TAg expression equivalent to that of mPyV TAg required for mPyV DNA replication in murine cells can be achieved by DNA transfection but still do not support BKV DNA replication (Fig. 2). These results, together with our previous findings that expression of very low levels of BKV TAg in human RPTE cells suffice for viral replication and progeny production (1), suggest that BKV TAg levels are not the limiting factor for viral DNA replication in murine cells.

Analyses of BKV TAg and mPyV TAg-dependent replication of their cognate origin-containing DNAs in combination with heterologous flanking sequences revealed that the BKV core origin and TAg are primary determinants of the restriction of BKV replication in murine cells. To study the molecular basis of this restriction, a robust BKV TAg-dependent DNA replication was established with archetype BKV DNA sequences and analyzed with human and murine cell extracts. As was observed with cellular assays of BKV replication, murine extracts also did not support BKV TAg-dependent replication. Furthermore, addition of replication-active human DNA polymerase α -primase, RPA and topoisomerase I to the murine extracts did not promote BKV DNA replication (Fig. 4), in contrast to similar studies of SV40 and mPyV DNA replication in heterologous systems (10, 99). Additional analyses of BKV TAg-dependent DNA replication with a monopolymerase system comprising these three human proteins revealed that murine cell extracts, but not human extracts, inhibit BKV DNA replication at an early stage (Figs. 5 and 6), perhaps during the unwinding of the core origin by BKV TAg. Such inhibition is not observed with SV40 TAg-dependent DNA synthesis in the monopolymerase system. The inhibitory activities may associate with and/or modify BKV TAg so as to interfere with its origin-binding or unwinding activities, and are consistent with the cell-based replication assays that indicate the

BKV core origin sequences bound by TAg are of primary importance for the restriction of replication in murine cells.

The absence of BKV TAg-dependent DNA synthesis in murine extracts, even with the addition of human replication proteins suggests that the restriction of BKV DNA replication in murine extracts differs from that observed for JCV and SV40 DNA replication. It is noteworthy that JCV TAg is reported to support replication of DNAs with its cognate origin in murine extracts (94). These differences also might be reflected by the origin selectivity of the virus TAg proteins for their cognate origins. All the experiments with murine extracts were carried out under conditions optimal for mPyV DNA replication, even though factors such as RPA and topoisomerase I are rate limiting (Fig. 4). Moreover, the stimulation of incorporation of dNMPs into mPyV DNA by RPA and topoisomerase I, and into SV40 DNA by human DNA polymerase α -primase indicate that these human proteins are functional in murine extracts.

One possible source of the restriction of BKV replication in murine cells and extracts might be the inability of the BKV TAg to promote dissociation of association of properly assembled replication complexes. Polyomavirus TAg DnaJ domains promote inactivation of pRb family proteins (reviewed in (37, 38, 101)) and facilitate oligomerization and DNA binding at the origin (114). Because of their homologous sequences and structures (38, 101), we can assume that DnaJ domain/LxCxE motif of BKV TAg should have similar functions, but might be modulated differentially in different milieu. For example, the SV40 TAg DnaJ domain is required for efficient SV40 DNA replication in CV-1P cells where the requirement for DnaJ domain is independent of pRb binding (11), while mPyV replication in murine cells only requires the DnaJ domain in quiescent cells, but not in replicating cells (31). Also, substitution of the JCV DnaJ domain for the DnaJ domain of SV40 TAg causes a dramatic decrease in DNA replication efficiency in BSC40 cells (102). More strikingly, JCV TAg is less efficient than its SV40 counterpart at binding pRb and DNA, as well as in oligomerization in Rat2 cells (106), even though they have very homologous J domains and well conserved LxCxE motifs through which they interact with pRb family proteins. These observations suggest that the DnaJ domains/LxCxE motifs of different TAg vary in

how they coordinate viral DNA replication in different cellular environments. It is very possible that BKV TAg DnaJ domain's function in promoting hexamer assembly and DNA binding is limited in murine cells. In addition, the pRb family proteins have been shown to inhibit SV40 and mPyV DNA replication (2, 82, 109) as well as cellular DNA replication (48), by competing with TAg for DNA polymerase α interaction (2) or CDK2/cyclinA-dependent phosphorylation (82). Since the DnaJ domain modulates pRb family proteins interaction with TAg proteins, it is possible that murine cellular factors effectively upregulate BKV DnaJ domain function in modulating pRb-mediated inhibition, but have little effect on mPyV, SV40 or JCV DnaJ domains. These ideas are presently being tested.

Although introduction of the human replication proteins did not restore BKV replication in murine extracts, one cannot rule out the possibility that the lack of BKV replication in murine cells is mediated by an incompatibility of components of the host DNA replication complex. For instance, murine initiation factor(s) might stably complex with BKV TAg, forming an inactive chimeric complex analogous to what has been observed with SV40 replication inhibition by polypeptides representing the protein-protein interaction regions of replication proteins (104, 105). Later steps of DNA replication involving other components like PCNA, RF-C, and DNA polymerase δ/ϵ required for elongation and polymerase switching might also contribute to the restriction of BKV DNA replication.

The replication assays using chimeric templates suggest that sequences flanking the core-origin are unlikely to be primary determinants of the restriction of replication. However, chimeric templates might lack some important "cross-talk" interactions between core-origin flanking sequences and the core-origin, which could be vital for DNA replication *in vivo*. For example, AP-1 stimulates both mPyV and SV40 DNA replication (32, 33, 44, 62, 108) but inhibits JCV replication (49, 80). NF-1 also has been shown to stimulate SV40 DNA replication (17, 69, 108); however, a closer look into different members of NF-1 family proteins revealed that NF-1D predominantly expressed in permissive glial cells stimulated JCV virus replication (67), while NF-1A predominantly expressed in non-permissive progenitor and HeLa cells restricted JCV

replication in these cells (79). NF-1 binding sites have been identified on BKV origin flanking sequences (13-15, 60), and AP-1 binding sites were reported in enhancers of BKV strains (60, 61).

Recently, DNA replication of SV40 and mPyV has been shown to activate and utilize ATM-mediated DNA damage response (22, 88), which can be detrimental for viral DNA replication due to a block to cell cycle progression at the G1/S checkpoint. To override the DNA damage response triggered by viral infection, SV40 TAg targets subunits of MRN complex for degradation through its interaction with CUL7, an E3 ubiquitin ligase (46, 117, 120). It is possible that BKV TAg does not interact with murine CUL7 and therefore cannot mediate the degradation of the murine MRN complex, whereas this mechanism would be functional in human cells and extracts. In addition, components of DNA replication machinery also participate in the DNA repair pathway (21, 76, 84). It is possible that the DNA damage response triggered by BKV infection of murine cells might differently modulate these components, causing restriction of BKV replication.

Although BKV is closely related to SV40 and JCV, our data indicate there to be distinct sites at which host specific replication factors may conflict with viral DNA replication. Detailed analysis of the restriction of BKV replication in murine cells may point to processes that maintain BKV DNA replication at low levels until stimulated in the transplant setting to activate replication.

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Figure 1.

Lack of BKV DNA replication in murine cells during viral infection. Human RPTE cells or murine 3T3 cells were infected with BKV. **(A)** Total cell lysates were harvested at 4 and 7 dpi and proteins (15 μ g) were subjected to western blot and probed for expression of TAg and GAPDH (a loading control). The numbers above the lanes indicate the days after infection at which the cell lysates were harvested. M: mock infected lysate. **(B)** Low molecular weight DNA was isolated at 0, 4, and 7 dpi and analyzed by real-time PCR. Data are presented as genome copy number per reaction, normalized to the control plasmid pRL-Null (control for purification efficiency). Samples were assayed in triplicate; results are representative of two independent experiments.

Figure 2.

DNA replication in human and mouse cells. **(A)** *In vivo* DNA replication of BKV and mPyV. Vectors expressing BKV TAg (lanes 1 to 8) or mPyV TAg (lane 9) were co-transfected into human HEK293 (lanes 1 to 4) or mouse TCMK-1 cells (lanes 5 to 9) together with plasmids containing the complete BKV origin (lanes 1 and 5, B-B-B), complete mPyV origin (lane 9, P-P-P), and BKV-mPyV chimeric origins (lanes 2 to 4 and 6 to 8). At 48 h after transfection, DNA was isolated and analysed by Southern blotting. DNA replication products are marked by arrows. **(B)** Vectors expressing Flag-tagged BKV TAg or Flag-tagged mPyV TAg were transfected into mouse TCMK-1 cells, and harvested after 48 h. Crude extracts were prepared and Flag-tagged viral TAGs were immunoprecipitated and subjected to SDS-PAGE, and western blot using anti-Flag antibody to detect TAGs, which are marked by arrows.

Figure 3.

Purification and activities of recombinant BKV TAg. **(A)** BKV Tag samples were analyzed by SDS PAGE and protein staining using colloidal Coomassie brilliant blue (left panel) or western blotting using polyclonal TAg-specific antibodies (right panel). In the left panel, 5 μ g of crude extract and flow through were loaded in lanes 1 and 2, respectively, about 0.1 μ g of protein from wash fractions was loaded in lanes 3 and 4, whereas in lane 5, 0.5 μ g of protein eluted from TALON[®] resin was analyzed. In the

right panel, the protein amounts were reduced: in lanes 1 and 2, 1 μg of crude extract and flow through, respectively, were loaded, lanes 3 and 4 show two wash fractions (about 0.1 μg of protein each), and in lane 5, 0.05 μg of protein eluted from TALON[®] resin was analyzed. **(B)** DNA synthesis in the presence of recombinant BKV Tag was measured by incorporation of dNMPs into DNA. Increasing amounts (0, 7, 13, and 26 μg) of High Five[™] cell extracts containing recombinant BKV TAg (about 0, 0.35, 0.65, and 1.3 μg , respectively) were added to HeLa hypotonic extracts in the presence of DNA with a BKV origin of replication (250 ng of B-B-B, ori+, columns 1-4) or empty vector (ori-, columns 5-8). **(C)** Cell-free DNA replication in the presence of BKV and SV40 TAg. DNA replication in presence of 200 ng of purified recombinant BKV TAg or SV40 TAg as indicated was measured using 40 μg of HeLa extract and 500 ng of DNA containing SV40 or BKV origin of replication (ori+) or an empty vector (ori-). Incorporation of dNMPs into DNA was measured by scintillation counting. The DNA synthesis was determined in duplicate and repeated three times. The average of these experiments and the standard deviations are presented.

Figure 4.

Modulation of polyomavirus DNA replication in murine cell extracts by human replication factors. *In vitro* DNA replication in the presence of equal amounts of purified recombinant BKV (grey bars), mPyV (open bars), and SV40 TAg (shaded bars) using mouse FM3A cell extracts. Columns 1 to 4 show DNA synthesis in the presence of mouse cell extracts and either mPyV TAg/P-P-P, BKV TAg/B-B-B, or SV40 TAg/pOriSV40. Incorporation of dNMPs into P-P-P in the absence of TAg served as a negative control (black bar). DNA synthesis with mouse cell extract and additional human DNA polymerase α -primase (2 units of hPol α -primase (72)) are depicted in columns 5 to 7 (mPyV TAg/P-P-P, BKV TAg/B-B-B, and SV40 TAg/pOriSV40, respectively). The DNA synthesis of mouse cell extracts with additional 0.5 μg of human RPA (hRPA) using mPyV TAg/P-P-P and BKV TAg/B-B-B are presented in columns 8 and 9, respectively, whereas the influence of human topoisomerase I (hTopo I, 120 ng) on the DNA synthesis in the presence of mouse cell extracts with mPyV TAg/P-P-P and BKV TAg/B-B-B are shown in columns 10 and 11, respectively. All assays were carried out in triplicate and the results presented are the average

of two independent experiments.

Figure 5.

DNA synthesis with purified human proteins in the presence of BKV and SV40 TAg. **(A)** Incorporation of dNMPs into DNA containing a BKV (column 2) or SV40 (column 4) origin of replication in the presence of 200 ng of the respective viral TAg and 100 ng of purified human DNA polymerase α -primase, 50 ng topoisomerase I and 1000 ng RPA was measured (monopolymerase DNA replication system). Vectors without functional viral origin, ori-, served as negative controls (columns 1 and 3). **(B)** The effect of human and mouse proteins on DNA synthesis by human DNA polymerase α -primase was determined with a BKV origin of replication. The incorporation of radioactive dNMPs using BKV origin of replication as a template was measured in the presence of buffer but no additional proteins, or 15 μ g human or mouse cell extracts (columns 1, 2 and 3, respectively). DNA synthesis in the presence of DNA lacking an origin of replication served as a negative control (column 4). **(C)** The effect of human and mouse proteins on the DNA synthesis by human DNA polymerase α -primase was determined with a SV40 origin of replication. The incorporation of radioactive dNMPs was determined in the presence of buffer but no additional proteins, or 15 μ g human or mouse cell extracts (columns 1, 2 and 3, respectively). DNA synthesis in the presence of DNA lacking an origin of replication served as a negative control (column 4). Incorporation of dNMPs into DNA was measured by scintillation counting. DNA synthesis was determined in duplicate and repeated three times. The average of these experiments and the standard deviations are presented.

Figure 6.

DNA replication with purified human proteins in the presence of BKV TAg and mouse cell extracts. The replication of polyomavirus DNA was biochemically separated into three consecutive reaction steps as presented in panel **A**: the unwinding, initiation, and elongation reactions. In the presence of RPA, topoisomerase I, ATP and an ATP-regenerating system, viral TAg unwinds viral DNA at 37°C for 30 min (unwinding reaction). To synthesize primers at the unwound origin of DNA replication (initiation reaction), human DNA polymerase α -primase and the three remaining ribonucleotides were added and oligoribonucleotide primers are synthesized during the incubation at

37°C for 30 min, whereas no DNA can be synthesized since dNTPs are lacking. Finally dNTPs, which include radioactively labelled dCTP to monitor DNA synthesis via scintillation counting, are added and DNA is synthesized at 37°C for 30 min (elongation reaction). Mouse cell extracts capable of supporting mPyV DNA replication were added to the reactions prior to the specified step (as indicated by the arrows). The addition of buffer served as control for the influence of salt and dilutions. Panel B shows the results of the monopolymerase assay using BKV TAg and template containing the BKV origin of replication. Panel C shows the results of the monopolymerase assay using SV40 TAg and template containing the SV40 origin of replication. In panels **B** and **C**, columns 1 and 2 represent dNMP incorporation into DNA in the absence and presence of TAg, respectively, but without mouse proteins. In column 3, mouse cell extracts were added to reaction components before the addition of human DNA polymerase α -primase and ribonucleotides (prior to unwinding of DNA). In column 4, mouse cell extracts were added after the unwinding reaction but before the addition of human DNA polymerase α -primase (prior to initiation of DNA replication). For column 5, mouse cell extracts were added after initiation of DNA replication but before addition of dNTPs (prior to elongation). Incorporation of dNMPs into DNA was measured by scintillation counting. DNA synthesis was determined in duplicate and repeated three times. The average of these experiments and the standard deviations are presented.

Figure 7.

Comparison of the replication of DNAs with different origins by polyomavirus TAg proteins *in vitro*. Replication assays were carried out as described in Materials and Methods. Incorporation of dNMPs into DNA was measured in the presence of the indicated TAg proteins (panels A, B and C) and HeLa extracts. Panel D presents mPyV TAg-dependent DNA replication in FM3A extracts. DNA synthesis in the presence of BKV, JCV, SV40, and mPyV origin-containing DNA but without (w/o) the cognate TAg, as well as DNA synthesis of plasmid DNA without a viral origin in the presence of the indicated TAg, served as negative controls in all panels. All assays were carried out in triplicate and the results presented are the average of two independent experiments.

Fig. 1

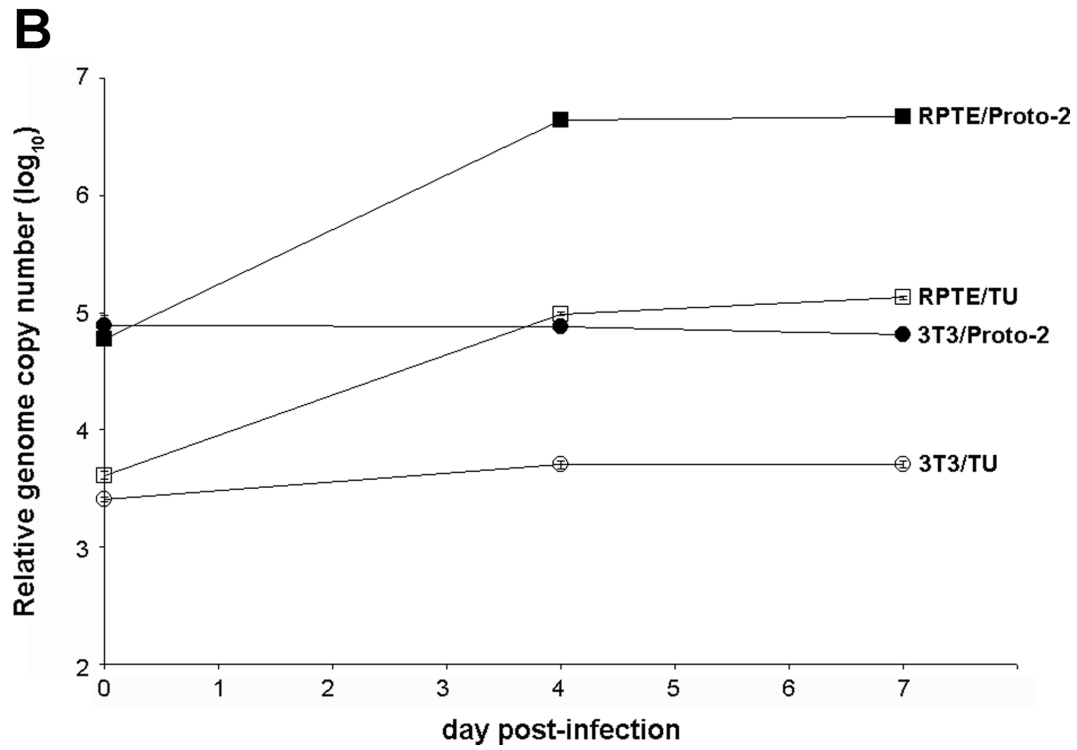
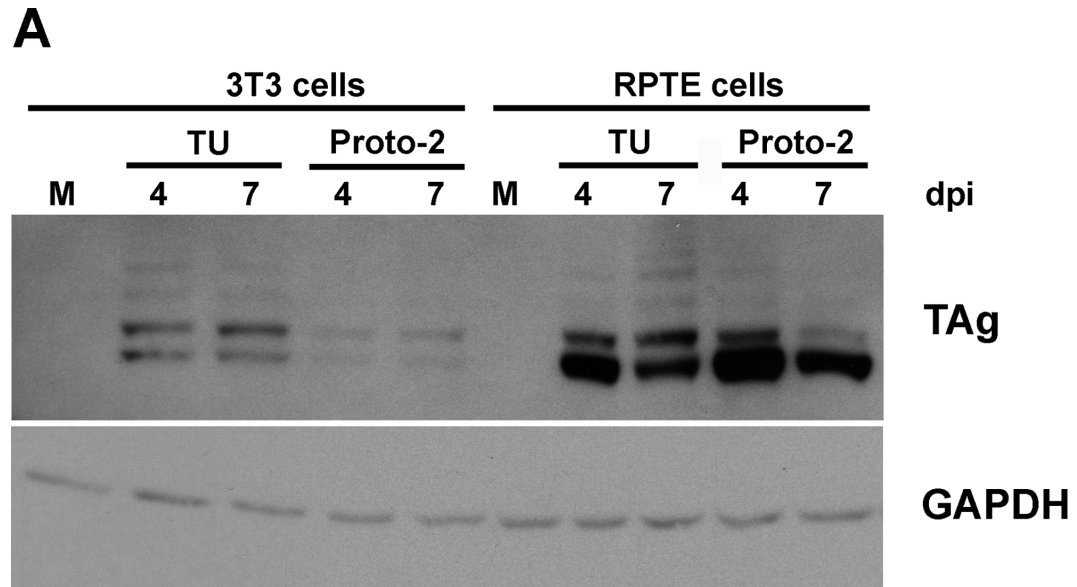


Fig. 2

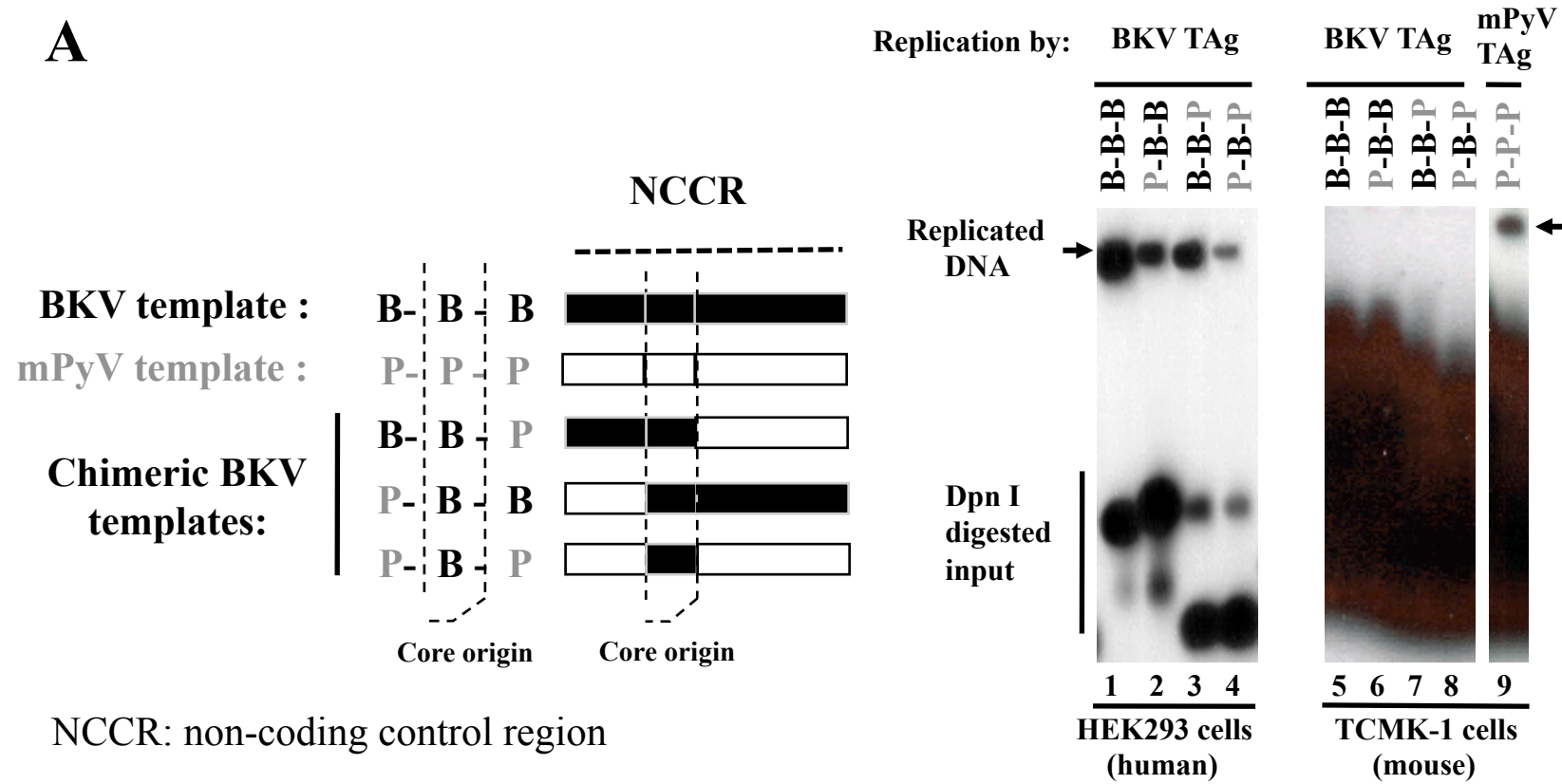


Fig. 2

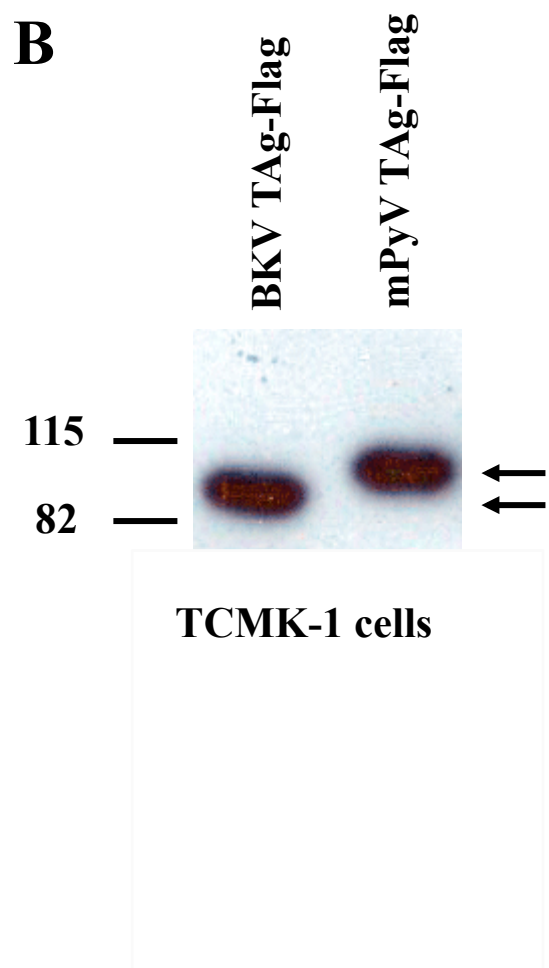


Fig. 3

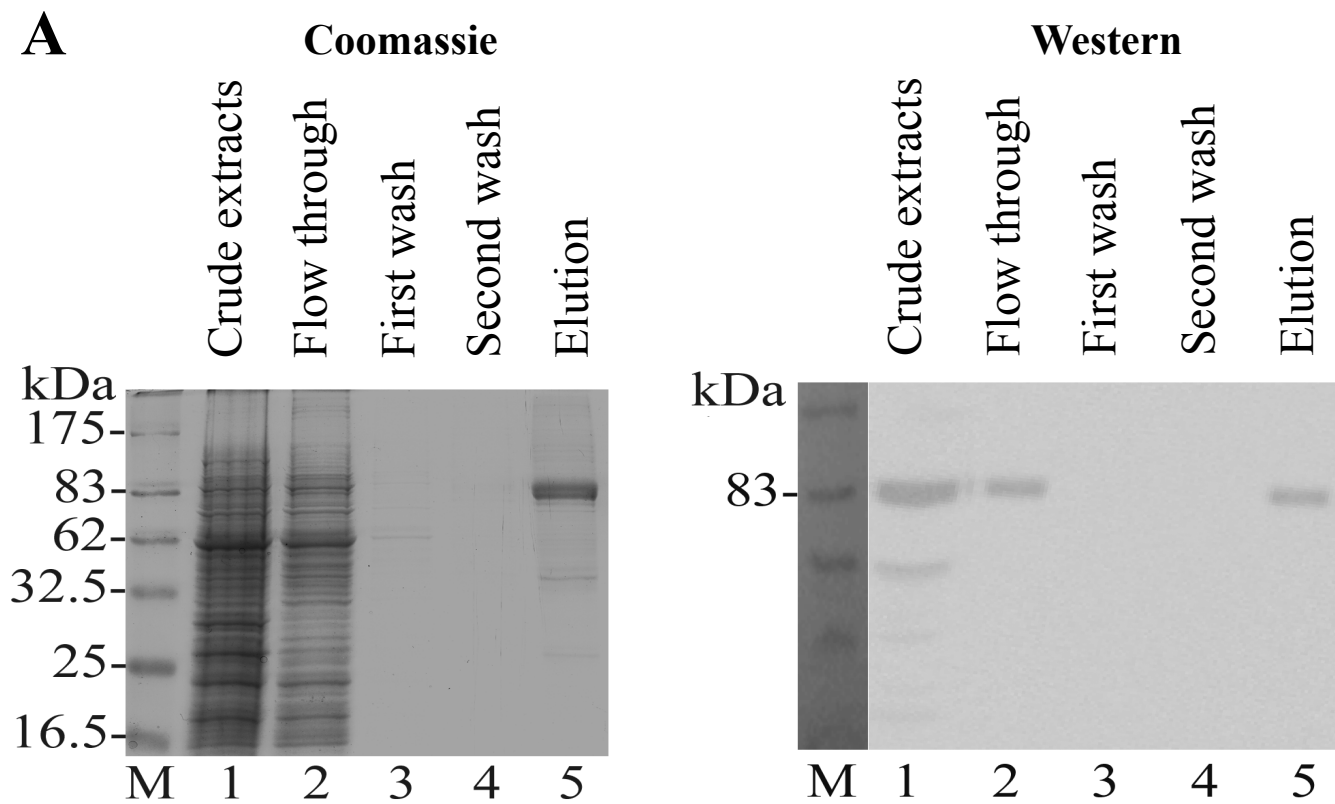


Fig. 3

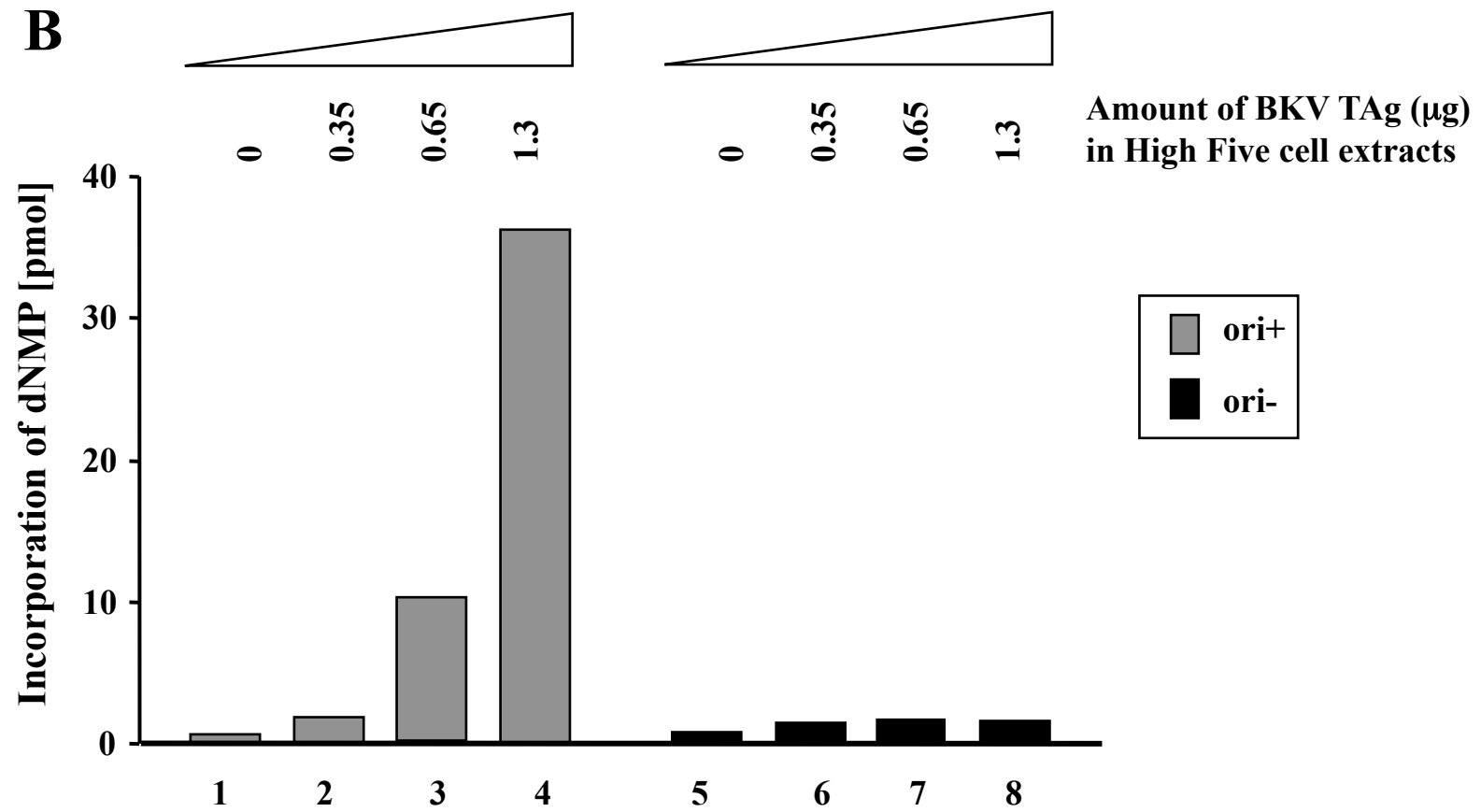


Fig. 3

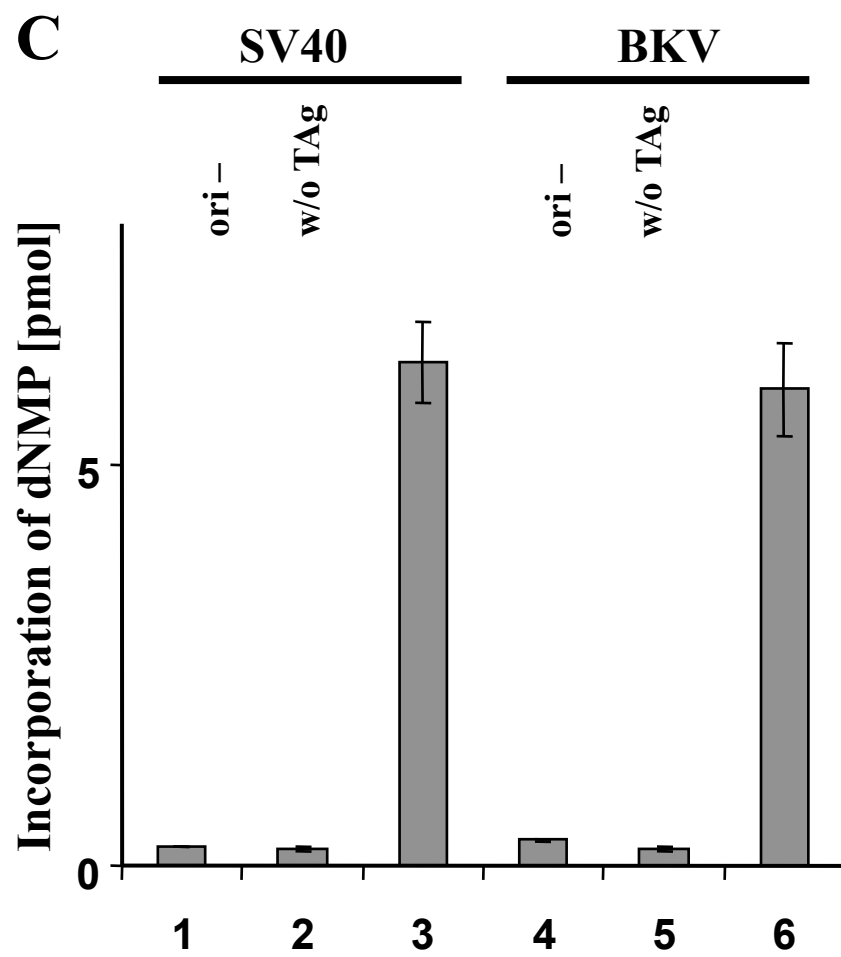


Fig. 4

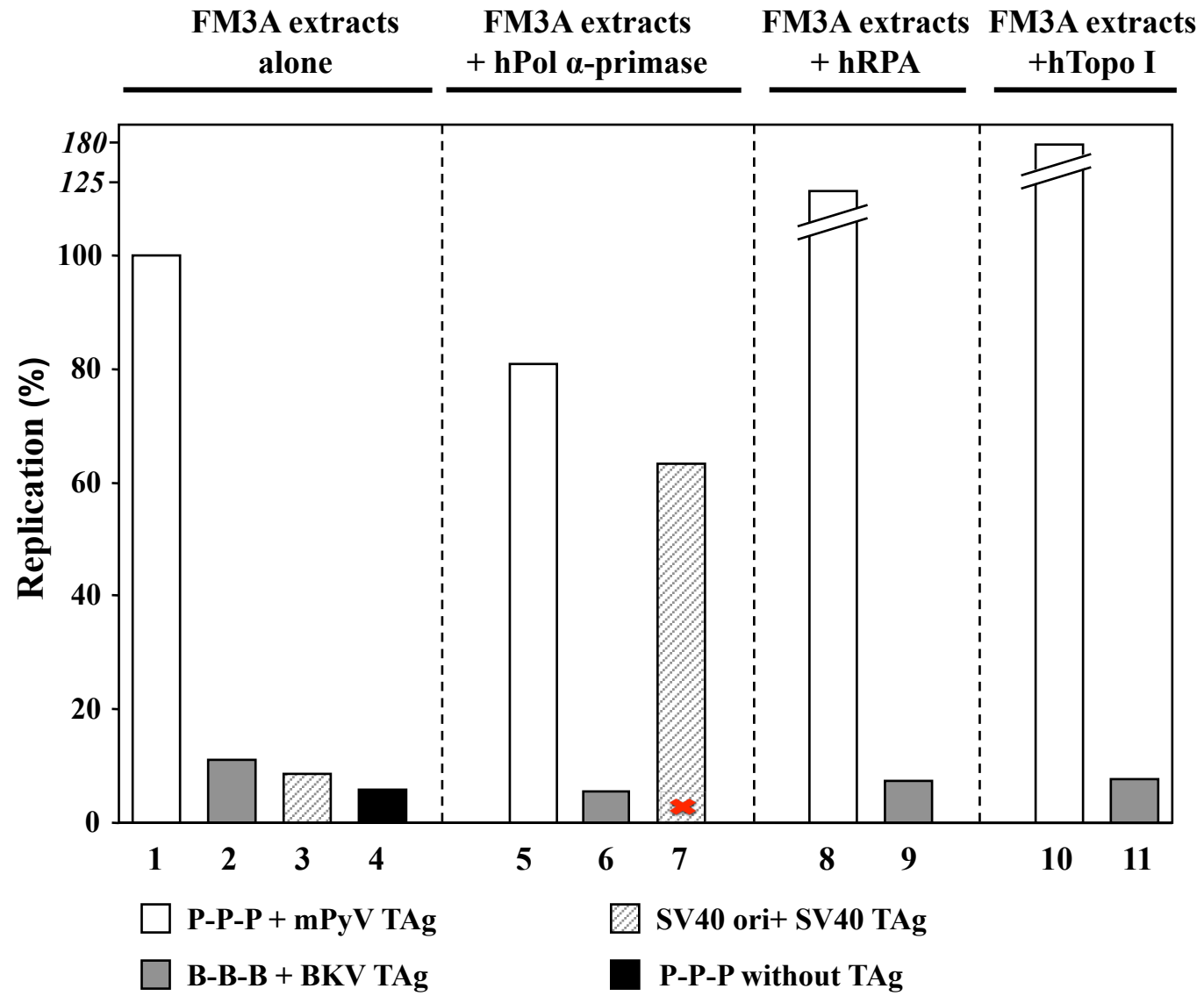


Fig. 5

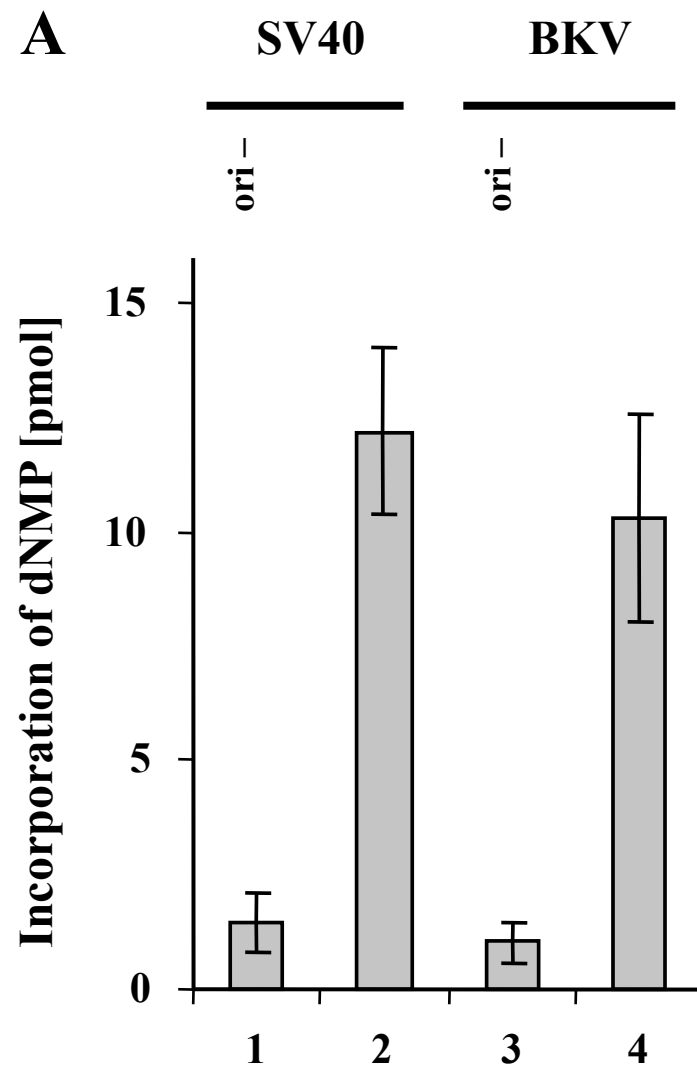


Fig. 5

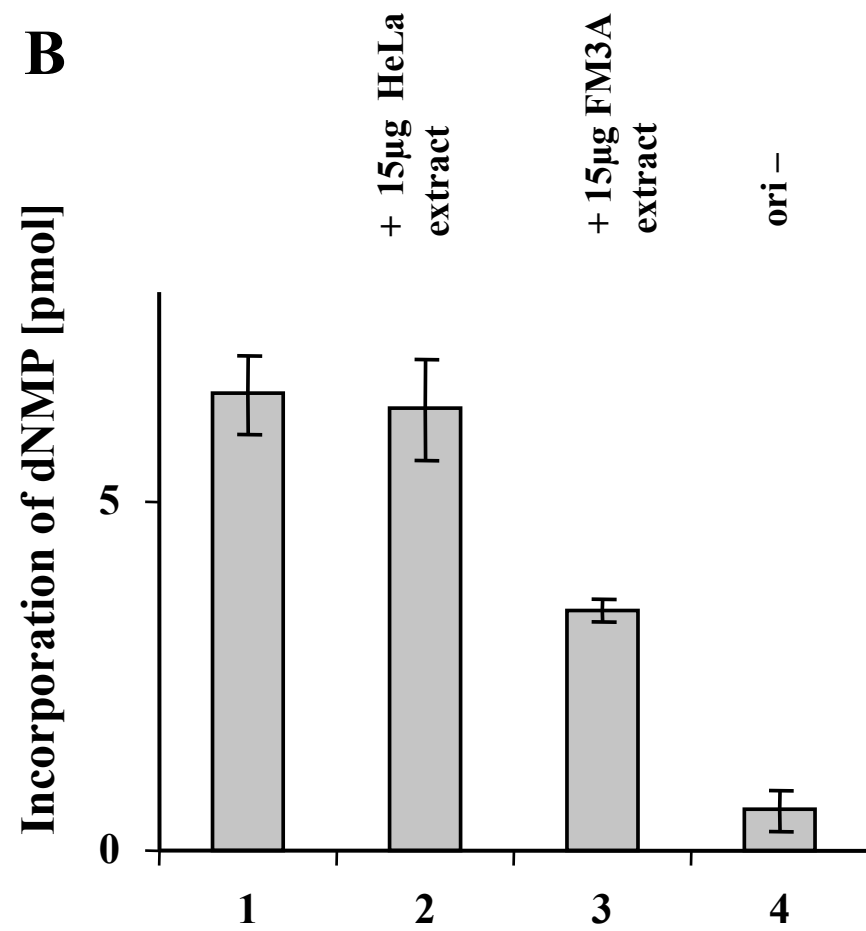


Fig. 5

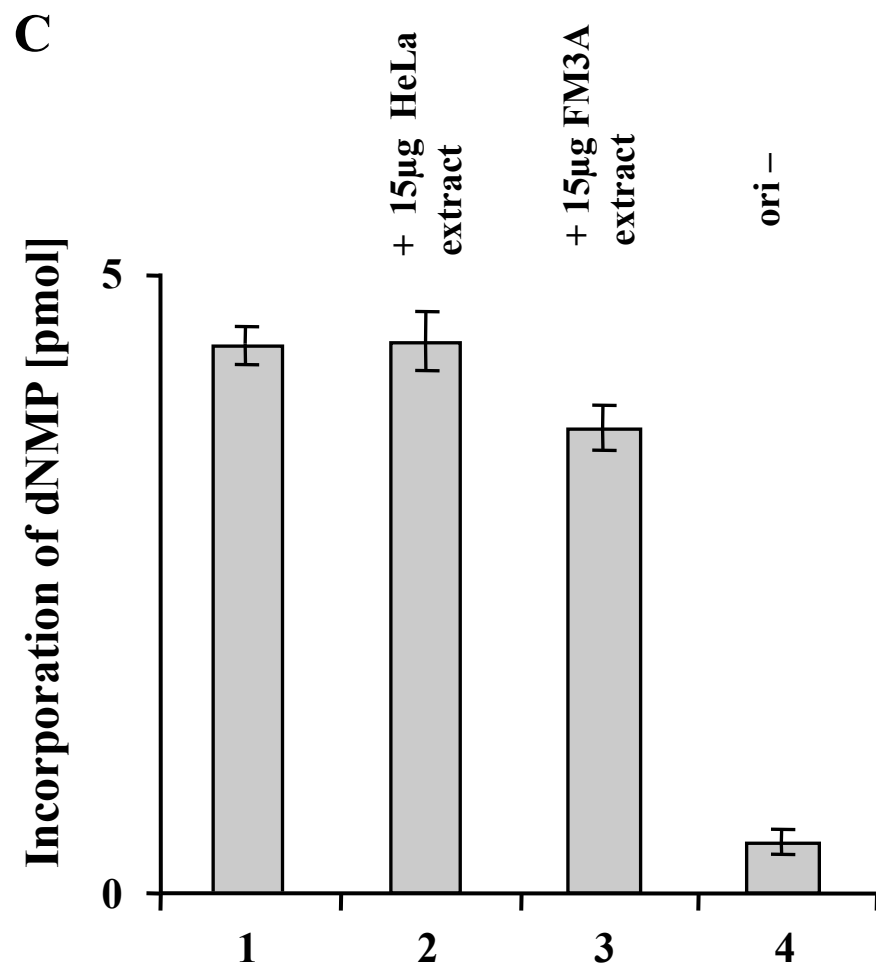


Fig. 6

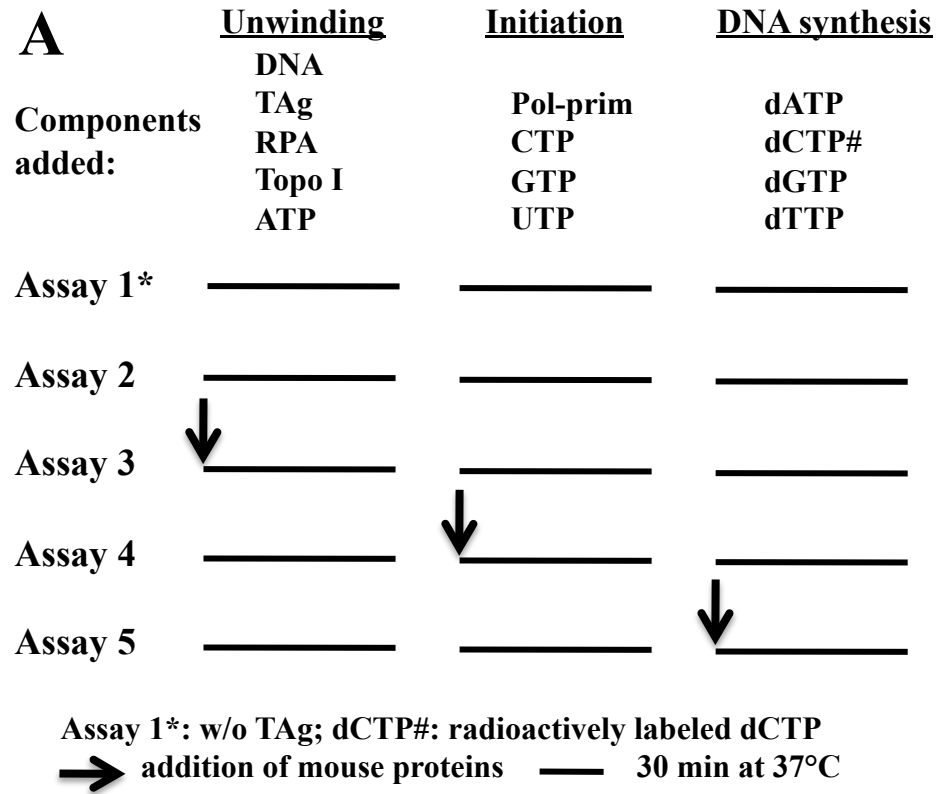


Fig. 6

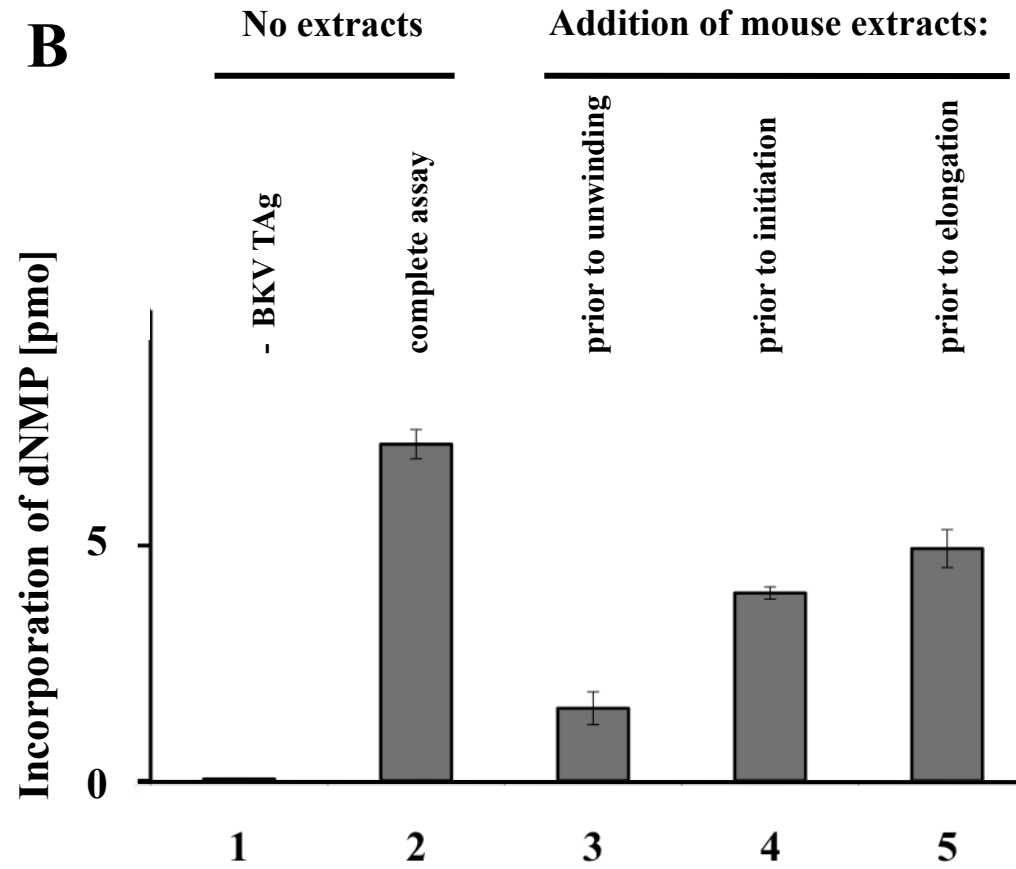


Fig. 6

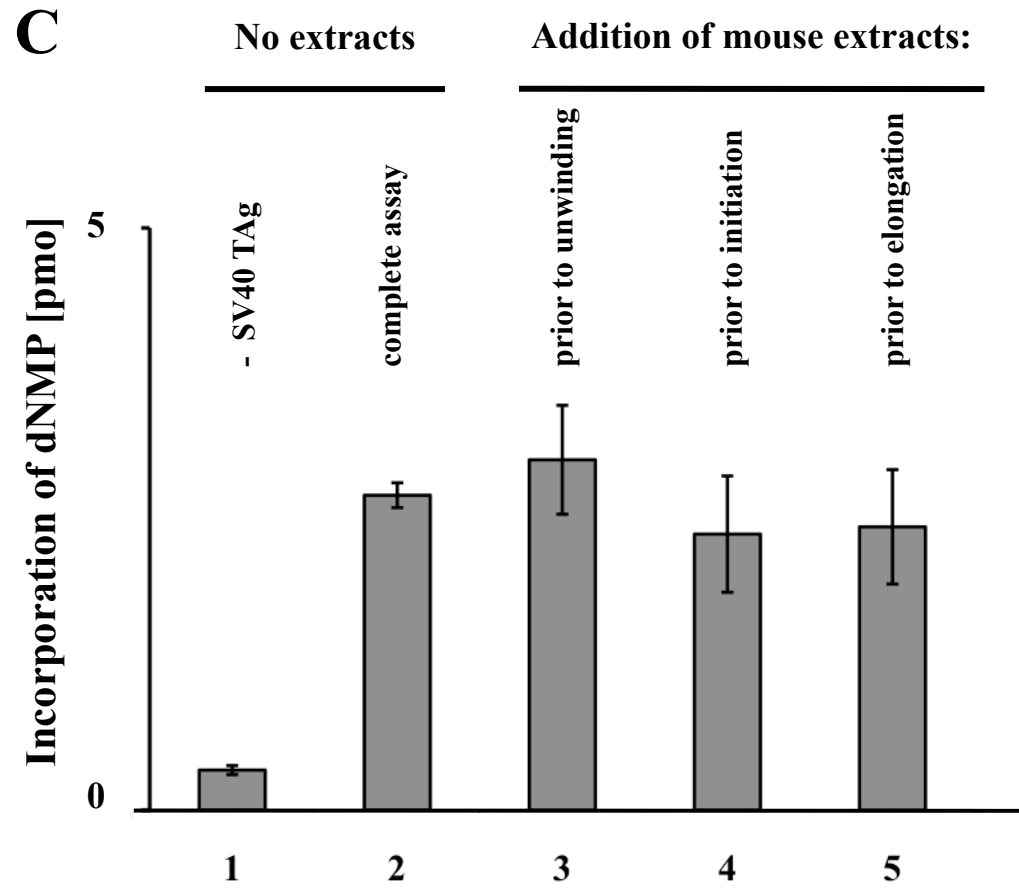
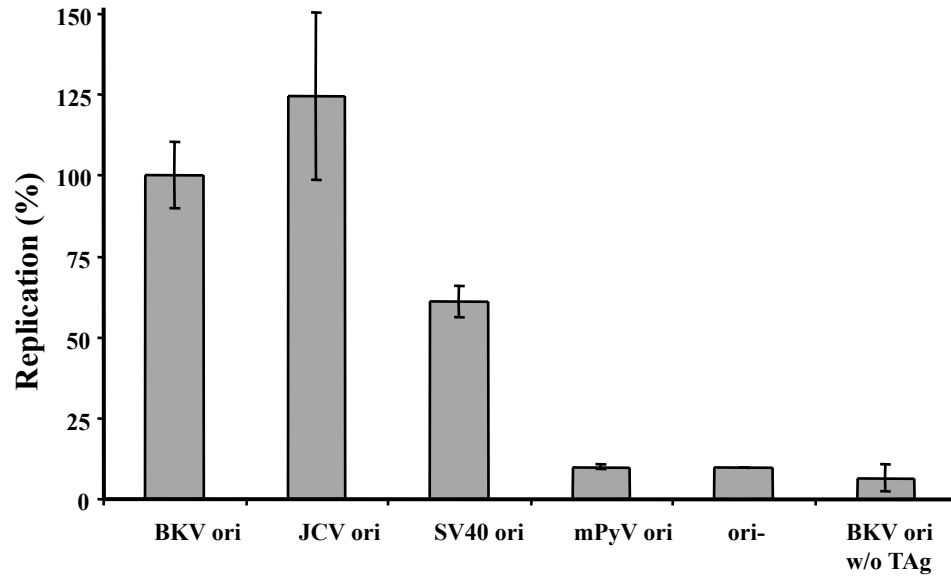
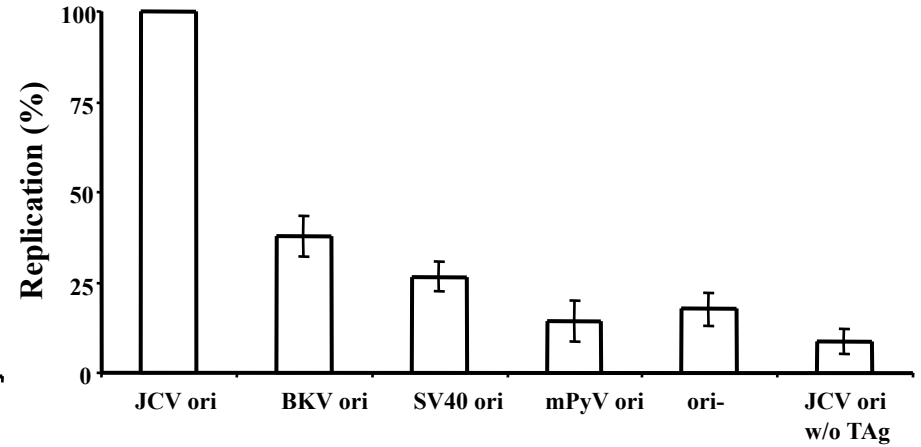


Fig. 7

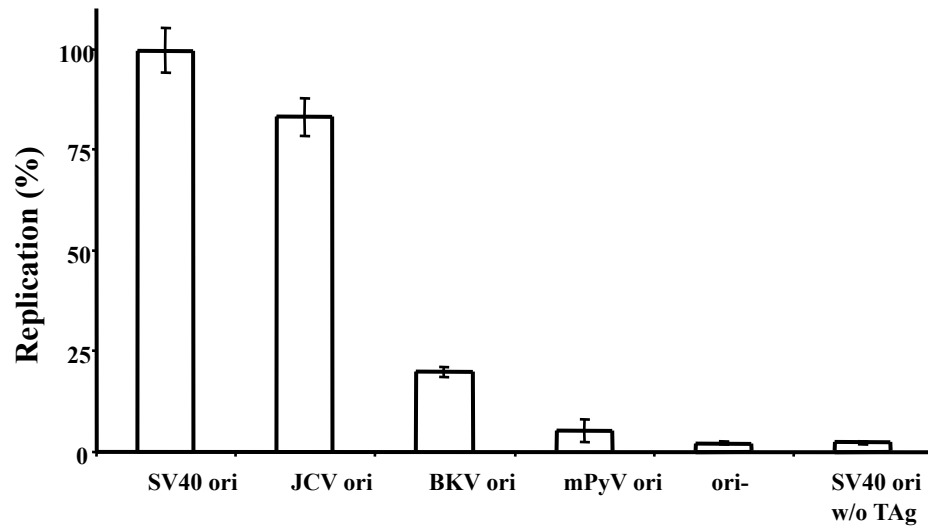
A BKV TAG: DNA Replication in Human Cell Extracts



B JCV TAG: DNA Replication in Human Cell Extracts



C SV40 TAG: DNA Replication in Human Cell Extracts



D mPyV TAG: DNA Replication in Mouse Cell Extracts

