BK virus early RNA transcripts in stably transformed cells: enhanced levels induced by dibutyryl cyclic AMP, forskolin and 12-O-tetradecanoylphorbol-13-acetate treatment

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The stably BK virus (BKV)-transformed hamster cell line BKT-1B and control BHK-21 cells were treated with dibutyryl cAMP (bu2cAMP), the adenylate cyclase activator forskolin, and the tumour-promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA). Cultures were stimulated for 30 min (short) and for 24 h (long). Northern blot analysis showed that for bu2cAMP and TPA both short and long stimulation resulted in significant increases in the levels of BKV early transcripts. Short exposure to forskolin resulted in a moderate increase and long exposure in a definite increase. In all cases the increased levels were maintained for at least 24 h after short stimulation was stopped. Experiments including the transcription inhibitor actinomycin D revealed that the enhanced levels of early BKV expression after treatment with the stimuli were due to induced RNA synthesis rather than to stabilization of the RNA. No DNA amplification of the early BKV sequences could be detected in the induced cells. The results are discussed with regard to possible roles for a cAMP-responsive element and a phorbol ester-responsive element, shown by sequencing to be present in the control region of the integrated BKV genome of the BKT-1B cells.

Introduction

The role of protein phosphorylation in mediating a cellular response to extracellular signals has been well studied. Phosphorylation is carried out by protein kinases which often respond directly to second messengers generated within the cell after the signalling molecule has bound to a specific membrane receptor. cAMP and diacylglycerol (DAG) are two such second messengers. cAMP activates specific cAMP-dependent protein kinases (protein kinase A, PKA; for reviews see McKnight et al., 1988; Taylor, 1989), whereas protein kinase C species (PKC) are transiently activated by DAG (Nishizuka, 1986). The expression of many eukaryotic cellular and viral genes is regulated by cAMP (for a review see Roesler et al., 1988). Analysis of the transcriptional control region of these genes reveals the consensus sequence TGACGTCA (Montminy et al., 1986). This sequence is called the cAMP-responsive element (CRE) and it has been shown that a nuclear CRE-binding protein (CREB) binds it (Montminy & Bilezikjian, 1987).

Phorbol and certain phorbol esters are tumour-promoting agents (Blumberg, 1981). They can induce an altered programme of gene expression. Such phorbol-responsive genes include protooncogenes (for a review see Hollstein & Yamasaki, 1987), cellular genes (Angel et al., 1987 and references therein) and genes of the polyomaviruses simian virus 40 (SV40) (Imbra & Karin, 1986) and the mouse polyomavirus (Wasylyk et al., 1987; Yamaguchi et al., 1989). Phorbol ester-responsive genes contain the conserved sequence TGAGCCA (Angel et al., 1987), referred to as the phorbol ester-responsive element (TRE). The transcription activator protein AP-1 binds to the TRE (Angel et al., 1987; Lee et al., 1987). It has been demonstrated that AP-1 is identical to or closely related to the c-jun oncogene product (Angel et al., 1987; Bohmann et al., 1987) or the previously identified p39 cellular protein (Rauscher et al., 1988a; Sassone-Corsi et al., 1988a).

BK virus (BKV) is a human polyomavirus first isolated from the urine of a renal allograft patient (Gardner et al., 1971). The genome consists of three functional regions. The early region encodes the large T and small t antigens. The late region carries the genetic information for the three viral capsid proteins VP1, VP2 and VP3 as well as the putative agnoprotein. The non-coding region contains the origin of replication and the promoter/enhancer contains sequences for early and late transcription. The enhancer sequences demonstrate the highest diversity between polyomaviruses and between different BKV strains (Yoshiike & Takemoto, 1986 and references therein).

The BKV (WW) strain (Rubinstein et al., 1987) might
be considered the ancestral strain from which the other strains have been derived by deletions and duplications. Markowitz & Dynan (1988) proposed division of the control region of the WW strain into three blocks, labelled P, Q and R. These blocks contain 68, 39 and 63 bp respectively. The Gardner strain has a triplicated P block (with a 18 bp deletion in the middle P block), a Q block and has lost the R block.

A CRE-like motif with the sequence TGACCTCA is present in every P block (Cassill et al., 1989; Deyerle & Subramani, 1988; our unpublished results). The TRE-like sequence are located at the P-P and P-Q junctions (Cassill et al., 1989; Deyerle & Subramani, 1988; Markowitz & Dynan, 1988; our unpublished results).

The BKV TRE motifs at the P-P and the P-Q junctions have the sequences TGACTCA and TGACCTCA respectively. A single 68 bp repeat (P block) showing the location of the putative CRE and TRE is presented in Fig. 1. It has recently been shown that Jun/AP-1 binds to the TRE-like sequence at the P-P junction (Markowitz & Dynan, 1988) and deletion studies in the BKV promoter/enhancer region suggest a putative role for these sequences in both early and late transcription in different cell lines (Cassill et al., 1989; Deyerle & Subramani, 1988).

These findings prompted us to investigate the possible involvement of PKA and PKC pathways in BK viral expression. We used the BKT-1B cell line (Näse et al., 1975; Stenn et al., 1976), a continuous cell line established from a BKV (Gardner)-induced hamster fibrosarcoma.
We employed the cAMP analogue dibutyryl cAMP (Bu₂cAMP), the adenylate cyclase activator forskolin (Seamon & Daly, 1986) and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) in stimulation experiments.

In this paper we report that short (30 min) and long time (24 h) induction of BKT-1B cells with Bu₂cAMP and TPA resulted in enhanced levels of early BKV RNA. The elevated levels were maintained for at least 23-35 h after a 30 min stimulation. A forskolin response seemed to require a longer stimulation time. Augmented early BKV RNA levels were prevented by the transcription inhibitor actinomycin D. The increased transcript levels did not result from gene amplification. Sequencing the control region of the integrated BKV genome revealed the presence of putative CRE and TRE sequences, and that the control region was significantly different from that of the prototype (Gardner) BKV.

**Methods**

*Cell lines and experimental culture conditions.* BHK-21 cells were obtained from the ATCC and cultured in Eagle's MEM supplemented with 1% HEPES, 2 mM-glutamine, 200 units of penicillin/ml, 100 mg streptomycin/ml and 10% foetal calf serum (FCS; Gibco). BKT-1B cells (a kind gift from Dr R. McIntyre, Kuopio, Finland) were grown to express the large T antigen constitutively (Näse et al., 1977). These cells were shown to require a longer stimulation time. Augmented early BKV RNA levels were prevented by the transcription inhibitor actinomycin D. The increased transcript levels did not result from gene amplification. Sequencing the control region of the integrated BKV genome revealed the presence of putative CRE and TRE sequences, and that the control region was significantly different from that of the prototype (Gardner) BKV.

**Stimulation experiments.** Forskolin, TPA and Bu₂cAMP (Sigma) were added to a final concentration of 100 μM, 50 ng/ml and 100 μM respectively. RNA was extracted following stimulation for 30 min or 24 h, or 23-35 h after a 30 min stimulation. Actinomycin D (Sigma) was used at a final concentration of 10 μg/ml.

**Plasmid construction.** The BKV prototype DNA was digested with EcoRI and ligated to the EcoRI site of the plasmid pUC19 (kindly provided by Dr T. Johansen). The recombinant plasmid was called pBK1913 and was subsequently used to subclone fragments of the BKV genome. The SacI–HindIII fragment spanning part of exon 2 of the large T antigen (nucleotides 3712 to 4685; numbering according to Yoshiike & Takemoto, 1986) was ligated to the SacI–HindIII sites of Riboprobe Gemini pGEM-3-blue vector (Promega Biotech) to generate plasmid pGEM-BKV3.

**DNA preparation and Southern blotting.** Genomic DNA was extracted according to standard procedures (Maniatis et al., 1982). Blotting was performed as described (Southern, 1975). Hybridization took place in 0.5× sodium phosphate pH 7.2/7% SDS (Church & Gilbert, 1984). BKV DNA was linearized with EcoRI, isolated from an agarose gel and purified by Geneclean. As a late probe pBK1913 was digested with SacI and HindIII and the EcoRI/HindIII fragment of 1428 bp (nucleotides 456 to 1884) was extracted and purified. Labelling was as described for DNA hybridization.

**Immunoperoxidase staining for BKV viral proteins.** Expression of viral early and late proteins in BKT-1B cells was examined by immunoperoxidase staining as described by Flagstad et al. (1986).

**Generation of ssDNA by the polymerase chain reaction (PCR).** The oligonucleotide primers (OriGene Tech) BKT-1 and BKT-2 with sequences 5' AAGGTCCTAGAGCTCATGAG 3' and 5' TGGGTAGGCAGAGGAGCAGCAGC 3' were complementary to the T antigen (BKT-1) and VP2 (BKT-2) coding regions of BKV. The two amplification primers were present in different molar amounts, 50 pmol of BKT-1 and 50 pmol of BKT-2. Under these conditions an excess of ssDNA will be produced and can be used directly in DNA sequencing reactions (Gyllensten & Erlich, 1988). The total volume of the PCR reaction was 100 μl and included primers, deoxynucleotides, reaction buffer, template DNA and 2-5 units of Taq polymerase (GeneAmp kit from Perkin-Elmer Cetus). This mixture was first heated to 94 °C for 4 min, and then subjected to 40 cycles of 1 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C, by using an EriCmp Thermocycler.

**Sequencing of ssDNA.** To eliminate dNTPs and oligonucleotide primers in the ssDNA PCR product, 50 μl of the reaction mixture was run on a Sephadex G50 spin column (Pharmacia). The ssDNA that eluted in the void volume was mixed with 2 ml of distilled H₂O₂, applied to a microconcentrator (Centricon 30; Amicon), and centrifuged to 2300 r.p.m. in a Sorvall HB4 rotor for 15 min. The retained material was mixed with 2 ml H₂O and centrifuged once more as described. The final retentate after this was brought up to a volume of 50 μl with water. Seven μl was mixed with 2 μl 5× Sequenase buffer and 1 μl (10 pmol) oligonucleotide primer and boiled for 5 min, incubated at 65 °C for 2 min, and cooled to room temperature in 30 min. Sequencing was according to the specifications of the manufacturer (Sequenase, US Biochemicals).

**Densitometric scanning.** Densitometric scanning of autoradiographic signals for quantification of both DNA and RNA bands was performed on a Hoefer Densitometry Scanner GS300 Transmittance (Reflantance; Hoefer Scientific Instruments). Hoefer GS365 data system software was used for calculations. Gaussian integration of areas under the peaks was used for quantification.
Results

BKV DNA is integrated in the BKT-1B genome, and the viral control region contains putative CRE and TRE motifs

We first wanted to establish the state of the viral DNA in the BKT-1B cell line. Total DNA was extracted, both undigested and EcoRI-digested DNA were separated on an agarose gel and Southern blotting was performed. The results are shown in Fig. 2. The extracted BKT-1B DNA was hybridized against total BKV DNA. A strong hybridization signal, corresponding to a 5-2 kb fragment, was found for the EcoRI-digested DNA. The BKV genome has a unique restriction site for this enzyme and this band probably represents linearized BKV DNA. Additional weaker bands were detected. No episomal DNA could be found in Hirt supernatants (Hirt, 1967). This indicates that the BKV DNA is integrated in the host chromosomes in multiple copies.

Rather than trying to subclone the integrated BKV DNA of BKT-1B cells into a sequencing vector we decided to approach sequencing by the PCR technique as described in Methods. By PCR a 725 bp BKV fragment was generated. This segment spans codon 16 for the large T and small t antigens to codon 11 in VP2 (CTA) plus the first base of the twelfth codon. This segment was sequenced (shown in Fig. 3a). The control region contains the P block with two point mutations compared to BKV (WW) (A→T and T→C; Rubinstein et al., 1987), the complete Q block and a R block with an insertion of 29 bp. Interestingly, this 29 bp region is derived from the P block (4 bp of late half) and part of the Q block (25 bp of early half). In addition the R block
has one point mutation (A→G) in the early part. We also noticed a point mutation outside the promoter/enhancer region. It is located in the palindromic sequence of the early side of the origin of replication. The P block contains the TGACCTCA CRE-like motif, and the junction between the P and the Q block forms the TRE-like element (ATGACTGGG). The insertion in the R block carries an almost complete TRE-like motif (TGACTGGG). A comparison of the control regions of BKV (WW), BKV (Gardner) and that found in BKT-1B cells with the position of the CRE-like and TRE-like sequences is shown in Fig. 3(b).

This sequence differs substantially from that of the Gardner strain although this strain was used originally to inject the newborn hamsters that provided the tumour tissue for establishing the BKT-1B cell line (see Discussion).

Long term (24 h) exposures of BKT-1B cells to bu2cAMP, forskolin and TPA enhances the levels of the BKV early transcripts

The sequencing data suggest that transcriptional regulation of BKV genes through PKA and PKC signal pathways may be targeted through CRE- and TRE-like elements in the BKV control region. Therefore cells were treated with the cAMP analogue bu2cAMP, the adenylyl cyclase activator forskolin (Seamon & Daly, 1986) or the tumour promoter TPA. Total RNA from both treated and untreated BKT-1B cells was analysed. As negative controls RNA from identically treated BHK-21 cells was employed. The total RNA was hybridized against the SacI-HindIII fragment which spans part of the large T antigen gene (as described in Methods). No hybridization signals were detected for BHK-21 RNA (Fig. 4, lanes 5 to 8). Analysis of RNA from BKT-1B cells revealed a major band of early BKV RNA corresponding to the 19S transcripts. The levels were enhanced in all the treated cultures (Fig. 4, lanes 2 to 4). Densitometric scanning of the autoradiographic signals illustrated that a 24 h incubation with 100 μM-bu2cAMP gave a twofold stimulation, whereas 100 μM-forskolin and 50 ng/ml TPA resulted in a 2.7-fold and threefold increase respectively. These results are summarized in Fig. 7. The values were standardized according to the untreated BKT-1B cells.

As an internal control for the Northern blots, the BKT-1B RNA was hybridized to a probe against 28S rRNA. The internal levels of 28S rRNA did not identify any differences between the different RNA isolations (see Fig. 4). Thus these results suggest that exposing BKT-1B cells to bu2cAMP, forskolin and TPA for 24 h resulted in a definite increase in early BKV transcripts.

Total RNA from unstimulated and stimulated BKT-
Fig. 4. Northern blot analysis of total RNA extracted from unstimulated and stimulated BKT-1B and BHK-21 cells. Stimulation was for 24 h with bu2cAMP, forskolin or TPA (see Methods). Hybridization was with a probe containing part of the large T antigen-coding region. Lanes 1 to 4, RNA from BKT-1B cells; lanes 5 to 8, RNA from control BHK-21 cells. Lanes 1 and 5, unstimulated cells; lanes 2 and 6, bu2cAMP-stimulated cells; lanes 3 and 7, forskolin-stimulated cells; lanes 4 and 8, TPA-stimulated cells. Densitometric scanning analysis is presented in Fig. 7. Hybridization to 28S rRNA cDNA probe is shown. The sizes of the RNA size markers are indicated in kb.

1B cells were also hybridized against a 1428 bp BKV HindIII–EcoRI fragment (nucleotides 456 to 1884, numbering according to Yoshiike & Takemoto, 1986) spanning part of the late coding region of BKV. This fragment contains overlapping sequences of all three capsid protein genes. No hybridization signals could be detected even after extended exposure times (results not shown).

Constitutive expression of BKV large T antigen was demonstrated by immunoperoxidase staining (Flegstad et al., 1986) employing the cross-reacting monoclonal antibody PAbl614 directed against the SV40 large T antigen (Ball et al., 1984). It is, however, not possible by this method to quantify the intensity of staining. Staining with rabbit antisera directed against the viral capsid protein revealed no signals (data not shown).

**Short term stimulation with bu2cAMP and TPA enhances BKV early RNA levels, whereas only a moderate increase was measured for forskolin**

Genes that are transcriptionally induced by cAMP can be divided into two general categories. Group 1 genes are rapidly regulated by cAMP, whereas for group 2 the transcript level is increased only after extended incubation time with cAMP (Roesler et al., 1988). In order to approach this time-dependent difference in response, we performed an experiment similar to that described above with exposure for 30 min. The results of RNA analyses are presented in Fig. 5.

Increased levels of early BKV transcripts were detected. Densitometric analysis of the hybridization signals revealed a 4.5-fold increase with bu2cAMP and a threefold increase for TPA (Fig. 5, lanes 2 and 4 respectively). Only a moderate enhancement was found for forskolin (Fig. 5, 1.5-fold, lane 3). The densitometric results are summarized in Fig. 7.

Hence, the early BKV genes seemed to be rapidly regulated by the cAMP analogue. A rapid induction by TPA was also demonstrated, whereas short time stimulation with forskolin resulted in only a moderate increase of the early BKV RNA.

Fig. 5. Northern blot analysis of total BKT-1B RNA after 30 min stimulation. Lane 1, unstimulated cells; lane 2, bu2cAMP-treated cells; lane 3, forskolin-exposed cells; lane 4, TPA-treated cells. Densitometric scanning analysis results are shown in Fig. 7. Hybridization to 28S rRNA cDNA probe is indicated. Size markers are shown (in kb).
Enhanced levels of early BK virus transcripts

Fig. 6. Northern blot analysis of BKT-1B RNA extracted 23.5 h after a 30 min stimulation. The position of the lanes is as in Fig. 4. Hybridization with a 28S rRNA cDNA probe is shown. Densitometric scanning results are summarized in Fig. 7. Size markers are shown (in kb).

Enhanced early BKV RNA levels are maintained for at least 24 h after treatment

The mRNA produced by cAMP-regulated genes appears to have a relatively short half-life (Roesler et al., 1988). This prompted us to analyse early BKV RNA levels 23.5 h after a 30 min stimulation with bu2cAMP, forskolin or TPA. After the incubation period the medium was removed, the cells were washed twice with medium and fresh medium was added. Unstimulated cells were treated in the same way. Total RNA was extracted 23.5 h later and analysed for the levels of early BKV transcripts. The results are shown in Fig. 6. Increased levels of early viral RNA were found for bu2cAMP, forskolin and TPA (lanes 2, 3 and 4 respectively). A 2.3-fold, 2.4-fold and 1.6-fold increase was measured by densitometric scanning (see Fig. 7). In contrast to the previous experiment, clearly higher levels were found after short stimulation with forskolin (1.5-fold compared to 2.4-fold).

These results indicated that the BKV early genes responded to short-time stimuli of bu2cAMP and TPA and that the increased levels were maintained for several hours. The original increased levels after a 30 min stimulation dropped to about half (4.5-fold to 2.3-fold for bu2cAMP and threefold to 1.6-fold for TPA). For forskolin, however, the levels increased even after it had been removed from the medium (1.5-fold to 2.4-fold).

Fig. 7. The relative levels of BKV early RNA in untreated and treated BKT-1B cells was determined by densitometric scanning of the autoradiograms shown in Fig. 4 to 6. The BKV RNA concentration in unstimulated cells was arbitrarily set at 1. The open bars represent relative RNA levels after a 24 h stimulation. The black bars give BKV early RNA levels after a 30 min stimulation and the hatched bars represent 23.5 h after a 30 min stimulation.

Increased early BKV RNA levels were not due to amplification of the early BKV sequences in the BKT-1B cells

To determine whether higher levels of BKV early transcripts were due to gene amplification, in situ filter DNA hybridization was performed. Equal amounts of BKT-1B cells were loaded on the slots and afterwards hybridized to a BKV early region DNA probe (see Methods). Densitometric scanning showed no differences in hybridization signals (see Fig. 8). These results indicate that increased RNA levels from the early BKV genes did not result from DNA amplification.

Enhanced early BKV RNA levels are the result of increased transcription rather than stabilization of the RNA

Enhanced RNA levels might result from increased transcription of the genes or from stabilization of the specific messengers. To examine this question, experiments were performed in the presence of the transcrip-
tion inhibitor actinomycin D. Since bu2cAMP and forskolin both exhibit their influence through the PKA pathway and only a moderate effect was seen by short-time forskolin treatment, we decided not to test the latter.

BKT-1B cells were treated for 30 min with 100 μM-bu2cAMP or 50 ng/ml TPA in the presence or absence of 10 μg/ml actinomycin D. Unstimulated BKT-1B cells were also incubated in the presence or absence of actinomycin D. Total RNA was purified and analysed by Northern blotting for their levels of early BKV RNA. The results are shown in Fig. 9. Actinomycin D reduced the levels of early viral transcripts (lanes 2, 4 and 6). In the absence of the transcription inhibitor increased hybridization signals were detected. Hybridization with a 28S rRNA cDNA probe revealed no differences between the RNA isolated from the different treated BKT-1B cells.

These results suggest stimulation of the PKA pathway by bu2cAMP or the PKC pathway by TPA induced transcription of the early BKV genes in the stably transformed BKT-1B cell line. However, we cannot exclude the possibility that enhanced levels of early viral transcripts were partially due to stabilization of the transcripts.

**Discussion**

Expression of the early BK virus genes in the stably BKV transformed hamster cell line BKT-1B was induced by activators of the PKA pathway (bu2cAMP and forskolin) and the PKC pathway (TPA). Increased levels of early BKV transcripts were found both after short stimulation (30 min) and long stimulation (24 h) although the stimulatory effect with forskolin after 30 min was only moderate. The early BKV RNA levels after short time stimulation with bu2cAMP and TPA were reduced to about half 23.5 h after induction but were still elevated compared to untreated BKT-1B cells. For forskolin, an adenylate cyclase activator (Seamon & Daly, 1986), higher viral RNA levels were found 24 h after a 30 min stimulation than immediately after a 30 min induction. This suggests that forskolin has a more prolonged effect. The BKV RNA levels after 24 h stimulation with forskolin were even higher. One possible explanation for these findings is that the adenylate cyclase activated by forskolin may have a longer activity period than the protein kinases induced by cAMP, the intracellular concentrations of cAMP may remain elevated following forskolin treatment, and hence cAMP-controlled signal transduction is left in the induced state.

In contrast with our results, transcription of the protooncogene c-fos was induced by forskolin after a 1 hr
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