Epstein–Barr virus BART gene expression

Maha Al-Mozaini,1,2 Gustavo Bodelon,1 Claudio Elgueta Karstegl,1 Boquan Jin,3 Mohammed Al-Ahdal2 and Paul J. Farrell1

1Department of Virology, Imperial College London, Norfolk Place, London W2 1PG, UK
2Molecular Virology Department, BMR King Faisal Hospital and Research Center, PO Box 3354 MBC-03, Riyadh 11211, Saudi Arabia
3Department of Immunology, The Fourth Military Medical University, Xi'an 710032, PR China

Introns from the Epstein–Barr virus (EBV) BART RNAs produce up to 20 micro RNAs (miRNAs) but the spliced exons of the BART RNAs have also been investigated as possible mRNAs, with the potential to express the RPMS1 and A73 proteins. Recombinant RPMS1 and A73 proteins were expressed in Escherichia coli and used to make new monoclonal antibodies that reacted specifically with artificially expressed RPMS1 and A73. These antibodies did not detect endogenous expression of A73 and RPMS1 proteins in a panel of EBV-infected cell lines representing the different known types of EBV infection. BART RNA could not be detected on Northern blots of cytoplasmic poly(A)+ RNA from the C666.1 NPC cell line and BART RNA was found to be mainly in the nucleus of C666.1 cells, arguing against an mRNA role for BART RNAs. In contrast, some early lytic cycle EBV mRNAs were found to be expressed in C666.1 cells. Artificially expressed A73 protein was known to be able to bind to the cellular RACK1 protein and has now also been shown to be able to regulate calcium flux, presumably via RACK1. Overall, the results support the conclusion that the miRNAs are functionally important products of BART transcription in the cell lines studied because the A73 and RPMS1 proteins could not be detected in natural EBV infections. However, the possibility remains that A73 and RPMS1 might be expressed in some situations because of the clear potential relevance of their biochemical functions.

INTRODUCTION

The BART RNAs are a heterogeneously spliced group of Epstein–Barr virus (EBV) RNAs transcribed rightward from position 138 352 to 160 531 on the EBV wild-type genetic map (Sadler & Raab-Traub, 1995; Smith et al., 2000; de Jesus et al., 2003). BART RNAs have been detected in peripheral blood of normal EBV carriers (Chen et al., 1996) and in all EBV-associated diseases that have been examined, including Burkitt’s lymphoma (Tao et al., 1998), gastric carcinoma (Sugiura et al., 1991), oral hairy leukoplakia (Webster-Cyriaque & Raab-Traub, 1998), nasal natural killer and T cell lymphomas (Chiang et al., 1996; van Gorp et al., 1996), Hodgkin’s lymphoma (Deacon et al., 1993) and hepatocellular carcinomas (Sugawara et al., 1999). Most of the viral micro RNAs (miRNAs) that are expressed in EBV latent infections are derived from the BART RNAs (Cai et al., 2006; Griffiths-Jones et al., 2006; Grundhoff et al., 2006; Pfeffer et al., 2004). The BART miRNAs are thought to be derived mainly from introns prior to splicing of the BART primary transcripts (Edwards et al., 2008). Few functional targets have yet been identified for the EBV miRNAs but there is evidence that miR BART2 can regulate the EBV DNA polymerase gene (Barth et al., 2008) and miR BART 1-5p and 17-5p can regulate EBV LMP1 (Lo et al., 2007).

The BART RNAs (also known as complementary strand transcripts or BARF0 RNAs) were originally identified by analysis of cDNA libraries established from the nude-mouse-passaged nasopharyngeal carcinoma (NPC) cell line C15 (Gilligan et al., 1990; Hitt et al., 1989). A 4.0 kb cDNA clone (RPMS1, Smith et al., 2000) containing six exons was isolated that spanned the whole BART region but many other spliced partial cDNA clones of BART RNA containing combinations of the 16 different alternate exons have been described previously (Sadler & Raab-Traub, 1995). Prior to the discovery of the EBV miRNAs, some open reading frames (ORFs) in the spliced BART cDNAs were investigated as potential protein-coding sequences, if the BARTs were acting as mRNA. We have previously studied the RPMS1 and A73 ORFs, which could be translated in vitro from the spliced BART cDNAs isolated from C15 NPC cells (Smith et al., 2000). RNase protection assays confirmed that the RNAs covering the RPMS1 and A73 ORFs represent significant portions of the BART family of transcripts (de Jesus et al., 2003) and biochemical activities...
of the artificially expressed RPMS1 and A73 proteins have been identified that could be relevant to the role of the virus in cancer (de Jesus et al., 2003; Smith et al., 2000; Zhang et al., 2001). Thus, RPMS1 was found to antagonize transcription activation by Notch 1 or EBNA2 by virus in cancer (de Jesus et al., 2000). A73 was found to bind to the RACK1 protein (Smith et al., 2000), which was known to act as a scaffold in Src family kinase signalling but has more recently been shown to bind and regulate calcium release from intracellular stores by enhancing the affinity of inositol 1,4,5-trisphosphate receptor (IP3R) binding for inositol 1,4,5-trisphosphate (IP3) (Patterson et al., 2004). Although there is some evidence of an immune response to the protein products of BART RNAs in EBV-infected humans (Gilligan et al., 1991; Kienzle et al., 1998), the RPMS1 and A73 proteins have not been identified in a natural EBV infection so their existence in real EBV infections remains uncertain.

BART promoter activity could be readily detected in transfection assays only if EBV sequences both upstream and downstream of the first exon were included in the promoter construct (de Jesus et al., 2003). Although most of the EBV genome in C15 NPC tumour DNA is heavily methylated, an unmethylated region was found around the EBER2.5 and AFB1 are EBV-positive lymphoblastoid cell lines (LCLs) derived from this region of the genome using the NPC cell line C666.1 (Cheung et al., 1999). New monoclonal antibodies (mAbs) that recognize artificially expressed A73 and RPMS1 were used to search for these proteins in EBV-infected cells and the A73 protein was shown to be able to regulate calcium flux. The results support the conclusion that the miRNAs are functionally important products of BART transcription in the cell lines studied because the A73 and RPMS1 proteins could not be detected in natural EBV infections. However, the possibility remains that A73 and RPMS1 might be expressed in some situation because the A73 and RPMS1 proteins could not be detected in a natural EBV infection so their existence in real EBV infections remains uncertain.

In this paper, we investigated further EBV methylation around the BART promoter and expression of viral RNAs derived from this region of the genome using the NPC cell line C666.1 (Cheung et al., 1999). New monoclonal antibodies (mAbs) that recognize artificially expressed A73 and RPMS1 were used to search for these proteins in EBV-infected cells and the A73 protein was shown to be able to regulate calcium flux. The results support the conclusion that the miRNAs are functionally important products of BART transcription in the cell lines studied because the A73 and RPMS1 proteins could not be detected in natural EBV infections. However, the possibility remains that A73 and RPMS1 might be expressed in some situation because of the clear potential relevance of their biochemical functions.

**METHODS**

**Cell lines.** 293 and HeLa are EBV-negative epithelial cell lines and Ramos and Akata 31 are EBV-negative Burkitt’s lymphoma (BL) cell lines. C2 + Obaji, C2 + BL16, A. Aluoch, WeiBi1, E1, B122, B142, EREB2.5 and AFB1 are EBV-positive lymphoblastoid cell lines (LCLs) made with a variety of different EBV strains (type 1 or type 2) and cord or peripheral B cells (Spender et al., 2006). C15 and C17 are EBV-positive NPC xenografts grown in nude mice (Busson et al., 1988) and C666.1 (Cheung et al., 1999) is an EBV-positive NPC cell line. TE1 is a human monocye cell line containing EBV (Masy et al., 2002) and Mak, Daudi, P3HR1, Jijoye, Baji, Namalwa and Akata2003 are EBV-positive BL cell lines. Cells were cultured in Dulbecco’s minimal essential medium or RPMI 1640 with 10% fetal calf serum. Transfection into 293 cells was carried out using Lipofectamine 2000 (Invitrogen).

**DNA methylation analysis, Northern blotting and RT-PCR.** For Southern blotting, 20 μg samples of DNA extracted from cells C666.1, LCL BM + Akata and BL line Akata were digested with either HpaII or MspI, and electrophoresed on a 1% agarose gel and Southern blots were hybridized with the probes labelled by random priming. For Northern blots, RNA was treated with 8.6% (w/v) glyoxal in DMSO for 10 min at 50 °C, electrophoresed on a 1.4% agarose gel in 10 mM sodium phosphate pH 7.0 and blotted to nitrocellulose or nylon membranes. Probes were labelled by random priming.

RT-PCR used cDNA made by reverse transcription using the Protoscript kit (New England Biolabs) and an oligo(dT) primer. The PCR primers for the BART-PCR were exon 3B–5 5'-CCGA-TTCAGGAGCGTTTAGC-3' and 5'-GCCGGCTCATTCCCCAGTGT-TC-3', exon 5–7 5'-GCCCAACATACCCCTTGTTATC-3' and 5'-AAGGCTTGCCTTTCACACACAG-3'. GAPDH primers were 5'-TGCTCTGGACACCACACT-3' and 5'-GCCGGCTTGCACACC-ACCTTC-3'. PCR involved an initial denaturation at 95 °C for 5 min, then 30 cycles of 94 °C, 90 s; 50 °C, 90 s; 72 °C, 2 min followed by 5 min at 72 °C to complete the reaction.

**Expression of A73 and RPMS1.** PCR-amplified A73 or RPMS1 ORFs were cloned into the pCR72/TOPO expression vector, with or without a FLAG tag, for expression by in vitro translation or transfection. The A73 PCR products were also cloned into the pCR72/TOPO plasmid vector in-frame with the 6× His tag of the vector for expression in BL21 (DE3) pLysS competent Escherichia coli. Protein expression was induced with 1 mM IPTG. The RPMS1 ORF was cloned as a maltose-binding protein (MBP) fusion in the pMAL vector (New England Biolabs), expressed in E. coli and purified on amylose resin (New England Biolabs).

**A73 and RPMS1 mAbs.** Hybridomas produced from mice immunized with the purified His-tagged A73 were initially screened for their ability to detect the His-tagged A73 but not a control His-tagged protein. Hybridomas raised against MBP–RPMS1 were screened for the ability to recognize MBP–RPMS1 but not MBP. Selected antibodies of high titre were then tested in Western immunoblotting and immunofluorescence assays, examples of which are shown in Fig. 3. Western blotting used total protein extract (40 μg) from transfected 293 cells, or A73 or RPMS1 in vitro translated in wheatgerm extracts. The extracts were loaded on to 12% SDS-PAGE gels, the proteins were then transferred to PVDF or nylon membranes for Western blotting.

For testing the mAbs by immunofluorescence, transfected cells were plated on sterilized coverslips coated with poly-lysine D. The transfected cells were fixed with paraformaldehyde and incubated with the hybridoma supernatant. For controls, the cells were stained with anti-FLAG antibody for 60 min at 4 °C. Anti-A73, anti-RPMS1 mAbs and anti-FLAG staining were visualized by incubating with fluorescein isothiocyanate-conjugated anti-mouse IgG for 60 min at 4 °C.

**Stably transfected cell lines expressing A73 or RPMS1.** One day prior to transfection, each well of a six-well plate was seeded with 0.5–1 × 10^5 293 cells, so that they would reach 70–80% confluency for the transfection assay. A 3 μg sample of each construct DNA was diluted in 250 μl Opti-MEM I medium without serum and mixed gently. A 10 μl volume of Lipofectamine 2000 (Invitrogen) was added to 240 μl Opti-MEM I medium without serum, mixed gently and incubated for 5 min at room temperature. The two solutions were combined, mixed gently and incubated for 20 min at room temperature.

In this paper, we investigated further EBV methylation around the BART promoter and expression of viral RNAs derived from this region of the genome using the NPC cell line C666.1 (Cheung et al., 1999). New monoclonal antibodies (mAbs) that recognize artificially expressed A73 and RPMS1 were used to search for these proteins in EBV-infected cells and the A73 protein was shown to be able to regulate calcium flux. The results support the conclusion that the miRNAs are functionally important products of BART transcription in the cell lines studied because the A73 and RPMS1 proteins could not be detected in natural EBV infections. However, the possibility remains that A73 and RPMS1 might be expressed in some situation because of the clear potential relevance of their biochemical functions.
Flow cytometry calcium ion measurement. The protocol was derived from Mullins molecular laboratory research methods (University of Washington School of Medicine, Department of Microbiology, USA) and was performed by the flow cytometry facility of King Faisal Hospital & Research Center. Cells (10^6 ml^{-1}) were stained with 7 µl Indo-1 dye ml^{-1}, wrapped in foil and incubated for 45 min in a 37 °C water bath. The cells were gently mixed every 10–15 min to ensure even loading. The Indo-1-stained cells were mixed with culture medium and centrifuged at 900 g for 8 min, then resuspended in culture medium at 5 × 10^6 ml^{-1}. Samples (0.8 ml) of culture medium were prewarmed in several 5 ml FACS tubes to 37 °C and then 0.2 ml Indo-loaded cells (1 × 10^6) was added to each tube and warmed at 37 °C for 3–5 min before running at 37 °C on a LSR flow cytometer (Becton Dickinson). The cells were run at >400 cells s^{-1} to establish a baseline of [Ca^{2+}] for 40–60 s before adding stimulant. UTP stimulant (to 100 µM) was added and data collected for another 5 min. The ionomycin response was then tested with 10 µmol ionomycin. After running the ionomycin, the FACS line was flushed with DMSO or 10% bleach, then for 1 min with culture medium to remove any residual ionomycin.

RESULTS

EBV BART promoter region methylation
Abundant BART miRNA expression has been reported in C15 NPC tumour cells and in the C666.1 NPC cell line but much lower levels were detected in latency I BL cell lines or LCLs (Cai et al., 2006), consistent with the high levels of BART RNA transcription in the NPC cells. Absence of DNA methylation in a region of the genome can be an indicator of locations where transcription factors required for promoter activity are bound and we previously showed that EBV DNA is locally unmethylated around exon I of the BART gene in the C15 NPC tumour (de Jesus et al., 2003). We have therefore now extended the methylation study to C666.1 NPC cells and to B cell lines carrying EBV. DNA from C666.1 cells, Akata BL cells and an LCL (BM + Akata) was digested with HpaII or MspI and fractionated on a 1 % agarose gel. Using a series of probes that cover the BART promoter region, we found that C666.1 cells also have a locally unmethylated region of DNA around the BART promoter, extending from about nt 138 180 to 139 050 on the EBV-wt map (Fig. 1a). For example, probes 5 and 6 in the hypomethylated region give mostly the same sized bands with the two enzymes, whereas other probes show no digestion with methylation-sensitive HpaII enzyme (Fig. 1a). The region of hypomethylation in this analysis extends from just upstream of the transcription start to about 570 bp downstream of the end of exon I and is similar to that found in C15 tumour cells.

LCLs have a very low expression of BART transcripts but restriction digestion showed the DNA to be unmethylated across the whole region (Fig. 1b). EBV DNA from Akata BL cells was also analysed; it showed a pattern of partial methylation (Fig. 1c). Some B cell lines have low levels of spontaneous EBV lytic replication but Gardella gel analysis of these cell lines showed predominantly closed circular DNA (Fig. 1d) with very little linear EBV DNA, suggesting that the unmethylated EBV DNA in the B cell lines was not a consequence of lytic replicating viral DNA. It therefore seems that the lack of expression of BART RNAs in the B cell lines is most likely a consequence of a lack of a transcription factor required for the activity of the BART promoter or another epigenetic modification rather than genome methylation preventing expression.

Northern blots of the BART region cytoplasmic poly(A)^+ RNA from C666.1 cells
Determining whether the BART transcripts can be detected in cytoplasmic poly(A)^+ RNA would be highly relevant to whether the BART RNAs could act as miRNA, to express for example the RPMsi or A73 proteins. Previous Northern blotting studies of BART RNAs in C15 cells (Gilligan et al., 1990; Hitt et al., 1989) detected several RNA species (the most abundant about 4.8 kb in length), perhaps corresponding to some of the cDNAs that have been cloned (Sadler & Raab-Traub, 1995; Smith et al., 2000). It is difficult to apply standard nuclear/cytoplasmic fractionation methods to xenograft samples from the C15 tumour (although this was used by Hitt et al., 1989), so most analyses of BART RNAs have used total cell RNA extracted with guanidinium isothiocyanate. The more recent availability of the C666.1 NPC cell line allows conventional nuclear/cytoplasmic fractionation prior to RNA extraction. BART transcripts are readily detected by RT-PCR in total cell RNA from C666.1 cells and the BART miRNAs are also easily detected by Northern blotting (Cai et al., 2006; and confirmed by us, data not shown).

Cytoplasmic poly(A)^+ RNA from C666.1 cells was tested by Northern blotting with various probes covering the BART region, the LMP-2A gene and the BamHI H region (Fig. 2). No specific bands corresponding to the BART transcripts could be detected with pDH1 or pBamH1A, which would be expected to hybridize to the BART transcripts. For comparison with the established EBV transcription map, RNA from B95-8 cells with or without 12-O tetradecanoyl phorbol-13-acetate (TPA) lytic-cycle-induction was analysed in parallel. A higher loading of C666.1 RNA was used to facilitate detection of transcripts (note the higher actin and GAPDH signals) but the only RNA from the vicinity of the BART region that could be detected in C666.1 cytoplasmic poly(A)^+ RNA co-migrated with the BALF2 lytic cycle RNA that has been characterized previously in TPA-treated B95-8 cells (see
probes DH1, DH2 and BamHI A in Fig. 2). Certain other lytic-cycle RNAs could be detected in the cytoplasmic poly(A)^+ C666.1 RNA, for example the BHLF1 transcript in BamHI H (Fig. 2). It thus appears that C666.1 cells express a subset of early lytic cycle RNAs, consistent with some other reports (Cabras et al., 2005; Feng et al., 2000; Martel-Renoir et al., 1995), but we could find no evidence for discrete BART transcripts in the cytoplasmic poly(A)^+ RNA. The apparently abundant nature of BART transcripts detected by RT-PCR in total cell RNA could be reconciled with this result if most of the BART RNA is heterogeneous in size and in the nucleus, acting as a precursor for the production of the BART miRNAs. The predominantly nuclear location of the BART RNAs was confirmed by using RT-PCR to detect spliced BART RNA in nuclear and cytoplasmic fractions of C666.1 cells (Fig. 2c). Priming cDNA synthesis with oligo(dT) and RT-PCR for either the spliced exons 3B–5 or 5–7 BART structures showed that the RNA was almost completely in the nucleus. The same cDNAs were tested for the cell GAPDH gene as a control and this showed the expected predominantly cytoplasmic distribution with a significant amount of nascent RNA in the nucleus, the nuclear fraction showing some longer molecules, presumably derived from incompletely spliced precursors that would be expected in the nuclear fraction (Fig. 2c). An EBV-negative cell line, BL2, acted as a negative control for the BART PCRs.

**New A73 and RPMS1 mAbs do not detect endogenous A73 or RPMS1**

Searching directly for the potential A73 and RPMS1 proteins has been constrained by the lack of antibodies specific for them. A His-tagged A73 protein was found to express well in *E. coli* and was purified by immobilized-
Fig. 2. Analysis of cytoplasmic and nuclear RNA from C666.1 cells for mRNAs mapping in the BART region. (a) The location of the BamHI A restriction fragment (154 289–166 156) and the DH1 (159 395–163 307), DH2 (163 307–166 023) and DH3 (166 023–168 575) segments of the EcoRI Dhet fragment are illustrated against part of the EBV genetic map overlapping the BART transcripts. Exons 5 and 7 of the BART RPMS1 cDNA [used in (c)] are marked. Probe coordinates are from the EBV-wt sequence, GenBank accession no. AJ507799. (b) Northern blots of cytoplasmic poly(A)+ RNA from C666.1 cells, B95-8 cells or B95-8 cells treated for 3 days with 30 ng TPA ml−1 are shown. Size markers are a HindIII digest of bacteriophage lambda DNA, sizes are in kb. BART-region probes are illustrated in (a) and additional probes used were the cDNA for LMP2A and the BamHI H restriction fragment (36 560–42 565). All probes were derived from B95-8 EBV and were labelled by random priming. (c) RT-PCR analysis of BART RNA comparing nuclear and cytoplasmic RNA from C666.1 cells. Total cell RNA (T) from EBV-negative BL2 cells or RNA from C666.1 NPC cells nuclear (N) or cytoplasmic (C) fractions was reverse transcribed and the cDNA subjected to PCR using primers spanning BART exons 3B–5 or 5–7 or GAPDH as a positive control. The PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide. Markers (M) are a 100 bp ladder.
Various attempts to produce RPMS1 recombinant fusion protein using a His tag or GST fusion approach gave proteins that were unstable in *E. coli* but a fusion of RPMS1 with MBP was successfully expressed in *E. coli* and purified. These proteins were used for mAb production in mice, giving rise after screening to three A73 mAbs (6-A73, 12-A73 and 16-A73) and two RPMS1 mAbs (LX172-1 and LX172-2). As a positive control to test the specificity of the antibodies, A73 and RPMS1 were expressed by *in vitro* translation in wheatgerm extracts or by transfection of expression plasmids into 293 cells. The specific detection of trans-

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**Fig. 3.** mAbs to A73 and RPMS1. (a) The selected mAbs were tested by Western blotting *in vitro*-translated A73 or RPMS1 (C, negative control translation; A-A73; R1 and R2, RPMS1). (b) A73 or RPMS1 mAbs were also tested for specificity in immunofluorescence on 293 cells transfected with A73-FLAG pcDNA3.1 or RPMS1-FLAG pcDNA3.1, respectively. The A73 panel shows cytoplasmic detection of A73 and the RPMS1 panel shows nuclear location of transfected RPMS1. The antibodies did not detect a signal in cells transfected with the empty vector (Control). (c) A73 and RPMS1 antibodies were tested on Western blots of cell extracts from many different EBV-infected cell lines, with positive controls for A73 or RPMS1 made by *in vitro* translation or transfection.
fected A73 or RPMS1 by Western blotting was demonstrated for each of these antibodies (Fig. 3a). The antibodies also worked in immunofluorescence, confirming the nuclear location of RPMS1 and cytoplasmic location of A73 when the expression cDNAs were transfected into 293 cells (Fig. 3b).

The A73 and RPMS1 mAbs were then tested by Western blotting cell extracts from many different cell lines containing EBV with various EBV-negative cell lines as controls. Positive controls for A73 and RPMS1 detection made by in vitro translation were included in the Western blots. Examples are shown in Fig. 3(c). No signal corresponding to endogenous expression of A73 or RPMS1 protein could be detected in these panels of cell lines (Fig. 3c). In some extracts, bands migrating at much higher molecular mass than the marker A73 or RPMS1 were detected but these were also present in cell lines lacking EBV and were therefore cross-reactions with cell proteins.

To investigate whether A73 or RPMS1 could be detected in archival tumour biopsy samples, EBV-positive NPC and BL cases from King Faisal Hospital and Research Centre, Riyadh, Saudi Arabia (confirmed EBER-positive by in situ hybridization) were tested with antibodies to A73 or RPMS1 but no specific signal was observed (data not shown). Similar negative results on NPC biopsies were communicated to us from a collaborating laboratory in Hong Kong to whom we had provided samples of the A73 antibodies (J. Nicholls, personal communication).

RACK1 interaction with A73

We previously found that A73 is a cytoplasmic protein, which associates with the cellular RACK1 protein (Smith et al., 2000). RACK1 was originally identified as a protein kinase C-binding protein (Ron et al., 1994), and has been shown to associate with Src (Chang et al., 1998) and integrin B protein (Liliental & Chang, 1998), but recently RACK1 protein has also been shown to play a major role in cell calcium signalling (Patterson et al., 2004). RACK1 can bind and regulate calcium release from intracellular stores by enhancing the affinity of IP3R binding for IP3 (Patterson et al., 2004). UTP (100 μM) can be used to specifically activate the IP3 receptor and we therefore tested whether A73 would be able to modify calcium signalling in transfected cells. Ionomycin is a non-specific stimulus that will open all the calcium channels within the cell and cause maximal calcium release as a positive control.

293 cells were stably transfected to produce cell lines containing A73-FLAG or the empty vector or RPMS1-FLAG (as negative controls). These cells were then subjected to a FACS analysis measuring calcium mobilization within the cell using the UV-excited dye Indo-1, which fluoresces at a different wavelength when bound to calcium compared with when it is unbound. Expression of the FLAG-tagged proteins was confirmed by Western blotting and by immunofluorescence at the time of the calcium flux assays. The results from three separate experiments were averaged and standard errors determined to give the summary shown in Fig. 4. All the cell lines tested responded equally strongly to the non-specific inducer ionomycin, but only the cells expressing A73 responded to 100 μM UTP giving an average signal of 25.9 compared with the unstimulated background of 9.4 (Fig. 4).

DISCUSSION

Several lines of evidence suggest that the promoter for the BART RNAs requires sequences downstream of the transcription start site for its activity. The upstream region showed very weak activity in reporter assays and we subsequently found that plasmids also containing 250–500 nt of the downstream sequence gave strong transcription in transfection assays from the correct transcription start point. The presence of a specific region of locally hypomethylated EBV DNA in two NPC cell lines, which have strong expression of BART RNAs, is consistent with this (Fig. 1; de Jesus et al., 2003).

Previous studies on potential proteins encoded by the BART RNAs, particularly RPMS1, A73 and BARF0, have been eclipsed in recent years by the discovery of the BART miRNAs, which convincingly occupy the previous gap in this part of the genetic map of EBV. The close correlation between BART RNA expression and BART miRNA expression makes it likely that the BART transcripts are the source of the miRNAs (Cai et al., 2006; Edwards et al., 2008) but we investigated whether the BART transcripts

![Fig. 4. Calcium flux in response to UTP stimulation of cells expressing A73. Stably transfected 293 cells expressing A73 were compared with control 293 cells, 293 cells containing the vector (V) or 293 cells expressing RPMS1. The calcium flux measured by integrated Indo-1 fluorescence relative to the unstimulated level over 500 s after either UTP or ionomycin treatment is shown. White bars are without stimulus, black bars are with ionomycin or UTP, as indicated. The means ± SD of three separate determinations are shown.](http://vir.sgmjournals.org)
also give mRNAs from which RPMS1 and A73 are translated. No evidence was found for this in the cell lines tested. We could not detect discrete sized RNAs corresponding to the BARTs in cytoplasmic poly(A)⁺ RNA from C666.1 cells on Northern blots (Fig. 2) and new mAbs, which we verified to be able to detect artificially expressed A73 and RPMS1 proteins, did not detect endogenous A73 and RPMS1 in any cell line tested (Fig. 3). Evidence for expression of the potential BARF0 protein is also lacking (Kienzle et al., 1999; Smith et al., 2000). It is possible that A73 and RPMS1 proteins might have a rapid turnover so the steady-state levels might be below the levels detectable by our antibodies but there was no indication of exceptional protein instability of the A73 or RPMS1 proteins made by transfection of expression constructs. The fact that most of the BART RNA appears to be in the nucleus of C666.1 cells (Fig. 2c) also argues against them acting as mRNAs in these cells.

However, there is some evidence that the A73 and RPMS1 ORFs might be expressed in some circumstance that we have not yet tested. Firstly, the protein sequences of the A73 and RPMS1 ORFs and most of the BART splice junctions are conserved in all the sequenced isolates of EBV. These include EBV type 2 from the AG876 cell line (Dolan et al., 2006), the GD1 strain from a Cantonese NPC patient (Zeng et al., 2005), the related non-human-primate virus strains Rhesus-LCV (Rivailler et al., 2002b) and the new world non-human primate marmoset strain of EBV known as Callitrichine LCV (Rivailler et al., 2002a).

Secondly, the clear biochemical functions identified for the A73 and RPMS1 proteins could fit well with a role in EBV biology and the diseases associated with EBV. RPMS1 was previously found to be a very effective antagonist of Notch-IC transcription activation. Notch activity affects several stages of lymphocyte development and proliferation, and is associated with differentiation of epithelial cells; RPMS1 might be expected to antagonize these processes. A recent study reported that RPMS1 enhanced anchorage-independence of growth of transfected 293 cells and produced tumours in nude mice (Li et al., 2005).

Thirdly, intracellular calcium regulation is crucial for cells and is primarily controlled through the IP3 receptor (Nishiyama et al., 2000). RACK1 has been shown to physiologically bind and regulate calcium release from intracellular stores by enhancing the affinity of IP3 receptor binding for IP3 (Patterson et al., 2004). Overexpression of RACK1 markedly increases calcium release. Depletion of RACK1 by small interfering RNA profoundly reduces and in some instances virtually abolishes calcium release. Thus, RACK1 is required for normal agonist-induced calcium release. The ability of A73 to interact with RACK1, possibly affecting Src signal transduction has been discussed previously (Smith et al., 2000). The novel effect we have found of A73 on calcium flux (Fig. 4) is most likely mediated through interaction of A73 with RACK1 and the ability of RACK1 to enhance the affinity of IP3R binding to IP3 (Patterson et al., 2004).

One of the most consistent features of NPC patients is a very strong humoral immune response to EBV lytic cycle antigens, surprising in view of the predominantly latent infection that is observed in the NPC cells (Rickinson & Kieff, 2007). Rising antibody titres have been used as a diagnostic tool for patients at risk of NPC and EBV antibodies and plasma DNA can be used to monitor treatment and relapse. Our Northern blotting of C666.1 cytoplasmic poly(A)⁺ RNA is consistent with there being a low level of early lytic cycle gene expression, perhaps in just a proportion of NPC tumour cells. It was notable that we readily detected the mRNA for BALF2 but not for some other early lytic cycle RNAs that should also be seen with the same probes (see BamHI A probe Northern blot). There thus appears to be a partial or abortive early lytic cycle pattern of gene expression in these cells, as some other investigators have reported for NPC tumour cells using other methods (Cabras et al., 2005; Feng et al., 2000; Martel-Renoir et al., 1995).

The results presented in this paper support the conclusion that the miRNAs are functionally important products of BART transcription in the cell lines studied because the A73 and RPMS1 proteins could not be detected in natural EBV infections. However, the possibility remains that A73 and RPMS1 might be expressed in some situations because of the clear potential relevance of their biochemical functions.

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