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Response to cAMP levels of the Epstein–Barr virus EBNA2-inducible LMP1 oncogene and EBNA2 inhibition of a PP1-like activity

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The expression of the Epstein–Barr virus LMP1 oncogene is regulated by viral and non-viral factors in a tissue dependent fashion. The virus encoded transcription factor EBNA2 induces its expression in human B-cells. However, this induction also requires the contribution of cellular and/or other viral factors. In nasopharyngeal carcinoma cells and in cells from Hodgkin's lymphoma, LMP1 gene transcription is independent of viral products. Here we show that the effect of a factor binding to a cAMP responsive-like element (CRE) in the LMP1 gene transcription regulatory sequence (LRS) is essential for efficient promoter activity in the DG75 B-cell line and that elevation of cAMP levels in the cells induces LRS-derived CAT activity in a CRE dependent fashion. Incubation of two EBV-immortalized B-cell lines expressing endogenous EBNA2A with 8-Br cAMP increased the levels of the latency associated 66 kDa LMP1 within 2 h. Interestingly, LMP1 expression in DG75 cells conferred resistance to the inhibitory effect of 8-Br cAMP on cell proliferation. The protein phosphatase 1 and 2A (PP1 and PP2A, respectively) inhibitor okadaic acid also stimulated LRS–CAT activity in DG75 cells. EBNA2A from an EBV-immortalized B-cell line co-immunopurified with a PP1-like protein. An EBNA2A fragment spanning residues 324–436 fused to the GST protein specifically rescued a PP1/PP2A-like component from DG75 cell extracts. This GST–EBNA2A fusion product inhibited a PP1-like activity in nuclear extracts from these cells.

Key words: cAMP/EBNA2/Epstein–Barr virus/latent membrane protein 1/protein phosphatase 1

Introduction

The accomplishment of Epstein–Barr virus (EBV) adaptation to its host environment is evident by the fact that the virus is detected in >95% of the adult human population. After primary infection, whether silent or producing the symptoms of infectious mononucleosis, the virus persists in the healthy host for life by mechanisms which are not yet fully understood. Several studies indicate that the principal carriers of the latent virus are long term-living B-cells (Gratama *et al.*, 1988; Niedobitek *et al.*, 1992, 1993). A well documented feature of the virus is its ability to immortalize resting B-cells *in vitro* (Nilsson *et al.*, 1971). The latent membrane protein 1 (LMP1) and the nuclear antigen EBNA2 are two of the viral proteins essential for this process (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989; Kaye *et al.*, 1993).

LMP1 is detected in several types of infected B-cells including *in vitro* immortalized latently infected cells (LCLs) and the phenotypically similar group III Burkitt's lymphoma (BL) cells (Rowe *et al.*, 1987) together with the full pattern of viral latent membrane proteins (LMP2A and 2B) and of latent nuclear antigens (EBNA 1–6) (reviewed in Sinclair and Farrell, 1992). LMP1 is also present in the majority of undifferentiated nasopharyngeal carcinomas (Fåhraeus *et al.*, 1988; Young *et al.*, 1988) and in cells from Hodgkin's lymphoma (Pallesen *et al.*, 1991; Deacon *et al.*, 1993) in which the only EBV nuclear antigen detected is EBNA1. LMP1 has cell transforming capacity in rodent fibroblasts (Wang *et al.*, 1985; Baichwal and Sugden, 1988, 1989) and in epithelial cell lines where it affects differentiation and expression of morpho-regulatory molecules (Dawson *et al.*, 1990; Fåhraeus *et al.*, 1990b, 1992). The expression of LMP1 in EBV-negative B-cell lines has been associated with up-regulation of vimentin, adhesion molecules, activation markers (Birkenbach *et al.*, 1989; Wang *et al.*, 1988, 1990; Peng and Lundgren, 1992) and the NFκB transcription factor (Hammarskjöld and Simurda, 1992). Another interesting finding is the association of LMP1 with the expression of *bcl-2* and with the inhibition of apoptosis in B-cells (Henderson *et al.*, 1991).

The type A strains of EBV are significatively more efficient than the type B EBV strains in their ability to immortalize B-lymphocytes (Rickinson *et al.*, 1987). EBNA2 is a key determinant for this difference (Cohen *et al.*, 1989). EBNA2 is a pleiotropic transcription activator involved in the induction of cellular and EBV proteins, including LMP1, in B-cells (reviewed in Sinclair and Farrell, 1992; Sinclair *et al.*, 1994). The molecular features of the EBNA2 activity are beginning to be understood. The acidic domain near the C-terminus of the protein is interchangeable with the transcription stimulatory acidic domain of VP16 (Cohen, 1992). Deletion and linker insertion mutations of type A EBNA2 (EBNA2A) suggest that other parts of this 487 amino acid long protein are also important in controlling transcription and promoting B-cell transformation (Cohen *et al.*, 1991). An EBNA2 fragment spanning positions 252–425 has been shown to bind to the recombination signal binding protein J*k* (RBPJ*k*) which targets the association of EBNA2A with the core sequence CNGTGGGAA of the EBV C-promoter (Ling *et al.*, 1993; Henkel *et al.*, 1994). This DNA

sequence also mediates EBNA2 responsiveness in the EBV LMP2A promoter (Zimber-Strobl *et al.*, 1993) and in the CD23 cellular promoter (Ling *et al.*, 1994). An EBNA2 fragment including positions 243–336 is also capable of binding RBPJ*k* (Grossman *et al.*, 1994).

The 60–66 kDa LMP1 is translated from a transcript initiated at the ED-L1 promoter (Hudson *et al.*, 1985). A DNA fragment spanning residues +40 to −634 relative to the ED-L1 promoter transcription initiation site has been defined as the LMP1 transcription regulatory sequence (LRS) (Fåhraeus *et al.* 1990a). Coupling of this fragment to the chloramphenicol acetyltransferase (CAT) reporter gene mediates EBNA2A stimulated transcription in B-cells and EBNA2A independent expression in epithelial cell lines, resembling the regulation of LMP1 expression *in vivo* (Fåhraeus *et al.*, 1993). A complex set of positive and negative *cis*-acting elements determines transcription from this promoter. Weak binding of the EBNA2A 252–425 fragment to an LRS oligonucleotide containing the CNGTGGGAA sequence has been detected *in vitro* (Ling *et al.*, 1994). This sequence is located between positions −215 and −223 of the LRS in the opposite orientation to that in other EBNA2 responsive promoters. However, an EBNA2 effect can also be detected in other regions of the LRS (Fåhraeus *et al.*, 1990a; Tsang *et al.*, 1991; A.Sjöblum, A.Jansson, T.Nilsson, S.Laín and L.Rymo, submitted).

EBNA2 and EBNA5 are the first EBV products detected after infection of human resting B-cells and they cooperate to cause G_0 to G_1 transition (Sinclair *et al.*, 1994). Nevertheless, LMP1 expression is delayed many hours until cells reach the G_1/S boundary (Allday and Farrell, 1994 and references therein). Absence of LMP1 expression in EBNA2-positive cells has been reported on several other occasions (Walls *et al.*, 1989; Azim *et al.*, 1990; Cordier *et al.*, 1990). These observations show that a permissive cellular environment is necessary to detect LMP1 expression. Interestingly, a peak in cellular cAMP levels during mid or late G_1 phase of the cell cycle is a general phenomenon (Friedman, 1982).

The CRE is a binding site for homo- and heterodimers of the CREB/ATF family of transcription factors. The activity of some of these factors has been shown to respond to the stimulation of the cAMP signalling pathway through their phosphorylation by cAMP-dependent protein kinase (PKA) (reviewed in Meyer and Habener, 1993). Serine/threonine protein phosphatases are crucial components of cellular signal transduction pathways (reviewed in Mumby and Walter, 1993). The PKA-phosphorylated Ser133 necessary for the activity of the CRE binding factor CREB is a substrate for PP1 and PP2A *in vitro* (Hagiwara *et al.*, 1992; Wadzinski *et al.*, 1993). However, microinjection of PP1, but not PP2A, blocks CRE regulated gene expression (Hagiwara *et al.*, 1992; Alberts *et al.*, 1993). Furthermore, it has also been demonstrated that the activation of CREB by PKA is maintained by specific inhibition of PP1-like activity (Hagiwara *et al.*, 1992; Alberts *et al.*, 1994). Full activation of a nuclear species of PP1 requires phosphorylation by PKA (Van Eynde *et al.*, 1994) indicating that this nuclear PP1 activity is induced by an increase in cellular cAMP levels. PP1 and PP2A are also involved in regulating cell growth and division, the function of the tumour supressor gene product RB and the activity of RNA polymerases (reviewed in Mumby and Walter, 1993; Walter and Mumby, 1993). The role of PP2A in cell transformation by small DNA tumor viruses has been also described (Kleinberger and Shenk, 1993; Walter and Mumby, 1993).

Here we aimed to reveal the significance of a CRE-like sequence in LRS and to study the effect of the cAMP signalling pathway and of protein phosphatases on LMP1 expression. We also show an association between EBNA2A and PP1 and the inhibition of a PP1-like activity from B-cell nuclear extracts by an EBNA2A protein fragment. These results permit the assignment of a biochemical function to EBNA2A indicating the existence of a relationship between the EBNA2A effect in B-cells and the cAMP signal transduction pathway and constitute, to our knowledge, the first evidence for the inhibition of a PP1-like nuclear activity by a viral protein.

Results

Analysis of LRS constitutive activity

Figure 1A represents schematically the positive and negative *cis*-acting elements in the LRS defined by transfecting the Burkitt's lymphoma group I, EBV-negative DG75 B-cells with plasmids containing different fragments of LRS coupled to the CAT reporter gene and with the EBNA2A expression vector pEΔA6 (Fåhraeus *et al.*, 1990a, 1993; A.Sjöblom *et al.*, submitted). The region near the transcription initiation site contains a putative TATA-box and a cAMP responsive-like element (CRE) (Ghosh and Kieff, 1990; Karin and Smeal, 1992). Between these two putative *cis*-acting elements, a GC sequence matching the consensus for the ubiquitous transcription factor Sp1 binding site was noticed. Further analysis of this region revealed that a construct containing the DNA fragment spanning from nucleotides +40 to −35 relative to the transcription initiation site [pgLRS(−35)CAT], and therefore excluding the putative Sp1 binding site and the CRE, is barely active in DG75 B-cells (Figure 1B). A similar behaviour was observed with a construct only disrupting the DNA fragment containing the CRE-like sequence [pgLRS(−41)CAT]. As previously described for B-cells and epithelial cells (Fåhraeus *et al.*, 1990a, 1993), the pgLRS(−54)CAT construct is clearly active in DG75 cells in the absence of EBNA2A, accounting for the so-called constitutive activity of LRS. pgLRS(−54)CAT includes the Sp1 and the CRE-like motifs. The activity of this construct did not change when it was co-transfected with an Sp1 expression vector (kindly provided by R.Tjian) into the Sp1-negative Schneider cells (data not shown). These results demonstrate that the inclusion of the LRS fragment containing the putative CRE is important for LRS constitutive activity in DG75 cells. Inclusion of further upstream sequences [pgLRS(−106)CAT] inhibits the constitutive activity in B-cells and in epithelial cells (Fåhraeus *et al.*, 1990a, 1993). In B-cells, EBNA2 expression overrides the effect of the negative elements (Fåhraeus *et al.*, 1990a, 1993). Here we report that a negative effect on constitutive LRS-derived CAT activity is observed with an LRS construct spanning nucleotides +40 to −72 [pgLRS(−72)CAT].

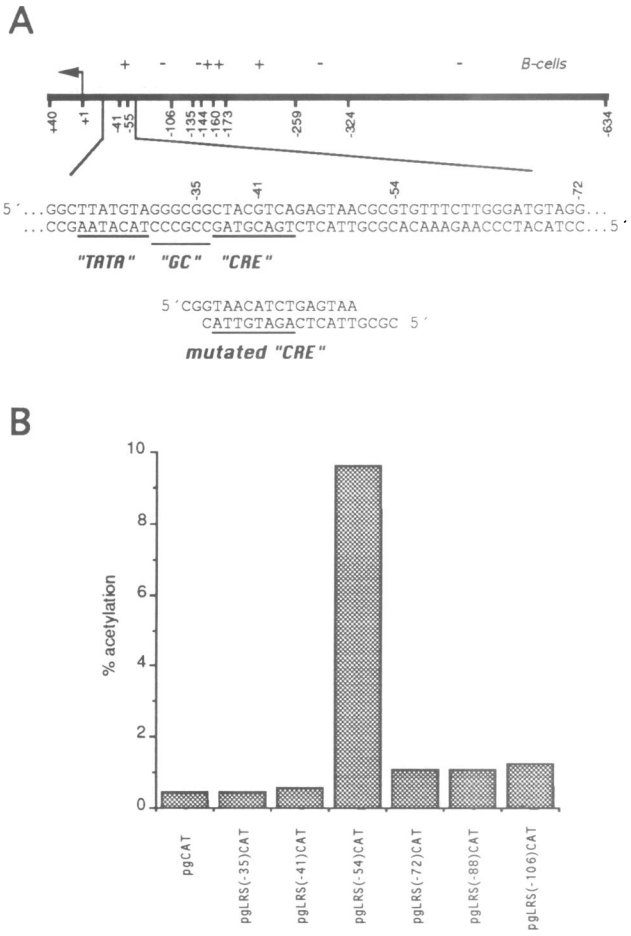

Fig. 1. (**A**) Schematic representation of the LMP1 regulatory sequence (LRS) showing the *cis*-elements acting positively and negatively in B-cells in the presence of EBNA2A (Fåhraeus *et al.*, 1993; A.Sjöblom *et al.*, submitted). The sequence of the region spanning nucleotides −21 to −72 relative to the transcription initiation site (+1) of the ED-L1 promoter of the B95-8 EBV strain is specified. This region includes the putative TATA-box, GC-box and CRE. The sequence of the oligonucleotide pair used to introduce the mutation in the CRE is written below. (**B**) Constitutive LRS-derived CAT activity in DG75 cells transfected with the indicated LRS–CAT constructs.

Participation of the putative CRE in the transcriptional activity of LRS

Three LRS–CAT constructs were chosen in order to study the role of the CRE-like sequence on the transcriptional regulation of the LMP1 gene. (i) pgLRS(−55)CAT is, apart from pgLRS(−54)CAT, the shortest construct including the putative CRE. This construct behaves like pgLRS(−54)CAT but is technically more adequate for introducing a mutation in the CRE. (ii) pgLRS(−259)CAT construct gives the highest LRS–CAT-derived activity in DG75 cells in the presence of EBNA2A (A.Sjöblom *et al.*, submitted). (iii) pgLRS(−634)CAT includes the complete LRS (Fåhraeus *et al.*, 1990a).

As shown in Figure 2A, mutation of the CRE-like sequence in the LRS (see Figure 1A) reduced the activity of the three LRS–CAT constructs in DG75 cells in the absence and in the presence of EBNA2A demonstrating that the intact CRE-like sequence is important for LRS promoter activity in these cells.

Co-transfection of DG75 cells with a plasmid encoding the CREB antagonist CREMβ (Foulkes *et al.*, 1991) also

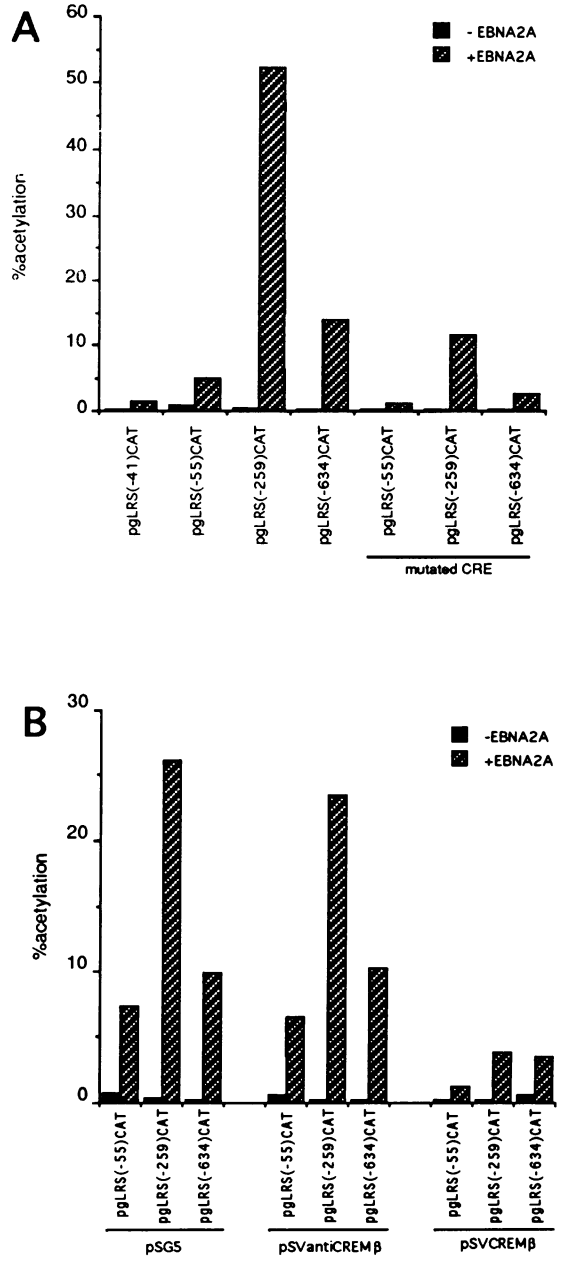

Fig. 2. (**A**) Effect of the CRE mutation (see Figure 1A) on the activity of three LRS–CAT constructs in DG75 together with the EBNA2A expression vector or with the corresponding control plasmid.
(**B**) Inhibiton of reporter activity by co-transfection of DG75 cells with LRS–CAT constructs together with 5 μg of the CREMβ expression vector (pSVCREMβ) in the presence or absence of EBNA2A. The same amount in moles of plasmid pSG5 or pSVantiCREMβ was used as control for CREMβ expression.

caused a significant reduction of LRS–CAT activity derived from any of the three constructs tested both in the presence and in the absence of EBNA2A (Figure 2B). The result shows that a factor described as binding specifically to the CRE affects LRS promoter activity.

8-Br cAMP induction of LRS–CAT activity

In order to investigate the response of the LMP1 ED-L1 promoter activity to increased cAMP levels, LRS–CAT transfected cells were incubated with the cell membrane

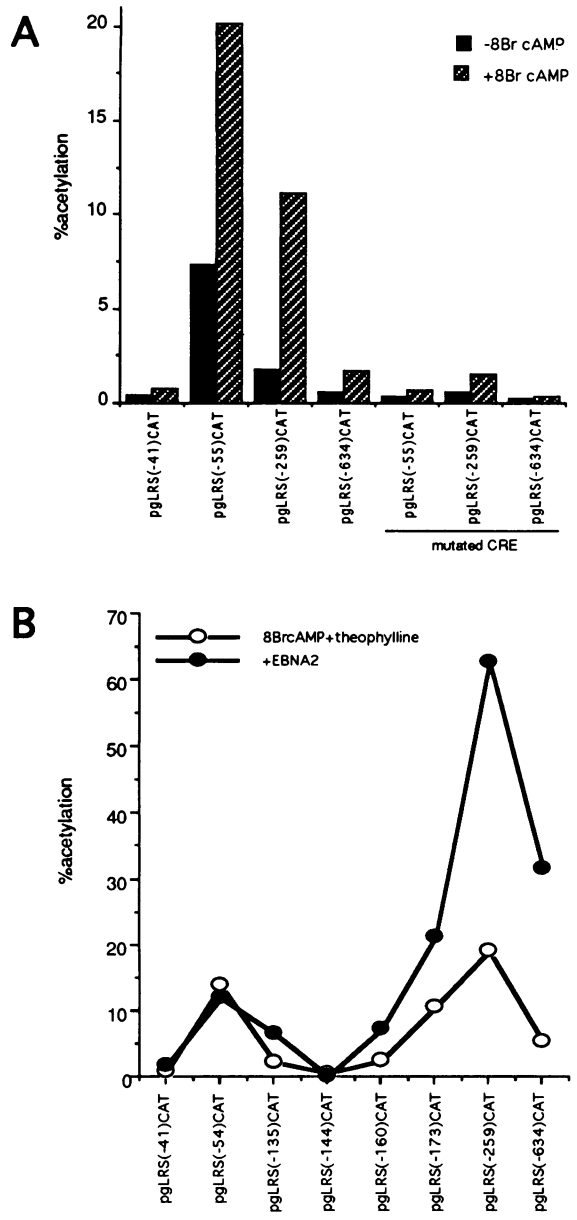

Fig. 3. (**A**) Activity of LRS−CAT constructs in DG75 cells incubated with 8-Br cAMP. (**B**) Comparison of the LRS−CAT activity patterns obtained by co-transfection with pEΔA6 and by induction with 8-Br cAMP and theophylline.

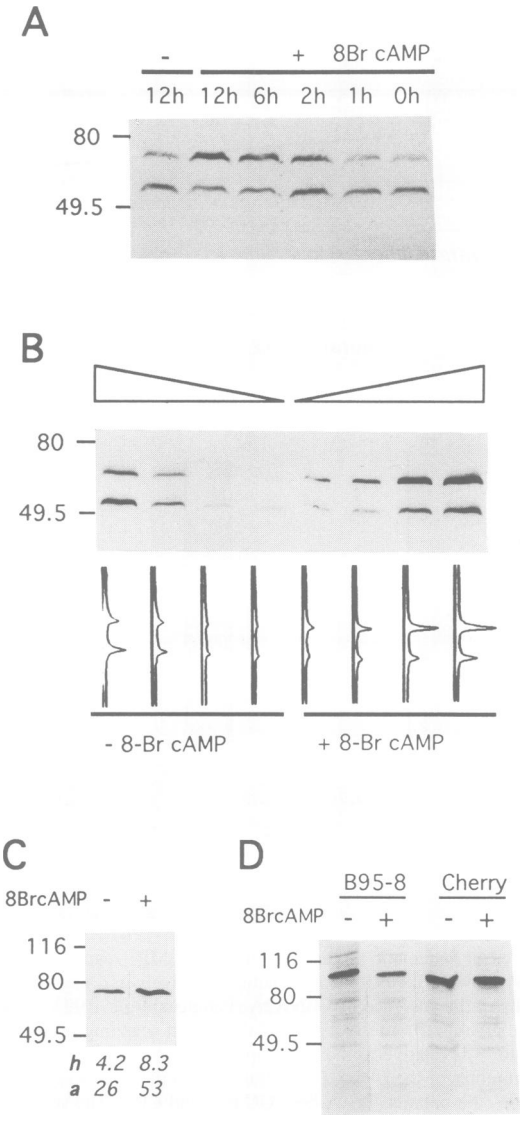

Fig. 4. (**A**) Time course analysis of LMP1 expression in B95-8 cells after incubation with or without 4 mM 8-Br cAMP. Whole cell extracts corresponding to 0.75×10^6 cells were subjected to immunoblot analysis with a mixture of monoclonal antibodies raised against LMP1. (**B**) Increasing amounts of extract from cells incubated for 6 h with or without 8-Br cAMP were loaded onto the gel. The intensity of the bands obtained was quantified as described in Materials and methods. (**C**) Effect on LMP1 expression in Cherry cells after 6 h of incubation with 4 mM 8-Br cAMP. The height (h) and area (a) assigned to these bands by densitometric analysis is specified below. (**D**) Immunoblot analysis of the EBNA2 protein in B95-8 and Cherry cells before and after incubation with 4 mM 8-Br cAMP using the PE2 mAb. The electrophoretic mobilities of the molecular weight markers are specified on the left side of each panel.

permeable cAMP analogue, 8-Br cAMP. Figure 3A shows the effect of 8-Br cAMP on the activity derived from the different LRS−CAT constructs containing an intact or a mutated CRE-like sequence in DG75 cells. The results demonstrate that LRS-derived CAT activity is induced by an increase in the cAMP levels in the absence of EBNA2 and that the effect of 8-Br cAMP depends on the CRE-like sequence in the LRS. When the cAMP phosphodiesterase inhibitor theophylline was added together with 8-Br cAMP, LRS−CAT activity was further increased and the relative activity of the different constructs was maintained. Induction by cAMP and EBNA2A produced qualitatively similar patterns of CAT activity in DG75 cells transfected with a larger series of LRS−CAT constructs (Figure 3B), indicating that stimulation of the cAMP signalling pathway could, to some extent, overlap with the EBNA2A strategy.

Nevertheless, in our conditions EBNA2A was clearly more efficient than 8-Br cAMP (and theophylline) in inducing the longer LRS−CAT constructs which include the potent negative *cis*-acting elements.

8-Br cAMP induction of LMP1 expression in EBNA2A-positive B-cells

In order to study the effect of cAMP on endogenous LMP1 expression, EBV-positive cell lines were treated with 8-Br cAMP for different time intervals. Figure 4A shows the induction of the 66 kDa LMP1 in B95-8 cells

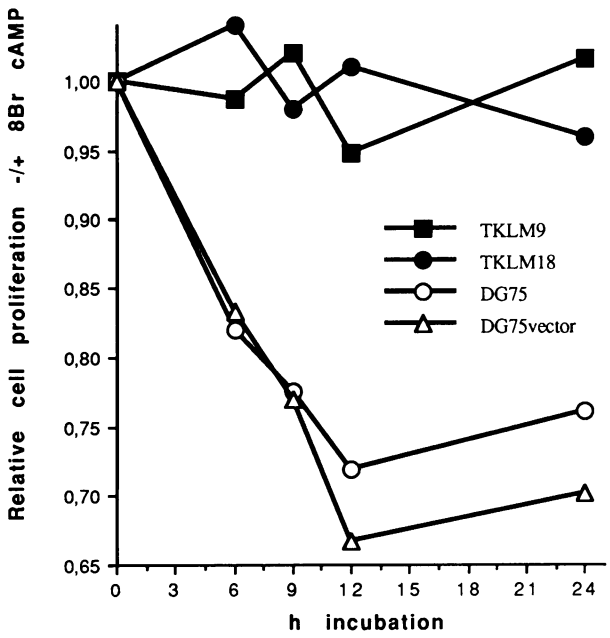

Fig. 5. Effect of 4 mM 8-Br cAMP on proliferation of DG75 cells, two lines of DG75 cells stably transfected with an LMP1 expression vector (TKLM9 and TKLM18) and one DG75 cell line stably transfected with a control plasmid (DG75 vector).

detected by immunoblotting. An increase in the intensity of the band corresponding to this protein could be observed within 2 h of treatment. After 6 h, the treated cells contained approximately two to three times the amount of LMP1 in non-induced cells (Figure 4B). The level of the 55 kDa LMP1, probably derived from the downstream ED-L1A promoter and associated with the viral lytic cycle (Hudson *et al.*, 1985), was not affected by the 8-Br cAMP treatment. A similar induction of the 66 kDa LMP1 was observed in Cherry cells, in which a faster migrating LMP1 is not detected (Figure 4C). It is noteworthy that these two B-cell lines express EBNA2A and that the amounts of this antigen were not increased by the addition of 8-Br cAMP (Figure 4D).

Protection from the inhibitory effect of 8-Br cAMP on cell proliferation by LMP1 expression

In the previous experiments we observed that treatment of DG75 cells with 8-Br cAMP markedly inhibited their proliferation, although unlike the okadaic acid treatment (see below), it did not kill the cells as determined by trypan blue exclusion. This inhibition of cell proliferation by cAMP was not noticeable with the EBV-positive LCLs B95-8 and Cherry (data not shown). In order to test if the resistance to inhibition of cell growth could be related to the expression of LMP1, two exponentially growing DG75 cell lines stably expressing LMP1 (Cuomo *et al.*, 1990) were exposed to the same amount of 8-Br cAMP. Interestingly, the LMP1-expressing DG75 cells were able to grow in the presence of the cAMP analogue as rapidly as in its absence (Figure 5). The proliferation of DG75 cells and DG75 cells transfected with a control vector (Cuomo *et al.*, 1990) was clearly diminished by this treatment. This shows that LMP1 expression in DG75 cells counteracts the inhibitory effect of increased levels of cAMP on cell growth.

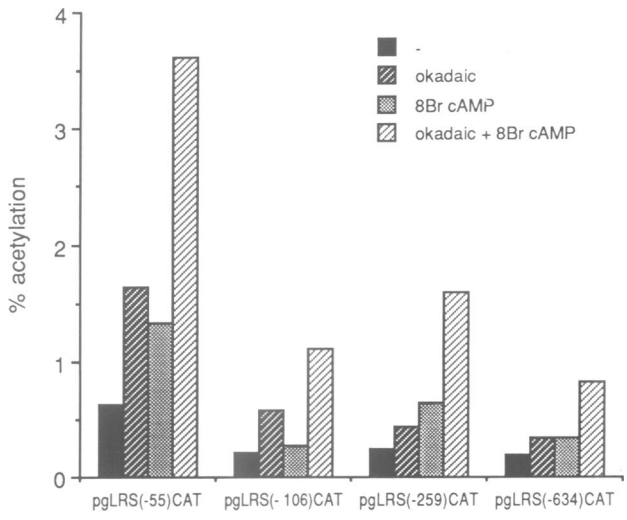

Fig. 6. CAT enzymatic activity in DG75 cells transfected with different LRS–CAT constructs and incubated with okadaic acid and/or 8-Br cAMP for 16 h.

Okadaic acid induction of LRS–CAT activity

Serine/threonine protein phosphatase activities play an important role in the cAMP signalling pathway and affect the phosphorylation status of factors involved in transcription (Hagiwara *et al.*,1992; Wadzinski *et al.*,1993; Mumby and Walter, 1993). In order to test the possible role of these phosphatases in regulating the LMP1 ED-L1 promoter, LRS–CAT transfected DG75 cells were treated with the PP1 and PP2A specific inhibitor okadaic acid (reviewed in Holmes and Boland, 1993). The inhibitory effect of okadaic acid on cell viability (Hagiwara *et al.*, 1992) made it necessary to shorten the incubation time after transfection. Nevertheless, a significant increase of LRS-derived CAT activity could be repeatedly observed (Figure 6). The stimulation of LRS-derived CAT activity was larger when cells were also treated with 8-Br cAMP, but even in these conditions, the longer constructs were not as efficiently induced as with co-transfection with the EBNA2A expression plasmid. Okadaic acid did not induce the activity of constructs carrying a mutated CRE (not shown). These results show that inhibition of the serine/threonine protein phosphatases PP1 and/or PP2A induces LRS-derived CAT activity.

EBNA2A association with protein phosphatase 1

Previous immunoaffinity purification experiments have shown that EBNA2A is associated with a protein of ~31 kDa (Dillner *et al.*, 1988) and with an enzymatic activity postulated to be related to a serine/threonine protein phosphatase activity (Randahl *et al.*, 1992). The molecular weight of the catalytic subunits of PP1 and PP2A have been described as ranging between 33 and 37.5 kDa (Cohen, 1989). We tested the possibility of an association between EBNA2A and the catalytic subunit of one of the protein phosphatases inhibited by nanomolar concentrations of okadaic acid. Whole cell extracts from the EBNA2A-expressing LCL Cherry and the EBV-negative B-cell line DG75 were fractionated by immunoaffinity chromatography using the 5B6 mAb against an 18 amino acid peptide of the C-terminal domain of EBNA2A by the method described by Dillner *et al.* (1988).

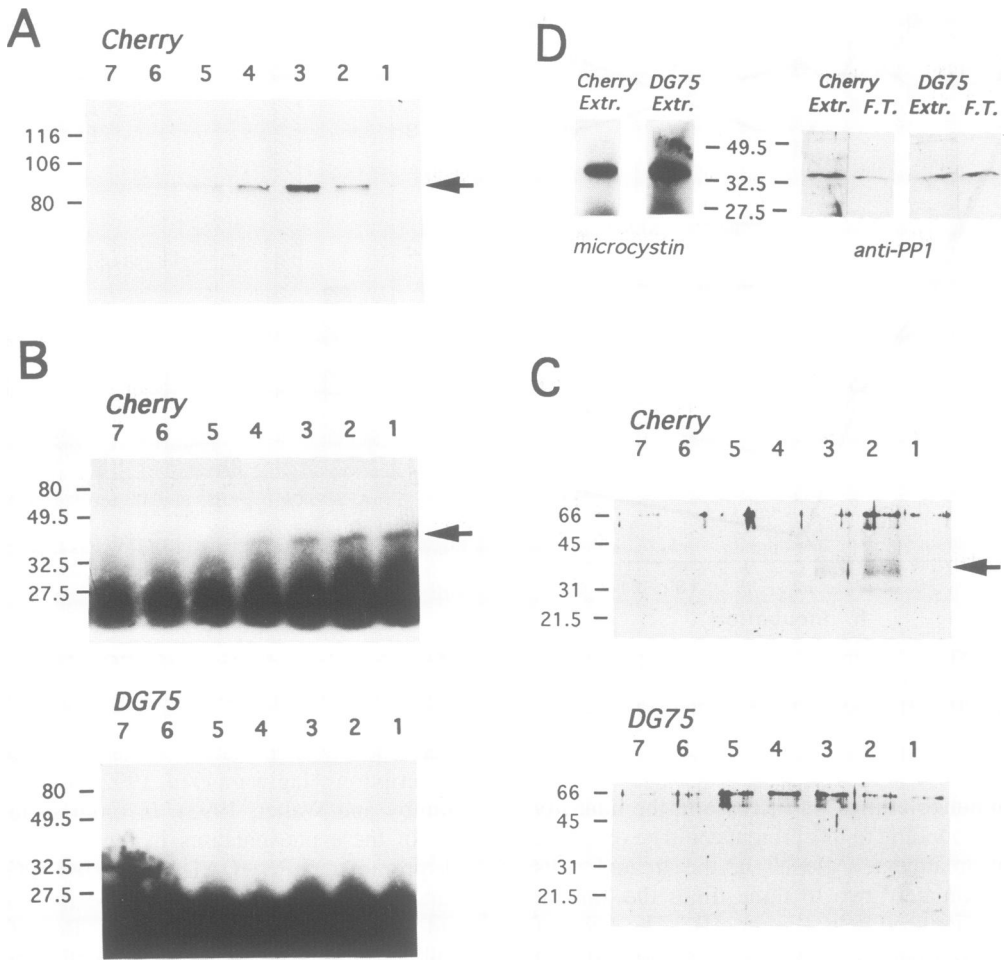

Fig. 7. Whole cell extracts from Cherry cells and DG75 cells were subjected to immunoaffinity chromatography purification using the EBNA2A mAb 5B6. Fractions were analysed by immunoblotting using the anti-EBNA2 mAb PE2 (**A**), labelled with [^{125}I]microcystin-YR (**B**) or immunoblotted using the PP1 specific polyclonal antibodies (**C**). (**D**) [^{125}I]microcystin-YR labelling of Cherry and DG75 whole cell extracts (Extr.) and anti-PP1 immunoblot of Cherry and DG75 whole cell extracts (Extr.) and flow-through fractions from immunoaffinitty columns (F.T.). The electrophoretic mobilities of the molecular weight markers are specified beside each panel and relevant bands are marked with arrows.

Another EBNA2A specific mAb, PE2, was used to identify the presence of EBNA2A in fractions derived from Cherry cell extracts by immunoblotting (Figure 7A). Using this technique, EBNA2A does not co-immunopurify with any other known EBV antigen (Randahl *et al.* 1992). An aliquot of each fraction was exposed to [^{125}I]microcystin-YR which specifically interacts with the catalytic subunits of PP1 and PP2A (Holmes and Boland, 1993). By this procedure it was possible to detect a [^{125}I]microcystin-YR labelled component of ~35 kDa in fractions derived from Cherry cells (Figure 7B). Although repeatedly tested, this band could not be detected in fractions derived from the EBV-negative B-cell line DG75 although the original amount of [^{125}I]microcystin-YR labelled protein was similar in Cherry and DG75 whole cell extracts (Figure 7D).

Furthermore, a fragment spanning positions 324–436 of the B95-8-derived EBNA2A sequence fused to the glutathione *S*-transferase (GST) protein specifically interacted with a component in DG75 whole cell extracts which is strongly labelled with [^{125}I]microcystin-YR and has an electrophoretic mobility similar to that of the PP1 and PP2A catalytic subunits (Figure 8). Notably, the [^{125}I]microcystin-YR labelled 35 kDa band was not detected using the GST protein or a GST fusion product that includes amino acids 383–487 of EBNA2A. The results obtained using these two regions of the EBNA2A sequence (324–436 and 383–387) are shown since it is known that deletions within them severely impair virus mediated B-cell transformation and LMP1 up-regulation (Cohen *et al.*, 1991) as well as LRS-derived CAT activity in B-cells (L.Rymo, unpublished results). No efficient binding to a microcystin-YR labelled component was detected using GSTΔEBNA2 fusion proteins not containing the EBNA2A 324–436 fragment.

Given that microcystin is described as interacting with either PP1 or PP2A (reviewed in Holmes and Boland, 1993) we used commercially available antibodies specifically recognizing each of these protein phosphatases to analyse the fractions obtained by immunoaffinity chromatography as described above. A band with similar electrophoretic mobility to the one detected with [^{125}I]microcystin-YR reacted with PP1 specific antibodies in the fractions derived from Cherry cells (Figure 7C). This band was diffuse, probably due to the concentration of the samples by precipitation needed to perform the Western analysis. Again, this band could not be detected in equivalent fractions derived from EBV-negative DG75

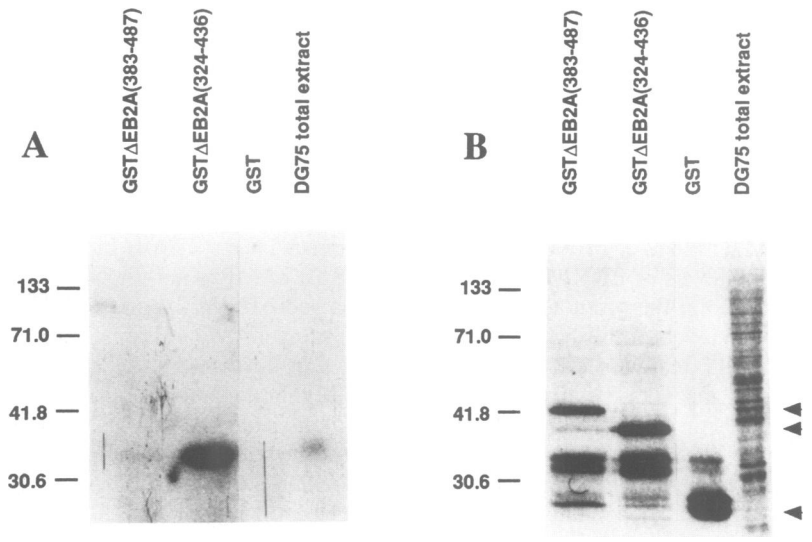

Fig. 8. (**A**) SDS–PAGE analysis of the [^{125}I]microcystin-YR labelled proteins after incubation of GST, the GSTΔEB2(324–436) or the GSTΔEB2(383–487) fusion proteins purified from bacteria with DG75 total cell extracts. (**B**) SDS–PAGE and Coomassie staining of the same samples. The bands corresponding to GST and the GSTΔEB2A fusion proteins are marked with arrows. The electrophoretic mobilities of the molecular weight markers are specified beside each panel.

cells even though Cherry and DG75 whole cell extracts contained comparable amounts of protein reacting with anti-PP1 (Figure 7D). A reduction of the amount of the 35 kDa band detected by immunoblotting with the PP1 specific polyclonal antibodies was observed in the flow-through fraction obtained after affinity chromatography of the Cherry whole cell extracts on the 5B6 mAb column. This reduction was not observed with DG75 whole cell extracts (Figure 7D).

Inhibition of a PP1-like activity from DG75 nuclear extracts

The stimulation of LRS-derived CAT activity by okadaic acid indicated that it is possible that, at least to some extent, EBNA2A mediates its activity on LRS by inhibiting either PP1 or PP2A-like activities presumably located in the nucleus. PP1 and PP2A are the only protein phosphatases which efficiently dephosphorylate phosphorylase-a in the absence of divalent cations (MacKintosh, 1993). Using this substrate and the GST–EBNA2 fusion constructs tested above we showed that, in agreement with the results obtained by microcystin-YR labelling, the GSTΔEB2A-(324–436) fusion product was able to inhibit a PP1- or PP2A-like activity in DG75 nuclear extracts (Figure 9). PP2A is described to be completely inhibited by 1–2 nM okadaic acid. Therefore it can be assumed that the phosphorylase-a protein phosphatase activity detected in the DG75 nuclear extracts in the presence of 2 nM okadaic acid is mainly due to the PP1-like activity in the DG75 nuclear extracts. Accordingly, this remaining activity could be largely inhibited by the 46 amino acid PKA phosphorylated fragment of PP1-specific inhibitor-1 peptide (kind gift from Dr C.Holmes). This PP1-like activity was tested in the presence of GST, the GSTΔEB2A(383–487) or the GSTΔEB2A(324–436) fusion product. Only the product including amino acids 324–436 of EBNA2A was capable of inhibiting the PP1-like activity in DG75 nuclear extracts.

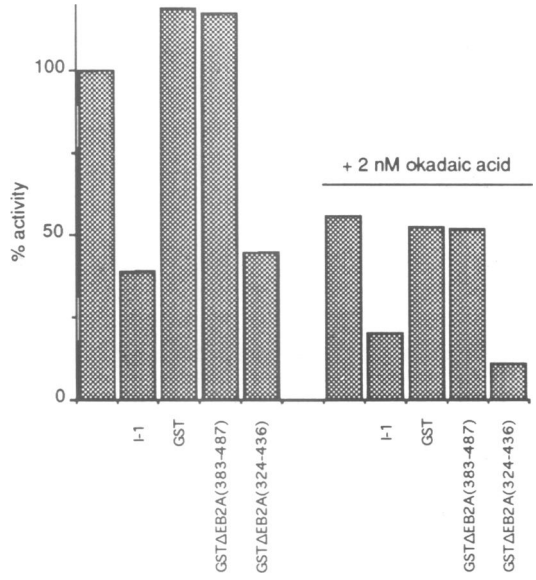

Fig. 9. Protein phosphatase activity on phosphorylase-a in the absence of divalent cations of DG75 nuclear extracts incubated with the 46 amino acid inhibtor-1 peptide (I-1), the GST protein, the GSTΔEB2(383–487) or the GSTΔEB2(324–436) fusion proteins, in the absence and in the presence of 2 nM okadaic acid.

Discussion

We have established the importance of a cAMP responsive-like element for LRS-derived CAT activity by showing that mutation of this element severely impairs the promoter activity in the DG75 B-cell model system. We also present evidence showing that the effect of this CRE responds to cAMP levels. The expression of a factor described to bind specifically to cAMP responsive elements (CREMβ) impairs LRS-derived CAT activity. This inhibition can be observed within the smallest LRS deletion construct containing the CRE. Treatment of cells with 8-Br cAMP

(and theophylline) raises LRS-derived CAT activity and an intact CRE in LRS is necessary for this effect.

The location of the CRE around position −41 in the LRS and the high turnover of the LMP1 gene product (Baichwal and Sugden, 1987; Mann and Thorley-Lawson, 1987) correlate well with the properties of other cAMP regulated genes (Roesler *et al.*, 1988). The increase of the LMP1 levels in the two EBV immortalized lymphoblastoid cell lines B95-8 and Cherry within 2 h of 8-Br cAMP induction without affecting the levels of EBNA2 supports the idea that the effect of cAMP on the promoter activity is not due to an increment on the levels of this transcription factor. Notably, the 8-Br cAMP treatment only induced the 66 kDa LMP1 protein in the B95-8 cells with no detectable effect on the expression of the 55 kDa band. The smaller size B95-8 LMP1 is probably derived from a transcript initiated from the ED-L1A promoter and has been associated with the viral lytic cycle (Hudson *et al.*, 1985). The different regulation of these two variants of LMP1, also observed by Contreras-Salazar *et al.* (1990), is indicative of their separate functions in the viral strategy. Although EBNA2 induces the expression of LMP1 in B-cells, there are several reports demonstrating EBNA2 independent induction of LMP1 in this cell type (Contreras-Salazar *et al.*, 1990; Rowe *et al.*, 1992; Cordier-Bussat *et al.*, 1993). These observations suggest that other cellular and/or viral factors play a crucial role in LMP1 gene regulation in B-cells. Another EBV factor, EBNA6, is involved in maintaining LMP1 expression at high levels in G_1-arrested Raji B-cells (Allday *et al.*, 1993; Allday and Farrell, 1994). In our conditions, treatment of the EBNA2-negative B-cell line Akata and of the EBNA6-positive, EBNA2-negative B-cell lines P3HR1 and Daudi with 8-Br cAMP (and theophylline) did not stimulate expression of either form of LMP1. Accordingly, a relatively low effect of 8-Br cAMP (and theophylline) is observed in DG75 cells transfected with the complete LRS−CAT construct containing the potent *cis*-acting negative elements in LRS as compared with the effect of co-transfecting with the EBNA2A expression plasmid. A solitary increase in cAMP levels has been described to be relatively ineffective in stimulating CRE-dependent transcription in B-cells, possibly as a result of rapid degradation of cAMP or abundant phosphatase activity against CRE binding factors (Xie *et al.*, 1993). Also, efficient transcription from complete promoters is likely to require the cooperation of factors that respond to different signals and mediate their effect through different DNA sequences. The CRE-containing human interferon β promoter is an illustrative example of such a combinatorial mechanism (Maniatis *et al.*, 1992). With these results, we conclude that the elevation of cAMP levels in B-cells increases endogenous LMP1 expression although the effect of other factors in LCLs or at least of EBNA2 is necessary in order to detect this induction.

As previously described for other transformed B-cells (reviewed in Friedman, 1982), an increase of cAMP levels in DG75 cells reduced their rate of proliferation. However, we noticed that EBV-transformed B-cells (LCLs) were resistant to this inhibitory effect. Comparison between the effect of 8-Br cAMP on DG75 cells, on a DG75 cell line stably carrying a control plasmid and on two DG75 cell lines stably expressing LMP1 showed that the expression of this oncogene confers resistance to the inhibition of proliferation by 8-Br cAMP. The relevance of these observations *in vivo* is an interesting question due to the importance of the cAMP mediated signals pathway in the differentiation and survival of B-cells (Knox *et al.*, 1993; Newell *et al.*, 1993). cAMP levels seem to be involved also in the maintenance of the virus latent stage opposing the effects of ligation of sIg or treatment with TPA (Daibata *et al.*, 1990). Induction of LRS−CAT activity by 8-Br cAMP is not impaired in LMP1-expressing DG75 cells (not shown), suggesting that the effect of LMP1 in inhibiting cAMP response on cell growth is not within the transduction of the signal. Rather, LMP1 acts by modifying the activity of other factors participating in the regulation of cell growth.

Another set of results described in this work regards the association between EBNA2A and PP1 and the inhibitory effect conferred by the EBNA2A 324–436 fragment on a PP1-like activity in DG75 nuclear extracts. An EBNA2A associated component binding to the PP1/PP2A specific inhibitor microcystin-YR and reacting with anti-PP1 antibodies was detected in the EBV-transformed Cherry B-cell line. The specificity of this association is sustained by the lack of this component in samples derived from the EBNA2-negative cell line DG75 while the initial levels in the extracts from Cherry and DG75 cells are similar. A PP1/PP2A-like component could be specifically rescued from DG75 cell extracts using the GSTΔEB2A(324–436) fusion product. Furthermore, a PP1/PP2A-like activity from DG75 nuclear extracts was specifically inhibited by the GSTΔEB2A(324–436) fusion product. This enzymatic activity was shown to correspond to a PP1-like protein due to its resistance to 2 nM okadaic acid and to its inhibition by the inhibitor-1 peptide. These results strongly indicate that the [^{125}I]microcystin-YR component bound by the GSTΔEB2A(324–436) fusion product and associated with the Cherry EBNA2A actually corresponds to a PP1-like protein as suggested by Western analysis. Unlike the PKA phosphorylated inhibitor-1 peptide, the 324–436 EBNA2A fragment did not inhibit partially purified PP1 from rabbit skeletal muscle (not shown) indicating the requirement for modification of the EBNA2A polypeptide or the presence of a targeting factor in the DG75 nuclear extracts. The description of PP1 regulatory factors and of factors that target PP1 to specific complexes is proving to be important in understanding this protein phosphatase function (reviewed in Hubbard and Cohen, 1993)

The B95-8 EBNA2A 324–436 region overlaps with the EBNA2 fragments reported to interact with RBPJκ (Ling *et al.*, 1993, 1994; Grossman *et al.*, 1994). However, the amino acid sequence motif around positions 323–324 of EBNA2A, essential for RBPJκ binding, is disrupted in the GSTΔEB2A(324–436) construct. This fragment contains the sequence equivalent to the 333–425 fragment of the W91 strain EBNA2 whose deletion abolishes virus mediated B-cell transformation and LMP1 up-regulation (Cohen *et al.*, 1991). Deletion of fragments within positions 324 and 436 of the B95-8 EBNA2A sequence also impairs its capability to induce LRS-derived CAT activity in B-cells (L.Rymo, unpublished results). The 122–344 fragment involved in EBNA2A oligomerization (Tsui and Schubach, 1994) also overlaps with a small segment of

the EBNA2A fragment that confers inhibition of the nuclear PP1-like activity.

Together with the transcription activation capacity of the acidic C-terminal domain of EBNA2 (Cohen, 1992), the interaction of EBNA2 with PP1 presented here might play an important role in EBNA2's effect on LMP1 gene transcription. The activation of LRS-derived CAT activity by okadaic acid demonstrates the involvement of a PP1 and/or PP2A protein phosphatase-like activity in the inhibition of expression from this promoter. Therefore, it is tempting to speculate that one of EBNA2A's functions in promoting transcription from LRS is to diminish the effect of a PP1-like activity on a transcription factor. PP1 activity is an important regulator of CREB and inhibition of PP1 activity ensures the maintenance of its transcription activating capacity (Hagiwara *et al.*, 1992; Alberts *et al.*, 1994). However, more data are necessary in order to determine if the EBNA2A–PP1 association actually has an influence on the CRE and/or on other promoter elements in the LRS. It is also possible that the nuclear PP1-like activity inhibited by the EBNA2A fragment has an effect on RNA polymerase II as described for the *SIT4* protein phosphatase of *Saccharomyces cerevisiae* (Arndt *et al.*, 1989; Stettler *et al.*, 1993). The loss of *SIT4* protein phosphatase activity is thought to result in the hyperphosphorylation of RNA polymerase II leading to the formation of an active species with a less stringent requirement for accessory factors (reviewed in Mumby and Walter, 1993). It has been speculated that phosphorylation of the three nuclear RNA polymerases is part of a cAMP dependent kinase/phosphatase cascade (Stettler *et al.*, 1993).

The variation of intracellular cAMP levels during the cell cycle and the importance of cAMP mediated signals in B-cell differentiation suggest that the observations on the response of LMP1 expression to the cAMP signal transduction pathway will be important in understanding the initial steps of EBV mediated cell immortalization and the mechanisms for survival of EBV infected B-cells. In view of the results presented, it is also conceivable that one of the consequences of EBNA2A inhibition of a nuclear PP1 is to promote transcription from the LMP1 gene promoter. Determination of the specific factor(s) affected by the EBNA2A mediated inhibition of PP1-like nuclear activity and of the nature of this association are interesting questions which are currently being addressed.

Materials and methods

Cell lines, culture conditions and cell proliferation analysis

The DG75 cell line derives from an EBV-negative Burkitt's lymphoma (Ben-Bassat *et al.*, 1977). DG75 cell lines expressing LMP1 under the control of the thymidine kinase promoter (clones TKLM9 and TKLM18) were kindly obtained from Dr P.Trivedi and are described in Cuomo *et al.* (1990). B95-8 and Cherry are two EBV-immortalized lymphoblastoid cell lines (Miller and Lipman, 1973; Randahl *et al.*, 1992). All cell types were cultured in RPMI 1640 medium with 10% fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin. For the analysis of cAMP mediated inhibition of cell proliferation, cells were diluted in culture medium with fetal calf serum to a density of 3×10^5 cells/ml 12 h before the addition of 8-Br cAMP up to 4 mM. Cell counting was carried out in a Burker chamber. Cell viability was determined by trypan blue exclusion.

Plasmid constructions

The LRS–CAT reporter plasmids include DNA fragments derived from the B95-8 EBV strain genome (Baer *et al.*, 1984) spanning nucleotides +40 to different upstream positions relative to the ED-L1 promoter transcription initiation site (+1). pgLRS(−634)CAT, pgLRS(−324)CAT, pgLRS(−144)CAT, pgLRS(−106)CAT pgLRS(−54)CAT and pgCAT have been described (Fåhraeus *et al.*, 1993). Construction of pgLRS(−259)CAT is described in A.Sjöblom *et al.* (submitted). pgLRS(−135)CAT, pgLRS(−160)CAT and pgLRS(−173)CAT are also described by A.Sjöblom *et al.* (submitted) where they are named as pgLRS(−217)m15, pgLRS(−217)m11 and pgLRS(−217)m6 respectively. pgLRS(−35)CAT, pgLRS(−41)CAT and pgLRS(−72)CAT contain LRS fragments spanning from position +40 to the *Aci*I site at position −35, to the unique *Mae*II site at position −41 and to the *Hae*III site at position −72 of the LRS, respectively. pgLRS(−55)CAT was constructed by deleting the *Mlu*I–*Sal*I fragment of pgLRS(−634)CAT and filling protruding ends with Klenow enzyme. CRE mutation constructs were obtained by inserting the double stranded synthetic oligonucleotide described in Figure 1A between the *Mlu*I site at position −54 and the *Aci*I site at position −35 of LRS in the corresponding pgLRS–CAT plasmids. EBNA2A expression vector pEΔA6 and the corresponding control plasmid pSV2gpt have been described previously (Fåhraeus *et al.*, 1993). pSVCREMβ, was a kind gift from Dr P.Sassone-Corsi and is described by Foulkes *et al.* (1991). Control plasmids for CREMβ expression were pSG5 (Green *et al.*, 1988) and pSVantiCREMβ which was obtained by changing the orientation of the *Eco*RI–*Eco*RI fragment of pSVCREMβ.

pGSTΔEB2A(383–487) and pGSTΔEB2A(324–436) were made by cloning the *Sph*I–*Dra*I (nucleotides 49650–50305) fragment and the *Bst*XI–*Bgl*II (nucleotides 49471–49811) fragment of the EBV B95-8 genome, corresponding to EBNA2A amino acids Pro383–Gln487 and Trp324–Ala436, respectively, into the pGEX2TK vector (Kaelin *et al.*, 1992). The expression of GSTΔEB2A recombinant proteins in bacteria was checked by Western analysis. All manipulations for plasmid constructions were carried out by standard procedures (Sambrook *et al.*, 1989) and all constructs were verified by sequencing.

Transfections and chloramphenicol acetyltransferase assays

DG75 cells (5×10^6) were transfected with 10 μg of the LRS–CAT reporter plasmids and eventually with 5 μg of the EBNA2A expression vector pEΔA6 or the same amount in nmoles of the pSV2-gpt control plasmid using the DEAE–dextran method as described by Ricksten *et al.* (1988). Transfected cells were harvested after 48 h and CAT activity was assayed (Ricksten *et al.*, 1988). Where indicated, 8-Br cAMP (SIGMA) and theophylline (SIGMA) were added to the 10 ml of transfected cells in culture medium to a concentration of 4 and 1 mM, respectively and incubated for 48 h before counting and harvesting. When analysing okadaic acid induction, 10 nM okadaic acid (SIGMA) with or without 4 mM 8-Br cAMP was added to the transfected cells 4 h post-transfection. Cells were harvested and assayed for CAT activity after only 16 h of incubation due to the high toxicity of okadaic acid. All transfections were carried out at least three times giving qualitatively equivalent results.

Immunoaffinity chromatography purification

Whole cell extracts from Cherry and DG75 cells were obtained and subjected to immunoaffinity chromatography with the 5B6 anti-EBNA2 mAb essentially as described by Dillner *et al.* (1988). All steps were carried out at +4°C. In summary, Cherry or DG75 cells were sonicated in lysis buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM DTT and 1 μg/ml aprotenin) containing 400 mM NaCl and centrifuged at 25 000 *g* for 30 min. Supernatants were applied to a protein A–Sepharose pre-column and the resulting flow-through was applied on a 2 ml 5B6 mAb–protein A–Sepharose column prepared as described (Harlow and Lane, 1988). After extensive washing with lysis buffer containing 150 mM NaCl the column was eluted with a pH 3 shock. 20 μl aliquots of the 1 ml fractions obtained were subjected to microcystin labelling (see below). 200 μl of each fraction were precipitated with 2 vol ethanol and analysed by immunoblotting. Similar results were obtained when using the method described by Randahl *et al.* (1992).

Immunoblotting and microcystin-YR labelling

Whole cell extracts or samples obtained from the immunoaffinity purification were subjected to SDS–PAGE and immunoblotting (Kallin *et al.*, 1986). LMP1 and EBNA2 were detected using the CS1–4 mixture of monoclonal antibodies (DAKO) and the PE2 monoclonal antibody, respectively. The bands corresponding to LMP1 and EBNA2 were quantified by the Macintosh Scan Analysis densitometer system. PP1 was detected using commercial rabbit polyclonal antibodies specific for PP1-alfa (UBI) which recognize this protein from human cells.

Microcystin-YR (Calbiochem) was labelled with ^{125}I using $Na^{125}I$ and iodogen and labelled toxin was purified using a C_{18} cartridge (R.W.MacKintosh, unpublished). 20 µl of each assay were pre-incubated 30 min at room temperature (RT) with 0.5% β-mercaptoethanol. One µl of [^{125}I]microcystin-YR was added and samples were further incubated for 30 min at RT, boiled in SDS–PAGE sample buffer and separated on 12.5% SDS–polyacrylamide gels which were fixed and visualized by autoradiography. (Protocol is a personal communication from R.W.MacKintosh, University of Dundee.)

GSTΔEB2A expression and binding assay

Glutathione-*S* transferase fusion protein expression and purification were essentially carried out as described by Kaelin *et al.* (1992). In summary, fresh overnight cultures of *Escherichia coli* (HB101) transformed with either pGEX2TK or the pGSTΔEB2A recombinant plasmids were diluted 1:50 and incubated at 37°C until an OD_{600} of 0.6 was reached and induced with 1 mM IPTG and incubated at 37°C for 3 h. Cells were chilled on ice, collected by centrifugation, washed with 50 mM glucose, 25 mM Tris–HCl pH 8.0 and frozen in aliquots at −70°C. Pellets were resuspended in 3 ml/g bacteria of NETN buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 2 µg/ml aprotinin and 0.1% Tween-20). After sonication (5×10 s, 10 microns) on ice, lysates were centrifuged at 20 000 *g* for 30 min at +4°C. The supernatant, corresponding to ~5 mg of protein, was added to 100 µl of 50% glutathione–Sepharose (Pharmacia) previously washed three times with NETN buffer containing 0.5% powdered milk and incubated by rocking 30 min at +4°C. Beads were washed three times with NETN buffer and 100 µl of DG75 whole cell extract, corresponding to 1 mg of protein, were added to the beads and incubated by rocking 60 min at +4°C. Samples were washed five times with NETN buffer. For microcystin-YR labelling experiments, the material bound to the beads was eluted with 400 µl of 2 mM glutathione (reduced form) in 0.1 M Tris–HCl and 120 mM NaCl. Supernatants were precipitated with 2 vol ethanol and resuspended in 20 µl of NETN buffer. A 10 µl aliquot was taken and labelled with [^{125}I]microcystin-YR as described above. The amount of fusion protein used in each binding assay was estimated by Coomassie staining.

Phosphorylase-a protein phosphatase activity assays

Protein phosphorylase-b was phosphorylated to render phosphorylase-a with phosphorylase kinase using the commercially available kit from GIBCO. PP1/PP2A activity assays were performed as described by the manufacturers in a total volume of 60 µl with 2 µl of DG75 nuclear extracts (2 mg protein/ml) prepared by the method described by Dignam *et al.* (1983). The activity obtained with this amount of nuclear extract was <30%, as recommended for this system. Where indicated, okadaic acid was added to the nuclear extracts before the 20 µl of phosphorylase-a substrate. The final concentration of okadaic acid in the 60 µl reaction mixture was 2 nM. Washed glutathione–Sepharose beads bound to GST or to the GSTΔEB2 fusion proteins as described above were resuspended in 20 µl protein phosphatase A+B buffer (GIBCO). For inhibition assays, 5 µl of either of these suspensions were added to the protein phosphatase reaction mixture before the phosphorylase-a substrate. In the case of inhibition with the PP1 specific inhibitor-1 peptide, a total of 4 ng of the 46 amino acid PKA phosphorylated inhibitor-1 peptide (kind gift from Dr C.B.Holmes) was added to the reaction mixture before the phosphorylase-a substrate.

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Note added

While this manuscript was being submitted we learned that Dr G.Howe, Department of Laboratory Medicine, Yale University, has obtained results in agreement with those presented here concerning induction of the LMP1 expression by cAMP.