
Repression of human cytomegalovirus gene expression associated with a novel immediate early regulatory region binding factor

S.L.Shelbourn, S.K.Kothari, J.G.P.Sissons and J.H.Sinclair

Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, UK

Received August 9, 1989; Revised and Accepted September 25, 1989

ABSTRACT

A major determinant controlling reactivation of persistent viruses is likely to be the level of cellular factors which regulate virus transcription. Human cytomegalovirus (the largest human herpesvirus) does not replicate in human teratocarcinoma (T2) cells due to a block in transcription of immediate early (IE) gene expression, but these cells become permissive upon retinoic acid induced differentiation. We have analysed changes in DNA binding factors to the major IE promoter/regulatory region of HCMV that occur during differentiation of T2 cells to a permissive phenotype. We show that undifferentiated T2 cells contain a specific nuclear factor that binds to a far upstream region of the major IE regulatory region. Differentiation of T2 cells is associated with a major decrease in this factor and deletion of its specific binding site from IE expression vectors also results in increased levels of expression in undifferentiated cells. Consequently, this novel factor present in undifferentiated cells is a candidate for a differentiation specific negative regulator of HCMV IE gene expression which binds to an element upstream of the major IE enhancer.

INTRODUCTION

HCMV is the largest known herpesvirus and a ubiquitous pathogen ⁽¹⁾. Primary infection with HCMV is usually asymptomatic after which the virus establishes persistent infection. Reactivation is associated with immunosuppression and transplant surgery and results in a wide spectrum of diseases. However the molecular mechanisms of latency and reactivation are unclear.

Embryonal carcinoma cells which differentiate in response to retinoic acid (RA), serve as a useful model system to study factors that regulate viral gene expression. For instance, HCMV does not infect undifferentiated human teratocarcinoma (T2) cells ⁽²⁾. This non-permissiveness for HCMV infection results from a block in viral immediate early (IE) gene expression ^(3,4). However, RA-induced differentiation lifts this repression permitting IE gene expression. Recent work ⁽³⁾ has shown that DNase 1 hypersensitivity of the major IE promoter/regulatory region upstream of the enhancer is induced upon differentiation of T2 cells, suggesting that DNA/protein interactions distal to the enhancer are important for IE gene expression in differentiated cells. Similarly, in agreement with Nelson et al ⁽⁵⁾, we have shown that pEs*Cat* (figure 1B), a CAT expression vector under the control of approximately 2 kb of the major IE promoter/regulatory region, is not expressed in undifferentiated T2 cells ⁽⁶⁾. However deletion of the modulator binding region ⁽⁷⁾ containing DNA sequences upstream of the enhancer (pIEP1*Cat*, see figure 1C), removes this repression permitting expression ^(5,6). As this deletion coincides with the sites of induced DNase 1 hypersensitivity in differentiated T2 cells ⁽⁷⁾, it is likely that undifferentiated cells contain negative factors which bind distal to the enhancer and repress

IE gene expression. Here we show that differentiation of T2 cells to a permissive phenotype for HCMV expression is correlated to the disappearance of a specific cellular factor which binds upstream of the major IE enhancer and that deletion of this binding site from IE expression vectors results in expression in undifferentiated cells. These sequences, therefore, represent a novel cell specific negative regulator of expression of the HCMV major IE gene. Such regulators of IE gene expression may be critical determinants for reactivation of latent virus.

MATERIALS AND METHODS

Cell Lines

The cell line NT2D1⁽²⁾ was maintained in DMEM supplemented with 10% FCS. Cells were split 1:3 every three days. Differentiation was induced by addition of 10⁻⁶M all-trans retinoic acid for 5 days.

Plasmids, DNA transfection and cat assays

The plasmids pEscat and pIEP1cat have been described⁽⁶⁾ pEsd12cat and pEsd16cat were constructed by *ECORV* deletion of pEscat. pUC5'ES was constructed by insertion of the *HincII* fragment of pEscat into pUC9. For DNA transfection, approximately 5 × 10⁶ cells were transfected with 100 ng of pEscat, pIEP1Cat, pEsd12cat or pEsd16cat by calcium phosphate precipitation⁽⁶⁾. For co-transfection, 100ng of pEscat with varying amounts of pUC5'ES were always made to 10 μg of total DNA by addition of pUC19 plasmid DNA. Cells were harvested 48h post-transfection and assayed for CAT expression⁽⁸⁾.

Mobility shift assays

Approximately 10 μg of nuclear protein extracts, prepared essentially as described by Henninghausen and Lubon⁽⁹⁾, were analysed for their ability to retard approximately 10 ng of probe, labelled by filling in with ³²P dCTP and Klenow as described⁽⁹⁾. Binding reactions contained 5 μg of poly dIdC and were incubated for 30 min. at room temperature with probe prior to loading on 8% polyacrylamide gels in 0.5% TBE. Where indicated, approximately 1 μg of cold competitor was added to the binding reactions for 10 min. prior to addition of probe.

The 3' dyad probe was; GATTTTTGGGCATACGcgatatctg,
ctaaaaccCGTATGCGCTATAGAC

The 5' dyad probe was; CTGATATCGCCATTTTTcctaaaagt
gactatagcGGTAAAAAGTTTTTCAA

The NF1 1 probe was;CAAGCTGGCACATGGCCAaatgca
gtccaCCGTGTACCGGTTTACGA

The AP1 probe was; gcatcTATACATTGAATCAATATG
CGTAGATATGTAACCTTAGTtatac

where nucleotides filled in by Klenow are shown in lower case.

RESULTS

The promoter/regulatory region of the HCMV major IE transcription unit contains the strongest known enhancer consisting of 17, 18, 19 and 21 bp imperfect repeat elements

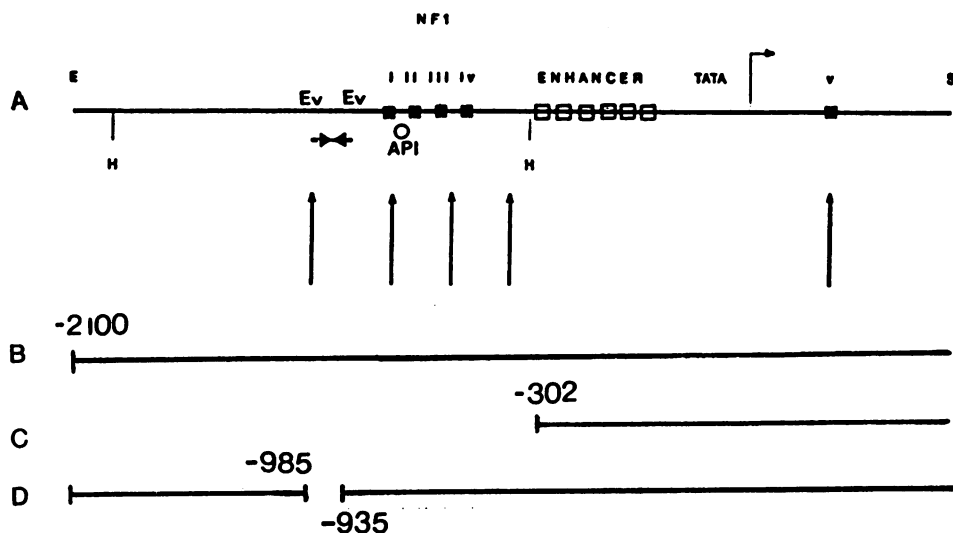


Figure 1. Immediate early expression vectors. (A) The upstream promoter/regulatory region of the major IE gene of HCMV is shown. ■ are NF1 binding sites, □□ is the enhancer, ○ is a consensus AP1 binding site, ◀▶ is the imperfect dyad symmetry, the curved arrow is the start of IE1 transcription and the straight arrows are RA-induced DNase1 hypersensitive sites ⁽⁷⁾. Restriction enzyme sites are; E = ECOR1, H = HINCII, S = SST1, and EV = ECORV. B) pEScat. C) pIEP1cat D) pESd12cat and pESd16cat.

⁽¹⁰⁾. Defined sequences upstream from the enhancer include a cluster of NF1 binding sites, an imperfect dyad symmetry, the start of IE1 transcription and at least one AP1 binding site ⁽¹¹⁾ (also see fig. 1A). To determine specific sites of interaction between the regulatory region of the major

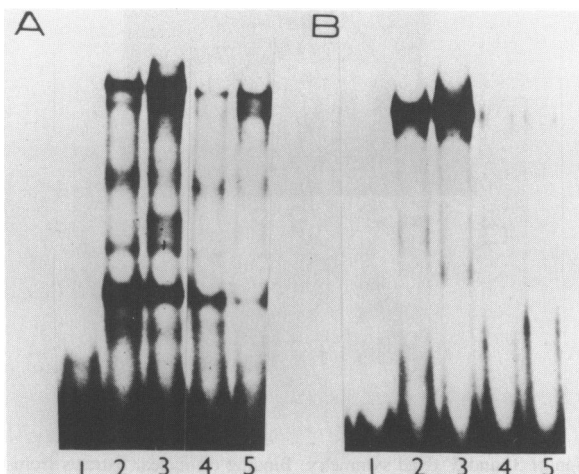


Figure 2. Gel retardation of NF1 and AP1 sites. Binding of nuclear extracts from undifferentiated cells (tracks 2, and 4) or differentiated cells (tracks 3, and 5) to the NF1 1 (A) or AP1 (B) sites were analysed by mobility shift assays. Tracks 2 and 3 contained no additional poly dIdC, tracks 4 and 5 were preincubated with approximately 1 μg of cold NF1 competitor (A) or cold AP1 competitor (B). Track 1 is free probe.

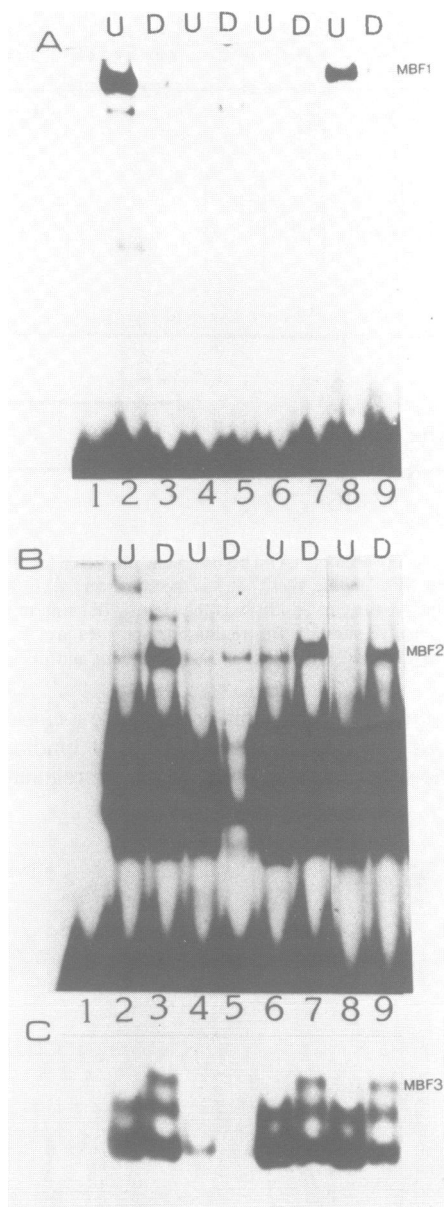


Figure 3. Gel retardation of 3' and 5' dyad symmetry. Binding of nuclear extracts from undifferentiated cells (tracks 2,4,6, and 8) or differentiated cells (tracks 3,5,7 and 9) to the 3'(A) or 5'(B) dyad symmetry were analysed by mobility shift assays. Tracks 2 and 3 contain no additional poly dIdC, tracks 4 and 5 were preincubated with approximately 1 µg of cold 5' dyad competitor, tracks 6 and 7 were preincubated with approximately 1 µg of

IE gene and protein factors in T2 cells, we carried out mobility shift assays using extracts from undifferentiated T2 cells and T2 cells induced to differentiate for 5 days with 10^{-6} M retinoic acid. We used annealed, double stranded synthetic oligonucleotide sequences containing the NF1 1 site, the putative AP1 binding site as well as the 5' or 3' half of the imperfect dyad symmetry to ask whether changes in DNA binding factors to these sequences accompanied differentiation of T2 cells to a permissive phenotype. We detected no major differences in binding factors to the NF1 1 probe between differentiated or undifferentiated T2 nuclear extracts (figure 2A). Similarly no major changes in binding factors to the putative AP1 binding site appeared to accompany differentiation of T2 cells by RA (figure 2B). However, major quantitative and qualitative differences in DNA binding factors to the 5' and 3' halves of the dyad symmetry were observed between nuclear extracts from differentiated and undifferentiated cells.

Analysis of the 3' half of the imperfect dyad symmetry (figure 3A) shows that undifferentiated T2 cells contain specific binding factors (which we have termed modulator binding factor 1 or MBF1) which generate a single major nucleoprotein complex. Retinoic acid induced differentiation, however, results in a major decrease in the presence of this factor.

Similarly, analysis of the 5' half of the dyad symmetry (fig 3B) also shows the presence of an identical complex to that observed with the 3' half of the dyad symmetry but at lower levels. But in addition, differentiation of T2 cells leads to a major increase in two major nucleoprotein complexes (MBF2 and MBF3) specific only for the 5' half of the imperfect dyad symmetry (fig. 3B and fig. 3C which is a shorter exposure of the heavily overexposed faster migrating complex in fig. 3B). The two fastest migrating complexes in fig. 3b are present in both differentiated and undifferentiated cells. Competition experiments between the 5' and 3' half of the dyad symmetry (fig. 3) confirm that the factors that appear upon differentiation (MBF2, MBF3) are specific for the 5' end of the dyad whereas the factor that disappears upon differentiation (MBF1) binds to both the 5' and 3' ends of the dyad symmetry. This suggests that this imperfect dyad symmetry contains asymmetrical binding sites for MBF2 and MBF3 but also contains a symmetrical binding site for the factor MBF1, which is lost upon differentiation, and is a candidate for a negative regulator of expression of the major IE gene.

We therefore tested this directly by construction of two derivatives of pEScat (pESd12cat and pESd16cat) in which the imperfect dyad symmetry had been deleted by digestion with *ECORV* (see fig. 1D). Figure 4A shows that pEScat is expressed poorly in undifferentiated cells but this repression is lifted if the cells are allowed to differentiate. Fig 4A also shows that deletion of the imperfect dyad symmetry in pESd12cat and pESd16cat does indeed result in expression of these vectors in undifferentiated cells to levels approaching that of pIEP1cat, confirming the imperfect dyad symmetry as a site for a differentiation specific repressor of IE gene expression. However, as pESd12cat and pESd16cat do not result in identical levels of CAT activity in undifferentiated T2 cells as pIEP1cat we cannot rule out the possibility that other DNA sequences act in concert with the dyad symmetry to repress IE expression or indeed other repressor sites of IE expression exist within this control region.

cold 3' dyad competitor and tracks 8 and 9 were preincubated with an additional 1 μ g of poly dIdC prior to incubation with probe. Track 1 is free probe. Figure 3C) is a reduced exposure of the faster moving over-exposed complex in fig. 3B. U is undifferentiated and D differentiated cell nuclear extracts.

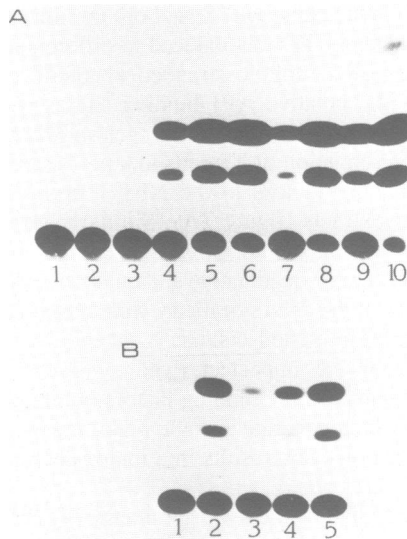


Figure 4A. Immediate early *cat* expression in T2 cells. Undifferentiated T2 cells (tracks 1,3,5 and 7) or differentiated cells (tracks 2,4,6 and 8) were transfected with 1 μ g of pES*cat* (3 and 4), pIEP1*cat* (5 and 6), pESd12*cat* (7 and 8) or pESd16*cat* (9 and 10) by calcium phosphate coprecipitation. Tracks 1 and 2 are control, untransfected undifferentiated or differentiated cells respectively.

Figure 4B. Titration of repressors from T2 cells. Undifferentiated (tracks 1,3,4 and 5) or differentiated (track 2) T2 cells were transfected with 1 μ g of pES*cat* together with 0.1 (track 3), 1.0 (track 4) or 10 μ g (track 5) of pUC5'ES. All transfections were made up to 10 μ g of total DNA by addition of pUC19 plasmid.

In order to confirm this and to ensure that increases in expression of these deletion vectors in undifferentiated cells could not be attributed to generation of novel DNA binding sites for positive factors, we asked whether co-transfection of undifferentiated cells with the plasmid pUC5'ES containing the HincII fragment from pES (see fig. 1A which contains the imperfect dyad symmetry) could titrate out putative negative factors in undifferentiated cells and permit expression of the IE expression vector pES*cat*. Figure 4B shows that co-transfection of undifferentiated T2 cells with increasing amounts of pUC5'ES, results in corresponding increases in pES*cat* expression which is direct evidence for this region containing sites which bind trans-acting factors that negatively regulate IE gene expression.

DISCUSSION

Differentiation specific trans-acting factors have been implicated in cell specific regulation of the polyoma⁽¹²⁾ and immunoglobulin⁽¹³⁾ enhancers. However our results suggest that expression of the HCMV major IE promoter/enhancer in differentiated and undifferentiated cells is regulated by binding of transcription factors to sequences distal to the enhancer in a region of imperfect dyad symmetry. We detected no major changes in nuclear factors binding to the NF1 1 site or the putative AP1 binding site of the major IE promoter regulatory region between differentiated or undifferentiated T2 cells. This suggests that changes in NF1 or AP1 do not play a major role in differentiation induced permissiveness for IE gene expression by RA in human teratocarcinoma cells.

In contrast, major changes in nuclear binding factors to the imperfect dyad symmetry were observed. The HCMV IE imperfect dyad symmetry binds factors that appear upon

differentiation (MBF2 and MBF3). However, this binding does not appear essential for expression *in vitro* as pIEP1*cat* is expressed well in both differentiated and undifferentiated cells. The T2 specific cell factor MBF1, which also binds to the imperfect dyad symmetry, is a candidate for a negative regulator of IE gene expression. The presence of this factor correlates well with lack of expression of the major IE promoter. Similarly, deletion of this binding site from IE expression vectors results in increased expression in undifferentiated T2 cells and this region of the promoter can titrate out negative factors present in T2 cells that normally repress IE expression. We can distinguish no homology between these sequences and known binding sites for PEA2⁽¹²⁾ which has been implicated in repression of the polyoma enhancer in murine embryonal carcinoma cells (EC). We have detected some homology between the IE imperfect dyad symmetry (ATTTTGGGCAT) and NFA3 (ATTTGCAT)⁽¹³⁾, an octamer binding factor associated with repression of the immunoglobulin enhancer in murine EC cells. However, our preliminary data suggests that the octamer motif does not compete with the 3' dyad symmetry in mobility shift assays (unpublished).

We have also observed a complex similar in mobility to MBF1 in the promonocytic HL60 cell line, which express pES*cat* poorly, which disappears upon PMA stimulation of these cells to monocytes and is correlated to increased expression of pES*cat*. in these cells (Sinclair unpublished). Consequently this factor may be ubiquitous to non-permissive cell lines.

We do not know by what mechanism MBF1 decreases upon differentiation. It could be either a reduction in protein expression or a modification of the protein. However, changes in the expression of such negative regulators in HCMV infected cells may play a major role in the determination of viral latency or reactivation.

ACKNOWLEDGEMENTS

We would like to thank Drs. Gavin Wilkinson and Alan Akrigg for pIEP1*cat*, Anne Jones for typing the manuscript and Linda Bryant and Joan Baillie for excellent technical assistance. This work was supported by grants from the MRC and Wellcome Trust.

REFERENCES

1. Alford, C.,A. and Britt, W., J. (1985) Cytomegalovirus. p.629–660. In B. N. Fields (ed.), Virology. Raven Press, New York
2. Gonczol, E., Andrews P.W. and Plotkin, S.A. (1984) Science 224 159–161.
3. Nelson, J.A. and Groudine, M. (1986) Mol. Cell Biol. 6 452–461.
4. Lafemina, R. A. and Hayward, G. S. (1986) J. Virol. 58 434–440
5. Nelson, J.A., Reynolds-Kohler, C. and Smith, B.A. (1987) Mol. Cell. Biol. 7 4125–4129.
6. Shelbourn, S.L., Sissons, J.G.P. and Sinclair, J.H. (1989) J. Gen Virol. 70 367–374
7. Lubon, H., Ghazal, P., Hennighausen, L., Reynolds-Kohler, K., Lockshin, C. and Nelson, J. (1989) Mol. Cell Biol. 9 1342–1345
8. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell Biol. 2 1044–1051
9. Hennighausen, L. and Lubon, H. (1987) Methods in Enzym. 152 721–731.
10. Boshart, M., Weber, F., Jahn, G., Dorch-Hasler, K., Fleckenstein, B. and Schaffner, W. (1985) Cell 41 521–530
11. Hennighausen, L. and Fleckenstein, B. (1986) EMBO J. 5 1367–1371
12. Wasyluk, B., Imler, I., Chatton B., Schatz, C. and Wasyluk, C. (1988) Proc. Natl. Acad. Sci. (USA) 85 7952–7956
13. Lenardo, M. J., Staudt, L., Robbins, A. K., Mulligan, R. C. and Baltimore, D. (1989) Science 243 544–546

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.