The 21bp repeat element of the human cytomegalovirus major immediate early enhancer is a negative regulator of gene expression in undifferentiated cells

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ABSTRACT

The major immediate early regulatory region of human cytomegalovirus (HCMV) has a complex set of DNA sites through which both cellular and viral factors coordinately regulate immediate early gene expression. In undifferentiated human teratocarcinoma (T2) cells we have previously shown that major immediate early gene expression is repressed by a differentiation specific nuclear factor MBF1, which binds to the imperfect dyad symmetry located upstream of the enhancer. However, upon differentiation MBF1 decreases resulting in immediate early gene expression. In this study we show, by mobility shift analysis that the same or similar factor(s) also binds to the 21bp repeat of the major immediate early enhancer. Deletion of this 21bp repeat from the immediate early enhancer expression vectors results in increased CAT expression in undifferentiated T2 cells, to levels similar to that in differentiated cells. Consequently, the 21bp repeat of the HCMV enhancer also acts to negatively regulate major immediate early enhancer function in non-permissive cells.

INTRODUCTION

HCMV is a 230kb double stranded DNA virus and belongs to the herpesvirus family. Upon infection, HCMV expression undergoes three phases of gene expression, immediate early (IE), early (E) and late (L). IE expression is known to be essential for regulation of both early and late phases of HCMV gene expression and the most abundant IE transcript, coding for the major IE1 product, is regulated at the transcriptional level (1). The regulatory region of the major IE gene is fairly well characterised and several sites to which host cell factors bind have been defined (2–5). It contains one of the strongest known enhancers comprising 17bp, 18bp, 19bp and 21bp repeat elements, arranged in a random array with respect to each other (6). The 18bp and 19bp motifs have been extensively characterised and are known to bind NFkB and cyclic AMP-responsive element binding protein, respectively (4,5). However, little is known about the function of the 17bp and 21bp repeat motifs. We have used human teratocarcinoma cells as a model system in which to analyse the cis- and trans-acting factors necessary for HCMV IE gene expression in permissive and non-permissive cells. HCMV can replicate in retinoic acid (RA) differentiated human teratocarcinoma cells but not in undifferentiated T2 cells (7). The non-permissiveness of undifferentiated T2 cells for HCMV infection is due to a block in the major IE gene expression (8–10) which is negatively regulated via modulator sequences located between −750 and −1145, upstream of the IE promoter/enhancer (11,12). Recently, we showed the presence of differentiation specific modulator binding factor 1 (MBF1), which formed a complex with an imperfect dyad symmetry upstream of the enhancer of the major IE gene. Upon differentiation of T2 cells with retinoic acid, MBF1 decreased significantly and this correlated with up-regulation in major IE gene expression. Deletion of the imperfect dyad symmetry also resulted in increased level of expression in non-permissive T2 cells (12). Consequently, it was apparent that this region acted to negatively regulate major IE expression in non-permissive, undifferentiated T2 cells. However, it was also apparent from further deletion analysis that sites other than this imperfect dyad symmetry were also repressing expression of IE vectors in undifferentiated T2 cells. We have, therefore, analysed the enhancer of the HCMV major IE promoter and find that the 21bp repeat present in the enhancer binds factors similar to the imperfect dyad symmetry. Similarly, deletion of 21bp repeat motif from pIEP1*cat* construct results in increased expression in undifferentiated non-permissive T2 cells. Consequently, the 21bp repeat motif of the HCMV IE enhancer is also a site of negative regulation of IE gene expression in undifferentiated cells and binds differentiation specific negative factors similar if not identical to the previously defined imperfect dyad symmetry binding factor MBF1.

MATERIALS AND METHODS

Cell lines

The cell line NT2D1 (13) was maintained in DMEM supplemented with 10%FCS. Cells were split 1:3 every three

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days. Differentiation was induced by addition of 10^{-6}M alltrans retinoic acid for 5 days.

Plasmids, DNA transfections and CAT assays

The plasmids pES*cat* and pIEP1*cat* have been previously described (10,12). Plasmid pIE21*cat* contained 525bp upstream sequence which includes the full major IE enhancer and was constructed by amplification of this region by polymerase chain reaction and subsequent cloning into a promoter deletion of pIEP1*cat*. pIE21A*cat* is equivalent to pIEP1*cat* but contains a single 21bp repeat element constructed by ligation of a double stranded synthetic oligonucleotide into pIEP1*cat*. pIE12A*cat* is identical to pIE21A*cat* except the 21bp repeat is in the anti-sense orientation. Both these vectors were confirmed by DNA sequencing.

For DNA transfection, approximately 5×10^6 cells were transfected with 100ng of pES*cat*, pIEP1*cat* or pIE21*cat* by calcium phosphate precipitation (10). Cells were harvested 48h post-transfection and assayed for CAT expression (14). The experiments shown are typical results which have been reproduced in three independent experiments.

Mobility shift assays

Approximately 10μg of nuclear protein extracts, prepared essentially as described by Hennighausen and Lubon (15), were analysed for their ability to retard approximately 10ng of probe, labelled by filling in with ^{32}P dCTP and klenow as described (12). Binding reaction contained 5μg 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 1μg of additional cold competitor was added to the binding reaction for 10 min. prior to addition of probe. Below are the sequences of the 3′ half of the dyad and 21bp repeat motif probes, where nucleotides filled in by Klenow are shown in the lower case.

The 3′ dyad probe was;

GATTTTTGGGCATACGcgatatctg
ctaaaaaccCGTATGCGCTATAGAC

The 21bp element probe was;

ACTTACGGTAAATGGCCCGCCTGGCTgaccg
tgaatGCCATTTACCGGGCGGACCGACTGGC

Sequences of competitor oligonucleotides are shown in figure 4C.

RESULTS

Our previous work showed that deletion of an imperfect dyad symmetry (−912bp to −963bp) upstream of the enhancer of the HCMV major IE promoter in CAT reporter constructs resulted in increased level of expression in undifferentiated T2 cells (12). However, comparison of the levels of expression of this deletion vector with pIEP1*cat*, which contains only 302bp of upstream sequence, suggested that other sequences beside the dyad symmetry could be sites of negative regulation of IE expression in T2 cells. Defined DNA sequences absent from the maximally expressed pIEP1*cat* vector but present in the repressed expression vector pES*cat* include NF1 sites, a putative AP1 site and the 21bp repeat motif of the HCMV IE enhancer (see figure 1D). We previously ruled out the NF1 sites and the putative AP1 site as regions of negative regulation of IE expression in undifferentiated

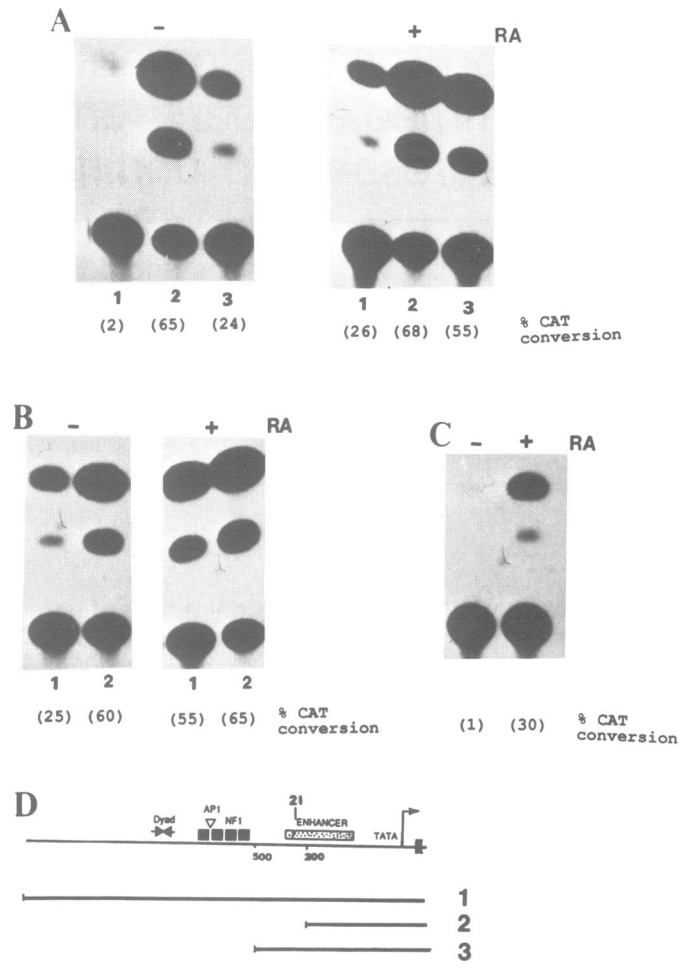

Figure 1. Expression of IEcat vectors. A) pES*cat* (1), pIEP1*cat* (2) and pIE21*cat* (3) were transfected into undifferentiated T2 cells (−RA) or differentiated T2 cells (+ RA) cells, and assayed 48h post-transfection for CAT activity. % CAT conversions are shown in brackets. B) pIE21A*cat* containing an additional single 21bp repeat (1) or pIEP1*cat* (2) were transfected into T2 (−RA) or differentiated T2 cells (+RA) and assayed 48h post-transfection for CAT activity. % CAT conversions are shown in brackets. C) pIE12A*cat* containing an additional single anti-sense 21bp repeat was transfected into T2 (−RA) or differentiated T2 cells (+RA) and assayed 48h post-transfection for CAT activity. % CAT conversions are shown in brackets. D) Expression vector deletions are shown as are the location of the 21bp repeats in the enhancer.

T2 cells (12). Consequently, we asked whether the 21bp repeat elements present within the enhancer could act to negatively regulate HCMV IE expression in T2 cells.

Figure 1A, lane 2 confirms that pIEP1*cat*, which contains only 302bp of upstream promoter region of the major IE gene but lacks the 21bp repeat elements, is maximally expressed in T2 cells. However, pIE21*cat* (figure 1A, lane3), which contains the full IE enhancer including three 21bp repeat elements is expressed at significantly lower levels than pIEP1*cat* in T2 cells. This strongly suggests that the 21bp repeat motifs act to negatively regulate IE expression in undifferentiated T2 cells. In order to confirm this we constructed a derivative of the maximally expressed pIEP1*cat* which contained a synthetic 21bp repeat element. Figure 1B (lane 1, −RA) shows that this construct (pIE21A*cat*) is also expressed at significantly lower levels in T2 cells than pIEP1*cat*, whereas it is still expressed at high levels in differentiated T2 cells (lane 1, +RA). Consequently, this

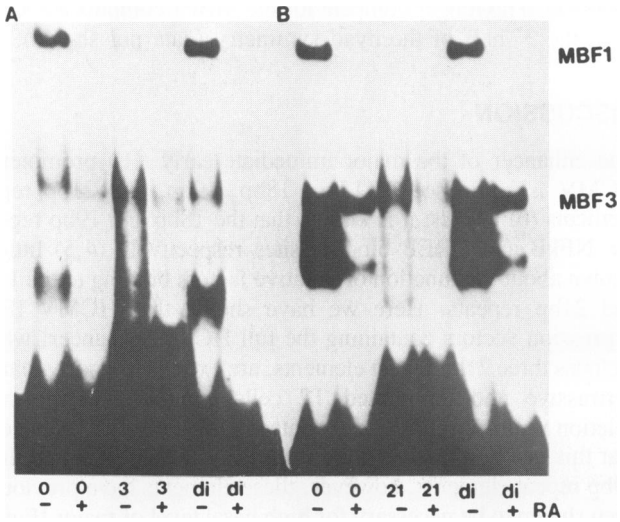

Figure 2. Mobility shift assays. Band shift assays of the 3′ half of the imperfect dyad symmetry (A) or the 21bp repeat element (B) using undifferentiated (−RA) and differentiated (+RA) T2 cell nuclear extracts. Binding assays with no additional competitor (0) or with 1μg specific cold 3′ half of the dyad (3), 21bp repeat (21) or 1μg of non-specific poly dIdC DNA (di) are shown. The autoradiograph of panel A was exposed for four times longer than panel B.

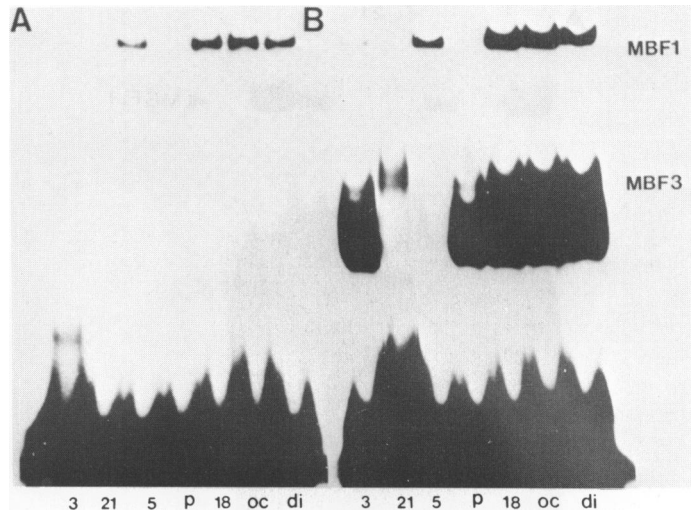

Figure 3. Competition assays. Band shift assays of the 3′ dyad symmetry (A) or the 21bp repeat (B) using undifferentiated T2 nuclear extracts. Binding assays with 1μg specific cold 3′ half of the dyad (3), 5′ half of the dyad (5), 21bp repeat (21), HCMV 18bp enhancer repeat (18), 200ng of the octomer motif (oc), 200ng of the polyoma PEA3 motif (P) or 1μg of non-specific poly dIdC DNA (di) are shown. The autoradiograph of panel A was exposed for four times longer than panel B.

confirms that in undifferentiated, non-permissive, T2 cells the 21bp repeat acts to negatively regulate expression of the major IE promoter. Figure 1C shows that the 21bp repeat motif also acts as a negative regulator in an anti-sense orientation. Indeed, the level of repression in T2 cells of CAT expression from pIE12A*cat* (a vector equivalent to pIE21A*cat* but with the 21bp repeat in opposite orientation) is much higher than pIE21A*cat*. As with pIE21A*cat* this vector is still expressed to high levels in differentiated T2 cells.

We next carried out mobility shift assays using the 21bp repeat as a DNA probe with nuclear extracts from undifferentiated and differentiated T2 cells to analyse changes in nuclear factors binding to this region, associated with cellular differentiation and a change from non-permissive to a permissive phenotype. Figure 2B shows that T2 cells contain specific factors which bind to the 21bp repeat motif. One of these complexes migrated with a previously defined complex, MBF1, seen in T2 cells which binds to the 3′ half of the imperfect dyad symmetry (figure 2A and ref. 12). Furthermore, the 21bp repeat specific complex is significantly reduced in differentiated T2 cells as is seen with the 3′ dyad symmetry specific complex MBF1. The faster migrating complexes specific for the 21bp repeat motif also appear to be similar to complexes specific for the 5′ half of the imperfect dyad symmetry, which includes a factor we have termed MBF3 (12) and see below.

To determine the specificity of these complexes, we carried out cross competition band shifts using the 21bp repeat element and the 3′ half of the dyad symmetry as DNA probes together with cold specific or non-specific competitors. Figure 3B shows that the slow migrating complex observed with the 21bp repeat motif in T2 cells and with mobility similar to the 3′ dyad symmetry specific MBF1 complex is indeed competed for by cold 3′ dyad oligonucleotide (see track marked '3') but not by the 18bp repeat of the HCMV enhancer, the octomer motif (16) or poly dIdC. Similarly cold 21bp repeat competes specifically for the 3′ dyad specific MBF1 complex (see track marked '21', figure 3A). This strongly suggests that the similar if not identical factors bind to the 21bp repeat motif and the 3′ half of the imperfect dyad symmetry. These cross competition assays also suggest that the faster migrating complexes observed between the 21bp repeat and undifferentiated T2 nuclear extracts comprise factors similar to those seen complexed to the 5′ half of the dyad symmetry as cold 5′dyad competitor specifically competes for these faster migrating 21bp specific complexes. Cold 5′ dyad competitor, however, competes less efficiently for the MBF1 like complex of the 21bp repeat and the 3′dyad symmetry which is consistent with the very low levels of MBF1 complex observed with undifferentiated T2 nuclear extracts and the 5′ half of the dyad symmetry (see ref. 12).

We have also observed that the polyoma PEA3 motif (17) competes well for the MBF1 like complex formed between the 21bp repeat or the 3′dyad symmetry (see tracks marked P). PEA3 is known to be important for activation of the polyoma virus enhancer (18). We are presently determining whether ets-1, the PEA3 binding factor (17), has any role in repression of the HCMV IE promoter in T2 cells.

DNA sequence comparisons between the 3′ half of the imperfect dyad symmetry, the 21bp repeat motif and the PEA3 motif show some similarities but no absolute homologies (see figure 4C). As yet we do not know the exact DNA sequences which complex with MBF1 in the 3′ half of the dyad symmetry or the 21bp repeat motif. So far, we have been unsuccessful in generating DNAse1 footprints of these regions with undifferentiated T2 nuclear extracts as it is known that the dyad symmetry is refractory to DNase1. Similarly, as more than one factor binds to the 21bp repeat, a foot print of this region would not unequivocally define the site of interaction of MBF1. However, cold competition assays (figure 4) using mutant 21bp oligonucleotides (figure 4C) showed that oligonucleotides with mutations at the 3′ or 5′ end (mutant oligonucleotides 21B and 21C) of the 21bp repeat still competed well for both the

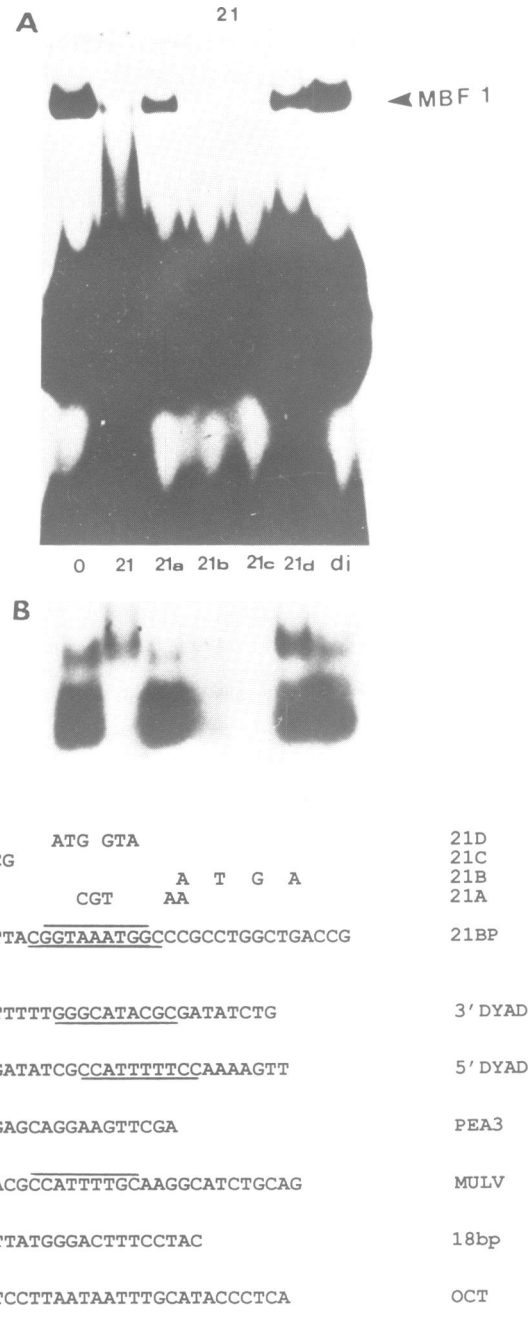

Figure 4. Mutant 21bp competition assays. (A) Band shift assays of the 21bp repeat with undifferentiated T2 nuclear extracts. Binding assays were carried out with no additional competitor (0) or 1μg of cold 21bp repeat mutations (A–D, see also figure 4C) or 1μg of non-specfic poly dI-dC (di). Figure (B) is a lower exposure of the fast migrating complexes in figure (A). (C) DNA sequence comparisons; The sequence of the 21 mutants are shown as well as the sequences of the 3′ dyad symmetry, the 5′dyad symmetry, the HCMV 21bp repeat, the polyoma PEA3 motif, the HCMV 18bp repeat, the octomer motif and the Murine Leukemia virus (MuLV) negative regulatory element. Regions of homology are over and underlined.

MBF1-like 21bp specific complex and the faster migrating 21bp specific complexes, whereas mutations within the GGTAAATGG region of the 21bp repeat (mutant oligonucleotides 21A and 21D) no longer competes for either the MBF1 or the faster migrating complexes. Similarly, as expected, these mutant 21bp repeat motifs also no longer compete for the MBF1 complex associated with the 3′ half of the dyad symmetry (data not shown).

DISCUSSION

The enhancer of the major immediate early (IE) promoter of HCMV is composed of 17bp, 18bp, 19bp, and 21bp repeat elements (6). Whilst it is known that the 18bp and 19bp repeats are NFkB and CREB binding sites respectively (4,5) little is known about the function or putative factors binding to the 17bp and 21bp repeats. Here we have shown that HCMV IE*cat* expression vectors containing the full HCMV enhancer, which includes three 21bp repeat elements, are expressed poorly in non-permissive undifferentiated T2 cells compared to enhancer deletion vectors which do not contain 21bp elements. We accept that this deletion also results in removal of one 18bp and three 19bp repeat elements, however, these elements have previously been shown to be necessary for high basal level of major IE gene expression (5). Also, expression of IE*cat* vectors containing only 17bp,18bp and 19bp repeats is considerably reduced in T2 cells in the presence of a single 21bp repeat element. This 21bp repeat element works in either sense or anti-sense orientation to repress expression from the major IE promoter. However, we do not know why repression is higher in the anti-sense, pIE12A*cat* than pIE21A*cat* in T2 cells.

Analysis of nuclear factors which bind to the 21bp repeat element by mobility shift assays also showed that a factor similar to MBF1 binds to the 21bp repeat. Like MBF1 which binds to the 3′ half of the HCMV IE imperfect dyad symmetry, this factor which is specific for the 21bp repeat motif is present in undifferentiated T2 cells and decreases substantially when these cells are induced to differentiate to a permissive phenotype for IE expression and HCMV infection with retinoic acid. Cross competition experiments confirm that this factor bears all the characteristics of MBF1. The differences in the amounts of MBF1 or MBF1-like complex in undifferentiated T2 cells binding to the 3′ half of the imperfect dyad symmetry and the 21bp repeat (note that panels A of figures 2 and 3 were exposed for four times longer than panels B) probably reflects the strength of binding of MBF1 to these elements, such that binding to the 21bp repeat is stronger than to the 3′ half of the dyad symmetry.

Our experiments suggest that the probable recognition site for MBF1 in the 21bp repeat element includes the sequence $^{5'}$ GGTAAATGG $^{3'}$. Comparison of the DNA sequence of the 3′ half of the dyad symmetry with the PEA3 motif and the 21bp repeat motif shows no absolute homology but the 3′ dyad does contain a sequence, $^{5'}$ GGCATACGC $^{3'}$. Confirmation that MBF1 binds to this region will require a similar analysis of mutant 3′ dyad competitors, which is in progress.

It would also appear that the faster migrating complexes of the 21bp repeat may also bind to the same region as MBF1 because mutations in the 21bp repeat which no longer compete for MBF1 also no longer compete for these bands. However these complexes are present in both undifferentiated and differentiated T2 cells and therefore probably do not play a role in differentiation specific repression of IE expression in permissive and non-permissive cells.

We do not know the mechanism by which MBF1 interacting with the dyad symmetry and the enhancer could negatively regulate major IE expression. Whether MBF1 acts to allow interaction of the 21bp repeat and the imperfect dyad symmetry by protein bridging effects (19) is open to speculation. However,

we would point out that competition of 3′ dyad probe or 21bp repeat probe with 21bp cold competitor or 3′ dyad cold competitor respectively removes binding of the complex and does not result in a supershift of the complex which would be expected to occur if MBF1 could bind both DNA motifs simultaneously.

We do not know the identity of the cellular factor MBF1. Analysis of the DNA sequence of the 21bp repeat which is the putative binding site of MBF1 does not show homology to DNA target motifs for any known DNA binding protein. However, it is interesting that repression of murine leukaemia virus (MuLV) in non-permissive cells has been correlated to the presence of a cellular factor(s) binding to a 5′ CGCCATTTT 3′ motif within the viral LTR (20). This shows good homology to the region of the HCMV 21bp repeat which we believe binds MBF1 (see figure 4C).

Also, previous work has shown that the polyoma virus enhancer is regulated by differentiation specific cellular factors binding to defined motifs within the viral enhancer (21). Our observations that the PEA3 motif of the polyoma virus enhancer may also bind MBF1 is intriguing. We can determine little sequence consensus between the 21bp repeat and the PEA3 motif. Although, a GGTA motif is present in the putative site of interaction of the 21bp repeat with MBF1 and this is similar to the GGAA motif which is believed to be essential for *ets*1 binding to the PEA3 motif. We are, presently, determining whether *ets*1 plays any role in this negative regulation of HCMV IE expression in non-permissive cells.

In summary, we have shown that the novel factor MBF1 which acts to negatively regulate HCMV major IE expression by interacting with the imperfect dyad symmetry also interacts with the 21bp repeat motif of the HCMV enhancer. Deletion of the 21bp repeats from HCMV enhancer also results in increased expression in undifferentiated T2 cells, confirming that this enhancer element acts as a site of negative regulation in non-permissive cells. The analysis of such differentiation specific cellular factors that regulate HCMV gene expression and their site of interaction may offer an insight into the mechanism of latency and reactivation of HCMV *in-vivo*.

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