cAMP response element of murine cytomegalovirus immediate early gene enhancer is transactivated by \textit{ras} oncogene products

Mirella Gaboli, Alessandra Angeretti, David Lembo, Marisa Gariglio, Giorgio Gribaudo and Santo Landolfo*

Institute of Microbiology, Medical School of Novara, University of Turin, and Immunogenetics and Experimental Oncology Center, CNR, 10126 Torino, Italy

Products of \textit{ras} oncogenes strongly stimulate the activity of the reporter gene, chloramphenicol acetyltransferase (CAT), driven by a 1.2 kb fragment of the murine cytomegalovirus (MCMV) immediate early (IE) gene enhancer (pCMVCAT). To define the role of proteins binding to the unique cAMP response element (CRE) present in the IE enhancer, NIH 3T3 cells were cotransfected with \textit{rasZip6} plasmid, a mammalian expression vector containing a \textit{v-Ha-ras} eDNA, together with pAACMVCAT (pCMVCAT without the CRE sequence). Lower stimulation of CAT activity was indeed observed upon deletion of the CRE sequence. Decreased levels of pAACMVCAT were also observed in cell lines carrying stably transfected \textit{ras} oncogenes. Further support for the role of the CRE sequence in MCMV enhancer activation comes from the finding that \textit{v-Ha-ras} expression increases the activity of a reporter gene, \(\beta\)-galactosidase, driven by three tandem copies of CRE sequence about six-fold. Moreover, this transactivation was prevented by cotransfection of the dominant inhibitor mutant Ha-\textit{ras} (Leu-61; Ser-186) and was not suppressed by cotransfection of Ha-\textit{ras} (Asn-17), suggesting that the effect is due to activated ras protein, rather than normal p21 \textit{ras}. Finally the transactivation observed is accompanied by an increase in nuclear proteins binding to a labelled oligonucleotide homologous to the CRE sequence, as shown in a gel retardation assay. These results suggest that the CRE element contributes to the transactivation of the MCMV IE gene enhancer by \textit{ras} oncogenes.

Introduction

The murine cytomegalovirus (MCMV), a beta-herpesvirus has a large DNA genome of 230 kbp encoding over 100 genes grouped into immediate early (IE), early (E) and late (L) genes (Ebeling \textit{et al.}, 1983; Keil \textit{et al.}, 1984). The three IE genes (IE1, IE2 and IE3) are expressed in the absence of prior viral protein synthesis, and the products are directly involved in the regulation of gene expression throughout infection (Keil \textit{et al.}, 1987; Messerle \textit{et al.}, 1991, 1992). Transcription of IE genes is regulated by a strong viral enhancer composed of an array of repeated sequence motifs containing several consensus binding sites for cellular transcription factors, including one Sp-1 site, nine NF-\(\kappa\)-B sites, of which seven partially overlap AP-1 sites, and one CREB/ATF-binding site, TGACGTCA, (Dorsch-Häsler \textit{et al.}, 1985). This motif is repeated five times within the human CMV enhancer and is an essential element for enhancer-dependent expression in uninfected HeLa cells (Boshart \textit{et al.}, 1985; Stinski & Roehr, 1985). Sambucetti \textit{et al.} (1989) have demonstrated that infection induces interaction of nuclear factors with the repeated elements of the IE enhancer. The most prominent of these are the NF-\(\kappa\)-B, AP-1 and CREB/ATF factors. It is therefore conceivable that the MCMV IE gene enhancer is also regulated by the mouse counterpart of these factors and signalling pathways.

Products of \textit{ras} genes, such as p21\textit{ras}, play a fundamental role in basic cellular functions (for review see Barbacid, 1987; Downward, 1992; Boguski & McCormick, 1993). In yeast, there is evidence that Ras has a mechanism of activation mediated through protein kinase A (PKA), but in higher eukaryotic cells, examination of some types of mammalian adenylyl cyclase suggests that it is not regulated by Ras proteins. Although a ras-dependent phosphorylation of ATF/CREB proteins has not yet been demonstrated, evidence suggesting ATF/CREB responsiveness to p21\textit{ras} has been presented (Kedar \textit{et al.}, 1990; Galien \textit{et al.}, 1991). ATPs/CREBs proteins are a multigene family of transcriptional transactivators that provide trans-
criptionally productive interactions when bound to the CREs of gene promoters, and whose binding and/or transcriptional activation functions are modulated by phosphorylations catalysed predominantly by cAMP-dependent PKA (Habener, 1990; Englander & Wilson, 1992; de Groot & Sassone-Corsi, 1993; Masson et al., 1993).

We have previously demonstrated that the expressed v-Ha-ras product stimulates IE enhancer activity (Lembo et al., 1994). However, an initial attempt to locate ras responsive elements has indicated that the stimulation of the overall enhancer activity is the result of the coordinated interactions of transcription factors with each other and their cognate binding sites.

This paper examines the role of one of these families, namely the ATF/CREB factors in the transactivation of MCMV IE gene enhancer by ras oncogenes. A transient transfection assay was used to demonstrate that a CRE sequence, homologous to the ATF binding site and cAMP sensitive, does indeed contribute to this transactivation.

Methods

Cell lines. NIH 3T3 (ATCC) 115/14 (a clone derived from NIH 3T3 carrying an amplified human c-Ha-ras, kindly provided by Dr M. Barbacid, National Cancer Institute (NCI), Frederick, MD, USA), and 226-4-1 (a clone derived from NIH 3T3 transfected with a point-mutated human c-Ki-ras kindly provided by Dr Ottolenghi, Milan, Italy) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum.

Oligonucleotide probes. Synthetic double-stranded oligonucleotide, corresponding to a copy of the 19 bp repeat element within the enhancer of CMV's immediate early genes with an internal CREB/ATF-binding site, was synthesized on an Applied Biosystems 308A DNA synthesizer. The oligonucleotide was as follows: 19 bp repeat, 5'-CACATTGAGCTCAATGGTG-3', with the complementary strand 5'-AGCCCATTGACGTCAATGGTG-Y. When required, the oligonucleotide was labelled at the 5'-end with [γ-32P]ATP by T4 polynucleotide kinase, annealed and purified by polyacrylamide gel electrophoresis.

Plasmids. pCMVCAT contains an 1.2 kb PstI–NdeI segment from the HindIII fragment L of MCMV DNA, positioned upstream from the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. This segment contains the IE enhancer and the IE1-3 promoter of MCMV (Gribaudo et al., 1993). pAACMV CAT was prepared from pCMVCAT by removing the unique CRE/ATF-binding site, using internal restriction enzyme sites PstI (−259 nt) and XhoI (−146 nt). The retroviral vector prasZip6 is derived from the pZipNeoSV(x) and contains the v-Ha-ras gene cloned in the BamHI site. Plasmids prasZip6 and pZipNeoSV(x) were kindly provided by G. Dotto (Boston, USA) pRSV-ras(Asn-17) and pRSV-ras(Leu-61; Ser-186) are eukaryotic expression vectors, in which dominant inhibitory mutants the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. Ha-ras(Asn-17)gene and Ha-ras(Leu-61; Ser-186) coding region are under the control of the Rous sarcoma virus LTR (Medema et al., 1991). Plasmid pON407, containing the MCMV IE1-3 promoter up to −146 nt linked to lacZ, and pON407.1983, in which three copies of the 19 bp repeat were inserted upstream of the MCMV sequences (Cherrington & Mocarski, 1989), were kindly provided by Dr E. Mocarski (Stanford University, CA, USA).

Nuclear extracts. Confluent NIH 3T3, 115/14 and 226-4-1 cells were harvested after being deprived for 24 h. Nuclear extracts were prepared according to Dignam et al. (1983). Buffers A and C are those described in the paper, but to minimize proteolysis 0.2 mm-PMSF, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml aprtinin and 0.1 mm-benzamidine were added.

Electrophoretic mobility shift assays (EMSA). Protein–DNA binding reactions were carried out in a final volume of 20 μl of solution containing 15 μg of nuclear extracts, 1 μg poly (dI:dC) and 5 x 105 c.p.m. of labelled probe in the presence of 10 mm-HEPES pH 7.9, 100 mm-NaCl, 1 mm-EDTA and 1 mm-DTT. Reaction mixtures were incubated at 22 °C for 30 min and loaded immediately onto a 6% non-denaturing polyacrylamide gel. Competitors were added just before the radiolabelled probe. Gels were run in 0.5 x TBE buffer, dried and autoradiographed on X-ray film.

Transfections, CAT assays and β-galactosidase assays. All plasmids were purified twice by caesium chloride centrifugation. For transient gene expression assay, cells were plated the day before transfection in growth medium supplemented with 10% newborn calf serum at a density of 2.5 x 104 cells/60 mm diameter dish. Four h before transfection, the medium was replaced by fresh DMEM containing 10% newborn calf serum. Cells were transfected by the calcium phosphate procedure and the amount of DNA in each precipitate was normalized to 12 or 15 μg with carrier DNA. Eighteen h after transfection, cells were washed twice with DMEM, cultured in medium supplemented with 0.5% newborn calf serum, and harvested 24 h later. Cell extracts were prepared according to Gorman et al. (1982). CAT activity was assayed by incubation of cell extracts, after appropriate dilution, with acetyl coenzyme A (Sigma) and [3H]choloramphenicol for 30 min. Acetylated products were separated by thin-layer chromatography, visualized by autoradiography, excised from the plates and counted in a liquid scintillation counter. CAT activity is expressed as the percentage of input chloramphenicol converted into the 1- and 3'-monoa cetylated forms. β-Galactosidase activity was assayed by adding diluted samples to an equal volume of the assay 2 x buffer (200 μm-sodium phosphate, pH 7.3, 2 mm-MgCl2, 100 mm-2-mercaptoethanol, 133 mg/ml o-nitrophenyl-β-D-galactopyranoside, ONPG). Samples were incubated at 37 °C until a yellow colour was present and the reaction was terminated by adding 1 m-sodium carbonate to a final concentration of 0.625 m. Absorbance at 420 nm was read with a spectrophotometer and compared to a β-galactosidase standard curve to allow quantification of activity in the samples.

Results

Role of CRE element in transactivation of the MCMV IE gene enhancer by v-Ha-ras or activated c-Ha-ras products

To determine the contribution of the unique CREB/ATF-binding site, (from −231 nt to −223 nt) to MCMV IE gene enhancer transactivation, the pAACMV CAT derived from pCMVCAT plasmid by deleting the 113 nt containing the CREB/ATF-binding site (from position −259 nt to −146 nt) was co-transfected in NIH 3T3 with the expression vector prasZip6, at the molar ratios of 1:4 (reporter/expression vector). As a control, the pAACMV CAT was co-
transfected with the pZipNeoSV(x), which lacks the v-Ha-ras cDNA, at the same molar ratios. As shown in Fig. 1, cotransfection of pCMVCAT construct with prasZip6 resulted in a sevenfold increase of CAT activity, whereas cotransfection of pAACMVCAT resulted in a 4.4-fold increase. The basal activities of the two constructs were almost comparable. Since CAT activity decreases when the CRE is removed from the IE enhancer, it may be supposed to play a part in the response to v-Ha-ras.

To determine whether MCMV IE enhancer transactivation throughout the CREB/ATF element could also be observed in cells stably transfected with activated ras proto-oncogenes, 115/14 and 226-4-1 cell lines, which stably express the amplified c-Ha-ras or the point-mutated c-Ki-ras, were transfected with pCMVCAT or pAACMVCAT. As shown in Fig. 2, CAT activities from cells with pAACMVCAT were lower than those from cells transfected with pCMVCAT. Although these decreases are not very high, they are significant (as shown by standard deviations) and reproducible. Altogether these results suggest that the ATF family may partly contribute to Ras transactivation of MCMV IE enhancer in ras-transformed cells.

Finally, it should be noted that the efficiency of transfection, evaluated by molecular hybridization as
Fig. 3. Effect of v-Ha-ras expression on MCMV IE gene enhancer CRE sequence. NIH 3T3 cells were transfected with either pON407 (2 μg) or pON407.19R3 (2 μg), in presence or absence of prasZip6 (8 μg). In controls, the amount of DNA was normalized with the parental vector, which does not contain the v-Ha-ras gene. Cells were serum-deprived for 24 h before assaying β-galactosidase activities (see Methods). Fold activation reflects the level of β-galactosidase activity measured from transfection of the indicated plasmids divided by the level measured from mock-transfected cells. Means and standard deviations are represented in the diagram.

previously described (Lembo et al., 1994), is comparable for the three cell lines (data not shown).

ras transactivation of three tandem copies of the MCMV CREB/ATF-binding site

To confirm further that activated p21ras could transactivate the MCMV IE enhancer through the CREB/ATF-binding site, plasmid pON407.19R3, which contains three tandem copies of the 19 bp CRE, was cotransfected in NIH 3T3 cells with either the prasZip6 or its counterpart without the v-Ha-ras, pZipNeoSV(x). As shown in Fig. 3, activated p21ras increases the β-
galactosidase activity of pON407.19R3 about fivefold, whereas it only slightly modifies the reporter activity of pON407. It should be noted that pON407 contains a CREB CS site (ACGTCA, from position −131 nt to position −136 nt), which may be responsible for the 19-fold increase of β-galactosidase activity observed when an activated p21ras is expressed.

Effect of dominant inhibitory Ha-ras mutations on CRE transactivation by v-Ha ras product

To evaluate the effects of p21ras inhibitors on pON407.19R3 basal and v-Ha-ras-induced expression, in NIH 3T3 cells, two dominant inhibitory ras mutant proteins, which selectively inhibit the activity of either cellular or oncogenic ras, have been studied (Stacey et al., 1991; Medema et al., 1991). The first, ras(Asn-17), has a single amino acid substitution at position 17, where serine is changed to asparagine, causing a preferential affinity for GDP, resulting in an inactive form of ras which interferes with the endogenous c-ras signalling system (Feig & Cooper, 1988; Bortner et al., 1993; Quilliam et al., 1994). The second dominant inhibitory ras mutant, ras(Leu-61; Ser-186) has a serine at position 186 replacing a cysteine, and inhibits the function of p21ras by competition for its cellular target (Gibbs et al., 1989; Michaeli et al., 1989).

As shown in Fig. 4(a), the β-galactosidase activity was unchanged when pON407.19R3 was cotransfected with prasZip6 and pRSV-ras(Asn-17), compared with the activity observed with the activated ras only. A lack of function of our pRSV(Asn-17) was ruled out by testing the activity of this vector on quiescent NIH 3T3 cells.

Fig. 4. Effect of dominant inhibitory Ha-ras mutants on v-Ha-ras transactivation of MCMV IE gene enhancer CRE sequence. (a) NIH 3T3 cells were transfected with pON407.19R3 (1.5 μg) and, where indicated were cotransfected with prasZip6 (6 μg), with pRSV-ras(Asn-17) (6 μg) or with both plasmids. Cellular extracts prepared from 24 h serum-deprived cells were assayed as described in Methods. β-Galactosidase activity is represented as fold activation with respect to the β-galactosidase activity of mock-transfected cells. The experiment was repeated three times and the average results and standard deviations are shown. (b) NIH 3T3 cells were transfected with pON407.19R3 (1.5 μg) and, where indicated were cotransfected with prasZip6 (6 μg), with pRSV-ras(Leu-61; Ser-186) (6 μg) or with both plasmids. Experimental conditions were those described in (a). β-Galactosidase activity is represented as fold activation with respect to the β-galactosidase activity of mock-transfected cells. Means and standard deviations are represented.
transfected with pCMV-CAT and treated with platelet-derived growth factor (PDGF), which is known to activate cellular p21ras by increasing the ratios of p21ras-GTP/p21ras-GDP (Satoh et al., 1990a, b; Gibbs et al., 1990). A strong inhibition of CAT activity following PDGF stimulation upon cotransfection of pRSV(Asn17) was indeed observed (data not shown). By contrast, when an expression vector for Ha-ras(Leu-61; Ser-186) was cotransfected with prasZip6, we observed inhibition of pON407.19R3 transactivation by oncogenic ras (Fig. 4b). This inhibition is dose-dependent since increasing concentrations of pRSVras(Leu-61; Ser-186) were able to completely antagonize the v-Ha-ras transactivation of pON407.19R3. Moreover, the pRSV vector itself, containing the RSV promoter alone, used as control in transfection experiments did not repress the v-Ha-ras transactivation of pON407.19R3 at the molar ratios employed (data not shown).

Taken as a whole, these data suggest that CREB/ATF-binding site activation is restricted to an activated form of ras, and that the normal ras product plays a role in the regulation of basal expression of this enhancer sequence.

**Agonistic effect of cAMP and v-Ha-ras product on the MCMV CRE element**

Stammlinger et al. (1990) have demonstrated that the HCMV IE gene enhancer basal activity can be considerably augmented by elevated levels of intracellular cAMP in a cell type-specific manner and that the 19 bp repeat is responsible for this effect. To confirm that the CRE sequence of MCMV IE gene enhancer is responsive to cAMP in NIH 3T3 cells we next tested the effect of forskolin on pON407 and pON407.19R3 constructs. Eighteen h after transfection, cells were treated with 8 μM-forskolin and β-galactosidase activities were assayed 24 h later. As shown in Fig. 5, pON407.19R3 β-galactosidase activity was enhanced, whereas no stimula-

![Fig. 5. Effect of forskolin treatment on pON407 and pON407.19R3. NIH 3T3 cells were transfected as indicated, serum-deprived and treated for 24 h with 8 μM-forskolin in DMSO or with 0.2% DMSO only. β-Galactosidase levels were measured and expressed as a ratio to that in mock-transfected cells. Means and standard deviations are shown in the diagram.](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>prasZip6</th>
<th>prasZip6</th>
<th>Ratio (v-Ha-ras)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>4.82</td>
<td>4.82</td>
</tr>
<tr>
<td>Forskolin</td>
<td>4.64</td>
<td>6.84</td>
<td>1.41</td>
</tr>
<tr>
<td>Ratio</td>
<td>4.64</td>
<td>6.84</td>
<td>1.41</td>
</tr>
<tr>
<td>(+ v-Ha-ras)</td>
<td>4.64</td>
<td>6.84</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Table 1. Regulation of the CRE sequence of MCMV IE gene enhancer by v-ras and forskolin treatment is agonistic*

* NIH 3T3 cells were transfected with 2 μg of pON407.19R3, and cotransfected with prasZip6 (8 μg) (+) or with the parental vector (−). Thus total DNA was kept constant at 10 μg in these studies. Forskolin treatment (8 μM) is indicated, whereas no indication stands for DMSO treatment. Results represent the average of three experiments, these deviated from the mean by less than 10%. To obtain fold induction of MCMV CRE sequence resulting from cotransfection with prasZip6, the pON407.19R3 activity in presence of v-Ha-ras was divided by that obtained in the absence of v-Ha-ras for each condition.

To investigate the factors which bind to the CRE sequence of the MCMV, NIH 3T3, 115/14 and 226-4-1 cell lines were initially grown in 10% calf serum. Thereafter the cultures were serum deprived for 24 h and

**ras oncogenes modulate ATFs in fibroblast nuclear extracts**

To investigate the factors which bind to the CRE sequence of the MCMV, NIH 3T3, 115/14 and 226-4-1 cell lines were initially grown in 10% calf serum. Thereafter the cultures were serum deprived for 24 h and...
nuclear extracts prepared. As shown in Fig. 6, when a radiolabelled oligonucleotide corresponding to the CRE was mixed with the nuclear extracts and analysed in EMSA, a strongly retarded complex was observed with 115/14 and 226-4-1 cell lines. By contrast, only a faint band was generated with extracts from NIH 3T3 cells. Moreover, nuclear extracts from NIH 3T3 cells transfected with rasZip6 contain a CRE-binding activity identical to that observed in nuclear extracts from ras-transformed cells (data not shown). These results suggest that high activity of MCMV IE enhancer in 115/14 and 226-4-1 cell lines is associated to an higher availability of ATF/CREB factors than in NIH 3T3 cells.

Discussion

The expression of murine cytomegalovirus IE genes is under the control of a strong enhancer containing repeated sequences that act in an additive manner (Gribaudo et al., 1993; Lembo et al., 1994). In the HCMV IE gene enhancer, the 19 bp repeat, containing a sequence similar to the CRE, is essential for enhancer-dependent expression of the major immediate early gene in HeLa cells (Boshart et al., 1985; Hunninghake et al., 1989; Stamminger et al., 1990).

The data presented in this paper demonstrate that also in the MCMV enhancer, the unique CRE contributes at least in part to the enhancer activation by activated ras. In this regard, we show by transient transfection assays that deletion of 113 nt, including the CRE, from MCMV IE gene enhancer reduces transactivation by ras oncogene products, but does not impair basal activity. In addition, the product of the v-Ha-ras gene activates three tandem CRE/ATF-binding sites linked to a reporter gene controlled by MCMV IE gene promoter (sequence up to –146 nt from transcription start). Finally, two dominant negative mutants of ras, Ha-ras Asn-17, which interferes with the endogenous c-ras signalling system, but not with activated ras proteins, and Ha-ras(Leu-61;Ser-186), which has been proposed to inhibit the function of p21ras by competition for its cellular target, have opposite effects on MCMV IE gene CRE sequence activation by v-Ha-ras. In fact, Ha-ras(Asn-17) mutant cannot prevent pON407.19R3 transactivation by v-Ha-ras, whereas Ha-ras(Leu-61;Ser-186) inhibits the effect of this activated Ras. The sum of these results demonstrates that oncogenic, rather than normal p21ras, is involved in MCMV IE gene enhancer CREB/ATF-binding site activation by ras. In line with our results, Kedar et al. (1990) found that in NIH 3T3 cells the β-polymerase gene promoter stimulation by ras products was dependent on the presence of a CRE, i.e. GTGACGTCACC, the cognate binding site of CREB/ATF family members. Galien et al. (1991) showed that ras oncogene activates the intracisternal A particle long terminal repeat (IAP LTR) promoter through a CRE. Moreover, by using forskolin, an activator of adenylyl cyclase leading to cellular cAMP level elevation, we established that in NIH 3T3 fibroblasts transfected with pON407.19R3 the same motif is responsive to the v-Ha-ras gene product and to cAMP. In addition, ras oncogenic proteins and PKA pathways appear to be agonistic in regulating MCMV IE gene CRE/ATF element enhancer activity, since forskolin treatment significantly increased pON407.19R3 transactivation by v-Ha-Ras protein and pON407.19R3 expression in cells stably transfected with amplified c-Ha-ras or point mutated c-Ki-ras.

A relationship between ras and PKA signalling pathways is still controversial. ras oncogene proteins, but not normal counterparts, induce terminal differentiation of PC12 cells into neuron-like cells by a mechanism independent of the cAMP/PKA pathway. Moreover, microinjection of anti-p21ras antibodies did not inhibit neurite formation by cAMP (Barbacid, 1987). By contrast, exposure of rat thyroid cells to oncogenic p21ras results in dedifferentiation due to down-regu-
lation of nuclear cAMP-dependent PKA (Avvedimento et al., 1991; Gallo et al., 1992). The ras and PKA pathways are also mutually antagonistic in regulating rat prolactin promoter activity (Conrad & Gutierrez-Hartmann, 1992). However, none of these authors has identified either the Ras response element (RRE) or the CRE, and this may explain their observation. According to Avvedimento et al. (1991) and Conrad & Gutierrez-Hartmann (1992), in fact, their promoter sequences do not contain any previously defined RREs, nor classical CRE or AP-1 sites. Our results, by contrast, focus on the identity between RRE and CRE sequences, suggesting that CREB/ATF proteins are the nuclear end-point of both oncogenic p21ras and cAMP pathways, at least with respect to MCMV IE gene enhancer CRE. In support to this conclusion, we demonstrated by EMSA that nuclear extracts from ras-transformed NIH 3T3 cells contain a much higher CRE-binding activity than normal NIH 3T3 fibroblasts, suggesting that the expression of oncogenic p21ras increases nuclear activated CREB/ATF proteins. This higher binding activity with extracts from ras-transformed NIH 3T3 cells seems to be generated by a post-translational modification as suggested by Northern blot analysis and resistance to cycloheximide treatment. In the former case the amount of CREB/ATF mRNA from ras-transformed cells was comparable to that found in NIH 3T3 cells. In the latter case treatment of the ras-transformed cells with cycloheximide for at least 18 h did not impair the amount of proteins binding to the CRE-binding site (data not shown). Taken as a whole, these findings underscore the likelihood that MCMV IE gene enhancer expression is modulated by oncogenic Ras proteins through the CREB/ATF-binding sites. However, because of the artificiality of the system any conclusion about the contribution of ATF proteins to CMV replication in vivo is still premature.

We thank GianPaoolo Dotto, Boston, MA, USA for the prsZip6 and pZipNeoSV plasmids, Dr Johannes Bos, Utrech, The Netherlands for the pRSV ras ASn-17 and pRSV ras(Leu-61; Ser-186) plasmids, Dr Edward S. Mocarski, Stanford, CA, for pON407 and pON407.19R3 plasmids. This work was supported by grants from the Italian National Research Council (P.F. 'A.C.R.O.' and 'F.A.T.M.A. ') and from the Associazione Italiana per la Ricerca sul Cancro. D.L. was supported by a fellowship from the Italian A.I.D.S. Project.

References


mapping of viral RNA synthesized at immediate early times after infection. *Journal of Virology* 50, 784–795.


(Received 6 July 1994; Accepted 21 November 1994)