Trans-activation of the mouse cytomegalovirus immediate early gene enhancer by ras oncogenes

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The ras gene family encodes 21K proteins that reside on the inner face of the plasma membrane and bind GTP and GDP with an equally high affinity. Cotransfection of NIH 3T3 cells with a mammalian expression vector containing a viral Harvey-ras (v-Ha-ras) cDNA, together with a plasmid (pCMVCAT) carrying the immediate early (IE) enhancer of the murine cytomegalovirus (MCMV) linked to the chloramphenicol acetyltransferase (CAT) reporter gene strongly stimulated CAT activity. Basal levels of pCMVCAT expression as well as trans-activation by v-ras plasmid were both inhibited by cotransfection of an expression vector containing the dominant inhibitory mutant gene Ha-ras Asn-17. This indicates that the p21V ~' protein is responsible for these activities. High pCMVCAT activation was also observed in cell lines carrying stably transfected ras oncogenes, activated by point mutation or amplification. To define the cis-acting DNA elements in the MCMV IE enhancer responsible for this trans-activation by p21ras protein, we constructed several plasmids containing the CAT gene under control of MCMV IE enhancers that were deleted in different regions. The CAT assays demonstrated that several sequences were responsive to p21 protein. These sequences are scattered throughout the IE enhancer, upstream of the transcription start site, and contain responsive elements that are homologous to the binding sites for cellular transcription factors such as NFkB, AP1, ATF and SP1. Activation of the p21ras protein may thus be one of the signals that regulate IE genes transcription during MCMV infection.

Introduction

The murine cytomegalovirus (MCMV), a betaherpes-virus, has been widely studied as a model of viral infection since many aspects of its biology, replication and pathogenesis resemble those of human cytomegalovirus (HCMV) (Hudson, 1979; Koszinowski et al., 1990; McDougall, 1990; Mocarski, 1991). It has a large DNA genome of 230 kbp encoding over 100 genes, grouped into immediate early (IE), early and late genes (Ebeling et al., 1983; Mercer et al., 1983). The three IE genes (IE1, IE2 and IE3) are expressed in the absence of prior viral protein synthesis, and their products are directly involved in the regulation of gene expression throughout infection (Keil et al., 1987; Manning & Mocarski, 1988; Messerle et al., 1992). Transcription of IE genes is regulated by a strong viral enhancer (Dorsch-Hasler et al., 1985; G. Gribaudo & S. Landolfo, unpublished results). This regulatory DNA element is composed of an array of repeated sequence motifs containing several consensus binding sites for cellular transcription factors such as nine NFkB binding sites, including seven that partially overlap with AP1 sites and one ATF binding site. In addition it also has DNA binding sites such as GC, CAAT and TATAA, which are recognized by basal transcription factors. IE gene transcription seems to be regulated by the interaction of cellular transcription factors with the IE enhancer.

Sambucetti et al. (1989) have characterized sequence-specific HCMV DNA-binding proteins that interact with some of the repeated elements of the IE enhancer during the IE phase of infection, and have shown that the most prominent induced factor corresponds to NFkB. However, other proteins were induced to bind to the enhancer repeated elements. These include one protein specific for a factor that might be AP1 and another specific for a protein that might be CREB/ATF. MCMV IE gene transcription may therefore also be under the control of some of the transcription factors described for the HCMV.

Products of proto-oncogenes [cellular (c-) oncogenes] seem to play an essential role in the control of transcription, replication and differentiation (Bishop, 1985a, b; Bortner et al., 1993; Varmus, 1984). A rapid and transient increase in the levels of c-oncogene RNAs for the c-fos, c-jun and c-myc genes has been noted between 20 and 60 min following the exposure of human embryonic fibroblasts to HCMV (Boldogh et al., 1991a).
Further studies indicated that the increase in c-oncogene RNA levels is the result of transcriptional activation of these genes (Boldogh et al., 1991b).

The biochemical and biological properties of the p21ras proteins encoded by the ras gene family [Harvey (Ha), Kirsten (Ki) and neuroblastoma], are altered after amplification or single mutations at defined positions (Barbacid, 1987; Downward, 1992; Marx, 1993). Through the protein kinase C (PKC) pathway, these oncogenes drive the induction of transcription of genes that have the cis-acting DNA element recognized by the transcription factor complex AP1–Jun in their promoter (Imler et al., 1988; Oku et al., 1990). In addition, PKC activation by p21ras proteins stimulates the binding of the NFκB transcription factor to its own binding site (Arenzana-Seisdedos et al., 1989). Indeed it has been reported that expression of oncogenic ras in human teratocarcinoma T2 cells induces partial differentiation and permissiveness for human cytomegalovirus infection (Shelbourn et al., 1989). From this it was concluded that p21ras-induced changes in cellular factors, associated with early events along the differentiation pathway of T2 cells, could be important for the expression of HCMV major IE genes and permissive infection.

Here we demonstrate that p21ras trans-activates the MCMV IE enhancer–promoter in mouse fibroblasts. Moreover, by mutant deletion analysis we show that different DNA sequences, scattered throughout the IE enhancer, and homologous to the binding sites of cellular transcription factors such as NFκB, AP1, ATF and SPI, are the responsive elements to the activated p21ras.

**Methods**

*Plasmids.* The pCMVCAT plasmid contains a 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. The pCMVLacZ plasmid contains the 1.2 kb *PstI–NdeI* segment positioned upstream from the bacterial β-galactosidase reporter gene. Deletion constructs p611CAT, p285CAT, p146CAT and p102CAT were prepared from pCMVCAT using internal restriction sites (Gribaudo et al., 1993). Plasmid pb250CAT contains the 450 bp *XhoI–EcoRI* segment from pCMVCAT cloned into the polylinker of the pBluescript SK(+) vector (Stratagene). The retroviral vector prasZip6 is derived from pZipNeo SV(x) and contains the viral (v)-Ha-ras gene cloned in the *BamHI* site. The prasZip6 and

**Fig. 1.** Dose-dependent stimulation of the MCMV IE enhancer by the v-Ha-ras oncogene, indicated by CAT activity. Thin-layer chromatographs (i) of the results of transfection of NIH 3T3 cells with pCMVCAT alone (2 μg; lanes 1) or 2 μg of pCMVCAT cotransfected with increasing amounts of prasZip6 (a) or pZipNeo (b) vectors: lanes 2, 2 μg; lanes 3, 4 μg; lanes 4, 8 μg; lanes 5, 12 μg. The percentage acetylation (see Methods) is given below each chromatograph and represented graphically in (ii) with equivalent lane numbers. The results were similar in at least four repetitions.
pZipNeoSV(x) vectors were kindly provided by G. Dotto (Boston, Mass., U.S.A.). The ability of prasZip6 to produce a transforming p21ras protein in NIH 3T3 cells has been shown elsewhere (Dotto et al., 1985). pRSVrasAsn-17 is an expression plasmid in which the dominant inhibitory mutant Ha-ras Asn-17 gene (with a serine to asparagine change in amino acid position 17 of the corresponding p21ras protein) is under the control of the Rous sarcoma virus long terminal repeat (LTR) element (Feig & Cooper, 1988; Cai et al., 1990; Medema et al., 1991). Plasmids pSV2CAT and TAR-ICAT, containing the simian virus 40 (SV40) and human immunodeficiency virus (HIV) enhancer-promoters respectively upstream from the CAT gene, have been described elsewhere (Gorman et al., 1982; Moch et al., 1992). pCH110 is a plasmid containing the β-galactosidase-coding sequences under the control of the SV40 early promoter (Hall et al., 1983).

Cell lines, transfections and CAT assays. NIH 3T3 cells (from the ATCC), 115/14 cells (a clone derived from NIH 3T3 cells carrying an amplified human c-Ha-ras kindly provided by Dr M. Barbaic, National Cancer Institute, Frederick, Md., U.S.A.), and 226-4-1 cells (a clone derived from NIH 3T3 cells transfected with a point-mutated human c-Ki-ras, kindly provided by Dr S. Ottolenghi, Milan, Italy) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum. All plasmids were purified twice by caesium chloride centrifugation. For a transient gene expression assay, cells were plated the day before transfection in growth medium at a density of 3 x 10^5 cells/60 mm diameter dish. At 4 h before transfection the medium was replaced by fresh medium. Cells were transfected by the calcium phosphate method and the amount of DNA in each precipitate was standardized to 12 μg with carrier DNA. After transfection, cells were cultured in DMEM containing 0.5% calf serum and harvested 48 h later. Cell extracts were prepared according to Gorman et al. (1982). All extracts were assayed after an appropriate dilution by incubation with acetyl coenzyme A (Pharmacia) and [14C]chloramphenicol for 30 min. Acetylated products were separated by TLC, visualized by autoradiography, excised from the plates and counted in a liquid scintillation counter. CAT activity was expressed as the percentage of added chloramphenicol converted into the 1'- and 3'-monoaacylated forms. In Results, CAT activity in the different cell lines is expressed as percentage conversion per unit of β-galactosidase to correct for differences in the efficiency of transfection and protein content in the various extracts.

Analysis of DNA and molecular hybridization. As a control for transfection, whole-cell DNA from transfected cells was harvested at various times after transfection (Sambrook et al., 1989). DNA (10 μg) was blotted onto nitrocellulose with a slot-blot device (Schleicher & Schuell). Plasmid DNAs were visualized by hybridization with a CAT-specific probe obtained by digesting pSV2CAT with HindIII and BamHI. DNA filters were prehybridized in 50% formamide, 5 x SSC, 50 mM-sodium phosphate pH 6.8, 4 x Denhardt's solution, 0.1% SDS and 250 μg/ml of sonicated salmon sperm DNA at 42 °C overnight. Hybridizations were carried out under the same conditions, using 2 x 10^6 c.p.m. of random primed DNA probe per ml for 18 h (sp. act. ranging from 1 x 10^6 to 3 x 10^6 c.p.m./μg). After hybridization, the filters were washed twice in 2 x SSC containing 0.1% SDS at room temperature for 30 min and twice in 0.1 x SSC containing 0.1% SDS at 60 °C for 30 min. They were then dried and subjected to autoradiography using Hyperfilm-MP (Amersham) with intensifying screens at -80 °C.

RNA isolation and RNase protection analysis. Total cellular RNA was isolated according to Chirgwin et al. (1979). RNA was analysed for CAT-specific transcripts by a quantitative RNase protection assay, using a T3 RNA polymerase-generated antisense RNA probe derived from the plasmid p250CAT. This was approximately 0.5 kb in size and covered about 304 nucleotides of the CAT gene and the IE1/3 leader sequence in pCMVCAT. It therefore allowed identification of correctly initiated transcripts from its MCMV IE1/3 promoter. RNA (30 μg) was hybridized with the probe overnight at 45 °C in 30 μl of hybridization buffer containing 80% formamide, 40 mM-PIPES pH 6.4, 400 mM-NaCl and 1 mM-EDTA. Single-stranded RNA was digested for 1 h at 30 °C with 2 μg/ml of RNase T1 (Pharmacia) in RNase buffer (300 mM-NaCl, 5 mM-EDTA and 10 mM-Tris–HCl pH 7.5). After ethanol precipitation, samples were analysed on denaturing 6% polyacrylamide gels. Correctly initiated transcripts from the MCMV IE1/3 promoter resulted in a signal of 304 nucleotides.

Results

Expression of the v-Ha-ras oncogene stimulates the activity of the MCMV IE enhancer

To test the ability of the expressed v-Ha-ras product to stimulate IE enhancer activity, we performed CAT assays with cell extracts obtained after cotransfection of the reporter pCMVCAT with increasing amounts of the expression vector prasZip6. CAT activity was enhanced at a reporter to effector molar ratio of 1:2 and the
Inhibition of MCMV IE enhancer stimulation by the Ha-ras Asn-17 mutant

Previous studies have shown that the activity of p21ras could be down-regulated by its dominant inhibitory mutants (Cai et al., 1990; Feig & Cooper, 1988; Medema et al., 1991). We therefore tested whether stimulation of the MCMV IE enhancer by v-Ha-ras could be blocked by expression of the dominant inhibitory mutant Ha-ras Asn-17. As shown in Fig. 3, cotransfection of NIH 3T3 cells with pCMVCAT and v-Ha-ras further induced CAT expression, whereas addition of Ha-ras Asn-17 revealed competition between v-Ha-ras and its mutant. Cotransfection of 4 µg or 6 µg of Ha-ras Asn-17 and 4 µg of v-Ha-ras blocked stimulation of pCMVCAT (from 81% to 12% CAT conversion), whereas 8 µg of Ha-ras Asn-17 almost completely prevented pCMVCAT activity. This suggests that the mutant blocks interaction of v-Ha-ras with the effector molecule(s) responsible for IE enhancer stimulation. The pRSVNeo vector lacks this mutant and did not impair activation of pCMVCAT when cotransfected instead of pRSVrasAsn-17 (data not shown).

A trans-acting factor involved in activation of the MCMV IE enhancer

Increasing the amount of v-Ha-ras reproducibly increased stimulation of CAT transcription by pCMVCAT, suggesting that ras overexpression directly or indirectly results in production of limiting factor(s) that interact with the MCMV enhancer. We therefore performed competition experiments, by including in the transfection a two- or fourfold molar excess (relative to pCMVCAT) or pCMVLacZ, which contains the IE enhancer driving the bacterial lacZ gene, or a fourfold molar excess of a vector containing the lacZ gene without the MCMV enhancer. As expected, the lacZ gene alone had no effect on pCMVCAT activity in the presence of prasZip6 (Fig. 4). In contrast, pCMVLacZ decreased v-Ha-ras stimulation of the IE enhancer by approximately 2.5-fold (from 40% to approximately 16% CAT conversion) at both molar ratios. Moreover, the pCMVLacZ vector itself did not significantly repress pCMVCAT activity at the molar ratios used in the experiment (data not shown). These results show that v-Ha-ras stimulation involves one or more trans-acting factors that can be titrated by MCMV enhancer sequences.

Stimulation of the MCMV enhancer–promoter by stably transfected amplified c-Ha-ras or point-mutated c-Ki-ras

NIH 3T3 cells and their transfected counterpart cell lines 115/14 and 226-4-1 (expressing the amplified c-Ha-ras and the point-mutated c-Ki-ras respectively) were trans-
fected with the plasmid pCMVCAT to determine whether the oncogenic p21ras proteins constitutively expressed in 115/14 and 226-4-1 cells could enhance expression of the CAT gene under the control of MCMV IE enhancer. As shown in Fig. 5 (lanes 1 to 3), transcription from the IE regulatory element was stimulated when pCMVCAT was transfected into 115/14 or 226-4-1 cells. This gave 95% or 88% CAT conversion respectively, compared to the 33% conversion observed in NIH 3T3 cells. As before, transfection of 8 μg pRSVrasAsn-17 significantly decreased stimulation of pCMVCAT in both 115/14 cells (reduction from 95% to 20% conversion) and 226-4-1 cells (88% down to 6% conversion), suggesting that it also blocks the interaction of the product of a ras oncogene activated by amplification or point mutation with effector molecule(s) responsible for MCMV IE enhancer stimulation (Fig. 5, lanes 4 to 6).

It should be noted that the efficiency of transfection, evaluated by molecular hybridization as described in Methods, is comparable for the three cell lines (data not shown).

Activation of different viral promoters by v-Ha-ras expression

The specificity of the effects of ras expression was assessed by examining the influence of v-Ha-ras on the activities of other viral promoters. In addition to pCMVCAT, cotransfection of v-Ha-ras enhanced the activity of promoter–CAT constructions with the HIV LTR and SV40 promoters (Fig. 6). The possibility that v-Ha-ras activity is directed to promoter sequences is proven by the lack of activation of the pSVOCAT construct, from which the SV40 promoter is missing. These results demonstrate that v-ras is a rather promiscuous stimulator of viral regulatory sequences.

Identification of the sequence motifs of the MCMV IE enhancer responsive to v-Ha-ras

The MCMV IE enhancer (NdeI fragment, 1200 nucleotides long) contains nine types of repetitive sequence elements that are homologous to NFκB binding sites. Seven of these partially overlap with API binding sites (G. Gribaudo & S. Landolfo, unpublished results). In
HCMV these repetitive elements contribute to the constitutive activity of the IE enhancer (Sambucetti et al., 1989). Moreover, consensus sequences for other transcription factors such as ATF/CREB, IRF-1 and SP1 are located immediately upstream from the transcription start site (Keil et al., 1987; G. Gribaudo & S. Landolfo, unpublished results). In an initial attempt to locate ras-responsive elements in the MCMV IE enhancer, a series of 5'-deletion constructs was cotransfected with prasZip6 and CAT activities were determined 48 h later (Fig. 7). Removal of six NFkB/API binding sites (between nucleotide positions −1200 and −611) reduced the responsiveness to p21*v-ras protein from 5-2-fold (pCMVCAT) to 3-2-fold (p611CAT). Removal of the remaining three sites (positions −611 to −285) reduced the response to a 2.5-fold increase. Deletion of 139 nucleotides containing a CREB/ATF binding site (from −285 to −146; p146CAT) virtually blocked responsiveness (reduction to 1.5-fold), suggesting that this cis-acting DNA element may also be important for ras trans-activation. Finally the shortest construct used (p102CAT), which contains an SP1 binding site and the TATAA element, displayed very low but reproducibly detectable levels of responsiveness (1.9-fold).

It should be also noted that basal CAT activity increased from 13% (pCMVCAT) to 35% (p285CAT) suggesting that DNA sequences with a negative effect on basal transcription of the MCMV IE enhancer may be also present between nucleotide positions −285 and −1200.

Discussion

The present results show that transiently transfected v-Ha-ras, permanently transfected amplified c-Ha-ras or point-mutated c-Ki-ras efficiently trans-activate expression of the MCMV IE enhancer located between the IE1/3 and IE2 promoters. It controls the expression of IE polypeptides required for efficient activation of the promoter of the next temporal class, the early genes (Dorsch-Hasler et al., 1985; Keil et al., 1987). Therefore these proteins play a key role in the progression of the MCMV lytic cycle, and their cellular levels may be...
critical in determining whether MCMV replication proceeds.

Activation of MCMV enhancer functions by p21ras was rather strong and dose-dependent. At a molar ratio of reporter to trans-activator plasmid of 1:4, an increase in CAT conversion from 16% to 84% (greater than fivefold stimulation) was detected. A slightly lower but still very significant stimulation was observed when the reporter plasmid was introduced into 115/14 and 226-4-1 cell lines stably expressing the activated p21ras. Induction could also be found when RNA was analysed, suggesting that the mechanism responsible is at the transcriptional level. To prove definitively that activation is affected by the p21ras protein, we used the Asn-17 dominant inhibitory mutant of c-Ha-ras, which has an asparagine instead of a serine in the p21Ha-ras protein (Feig & Cooper, 1988; Cai et al., 1990; Medema et al., 1991). In NIH 3T3 cells, cotransfection of Ha-ras Asn-17 plasmid with the v-ras expression vector completely blocked the activity of the IE enhancer. Stimulation by the amplified c-Ha-ras gene or point-mutated c-Ki-ras gene was also completely inhibited by cotransfection of Ha-ras Asn-17 into the 115/14 or 226-4-1 cell lines. These results indicate that p21ras trans-activates the MCMV IE enhancer.

Previous work has established that the p21ras protein stimulates the activity of viral enhancers such as those of HIV (Arenzana-Seisdedos et al., 1989), SV40 (Chin et al., 1992), Rous sarcoma virus (Chin et al., 1992) and polyoma virus (Waslyk et al., 1987; Satake et al., 1988), as well as enhancers of cellular genes (Owen & Ostrowski, 1987; Chin et al., 1992; Bortner et al., 1993). The results reported in this study extend these findings to the MCMV IE enhancer. The proteins encoded by ras genes bind guanine nucleotides, possess an intrinsic GTPase activity and are associated with the inner surface of the plasma membrane (Barbacid, 1987; Downward, 1992; Marx, 1993). They are similar to the G proteins, which control the transduction of a wide array of extracellular signals from the plasma membrane to the nucleus. Therefore, it is not surprising to find that the p21ras protein is a rather promiscuous stimulator of many different promoters.

To locate the ras-responsive sequences, we prepared a series of IE enhancer deletions and cloned the resulting fragments into CAT reporter vectors. The CAT activities of these constructs demonstrated that overall enhancer activity was the result of the coordinated interactions of transcription factors with each other and with their cognate binding sites. However the relative contribution of each factor to the response of the enhancer to p21ras does not appear to be equivalent. Removal of the nine repetitive NFkB binding sites, seven of which partially overlap AP1 binding sites, significantly reduced IE enhancer trans-activation by p21ras. Involvement of NFkB/AP1 binding sites in HMCV enhancer activation has been shown in two other systems; Sambucetti et al. (1989) reported that these motifs are the responsive elements for trans-activation by the major IE protein, and Moch et al. (1992) demonstrated trans-activation by p40ras of the human T cell leukaemia virus type 1 via repetitive elements homologous to target sites for transcription factor NFkB. A direct link between NFkB activation and p21ras has been provided by Dominguez et al. (1993). Microinjection of either p21ras or phospholipase C into oocytes from Xenopus laevis promoted a significant translocation to the nucleus of an NFkB-like activity. Addition of a peptide Z, containing a sequence identical to that of the z isofrom of PKC dramatically inhibited this activation of NFkB by p21ras.

A contribution to p21ras trans-activation was also made by a cAMP-responsive cis-acting DNA element containing a CREB/ATF binding site (from positions -285 to -146), the removal of which drastically reduced basal activity (from 35% to 7.6% CAT conversion) and further impaired trans-activation by p21ras of the MCMV IE enhancer in NIH 3T3 cells. Kedar et al. (1990) have also found that in NIH 3T3 cells the b- polymerase gene promoter is trans-activated by p21ras via a CREB/ATF element. Galien et al. (1991) have observed that the ras oncogene activates the intracisternal A particle (IAP) LTR promoter through a cAMP-responsive element. Finally a low but significant level of responsiveness to p21ras was still observed with the shortest fragment containing the SPI binding site and the TATAA element, suggesting that the SPI element may contribute to ras oncogene responsiveness. Similar conclusions have been drawn for the IAP and HIV LTRs, where removal of SPI binding sites reduced activation by p21ras (Arenzana-Seisdedos et al., 1989; Galien et al., 1991).

Taken as a whole, the results reported in this study demonstrate that ras genes trans-activate the MCMV IE enhancer and provide new insights into the molecular mechanisms exploited by MCMV during the early phases of infection.

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