Human cytomegalovirus infection induces cellular thymidylate synthase gene expression in quiescent fibroblasts

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Introduction

Human cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus that generally causes benign or asymptomatic infections. However, in immunocompromised hosts, such as transplant recipients and AIDS patients, and in immunologically immature individuals, it can cause life-threatening diseases. Furthermore, HCMV is the major viral cause of birth defects in humans (Fortunato & Spector, 1999; Griffith, 2000; Mocarski & Courcelle, 2001). Its success as an opportunistic pathogen depends largely on its ability to establish latent lifelong infections, counteract host defence mechanisms and replicate in a wide variety of cells and tissues, including differentiated, post-mitotic cells (Hengel et al., 1998; Hengel & Weber, 2000; Lalani et al., 2000). The absence of functional virus-encoded dNTP-synthesizing enzymes such as thymidine kinase, dihydrofolate reductase (DHFR), thymidylate synthase (TS) and ribonucleotide reductase (Chee et al., 1990; Rawlinson et al., 1996) compels HCMV to depend mainly on host-cell metabolism to ensure a sufficient supply of dNTPs for its DNA replication. However, these enzymes are expressed at very low levels in post-mitotic cells. To explain this apparent paradox, it has been hypothesized that HCMV infection of quiescent cells induces biochemical pathways that are required for DNA replication, including enzymes involved in nucleotide metabolism. Subsequently, to gain selective access to the newly synthesized dNTPs, the virus prevents replication of the host cell DNA by blocking cell-cycle progression at the beginning of the S phase (Fortunato et al., 2000; Wiebusch & Hagemeier, 2001).

TS is an essential enzyme that catalyses the de novo biosynthesis of thymidylic acid (dTMP) by the reductive transfer of the methylene group from 5,10-methylenetetrahydrofolate to the 5 position of the substrate, deoxyuridylic acid, to form dTMP and dihydrofolate. TS activity is associated with cell proliferation and its inhibition in rapidly proliferating cells leads to inhibition of DNA synthesis and cell death. For this reason, TS is an important target enzyme in cancer chemotherapy. Both substrate and cofactor analogues have
been widely used as anti-neoplastic agents. TS mRNA and enzyme levels are very low in quiescent cells, but increase at the G1–S border during a serum-induced transition from the resting (G0) phase to the S phase (Jenh et al., 1985; Ayusawa et al., 1986). TS mRNA content is primarily controlled at the post-transcriptional level in growth-stimulated cells, since nuclear run-on transcription assays have revealed that TS gene transcription in human and mouse cells does not change during the G1–S transition (Ayusawa et al., 1986; Ash et al., 1995). Studies with transfected TS minigenes have shown that both the TS essential promoter region and an intron in the transcribed region are required for proper S-phase regulation, suggesting that some form of communication between the TS promoter and the RNA processing machinery may be important for regulation of TS mRNA production in growth-stimulated cells (Kaneda et al., 1992; Takayanagi et al., 1992; Ash et al., 1993, 1995; Johnson, 1994; Ke et al., 1996).

We have been studying the effects of CMV infection on the expression of enzymes involved in dTMP biosynthesis, since elucidation of the molecular mechanisms of virus-mediated regulation could lead to the design of molecules with antiviral activity. In the murine system, we have observed that murine CMV (MCMV) replication in quiescent NIH 3T3 cells increases the expression of enzymes involved in dTMP biosynthesis, namely folic acid synthetase (TPS), DFR and TS (Lembo et al., 1998; Gribaudo et al., 2000; Cavallo et al., 2001). Here we report that HCMV infection of quiescent human embryonic lung fibroblasts induces the expression of TS mRNA and protein, and that TS activity is required for efficient HCMV DNA synthesis. Regulation of TS gene expression takes place at the transcriptional level, and several DNA elements within the TS promoter are necessary for its increase. Lastly, HCMV and MCMV transactivate the corresponding cellular TS promoters by different mechanisms.

Methods

■ Cells and culture conditions. Low-passage human embryonic lung fibroblasts (HEL) were grown in minimal essential medium (MEM) (Gibco/BRL) supplemented with 10% foetal calf serum (Gibco/BRL). NIH 3T3 murine fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum. Quiescent cells (arrested in G0/G1 phase) were obtained by incubating confluent monolayers for 48 h in medium containing 0.5% serum (low-serum medium). Flow cytometry demonstrated that more than 90% were growth-arrested.

■ Virus preparation and infections. HCMV (strain AD169; ATCC VR-538) was passaged in HELF cells. MCMV (strain Smith; ATCC VR-194) was propagated in NIH 3T3 cells. Both viruses were prepared in low-serum medium. Quiescent HELF or NIH 3T3 cells were infected with HCMV or MCMV, respectively, at an m.o.i. of 5, unless otherwise stated. Mock-infected control cultures were exposed to an equal volume of mock-infecting fluid. At the end of the adsorption period, which was defined as 0 h post-infection (p.i.), the low-serum medium removed before infection was restored to the cultures to avoid stimulation due to the addition of fresh serum growth factors. UV-inactivated HCMV was prepared with one pulse of 12 J/cm² UV light. The UV-inactivated HCMV did not replicate and produce detectable levels of immediate-early (IE) gene products.

For infection in the presence of cycloheximide (CHX), quiescent HELF cells were pretreated for 1 h with 100 µg/ml CHX before infection and then maintained in the medium throughout the infection. For infection in the presence of phosphonoformic acid (PFA), PFA was added to the medium at a final concentration of 200 µg/ml at the time of infection. RNA was then harvested at 24 (CHX) or 48 (PFA) h p.i.

■ Northern blot analysis. Total RNA or poly(A)⁺ mRNA was fractionated on a 1% agarose, 2 M formaldehyde gel and then blotted on to a nitrocellulose membrane (Hybond-C-extra; Amersham). The filters were hybridized to random-primed radiolabelled probes corresponding to the human TS cDNA (Ayusawa et al., 1984) and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA as a control.

■ Plasmids. Plasmids containing the indicated regions of the human TS promoter (numbered relative to the A of the translational start codon; see Fig. 3) driving expression of the luciferase indicator gene were constructed in the following manner. Plasmids pH977TS 161/–98 Luc, pH977TS 161/–141 Luc and pH977TS 140/–98 Luc were constructed by inserting the HindIII–Nhel fragment of the corresponding TS minigene (Dong et al., 2000) into the HindIII and Nhel sites of pGL3 basic-EP, which is the same as pGL3 basic (Promega) except that the multiple cloning sequence was modified by the insertion of a synthetic DNA fragment to give a new multiple cloning sequence (Kml, HindIII, SacI, XbaI, Nhel, Smal, BgIII). Plasmid pH977TS 161/–141 Sp1 mut. Luc was constructed by inserting synthetic oligonucleotides with the desired nucleotide changes into the HindIII and Nhel sites of pGL3 basic-EP. Plasmid pH977TS 243/–30 Luc was constructed by PCR-amplifying the indicated region of the human TS promoter and inserting it into the Nhel site of pGL3 basic that had been modified by deleting 13 nucleotides between the BgIII and HindIII sites (Dong et al., 2000). The promoter regions of all constructs were sequenced to verify the presence of the desired promoter regions and/or mutations and the absence of undesired changes. Plasmids pH977TS 243/–70 Luc, pH977TS 243/–98 Luc and pH977TS 243/–141 Luc were as described previously (Dong et al., 2000). pTSWTGL3 was constructed by inserting the mouse TS promoter and 5′ flanking region from the Xbal site at –985 to an engineered BgIII site at –11 into the Nhel and BgIII sites of pGL3 basic. pTSWTGL3 (–113) was the same as pTSWTGL3, except that the potential E2F element just upstream from the mouse TS essential promoter region (–113GATTCTTGGCGGCC–163) was mutated to –113GATTGCTAGGCC–163 (Geng & Johnson, 1993). pSGL2 and pSGL86 contained cDNAs corresponding to the HCMV IE1 72 kDa and IE2 86 kDa proteins, respectively. Their expression was driven by the simian virus 40 (SV40) early promoter (Kucher et al., 1993). p729CAT contained the CAT reporter gene driven by the HCMV UL112/113 early promoter (Staprans et al., 1988).}

■ Transient transfection and reporter gene assays. Cells were transfected by the calcium phosphate procedure, as previously described (Gribaudo et al., 2000). The transfected cells were washed twice with medium and incubated in MEM supplemented with 0.5% foetal calf serum (low-serum medium) for 48 h. Luciferase and CAT activity were assayed as previously described (Gribaudo et al., 1995). Reporter gene activity was normalized to the amount of plasmid DNA introduced into recipient cells, as previously described (Abken & Reifenrath, 1992).

■ Immunoblotting. Whole-cell protein extracts were prepared as previously described (Gribaudo et al., 2000). Proteins were separated by SDS–PAGE and then transferred to Immobilon-P membranes (Millipore). Filters were immunostained with the mouse anti-TS human mAb (Johnston et al., 1991) (clone TS106; Labvision Neomarkers), the mouse
anti-HCMV IEA mAb (E13 clone; Argene Bio-Soft) recognizing both the IE1 72 kDa and IE2 86 kDa proteins, or the mouse anti-actin mAb (Boehringer) at room temperature for 1 h. Immune complexes were detected with sheep anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham) and visualized by enhanced chemiluminescence (Super Signal; Pierce).

**Inhibition of viral DNA synthesis.** To evaluate the inhibition of HCMV DNA synthesis, cells were grown to confluence, incubated in low-serum medium for 48 h and infected with HCMV at an m.o.i. of 1. The infected cultures were treated in low-serum medium with different concentrations of N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid (raltitrexed, Tomudex, formerly ZD1694; Zeneca). At 96 h p.i. cells were harvested and total DNA was isolated. Dot-blot hybridization was then performed using 32P-labelled probes prepared from the PstI–BamHI DNA fragment of the HCMV IE1 gene (exon 4) and mouse G3PDH cDNA. The membranes were autoradiographed and the hybridization signals were quantified using a phosphomager.

**Results**

**Inhibition of cellular TS activity blocks HCMV DNA synthesis**

To determine whether cellular TS activity is required for HCMV replication, we analysed the effects of raltitrexed, a TS inhibitor, on HCMV DNA synthesis. Quiescent HELF cells were infected at an m.o.i. of 1. After virus adsorption, raltitrexed was added at concentrations ranging from 10⁻⁵ to 10 µM. After 96 h p.i., viral DNA levels were measured by dot-blot analysis using a radiolabelled viral DNA probe. As shown in Fig. 1, addition of raltitrexed greatly reduced HCMV DNA levels. The drug EC₅₀ was 0.03 µM, while its 50% cytotoxic concentration was > 10 µM. These results demonstrated that inhibition of TS activity impairs HCMV DNA synthesis in quiescent cells and that this inhibition is not due to generalized cellular toxicity.

**HCMV infection upregulates TS gene expression in quiescent HELF cells**

Having established that TS activity is required for efficient HCMV DNA synthesis in quiescent fibroblasts, we analysed TS gene expression in serum-arrested HELF cells during productive HCMV replication. Poly(A)⁺ RNA was prepared at different times p.i., and TS mRNA content was measured by Northern blot analysis. As shown in Fig. 2(A), HCMV infection increased TS mRNA levels by more than eightfold by 24 h p.i. compared with mock-infected cells.

To identify which phase of viral gene expression was temporally associated with the upregulation of TS mRNA, we infected quiescent fibroblasts in the presence of CHX to block protein synthesis so that we could monitor the effect of the binding and entry of HCMV on TS expression. Alternatively, the infection was carried out in the presence of PFA to inhibit viral DNA replication and restrict viral gene expression to IE and E genes. Thereafter, total RNA was prepared at 24 (CHX) or 48 (PFA) h p.i., and TS mRNA content was measured by Northern blot analysis. As shown in Fig. 2(B), the increase in TS mRNA content occurred only in the presence of PFA treatment, indicating that TS gene expression was regulated by newly expressed IE and/or E proteins, rather than by the binding and entry of the virus particle or by the action of virion proteins within the infected cell.

To determine whether viral infection would also lead to an increase in TS protein, cell extracts were prepared at different times p.i. and analysed by immunoblotting with an anti-TS mAb. As shown in Fig. 2(C), TS protein was undetectable in mock-infected cells, started to increase at 24 h.p.i. in infected cells and peaked at 72 h p.i. In contrast, it was undetectable in cells infected for 48 h with UV-inactivated HCMV, further demonstrating that newly synthesized viral proteins are required to induce cellular TS expression. The efficacy of UV treatment was confirmed by the lack of viral IE gene expression. These results confirmed that the increases in TS mRNA and protein levels during HCMV replication in quiescent HELF cells depend on viral gene expression.

**HCMV infection transactivates the TS promoter in quiescent HELF cells**

To determine whether the increase in TS mRNA was a consequence of stimulation of TS promoter activity, we analysed the effects of HCMV infection on the expression of a transiently transfected luciferase reporter gene driven by the human TS promoter. HELF cells were transiently transfected
expression of an indicator gene (Dong et al., 2000). Transfected cells were serum-starved and then infected with HCMV or UV-inactivated virus, mock-infected or stimulated with serum. At different times p.i., cells extracts were prepared and assayed for luciferase activity. As shown in Fig. 4, HCMV infection led to a sixfold increase in luciferase activity by 12 h, a 26-fold increase by 24 h and an 18-fold increase by 48 h p.i. UV-inactivated virus did not affect luciferase activity, demonstrating that HCMV-mediated transactivation requires de novo viral protein expression. As expected, serum stimulation did not increase the reporter activity since, as previously observed, the S-phase-specific human TS expression requires the presence of both the promoter region and a spliceable intron (Takayanagi et al., 1992). Taken together, these results demonstrate that HCMV regulates TS gene expression primarily at the transcriptional level using mechanisms that are different from those employed by growth-stimulated cells.

Transactivation of the human TS promoter by HCMV requires the essential promoter region and sequences between −98 and −70

To determine the locations of nucleotide sequences that are important for the HCMV-mediated regulation of TS promoter activity, we first performed a promoter deletion analysis. We have previously shown that the sequences responsible for high-level promoter activity of the human TS gene are located between −243 and +30 relative to the AUG start codon (Dong et al., 2000). Our first goal was to establish the 3′ boundary of the sequences that are important for virus induction of the TS promoter. To accomplish this, the 5′ end of the promoter region was kept at position −243 and the 3′ boundary was varied from +30 to −141 (Fig. 3). HELF cells were transfected with the different constructs, serum-starved and then infected with active or UV-inactivated HCMV. At 24 h p.i., cell extracts were prepared and assayed for luciferase activity. The results (Fig. 5) show that deletion of the sequences between +30 and −70 had little effect on HCMV-mediated transactivation. In contrast, deletion of sequences downstream from −98 led to a threefold reduction in reporter activity, suggesting that a positive-acting promoter element regulating TS gene expression in response to HCMV infection is located between −98 and −70. Further deletion of sequences downstream of −141 (pTS −243/−141 Luc) did not significantly reduce the luciferase activity relative to that of pTS −243/−98 Luc.

To establish the 5′ boundary of the regulatory elements necessary for virus stimulation of TS promoter activity, we constructed two more plasmids, pTS −161/−98 Luc and pTS −140/−98 Luc, which retained the region of the TS promoter containing most of the transcription initiation sites (Fig. 3). As shown in Fig. 5, deletion of the sequences between −243 and −161 had no effect on the virus induction of promoter activity since both plasmids were transactivated eightfold by virus infection. However, deletion of sequences

with the indicator plasmid pHS −243/+30 Luc, which corresponds to an intronless luciferase gene driven by the promoter and adjacent sequences of the human TS gene (Fig. 3). These promoter sequences are sufficient to drive high-level

Fig. 2. HCMV infection stimulates TS gene expression in quiescent fibroblasts. (A) HCMV infection increases TS mRNA content. HELF cells were serum-starved for 48 h and then infected with HCMV (m.o.i. of 5) or mock-infected. Poly(A)+ RNA was isolated and purified at the indicated times p.i. and 2 µg was analysed by Northern blot as described in Methods. The filter was sequentially hybridized with human TS and G3PDH radiolabelled probes. Hybridization signals were quantified by a phosphorimager. (B) HCMV immediate-early and early gene expression are required to increase TS mRNA levels. Quiescent HELF cells were infected with HCMV (m.o.i. of 5) in the presence of CHX or PFA or were left untreated. Total RNA was isolated and purified at 24 (CHX-treated) or 48 (PFA-treated) h p.i., and 20 µg of total RNA was analysed by Northern blot. The filter was sequentially hybridized with human TS and G3PDH radiolabelled probes. Hybridization signals were quantified by a phosphorimager. (C) HCMV increases TS protein levels. Quiescent HELF cells were infected with HCMV or UV-inactivated HCMV, or mock-infected. Total cell extracts were prepared at the indicated times p.i. and fractionated by SDS–PAGE (50 µg protein per lane). Proteins were analysed by immunoblotting with the anti-TS mAb or with the anti-IEA mAb, as described in Methods. Actin immunodetection with a mAb was used as an internal control. Cell extracts were prepared from mock-infected cells (Mock), from cells infected with HCMV for 24, 48 or 72 h, and from cells infected with UV-treated HCMV for 48 h (48 h UV).
HCMV stimulates thymidylate synthase expression

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Fig. 3. Sequence of the human TS promoter region. The sequence of the human TS gene (Takeishi et al., 1989) between -250 and +51 relative to the A of the translational start codon (***) is shown. The sequence of the TS promoter region analysed in the present study is identical to the published sequence except that the boxed region (one of the repeated sequences) is missing and there is a G→A change at -39. The differences are presumably the result of sequence polymorphisms and do not appear to affect TS promoter activity (Dong et al., 2000). Direct and inverted repeat sequences are indicated by arrows. Potential binding sites for several transcription factors are shown below the sequence. Sp1 mutation indicates the nucleotide changes (underlined) that inactivated the Sp1 element.

Fig. 4. HCMV infection transactivates the human TS promoter in quiescent cells. HELF cells were transiently transfected with 2 µg of the indicator construct phTS -243/+30 Luc (Fig. 3) and 10 µg carrier DNA (pBluescript SK). After 18 h, cells were washed and growth-arrested in low-serum medium for 48 h. Thereafter, transfectants were infected with HCMV, UV-inactivated HCMV, mock-infected or serum-stimulated. Total cytoplasmic extracts were isolated at the indicated times p.i. and assayed for luciferase activity. Reporter gene activity was normalized to the amount of plasmid DNA introduced into recipient cells as determined by dot blot analysis. The normalized luciferase activity is expressed as fold induction relative to basal levels measured in cells transfected with phTS -243/+30 Luc and then mock-infected, which was set at 1. The experiment was repeated twice and representative results are shown.

between -161 and -140 (phTS -140/-98 Luc) almost completely prevented virus induction of promoter activity. These results suggested that the sequences between -161 and -140 are important for stimulation of TS promoter activity in response to HCMV infection. To confirm this conclusion, the 20-nucleotide region was inserted upstream of the luciferase gene to obtain the phTS -161/-141 Luc plasmid. As shown in Fig. 5, HCMV infection led to a more than fivefold increase in luciferase activity with this plasmid. Previous studies have demonstrated that the sequence between -161 and -141 is highly conserved between the human and mouse TS promoter (Horie & Takeishi, 1997) and is the minimum essential region for TS promoter activity in proliferating cells (Dong et al., 2000).

We have recently observed that MCMV infection transactivates the mouse TS promoter and that this transactivation was abolished by inactivating the functional E2F element immediately upstream of the mouse TS essential promoter region. These observations indicated an E2F-dependent mechanism for induction of the mouse TS promoter in response to
Fig. 5. Human TS promoter deletion analysis. HELF cells were transiently transfected with 2 µg of the indicated phTS constructs (Fig. 3) and 10 µg carrier DNA (pBluescript SK). After 18 h, cells were washed and growth-arrested in low-serum medium for 48 h. Thereafter, transfectants were infected with HCMV or UV-irradiated HCMV or were mock-infected. Total cytoplasmic extracts were isolated at 24 h p.i. and assayed for luciferase activity. Reporter gene activity was normalized to the amount of plasmid DNA introduced into recipient cells as determined by dot-blot analysis. The normalized luciferase activity is expressed as fold induction relative to basal levels measured in cells transfected with the different phTS constructs and then mock-infected, which was set at 1. The data shown are the averages of three experiments ± SEM (error bars).

MCMV infection (Gribaudo et al., 2000). The presence of two potential E2F binding sites within the inverted repeat element between −128 and −98 of the human TS promoter (Fig. 1) raised the possibility that these elements may contribute to the regulation of human TS promoter activity in response to HCMV infection. We recently found that recombinant E2F is able to bind to the human TS promoter region between −134 and −105 in EMSA assays (data not shown). Unexpectedly, we found that the E2F elements were not necessary for the HCMV-mediated increase in TS promoter activity since a similar increase in luciferase activity was observed with phTS −161/−141 Luc, which lacks the E2F elements, as with phTS −161/−98 Luc, which retains the E2F elements (Fig. 5).

Taken together, these results indicate that the −98/−70 element and the minimal essential TS promoter segment play important positive roles in the response of the human TS promoter to HCMV infection. Furthermore, in contrast to our earlier observations in the murine system, stimulation of the
human TS promoter by HCMV does not appear to involve an E2F-dependent mechanism.

**HCMV IE1 protein transactivates the human TS promoter**

We have previously observed that the expression of MCMV IE1 protein activates the mouse TS promoter and that this activation is dependent on the integrity of the E2F element (Gribaudo et al., 2000). To examine the potential role of the HCMV major immediate-early proteins in the regulation of the human TS promoter, we co-transfected an expression vector for the IE1 72 kDa or the IE2 86 kDa protein with different human TS constructs into HELF cells. The major HCMV IE proteins were expressed under the control of the early SV40 promoter to avoid the potential complication of negative autoregulation of the HCMV IE promoters by the IE2 protein (Klucher et al., 1993). Fig. 6 demonstrates that only the IE1 product transactivated the human TS promoter, although to a lesser extent than that observed with virus infection. The magnitude of activation was 4.5-fold for phTS —243/+30 Luc, 3.5-fold for phTS —243/—70 Luc, threefold for phTS —243/—98 Luc and 2.5-fold for phTS —243/—141 Luc relative to the control. None of the other constructs analysed was significantly transactivated by IE1 (data not shown).

The ability of the IE1 or the IE2 constructs to express functional proteins was verified by co-transfection with the p729CAT indicator plasmid containing the HCMV UL112/113 early promoter. As previously reported (Klucher et al., 1993), the cDNA construct expressing the IE2 protein transactivated the early promoter, whereas when both the IE1 and IE2 constructs were transfected together, a small but reproducible synergistic effect was observed (data not shown).

**Differential regulation of mouse and human TS promoters by CMV infection**

As indicated above, activation of the mouse TS promoter by MCMV is mediated through the E2F site in the mouse TS promoter (Gribaudo et al., 2000), whereas activation of the human TS promoter by HCMV does not depend on the presence of the E2F sites. This discrepancy prompted us to see whether HCMV regulates the mouse TS promoter by a different mechanism than that used by MCMV. We reasoned that if HCMV were able to control the mouse TS promoter through the E2F element, differences in the architecture of the regulatory elements between the human and mouse TS promoter might account for the different observations. However, if the mouse TS promoter were not regulated in the same manner, this would indicate that the two viruses exploit different molecular mechanisms.

To explore these possibilities, HELF cells were transiently transfected with the pTSWTGL3 and pTSWTGL3(—110) constructs, serum-starved and then infected with HCMV for 24 h. The pTSWTGL3 contains the luciferase gene driven by the wild-type promoter and 5’ flanking region of the mouse TS gene. pTSWTGL3(—110) is the same as pTSWTGL3 except that the E2F binding site at —110 (TCTGGCGG) has been mutated to TCGCTAGC (Geng & Johnson, 1993). Fig. 7(A)
shows that pTSWTGL3 and pTSWTGL3(-110) were trans-activated by HCMV infection by fivefold and by more than 11-fold respectively. Surprisingly, inactivation of the E2F binding site led to a more pronounced response to HCMV infection than that measured with the wild-type construct.

We then performed the reciprocal experiment. Murine NIH 3T3 cells were transiently transfected with the human TS promoter constructs phTS — 161/ — 98 Luc or phTS — 161/ — 141 Luc, serum-starved and then infected with MCMV for 24h. As shown in Fig. 7(B), MCMV infection increased the luciferase activity by about twofold in cells transfected with phTS — 161/ — 98 Luc, which retains the E2F elements. However, the increase was almost completely abolished in cells transfected with phTS — 161/ — 141 Luc, which lacks the E2F elements. This indicates that MCMV is able to regulate (at least in part) the human TS promoter through an E2F-dependent mechanism. Taken together these results confirm that MCMV and HCMV activate the TS promoter by different mechanisms.

**Discussion**

During natural infection, HCMV productively replicates in terminally differentiated epithelial, endothelial and smooth muscle cells despite the absence of virus-encoded enzymes involved in the de novo biosynthesis of dTMP. In addition, infection of fibroblasts with HCMV leads to an expansion of cellular dTTP pools (Suzuki et al., 1985; Biron et al., 1986). These observations suggest that HCMV is able to stimulate the expression of cellular enzymes involved in the synthesis of dTMP. In the present study, we show that HCMV infection of quiescent fibroblasts stimulates the transcription of the cellular TS gene and that TS activity is required for efficient viral DNA synthesis. The dependence of HCMV replication on TS activity is supported by the results obtained with the folate analogue raltitrexed, a powerful TS inhibitor that prevents DNA synthesis and repair by blocking the de novo synthesis of dTMP. We have shown that raltitrexed inhibited HCMV DNA synthesis in quiescent cells, indicating that induction of TS activity is required for viral DNA replication. In line with this, we have previously observed that raltitrexed had little effect on MCMV and HCMV IE gene expression, although it strongly inhibited late gene expression as well as the replication of the virus in quiescent cells (Lembo et al., 2000). Taken together, these observations suggest that raltitrexed inhibits HCMV replication by blocking viral DNA synthesis. We also found that raltitrexed is far more detrimental to CMV DNA replication than to the survival of uninfected quiescent cells. This is probably due to the fact that TS is present at very low levels and is irrelevant for the survival of quiescent cells (Jenh et al., 1985; Johnson, 1994), but is required for viral DNA replication. These observations suggest that drugs that target TS may be highly effective in treating CMV infections.

Several laboratories have recently shown that HCMV infection blocks cell-cycle progression and cellular DNA replication while activating the cellular DNA synthetic machinery. These alterations create a favourable environment for high-level viral DNA replication, since they provide the viral DNA polymerase with dNTPs while preventing competition by cellular DNA synthesis (Fortunato et al., 2000; Flemington, 2001). Activation of host genes important for DNA synthesis has been reported to depend on binding of CMV to the cell surface or expression of viral IE proteins (Fortunato et al., 2000). Here we have shown that: (i) TS mRNA upregulation was prevented in the absence of protein synthesis; and (ii) inactivation of HCMV by UV exposure abolished both the induction of TS protein and the activation of the TS promoter. These results indicate that viral IE and E gene expression, rather than interaction of viral particles with the cell surface, is required to stimulate TS gene expression. Furthermore, we have shown that the product of the IE1 gene partially transactivated the TS promoter and that the virus-dependent activation of TS transcription coincided with expression of the IE1 protein. However, the difference between the levels of transactivation of the human TS promoter in HCMV-infected and in IE1-transfected cells suggests that viral IE and/or E gene products other than IE1 contribute to the overall response of the human TS promoter to virus infection.

To map the sequences important for activation of the TS promoter by HCMV, we assessed its ability to stimulate TS promoters modified by deletions or point mutations. Two positive-acting elements were identified. The first was located between — 70 and — 98 (relative to the AUG start codon) and corresponded to one of the direct repeats of a 28-nucleotide G/C-rich sequence. The human TS promoter is polymorphic, containing either two or three tandem direct repeats in addition to a single inverted copy of the repeat (Horie et al., 1995; Marsh et al., 1999). The promoter examined in this study had two copies of the direct repeat sequence. We showed that deletion of the downstream direct repeat had little effect on TS promoter activity following HCMV infection, but elimination of both direct repeats significantly reduces expression of the reporter gene in virus-infected cells. Earlier studies did not identify potential binding sites for transcription factors within the repeated sequences (Horie et al., 1992). In addition, we have shown that deletion of all of the direct repeats has little effect on expression of reporter genes driven by the TS promoter in proliferating cells (Dong et al., 2000). Therefore, the direct repeat may contribute to the human TS gene expression only under certain conditions, such as virus infection. The repeated sequences have also been reported to affect the efficiency of translation of TS mRNA (Kaneda et al., 1987; Horie et al., 1995; Kawakami et al., 2001). For this reason, it is not clear if the repeated sequence has a positive effect on TS promoter activity or on mRNA translation following HCMV infection.

The second positive-acting element was located between — 161 and — 141. This segment has been defined as the TS
essential promoter region, since it is both necessary and sufficient for efficient promoter activity in proliferating cells. This region contains binding sites for Ets, Sp1 and LSF transcription factors, and inactivation of any of these motifs leads to a significant decrease in promoter activity (Dong et al., 2000; Powell et al., 2000). Previously, it was shown that Sp1 binds to its cognate motif within the essential region of the human TS promoter (Horie & Takeishi, 1997) and that an increase in Sp1 DNA binding activity occurs in HELF cells during HCMV infection (Yurochko et al., 1995, 1997). However, mutation of the Sp1 site (Fig. 3) in the pHTS −161/−141 Luc construct had no significant effect on stimulation of TS promoter activity following virus infection (data not shown), suggesting that the Sp1 site is not important for the response to HCMV infection. It remains to be established whether the Ets and/or the LSF elements may be required for the stimulation of the TS essential promoter region by HCMV. Relevant to this, the DNA-binding activity of an Ets family member, Elk-1, was found to increase in HCMV-infected fibroblasts (Chen & Stinski, 2000).

The human TS promoter has two E2F-binding sites within the inverted repeat sequence downstream from the essential promoter region (Dong et al., 2000). However, deletion of both of these E2F motifs did not significantly diminish the expression of the reporter gene following HCMV infection, indicating that these sequences do not have a significant role in the response of the TS promoter to the virus. This is surprising since it has been reported that HCMV infection results in an E2F-dependent activation of the DHFR promoter (Wade et al., 1992; Margolis et al., 1995) and since an E2F element is necessary for the activation of the mouse TS promoter by murine CMV (Gribaudo et al., 2000).

These observations raised the possibility that MCMV and HCMV may activate the TS promoter by different mechanisms. To explore this possibility, we examined the effect of MCMV and HCMV on the expression of the human or mouse TS promoters, respectively. We found that the murine TS promoter with a mutated E2F site was activated to a higher extent than the wild-type promoter when introduced into human cells that were then infected with HCMV. In contrast, deletion of both E2F sites of the human TS promoter abolished its response to MCMV when the construct was introduced into NIH 3T3 cells. These results suggest that MCMV activates the TS promoter through an E2F-dependent pathway, whereas HCMV relies on a different mechanism.

TS catalyses the de novo synthesis of dTMP by the reductive methylation of dUMP, and availability of the substrate may thus be rate-limiting for dTMP biosynthesis. There are several pathways by which dUMP can be produced in the cell, although deamination of dCMP by deoxycytidylate deaminase (dCMP deaminase) appears to be the most important route. dCMP deaminase activity is much higher in rapidly dividing cells than in quiescent cells and is expressed at the highest levels during the S phase of the cell cycle (Maley & Maley, 1990). We have recently observed that HCMV infection of quiescent HELF cells also leads to an increase in dCMP deaminase gene expression (data not shown). These findings, along with previous studies showing increased DHFR activity during HCMV infection (Lembo et al., 1999), demonstrate that HCMV is able to coordinately activate the expression of multiple cellular enzymes involved in the synthesis of dTMP, thereby releasing the virus from the requirement of an S-phase environment for its replication.

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References


HCMV stimulates thymidylate synthase expression


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