High level transient expression of the murine coronavirus haemagglutinin–esterase

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We have expressed the murine coronavirus haemagglutinin–esterase protein in a vaccinia virus/T7 RNA polymerase system. The levels of expression observed are significantly higher than those found in virus-infected cells. The expressed protein has both receptor-destroying (esterase) and receptor-binding (haemadsorption) activities. The use of this system will greatly facilitate analysis of the structure–function relationships of this protein.

Introduction

The coronaviruses are a group of enveloped, positive-strand RNA viruses associated with diseases of economic importance in both animals and humans (Wege *et al.*, 1982). Among the most intensively studied members of the group are the murine coronaviruses, collectively known as murine hepatitis virus (MHV). These viruses are characterized by their ability to cause a variety of different diseases depending on the virus isolate, the host animal and the route of infection. MHV-based animal models have been established for neurological, enteric and hepatic diseases (Lamontagne *et al.*, 1989; Barthold & Smith, 1989; Perlman *et al.*, 1990) and it is hoped that they will help to elucidate important aspects of viral pathogenesis.

The MHV genome is a 32 kb RNA which encodes four major structural proteins: the nucleocapsid protein, N (50000 to 60000 M_r), the membrane glycoprotein, M (23000 to 26000 M_r), the surface glycoprotein, S (180000 M_r) and the haemagglutinin–esterase glycoprotein, HE (65000 M_r) (Siddell, 1982; Pachuk *et al.*, 1989). The sequence of each structural protein gene is known and biological functions have been ascribed to the proteins themselves (for a recent review, see Spaan *et al.*, 1988). It is reasonable to assume that determinants on each of these proteins will be associated with changes in viral pathogenicity.

The MHV HE gene encodes a polypeptide of 439 amino acids which is *N*-glycosylated at nine or 10 positions (Shieh *et al.*, 1989; Yokomori *et al.*, 1989). In the virion, the HE protein is composed of disulphide-linked homodimers of 120000 M_r (Siddell, 1982). The homodimers are anchored in the virus membrane and form the short spike structures seen in negatively stained preparations (Sugiyama & Amano, 1981).

As has been noted previously, the predicted amino acid sequence of the MHV HE protein shows about 75% similarity with the bovine coronavirus (BCV) HE protein and, more remarkably, 30% similarity with the HEF_1 subunit of the influenza C virus HEF protein (Luytjes *et al.*, 1988; Parker *et al.*, 1989; Kienzle *et al.*, 1990). Both the BCV and influenza C virus proteins have haemagglutinin activity and an acetylesterase activity specific for *N*-acetyl-9-*O*-acetylneuraminic acid (Herrler *et al.*, 1985; King *et al.*, 1985; Vlasak *et al.*, 1987, 1988*a*, *b*). These activities have been sought in MHV virions, and indeed Yokomori *et al.* (1989) have shown that the presence of the HE protein in the MHV virion is correlated with the presence of an acetylesterase activity. In contrast, there are conflicting reports of a haemagglutinin activity associated with MHV virions (Sugiyama & Amano, 1980; Walker & Cleator, 1980; Yokomori *et al.*, 1989).

In the experiments reported here, we have transiently expressed the MHV HE protein at high levels using a recombinant vaccinia virus/T7 RNA polymerase system. This has allowed us to demonstrate directly the functions associated with the MHV HE protein and provides a system to analyse the structure–function relationships of this protein.

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D00764.

Methods

Cells and viruses. HeLa cells (ATCC CCL2), Sac(−) cells (Weiland *et al.*, 1978) and DBT cells (Kumanishi, 1967) were grown in monolayers in MEM containing 10% heat-inactivated foetal calf serum (FCS), glutamine, antibiotics and non-essential amino acids. Sac(−) cells were also grown in suspension culture in modified MEM containing 5% FCS and antibiotics.

The MHV used in this study was derived from material originally supplied as a suckling mouse brain homogenate, MHV JHM SMB7, by Dr L. Weiner. This material was propagated twice in suckling mouse brain, plaque-purified three times, and then grown for approximately 20 passages at low m.o.i. in Sac(−) cells. This virus population, referred to as MHV JHM in our publications prior to 1986, was used to isolate MHV JHM Wb1, which was plaque-purified twice on DBT cells and amplified by minimal low m.o.i. passage to stocks of approximately 2×10^7 $TCID_{50}$ per ml. The MHV A59 isolate was obtained from Dr P. Carthew and was plaque-purified twice in DBT cells and amplified as described above to a stock of 2×10^7 $TCID_{50}$ per ml. MHV virus purification was done as described by Wege *et al.* (1979).

The recombinant vaccinia virus, vTF7-3 (Fuerst *et al.*, 1986) was plaque-purified and grown to stocks of approximately 2×10^9 p.f.u. per ml in HeLa cells as described by Mackett *et al.* (1985). Under the conditions used, this virus does not exhibit haemagglutinin or fusion activities, both non-essential functions for virus replication (Ichihashi & Dales, 1971). Influenza C virus (JHB/1/66) was grown in embryonated eggs and purified as described by Herrler *et al.* (1985).

cDNA cloning and sequence analysis. Poly(A)-containing RNA was isolated from MHV-infected Sac(−) cells as described previously (Siddell *et al.*, 1980). cDNA synthesis was essentially by the method of Gubler & Hoffman (1983) using the MHV-specific oligonucleotide 5′ TTA GAT TAT GCC TCA TGC 3′ (complementary to positions 2169 to 2186 in Fig. 1 of Shieh *et al.*, 1989) as a first-strand primer. The synthesized double-stranded cDNAs were treated with T4 DNA polymerase, ligated into *Sma*I-linearized pBluescript II, KS+ DNA and used to transform competent *Escherichia coli* JM103 cells.

Recombinant clones were identified by colony hybridization with the MHV-specific oligonucleotide 5′ AAT ACG ACC GGT AAT GGG 3′ (complementary to positions 2070 to 2087 in Fig. 1 of Shieh *et al.*, 1989). The cDNA insert of clone 2/C3 was sequenced and found to extend from the primer position to a position 312 nucleotides from the 5′ end of the ns2 gene (Schwarz *et al.*, 1990). The plasmid DNA of the 2/C3 clone was linearized with *Eco*RV and treated for various times with nuclease Bal 31 [6 μg of DNA, 10 units (U) Bal 31, 30 °C, 15 to 90 s]. The truncated DNAs were cut with *Xba*I, ligated to the *Xba*I site of *Xba*I/*Sma*I-cut pBluescript II, KS+, treated with DNA polymerase I (Klenow fragment), self-ligated and used to transform competent *E. coli* TG1 cells.

Colony hybridizations with two MHV-specific oligonucleotides, 5′ ACT GCC CAT TTC AAC AAA AT 3′ (complementary to positions 852 to 871 in Fig. 1 of Shieh *et al.*, 1989) and 5′ AGT GTA AAA ACA AAT CAA C 3′ (complementary to positions 811 to 829 in Fig. 1 of Shieh *et al.*, 1989) were used to identify a clone, pBS+/MHV2-1, which was partially sequenced and contained a cDNA insert extending from seven nucleotides upstream of the HE gene initiation codon to a position three nucleotides downstream of the termination codon.

Finally, the pBS+/MHV2-1 cDNA was excised with *Xba*I and *Eco*RI, treated with DNA polymerase I (Klenow fragment) and ligated into the *Bam*HI site of the vaccinia virus transfer vector pTF7-5 (Fuerst *et al.*, 1987). This construct, pTF7-5/HE, was partially sequenced and contained the complete open reading frame (ORF) of the MHV JHM Wb1 HE gene with the initiation codon located 53 nucleotides downstream of the bacteriophage T7 promoter. Plasmid purification, agarose gel electrophoresis and standard recombinant DNA procedures were done as described by Sambrook *et al.* (1989). Colony hybridizations were done as described by Woods (1984).

DNA sequencing was done by the chain termination method (Sanger *et al.*, 1977). The 2/C3 cDNA insert was sequenced from single-stranded DNA templates after subcloning into M13mp9. The partial sequencing of pBS+/MHV2-1 and pTF7-5/HE was done on double-stranded DNA templates. In all, 17 MHV-specific oligonucleotides, two pBluescript-specific oligonucleotides and the M13 universal primer were used. Sequence data were assembled by the programs of Staden (1982) and analysed with the programs of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984). Oligonucleotides were synthesized by phosphoramidite chemistry and purified by gel electrophoresis.

Virus infection, labelling and cell lysates. DBT cell monolayers were infected with MHV at an m.o.i. of 6 as previously described (Siddell *et al.*, 1980). To label intracellular polypeptides, the medium was replaced 12 h after infection for 1 h with medium containing 2% dialysed FCS and 100 μCi of [^{35}S]methionine (SJ.204, 800 Ci/mmol; Amersham Buchler) per ml. To prepare cell lysates, labelled or unlabelled cell monolayers were washed twice with ice cold phosphate-buffered saline (PBS), scraped into PBS and pelleted at 800 *g* for 2 min. The cells were then lysed at 4 °C in 50 mM-Tris-HCl pH 7·5, 100 mM-NaCl, 0·2% NP40, 500 U of aprotinin per ml (TNPA buffer). The cytoplasmic lysates were centrifuged at 10000 *g* for 2 min and the supernatants were stored at −70 °C.

Transient expression. DBT cell monolayers were infected with the vaccinia virus recombinant vTF7-3 (Fuerst *et al.*, 1986) at an m.o.i. of 30 p.f.u./cell. The virus-containing medium was removed 2 h after infection and replaced with 20 μg of pTF7-5/HE DNA coprecipitated with calcium phosphate as described by Sambrook *et al.* (1989). The infected/transfected cells were incubated at 37 °C for 6 h and labelled or unlabelled cytoplasmic lysates were then prepared as described above. Tunicamycin (2 μg/ml) was added to the culture medium after the DNA transfection procedure.

Immunoprecipitation and SDS-PAGE. Cytoplasmic lysates were incubated with two volumes of TNPA buffer containing 1 mg of bovine serum albumin per ml and 1 volume of hybridoma tissue culture supernatant for 2 h at 4 °C. The immunocomplexes were absorbed to a suspension of Protein A–Sepharose and washed three times with TNPA buffer. The complexes were then either resuspended in PBS and assayed for esterase activity (see below), or heated in reducing sample buffer for 5 min at 55 °C. After removal of the Sepharose, the complexes were heated at 100 °C for 2 min and analysed by SDS–PAGE. The surface glycoprotein-specific monoclonal antibody (MAb) E2-A1 (Wege *et al.*, 1984) and the HE-specific MAb, α65 (Wege *et al.*, 1987) were kindly provided by H. Wege. SDS-PAGE of lysates and immunoprecipitates was done under reducing conditions on 15% polyacrylamide gels according to the method of Laemmli (1970).

Esterase and haemadsorption assays. Purified virus or immunocomplexes were resuspended in PBS and incubated with 1 mM-*p*-nitrophenyl acetate at room temperature. Hydrolysis of the substrate was monitored at 405 nm. The inhibition of esterase activity by di-isopropyl fluorophosphate (DFP) was assayed by preincubating purified MHV with 1 mM-DFP in PBS for 30 min at 4 °C prior to the esterase activity determination. Haemadsorption by DBT cells expressing the MHV HE protein was assayed by incubation with a 2% (v/v) suspension of washed rat erythrocytes in PBS at 4 °C for 30 min, followed by exhaustive washing of the monolayers with ice-cold PBS.

Protein labelling with [^{3}H]DFP. Purified virus (12·5 μg) was incubated in PBS containing 100 μCi [^{3}H]DFP (2 Ci/mmol;

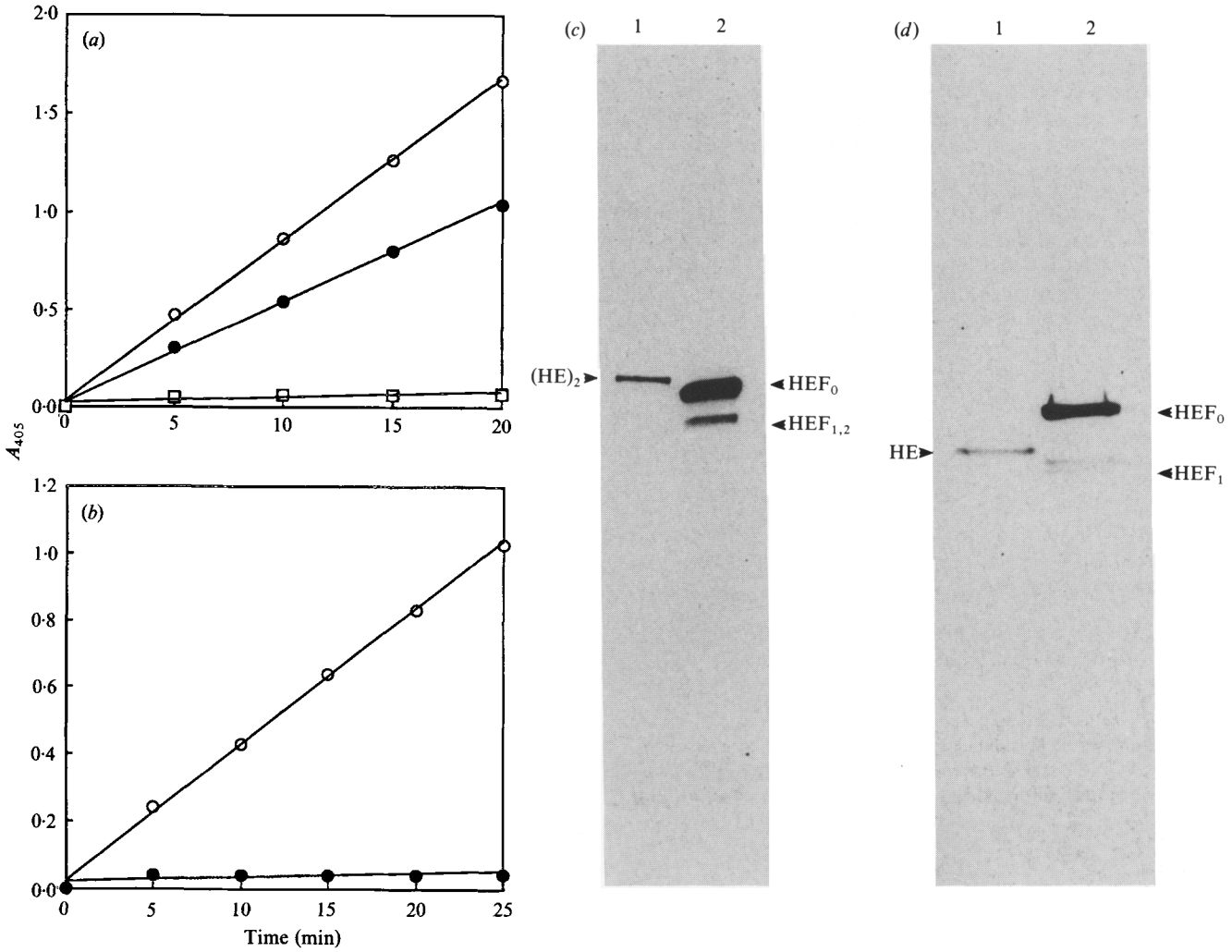

Fig. 1. (*a*) Esterase activity associated with MHV JHM, MHV A59 and influenza C virus. Purified MHV JHM, 20 μg (●), MHV A59, 20 μg (□) and influenza C virus, 0·2 μg (○) were incubated (in 1 ml) with 1 mM-*p*-nitrophenyl acetate and hydrolysis of the synthetic substrate was monitored at 405 nm. (*b*) Inhibition of the MHV JHM-associated esterase activity with DFP. Ten μg of purified MHV JHM was preincubated with 1 mM-DFP (●) or PBS (○) at 4 °C for 30 min and then assayed for esterase activity as described above. (*c*) and (*d*) [^{3}H]DFP labelling of MHV JHM virions. MHV JHM virions (lanes 1) or influenza C virions (lanes 2) were labelled with [^{3}H]DFP, electrophoresed on 10% SDS–polyacrylamide gels under reducing (*d*) or non-reducing (*c*) conditions, and autoradiographed. The identification of the labelled proteins is based on their known M_r and electrophoretic mobility.

Amersham Buchler) per ml for 30 min at 4 °C. The mixture was then layered onto a 600 μl cushion of 15% sucrose and centrifuged at 40000 *g* for 30 min. The pellet was resuspended in 20 μl of sample buffer and analysed by SDS–PAGE under reducing or non-reducing conditions on 10% polyacrylamide gels as described by Herrler *et al.* (1988*a*)

Results

The two most intensively studied MHV strains are MHV A59 and MHV JHM. Recently, sequence analysis (Luytjes *et al.*, 1988) has shown that the MHV A59 genome has undergone mutation and no longer has a functional HE gene. Also, Lai and co-workers (Shieh *et al.*, 1989) have shown that different MHV JHM isolates exhibit very different levels of HE gene expression. For this reason, we decided to confirm that the MHV JHM Wb1 isolate does indeed have a functional HE gene.

Fig. 1 (*a*) shows the esterase activity associated with purified MHV JHM Wb1, MHV A59 and influenza C virions using a synthetic substrate. As expected, the influenza C virus preparation showed a high level of esterase activity, which is known to be associated with the surface glycoprotein (Herrler *et al.*, 1988*a*). The MHV JHM Wb1 isolate also has a readily detectable esterase activity. In contrast, no activity above the level of controls (PBS alone, data not shown) could be demonstrated for the purified MHV A59.

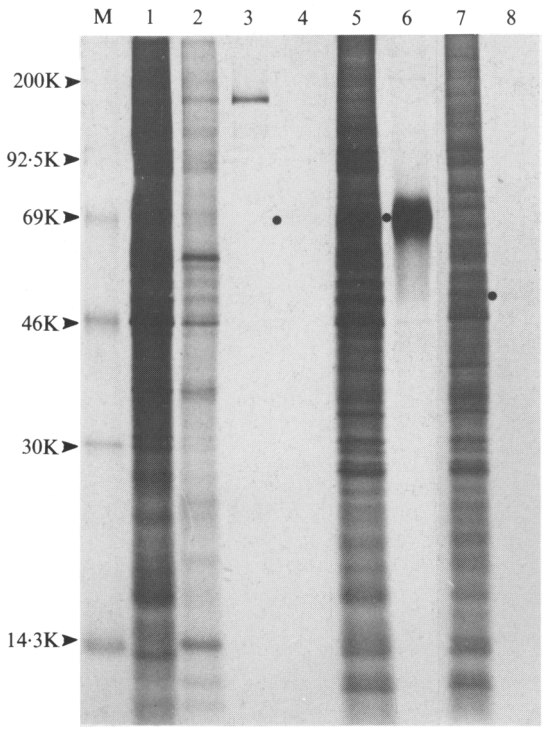

Fig. 2. Expression of the MHV HE gene in a vaccinia virus/T7 RNA polymerase system. DBT cells were infected with MHV JHM at an m.o.i. of 6 or with the recombinant vaccinia virus, vTF7-3, at an m.o.i. of 30. The MHV-infected cells were labelled with [^{35}S]methionine and cytoplasmic lysates were analysed directly or after immunoprecipitation. The vaccinia virus-infected cells were transfected with pTF7-5/HE DNA, labelled with [^{35}S]methionine with or without tunicamycin treatment and cytoplasmic lysates were analysed directly or after immunoprecipitation. Lane M, M_r Markers (CFA.626; Amersham Buchler); lane 1, uninfected DBT cell lysate; lane 2, MHV-infected DBT cell lysate; lane 3, MHV-infected DBT cell lysate immunoprecipitated with MAb E2-A1 (S protein-specific); lane 4, MHV-infected cell lysate immunoprecipitated with MAb α65 (HE protein-specific); lane 5, vaccinia-virus infected, pTF7-5/HE-transfected DBT cell lysate; lane 6, vaccinia virus-infected DBT cell lysate immunoprecipitated with MAb α65; lane 7, vaccinia virus-infected, pTF7-5/HE-transfected DBT cell lysate labelled in the presence of tuncamycin; lane 8, vaccinia virus-infected, pTF7-5/HE-transfected DBT cell lysate labelled in the presence of tuncamycin and immunoprecipitated with MAb α65. The position of the HE protein and apoprotein are indicated.

In a further experiment, the sensitivity of the MHV JHM-associated esterase activity to the serine esterase inhibitor DFP was assayed. As shown in Fig. 1(*b*), the esterase activity was completely inhibited when the virus was preincubated with 1 mM-DFP for 30 min. Finally, [^{3}H]DFP was used to confirm that it is indeed the MHV JHM HE protein which is associated with the esterase activity. As can be seen in Fig. 1(*c*) and (*d*), [^{3}H]DFP, which binds covalently to the serine of the esterase active site (Cohen *et al.*, 1967), labels only the MHV HE protein, either in its reduced monomer form (Fig. 1*d*, lane 1) or as a non-reduced homodimer (Fig. 1*c*, lane 1).

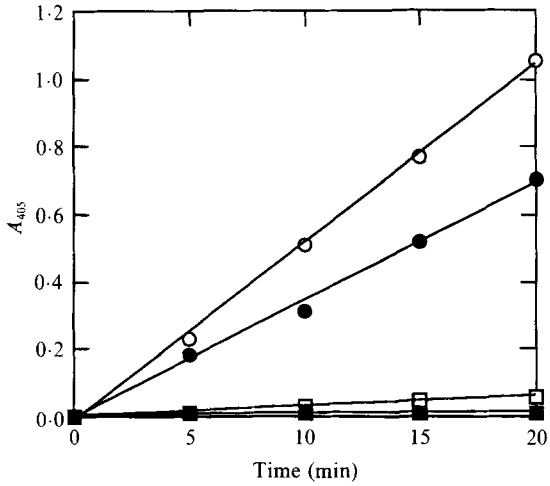

Fig. 3. Esterase activity associated with the expressed MHV JHM HE protein. Purified MHV JHM virus, 20 μg (○), the MAb α65–HE protein complex from vaccinia virus-infected, pTF7-5/HE-transfected DBT cells (●), the MAb α65–HE protein complex from MHV-infected DBT cells (□) and the MAb α65 complex from vTF7-3-infected cells (■) were incubated (in 1 ml) with 1mM-*p*-nitrophenyl acetate and hydrolysis of the synthetic substrate was monitored at 405 nm.

As a control, the binding of [^{3}H]DFP to the reduced and non-reduced forms of the influenza C virus HEF protein (Fig. 1*d*, lane 2 and Fig. 1*c*, lane 2) was also demonstrated.

Having confirmed that the MHV JHM Wb1 isolate has a functional HE gene, we isolated and sequenced a cDNA copy which contained the complete ORF of this gene. This analysis revealed an ORF of 439 amino acids which encodes a polypeptide of 49000 M_r with 10 potential *N*-glycosylation sites. The predicted HE protein sequence differs at only three positions (amino acid 133, A to R; amino acid 245, L to F; amino acid 247, C to S) from that determined by Shieh *et al.* (1989).

Transient expression of the MHV JHM Wbl HE gene

In order to express the MHV Wb1 HE gene in isolation from other viral gene products, it was placed under the control of the bacteriophage T7 promoter and transfected into DBT cells which had previously been infected with the recombinant vaccinia virus vTF7-3. The expression of the HE protein was then monitored by metabolic labelling and immunoprecipitation. Fig. 2 (lanes 5 and 6) shows that in the vaccinia virus/T7 system, the HE protein is dramatically over-expressed when compared to the levels of HE protein expressed during a high multiplicity infection of DBT cells (lanes 2 and 4). The electrophoretic mobility and immunoreactivity of the HE protein expressed in the vaccinia virus/T7 system suggests that it is correctly translated and processed. It is interesting to note that in the presence of tunicamycin (lanes 7 and 8) the amount of HE

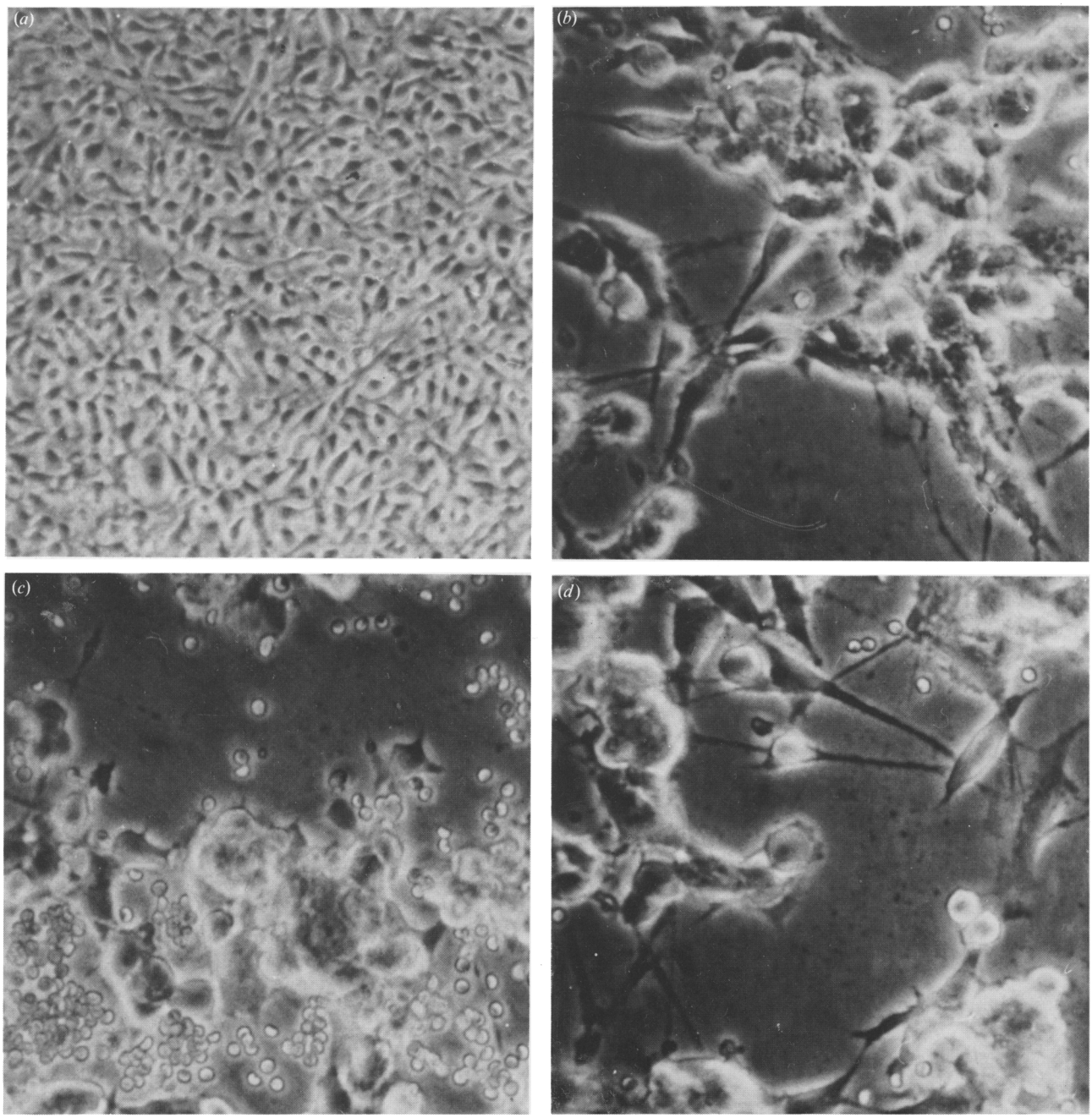

Fig. 4. Haemadsorption activity associated with the expressed MHV JHM HE protein. Uninfected DBT cells (*a*), vaccinia virus vTF7-3-infected DBT cells (*b*) and vaccinia virus vTF7-3-infected, pTF7-5/HE-transfected DBT cells (*c* and *d*) were chilled to 4 °C, incubated with rat erythrocytes and exhaustively washed at 4 °C (*a* to *c*) or at 37 °C (*d*) with PBS.

polypeptide expressed is greatly reduced. The size of the polypeptide synthesized is consistent with the size of the predicted HE apoprotein (approximately 49000 M_r) but apparently, in the absence of cotranslational glycosylation, the polypeptide is either rapidly degraded or poorly translated. It should also be mentioned that in the course of these studies we were unable to isolate a vaccinia virus recombinant which contained an integrated and expressed copy of the MHV HE gene (unpublished data). This may be due to the adverse effects of HE gene expression on vaccinia virus replication.

Functions of the MHV HE protein

Using the vaccinia virus/T7 system, it was possible to analyse directly the biological functions of the MHV HE

protein. Firstly, as expected, we were able to show that the HE protein functions as an esterase. The HE protein–MAb complex isolated from DBT cells which had been infected with vTF7-3 and transfected with pTF7-5/HE was clearly able to hydrolyse *p*-nitrophenyl acetate (Fig. 3). This activity was not found in cells which had been infected with vTF7-3 but not transfected. In MHV JHM-infected DBT cells, a low level of HE protein-associated esterase activity was detected, consistent with the low level of HE protein expression illustrated in Fig. 2.

The second biological function of the HE protein which was investigated was the ability to bind rat erythrocytes. These were chosen because they are known to contain the influenza C virus receptor determinant, *N*-acetyl-9-*O*-acetylneuraminic acid in high amounts (Rogers *et al.*, 1986). As is shown in Fig. 4(*c*), DBT cells which expressed the HE protein were able to bind rat erythrocytes at 4 °C. Elevation of the temperature to 37 °C resulted in the subsequent release of these erythrocytes (Fig. 4*d*). Uninfected DBT cells or cells infected with the recombinant vaccinia virus vTF7-3 did not have this activity (Fig. 4*a* and *b*). These results demonstrate that the HE protein synthesized in the vaccinia virus/T7 system is transported to the cell surface and they are consistent with the idea that the MHV HE protein has both receptor-binding and receptor-destroying activities.

Discussion

The experiments reported in this paper lead to a number of conclusions. Firstly, they provide the first formal proof that the esterase activity associated with MHV virions is a function of the HE protein. The specificity of this activity has not been determined, but by analogy to the influenza C virus HEF protein, it seems likely that the esterase substrate will be 9-*O*-acetyl residues on *N*-acetylneuraminic acid. This idea is supported by the structural analysis of the MHV HE gene which predicts an amino acid stretch, FGDSR (amino acids 42 to 46; Shieh *et al.*, 1989), identical to the amino acid sequence surrounding the residue which has been identified as the active site serine of the influenza C virus acetylesterase (Herrler *et al.*, 1988*b*; Vlasak *et al.*, 1989). Also, the release of bound erythrocytes from HE-expressing cells at 37 °C is consistent with this interpretation.

Secondly, the experiments conclusively demonstrate that the HE protein of MHV JHM has a receptor-binding activity for rat erythrocytes, despite the fact that a haemagglutinin activity associated with the JHM strain of MHV could not be shown (Yokomori *et al.*, 1989). This apparent discrepancy could be explained in two ways. On the one hand, it may be that the level of HE protein expression in the vaccinia virus/T7 system is sufficient to reveal an activity which is normally beyond the sensitivity of the assays employing purified virions. Alternatively, the expression of the HE protein in isolation may unmask the receptor-binding activity. This could be due, for example, to steric hindrance by other virion components.

Thirdly, it is evident that the vaccinia virus/T7 system offers a unique opportunity to analyse the structure–function relationships of the MHV JHM HE protein. In the heterologous system the level of HE gene expression is significantly higher than in virus-infected cells. This is particularly striking if the efficiency of the transfection and infection procedures is taken into account. We do not know the reason for this over-expression and similar constructs developed to express the MHV S and N proteins in the vaccinia virus/T7 system do not show this property. However, the elevated expression level will greatly facilitate the analysis of the functions associated with HE proteins which have been manipulated, for example, by site-directed mutagenesis.

The final and perhaps most important question arising from these studies is the biological role of the MHV HE protein in the infection process. Clearly, as the MHV A59 isolate does not express an HE protein its functions cannot be essential for replication in transformed murine cells. However, there are at least three lines of evidence which suggest that the HE protein may have an important function *in vivo*. Firstly, MAbs specific for the BCV HE protein are able to neutralize virus infectivity (Deregt & Babiuk, 1987). Secondly, Taguchi *et al.* (1986) were able to select MHV JHM isolates which expressed high levels of the HE protein by passage through neural cell cultures. Thirdly, MHV isolates which have not been maintained in tissue culture for prolonged periods of time also express relatively high levels of the HE protein (Sugiyama & Amano, 1980). Taken together, these data suggest that one or both of the known HE protein functions are relevant to the biological and possibly pathological properties of the virus. Experiments are in progress to investigate the role of the MHV HE protein in the tropism, virulence and cytopathology of murine hepatitis virus infections *in vivo*.

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