Characterization of proteins encoded by the short unique region of herpesvirus of turkeys by in vitro expression

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Nine open reading frames mapping in the short unique (Us) region of the genome of herpesvirus of turkeys (HVT) were expressed by in vitro transcription and translation. The observed Mrs of US10, SORF3 and US2 were as predicted from the sequence but there were discrepancies between the observed and predicted Mrs of US1, protein kinase, gI, gD and gE. These could be accounted for in most cases by post-translational and co-translational processing. Analysis of the synthesized products at different time points provided evidence for post-translational modification of HVT protein kinase. Translation in the presence of microsomal membranes resulted in co-translational processing of HVT gD, gI and gE by glycosylation and signal peptide cleavage.

Herpesvirus of turkeys (HVT) is classified as Meleagrid herpesvirus 1 and is serologically related to Marek’s disease virus (MDV) which causes T cell lymphomas in chickens (Churchill & Biggs, 1967; Payne, 1985). HVT is apathogenic in turkeys and chickens and is used extensively as a live vaccine against Marek’s disease (Witter et al., 1970). HVT and MDV have genome structures similar to those of alphaherpesviruses (Cebrian et al., 1982; Fukuchi et al., 1984; Igarashi et al., 1987) and have numerous genes that are homologous to and collinear with those of varicella-zoster virus and herpes simplex virus (HSV) (Buckmaster et al., 1988). Sequencing studies (Brunovskis & Velicer, 1992; Ross et al., 1991; Sakaguchi et al., 1992) have indicated that MDV Us encodes proteins homologous to HSV US1, US2, protein kinase (PK), US10 and glycoproteins gD, gI and gE but that three proteins (SORF1, SORF2 and SORF3) have no counterparts in HSV or any other mammalian herpesvirus. Using rabbit antisera directed against fusion proteins expressed in Escherichia coli, Brunovskis & Velicer (1992) characterized MDV US1, US10, US2, PK and glycoproteins gI and gE in extracts of infected duck embryo fibroblasts (DEF) by radioimmunoprecipitation assay (RIPA) but were unable to identify MDV gD. There is no information about the proteins encoded by the HVT short unique (Us) region.

Recently we reported on the sequence and gene organization of the US region and part of the terminal repeat region (TRs) of HVT (Zelník et al., 1993). We noted that the genes mapping in HVT Us were homologous to the MDV Us genes but that HVT did not have counterparts of MDV SORF1 and SORF2. Moreover, in contrast to MDV gE, which maps entirely in Us, HVT gE spanned the Us and TRs junction and identical C-terminal coding sequences were also present in the internal repeat region (IRs). This led us to predict that HVT could encode two related proteins (gE and gE*) with identical C-terminal amino acids but differing at their N termini (Zelník et al., 1993). In this study we have used the alternative approach of in vitro transcription and translation to characterize the proteins encoded by the open reading frames (ORFs) that we identified previously in HVT Us and have examined whether both gE and gE* can be synthesized. We have also characterized MDV gD since this glycoprotein had not been identified in MDV-infected cells previously.

The DNA templates covering individual ORFs used for in vitro transcription were pBluescript subclones of restriction enzyme fragments of HVT DNA (strain FC 126) used previously for sequencing HVT Us (Zelník et al., 1993). To study the expression of MDV PK and gD genes, a 5.5 kbp fragment derived from the Us region of the RB1B strain of MDV (Ross et al., 1991) was subcloned into pBluescript. The gene layout in HVT Us and the location of the RNAs transcribed in vitro are shown in Fig. 1. In vitro transcription was carried out in 100 μl reactions containing 5 μg of linearized DNA templates, 1 x transcription buffer (Promega), 0.5 mM rNTPs (each of ATP, CTP, GTP, UTP), 10 mM DT, 40 U RNasin (Promega) and 40 U of either T7 or T3 RNA polymerase (Promega). After incubation for 2 h at 37 °C, 50 U of RNase-free DNase I (Boehringer

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Mannheim) was added to the reaction mixture and incubation was continued for a further 15 min. The transcribed RNA was extracted with phenol/chloroform and precipitated with ethanol. Of the RNA preparations obtained, 1/50 to 1/20 was translated in vitro using either rabbit reticulocyte lysate or wheat germ extract (Promega) in 25 µl reactions according to the recommendations of the supplier. To study co-translational modifications (glycosylation and signal peptide cleavage), canine microsomal membranes (Promega) were added to the translation mixture. Synthesized proteins were labelled by adding 20 µCi [³²S]methionine (NEN Du Pont) to the translation reactions and 1 to 3 µl of the products were analysed by denaturing SDS–PAGE (Laemmli, 1970). Polyacrylamide gels (10 to 12 %) were stained with Coomassie Brilliant Blue, treated with Amplify solution (Amersham), dried and exposed to X-ray film for 12 to 24 h. MrS were estimated using high range protein molecular weight standards (Gibco BRL).

Table 1 shows the map positions of the DNA fragments used as templates for in vitro transcription and the number of bases from the start of transcription to the first ATG codon of each ORF. The observed and predicted MrS of proteins encoded by each ORF are also indicated. Although other ATG codons are present between the 5' end of the cloned fragments and the initiation codons, they do not interfere with the in vitro expression of the relevant ORFs because these ATG codons are not in frame and the presence of stop codons prevents the creation of larger ORFs.

The predicted Mr of HVT US1 is lower than that of the observed translation product, which was 23K (Fig. 2a, Table 1). Synthesis of this protein was not affected by addition of microsomal membranes. Treatment of in vitro translated products of HVT US1 with several phosphatases (calf intestinal alkaline phosphatase, lambda phosphatase and YOP₉₅ phosphatase) did not result in altered mobility of HVT US1 protein. Similarly, the size of in vitro translated US1 was not altered by pulse-labelling for 5, 10 or 15 min (data not shown). In the case of MDV, three possible isoforms of US1 protein were identified in infected cells by RIPA using MDV US1-specific serum (Brunovskis & Velicer, 1992). Two of them (Mr, 24K and 27K) were reported to be phosphorylated and of higher Mr than expected from the sequence. In our in vitro expression system, we identified a single HVT US1 band in SDS–PAGE. Differences between the predicted and observed mobilities of HSV-1 US1 have also been noted (Marsden et al., 1976; McGeoch et al., 1985). It has also been reported that HSV-1 US1 (ICP22, IE68) is a phosphoprotein (Pereira et al., 1977) that could affect expression of some late genes (Sears et al., 1985) and that its unusual mobility in PAGE could be due to its highly polar primary structure (McGeoch et al., 1985).

The predicted Mr of the 209 amino acid HVT US10 protein agrees with the observed mobility of the in vitro synthesized protein (Fig. 2b, Table 1). A phosphoprotein of similar Mr, was identified in MDV-infected cells using antisera against an MDV US10 fusion protein (Brunovskis & Velicer, 1992). The function of HSV-1 US10, a minor infected cell protein is unknown (Lee et al., 1982; McGeoch et al., 1985). In the case of HVT, the US10 protein is non-essential for virus replication in vitro and in vivo and has been used as a site for insertion and expression of foreign sequences (Morgan et al., 1992).

The Mr of the largest in vitro translated product of HVT SORF3 (Fig. 2c) is in agreement with that of the predicted protein (Table 1). Other lower MrS bands observed could be products arising from translation initiated from internal ATG codons present in the sequence of HVT SORF3. This phenomenon, which was also observed for other in vitro expressed HVT US proteins (see Fig. 2c to k), could be due to inefficient utilization of the first ATG codons of the ORFs that do not conform with Kozak's consensus sequence for translation initiation (Kozak, 1987). The function of HVT SORF3 or of its MDV homologue, SORF3 (Brunovskis & Velicer, 1992), is unknown.

The predicted Mr of the 282 amino acid HVT US2 protein agrees with the observed mobility of the product expressed in vitro (Fig. 2d, Table 1). Although the N terminus of HVT US2 is hydrophobic and is highly conserved in alpha-herpesviruses, it is unlikely to function as a signal peptide since the size of the protein was not affected when in vitro translation was carried out in the presence of microsomal membranes (lane M, Fig. 2d). MDV US2 has been identified as a protein having an Mr,
Table 1. In vitro transcription coordinates and Mr of HVT and MDV US ORF translation

<table>
<thead>
<tr>
<th>In vitro transcript</th>
<th>5' end</th>
<th>3' end</th>
<th>Bases from 5' end to AUG</th>
<th>Mr of translation products (x 10^2)</th>
<th>Predicted</th>
<th>Observed</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVT US1</td>
<td>40*</td>
<td>1118*</td>
<td>447</td>
<td></td>
<td>19</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>HVT US10</td>
<td>1118</td>
<td>2216</td>
<td>101</td>
<td></td>
<td>24</td>
<td>24</td>
<td></td>
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<tr>
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<td>1735</td>
<td>509</td>
<td></td>
<td>41</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>HVT US2</td>
<td>4618</td>
<td>3070</td>
<td>546</td>
<td></td>
<td>31</td>
<td>31</td>
<td></td>
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<tr>
<td>HVT PK</td>
<td>4083</td>
<td>5615</td>
<td>139</td>
<td></td>
<td>44</td>
<td>53,44</td>
<td>44K protein observed after short time synthesis</td>
</tr>
<tr>
<td>HVT gD</td>
<td>5712</td>
<td>7132</td>
<td>35 (208)‡</td>
<td></td>
<td>44</td>
<td>37,34,44</td>
<td>Several possible initiation codons</td>
</tr>
<tr>
<td>HVT gI</td>
<td>6931</td>
<td>8485</td>
<td>60</td>
<td></td>
<td>40</td>
<td>37,32</td>
<td>Several possible initiation codons</td>
</tr>
<tr>
<td>HVT gE</td>
<td>8056</td>
<td>10676</td>
<td>158</td>
<td></td>
<td>55</td>
<td>54,36</td>
<td></td>
</tr>
<tr>
<td>HVT gE*</td>
<td>359</td>
<td>146</td>
<td>37</td>
<td></td>
<td>314</td>
<td>C-terminal a.a.s identical with those of gE</td>
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</tr>
<tr>
<td>MDV PK</td>
<td>1069†</td>
<td>2501†</td>
<td>177</td>
<td></td>
<td>45</td>
<td>53,52</td>
<td></td>
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<tr>
<td>MDV gD</td>
<td>2863†</td>
<td>4585†</td>
<td>26</td>
<td></td>
<td>45</td>
<td>45,37,41</td>
<td>Several possible initiation codons</td>
</tr>
</tbody>
</table>

* Nucleotide positions in the DNA sequence of HVT US (Zelnik et al., 1993) corresponding to the 5' and 3' ends of each in vitro transcript. The 5' ends are defined by the restriction enzyme sites indicated or by the location of nested deletion clones (Δ). The 3' ends correspond to the restriction enzyme sites used to linearize DNA templates.

† Nucleotide positions and location of restriction sites defined by Ross et al. (1991).

‡ Constructs with different lengths of 5' untranslated sequences (alternative lengths in brackets) were also used but no effect on in vitro translation products was observed (data not shown).

§ 'g' indicates glycosylated forms obtained when in vitro translations were carried out in the presence of microsomal membranes.

of 30K in infected cells (Brunovskis & Velicer, 1992) and has been reported to be non-essential for MDV growth in vitro (Cantello et al., 1991).

The main product of HVT PK synthesized using either the rabbit reticulocyte lysate or wheat germ extract system has an Mr of 53K. This Mr was not affected by the addition of microsomal membranes to the translation mixtures (Fig. 2e). However, a discrepancy of approximately 9K between the predicted and observed Mr of HVT PK was noted (Table 1). For comparison, the MDV PK was also expressed in vitro. The results showed the presence of two polypeptides (Mr, 53K and 52K) that were of higher Mr than expected (Fig. 2f, Table 1). When translation of HVT PK was limited to 5 min only, a protein of the expected size (Mr, 44K, marked by an asterisk in Fig. 3a) was identified. However, after translation for 10 min, a modified HVT PK of Mr, 53K was predominant. This clearly demonstrated that HVT PK is post-translationally modified and that processing is independent of HVT infection and is possibly mediated by host cell factors. Similar discrepancies between the predicted and observed Mr were reported for PK encoded by the Us of HSV-1 (Frame et al., 1987). Sakaguchi et al. (1993) reported that polyclonal serum directed against MDV PK expressed in baculovirus identified two forms of PK (Mr, 44K and 45K, respectively) in MDV-infected cells. However, the antiserum used cross-reacted strongly with some host protein(s). It is possible that detection of the processed isoform of MDV PK (Mr, 53K) described here was masked in the previous study (Sakaguchi et al., 1993). The mobility of HVT PK, as that of HVT US1, was not affected after treatment of in vitro translation products with phosphatases (data not shown). In the HSV-1 system, PK encoded by US3 phosphorylates the product of US34 but the significance of this modification is unknown (Purves et al., 1991).

Several (in frame) ATG codons present at the 5' ends of both HVT and MDV gD ORFs (Zelnik et al., 1993;
Fig. 2. Autoradiograms of [35S]methionine-labelled in vitro translated polypeptides separated in 12% acrylamide SDS-PAGE gels: HVT US1, (a); HVT US10, (b); HVT ORF3, (c); HVT US2, (d); HVT PK, (e); MDV PK, (f); HVT gD, (g); MDV gD, (h); HVT gI, (i); HVT gE, (j) and HVT gE* (k). Proteins were synthesized using either rabbit reticulocyte lysate (lanes R) or wheat germ extract (lanes W). Lanes M show proteins synthesized in the presence of microsomal membranes.
Ross et al., 1991) could be responsible for the synthesis of numerous in vitro translation products (Fig. 2g and 2h). The Mr of in vitro synthesized HVT gD proteins ranged from 34K to 44K and those of MDV gD from 37K to 45K (Table 1). The addition of microsomal membranes decreased the mobility of synthesized proteins. The differences noted between the Mr of the main glycosylated forms of HVT gD and MDV gD could be due to different levels of glycosylation since HVT gD contains three potential N-linked glycosylation sites (Zelnik et al., 1993) whereas MDV gD has four such sites (Ross & Binns, 1991). To study further the nature of the co-translational modification of HVT gD, translation products were treated with endoglycosidase H, which removes N-linked carbohydrate residues from glycoproteins. Glycosylated HVT gD products (4 μl) were digested overnight at 37°C with 4 μM of endoglycosidase H (Boehringer) in a 20 μl reaction containing 100 mM-sodium citrate pH 5.5, 0.1% SDS, 100 mM-2-mercaptoethanol and 1 mM-PMSF. SDS-PAGE of the products revealed a new 41K protein (marked in Fig. 3b). The Mr of the endoglycosidase-treated protein was consistent with the predicted Mr of the primary product of HVT gD after subtracting 3K, which accounts for the Mr of the signal peptide. This result suggested that the addition of microsomal membranes to the translation reaction enabled co-translational modification of the HVT gD by glycosylation and cleavage of signal peptide.

HSV-1 gD is one of the major targets of neutralizing antibodies (Cohen et al., 1984; Para et al., 1985) and mediates penetration of target cells (Fuller & Spear, 1987). Similar functions have not yet been demonstrated for either HVT or MDV gD.

The ORF of HVT gi, like that of HVT gD, has several in-frame ATG codons and the profile of synthesized proteins was consistent with multiple in vitro translation initiation sites. The Mr of the main products were 37K and 32K respectively (Fig. 2i), indicating that the first ATG is not used efficiently during in vitro translation (Table 1). The addition of microsomal membranes to the translation mixture resulted in the synthesis of two glycosylated forms of HVT gi (Mr 47K and 44K) which were reduced to a protein of Mr 36K following digestion with endoglycosidase H (marked • in Fig. 3c). These results indicated that HVT gi was co-translationally modified in a similar way to HVT gD. It has previously been reported by Brunovskis et al. (1992) that MDV gi expressed in MDV-infected DEF is a glycoprotein (Mr 45K) and that the non-glycosylated precursor synthesized in the presence of tunicamycin (an inhibitor of glycosylation) has an Mr of 35K. It follows, therefore, that the non-glycosylated forms of HVT and MDV gIs are almost identical in size.

The in vitro transcript of HVT ge was derived from a DNA fragment spanning Us/TRs of HVT comprising 710 bp that mapped in Us and the rest in TRs (Zelnik et al., 1993). The HVT ge* transcript differed from the HVT ge transcript in the 5’ region (see Fig. 1). The Mr of the largest in vitro translation product of HVT ge was similar to the predicted estimate of 54K (Fig. 2j, Table
1). Similarly, the observed $M_\text{r}$ of gE* (Fig. 2k) was in agreement with the predicted $M_\text{r}$ of 37K (note that this protein is distinct from the non-specific 36K band present in the translation product of gE). Interestingly, as in the case of gI, we observed that the RNAs of HVT gE and gE* were more efficiently translated under standard conditions using wheat germ extracts than rabbit reticulocyte lysate. It is possible that in vitro translation of these HVT glycoproteins in the rabbit reticulocyte lysate system, in the absence of membranes, requires different conditions than those suitable for expression of the other ORFs of HVT. After addition of microsomal membranes to the rabbit reticulocyte lysate translation mixture, synthesis of HVT gE seemed to improve, resulting in a glycosylated product of $M_\text{r}$ 64K. In contrast, synthesis of gE* either with or without membranes did not proceed efficiently using rabbit reticulocyte lysate. Removal of carbohydrate residues from HVT gE with endoglycosidase H reduced the $M_\text{r}$ to 52K (Fig. 3d). It has been reported previously (Brunovskis et al., 1992) that two glycosylated forms of MDV gE ($M_\text{r}$ 62K and 72K) are synthesized in MDV-infected DEF cells and that treatment of infected cells with tunicamycin resulted in the synthesis of a 45K protein. It was also reported that anti-MDV gI sera co-precipitated MDV gI and gE (Brunovskis et al., 1992), suggesting that gI and gE of MDV can form a complex as reported for HSV-1 (Johnson & Feentra, 1987; Johnson et al., 1988). There is evidence that HSV-1 gE alone or associated with gI can bind to the Fc part of immunoglobulin G molecules (Bell et al., 1990; Johnson et al., 1988). It is not known whether gE and gI of HVT or MDV have similar properties.

Previously we identified a number of ORFs in HVT U₈ and predicted that they could encode proteins (Zelnik et al., 1993). In this study we have extended our earlier work and have characterized the proteins encoded by the potential ORFs using in vitro transcription and translation. The approach of expressing herpesvirus proteins in vitro has been successfully used to localize genes (Lee et al., 1982) and for analysis of their immunological and functional properties (Dorsky & Crumpacker, 1988; Matthews et al., 1983; Robertson et al., 1988). Our results have shown that the $M_\text{r}$ of several in vitro translated HVT U₈ proteins were as predicted from their primary structure and have provided evidence for post-translational modification in most cases where there were discrepancies between the observed and predicted $M_\text{r}$. In particular, we have obtained evidence of glycosylation and signal peptide cleavage in the case of glycoproteins gD, gI and gE. Evidence for post-translational modification of HVT PK was obtained but further work is required to determine the mechanism of processing and its significance in protein function. We have also been able to characterize MDV and HVT gD and have preliminary evidence that there may be several initiation codons in these molecules.

The results presented here have provided information that could help to identify and characterize HVT U₈ proteins in infected cells and facilitate further study of the control of their expression and of their functions during infection. This work was supported by the EEC BRIDGE program BIOT CT90-0173.

References


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