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The mouse mammary tumour virus long terminal repeat encodes a type II transmembrane glycoprotein

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Superantigens are products of bacterial or viral origin which stimulate large numbers of T cells as a consequence of the interaction of particular Vβ chains of the T cell receptor with class II major histocompatibility complex (MHC) molecules and superantigen on the stimulating cell. The Minor lymphocyte stimulatory (Mls) antigens, originally discovered as strong lymphocyte stimulatory determinants *in vitro* and subsequently shown to delete T cells expressing specific Vβ chains during development, have recently been shown to be genetically linked to endogenous mouse mammary tumour viruses (MTVs). This stimulation is effectuated by an unidentified product encoded by an open reading frame (orf) present in the 3′ long terminal repeat (LTR) of MTVs. Using *in vitro* translation in the presence of rough microsomal vesicles, we show that (i) the orf of MTV encodes a type II transmembrane glycoprotein (N-terminus intracellular, C-terminus extracytoplasmic), and (ii) a cotranslationally secreted orf protein is not produced. We have also isolated and sequenced several endogenous MTV orfs (MTV-1, MTV-6 and MTV-13) which are involved in the deletion of Vβ-bearing T cells; each of these sequences are nearly identical to each other. These observations, together with sequence comparisons of several orf genes, lead to a model of action of viral superantigens.

Key words: Mls/MMTV orf/superantigens/type II transmembrane glycoprotein

Introduction

Stimulation of T cells by superantigens results in the specific expansion of T cells which bear only particular Vβ gene segments. Two classes of superantigens have been described: the bacterial enterotoxins, which are secreted and act by binding directly to class II MHC molecules, and the self superantigens (reviewed in Acha-Orbea and Palmer, 1991; Herman *et al.*, 1991; Marrack and Kappler, 1990). Self-superantigens have been defined in the mouse as a group of dispersed genetic loci which, together with class II MHC molecules, cause the clonal elimination of T cells (Fry and Matis, 1988; Kappler *et al.*, 1988; MacDonald *et al.*, 1988; Pullen *et al.*, 1988; Woodland *et al.*, 1990). They include loci which were previously identified as Mls antigens by their ability to stimulate a primary mixed lymphocyte reaction n H-2 identical strains (Festenstein, 1976). Several of these loci have recently been shown to map to endogenous mouse mammary tumour viruses (MTVs) (Dyson *et al.*, 1991; Frankel *et al.*, 1991; Woodland *et al.*, 1991). In addition, a milk-borne MTV is capable of inducing T cell deletion (Marrack *et al.*, 1991). In particular, transfection of the orf gene present in the 3′ LTR of exogenous MTV stimulates T cells bearing Vβ14 and 15 (Choi *et al.*, 1991). Similarly, a related orf sequence from GR mice, when present as a transgene, results in the deletion of T cells expressing Vβ14 (Acha-Orbea *et al.*, 1991). Since the MTV orf can encode multiple products, due to multiple initiation sites (Dickson *et al.*, 1981; Racevskis and Prakash, 1984), the specific protein which is responsible for T cell stimulation is unclear. We therefore attempted to determine the properties of the proteins encoded by the MTV orf. In addition, we have isolated the endogenous MTVs which encode Mls-2[a] (MTV-13) and Mls-3 (MTV-6), each of which contributes to the deletion of Vβ3-bearing T cells (Frankel *et al.*, 1991), in order to determine the sequence polymorphism of these orfs and how this polymorphism contributes to their stimulatory specificity.

Results

In vitro translation of proteins encoded by the MMTV orf

In vitro translation in the presence of canine pancreatic microsomes was used to study the biosynthesis and processing of proteins encoded by the MTV C3H orf. Translation of RNA containing the entire orf in the absence of microsomes yields a predominant protein with a molecular weight of 36 kDa together with other major products of 25, 21 and 18 kDa (Figure 1, lane 3), consistent with previous reports (Dickson *et al.*, 1981; Racevskis and Prakash, 1984). These smaller products represent initiation at internal methionine codons which are in frame with the first methionine codon (cf. Figure 4). However, when translation is carried out in the presence of microsomes, a ladder of five closely spaced bands appears whose major component is 46 kDa while the 36 kDa protein is reduced in quantity; in contrast, none of the smaller products are similarly modified (Figure 1, lane 4). This suggests that only the 36 kDa protein was glycosylated and was therefore translocated to the lumen of the microsomes. Translation carried out in the presence of a competitive inhibitor of glycosylation (Benzoyl−Asn−Gly−Thr−NHMe), in which translocation and cleavage are unaffected (Lau *et al.*, 1983) (Figure 1, lane 5), or treatment of the translation products of C3H orf in the presence of microsomes with *N*-glycanase, which removes N-linked oligosaccharides (Figure 1, lane 9), confirms that the 46 kDa orf is glycosylated. Indeed, the C3H orf contains five potential sites of *N*-glycosylation at amino acids 79, 89, 93, 131 and 146 (cf. Figure 4). Glycosylation of a truncated orf protein has been described (Racevskis, 1986) and more recently expression of a full-

length orf in insect cells and by *in vitro* translation was shown to encode a 45 kDa glycoprotein (Brandt-Carlson and Butel, 1991). The foregoing experiments also indicate that the orf protein is not proteolytically cleaved during translocation, since the deglycosylated orf product has a molecular weight of 36 kDa, the size of the unglycoslated precursor of orf.

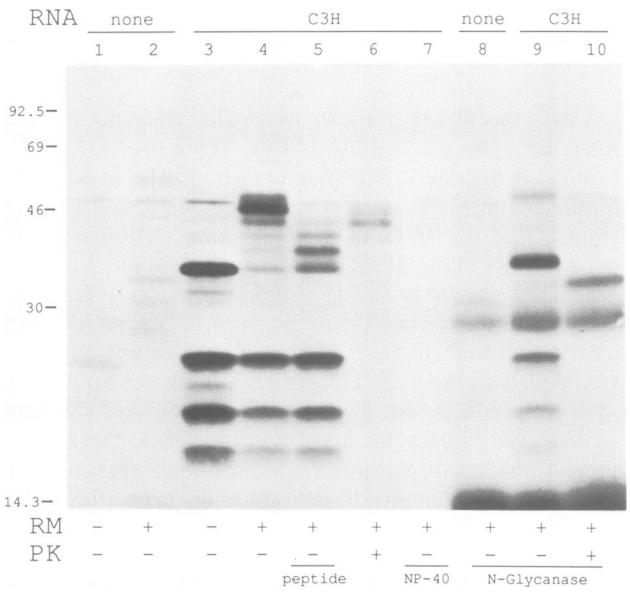

Fig. 1. Cell free translation and membrane insertion of C3H orf. Addition of *in vitro* transcribed C3H orf RNA is indicated above the figure. RM indicates addition of rough microsomes during translation. PK indicates post-translational treatment of the translation products with proteinase K. Additions of peptide (tripeptide inhibitor of glycosylation added during translation), NP-40 and treatment with *N*-glycanase are indicated. Molecular weight markers in kDa are indicated in the figure.

Protease digestion was then used to ascertain the membrane orientation of the glycosylated orf product. Treatment of the products of C3H orf RNA translated in the presence of microsomes with proteinase K resulted in a shift in the molecular weight of the glycoprotein ladder of ~3 kDa, as well as complete digestion of all non-glycosylated polypeptides (Figure 1, lane 6 versus 4). The loss of ~3 kDa is more clearly seen when these same samples were further treated with *N*-glycanase (Figure 1, lane 10 versus 9). Addition of detergent prior to the addition of proteinase K resulted in the digestion of all translation products (Figure 1, lane 7). These results suggest that the 46 kDa orf glycoprotein has a transmembrane topography; the majority of the protein is sequestered in the vesicles, therefore extracytoplasmic, and a tail of 30–40 aa is exposed on the external face of the vesicle, which corresponds to the cytosolic compartment. Since translocation of the orf protein occurs without cleavage, it is likely that the hydrophobic segment from amino acids 45–67 (which is bracketed by polar amino acids but includes the charged amino acids Lys51 and Glu64) serves as an internal uncleaved signal sequence which directs membrane anchorage of the protein. This predicts that the protease-sensitive tail is N-terminal.

Experiments employing N- and C-terminal deletions of the C3H orf gene demonstrate that the N-terminus of the orf protein is intracellular (Figure 2). If synthetic mRNAs encoding C-terminal deletions of C3H orf (C3HΔ247–319 and C3HΔ186–319) are translated in the presence of microsomes, each of the full-length translation products is translocated and glycosylated, and exposes a protease-

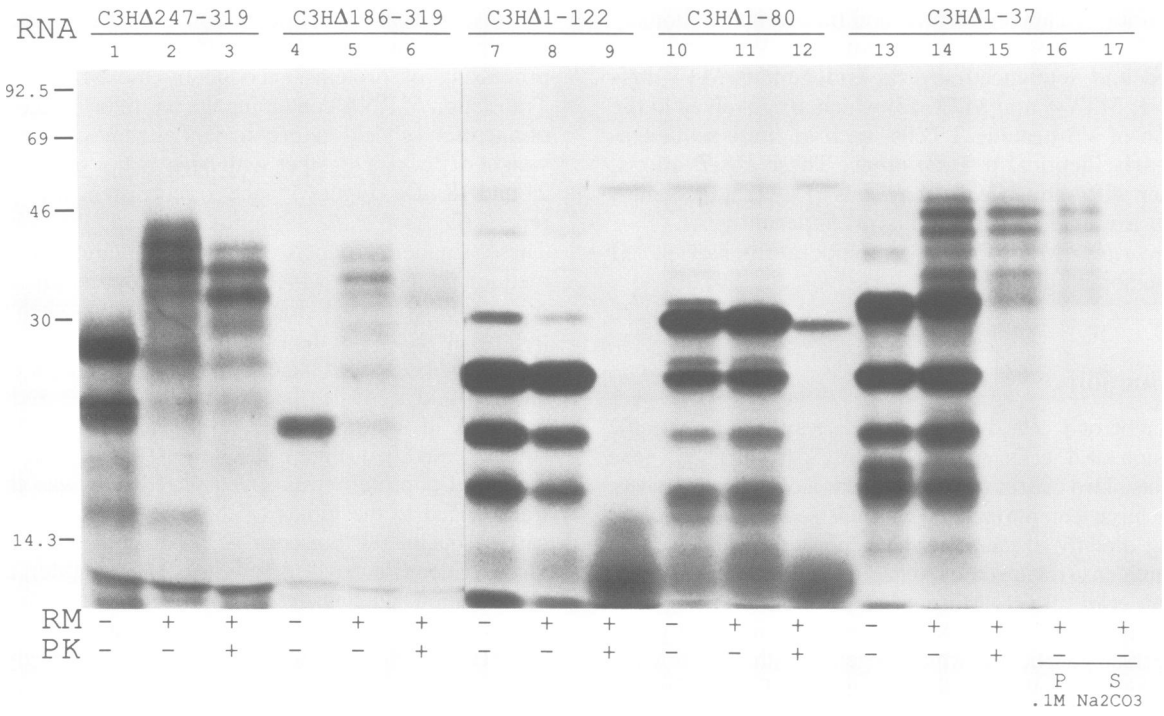

Fig. 2. Cell free translation and membrane insertion of amino- (C3HΔ1–37, C3HΔ1–80 and C3HΔ1–122) and C-terminal (C3HΔ247–319 and C3HΔ186–319) deletions of C3H orf. See Figure 4 for numbering of amino acid residues. *In vitro* transcribed RNAs were added as indicated above the figure. RM, addition of rough microsomes; PK, post-translation treatment with proteinase K. The pellet (P) and supernatant (S) fractions of microsomes containing C3HΔ1–37 after sodium carbonate (0.1 M Na_2CO_3) treatment are indicated. Molecular weight markers in kDa are indicated in the figure.

sensitive tail of a size similar to that observed for the 46 kDa orf protein (Figure 2, lanes 1−6). In contrast, deletions at the N-terminus (C3HΔ1−122, C3HΔ1−80), which remove the hydrophobic segment, result in the synthesis of proteins which are not associated with vesicles and are consequently digested by proteinase K (Figure 2, lanes 7−12). Finally, a deletion which results in initiation at Met38 (C3HΔ1−37), seven amino acids prior to the hydrophobic segment, results in the synthesis of a 32 kDa protein which is translocated, glycosylated and has no detectable exposed N-terminus.

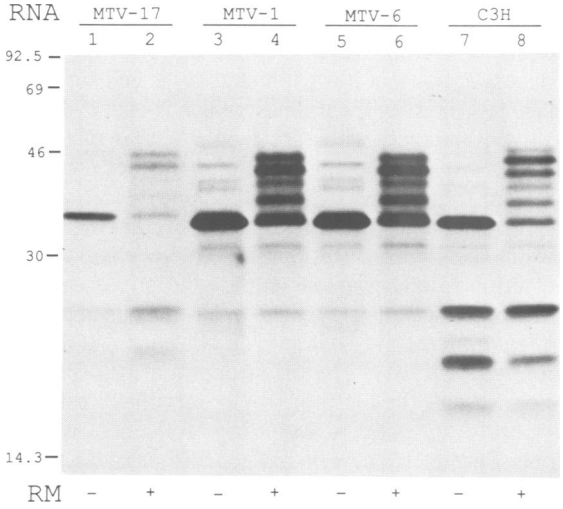

Fig. 3. Cell free translation and membrane insertion of endogenous orfs, MTV-17 (clone 53), MTV-1 (clone 62) and MTV-6, isolated from DBA/2J DNA. Molecular weight markers in kDa are indicated in the figure.

It was of interest to determine if orf proteins which arise from initiation at Met38 could be secreted, i.e. can the transmembrane anchor undergo processing by signal peptidase? For example, deletion of the N-terminal intracytoplasmic domain of the invariant chain, a type II transmembrane glycoprotein associated with class II MHC molecules, unmasks a signal sequence cleavage site in the transmembrane domain (Lipp and Dobberstein, 1986). Treatment of membranes with sodium carbonate at alkaline pH has been used to release secreted and peripheral membrane proteins from microsomal membrane preparations (Fujiki *et al.*, 1982). Thus, vesicles containing glycosylated C3HΔ1−37 were treated with sodium carbonate and then centrifuged to separate soluble from membrane bound components. As seen in Figure 2 (lanes 16 and 17) all the C3HΔ1−37 material is associated with membrane pellet, suggesting that this product remains membrane associated. Secretion requires excision of the signal peptide; the molecular weight of glycosylated C3HΔ1−37 is consistent with lack of cleavage. Furthermore, treatment of glycosylated C3HΔ1−37 with *N*-glycanase reduces its size to that of the 32 kDa unglycosylated precursor of C3HΔ1−37, indicating that no cleavage has occurred (data not shown). These data, together with the observation that initiation at Met38 is generally inefficient (cf. Figure 1 lane

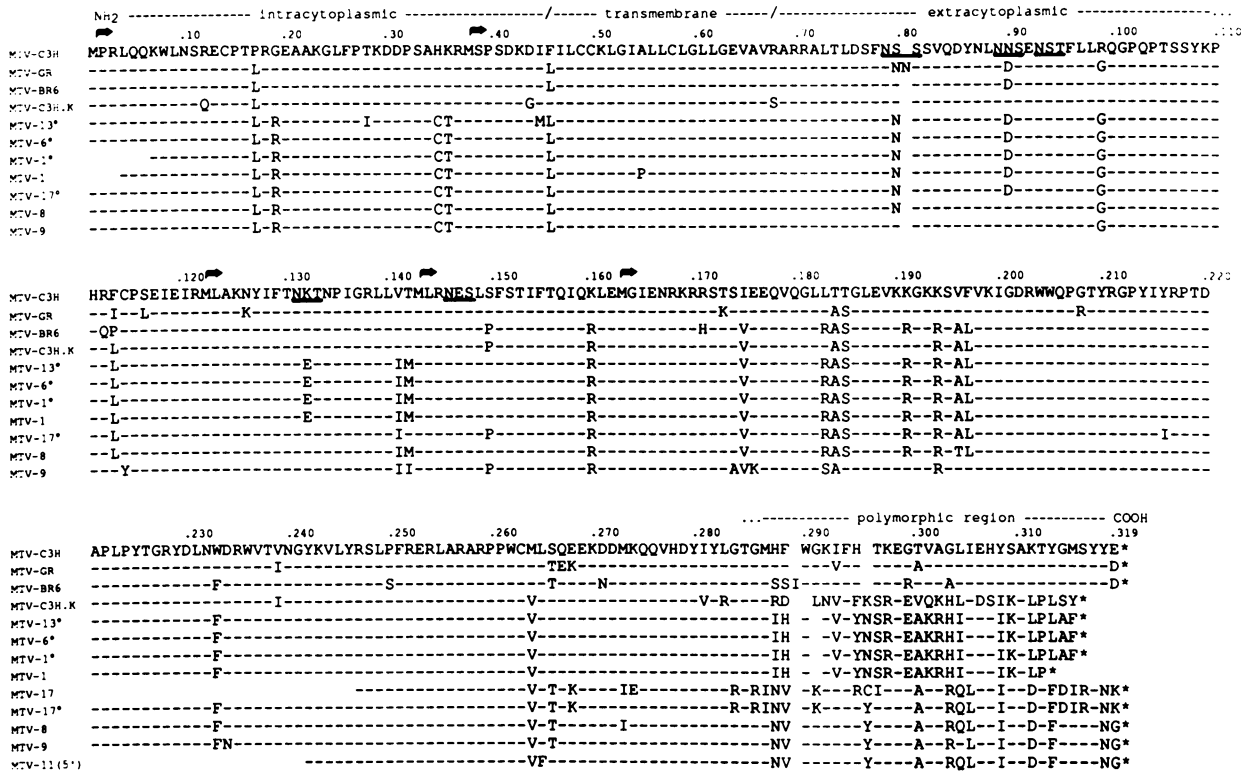

Fig. 4. Protein sequences (in the single letter amino acid code) of orf genes from various MTVs. Sequences denoted by the symbol ° were determined in this work. Other sequences were taken from the following references: MTV-C3H (Majors and Varmus, 1983) corrected in Choi *et al.* (1991) and herein, MTV-BR6 (Moore *et al.*, 1987), MTV-GR (Fasel *et al.*, 1982), MTV-C3H.K (Acha-Orbea *et al.*, 1991), MTV-1 (Crouse and Pauley, 1989), MTV-17 (Kuo *et al.*, 1988), MTV-8 (Donehower *et al.*, 1983), MTV-9 (King *et al.*, 1990), MTV-11 (Crouse and Pauley, 1989). The intracytoplasmic N-terminal (NH_2) tail, the transmembrane domain and the extracytoplasmic domain, containing the polymorphic C-terminus (COOH), are indicated above the MTV-C3H sequence. Potential sites of *N*-glycosylation (N−X−S/T) are underlined in the MTV-C3H sequence. Putative initiator methionines are indicated by an arrow above the MTV-C3H sequence and termination codons are marked by an *.

3, Figure 3), suggest that an orf product is not secreted cotranslationally.

We have also shown that endogenous MTV orfs encode glycosylated 46 kDa proteins (Figure 3). Three endogenous MTV LTRs, isolated from DBA/2J mice [MTV-17 (clone 53), MTV-1 (clone 62) and MTV-6] were used as templates for the preparation of RNA. Each of these RNAs directs the synthesis of a 36 kDa protein which becomes highly glycoslyated in the presence of microsomes. All the glycosylation sites among these orfs are highly conserved (Figure 4). Curiously, the level of proteins produced by internal initiation of the orfs is much reduced compared to MTV-C3H.

Sequence variation of endogenous MTV orfs

Sequence comparisons of orfs which are related in functional stimulatory capabilities (i.e. Vβ specificity) are nearly identical in sequence, but it is in the C-terminal 30 aa that variation is observed among the different orfs (Acha-Orbea *et al.*, 1991; Choi *et al.*, 1991) (Figure 4). For example, sequence similarity is observed for MTV-8, -9 and -11, which are all associated with Vβ11 tolerance, but this family differs in sequence at the C-terminus from MTV-1 and MTV-C3H (Choi *et al.*, 1991). There remain, however, several endogenous MTV orfs whose sequences have yet to be determined. In particular several MTV loci are associated with the deletion of $V\beta3^+$ T cells. MTV-6 has been shown to be genetically linked to $Mls\text{-}3^a$, which governs the strongest Vβ3 deletion, while MTV-13 is associated with $Mls\text{-}2^a$ (Pullen *et al.*, 1989; Frankel *et al.*, 1991). In addition, MTV-1 contributes to Vβ3 deletion (Frankel *et al.*, 1991).

In order to analyse the sequence polymorphism which contributes to this specificity, we isolated MTV-1, -6 and -13 from DBA/2J by amplifying the MTV orfs from size fractions of DNA which had previously been identified as containing these MTV sequences (Frankel *et al.*, 1991). Sequence analysis revealed that the protein sequences of MTV-1 and MTV-6 are identical to each other, while MTV-13 differs from MTV-1 and -6 by just two amino acids at positions 28 and 45 (Figure 4). Comparison of the protein sequences of MTV-1 from DBA/2J and MTV-1 from C3Hf/Se reveals that they are nearly identical (four differences). However, the DNA sequences of MTV-1, -6 and -13 have a sufficient number of differences among them to show that these sequences arise from the specific MTV loci and not as PCR artifacts. MTV-1, which is derived from either the 5′ or 3′ LTR, has nine nucleotide differences (including two noncoding) with MTV-6. Similarly, MTV-13 has nine nucleotide differences with MTV-1, while it has eight nucleotide differences when compared with MTV-6. Thus, it appears that MTV-1, -6 and -13 comprise a family of endogenous MTV loci which share sequence identity and therefore Vβ specificity. This family may contain another member which was isolated from a fraction of DNA which contained MTV-17 (see below). This sequence (of which only a single clone was analysed) is derived from a 3′ LTR fragment and differs from MTV-1 and -6 by three amino acids (data not shown); if it is expressed it could also contribute to Vβ3 deletion. Lastly, we have determined the sequence of MTV-17 which was isolated from a fraction of DBA/2J DNA containing either the 3′ LTR of MTV-17 or MTV-14. It is similar to a partial sequence of MTV-17 isolated from C57Bl/6 (five amino acid differences in the region sequenced). MTV-17 is highly similar to MTV-8, 9 and 11 in its C-terminus and thus represents another member of this family.

Discussion

The experiments described here present a type II transmembrane glycoprotein structure for MTV-encoded superantigens and have several implications for the biology of orf/Mls gene products. The transmembrane orientation of the orf gene product suggests that it is expressed at the cell surface such that the polymorphic C-terminus, which is extracytoplasmic, selectively interacts with specific Vβ chains of the T cell receptor. Sequence comparisons of orf proteins reveal that the C-terminus is nearly identical among MTVs with identical stimulatory specificities, but differs among orfs with different specificities. Residues of the Vβ chain which are involved in Mls interactions are located on a face of the T cell receptor which is thought to lie outside the putative binding site for MHC and antigen (Cazenave *et al.*, 1990; Pullen *et al.*, 1991). The orf structure also lends support to the idea that the orf protein can serve as a cell surface Vβ-specific adhesion molecule or 'coligand', which can augment the efficiency of antigen presentation (Janeway *et al.*, 1983; Janeway, 1990). In addition, the existence of an intracytoplasmic domain of the orf protein suggests the possibility of signalling upon interaction with the T cell. While the orf protein has not been observed at the cell surface, its structure as demonstrated here, is consistent with cell surface expression. This suggests that orf stimulates T cells as a consequence of cell surface expression and not as a processed peptide which interacts with MHC class II molecules. Furthermore, our results argue against the expression of a secreted form of the orf protein, whose mode of action is analogous to the bacterial enterotoxins.

Superantigen stimulation of T cells requires the presence of class II MHC molecules (Abe and Hodes, 1989). This fact and the effects of class II haplotypes on Mls stimulation (Kappler *et al.*, 1988) have been interpreted as evidence for a direct interaction of the orf protein with class II molecules. The transmembrane nature of the orf protein creates the possibility for contacts with class II molecules through either the intracytoplasmic, transmembrane or extracytoplasmic portions of the orf protein. Indeed, an effect of $Mls\text{-}1^a$ on the membrane mobility of class II molecules has been described (Mecheri *et al.*, 1990). Class II MHC−orf interactions may occur intracellularly, or alternatively, the orf protein may reach the cell surface independent of class II molecules. Furthermore, class II molecules may not be required for T cell stimulation. For example, $CD8^+$ T cells have been shown to be potent inducers of tolerance to $Mls\text{-}1^a$ (and $Mls\text{-}3^a$) (Webb and Sprent, 1990; Webb *et al.*, 1990), while they are generally thought to be class II negative. Similarly, T cell blasts, presumably class II negative, can stimulate Mls responses (Larsson-Sciard *et al.*, 1990).

Finally, intercellular transfer of Mls antigens *in vivo* has been observed in irradiation bone-marrow chimeras (Pullen *et al.*, 1988; Spieser *et al.*, 1989), although contested by others (Webb *et al.*, 1989). However, transfer of Mls *in vitro* has been difficult to demonstrate. Our results suggest that it is unlikely that orf proteins are readily transferred between cells. Perhaps they can be shed or cleaved from the membrane or rather that the orf gene is transferred through viral infection. Further studies on the biosynthesis and

expression of the orf gene should elucidate its role in T cell stimulation and tolerance as well as its role in viral physiology.

Materials and methods

In vitro translation and DNA constructs

RNAs were synthesized using either T7 or SP6 polymerase in the presence of 7mGpppA cap analogue and translated in a rabbit reticulocyte lysate as described (Gaspar *et al.*, 1991). Rough microsomal membranes (RM) were prepared from canine pancreas as described (Blobel and Dobberstein, 1975) and were added to the translation mix at a concentration of ~5 OD_{280}/ml. Post-translational proteolysis of the products of cell free translation was carried out using proteinase K (PK) at a concentration of 0.2 mg/ml for 10 min at 25°C, followed by the addition of phenylmethyl sulfonyl chloride. Nonidet P-40 (NP-40) was added at a concentration of 0.1% where shown. For inhibition of cotranslational addition of carbohydrate side chains, translations were carried out in the presence of oligosaccharide acceptor peptide, Benzoyl−Asn−Gly−Thr−NHMe (peptide) at a concentration of 500 mM. Treatment with *N*-glycanase at 10 U/ml (Genzyme) was carried out at 37°C for 8 h in 0.2 M $NaPO_4$ pH 8.8, 1.25% NP-40 after boiling in 1% SDS. The non-specific band which appears at ~29 kDa in all samples with *N*-glycanase is due to trapping of radioactivity by a cold protein present in the reaction mixture. For carbonate treatment (Fujiki *et al.*, 1982), microsomes were pelleted through a sucrose cushion and resuspended in 0.1 M Na_2CO_3 pH 11 for 30 min at 0°C; the reaction mixture was then centrifuged at 240 000 *g* for 1 h to separate membrane from soluble components. Aliquots of the translation mixes were electrophoresed on 12.5% polyacrylamide gels and processed for autoradiography.

The C3H orf and other orfs isolated from DBA/2J (see Figure 4) were cloned into pGEM-3zf(−) (Promega). C-terminal deletions were created by digesting plasmids prior to transcription with *Sau*3AI for C3HΔ247−319 or *Stu*I for C3HΔ186−319. N-terminal deletions (C3HΔ1−122 and C3HΔ1−80) were created by ligating an *Alu*I−*Bgl*II or a partial *Bsm*I−*Bgl*II fragment, respectively, of the C3H orf into a plasmid containing the T7 promoter followed by an initiator ATG and a polylinker (kindly provided by R.Andino). Thus for the N-terminal deletions C3HΔ1−122 and C3HΔ1−80, the protein is synthesized with the amino acid sequence Met−Ala−Ser−Gly−Thr prior to the orf sequence. C3HΔ1−37 was constructed by subcloning a *Fsp*I−*Bgl*II fragment of orf into pGEM-3zf(−).

Isolation of endogenous MMTV orf sequences

MTV-1, MTV-6, MTV-13 and MTV-17 were isolated from DBA/2J by the polymerase chain reaction. DBA/2J DNA was digested with *Pvu*II and electrophoresed on a 0.8% agarose gel. Size fractions of the DNA were used for PCR reactions using either primers which are specific for the MTV envelope (5′-GCAGTCAGATCTTAACGTGC-3′, 27 bp 5′ to the ATG for Met1) or the 5′ portion of the MTV LTR (5′-GGATCCGCCA-CCATGCCGCGCCTGCAGCAG-3′, which includes the ATG for Met1) and a primer specific for the 3′ portion of the LTR (5′-TTAGATCT-AAACATTTGATACC-3′, 104 bp 3′ of the termination codon in MTV-C3H). C3H orf was isolated by amplification of a plasmid containing MTV C3H envelope and 3′LTR (kindly provided by G.Shackelford). DNA sequences were determined from clones isolated in two separate amplification reactions. The identification of specific MTV was based on the prior determination of size fragments containing specific MTV as described by Frankel *et al.* (1991). All the MTV LTR orfs were isolated with the envelope primer except for MTV-1; therefore these orfs are derived from the 3′ LTR from which the orf message can be transcribed. MTV-6 was isolated from a fraction of DNA of ~2.8−3.3 kb, MTV-13 from a size fraction of ~6.0−6.6 kb. MTV-17 (clone 53) was isolated from a DNA fraction (~6.6−7.4 kb) containing the 3′ LTR of MTV-17 and MTV-14. This DNA fraction also contained a second clone which is related to the MTV-1, -6, -13 family (data not shown) and is possibly MTV-14.

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Note added in proof

The sequences reported in this paper have been deposited in the EMBL Data Library under the accession numbers X64553 (MTV-1), X64554 (MTV-6), X64555 (MTV-13) and X64556 (MTV-17). Similar results have recently been published [Pullen *et al.* (1992) *J. Exp. Med.*, **175**, 41−47; Choi *et al.* (1992) *J. Exp. Med.*, **175**, 847−852].