Use of a Cell-Free System To Identify the Vaccinia Virus L1R Gene Product as the Major Late Myristylated Virion Protein M25†

CHRISTINE A. FRANKE, ELIZABETH M. WILSON, AND DENNIS E. HRUBY*

Department of Microbiology, Center for Gene Research, Oregon State University, Corvallis, Oregon 97331-3804

Received 6 July 1990/Accepted 12 September 1990

A 25-kDa vaccinia virus (VV) virion protein, designated M25, is modified *in vivo* by covalent addition of myristic acid. The predicted amino acid sequences of all VV open reading frames which have been reported were searched for the sequence M-G-X-X-X-(S/T/A), which has been proposed to be the consensus recognition signal for cotranslational modification of proteins by *N*-myristyltransferase. This conserved signal was found at the amino terminus of a single locus, which corresponded to the leftmost rightward-reading open reading frame (L1R) initiating within the VV *Hind*III L DNA fragment. By using synthetic oligonucleotides in concert with polymerase chain reaction techniques, a chimeric gene consisting of open reading frame L1R fused to a bacteriophage T7 promoter was constructed and cloned into a plasmid vector. Transcripts derived from the wild-type expression plasmid (designated pL1_{G1}) were translated *in vitro* in a wheat germ extract to yield a polypeptide with an apparent molecular mass of 25 kDa. This polypeptide was labeled with either [³⁵S]methionine or [³H]myristic acid and comigrated with *in vivo*-labeled protein M25 on sodium dodecyl sulfate-polyacrylamide gels. Polyclonal antiserum generated in rabbits against a *trpE*:L1R fusion protein immunoprecipitated a 25-kDa protein labeled either *in vitro* (the L1R gene product, designated protein L1) or *in vivo* (from purified VV, protein M25), identifying the M25 protein as the gene product of open reading frame L1R. Chromatographic analysis of the protein L1-bound fatty acid moieties liberated after acid methanolysis resulted in recovery of greater than 99% of the fatty acid as myristate-associated label. Cell-free translation of proteins derived from a set of deletions from the carboxy terminus of the open reading frame L1R suggested that the site of myristylation maps near the amino terminus of protein L1. This hypothesis was supported by cell-free translation of mutant L1R transcripts in which the penultimate glycine codon had been altered by site-directed mutagenesis to encode either an aspartic acid (pL1_{D1}) or alanine (pL1_{A1}) residue. In both cases, the mutant transcripts were translated into a 25-kDa protein which could be labeled *in vitro* with [³⁵S]methionine but not with [³H]myristic acid. These data demonstrate that VV open reading frame L1R encodes a myristylated protein and provide evidence that the site of modification of protein L1 is the amino-terminal glycine residue.

In recent years, it has become increasingly recognized that covalent linkage of lipid moieties to protein molecules represents a major pathway by which cellular polypeptides are co- or posttranslationally modified. Although a variety of acyl groups have been found attached to proteins, the most common species are the saturated 14- and 16-carbon fatty acids, namely, myristic acid and palmitic acid (10, 32, 33, 40). Although myristate and palmitate are closely related in structure, there are distinct differences in the linkage and positions by which they are attached to substrate proteins. Myristate is typically attached to proteins cotranslationally via an amide bond to an N-terminal glycine residue. Analysis of the amino-terminal sequences of known myristylated polypeptides and studies *in vitro* using synthetic oligopeptides as substrates for *N*-myristyltransferase (NMT) purified from *Saccharomyces cerevisiae* (38) or rat liver (39) have elucidated fundamental structural requirements for myristylation of a polypeptide chain. Immediately after the initiation codon-derived methionine, which is removed from the polypeptide before myristylation, an amino-terminal glycine residue (at position 1) is the only conserved residue and is the acceptor residue of the myristate moiety. The only other discernible motif is the presence of a serine, threonine, or less commonly, alanine residue at position 5 in most my-

ristylated polypeptides. Additional sequence determinants may be important, and some restrictions seem to exist at positions 2 and 6 (for a review, see reference 40). In contrast, palmitate is usually attached posttranslationally to internal serine or cysteine residues via an ester or thioester bond, respectively, with little apparent conservation in the amino acid residues which flank the attachment site (21, 32).

Although the overall biological significance of protein acylation has yet to be determined, many acyl proteins appear to be associated primarily with membranes and the acyl group is believed to be responsible, at least in part, for membrane targeting (10, 15). Examples of palmitated protein species include both peripheral and transmembrane proteins, such as *N-ras*, vesicular stomatitis virus G protein, and the transferrin receptor. In contrast, many myristylated proteins, such as pp60^{v-src} and cyclic AMP-dependent protein kinase are peripheral membrane proteins. Note that several of these examples are viral proteins, which are representative of an increasing number of reported viral acyl proteins (10, 32). In view of this fact, together with the observation that vaccinia virus (VV) infection involves both viral and cellular membranes at a number of levels (7), examination of the potential role of protein acylation in the VV replication cycle should be both relevant and potentially important.

The existence of VV acyl proteins has been demonstrated by metabolically labeling VV-infected cells with either [³H]myristic acid or [³H]palmitic acid. Under these condi-

* Corresponding author.

† Oregon Agricultural Experiment Station technical paper 9291.

tions, a number of labeled protein species are evident in extracts made from cells late during the infection cycle which are absent in early extracts or extracts from uninfected cells (8). There are three proteins which are apparently palmitated, including a 40-, a 20-, and a major 37-kDa species (P37). The latter protein, P37, was previously reported by Hiller and Weber and appears to be a membrane protein that is specifically associated with extracellular enveloped virions (12). The gene that encodes this protein has been mapped to the *Hind*III F fragment and sequenced by Hirt and colleagues (13). There also appear to be three late myristylated VV proteins, two minor species with apparent molecular masses of 37 and 35 kDa, and a major protein of 25 kDa which has been designated M25 (8).

Protein M25 appears to be associated with the intracellular virion envelope. Linkage of the [³H]myristic acid label to M25 is resistant to treatment with 1 M hydroxylamine, suggesting that the acyl group is linked to the protein via an amide bond. The identity of the acyl group attached to M25 was established by high-pressure liquid chromatography (HPLC) analysis. Although protein M25 can be labeled *in vivo* only with a myristic acid label and not palmitic acid, analysis of the M25-associated lipid demonstrated that although initially most of the fatty acid linked to the protein was myristic acid, a considerable portion was apparently elongated to palmitic acid after addition of myristic acid to the protein substrate. The biological significance of this conversion remains to be investigated (8).

After it was demonstrated that polypeptide M25 represents a bona fide myristylated VV protein, it was of interest to map the viral gene which encodes protein M25 so that molecular genetics could be used to investigate the biochemistry and biology of this modification reaction within the context of virus-infected cells and to determine what role protein M25 plays during the VV replication cycle. In the experiments reported here, the VV L1R transcriptional unit was identified as the locus which encodes protein M25. In addition, cell-free translation and acylation systems were used to study the specificity and nature of myristylation of L1R-derived protein *in vitro*.

MATERIALS AND METHODS

Cells and virus. Growth, purification, and metabolic labeling of VV (strain WR; Western Reserve) were carried out as previously described (8, 14). Monolayers of BSC-40 (African green monkey kidney) cells were maintained in Eagle minimal essential medium (Earle salts; GIBCO Laboratories) supplemented with 10% heat-inactivated fetal bovine serum–2 mM L-glutamine–10 µg of gentamicin sulfate per ml at 37°C, 5% CO₂, and 95% humidity.

Computer analyses. The predicted amino acid sequences of all VV open reading frames (ORFs) in the Swiss-Prot 12 and Protein Identification Resource 21 data banks were searched by using the QUEST program of the IntelliGenetics Suite, Release 5.37, for the sequence G-X-X-X-(S/T/A), the proposed minimal consensus signal for N-myristylation of a protein.

PCR and construction of recombinant plasmids. To construct plasmids containing wild-type (pL1_{G1}) or mutant (pL1_{D1} and pL1_{A1}) sequences of ORF L1R abutted to a bacteriophage T7 promoter sequence, polymerase chain reaction (PCR) amplification primers were synthesized on an Applied Biosystems DNA synthesizer and purified by HPLC (1). The PCR was performed as previously described, with minor modifications (42). Briefly, 0.1 pmol of a DNA tem-

plate (pBR322:L; the VV *Hind*III L fragment cloned into the *Hind*III site of plasmid pBR322) and 300 pmol of each PCR oligonucleotide primer [PCR 5' primer, 5'-TAATACGACT CACTATAGGGAGAATGG (G/C/A/T)TGCCGCGGCAA G-3' (T7 promoter sequences are in boldface); PCR 3' primer, 5'-CGGATCCGTCAGTTTTGCATATCC-3'] in a volume of 100 µl were denatured at 94°C for 1.5 min (1 min in subsequent cycles) in 50 mM KCl–10 mM Tris hydrochloride (pH 8.4 at room temperature)–1.5 mM MgCl₂–dATP, dCTP, dGTP, and dTTP, each at 200 µM–2.5 U of *Taq* polymerase (Promega Biotec, Madison, Wis.). The reactions were annealed at 37°C for 2 min, and chain extension was carried out at 70°C for 3 min. An additional 25 cycles of denaturation, annealing, and polymerization were performed. The PCR products were extracted with phenol-chloroform and concentrated by precipitation with ethanol. The 784-bp PCR-amplified product was isolated as the major ethidium bromide-stained species from a 1% agarose (Tris-borate-EDTA) gel, phosphorylated by using polynucleotide kinase T4 (New England BioLabs), and ligated into the *Sma*I site of pUC119 (a derivative of pUC19 in which an M13 phage origin of replication was inserted; a gift of J. Vieira).

Construction of plasmids pGEM-*src* (6; the generous gift of M. D. Resh) and pT7:TKII (42) has been described elsewhere.

Construction of the pATH:L1R expression vector and production of anti-L1 serum. A pATH:L1R expression vector was assembled for production of a *trpE*:L1 fusion protein to serve as the antigen for production of a monospecific polyclonal antiserum to the predicted L1R gene product. Plasmid pL1_{G1} DNA was digested with restriction endonucleases *Nco*I and *Pvu*II, which cut the DNA between the codons for amino acid residues 46 and 47 of ORF L1R (250 residues total) and beyond the 3' end of the ORF in plasmid DNA sequences, respectively. The single-stranded termini of the DNA fragment that was liberated were converted to flush ends by the activity of the Klenow fragment of DNA polymerase I and cloned into the *Sma*I site of the pATH-2 *trpE* expression vector plasmid (34). Analytical restriction endonuclease digestions were used to verify that the DNA was inserted in a colinear fashion with the bacterial *trpE* gene. The resulting plasmid, pATH:L1R, therefore contained a chimeric ORF which consisted of the N terminus of the *trpE* gene fused in frame with the carboxy-terminal 204 codons of the VV L1R gene under control of the *trpE* transcriptional signals. The predicted molecular mass of the *trpE*:L1 fusion protein was approximately 60 kDa.

Induction of expression of the *trpE*:L1 fusion protein and preparation of *Escherichia coli* protein lysates were performed by previously described methods (24). The overexpressed *trpE*:L1 fusion protein was isolated by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The fusion protein was localized by staining with Coomassie brilliant blue, excised, emulsified in Freund complete adjuvant, and injected intradermally into female New Zealand White rabbits (approximately 2-kg body weight). The rabbits received two additional injections of *trpE*:L1 antigen in Freund incomplete adjuvant at 7 and 14 days, intradermally and intramuscularly, respectively. Approximately 200 µg of antigen was used per inoculation. The rabbits were bled by cardiac puncture 10 days after the last inoculation, and the serum was prepared for use in immunoassays.

In vitro transcription reaction and analyses. Plasmid DNAs to be used as templates for *in vitro* runoff transcription reactions were purified in accordance with the protocols of

the manufacturer of the Riboprobe system (Promega Biotech). Plasmids (pL1_{G1}, pL1_{D1}, pL1_{A1}, pT7:TKII, and pGEM-*src*) were linearized by digestion with restriction endonucleases (*Bam*HI for pT7:TKII and *Eco*RI in all other plasmids) and transcribed in vitro (23) with either RNA polymerase T7 (pL1_{G1}, pL1_{D1}, pL1_{A1}, and pT7:TKII) or SP6 (pGEM-*src*). The transcription reaction mixture contained ATP, CTP, and UTP, each at 800 μ M; 80 μ M GTP; and 800 μ M GpppG (New England BioLabs) as described previously (42). After incubation for 2 h at 37°C, the reaction mixture was precipitated with ammonium acetate and ethanol by standard procedures. The sizes and integrity of the transcripts were verified by denaturing formaldehyde-agarose gel electrophoresis (20).

In vitro translation and product analyses. The mRNAs produced in the in vitro transcription reactions were translated in mRNA-dependent wheat germ extract as specified by the manufacturer (Promega Biotec). Radiolabeling was performed with 0.5 μ Ci of L-[³⁵S]methionine (1,153 Ci/mmol; Amersham Corp.) per μ l in 20 μ M amino acids (minus methionine) or with 2 μ Ci of [9,10-³H]myristic acid (54 Ci/mmol; Amersham) which had been dried under nitrogen and suspended by sonication in water per μ l in 20 μ M amino acids. Reaction mixtures (15 μ l) were incubated at 25°C for 60 min. The radioactive polypeptides synthesized were routinely analyzed on 10% polyacrylamide slab gels containing SDS (35). Low-molecular-weight polypeptides were analyzed on a 20% T–0.5% C_{bis} acrylamide (pH 9.3) separating gel with a 10% T–4.8% C_{bis} acrylamide (pH 6.8) stacking gel (G. G. Guilian and J. Graham, Fed. Proc. 44:686, 1985). The gels were then impregnated with 2,5-diphenyloxazole and fluorographed at –70°C on Kodak XAR-5 film (2). To establish the relatedness of the 25-kDa polypeptide labeled by myristic acid in vivo and the gene product of ORF L1R, extracts of purified VV or translation reaction mixtures were suspended in radioimmune precipitation assay buffer and immunoprecipitated (24) with the *trpE*:L1 antiserum described above.

Analysis of protein-bound fatty acid. Neutral hydroxylamine and chloroform-methanol (2:1) treatment of labeled proteins, acid methanolysis, and HPLC analysis of protein-bound fatty acid were done as described previously (8).

RESULTS

Identification of a VV ORF containing a myristylation consensus sequence. Covalent attachment of myristic acid to two VV polypeptides (M_r s, ~25,000 and 35,000; designated M25 and M35, respectively) has been reported previously (8). Both myristylated polypeptides were expressed at late times during the virus replicative cycle and were present in purified intracellular virus particles. Neither the role that the modification of these polypeptides by myristate plays nor the biological function of these polypeptides has been determined. To address these questions, we sought to map the genes that encode these myristylated polypeptides and obtain their sequences, which might provide information regarding homologous functional domains of previously sequenced proteins and allow us to clone and manipulate the sequences responsible for modification by myristic acid to assess the biological consequences of such changes. As an initial approach, the predicted amino acid sequences of all VV ORFs in the Swiss-Prot and Protein Identification Resource data banks were searched for the sequence G-X-X-X-(S/T/A), a sequence which comprises the minimal consensus recognition signal for modification of proteins by NMT.

In each case previously studied, myristic acid has been found to be linked through an amide bond to the α -amino group of the amino-terminal glycine (33). While the minimal sequence was frequently found to be internal to protein sequences (data not shown), this conserved signal was found at the amino terminus of only a single VV protein sequence in the Protein Identification Resource data bank. The protein sequence containing the amino-terminal myristylation consensus sequence was the protein (Protein Identification Resource entry, QQVZF2) predicted on the basis of the nucleotide sequence of ORF F2 and is encoded within the VV *Hind*III L fragment (27). We shall refer to the ORF previously designated F2 as ORF L1R in accordance with the nomenclature proposed by Rosel et al. (30). ORF L1R corresponds to the leftmost rightward-reading ORF initiating within the VV *Hind*III L DNA fragment (Fig. 1) and is predicted to encode a protein with a calculated molecular weight of 27,250. ORF L1R contains the conserved nucleotide sequence (TAAATG) surrounding the translational initiation site which is characteristic of many VV late genes (30). Furthermore, proteins synthesized by in vitro translation of late VV mRNA hybridizing to this portion of the *Hind*III L DNA fragment contain a protein of ~27 kDa (41). The kinetic class of the gene and the predicted molecular weight of the polypeptide encoded by ORF L1R are in good agreement with those of myristylated VV protein M25.

In vitro synthesis and myristylation of polypeptide L1. Because the results presented in Fig. 1 suggested that ORF L1R of VV encodes the myristylated 25-kDa polypeptide previously designated M25 on the basis of in vivo studies (8), it was of interest to determine biochemically if this were the case. Towler and Glaser (36, 37) have previously shown that myristylation can be studied in vitro by using synthetic peptide substrates to detect NMT activity. By using this approach, NMT activity has been detected in many diverse eucaryotic cell types, notably, in rabbit reticulocytes and wheat germ lysates, and recently published studies (4, 6, 11) have reported synthesis of myristylated proteins in rabbit reticulocyte lysates. Therefore, the translational capabilities of cell-free systems shown to contain NMT activity, namely, rabbit reticulocytes and wheat germ extracts, were used to determine whether the gene product of ORF L1R was a substrate for N-myristylation in vitro.

Toward this end, a plasmid transcription vector containing a chimeric gene consisting of all of ORF L1R fused to a bacteriophage T7 promoter was constructed by using synthetic oligonucleotide primers to amplify the target sequences by PCR. The PCR 5' primer consisted of the nucleotide sequence for a bacteriophage T7 promoter adjacent to the first 16 nucleotides of ORF L1R, beginning with the ATG codon which encodes the methionine residue that initiates the ORF (Fig. 1). To mutagenize the putative myristate acceptor glycine residue of the polypeptide, four-fold degeneracy was introduced in the nucleotide sequence of the PCR 5' primer at position 2 of the codon that encodes the amino-terminal glycine residue (protein residue 1), such that mutations that encode alanine (A), valine (V), or aspartic acid (D) should be obtained along with the wild-type sequence. The PCR 3' primer consisted of 13 nucleotides upstream of and including the termination codon (TGA) of ORF L1R joined to a *Bam*HI restriction endonuclease site. The PCR primers were used to amplify the chimeric sequence from plasmid DNA containing the VV *Hind*III L fragment. The 784-bp PCR-amplified product was ligated into the *Sma*I site of pUC119, and dideoxynucleotide chain termination sequencing reactions were performed by using

FIG. 1. Genomic location of the L1 ORF and construction of L1 expression plasmids. The diagram at the bottom represents the *Hind*III restriction map of the VV genome. *Hind*III fragments are designated A to P on the basis of size. In the middle, the *Hind*III L fragment is expanded. The transcriptional pattern predicted on the basis of the nucleotide sequence analysis of this fragment (27) is illustrated by arrows which depict the location and orientation of each ORF. The ORFs are named in accordance with the conventions proposed by Rosel et al. (30). The relative locations of the oligonucleotide PCR primers used to amplify, mutagenize, and clone the *Hind*III L1 ORF are depicted (PCR 5' and PCR 3'). The PCR 5' primer contains a bacteriophage T7 promoter sequence. At the top, the structure of wild-type expression plasmid pL1_{G1} is diagrammed. The L1 ORF is abutted to a T7 promoter and inserted into the *Sma*I site of plasmid pUC119. Relevant restriction endonuclease sites within the plasmid polylinker are indicated (*Hind*III and *Eco*RI). The nucleotide sequence and the predicted amino acid sequence of the amino-terminal seven codons are displayed. The myristylation consensus recognition sequence is listed (X is any amino acid residue) and enumerated above pL1_{G1}, with the homology between the two sequences surrounded by a box. The mutant plasmids (pL1_{D1} and pL1_{A1}) are shown below (stippled boxes), with the nature of the nucleotide mutations and predicted residue substitutions indicated.

either the M13 universal or reverse sequencing primer to sequence the 3' and the 5' junctions to identify and verify the presence of wild-type and mutant sequences in the glycine (position 1) codon (31). The wild-type plasmid was designated pL1_{G1}, and the mutant plasmids were designated pL1_{A1} and pL1_{D1}, with the subscript in the nomenclature indicating the amino acid residue encoded in position 1, i.e., residue 1 after the initiator methionine (Fig. 1). No mutants containing the valine codon in position 1 were obtained from this amplification. Plasmids containing genes known to encode either a myristylated protein or a nonmyristylated protein served as controls for the expression vector constructed to study the L1R gene product. Transcription vector pGEM-*src* (6) consists of a restriction fragment containing the *src* gene, which encodes the well-characterized myristylated protein pp60^{v-src}, the transforming protein of Rous sarcoma virus (3, 17), cloned into a vector containing the bacteriophage SP6 promoter. Expression vector pT7:TKII (42) contains DNA sequences which encode the gene for the VV enzyme thymidine kinase, which is a nonmyristylated protein, abutted to a bacteriophage T7 promoter. Functional *src*, *tk*, and *L1* mRNAs were synthesized by in vitro transcription with either RNA polymerase SP6 or T7. The mRNA was used to program either rabbit reticulocyte lysates or wheat germ extracts, and the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis. Preliminary studies demonstrated that wheat germ extracts were more efficient than rabbit reticulocyte lysates for myristylation of pp60^{v-src} synthesized by in vitro translation (data not shown). Therefore, wheat germ extracts were used for the studies described here. In [³⁵S]methionine,

each mRNA served as the template for a single polypeptide band of an appropriate molecular size (Fig. 2) (*src*, 60 kDa; *tk*, 20 kDa; *L1*, 25 kDa). Translations performed with [³H]myristic acid resulted in incorporation of radiolabel into pp60^{v-src} and the L1 polypeptide but not the *tk*-encoded polypeptide (Fig. 2). The L1 protein synthesized in vitro comigrated with M25 protein from purified VV labeled in vivo.

The identity of the 25-kDa polypeptide labeled in vivo by [³H]myristic acid (M25) as the gene product of ORF L1R was further established by immunoprecipitation of either extracts of purified VV or the L1 translation reaction mixtures, radiolabeled with either [³H]myristic acid or [³⁵S]methionine, by using polyclonal antiserum to the *trpE*:L1R fusion protein (described in Materials and Methods). In the experiment shown in Fig. 3, a 25-kDa polypeptide was immunoprecipitated from either purified VV or cell-free translation reactions of the L1 mRNA by α -*trpE*:L1R serum. Preimmune serum did not react with this polypeptide species. Similar results were observed in immunoblots of the extracts (data not shown).

Analysis of L1 protein-bound fatty acids. Covalent attachment of myristate to the L1 polypeptide synthesized in vitro was investigated by experiments in which myristic acid-labeled translation reaction mixtures were delipidated by sequential extraction with chloroform-methanol (2:1) before SDS-polyacrylamide gel electrophoresis (8). The pattern of labeled proteins was unaffected by this treatment, except that the non-protein-bound radioactive lipid which migrates to the dye front during electrophoresis was absent after treatment (data not shown).

FIG. 2. Synthesis and myristylation protein L1 produced by in vitro translation. Wheat germ extracts were programmed with mRNA (–, no RNA; src, Rous sarcoma virus pp60^{src}; tk, VV thymidine kinase; L1, VV L1 ORF) synthesized in vitro by RNA polymerase SP6 or T7. Translation was performed with [³⁵S]methionine (left panel) or [³H]myristic acid (right panel). Purified intracellular VV was prepared from infected cells labeled continuously for 24 h postinfection with [³H]myristic acid (M25). Labeled translation products were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and visualized by fluorography. Migration of radioactive molecular size markers (MW) is indicated in the center. The markers were phosphorylase *b* (97.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21.5 kDa).

Fatty acids have been previously shown to be attached to proteins via both ester and amide linkages, and in general, some fatty acyl chain specificity of protein-lipid linkages has been observed. The linkage of the palmitic acid moiety to most of the acyl proteins studied has been identified as an ester or highly reactive thioester bond on the basis of its lability in response to alkaline methanolysis and sensitivity to the nucleophile hydroxylamine (19, 21). In contrast, the bond through which myristate is linked to proteins is resistant to treatment with hydroxylamine or alkaline methanolysis, suggesting amide linkage of this fatty acid to the acyl protein (25). To investigate the nature of the fatty acid linkage to the L1 polypeptide synthesized in vitro, the sensitivity of the myristate-labeled protein to cleavage by hydroxylamine was examined. Treatment of the L1 polypeptide with hydroxylamine did not substantially alter the myristate or methionine labeling pattern (data not shown). The resistance of the L1-bound myristate label to hydroxylamine treatment suggests that the fatty acid linked to these proteins is attached via an amide bond.

To determine whether the label associated with the L1 polypeptide was present as a fatty acid, rather than lipid metabolites, and to identify the moiety covalently bound to the polypeptide, individual fatty acid-labeled proteins were isolated by SDS-polyacrylamide gel electrophoresis. The identities and purity of the recovered proteins were confirmed by gel electrophoresis. In the experiment shown in

FIG. 3. Immunoprecipitation of purified VV virions and wheat germ translation reaction mixtures with α -L1 antiserum. Velocity gradient-purified VV metabolically labeled with either [³⁵S]methionine (S) or [³H]myristic acid (H) or wheat germ translation reaction mixtures programmed with L1 mRNA with [³⁵S]methionine (L1) were immunoprecipitated with α -L1 antiserum. Migration of radioactive molecular size markers is indicated at the left. The markers were phosphorylase *b* (97.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

Fig. 4, isolated polypeptide L1 from myristate-labeled in vitro translation reactions was subjected to acid methanolysis, followed by ether extraction as described previously (8) and the liberated fatty acids and fatty acid methyl esters were separated by reverse-phase HPLC. Fractions were collected and subjected to liquid scintillation counting. The distribution of radioactivity was compared with the absorbance elution profile of unlabeled fatty acid (myristate and palmitate) and fatty acid methyl ester (methyl myristate and methyl palmitate) standards which were cochromatographed to identify the radioactive acid methanolysis products. Acid methanolysis of the myristate-labeled L1 protein resulted in recovery of myristic acid and methyl myristate. Essentially no palmitic acid or methyl palmitate was detected. Most (99%) of the labeled lipid bound to L1 (isolated from myris-

FIG. 4. HPLC of L1 protein-bound fatty acids. Purified [³H]myristic acid-labeled L1 protein synthesized in vitro was subjected to acid methanolysis, and the released fatty acids and fatty acid methyl esters were separated by HPLC. Fractions (0.5 ml) were collected and subjected to liquid scintillation counting. The distribution of radioactivity was compared to the absorbance elution profile (A_{254}) of unlabeled fatty acid and fatty acid methyl ester standards which were cochromatographed to identify the radioactive acid methanolysis products. The fraction in which each fatty acid eluted is identified and marked by an arrow.

FIG. 5. Synthesis and myristylation of truncated L1 proteins. The structure of wild-type expression plasmid pL1_{G1} is diagrammed at the top, along with salient restriction endonuclease (R.E.) sites within the ORF. The *Eco*RI site lies external to the ORF. The plasmid DNA template was digested with each restriction endonuclease, and mRNA was synthesized in vitro with RNA polymerase T7. The successively truncated mRNAs were translated in wheat germ extracts with [³⁵S]methionine or [³H]myristic acid. A schematic representation of the predicted translation products is shown at the center. The unfilled box (*Eco*RI) represents full-length, protein L1 with the amino (N) and carboxyl (C) termini indicated. The stippled boxes below represent the truncated L1 proteins predicted to be synthesized from the correspondingly shortened mRNAs. The small black box at the N terminus of each predicted protein indicates the position of the proposed myristylation recognition consensus sequence (GAAAS). The positions of the initiator methionine, (M), and all other methionines encoded within the protein, M, are depicted. At the right, the predicted number of amino acid residues (AA) and M_s of the intact and truncated L1 proteins are indicated (minus the initiator methionine). At the bottom, translation products labeled with either [³⁵S]methionine (left panel) or [³H]myristic acid (right panel) were separated by SDS-polyacrylamide gel electrophoresis on a 20% T-0.5% C₁₈ acrylamide (pH 9.3) separating polyacrylamide gel and visualized by fluorography. Translation reactions are indicated by the restriction endonucleases used to generate the truncated template for RNA transcription (–, no RNA). Migration of radioactive molecular size markers (MW) is indicated at the center. The markers were carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa).

tate-labeled in vitro translation reactions) was found to exist as myristate moieties.

Mapping the site of myristylation of polypeptide L1. To identify the site of polypeptide L1 that is modified by myristate, two experimental approaches were used. In the first, wild-type expression plasmid (pL1_{G1}) DNA was digested individually with the restriction endonucleases listed

in Fig. 5 and mRNA was synthesized in vitro from the successively truncated DNA templates by using RNA polymerase T7. The nested set of truncated mRNAs were translated in wheat germ extracts in the presence of [³H]myristic acid or [³⁵S]methionine to produce a set of amino-coterminal L1 polypeptides sequentially deleted from the carboxyl terminus. The translation products were sepa-

rated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. Cell-free translation with [35 S]methionine resulted in synthesis of a major polypeptide of the approximate molecular size predicted in each case, except for the mRNA produced from the *Nco*I-truncated template, in which no polypeptide was labeled in the translation reaction. Since the only methionine residue that this truncated polypeptide is predicted to encode is the initiator methionine, this result suggests that the initiator methionine is removed by a methionine aminopeptidase in the wheat germ extract. This is in agreement with the finding of Towler et al. that before myristylation can take place, the initiator methionine at the N terminus of most newly synthesized apomyristyl proteins must be removed (38). A minor protein species whose migration showed that it was approximately 5 kDa smaller than the major translation product was present in the *Eco*RI, *Rsa*I, *Hae*III, and *Taq*I translation reactions but not in the *Hpa*II or *Nco*I reactions. This band likely results from an internal translational initiation event at an ATG between the *Hpa*II and *Taq*I sites. With [3 H]myristic acid, each truncated mRNA served as the template for a major labeled polypeptide band that comigrated with those that incorporated [35 S]methionine. Even the most highly truncated polypeptide (45 amino acids, resulting from *Nco*I digestion) was efficiently myristylated in vitro, suggesting that the site of myristylation mapped near the amino terminus of protein L1.

In most cases in which N-myristylation of a polypeptide has been observed, the substrate for the NMT-catalyzed acylation reaction is the glycine residue immediately adjacent to the initiation codon-derived methionine. To localize the site of myristylation of protein L1 further, point mutations were generated in the nucleotide sequence that codes for protein L1 to modify the N-terminal glycine residue (G_1) as described previously. The amino-terminal structures of the wild-type (pL1 $_{G1}$) and mutant (pL1 $_{A1}$ and pL1 $_{D1}$) L1 expression plasmids are depicted at the top of Fig. 6. To study the effects of the mutations on myristylation, wild-type and mutant plasmids were linearized by *Eco*RI and transcribed in vitro by using RNA polymerase T7. The mRNAs were translated in wheat germ extracts with [3 H]myristic acid or [35 S]methionine to produce a set of wild-type and mutant L1 polypeptides. The translation products were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and visualized by fluorography. With [35 S]methionine, each mRNA served as the template for a single polypeptide band of approximately 25 kDa that was immunoprecipitated with polyclonal antiserum to fusion protein *trpE*:L1R (Fig. 6; data not shown). In contrast, [3 H]-labeled protein L1 was detected only in the translation reaction from wild-type (G_1) mRNA. No [3 H]myristate labeling was detected in either mutant polypeptide L1 $_{A1}$ or L1 $_{D1}$ (Fig. 6). These results strongly implicate the amino-terminal glycine residue of polypeptide L1 as the acceptor residue for N-myristylation.

DISCUSSION

We have previously observed in vivo covalent attachment of myristic acid to an ~25-kDa VV virion protein, M25 (8). Our efforts to map the gene that encodes this protein were accelerated when a search of all VV ORFs in the data banks revealed that ORF L1R contains the minimal consensus recognition signal for NMT at the amino terminus of the predicted polypeptide. To determine whether this candidate ORF encodes M25, we used cell-free systems for *L1* mRNA

FIG. 6. In vitro synthesis and myristylation of wild-type and mutant L1 proteins. At the top, the amino-terminal structures of the wild-type and mutant L1 expression plasmids are depicted. The nucleotide and predicted amino acid sequences of the amino-terminal seven codons are displayed. The myristylation consensus recognition sequence is listed and enumerated above pL1 $_{G1}$ (wild type). The putative acceptor glycine residue (G_1) is highlighted by a lightly stippled box. The mutant plasmids (pL1 $_{D1}$ and pL1 $_{A1}$) are shown below, with the nature of the nucleotide mutations and predicted residue substitutions indicated within the darker stippled boxes. Wheat germ extracts were programmed with mRNA (–, no RNA; G_1 , pL1 $_{G1}$; D_1 , pL1 $_{D1}$; A_1 , pL1 $_{A1}$) synthesized in vitro by using RNA polymerase T7. Translation was performed with [35 S]methionine (left panel) or [3 H]myristic acid (right panel). Labeled translation products were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and visualized by fluorography. Migration of radioactive molecular size markers is indicated at the center. The markers were phosphorylase *b* (97.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21.5 kDa).

translation and myristylation. Recently published studies (4, 6, 11) have reported that myristylation occurs in rabbit reticulocyte lysates. However, some differences have been reported with regard to the specificity of these lysates for myristate over palmitate for acylation of *src*-encoded synthetic peptides, as well as the efficiency of myristylation of pp60 $^{v-src}$ synthesized by in vitro translation (6, 11). Wheat germ extracts have been also shown to contain NMT activity in studies using synthetic peptide substrates (6, 11). However, the NMT activity in wheat germ extracts exhibited specificity for myristate over palmitate, while retaining specificity for the peptide substrate (11). Since NMT had been detected in rabbit reticulocyte lysates and wheat germ extracts, we attempted to synthesize and myristylate both pp60 $^{v-src}$ and L1 in both of these translation systems. To program the translation mixture with a homogeneous population of mRNA, *L1* mRNA was synthesized by in vitro

transcription from a vector containing ORF L1R DNA. Addition of L1 mRNA to either nuclease-treated rabbit reticulocyte or wheat germ extract resulted in synthesis and myristylation of a single ~25-kDa polypeptide that comigrated with M25 labeled in vivo with [³H]myristic acid. However, both translation and myristylation were more efficient in wheat germ extracts than in rabbit reticulocyte lysates. The relationship between the gene product of ORF L1R and M25 was further established by immunoprecipitation of M25 (labeled in vivo) by antiserum to a *trpE*:L1 fusion protein.

Biochemical studies were conducted to examine the nature of the linkage of the fatty acid to the polypeptide, as well as to identify the acyl group attached. These studies indicated that the acyl group attached to the polypeptide was exclusively myristic acid and that the fatty acid was associated with the polypeptide in a hydroxylamine-resistant, presumably amide linkage. This is in agreement with the observations of Heuckeroth et al. (11) that NMT activity in wheat germ extracts exhibited specificity for myristate over palmitate. However, this is in contrast to what we previously observed when the fatty acid composition of M25 synthesized in vivo was determined (8). Approximately 50% of the fatty acid in M25 labeled during a 30-min pulse in VV-infected BSC-40 cells was myristate and 50% was palmitate. The biological significance of the apparent elongation of M25-associated myristic acid to palmitic acid which occurs in vivo remains to be established. The apparent differences between the M25 synthesized in vitro versus that made in vivo may be due to different specificities of the enzymatic acylation machinery present in wheat germ and BSC-40 cells. Likewise, it is possible that one of the consequences of VV infection is perturbation of the normal lipid metabolism pathways within infected cells. It will be of interest to investigate this question by using cloned L1R sequences to express protein M25 within the context of uninfected and infected cells to determine the nature of the added acyl groups.

When the predicted amino acid sequence of ORF L1R was compared with known protein sequences in current data banks, no striking homologies were evident. However, it was of interest that one of the proteins which appeared most similar to ORF L1R was fatty acid synthase, in particular, the acyl carrier protein domain. In view of (i) the apparent perturbation of acylation reactions which occurs in VV-infected cells (8), (ii) the unique mechanism of viral membrane formation which occurs around intracellular VV virions (7), and (iii) the biochemically distinct nature of the VV virion membrane, it is tempting to speculate that one or more virus-encoded proteins, such as M25, play a direct role in catalyzing assembly of the viral membrane within infected cells.

In all known cases, myristylation of a polypeptide occurs on the penultimate glycine residue, immediately subsequent to the initiation codon-derived methionine. Thus, before myristylation, removal of the initiating methionine is required. Although NMT does not appear to possess an intrinsic aminopeptidase (38), such an activity is known to catalyze removal of amino-terminal methionine residues in a cotranslational fashion (25). Translation of carboxy-truncated L1 polypeptides in wheat germ extracts mapped the site of myristylation to the amino-terminal 45 amino acid residues of the polypeptide. Failure to incorporate [³⁵S]methionine into this truncated polypeptide provided evidence that the initiator methionine is removed by an aminopeptidase activity in wheat germ. By converting the

glycine in position 1 that is obligatory for myristylation into either an alanine or aspartic acid residue, we created mutant forms of L1 that failed to incorporate myristate, providing genetic evidence that the penultimate glycine residue is the myristate-modified residue. Similar mutations of other myristylated proteins have also been seen to abrogate myristylation (5, 16, 18, 22, 28, 29). Furthermore, when a direct test for a protein function has been made, the modification by the myristyl group has proved to be essential for biological activity.

Both the role of protein M25 and the biological consequences of its posttranslational modification by covalent addition of myristic acid remain to be established. Interestingly, the gene that encodes protein M25 lies within the highly conserved central region of the VV genome (27), implying that it serves an important function in the viral life cycle. In support of this notion, even when using a dominant selection scheme (G418-*npt* gene; 9), we have been unable to isolate a recombinant virus containing an insertionally inactivated gene for L1R, suggesting that the activity of protein M25 is essential for viral replication in tissue culture. Experiments are in progress to elucidate the function of protein M25 during VV infection and how modification of nascent protein M25 affects its activity.

ACKNOWLEDGMENTS

We thank Anne Schuster for assistance with computer analyses, the Central Services Laboratory of the Center for Gene Research and Biotechnology for synthesis of synthetic deoxyoligonucleotide primers, and the Oregon State University Laboratory Animal Resource Center for assistance during preparation of anti-L1 serum. We especially thank Marilyn D. Resh, Department of Biology, Princeton University, Princeton, N.J., for the generous gift of the pGEM-*src* transcription vector.

This research was supported by a Public Health Service research grant (AI-20563) and a Research Career Development award (AI-00666) from the National Institutes of Health.

LITERATURE CITED

1. Becker, C. R., J. W. Efcavitch, C. R. Heiner, and N. F. Kaiser. 1985. Use of a reverse phase column for the HPLC purification of synthetic oligonucleotides. *J. Chromatogr.* **326**:293-299.
2. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-91.
3. Buss, J. E., and B. M. Sefton. 1985. Myristic acid, a rare fatty acid, is the lipid attached to the transforming protein of Rous sarcoma virus and its cellular homolog. *J. Virol.* **53**:7-12.
4. Chow, M., J. F. E. Newman, D. Filman, J. M. Hogle, D. J. Rowlands, and F. Brown. 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. *Nature (London)* **327**:482-486.
5. Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa. 1984. A short sequence in the p60^{src} N terminus is required for p60^{src} myristylation and membrane association and for cell transformation. *Mol. Cell. Biol.* **4**:1834-1842.
6. Dechaite, I., L. P. Casson, H.-P. Ling, and M. D. Resh. 1988. In vitro synthesis of pp60^{v-src}: myristylation in a cell-free system. *Mol. Cell. Biol.* **8**:4295-4301.
7. Essani, K., R. Dugre, and S. Dales. 1982. Biogenesis of vaccinia: involvement of spicules of the envelope during virion assembly examined by means of conditional lethal mutants and serology. *Virology* **118**:279-292.
8. Franke, C. A., P. L. Reynolds, and D. E. Hruby. 1989. Fatty acid acylation of vaccinia virus proteins. *J. Virol.* **63**:4285-4291.
9. Franke, C. A., C. M. Rice, J. H. Strauss, and D. E. Hruby. 1985. Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. *Mol. Cell. Biol.* **5**:1918-1924.

10. Grand, R. J. A. 1989. Acylation of viral and eukaryotic proteins. *Biochem. J.* **258**:625–638.
11. Heuckeroth, R. O., D. A. Towler, S. P. Adams, L. Glaser, and J. I. Gordon. 1988. 11-(Ethylthio)undecanoic acid. A myristic acid analogue of altered hydrophobicity which is functional for peptide N-myristylation with wheat germ and yeast acetyltransferase. *J. Biol. Chem.* **263**:2127–2133.
12. Hiller, G., and K. Weber. 1985. Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *J. Virol.* **55**:651–659.
13. Hirt, P., G. Hiller, and R. Wittek. 1986. Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. *J. Virol.* **58**:757–764.
14. Hruby, D. E., L. A. Guarino, and J. R. Kates. 1979. Vaccinia virus replication. I. Requirement for a host cell nucleus. *J. Virol.* **29**:705–715.
15. James, G., and E. N. Olson. 1990. Fatty acylated proteins as components of intracellular signaling pathways. *Biochem.* **29**:2623–2634.
16. Jones, T. L. Z., W. F. Simonds, J. J. Merendino, M. R. Brann, and A. M. Spiegel. 1990. Myristoylation of an inhibitory GTP-binding protein α subunit is essential for its membrane attachment. *Proc. Natl. Acad. Sci. USA* **87**:568–572.
17. Jove, R., and H. Hanafusa. 1987. Cell transformation by the viral src oncogene. *Annu. Rev. Cell Biol.* **3**:31–56.
18. Kamps, M. P., J. E. Buss, and B. M. Sefton. 1985. Mutation of N-terminal glycine of p60^{src} prevents both myristoylation and morphological transformation. *Proc. Natl. Acad. Sci. USA* **82**:4625–4628.
19. Kaufman, J. F., M. S. Krangel, and J. L. Strominger. 1984. Cysteines in the transmembrane region of major histocompatibility complex antigens are fatty acylated via thioester bonds. *J. Biol. Chem.* **259**:7230–7238.
20. Lehrach, G., D. Diamond, J. M. Woxney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16**:4743–4752.
21. Magee, A. I., and M. J. Schlesinger. 1982. Fatty acid acylation of eucaryotic cell membrane proteins. *Biochim. Biophys. Acta* **694**:279–289.
22. Marc, D., G. Drugeon, A. Haenni, M. Girard, and S. van der Werf. 1989. Role of myristoylation of poliovirus capsid protein VP4 as determined by site-directed mutagenesis of its N-terminal sequence. *EMBO J.* **8**:2661–2668.
23. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**:7035–7056.
24. Miner, J. N., and D. E. Hruby. 1989. Rifampicin prevents virosome localization of L65, an essential vaccinia virus polypeptide. *Virology* **170**:227–237.
25. Olson, E. N., D. A. Towler, and L. Glaser. 1985. Specificity of fatty acid acylation of cellular proteins. *J. Biol. Chem.* **260**:3784–3790.
26. Palmiter, R. D. 1977. Prevention of NH₂-terminal acetylation of proteins synthesized in cell-free systems. *J. Biol. Chem.* **252**:8781–8783.
27. Plucienniczak, A., E. Schroeder, G. Zettlmeissl, and R. E. Strecek. 1985. Nucleotide sequence of a cluster of early and late genes in a conserved segment of the vaccinia virus genome. *Nucleic Acids Res.* **13**:985–998.
28. Rein, A., M. R. McClure, N. R. Rice, R. B. Luftig, and A. M. Schultz. 1986. Myristylation site in Pr65^{gag} is essential for virus particle formation by Moloney murine leukemia virus. *Proc. Natl. Acad. Sci. USA* **83**:7246–7250.
29. Rhee, S. S., and E. Hunter. 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. *J. Virol.* **61**:1045–1053.
30. Rosel, J. L., P. L. Earl, J. P. Weir, and B. Moss. 1986. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the *HindIII* H genome fragment. *J. Virol.* **60**:436–449.
31. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing using chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
32. Schultz, A. M., L. E. Henderson, and S. Oroszlan. 1988. Fatty acylation of proteins. *Annu. Rev. Cell Biol.* **4**:611–647.
33. Sefton, B. M., and J. E. Buss. 1987. The covalent modification of eukaryotic proteins with lipid. *J. Cell Biol.* **104**:1449–1453.
34. Spindler, K. R., D. S. E. Rosser, and A. J. Berk. 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. *J. Virol.* **49**:132–141.
35. Studier, F. W. 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol.* **79**:237–248.
36. Towler, D., and L. Glaser. 1986. Acylation of cellular proteins with endogenously synthesized fatty acids. *Biochemistry* **25**:878–884.
37. Towler, D., and L. Glaser. 1986. Protein fatty acid acylation: enzymatic synthesis of an N-myristoylglycyl peptide. *Proc. Natl. Acad. Sci. USA* **83**:2812–2816.
38. Towler, D. A., S. P. Adams, S. R. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon. 1987. Purification and characterization of yeast myristoyl CoA:protein N-myristoyltransferase. *Proc. Natl. Acad. Sci. USA* **84**:2708–2712.
39. Towler, D. A., S. P. Adams, S. R. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon. 1988. Myristoyl CoA:protein N-myristoyltransferase activities from rat liver and yeast possess overlapping yet distinct peptide substrate specificities. *J. Biol. Chem.* **263**:1784–1790.
40. Towler, D. A., J. I. Gordon, S. P. Adams, and L. Glaser. 1988. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* **57**:69–99.
41. Weir, J. P., and B. Moss. 1984. Regulation of expression and nucleotide sequence of a late vaccinia virus gene. *J. Virol.* **51**:662–669.
42. Wilson, E. M., C. A. Franke, M. E. Black, and D. E. Hruby. 1989. Expression vector pT7:TKII for the synthesis of authentic biologically active RNA encoding vaccinia virus thymidine kinase. *Gene* **77**:69–78.