division leading to malignancy. Our data are thus consistent with the hypothesis that *ets* has an important function in the control of cell proliferation. Further study of the role of the *ets*-2 protein in *Xenopus* oocyte maturation may lead to an understanding of the involvement of this protein in oncogenesis.

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The Function of a Leader Peptide in Translocating Charged Amino Acyl Residues Across a Membrane

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Insertion of bacteriophage coat proteins into the membrane of infected bacterial cells can be studied as a model system of protein translocation across membranes. The coat protein of the filamentous bacteriophage Pf3—which infects *Pseudomonas aeruginosa*—is 44 amino acids in length and has the same basic structure as the coat protein of bacteriophage M13, which infects *Escherichia coli*. However, unlike the Pf3 coat protein, the M13 coat protein is synthesized as a precursor (procoat) with a typical leader (signal) sequence, which is cleaved after membrane insertion. Nevertheless, when the gene encoding the Pf3 coat protein is expressed in *E. coli*, the protein is translocated across the membrane. Hybrid M13 and Pf3 coat proteins were constructed in an attempt to understand how the Pf3 coat protein is translocated without a leader sequence. These studies demonstrated that the extracellular regions of the proteins determined their cellular location. When three charged residues in this region were neutralized, the leader-free M13 coat protein was also inserted into the membrane. Differences in the water shell surrounding these residues may account for efficient membrane insertion of the protein without a leader sequence.

THE INFORMATION SPECIFYING membrane insertion is located within proteins as transmembrane integration signals (*1*). For example, leader (signal) peptides are NH_2-terminal extensions that target a protein to the membrane. Some leader peptides are removed after membrane integration, whereas others are retained as uncleaved leader peptides or as start-stop sequences that anchor the protein in the bilayer. Each of these leader peptides contains a hydrophobic stretch of ~20 amino acid residues that is important for their function. The molecular mechanism of protein insertion has been extensively studied with the coat protein of bacteriophage M13, which inserts into the plasma membrane of *E. coli*. This protein is synthesized as a precursor (procoat) with a typical 23-residue NH_2-terminal leader peptide. Both the positively charged region of the leader peptide (*2*) and the apolar core are required for membrane insertion (*3*). In addition, features of the mature region of the protein are also essential (*4*). The hydrophobic domains of the leader and the mature region form a looped structure that allows the central segment of the protein to translocate across the membrane (*5*).

The major coat protein of bacteriophage Pf3, which infects *Pseudomonas aeruginosa*, is synthesized without a leader peptide (*6*) but has other structural features similar to the M13 coat protein (Fig. 1A). Both proteins have a hydrophobic region of ~20 amino acids that is flanked by an upstream acidic region and a short downstream basic region. However, there is no sequence similarity, either at the nucleotide or amino acid level (*7*).

To study membrane insertion of the Pf3 coat protein, we cloned into an expression vector (pUC9) a 184–base pair (bp) fragment of the Pf3 genome that encodes the major coat protein, and we used the resulting construct to transform *E. coli* (strain JM103). Protein synthesis was induced with isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM) and the cells were labeled with [^{35}S]methionine for 3 min. Detergent-solubilized proteins were immunoprecipitated and separated by polyacrylamide gel electrophoresis (PAGE). The Pf3 coat protein was immunoprecipitated with antibodies to Pf3 phage (anti-Pf3) but not with antibodies to M13 (anti-M13) (Fig. 1C). In a pulse-chase experiment, the apparent molecular size (4625 daltons) of the Pf3 coat protein did not change with time—as would be expected in view of the absence of a leader peptide (*8*).

To determine the cellular location of the Pf3 coat protein, we induced its expression from the plasmid pAN-1, which also overproduces a cytoplasmic fragment of ribulokinase (AraB). Cells induced to synthesize the coat protein were labeled with [^{35}S]methionine for 3 min and their outer membrane was permeabilized. Proteinase K was added externally to the cells for various times, and samples were analyzed by PAGE. Outer membrane protein A (OmpA) was digested by the protease as would be expected (Fig. 2A), whereas the cytoplasmic AraB fragment was only digested when the inner

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membrane was disrupted by detergent (Fig. 2B). The Pf3 coat protein was digested by proteinase K, indicating that it was inserted across the membrane (Fig. 2C). Externally added trypsin, however, did not attack the Pf3 protein (Fig. 2D). Because the lysine and arginine residues of the Pf3 coat protein are near the COOH-terminus, their inaccessibility by trypsin implied that the Pf3 coat protein was inserted into the membrane with its COOH-terminus facing the cytoplasm. When cells were osmotically shocked and the released proteins were analyzed (Fig. 2E), the periplasmic protein β-lactamase was found in the shock fluid, whereas the Pf3 coat protein was found in the cell fraction.

Membrane insertion of most bacterial proteins requires an energized membrane. Protonophores—which collapse the membrane potential—such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) block the translocation of the M13 procoat protein (*9*). To investigate whether the Pf3 coat protein requires a membrane potential for insertion, we treated *E. coli* expressing Pf3 coat protein with CCCP (30 μM) for 5 min and then labeled the cells with [^{35}S]methionine for 3 min. The location of the Pf3 coat protein was determined by the external addition of proteinase K. Most of the Pf3 coat protein was digested by the protease in energized cells (Fig. 3A). However, in CCCP-treated cells, the Pf3 coat protein was not accessible to the protease (Fig. 3B). OmpA accumulated in its precursor form (pro-OmpA) in CCCP-treated cells, and the externally added protease only degraded the mature protein (Fig. 3B), verifying that translocation across the membrane was sensitive to CCCP. Thus, Pf3 coat protein required a membrane potential for its insertion, as does pro-OmpA and the M13 procoat protein.

In contrast to most other membrane proteins, the M13 procoat inserts into the membrane independent of the Sec protein machinery, which includes SecA, SecB, SecY, and SecE. As a way of analyzing whether Pf3 coat protein was inserted into the membrane by a *sec*-dependent pathway, we used the plasmid encoding the Pf3 coat protein to transform a *secA*^{ts} strain (CJ105) and a *secY*^{ts} strain (CJ107) of *E. coli* (*10*). The cultures were grown at 30°C and shifted to the nonpermissive temperature (42°C) for 2 hours. Pf3 coat protein was fully accessible in CJ105 (Fig. 3C), CJ107 (Fig. 3D), and wild-type cells (*8*), and thus was not affected by the *sec* deficiencies. In contrast, membrane insertion of pro-OmpA was retarded in the *sec* mutants (Fig. 3, C and D). Membrane insertion of Pf3 coat protein is, therefore, independent of *secA* and *secY*, as is that of the M13 procoat protein.

In an attempt to understand why M13 coat protein has a leader peptide and Pf3 does not, we constructed hybrid M13 and Pf3 coat proteins (Fig. 1, A and B). The hybrid proteins (MPF1 to MPF6) all contained the hydrophobic and COOH-terminal region of the M13 coat protein, but differed in their periplasmic regions. We expressed the hybrid proteins in *E. coli* and identified them by immunoprecipitation (Fig. 1C). The proteins were of the expected molecular size and were recognized appropriately by anti-Pf3 or anti-M13. The immunoprecipitation experiments showed that the antigenic sites of both proteins were located within the first ten amino acid residues.

Synthesis of the hybrid proteins was induced and the cells were labeled with [^{35}S]methionine for 3 min. Each hybrid was tested by protease treatment for its ability to insert into the *E. coli* membrane within the 3 min labeling period (Fig. 4). The hybrid protein MPF1, in which the periplasmic acidic region of the M13 procoat was replaced by the first 18 amino acids of the Pf3 coat protein, was accessible to proteinase K (Fig. 4A). Thus, the hydrophobic region of the M13 procoat allowed efficient membrane translocation of the Pf3 NH_2-terminal region.

To determine the role of the acidic region of the M13 coat protein in translocation, we generated additional hybrid proteins. One mutant, MPF2, had the acid regions of the Pf3 and M13 coat proteins in tandem and was not digested by externally added proteinase K (Fig. 4B). The hybrid protein MPF3 contained the first 11 amino acid residues of Pf3 attached to 35 residues of the M13 procoat protein, was recognized by anti-Pf3 (Fig. 1C), and was efficiently inserted into the membrane (Fig. 4C). However, the hybrid protein MPF4, which consisted of the entire mature region of the M13 coat protein plus the first three amino acids from the Pf3 coat protein, failed to be translocated across the membrane (Fig. 4D). This suggested that the first 15 amino acid residues of the mature M13 coat protein required a leader sequence for translocation. Therefore, we mutated specific residues in this region to identify the amino acids that prevented membrane insertion. In MPF5 and MPF6, the two aspartic acid residues at positions +4 and +5 of the mature M13 coat

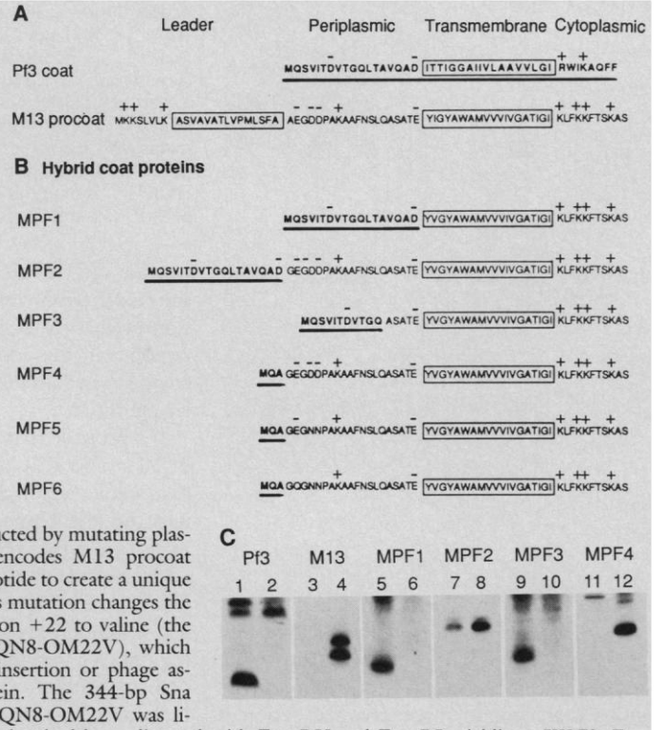

Fig. 1. (**A**) Amino acid sequences of the Pf3 coat and M13 procoat proteins. Hydrophobic domains are boxed and charged residues are indicated with the appropriate (+) or (−) sign. (**B**) Hybrid Pf3 and M13 coat proteins differed in their periplasmic regions. A 184-bp fragment [nucleotides 101 to 284 (*6*)] encoding the Pf3 coat protein (4630 daltons) was obtained by restriction digestion of Pf3 DNA with Mnl I. This fragment was cloned into the Sma I site of the pUC9 vector to give pJX-1. The hybrid protein MPF1 (4927 daltons) was constructed by mutating plasmid pQN8 (*16*), which encodes M13 procoat protein, by one oligonucleotide to create a unique Sna BI restriction site. This mutation changes the isoleucine residue at position +22 to valine (the resulting plasmid being pQN8-OM22V), which did not affect membrane insertion or phage assembly of the coat protein. The 344-bp Sna BI–Eco RI fragment of pQN8-OM22V was ligated with plasmid pJX-1 that had been digested with Eco RV and Eco RI, yielding pJX172. For MPF2 (7069 daltons), the 404-bp Nae I–Eco RI fragment of pQN8-OM1 (*17*) was ligated with pJX-1 that had been digested with Eco RV and Eco RI, yielding pJX-156. MPF3 (4869 daltons) and MPF4 (5556 daltons) were obtained by oligonucleotide-directed deletion of pJX-156. MPF5 and MPF6 were obtained by oligonucleotide mutagenesis of MPF4. (**C**) Immunoprecipitation analysis of hybrid coat proteins. *E. coli* strain JM103 containing plasmids with either wild-type or hybrid genes was grown at 37°C in M9 minimal medium. Protein synthesis was induced by IPTG in the presence of [^{35}S]methionine, and coat protein expression was analyzed by immunoprecipitation with anti-Pf3 (odd-numbered lanes) and anti-M13 (even-numbered lanes), SDS-PAGE, and fluorography (*16*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L. Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

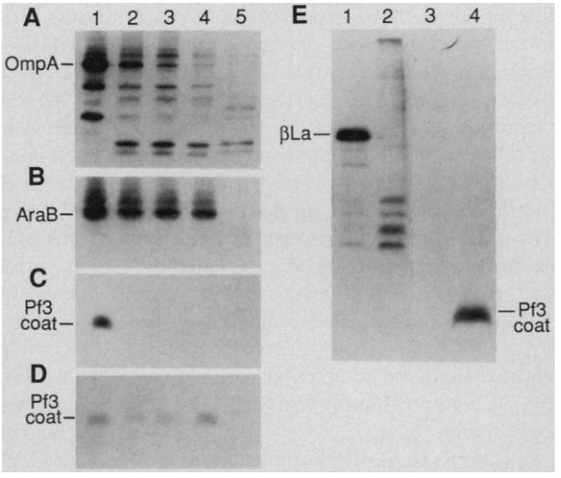

Fig. 2. Pf3 coat protein inserts into the *E. coli* cytoplasmic membrane. *E. coli* (strain JM103) cells expressing the Pf3 coat protein were labeled for 3 min with [^{35}S]methionine (100 μCi). The outer membrane was permeabilized and proteinase K (1 mg/ml) (**A** to **C**) or trypsin (100 μg/ml) (**D**) was added. Samples were removed at 0 (lane 1), 30 (lane 2), 60 (lane 3), and 120 min (lane 4). Samples in lane 5 were incubated with the proteases for 60 min after lysis with 2.5% Triton X-100. All samples were precipitated with trichloroacetic acid and immunoprecipitated with antibodies to OmpA (A), to AraB (B), and to Pf3 (C and D). (**E**) The labeled cells were subjected to osmotic shock and then separated from the shock fluid. Shock fluid (lanes 1 and 2) and shocked cells (lanes 3 and 4) were analyzed by immunoprecipitation with antibodies to β-lactamase (βLa) (lanes 1 and 3) and to Pf3 (lanes 2 and 4). The expression vector pING-1 (*18*) was used to study Pf3 membrane insertion because this plasmid encodes a cytoplasmic fragment of AraB. Cells were grown as described (*16*) and the outer membrane was permeabilized by the addition of an equal volume of 60 mM tris-HCl (pH 8), 40% (w/v) sucrose, and 20 mM EDTA. Proteinase K or trypsin digestion was performed at 0°C and terminated by the addition of 5 mM phenylmethylsulfonyl fluoride.

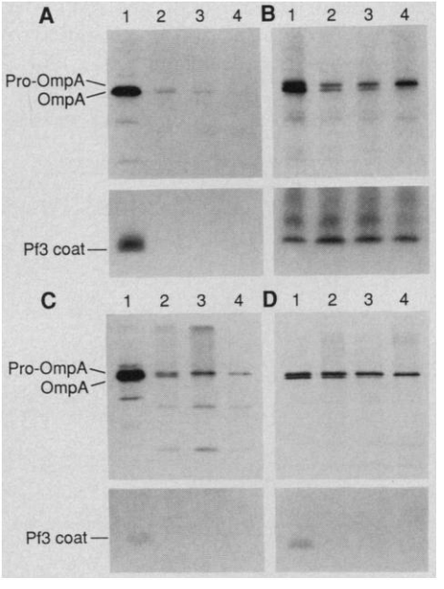

Fig. 3. Pf3 coat protein insertion requires an electrochemical membrane potential and is independent of *secA* and *secY*. Insertion of the Pf3 coat protein was analyzed by proteinase accessibility in the absence (**A**) and presence (**B**) of CCCP (30 μM) and in *secA*-deficient (**C**) and *secY*-deficient (**D**) strains. Wild-type cells (strain HJM 114) (500 μl) in the exponential growth phase containing plasmids coding for Pf3 coat protein were treated with ethanol (17 μl) (A) or CCCP (1 mM, in ethanol) (17 μl) (B), and grown for 5 min. Plasmid pJX-1 was transformed into *E. coli* strains CJ105 ($secA^{ts51}$) (C) and CJ107 ($secY^{ts24}$) (D) and grown at 30°C. At a culture density of 5×10^7 cells/ml, the incubation temperature was shifted to 42°C and growth was continued for 120 min. [^{35}S]Methionine (100 μCi) was added to all cells for 3 min, and the outer membrane of the cells was permeabilized in 30 mM tris-HCl (pH 8), 20% sucrose, and 10 mM EDTA. Proteinase K (1 mg/ml) was then added to the cells for 0 (lanes 1), 30 (lanes 2), 60 (lanes 3), and 120 min (lanes 4) at 0°C. After digestion, the samples were treated with phenylmethylsulfonyl fluoride (5 mM), precipitated with 20% trichloroacetic acid, and immunoprecipitated with antibodies to OmpA (upper panels) and to Pf3 (lower panels).

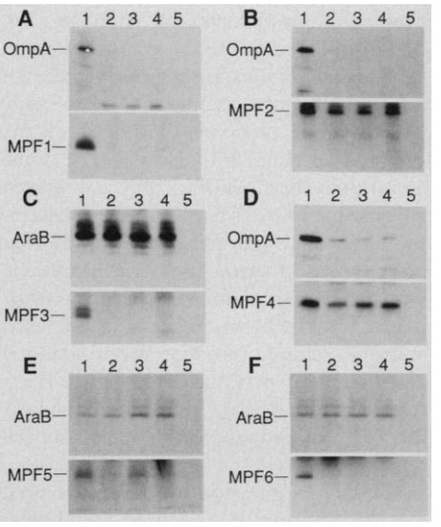

Fig. 4. Membrane insertion of Pf3-M13 hybrid proteins. Recombinant plasmids encoding hybrid proteins MPF1 (**A**), MPF2 (**B**), MPF3 (**C**), MPF4 (**D**), MPF5 (**E**), and MPF6 (**F**) were transformed into *E. coli* strain JM103 and grown at 37°C in M9 minimal medium containing 0.5% fructose. At a density of 2×10^8 cells/ml, the cultures were induced with IPTG and labeled for 3 min with [^{35}S]methionine (100 μCi). The outer membrane of the cells was then permeabilized with 30 mM tris-HCl (pH 8), 20% sucrose, and 10 mM EDTA. Proteinase K (1 mg/ml) was added and the cells were incubated at 0°C for 0 (lanes 1), 30 (lanes 2), 60 (lanes 3), and 120 min (lanes 4). As a control, the cells in one portion of each culture were lysed by addition of 2% Triton X-100, and digested with proteinase K for 30 min (lanes 5). The samples were then immunoprecipitated with antibodies to OmpA or AraB (upper panels), and with anti-Pf3 (A to C) or anti-M13 (D to F). Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

protein were mutated to asparagine residues. In addition, in MPF6, the glutamic acid residue at position +2 was mutated to a glutamine. Analysis of membrane insertion by protease treatment showed that most of the MPF5 (Fig. 4E) and MPF6 (Fig. 4F) proteins were inserted into the membrane. Thus, insertion of the M13 coat protein without its leader peptide may occur as long as the three negatively charged residues in the periplasmic region are neutralized. Replacement by the corresponding, but simpler, periplasmic region of the Pf3 coat protein (MPF1 and MPF3) made a leader peptide dispensable.

We conclude that small, uncharged passenger regions can be easily translocated across the membrane in the absence of a signal sequence, whereas more complex regions that contain many charged residues require a leader sequence. Transport of charged residues across the lipid bilayer may involve cotransport or removal of a water shell surrounding these charged residues, and will therefore require additional energy (*11*). Consistent with this view, charged amino acid residues inhibit membrane translocation of a number of proteins, particularly when they are located close to the leader peptide (*12*). Moreover, the orientation of the leader peptidase was inverted by a deletion of charged residues in the cytoplasmic region and the addition of four positively charged residues in the preceding extracellular region (*13*).

We propose that the hydrophobic stretch of 18 amino acids and the COOH-terminal hydrophilic region direct the Pf3 coat protein into the membrane. The structural arrangement of the two regions is reminiscent of a leader peptide, but with a reverse orientation because the NH_2-terminal region of the protein is transferred to the external periplasm of the cell. We suggest that the transmembrane domain promotes the translocation of the NH_2-terminal part of the Pf3 coat protein.

Membrane anchor sequences may have a spontaneous insertion activity that allows them to insert into the membrane before the more NH_2-terminal regions are translocated. Indeed, internal insertion sequences and stop-transfer sequences of a multispanning membrane protein insert independent of the signal recognition particle (*14*). In addition, sequences that flank a membrane anchor

contain positively charged residues mostly at the cytoplasmic side (*15*). This finding is in agreement with the structural arrangement of the membrane-spanning region of the Pf3 coat protein.

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Efficient Incorporation of Human CD4 Protein into Avian Leukosis Virus Particles

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Virus envelope (Env) proteins are thought to contain specific signals for selective uptake by virus particles. In the course of attempting to define these signals by testing virus incorporation of CD4-Env chimeric proteins, normal human CD4 was found to be efficiently and selectively assembled into avian leukosis virus particles in quail cells. Viruses bearing CD4 at their surface may be useful reagents in the design of retrovirus-mediated gene therapy for the acquired immune deficiency syndrome.

RETROVIRUS PARTICLES EFFICIENTLY incorporate viral envelope (Env) proteins from the cell surface and exclude the majority of host cell surface proteins, but the basis of this specificity is poorly understood. Retroviral Env proteins are made up of two polypeptide chains: surface (SU) and transmembrane (TM). Specific interactions between the transmembrane domain of TM Env proteins and matrix (MA) Gag proteins have been proposed to account for the selective uptake of Env proteins by avian leukosis virus (ALV) particles (*1*). On the basis of this hypothesis, we predicted that ALV might incorporate chimeric proteins comprising the extracellular region of human CD4 fused to sequences from the transmembrane and cytoplasmic tail domains of retroviral TM Env proteins.

Two chimeric *CD4-env* genes were constructed, designated *CR* and *CM*, which encode the extracellular domains of human CD4 fused in-frame with the transmembrane and cytoplasmic tail sequences of either the ALV TM protein or the TM protein of murine leukemia virus (MLV) (CR and CM, respectively, Fig. 1A). The *CM* chimeric gene was constructed because previous experiments have shown that MLV can provide Env proteins for ALV pseudotypes (*2*).

Incorporation of CR, CM, or wild-type CD4 proteins into ALV was tested by transiently expressing these proteins, by means of SV40-based expression vectors, in quail QT6 cells (*3*) previously infected with a replication-competent ALV vector. Immunofluorescence studies with CD4-specific antisera demonstrated transient cell surface expression of CD4, CR, or CM chimeric proteins in 5 to 10% of transfected cells (*4*). Transfected cells were metabolically labeled, and cell extracts and virions were assayed by immunoprecipitation. The production of the 95-kD ALV precursor Env protein (*5*) and CD4, CR, or CM proteins in transfected cells was confirmed by means of antisera specific for either viral Env or human CD4 proteins (Fig. 1B, lanes 1 to 6). Equivalent virus production from transfected cells was demonstrated by immunoprecipitation of Gag proteins from virus samples (Fig. 1B, lanes 10, 14, 18). The low concentrations of Env proteins detected in these experiments was due to inefficient

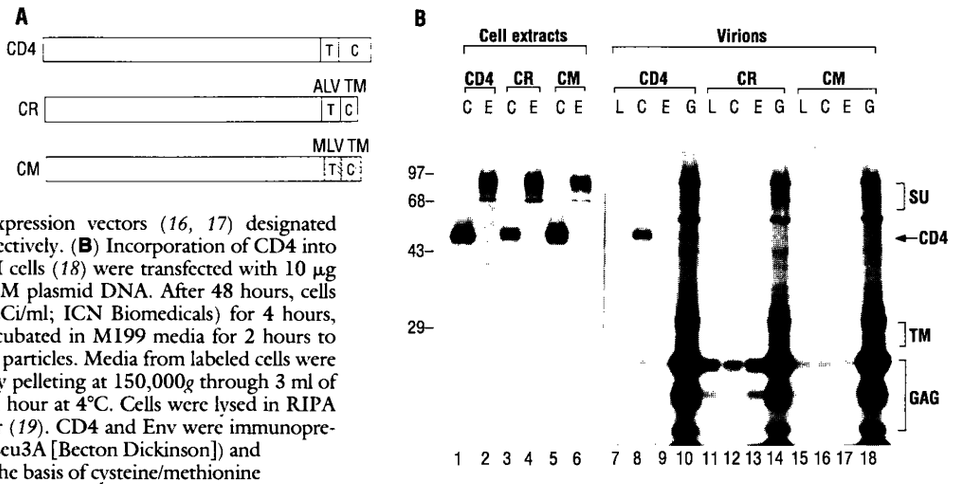

Fig. 1. (**A**) Structure of *CR* and *CM* chimeric CD4-Env proteins. *CD4-env* genes were constructed to encode the extracellular domains of human CD4 (*14*) fused in-frame with the transmembrane and cytoplasmic tail domains of either ALV (CR) or MLV (CM) transmembrane (TM) Env proteins (*15*). A wild-type *CD4* gene (*14*) and the *CM* and *CR* chimeric genes were expressed from SV40-based expression vectors (*16, 17*) designated pSVβCD4, pSV7dCM, and pSV7dCR, respectively. (**B**) Incorporation of CD4 into ALV particles. Approximately 3×10^6 QAH cells (*18*) were transfected with 10 μg of either pSVβCD4, pSV7dCR, or pSV7dCM plasmid DNA. After 48 hours, cells were incubated with [^{35}S]trans-label (350 μCi/ml; ICN Biomedicals) for 4 hours, washed in phosphate-buffered saline, and incubated in M199 media for 2 hours to allow the export of radioactively labeled virus particles. Media from labeled cells were cleared at 2000 *g*, and virions were purified by pelleting at 150,000*g* through 3 ml of 20% sucrose in a Beckman SW41 rotor for 1 hour at 4°C. Cells were lysed in RIPA buffer and viruses were lysed in NP-40 buffer (*19*). CD4 and Env were immunoprecipitated with C (the CD4-specific antibody Leu3A [Becton Dickinson]) and E [an ALV-Env specific antiserum (*20*)]. On the basis of cysteine/methionine compositions, the ratio of labeled TM:SU:CD4 proteins is 1: 1.4: 1.7. Viral Gag was immunoprecipitated with G [an ALV-Gag–specific antiserum (*4*)], and L [the CD8-specific monoclonal antibody Leu2A (Becton Dickinson)] was used as a negative control. Samples were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE) and fixed in 40% methanol/7% acetic acid. The gel was incubated in Amplify (Amersham), dried, and exposed to Kodak XAR-5 film at −70°C for 1 day (cell extracts) or 7 days (virions).