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20. Plasma vW AgII was purified with monoclonal antibodies to platelet vW AgII. The eluted (diethylamine, *p*H 11) vW AgII was then bound to a Mono-S column (Pharmacia) and eluted with a sodium acetate gradient. After Edman degradation (*29*), the amino acid sequence of vW AgII (0.5 μg, 50 pmol) was determined with a gas phase protein sequencer (Applied Biosystems 470A) according to the method of Hewick *et al.* (*31*). The resultant phenylthiohydantoin amino acids from each cycle were analyzed by HPLC through the use of phenylthiohydantoin analyzer (Applied Biosystems 120A). Amino acids at the 5, 7, and 12 positions were undetectable at this protein concentration.
21. The 100-kD glycoprotein (2.3 mg, 23 nmol) was incubated with human α thrombin (0.9 mg, 23 nmol) in 0.6 ml of buffer containing 20 m*M* imidazole HCl at *p*H 7.0, 150 m*M* NaCl, 2 m*M* $CaCl_2$, and 0.02% NaN_3. Proteolysis of the 100-kD polypeptide was greater than 95% complete after 16 hours at 23°C as determined by SDS-PAGE, which also revealed that the 75- and 26-kD fragments are linked by disulfide bonds. To remove thrombin from the covalently linked fragments, we chromatographed the reaction mixture with Sephacryl S-300 (1 by 31 cm) equilibrated in 20 m*M* imidazole HCl at *p*H 7.0, 0.3*M* NaCl, 0.1*M* lysine HCl, and 0.02% NaN_3 at a flow rate of 5 ml/hour. Fractions (0.5 ml) were collected and monitored for protein. The peak fractions of fragments derived from the 100-kD protein were pooled (1.4 mg, 3 ml). To this mixture was added 1/10 volume of 2*M* tris HCl (*p*H 8.2) and solid guanidine HCl to a final concentration of 6*M*. Dithiothreitol was then added to 10 m*M*, and the reaction mixture was incubated for 1 hour at 23°C. The reduced sample was alkylated with iodoacetamide (11 m*M* final concentration) for 1 hour at 23°C. The reduced and alkylated polypeptides were chromatographed on Sephacryl S-300 (1.5 by 20 cm) equilibrated in 0.1*M* tris-HCl at *p*H 8.2, 6*M* guanidine HCl, 0.15*M* NaCl, 10 m*M* dithiothreitol, and 0.02% NaN_3 at a flow rate of 5 ml/hour. Because of the volume of the sample it was necessary to perform the step in two successive column runs, with less than 2 ml applied per loading. The peak fractions of the purified 75- and 26-kD fragments were conservatively pooled to minimize any cross-contamination. Approximately 890 μg (12 nmol) of the large fragment and 350 μg (13 nmol) of the small fragment were recovered. The fragments were dialyzed extensively against water and lyophilized.
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AIDS Retrovirus (ARV-2) Clone Replicates in Transfected Human and Animal Fibroblasts

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A molecular clone of the AIDS-associated retrovirus (ARV-2) was transfected into human T lymphocyte and monocyte cell lines as well as mouse, mink, monkey, and human fibroblast lines. A replicating virus with cytopathic and biologic properties of ARV-2 was recovered from all the cell lines. The animal and human fibroblast cells are resistant to direct infection by ARV, and in these experiments virus production in the fibroblast lines, especially mouse, was reduced compared to human lymphocytes. However, human fibroblasts were more permissive to virus expression than mouse cells. These results show that, whereas the primary block to ARV infection in certain cells may occur at the cell surface, intracellular mechanisms can also participate in controlling virus replication. The results have relevance to vaccine development and encourage further work with modified molecular clones to examine regions of the ARV genome necessary for cytopathology and replication.

THREE SEPARATE ISOLATES OF THE retrovirus associated with acquired immune deficiency syndrome (AIDS) have been molecularly cloned and sequenced: the lymphadenopathy-associated virus (LAV), the human T cell lymphotropic virus type III (HTLV-III), and the AIDS-associated retrovirus (ARV) (*1*). In most studies of the AIDS retrovirus, investigators have used human T cell lines because other cells are resistant or less susceptible to infection. We now report that after transfection of a molecular clone of ARV-2 into human lymphocytes and into fibroblasts from several mammalian species, replicating ARV can be recovered. The studies indicate that infection by ARV is regulated by intracellular mechanisms as well as cell surface receptors.

The full-length proviral ARV-2 DNA represented in the molecular clone phage λ9B-7 (*2*) was used for these studies. Digestion of this DNA with the restriction endonuclease Eco RI produced two DNA fragments of ARV-2 that were cloned into the Eco RI site of the plasmid vector pSp65 (*3*) (Fig. 1). This plasmid, p9B-7, was also used.

The U937 human monocyte cell line and the Jurkat and HUT-78_{AG} human T cell lines (*4*) were grown in RPMI 1640 medium supplemented with 10 percent fetal calf

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serum, glutamine (2 m*M*), and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml). The NIH 3T3 (mouse), MiL (mink lung), COS-7 (African Green monkey), and RD-4 rhabdomyosarcoma (human) cells were grown in Eagle's minimal essential medium containing the same supplements.

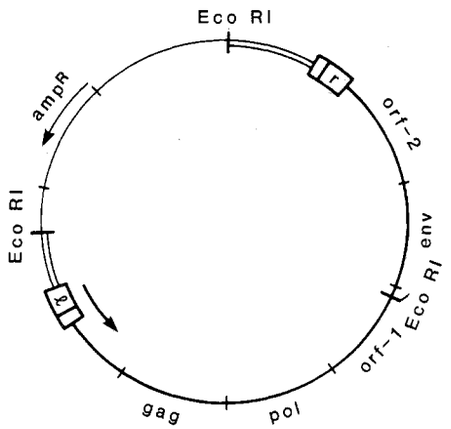


Fig. 1. Plasmid–ARV-2 molecular clone. The plasmid clone p9B-7 contains ARV-2 DNA in a proviral configuration. The heavy line represents ARV DNA, the double line is cellular DNA flanking the provirus, and the light line is the vector pSp65. The boxes are the (r) rightward and (ℓ) leftward long terminal repeats. Genetic regions of the virus are indicated (*gag, pol*, and *env* genes) together with the two open reading frames (*orf-1* and *orf-2*). Eco RI sites are designated. The bold arrow shows the direction of viral transcription; amp^R is the bacterial plasmid ampicillin resistance gene in the pSp65 vector.

We first assessed the biologic integrity of the molecular clone of ARV-2. HUT-78 and Jurkat T cells, as well as the U937 cells [which are also susceptible to ARV infection (*4*)], were transfected by standard techniques with either the λ9B-7 or p9B-7 clones (see Table 1). ARV production was detected in the Jurkat and U937 cells at 36 to 44 days after transfection by the presence of reverse transcriptase (RT) activity, which reached up to 20,000 counts per minute (cpm) per milliliter of culture fluid (*5*). Virus replication was detected at 5 days in the HUT-78 line, with RT activity reaching over 200,000 cpm/ml in 21 days. Virus from each culture was subsequently passed to mitogen-stimulated normal human peripheral mononuclear cells (PMC) obtained from healthy volunteers, and ARV was identified by an indirect immunofluorescence assay (IFA) for viral antigens (*6*). Reverse transcriptase activity increased to over 10^6 cpm/ml within 14 days after the virus from HUT-78 cells was passed to fresh human PMC, and up to 30 percent of the PMC showed staining for ARV-specific antigens as detected by human antiserum to ARV (*6*).

The virus produced in HUT-78 cells showed cytopathic effects (fusion, balloon degeneration) typical of AIDS retroviruses (*1*). Extracts of the infected HUT-78 cells and PMC contained all the antigens of ARV as demonstrated by immunoblotting (Fig. 2). These included the envelope proteins gp160, gp120, and gp41, and the *gag* proteins of molecular weight 55K, 25K, and 16K.

We next investigated whether the tropism displayed by ARV for certain cells is governed solely at the level of a cell membrane receptor for the virus. Evidence from several laboratories suggests that the CD4 (T4) complex could serve as this receptor (*7, 8*) although other molecules may be involved (*4, 8*). We transfected with p9B-7 the mouse, monkey, mink lung, and human (RD-4) fibroblast cells. Although these cells are not susceptible to direct infection with ARV (*4*), virus replication occurred after transfection in all these lines within 5 to 14 days; RT activity reached 10,000 to 20,000 cpm/ml.

The detection of virus was enhanced by cocultivation of the fibroblast cells with mitogen-stimulated normal human PMC added 5 to 18 days after transfection. The RT activity reached 5×10^6 cpm/ml in the cocultures of transfected MiL, COS-7, and RD-4 cells (Fig. 3) when fresh PMC were added every 3 to 6 days. However, the level of enhancement by PMC was much less with the transfected NIH 3T3 cells; the RT activity in the culture fluid reached only 18,000 cpm/ml (Fig. 3). This result appeared to reflect a lower level of infectious virus production in these cells compared to the monkey, mink, and human cells. The lower RT activity could not be explained by a relatively less efficient transfection frequency in mouse cells, since the number of neomycin-resistant colonies obtained from all transfected cultures was comparable (14 to 16 G418-resistant colonies per microgram of pSV2-neo). Moreover, virus pro-

Table 1. Recovery of ARV from cells transfected with a molecular clone of the virus. In experiment 1, 5×10^5 HUT-78 cells were transfected with 3 μg of λ9B-7 DNA and 20 μg of low molecular weight (MW) HUT-78 cell DNA by the calcium phosphate coprecipitation technique (*14*), treated for 2 minutes with dimethyl sulfoxide (30%), and then resuspended in RPMI 1640 medium containing 10% fetal calf serum and 1 μg/ml of Polybrene. In experiment 2, Jurkat or U937 cells (2×10^5) were treated with diethylaminoethyl-dextran (DEAE-D) (100 μg/ml for 15 minutes) (*15*) before being transfected with 2 to 3 μg of λ9B-7 and 20 μg of low MW salmon sperm DNA (15 to 30 minutes, 37°C), washed, and resuspended in RPMI 1640 medium as above. In experiment 3, 5×10^5 HUT-78 cells were transfected with 5 μg of plasmid vector DNA [pSp65-9B-7 (p9B-7)] by the DEAE-D technique (*15*). In all three experiments, ARV was initially detected by the presence of particle-associated RT activity in the culture supernatant (*5*). It was subsequently passed to mitogen-stimulated normal human PMC and further identified by IFA with human serum reactive with ARV (*6*). In experiments 4 and 5, NIH 3T3, COS-7, MiL, and RD-4 cells were transfected by the calcium phosphate and DEAE-D precipitation techniques (*14, 15*). In brief, 10 μg of p9B-7 DNA and 1.0 μg of pSV2-neo DNA (Bethesda Research Laboratory) were used per 3×10^5 cells in the absence of additional carrier DNA. Eighteen hours after transfection the cultures were split 1:3 and exposed to 200 μg/ml of G418 (Gibco). Resistant colonies were cultured for more than 1 month and assayed regularly for infectious ARV by the detection of RT activity in the supernatant (*5*) and by radioimmunoassay (RIA) with *gag* p25 protein (from K. Steimer, Chiron Corp.) (*16*). Further confirmation of ARV was made by cocultivation with normal PMC followed by IFA or immunoblot procedures.

Experiment	Clone	Culture	Cell type (species)	Transfection technique	Technique for virus identification
1	λ-9B-7	1192A	HUT-78 T (human)	$CaCl_2$-Hepes	IFA
2	λ-9B-7	1193A	Jurkat T (human)	DEAE-D	IFA
	λ-9B-7	1195A	U937 monocyte (human)	DEAE-D	IFA
3	p9B-7	4011	HUT-78 T (human)	DEAE-D	IFA, immunoblot
4	p9B-7	NIH-Ca	NIH 3T3 (mouse)	$CaPO_4$	IFA
	p9B-7	COS-Ca	COS-7 (monkey)	$CaPO_4$	RIA
	p9B-7	MiL-Ca	MiL (mink)	$CaPO_4$	RIA, immunoblot
5	p9B-7	COS-D	COS-7 (monkey)	DEAE-D	RIA, immunoblot
	p9B-7	RD-Ca	RD-4 (human)	$CaPO_4$	RIA, IFA

Table 2. Effect of ARV LTR promoter on induction of neomycin resistance in human and mouse cells. In these experiments, 10 μg of plasmid DNA was inoculated onto 5×10^6 cells by the calcium phosphate coprecipitation method (*13*). Subsequently, cells were plated and cultures were maintained for up to 2 weeks in the presence of G418 (*14*). Results show the number of G418 (neomycin)-resistant colonies produced by transfection in quadruplicate plates.

Cells (species)	Salmon sperm DNA	pSV-2/neo	pLTR-1/neo
RD (human)	0	135, 147, 111, 197	63, 43, 56, 70
NIH 3T3 (mouse)	0	77, 84, 48, 68	2, 2, 1, 3

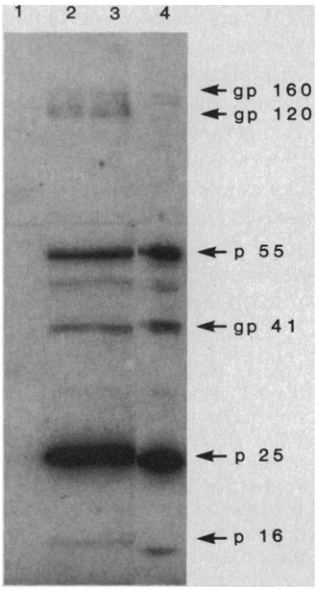


Fig. 2. Identification of ARV antigens in infected cells by immunoblot analysis. Extracts of normal PMC (lane 1) and PMC infected with virus recovered from transfected MiL cells (lane 2), COS-7 cells (lane 3), and HUT-78 T cells (lane 4) were prepared and subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gel. The fractionated proteins were then transferred to a nitrocellulose sheet by electroblotting for 4 hours at 300 mA (*17*). The blot was incubated for 2 hours in a blocking buffer containing 0.05*M* tris-HCl, *p*H 7.5; 0.5*M* NaCl, 3% nonfat dry milk (Carnation), 0.5% NP40, and 5 mg/ml of bovine serum albumin (Sigma). Serum positive for antibodies to ARV was then added to a final dilution of 1:100 in blocking buffer and allowed to react at 4°C overnight. The blot was subsequently washed and ^{125}I-labeled protein A (New England Nuclear) at a final dilution of 2×10^6 cpm/ml was added. After incubation for 30 minutes, the label was removed and the nitrocellulose blot was washed, dried, and exposed to Kodak XAR-5 x-ray film.

duction by the mouse, monkey, mink, and human RD-4 cells was measured in cultures containing the same number of cells. When examined by IFA, nearly all of the RD-4 cells contained ARV proteins, whereas the NIH 3T3 cells showed a faint fluorescence and quantitation was not possible. Data from other studies indicate that most cells transfected with two species of DNA take up both types (*9*). Thus, the neomycin resistance in the cells reflects the uptake of ARV-2 DNA.

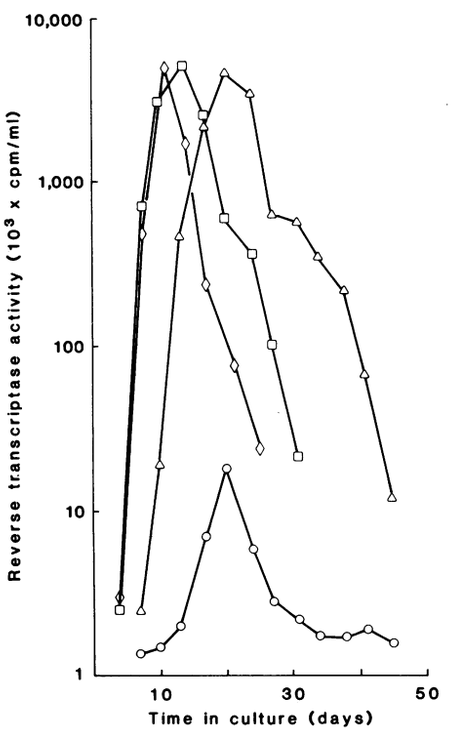
The virus recovered from all the cells was cytopathic for HUT-78 cells and extracts of the PMC cocultivated with transfected COS-7, MiL, and RD-4 cells contained all the antigens of ARV as demonstrated by immunoblotting (Fig. 2).

To determine further if intracellular restrictions were involved in the low replication of ARV-2 in rodent cells, we examined the promoter properties of the ARV-2 long terminal repeat (LTR). A plasmid construction containing the selectable marker neomycin under the control of the LTR (pLTR-1/neo) derived from cloned ARV-2 DNA (Fig. 4) was compared with respect to transfection and expression efficiency of pSV-2/neo, a plasmid containing the SV40 early promoter (*10*). Transfection with the SV40 promoter gave nearly the same frequency of neomycin resistance in mouse and human cells. In contrast, the ARV-2 LTR promoted neomycin resistance about one-third as efficiently in human cells as the SV40 promoter, and only about 1/50 as efficiently in NIH 3T3 cells (Table 2). These results support our observation (Fig. 3) that smaller amounts of infectious ARV are produced by rodent cells than by other animal and human cells.

These data indicate that, like the HTLV-III molecular clone already described (*11*), our molecular clone of ARV-2 is biologically active. This clone gives rise to replicating infectious virus that has the biologic characteristics of ARV, including cytopathology in HUT-78 cells and growth in high titer in PMC. These results indicate that ARV is the sole cause for these known properties of the AIDS retrovirus (*1*).

That the AIDS retrovirus can replicate in fibroblast cell lines and in cells of nonhuman origin has not, to our knowledge, been shown previously. Our results show that although virus infection is controlled primarily at the membrane level, cells resistant to direct infection by ARV can produce the virus if the genome is introduced in vitro by transfection. That ARV replication is also controlled intracellularly is indicated by the low level of infectious virus produced by the transfected monolayer cells, particularly those from mouse (Fig. 3), and the lower expression of the ARV LTR in mouse cells (Table 2). A similar influence of both membrane and intracellular factors in retrovirus infection has been observed with xenotropic and ecotropic mouse type C retroviruses (*12*). In our experiments, the cells of human origin, particularly T lymphocytes, were the most permissive for virus replication, but the mechanism responsible for this permissiveness is not known.

The ease of recovery of infectious ARV from transfected cells of different species suggests that this procedure may prove useful for elucidating the function of individual regions of the viral genome. By evaluating the effects of modified molecular clones of



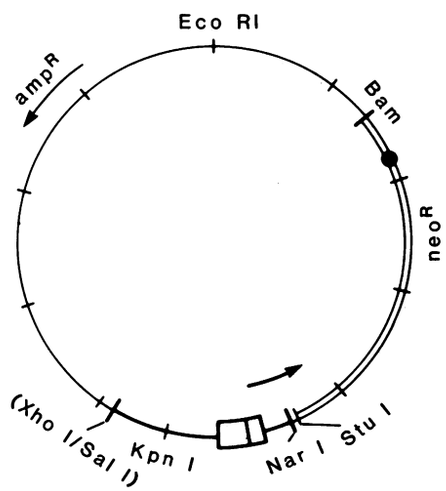


Fig. 3 (left). Detection of progeny ARV. Representative results from at least two experiments are shown. Mitogen-stimulated normal human peripheral mononuclear cells were cocultivated with the same number of G418-resistant cells obtained from clone p9B-7/pSV2-neo transfected NIH 3T3 (○), COS-7 (△), MiL (□), and RD-4 (◇) cells 14 to 18 days after transfection. After 4 days, the PMC were removed and cultured for over 2 months with addition of fresh PMC every 3 to 6 days. Culture media were collected periodically and particle-associated RT activity was assayed as described (*5*). Fig. 4 (right). Structure of the pLTR-1/neo plasmid. From the ARV-2 molecular clone, λ-7A, a DNA fragment bounded by an Xho-I site and an Nar I site and encompassing the LTR (box) was cloned into a modified pML vector (*3, 18*). A polylinker containing several restriction enzyme sites was placed in the Nar I site downstream from the LTR. From pSV2-neo (*10*), a DNA fragment encoding the *neo* gene together with the SV40 polyadenylation site was then cloned into the polylinker at the Nar I site to produce pLTR-1/neo. The heavy line is ARV DNA, the double line is the neomycin gene (*neo*^{R}), and the light line is the vector pSp65. Amp^R is the ampicillin resistance gene and ● is the SV40 polyadenylation site. The bold arrow shows the direction of transcription.

ARV it may be possible to find a means of eliminating the cytopathic effects of the virus or preventing its replication in human cells. This approach may also prove useful in attempts to induce the expression of certain ARV proteins that may be required for vaccine development.

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Mapping Epitopes on a Protein Antigen by the Proteolysis of Antigen-Antibody Complexes

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A monoclonal antibody bound to a protein antigen decreases the rate of proteolytic cleavage of the antigen, having the greatest effect on those regions involved in antibody contact. Thus, an epitope can be identified by the ability of the antibody to protect one region of the antigen more than others from proteolysis. By means of this approach, two distinct epitopes, both conformationally well-ordered, were characterized on horse cytochrome *c*.

THE INTERACTION OF ANTIBODIES to proteins with their respective antigens has been the subject of extensive studies. However, even where the covalent and tertiary structure of a protein antigen is known we rarely know the full identity and conformation of its antigenic sites. The two main approaches to this problem are peptide-binding studies and fine-specificity analyses, but inherent weaknesses limit their usefulness. The concept of using synthetic peptides to probe the antigenicity of a protein was first developed over 10 years ago (*1*). Antibodies that have stringent requirements for native conformation, however, or for surface contacts on the antigen that involve discontiguous regions of the polypeptide backbone, may not be detected by this method. Fine-specificity studies are done with panels of evolutionarily variant proteins. This approach has enabled immunodominant residues in globular proteins to be identified whose epitopes are conformationally dependent, such as in lysozyme, myoglobin, and cytochrome *c* (*2*). However, only one or two evolutionarily variant residues involved in antibody binding can be identified by this method.

These approaches have been used to study the specificities of two monoclonal antibodies, C3 and E8, that were derived from a mouse that had been immunized with cytochrome *c* (*3*). Fine-specificity studies indicated that the determinant for C3 was centered around residue 44. Assignment of the site for E8 was not definitive because the panel of naturally occurring cytochromes *c* was inadequate to distinguish binding at either of the two regions about residues 60 or 89. Further delineation of the antigenic sites with synthetic peptides and large cyanogen bromide–cleaved fragments of the protein was not possible since the antibodies did not bind them (*3*).

We therefore applied a novel approach to detect the surface regions of cytochrome *c* that contact these monoclonal antibodies. Since the regions of antibodies that bind antigen are resistant to proteolysis (*4*) while cytochrome *c* is readily proteolyzed (*5*), we hypothesized that the relative rates of release of peptides from cytochrome *c*–antibody complexes during proteolysis with trypsin, compared to release of peptides from unbound antigen, would indicate which parts of the antigen are in contact with the antibody.

Cytochrome *c* peptides are readily identified in a trypsin-digested protein mixture containing monoclonal antibodies by means of reversed-phase high-performance liquid chromatography (rHPLC). To demonstrate this a monoclonal antibody to dinitrophenol (anti-DNP) that was of the immunoglobulin G1 (IgG1) class and did not bind cytochrome *c* was added to horse cytochrome *c* in a relative amount that would have just saturated binding sites had the two components interacted. The mixture was digested with trypsin for 30 minutes and peptides were separated by rHPLC. One cytochrome *c* peak overlapped with an IgG peptide that had a substantial signal (Fig. 1, A and B), and therefore could not be quantified. This peak lies between peaks 34 and 37 in Fig. 1A and represents the sequence from residues 61 to 72 in the cytochrome *c* molecule. All other cytochrome *c* peptides, however, eluted at positions distinct from the IgG peptides or had significantly larger signals and were quantifiable. Although the IgG had been predigested with trypsin and fractionated on Sephadex G-75, small peptides were still present (Fig. 1B); however, the antigen-binding fragment that was resistant to proteolysis did not elute in the rHPLC gradient.

Essentially the same elution profiles were observed with anti-DNP and anti–cytochrome *c* (Fig. 1B). Fab's prepared from the antibodies also gave a similar profile (Fig. 1C), although the signals at 214 nm were lower. Trypsin (Fig. 1D) contributed little to the peptide signals seen in the total digest in Fig. 1A. Tryptic peptides of horse cytochrome *c* corresponding to the numbered peaks were identified from their amino acid composition and the sequence of horse cytochrome *c* (*5*). In the 30-minute digest of cytochrome *c* there was a peak (peak 39 in Fig. 1A) that was not present in a limit digest (18 hours). This peptide corresponds to residues 56–73 and was an intermediate digestion product in the formation of the peptides containing residues 56–60 and 61–72. Cytochrome *c*, itself, and any peptides larger than those shown did not elute in the gradient.

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