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# In Vitro Synthesis of A-Particle Structural Protein by Membrane-Bound Polyribosomes

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On the basis of association with endoplasmic reticulum membranes, poyribosomes isolated from mouse myeloma MOPC-104E were separated into two classes, membrane bound and free. The membrane-bound and free polyribosomes were then compared for their capacity to incorporate [$^{35}$S]methionine into A-particle proteins in vitro. As revealed by a radioimmunological assay method, labeling of A-particle protein occurred with the membrane-bound polyribosomes but not with the free polyribosomes. Peptide mapping of the immunoprecipitated, in vitro [$^{35}$S]methionine-labeled product confirmed that A-particle protein had been synthesized in vitro.

Intracisternal type A particles are virus-like structures, which form by budding at the membranes of the endoplasmic reticulum and appear to remain localized within the cisternae (5). They occur regularly in the early embryos of all mouse strains thus far studied and seem to represent the transient expression of a genetic element that is vertically transmitted in this species (2-4). A-particle synthesis is permanently activated in many transformed mouse cells (8), providing an opportunity to study the possible role(s) of these structures in the cellular economy. The relationship of intracisternal type A particles to known oncogenic RNA viruses is still unclear.

As isolated from various mouse tumor cells, intracisternal A particles contain a major structural polypeptide with a molecular weight of 73,000 (P73) and minor amounts of polypeptides with molecular weights of 46,000 and 30,000 (10). These proteins possess common antigenic determinants, and analysis of tryptic digests has indicated that common peptide sequences are present in all three (10, 11). It is not known whether P46 and P30 are synthesized independently or arise from cleavage of P73. A recent study on the synthesis and turnover of A particles in cultured neuroblastoma cells revealed that P73 was more rapidly synthesized and degraded than the average cell protein (9). In these cells, assembly of P73 into particulate form occurred very rapidly after synthesis, and the soluble pool of P73 was quite small. Similarly, very little of the total P73 in myeloma cells was recovered in the nonparticulate fraction (100,000 × *g* supernatant fluid) (18).

Attempts to detect newly synthesized P73 on polyribosomes after pulse labeling of cells were unsuccessful. To learn more about the site of synthesis of A-particle proteins, an in vitro protein-synthesizing system was developed using polyribosomes from mouse myeloma MOPC-104E, a tissue that contains numerous A particles. It was hoped that, by dissociating transport and assembly from synthesis, enough P73 could be accumulated on polyribosomes to make detection possible. The results of the current study show that A-particle protein is synthesized in vitro on membrane-bound polyribosomes.

## MATERIALS AND METHODS

**Preparation of polyribosomes.** A 2- to 3-g amount of MOPC-104E tumor was homogenized in 3 volumes of 35 mM Tris-hydrochloride (pH 7.5), 60 mM KCl, 4.5 mM magnesium acetate, and 1 mM dithiothreitol (solution A) containing 0.25 M sucrose, with 10 strokes in a loose-fitting, Teflon-glass homogenizer. The nuclei were then removed by low-speed centrifugation. Aliquots of the cytoplasmic extract were layered over 1-ml cushions of 1.5 M sucrose and 2 M sucrose in solution A which contained 50% 100,000 × *g* supernatant that had been passed through a Sephadex column (GS-100) (see below). After centrifugation in a Spinco SW50.1 rotor for 4 h at 200,000 × $g_{ave}$, membrane-bound polyribosomes collected at the interface between 1.5 and 2 M sucrose, and free poyribosomes passed to the pellet. Membrane-bound polyribosomes were mixed with solution A containing 50% GS-100 and then the solution was adjusted to 1% in sodium deoxycholate. Polyribosomes in this solution were collected as a pellet by centrifugation through 1 ml of solution A containing 1.5 M sucrose. The polyribosome pellets were suspended by gentle homogenization in solution A. Deoxycholate-treated polyribosomes (bound plus free) were prepared from mouse liver in the same way, by sedimentation through a 1.5 M sucrose cushion.

For preparation of the GS-100, the cytoplasmic

extract of myeloma was centrifuged at 100,000 × $g_{max}$ for 90 min, and the top two-thirds of the supernatant was collected. This supernatant was applied to a column of Sephadex G-25 and eluted with solution A.

**Cell-free protein synthesis.** The reaction mixtures for cell-free protein synthesis contained the following components in a 50-μl reaction volume: Tris-hydrochloride (pH 7.5), 35 mM; magnesium acetate, 4.8 mM; KCl, 130 mM; dithiothreitol, 1 mM; ATP, 1 mM; GTP, 0.2 mM; CTP, 0.6 mM; phosphocreatine, 8 mM; creatine kinase, 10 μg; [$^{35}$S]methionine (average of 5.3 × $10^5$ counts/min per pmol; Amersham/Searle), 25 pmol; 19 other L-amino acids, each at 0.05 mM; GS-100 protein, 160 μg; and polyribosome protein, 5 to 20 μg. For routine assays, the reaction mixtures were incubated for 10 min at 37 C; 0.2 ml of 0.1 M NaOH was added to stop the reactions, and the incubation was continued for 15 min. To measure methionine incorporation, reaction mixtures were precipitated by the addition of 1 ml of 10% trichloroacetic acid containing 0.01 M unlabeled L-methionine, and the precipitates were collected and washed on separate nitrocellulose filters (Millipore Corp., 0.45 μm). The filters were dissolved in 1 ml of methyl cellosolve, 10 ml of Triton-toluene scintillation mixture was added, and radioactivity was measured in a Beckman 250 liquid scintillation counter.

**Gel electrophoresis.** Samples were analyzed by electrophoresis in 6% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and 6 M urea as described previously (9).

**Preparation of A-particle standards.** Unlabeled SDS-inner shells were prepared from MOPC-104E gradient-isolated A particles as described previously (19). $^3$H-labeled P73 was prepared from neuroblastoma cells (clone N4) that had been labeled for 48 h with a mixture of amino acids (9). The Triton X-100–EDTA-resistant pellet from these cells, containing over 95% of the A-particle antigen, was analyzed by polyacrylamide gel electrophoresis as described previously (9), and the P73 peak was collected by elution from the gels.

Purified, $^{35}$S-labeled P73 was isolated from myeloma cells. MOPC-104E ascites cells were suspended in methionine-free, Dulbecco-Vogt-modified Eagle medium containing dialyzed fetal calf serum and labeled with L-[$^{35}$S]methionine (10 μCi/ml [average of 300 Ci/ mmol], Amersham/Searle) for 4 h. The Triton X-100–EDTA-resistant pellet was then isolated from these cells and further treated with 1% SDS to prepare an SDS-resistant pellet. This fraction was analyzed on gels, and the P73 peak was eluted as above. The protein was purified further by chromatography on a hydroxylapatite column by the procedure of Moss and Rosenblum (12). The P73 peak, eluting between 0.5 and 0.56 M sodium phosphate, was rechromatographed (K. K. Lueders, Ph.D. thesis, Univ. of Maryland, College Park, 1975), and the resulting peak, which eluted between the same sodium phosphate concentrations, was used as pure P73 for peptide mapping.

**Isotope dilution-radioimmunoassay for A-particle protein.** Radioactive A-particle protein was measured with an indirect immunoprecipitation reaction using rabbit anti-A-particle P73 serum, which has been described in detail elsewhere (8). The antiserum reacts with A particles to give a single precipitin line upon immunodiffusion in agar. SDS-inner shells from MOPC-104E were used as unlabeled antigen. Fractions were treated with 0.5% SDS and 1 mM dithiothreitol and heated at 100 C for 1 min to "unmask" antigen and then diluted to 1 ml with Hanks basic salt solution. After addition of 1.5 μl of anti-A-particle serum, the samples were incubated for 1 h at 37 C and overnight at 4 C. A 25-μl volume of sheep anti-rabbit immunoglobulin G serum (a gift from M. Mage, National Cancer Institute) was then added, and the samples were incubated for 1 h at 37 C and for 4 h at 4 C before the precipitates were collected by centrifugation at 2,000 × $g$ for 15 min at 5 C. The precipitates were washed two times with Hanks solution and taken up in a solution of 10 mM sodium phosphate, 1% SDS, and 1% 2-mercaptoethanol for counting in Triton-toluene scintillation mixture.

**Peptide mapping.** Peptide mapping was performed by the procedure of Marciani and Kuff (11) for A-particle SDS-inner shell proteins. Samples were reduced with 0.1 M 2-mercaptoethanol in 1% SDS and alkylated with iodoacetamide. The SDS was removed by precipitation of the proteins with chloroform-methanol (1:1, vol/vol) followed by extensive washing with acetone. Samples were treated with trypsin-TPCK (Worthington Biochemicals), lyophilized, and analyzed by two-dimensional, thin-layer chromatography, with electrophoresis preceding ascending chromatography. Peptides were localized by staining with 0.3% ninhydrin. Radioactive peptides were located by autoradiography.

## RESULTS

**Cell-free protein synthesis.** Under the conditions used, incorporation of [$^{35}$S]methionine into protein by free and membrane-bound polyribosomes was proportional to the amount of polyribosome protein in the reaction mixtures; this incorporation was greater per microgram of polyribosome protein for free polyribosomes than for bound polyribosomes. Absolute amounts of incorporation were 40 to 65 and 25 to 50 pmol of [$^{35}$S]methionine incorporated per mg of protein per 10 min for free and membrane-bound polyribosomes, respectively.

**Gel analysis of immunoprecipitated products labeled in vitro.** An indirect immunoprecipitation method was previously used to identify in vivo labeled P73 contained in particles isolated from neuroblastoma cells (9). This method was also applied in an effort to select A-particle proteins from the products labeled in the cell-free system. Aliquots of the reaction mixtures were precipitated with A-particle-specific serum and with preimmunization serum. The preimmunization serum precipitated approximately 2% of the total product from free

and bound polyribosomes. The A-particle-specific serum precipitated an additional 1 to 2% of the product from both free and bound polyribosomes. However, when the precipitates were analyzed by electrophoresis in SDS-polyacrylamide gels, no peak corresponding to [$^{35}$S]methionine-labeled P73 was seen in the resulting protein pattern. A possible explanation lay in the observed sensitivity of isolated P73 to proteolytic cleavage (Lueders, unpublished data). In control experiments, indirect immunoprecipitation of small amounts of authentic, $^{3}$H-prelabeled P73 resulted in conversion of part of the label to material that coelectrophoresed with P46, suggesting that P73 present among the cell-free products might also be degraded to smaller components. A-particle proteins in the size range of P30 and P46 are precipitated by A-particle-specific serum since they also contain the antigenic site (10); however, the lower-molecular-weight components are difficult to distinguish from other cell proteins in this size range, and the small increments in label precipitated from the in vitro products by A-particle-specific serum over that precipitated by prebleed might be attributable to differences in nonspecific binding by the two sera. These results indicated that identification of in vitro labeled A-particle proteins would not be possible on the basis of size.

**Isotope dilution-immunobinding assay for A-particle protein.** A radioimmunoassay based on isotope dilution was developed to gain specificity in the measurement of small amounts of A-particle protein labeled in vitro. In this assay, radioactive protein (e.g., the protein synthesized in vitro) was bound to A-particle-specific antibody, and the resulting complex was precipitated with a sheep antiserum to the A-particle antibody. In the presence of a constant and limiting number of antibody sites, labeled antigen can be displaced from the complex by the addition of increasing amounts of unlabeled A-particle protein, and the kinetics of this displacement will depend on the binding constant of the A-particle antigen-antibody complex and the total concentration of antigen in the system (labeled plus unlabeled).

To determine the binding capacity of the antibody, the binding of A-particle protein was examined at a constant antibody concentration. From a double-reciprocal plot of the data, it was calculated that the binding constant of the antiserum was $1.8 \times 10^{4}$ liters/mol (on the basis of P73). Theoretical isotope dilution curves for different amounts of labeled A-particle protein were then derived on the basis of the experimentally determined binding constant. They showed that, for all of the reactions containing labeled A-particle protein up to 3 $\mu$g, more than 90% of the radioactivity that was sensitive to isotope dilution would be displaced from the antigen-antibody complex after addition of approximately 50 $\mu$g or more of unlabeled A-particle protein. This provided a means for estimating the relative amount of labeled A-particle protein in the in vitro products. Moreover, the shapes of the theoretical isotope dilution curves were a function of the absolute amount of labeled A-particle protein in the sample. The shapes of these curves were not distinguishable for quantities of antigen of 0.15 $\mu$g and less.

The assay for radioactive A-particle protein was then tested with previously characterized $^{3}$H-labeled A-particle protein. Labeled P73 (1.8 $\mu$g) was reacted with anti-A-particle serum in the absence and the presence of increasing amounts of unlabeled A-particle protein. The results were converted to the form of an isotope dilution curve as follows. The radioactivity bound at the highest level of added unlabeled antigen (95 $\mu$g) was subtracted from the total bound in the absence of unlabeled antigen. This increment represented the total competed radioactivity. The radioactivity displaced at each concentration of unlabeled antigen was expressed as a percentage of the total competed radioactivity (Fig. 1). More than 90% of the labeled A-particle protein was displaced from the antibody precipitate by 48 $\mu$g of unlabeled A-particle protein, as expected, and the reduction in radioactivity bound to the antibody was similar to the theoretical isotope dilution curve calculated for reactions containing 1.8 $\mu$g of A-particle protein.

**Detection of A-particle-specific label on polyribosomes.** The competitive binding assay was used to determine whether labeling of A-particle proteins occurs on free or membrane-bound polyribosomes. [$^{35}$S]methionine-containing peptides synthesized in vitro by free and bound polyribosomes were reacted with anti-A-particle serum in the absence and the presence of increasing amounts of unlabeled A-particle protein (Table 1). In the absence of added unlabeled antigen, 3.9% of the material labeled in vitro by bound polyribosomes was precipitated. Of this, 41% (1.6% of total product) was competed by the addition of excess unlabeled antigen. From the specific activity of the methionine and the methionine content of the A-particle protein (8 mol/mol of P73) (10), it can be calculated that the amount of displaced label (3,028 counts/min, Table 1) corresponds to 0.035 ng of A-particle protein in this aliquot of in vitro product from bound polyribosomes. Of the

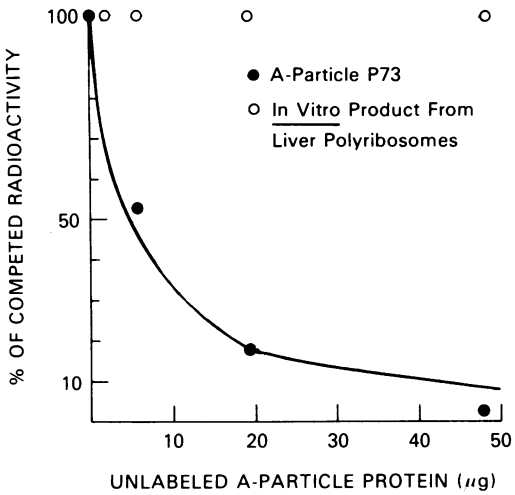


FIG. 1. *Competitive binding assay for A-particle protein. Dose response measurements for binding of [³H]amino acid-labeled P73 (2,500 counts/min, 1.8 μg of protein) (●), the theoretical isotope dilution curve for 1.8 μg of A-particle protein (——), and [³⁵S]methionine-labeled in vitro product from liver polyribosomes (92,500 counts/min, 0.32 pmol) (○). Each assay contained 1.5 μl of anti-A-particle serum; the unlabeled protein was MOPC-104E SDS-inner shells; 100% competed radioactivity represents the difference in radioactivity bound in the absence of added unlabeled antigen and 95 μg of added unlabeled antigen.*

TABLE 1. *Competitive binding assay for A-particle-specific proteins in the in vitro products*[a]

| Unlabeled antigen added (μg) | ³⁵S radioactivity in immunoprecipitate (counts/min) | |
|---|---|---|
| | Product from bound polyribosomes | Product from free polyribosomes |
| None | 5,007 | 3,674 |
| 1.9 | 4,224 | 3,915 |
| 5.7 | 3,474 | 3,910 |
| 48 | 3,074 | 3,685 |
| 95 | 2,979 | 3,705 |

[a] All reactions contained 1.5 μl of anti-A-particle serum. In vitro products added were as follows: product from 3 μg of free polyribosomes, 110,160 counts/min (0.20 pmol); product from 6 μg of bound polyribosomes, 129,425 counts/min (0.23 pmol). Unlabeled antigen was MOPC-104E SDS-inner shells.

material labeled in vitro by free polyribosomes, 3.3% was precipitated, and none of this radioactivity was displaced from the precipitate by the addition of increasing amounts of unlabeled antigen. By this criterion, A-particle-specific label was detected in the product from bound polyribosomes but not in that from free polyribosomes.

Mixing of the products from the bound and free polyribosomes did not interfere with detection of the A-particle proteins made by the bound polyribosomes. Thus, the failure to detect A-particle protein in the products from the free polyribosomes was not the result of an inhibitor in that fraction. To establish that the observed competition of label from the product of bound polyribosomes was not due to some form of nonspecific competition, the ³⁵S-labeled product synthesized in a cell-free system by polyribosomes isolated from mouse liver was tested in the isotope dilution assay. Mouse liver does not contain intracisternal A particles, but most of its polyribosomes are membrane bound (17). None of the protein labeled by isolated liver polyribosomes was competed from the antibody precipitate upon addition of unlabeled A-particle protein (Fig. 1). In other control experiments, preimmunization serum precipitated comparable amounts of labeled products from free and bound myeloma polyribosomes, and none of this radioactivity was displaced by an excess of added unlabeled antigen.

Data in Table 1 were converted to the form of an isotope dilution curve as described for P73 in Fig. 1. The data points for the in vitro product from bound polyribosomes (Fig. 2) fell very close to a theoretical isotope dilution curve calculated on the assumption that the labeled fraction contained 0.15 μg or less of A-particle protein. This was to be expected in view of the

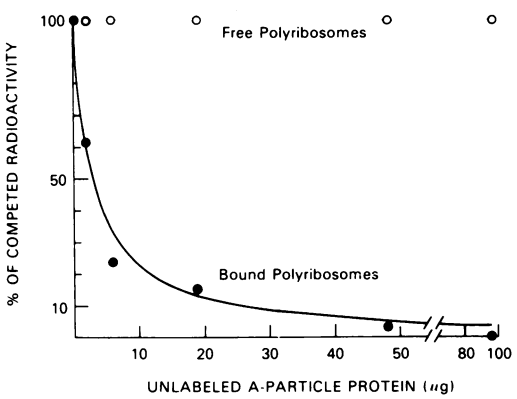


FIG. 2. *Dose response curves for in vitro products. The difference in radioactivity bound in the absence of unlabeled antigen and at the highest level of unlabeled antigen in Table 1 was taken as A-particle-specific label and represents 100% competed radioactivity. The percentage of competed radioactivity versus increasing amounts of unlabeled antigen is plotted for the in vitro products from bound polyribosomes (●), the theoretical isotope dilution curve for 0.15 μg of protein (——), and the in vitro products from free polyribosomes (○).*

amount of labeled A-particle protein calculated to be in the sample (0.035 ng) and the fact that isotope dilution curves for quantities of antigen equal to or less than 0.15 μg are indistinguishable, as mentioned earlier.

The relative amounts of A-particle protein were measured in the in vitro products from a variety of independently isolated preparations of free and bound polyribosomes, using the displacement of label from the immunoprecipitate by an excess of unlabeled antigen (Table 2). Labeling of A-particle protein was detected with bound polyribosomes in all cases but not with free polyribosomes. The percentage of the in vitro labeled protein that was specifically competed by unlabeled antigen ranged from 0.7 to 1.6% of the total incorporation, a value comparable to that of total cell amino acid incorporation into P73, which was approximately 1.4% (9).

**Peptide mapping of immunoprecipitated in vitro products.** To further identify the radioactive peptides made in the in vitro system by the bound polyribosomes, peptide maps of tryptic digests were prepared. The material labeled by bound polyribosomes was directly immunoprecipitated in the presence of 19 μg of carrier, unlabeled antigen as described previously (9), and the precipitate was analyzed in parallel with in vivo [$^{35}$S]methionine-labeled P73 that had been purified by hydroxylapatite column chromatography. Before tryptic digestion, an additional 150 μg of unlabeled A-particle protein was added to the labeled preparations. In the subsequent peptide maps, the peptides derived from immunoglobulin did not interfere with identification of those derived from the A-particle protein. The plates were then exposed to X-ray film to determine which peptides contained label. Autoradiographs of the tryptic maps of the two preparations are shown in Fig. 3. The four major labeled peptides present in authentic [$^{35}$S]methionine-labeled P73 (a through d) were also present in the immunoprecipitated products from bound polyribosomes, although one peptide contained much less label (d). This weakly labeled peptide may represent sequences from the N-terminal end of the protein, since the in vitro labeling appears to involve primarily the elongation of preexisting nascent chains with very little initiation of new chains (Lueders, Ph.D. thesis). There was one strongly labeled non-A-particle peptide present in the map of the immunoprecipitated in vitro product (Fig. 3, *). This peptide was also a prominent component in a peptide map of total in vitro product from bound polyribosomes and was not concentrated by immunoprecipitation. The A-particle peptides, on the other hand, were markedly concentrated by the immunoprecipitation. From the competitive binding assay it was expected that only 40% of the label in the immunoprecipitate would be A-particle proteins. This is consistent with the number and intensity of the $^{35}$S-labeled A-particle peptides found.

TABLE 2. *In vitro product analysis for A-particle-specific label*[a]

| Source of in vitro product | [$^{35}$S]methionine product (pmol added to assay) | Polyribosomal protein in reaction (μg) | A-particle-specific product | |
|---|---|---|---|---|
| | | | pmol of [$^{35}$S]methionine × $10^3$ | % of total |
| Bound polyribosomes | 0.80 | 31.7 | 5.7 | 0.7 |
| Bound polyribosomes | 0.65 | 9.0 | 5.2 | 0.8 |
| Bound polyribosomes | 0.20 | 3.7 | 1.8 | 0.9 |
| Free polyribosomes | 0.70 | 18.0 | ND | ND |
| Free polyribosomes | 0.72 | 10.0 | ND | ND |
| Free polyribosomes | 0.74 | 20.0 | 0.04 | 0.01 |

[a] The in vitro product in each assay represents product from independently isolated polyribosomes. Unlabeled antigen was 48 μg of SDS-inner shells. ND, None detected.

## DISCUSSION

Immunoprecipitation of radioactive proteins has been used in numerous cases for identification of products made in vitro (13, 14, 16). As pointed out previously, however, immunoprecipitation of A-particle proteins from solution involves an extensive precipitation of nonspecific proteins (9). This high background and the demonstrated cleavage of small amounts of P73 to material of lower molecular weight during the immunoprecipitation indicated that A-particle protein labeled in vitro could not be reliably identified by gel electrophoretic analysis of immunoprecipitates. The competitive binding assay was developed to give specificity to the immunoprecipitation of small amounts of these proteins. From the results of this assay and the peptide maps, it appears that A-particle proteins are labeled in vitro and that incorporation of label takes place primarily on polyribosomes that were originally membrane bound. The experiments do not exclude the possibility that some A-particle protein could be made on free polyribosomes in the cell.

Intracisternal A particles share a number of properties with known oncogenic RNA viruses

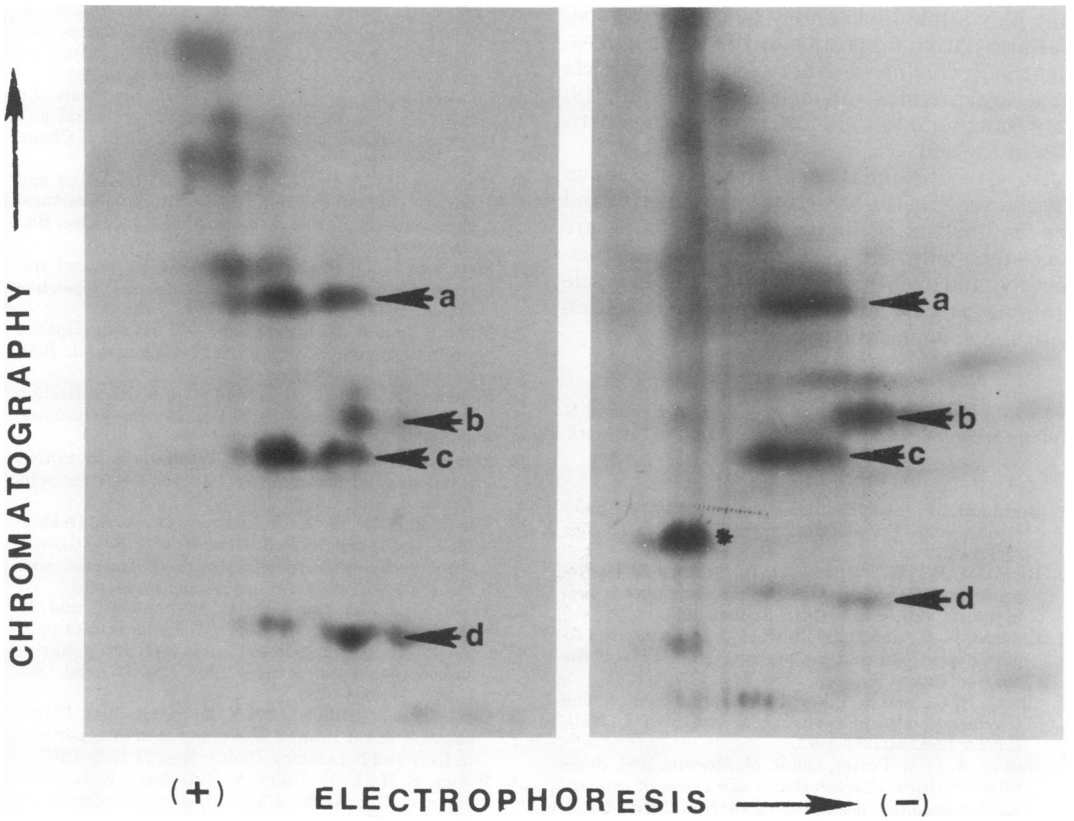


FIG. 3. *Autoradiographs of peptide maps of P73 isolated from labeled cells (left) and the immunoprecipitate of the in vitro product from bound polyribosomes (right). About 49,700 and 129,400 counts/min of $^{35}S$, respectively, were mixed with 150 μg of SDS-inner shells, digested with trypsin, and mapped as described in the text. Samples were applied to the origin, subjected to electrophoresis in one dimension, and chromatographed in the other. The chromatograms were stained with ninhydrin and then exposed to X-ray film for 21 days. The arrows on the autoradiographs (a through d) indicate the major labeled peptides in both P73 and the in vitro product. Corresponding ninhydrin-stained peptides from authentic A-particle antigen were also present. The splitting of spots a and c in the left-hand panel is an artefact.*

(20) and form by budding at the endoplasmic reticulum in a way similar to the budding of type C viruses at the plasma membrane (1). It should be noted that virus-specific mRNA in cells infected with Rauscher leukemia virus has been found on both free and bound polyribosomes (7), as is also the case with viral RNA and nascent polypeptides in murine sarcoma virus-leukemia virus-infected cells (16). However, in both studies bound polyribosomes had a higher content of both viral RNA and viral peptides than did free polyribosomes. Thus, the site of synthesis of C-particle and A-particle internal structural proteins appears to be the same (i.e., bound polyribosomes).

After synthesis, both C-particle and A-particle proteins are found to be associated with membranes (9, 15). C-particle precursor polypeptides are evidently cleaved while in association with membranes, and the viral structural proteins are then rapidly assembled into virus form at the plasma membrane (15). As revealed by electron microscopy, an early phase in A-particle assembly appears to involve thickening of discrete areas of the endoplasmic reticulum (6). This assembly into recognizable particle form occurs on areas of the endoplasmic reticulum devoid of ribosomes. Since no soluble pool of A-particle protein has been detected (9) and only small amounts of C-particle proteins have been found in the soluble phase of infected cells (15), these observations suggest that, in both cases, migration of the proteins from the site of synthesis on membrane-bound polyribosomes to the site of assembly occurs while the proteins are membrane associated and may involve some directional membrane flow. Repetitive sequences in the A-particle structural proteins

may play a role in assembly by conferring special associative properties to the polypeptides, and the firm adherence between the A-particle structural proteins and membrane proteins (19) may contribute to the localization of the particles in the cell.

These results and those of previous studies on the character of the structural proteins (10) and the metabolism of the particles in cells (9) are consistent with the model that synthesis, assembly, and eventual degradation of A-particle proteins are processes that occur in association with the endoplasmic reticulum.

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