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14. For each normal, term human placenta, a single cotyledon was dually perfused according to the method of H. Schneider, M. Panigel, and J. Dancis [*Am. J. Obstet. Gynecol.* **114**, 822 (1972)] and H. Schneider and A. Huch [*Contrib. Gynecol. Obstet.* **13**, 40 (1985)]. Briefly, a chorionic arterial (fetal influx) vessel and a venous (fetal efflux) vessel, supplying an intact lobe, were catheterized and perfused with Earle's balanced salt solution containing 3% dextran, molecular weight 77,000 (EBSS), aerated with 95% oxygen and 5% carbon dioxide. The fetally perfused cotyledon was cut from the placenta with generous circumscription and placed on a water-jacketed, temperature-controlled perfusion stage with the maternal decidua facing up. Four metal cannulae pierced the maternal basal plate to provide perfusion of the intervillous space (maternal influx). The perfusate leaving the intervillous space (maternal efflux) was allowed to accumulate to a volume of 3 to 5 ml, which was then pumped out of the perfusion stage. The flow rate in both maternal and fetal circulations was 6.3 ml/min. The perfusion stage, perfusate reservoirs, and all tubing were kept in a temperature-controlled perfusion case at 37°C. Perfusate reservoir volumes were monitored to detect leaks; pressures were below 50 mmHg throughout all successful perfusions. Antipyrine, detected by ultraviolet spectroscopy [B. B. Brodie, J. Axelrod, R. Soberman, B. B. Levy, *J. Biol. Chem.* **179**, 25 (1949)], was used as a diffusion marker for maternal-to-fetal transfer characteristics. The general perfusion protocol was as follows: 30 min of washout or equilibration (EBSS with no substrate; maternal perfusate nonrecirculated or open, fetal open); 30 min of blank perfusate preparation (EBSS with no substrate; maternal open, fetal recirculated or closed); 60 min of perfusion with EBSS plus 65 m*M* (300 mg/dl) ethanol in the maternal influx (maternal open, fetal closed; fresh fetal perfusate); 15 min of washout (EBSS with no substrate; maternal open, fetal open); 60 min of perfusion with EBSS plus 65 m*M* (300 mg/dl) ethanol and 25 μ*M* AcH in maternal influx (maternal open, fetal closed; fresh fetal perfusate).
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Antibodies to Asp-Asp-Glu-Asp Can Inhibit Transport of Nuclear Proteins into the Nucleus

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The signal sequence of simian virus 40 (SV40) large T-antigen for translocation into the nucleus is composed of positively charged amino acids Lys-Lys-Lys-Arg-Lys. Rabbit antibodies to a synthetic peptide containing the negatively charged amino acid sequence Asp-Asp-Asp-Glu-Asp were obtained. Indirect immunofluorescence of the antigens recognized by the antibody was punctate at the nuclear rim or the nuclear surface, depending on the plane of focus. The antibody blocked transport of nuclear proteins into the nucleus. The antigens recognized by the antibody were predominantly localized to the nuclear pores.

NUCLEAR PROTEINS HAVE SPECIFIC signal sequences that are necessary for their transport from the cytoplasm into the nucleus (*1*). Colloidal gold particles coated with nucleoplasmin and injected into the cytoplasm of *Xenopus* oocytes enter the nucleus through nuclear pores (*2*). Wheat germ agglutinin binds to *O*-linked *N*-acetylglucosamine (GlcNAc) residues of nuclear pore complex proteins and inhibits import of proteins into the nucleus in vivo (*3*) and in vitro (*4*). The nuclear targeting signal of SV40 large T antigen is composed of positively charged amino acids and has the characteristic sequence KKKRK (*1*, *5*). Other nuclear proteins are reported to have similar sequences (*6*). When the SV40 large T-antigen signal sequence is conjugated to nonnuclear proteins with molecular sizes of more than 68 kD (too large to enter the nucleus by passive diffusion) and these are injected into the cytoplasm of cells, these conjugates are transported into the nucleus within 30 min, without cell division (*7*). Thus, the signal sequence of SV40 large T antigen may be the active nuclear transport signal.

"Receptors" that interact with the signal sequence may have a negatively charged region electrostatically complementary to the positively charged signal sequence. The putative signal sequence-binding site might contain either DDDED or EEEDE. We therefore synthesized these peptides (Table 1) and prepared and purified antibodies to them (Fig. 1). The antibodies consisted almost exclusively of immunoglobulin G (IgG) and reacted specifically with the corresponding peptides (Fig. 1, A and B) (*8*), but did not react directly with iodinated nucleoplasmin (Fig. 1C). Almost the same result was obtained with bovine serum albumin (BSA) conjugated to the nuclear targeting sequence of either SV40 large T antigen or polyoma virus large T antigen (SV40 T–BSA or polyoma T–BSA).

We used indirect immunofluorescence to determine the intracellular locations of the antigens recognized by these antibodies. Both kinds of antibodies gave similar results, although 10 to 20 times as much anti-EEEDE as anti-DDDED was required for equal staining. In interphase cells anti-DDDED stained mainly the nucleus, with weak staining of the cytoplasm (Fig. 2A). At higher magnification (Fig. 2, C, D, and E), the immunofluorescence of the nucleus appeared punctate. Depending on the plane of focus, the punctate staining was seen at the nuclear rim (Fig. 2C) or the nuclear surface (Fig. 2, D and E). A punctate pattern was also seen in the cytoplasm close to the

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nucleus (Fig. 2E), although it varied in extent from cell to cell. These results were similar to those obtained by others, which suggested that the molecules recognized by their antibodies were localized in the nuclear pore complex (*9*).

At mitosis, the punctate pattern disappeared and the fluorescence became diffuse throughout the cell (without staining of chromatin); the punctate staining reappeared at telophase. Similar results were obtained with other cell lines such as human embryonic lung (HEL) cells, Madin-Darby bovine kidney (MDBK) cells, and Swiss 3T3 murine fibroblasts.

These antibodies were then examined for their effect on nuclear translocation of proteins. Anti-DDDED (5 mg/ml) almost completely inhibited the nuclear transport of nucleoplasmin, SV40 T–BSA, and polyoma T–BSA (Fig. 3A) (*10*). These results were

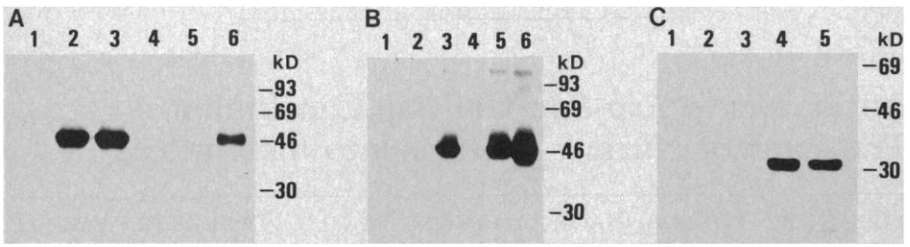

Fig. 1. Characterization of anti-DDDED and anti-EEEDE by immunoprecipitation. Synthetic peptides DDDED and EEEDE were chemically conjugated to keyhole limpet hemocyanin (KLH) as described (*3*, 7, *17*). Rabbit anti-DDDED and anti-EEEDE were prepared by intramuscular or subcutaneous injection of DDDED-KLH or EEEDE-KLH, and were absorbed with 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP)–activated Avid–gel F (Bioprobe International, Inc.) conjugated to 4-(*p*-maleimidophenyl) butylate (MPB)–KLH. Antibodies specific for DDDED and EEEDE were purified by affinity chromatography on DDDED- or EEEDE-conjugated Avid–gel F. Iodinated proteins (1.0 to 1.5 μg; 1.0×10^6 to 1.5×10^6 cpm/μg) were immunoprecipitated with antibody (30 to 40 μg) and Protein A–Sepharose CL-4B and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (10% separating gel) with subsequent autoradiography. (**A**) ^{125}I-labeled DDDED-ovalbumin immunoprecipitated with anti-DDDED (lanes 2 to 5) or nonimmune rabbit IgG (lane 1). The immunoprecipitates were formed in the presence of excess amounts of nonlabeled ovalbumin (lane 3), DDDED-ovalbumin (lane 4), or DDDED-KLH (lane 5). Lane 6, ^{125}I-labeled DDDED-ovalbumin. (**B**) ^{125}I-labeled EEEDE-ovalbumin immunoprecipitated with anti-EEEDE (lanes 2 to 5) or nonimmune rabbit IgG (lane 1). The immunoprecipitates were formed in the presence of nonlabeled EEEDE-KLH (lane 2), EEEDE-ovalbumin (lane 4), or ovalbumin (lane 5). Lane 6, ^{125}I-labeled EEEDE-ovalbumin. (**C**) ^{125}I-labeled nucleoplasmin immunoprecipitated with anti-DDDED (lane 1), anti-EEEDE (lane 2), nonimmune rabbit IgG (lane 3), or rabbit antibody to nucleoplasmin (lane 4). Lane 5, ^{125}I-labeled nucleoplasmin. The molecular sizes (in kilodaltons) of protein standards are indicated on the right in each panel.

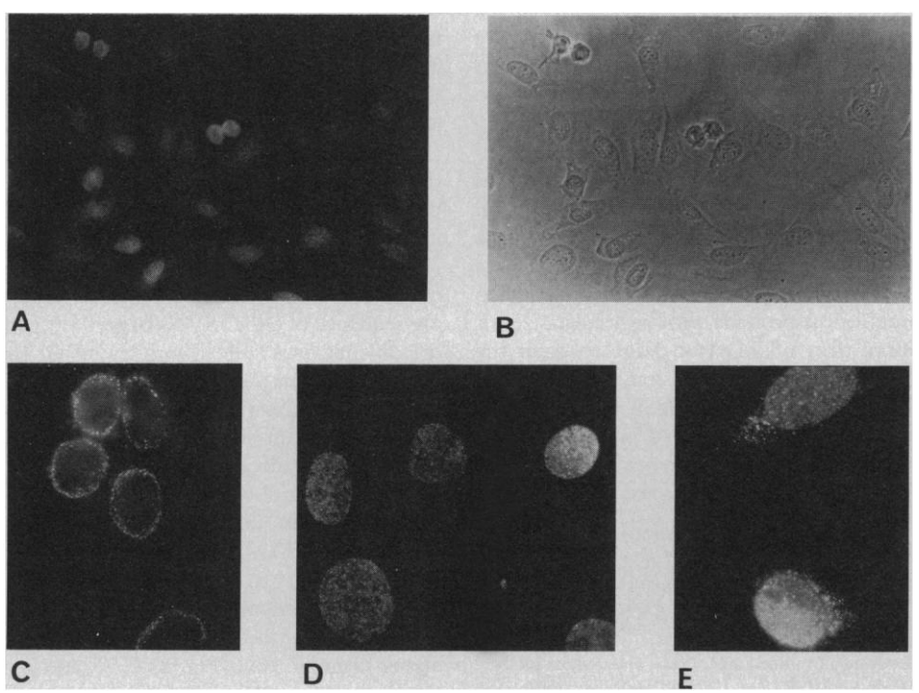

Fig. 2. Indirect immunofluorescence staining of F2408 rat fibroblast cells with rabbit anti-DDDED. F2408 cells were plated on cover slips and fixed with 3.7% formaldehyde at room temperature for 20 min, and then treated with methanol at −20°C for 5 min. Cover slips were incubated with affinity-purified anti-DDDED and then with fluorescein isothiocyanate (FITC)–conjugated goat antibody to rabbit IgG (anti-IgG). Cells were examined by phase contrast (**B**) and fluorescence (**A** and **C** to **E**) microscopy and photographed. (C), (D), and (E) show the cells at high magnification.

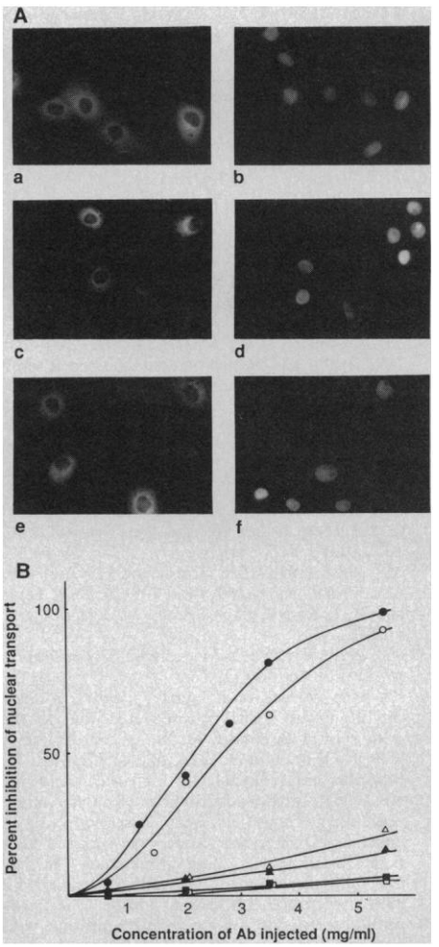

Fig. 3. Effects of various antibodies on nuclear transport of nucleoplasmin, SV40 T–BSA, and polyoma T–BSA. (**A**) Anti-DDDED (5 mg/ml) (a, c, e) or nonimmune rabbit IgG (b, d, f) was injected with nucleoplasmin (0.8 mg/ml) into the cytoplasm of HEL cells (*3*, *18*); the cells were incubated for 30 min at 37°C and then fixed as described in Fig. 2. To determine the subcellular location of the injected proteins, the cells were then incubated with a mixture of three kinds of mouse monoclonal antibodies to nucleoplasmin, or sheep antibody to BSA, and then with FITC-conjugated anti-IgG. (**B**) Various concentrations of anti-DDDED (●, ○), anti-EEEDE (▲, △), or nonimmune rabbit IgG (■, □) were injected into the cytoplasm of HEL cells with nucleoplasmin (0.8 mg/ml) (●, ▲, ■) or SV40 T–BSA (0.6 mg/ml) (○, △, □). After 30 min, the cells were treated as described above. Percent inhibition was determined from counts of the number of cells in which the fluorescence was found in the cytoplasm and the total number of cells injected. Counts were made on totals of 100 to 150 cells for each point.

reproduced with the cell lines F2408, MDBK, and Swiss 3T3 cells. To determine the concentration dependence of the inhibition by anti-DDDED and anti-EEEDE, we injected nucleoplasmin or SV40 T–BSA with various amounts of the antibodies into the cytoplasm of HEL cells. Nuclear transport of both nucleoplasmin and SV40 T–BSA was inhibited in a dose-dependent manner by co-injection of anti-DDDED (Fig. 3B). Since the solution injected into the cytoplasm was diluted about tenfold in the cells, and the antibody specific for the DDDED sequence accounted for 22% of the antibody in the preparation, the actual amount of anti-DDDED required for 50% inhibition was about 0.35 μ*M* (55 μg/ml). However, the anti-EEEDE preparation had only 1/10 to 1/5 the inhibitory effect of the anti-DDDED preparation (Fig. 3B), yet 65% of the antibody was specific. Thus, anti-EEEDE was about 1/15 to 1/30 as inhibitory as anti-DDDED.

Anti-DDDED reacted not only with the corresponding peptide, GGDDDEDGG (Fig. 1A), but was also almost as reactive with VEDDEDED. The affinity-purified antibody to VEDDEDED inhibited nuclear transport almost as effectively as anti-DDDED. Thus the antibodies specific for the DDED sequence were inhibitory. The inhibitory activity of the antibody in the cells was maintained at almost maximum levels for 3 hours, and then gradually decreased (*11*).

To further localize the antigen recognized by anti-DDDED, we fractionated rat liver cells and examined which fraction can absorb the inhibitory activity of the antibody. The inhibitory activity was partially absorbed by nuclei, more effectively by low-salt detergent–treated nuclear envelope and most effectively by high-salt detergent extract of the nuclear envelope (Table 2), known to contain the previously identified nuclear pore complex (*9*). In contrast, the inhibitory activity of anti-DDDED was only weakly absorbed by the cytoplasmic fraction or deoxyribonuclease (DNase) I extract. The immunoabsorption data and the immunofluorescence data (Fig. 2) suggest that the antigens recognized by anti-DDDED are predominantly localized to the nuclear pores.

Proteins of 69 and 59 kD were identified with anti-DDDED by immunoblotting rat liver nuclei lysates (Fig. 4A) (*12*). The intensity of the two protein bands decreased in the presence of excess amounts of DDDED peptides (Fig. 4A, lane 3). However, by immunoblotting rat liver cytoplasm, the nuclear envelope fraction, and the high-salt detergent extract of the nuclear envelope with anti-DDDED, we could not detect any specific bands under our conditions. We also ^{125}I-labeled the high-salt detergent extract nuclear envelope fraction (Table 2) and immunoprecipitated the iodinated proteins with the antibody. Proteins of 65, 54, 50, 43, and 34 kD were specifically immunoprecipitated (Fig. 4B). It remains to be determined whether all or some of the proteins detected by immunoblotting and immunoprecipitation actually participate in nuclear transport.

Two other techniques had been developed to produce antibody to a receptor without the use of the receptor as antigen. One is preparation of an anti-idiotype to the antibody to the ligand (*13*), as was done to produce antibodies to the insulin receptor and a receptor for protein import into chloroplasts (*13*). The other technique is based on the hydropathic complementarity of amino acids. The peptide encoded by the antisense DNA of the ligand is synthesized, and antibody to this "anticodon ligand" may bind the ligand's receptor. This procedure

Table 1. Amino acid sequences of the synthetic peptides. Peptides were synthesized with a model 430A synthesizer (Applied Biosystems, Inc.) (*15*).

Nomenclature	Sequence
DDDED	CGGDDDEDGG
EEEDE	CGGEEEDEGG
SV40 T	CPKKKRKVEDPC
Polyoma T	CDPPRTPVSRKRPRPAC

Table 2. Immunoabsorption of inhibitory activity of anti-DDDED. Cytoplasm was obtained as the 3000-rpm, 10-min supernatant of total rat liver homogenates. Rat liver nuclei were isolated from adult rats (*4*). Nuclei were treated with deoxyribonuclease I and centrifuged to yield supernatant (deoxyribonuclease I extract) and precipitates (nuclear envelope) (*16*). Treatments of the nuclear envelope were as described (*9*) with slight modifications. Low-salt detergent treatment: nuclear envelopes were treated with 50 m*M* octylglucoside, 10% sucrose, 10 m*M* triethanolamine, and 0.1 m*M* $MgCl_2$ (*p*H 7.5). High-salt detergent treatment: nuclear envelopes were treated with 50 m*M* octylglucoside, 0.5*M* NaCl, and 10 m*M* triethanolamine (*p*H 7.5). Samples were incubated for 30 min and centrifuged for 10 min at 18,000 rpm to yield a supernatant (extract) and a precipitate (treated nuclear envelope).

Fraction used for immunoabsorption	Absorption of inhibitory activity of anti-DDDED* (%)
(Anti-DDDED alone)	0
Cytoplasmic fraction†	8
Nuclei	31
DNase I extract†	9
Nuclear envelope	25
Low-salt detergent–treated nuclear envelope	41
High-salt detergent–treated nuclear envelope	23
High-salt detergent extract of nuclear envelope†	60

*After incubating 25 μg of anti-DDDED with 50 μg of proteins from each fraction (4°C, 12 hours), inhibitory activity of the antibody on the nuclear transport of nucleoplasmin was assayed by microinjection as described in Fig. 3. For each point 200 to 300 cells were counted. †Soluble fractions were conjugated with cyanogen bromide (CNBr)–activated Sepharose 4B after dialysis against 0.5*M* NaCl, 25 m*M* octylglucoside, and 0.1*M* $NaHCO_3$.

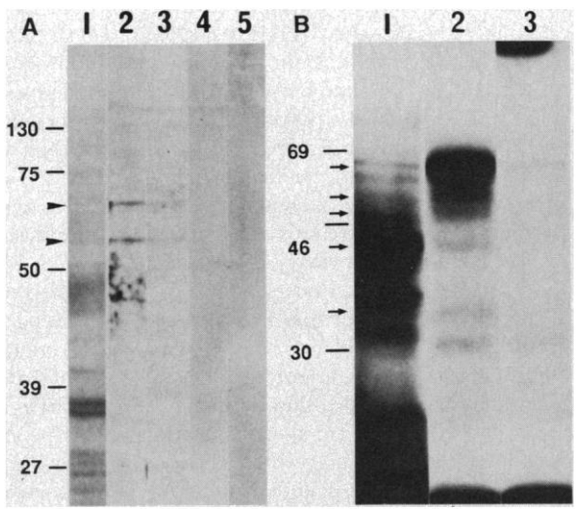

Fig. 4. (**A**) Immunoblot of cellular proteins identified with anti-DDDED and anti-EEEDE. Preparations subjected to SDS-PAGE (10% separating gel) are described in Table 2. The rat liver nuclear fraction (lanes 1 to 5) was subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose sheets. We used Coomassie brilliant blue staining of the gel (lane 1) and immunoblot staining (lanes 2 to 5). The nitrocellulose sheets were incubated in phosphate-buffered saline at 70°C for 1 hour immediately before incubation with the first antibodies (18.2 μg/ml): anti-DDDED (lanes 2 and 3), anti-EEEDE (lane 4), and nonimmune rabbit IgG (lane 5). Lane 3 was incubated with the antibody in the presence of DDDED-peptides (2 mg/ml). The sheets were washed and incubated with alkaline phosphatase–conjugated goat antibody to rabbit IgG. Arrowheads denote the positions of 69- and 59-kD proteins. The molecular sizes (in kilodaltons) of protein standards are indicated on the left. (**B**) ^{125}I-labeled high-salt detergent extract (lane 1) was incubated at 4°C overnight with 10 μg of anti-DDDED (lane 2) and normal rabbit IgG (lane 3) and was precipitated with Protein A–Sepharose CL-4B [incubation buffer: 0.5*M* NaCl, 1% Nonidet P-40, 15 m*M* octylglucoside, 5 mg/ml ovalbumin, and 10 m*M* triethanolamine (*p*H 7.5)]. Precipitates were subjected to SDS-PAGE (11% separating gel) followed by autoradiography. Arrows indicate positions of 65-, 54-, 50-, 43-, and 34-kD proteins. The molecular sizes (in kilodaltons) of protein standards are indicated on the left.

was used for the fibronectin receptor (*14*). Our approach with electrostatically complementary peptides is different from either of these techniques.

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8. The amount of specific antibody present in the preparations was determined by quantitative immunoprecipitation with peptide-conjugated ^{125}I-labeled ovalbumin (the average peptide-to-ovalbumin molar ratio was 5:1 as determined by amino acid analysis). The amounts of specific antibodies to the peptides DDDED and EEEDE (anti-DDDED and anti-EEEDE) in the preparations were 22.4% and 64.8%, respectively. The counts (cpm) of ^{125}I-labeled EEEDE-ovalbumin immunoprecipitated by anti-DDDED were about 65% of those by anti-EEEDE. On the other hand, the counts of ^{125}I-labeled DDDED-ovalbumin immunoprecipitated by anti-EEEDE were about 3.5% of those by anti-DDDED. These values were obtained by subtracting the value of control normal rabbit IgG.
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10. Cellular incorporation of [^{3}H]uridine, as assayed by autoradiography, indicated that the injection of anti-DDDED into the cytoplasm did not block the synthesis or release of RNA. Passive diffusion of the monomeric form of phycoerythrin (30 kD) was also unaffected by antibody.
11. Nucleoplasmin or SV40 T–BSA was injected at various times after injection of anti-DDDED (7 mg/ml) into the cytoplasm of cells into which anti-DDDED was injected, and 30 min later, the cells were treated as described in Fig. 3. Complete inhibition of nuclear transport was observed for 3 hours and then the inhibitory activity of the antibody in the cells gradually decreased. These results were supported by the fact that when anti-DDDED was injected into the cytoplasm it remained in the cells for about 3 hours and then gradually disappeared, as shown by injecting the antibody and then examining the cells by indirect immunofluorescence microscopy with goat anti-rabbit IgG.
12. From this and other gels, the sizes were calculated to be 69 and 59 kD. Although the DDED sequence is found in lamins A and C (amino acid positions 552 to 555) [F. D. McKeon, M. W. Kirshner, D. Caput, *Nature* **319**, 463 (1986)], these two proteins had molecular sizes slightly different from lamins A and C and anti-DDDED did not react with lamins A and C by immunoblotting assay under our conditions. Thus, these two proteins are distinct from lamins A and C.
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15. We used three karyophilic proteins such as nucleoplasmin and BSA conjugated with a synthetic peptide containing the nuclear localization signal sequence for SV40 large T antigen (SV40 T–BSA) or polyoma virus large T antigen (polyoma T–BSA). Polyoma T–BSA as well as nucleoplasmin and SV40 T–BSA entered the nucleus effectively when injected into the cell cytoplasm.
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Expression of a Calcium-Mobilizing Parathyroid Hormone–Like Peptide in Lactating Mammary Tissue

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A survey of rat tissues by RNA analysis, aimed at uncovering the physiological function of the parathyroid hormone–like peptide (PTH-LP) associated with hypercalcemia of malignancy, revealed the presence of a 1.5-kilobase messenger RNA encoding this peptide in lactating mammary glands. PTH-LP messenger RNA is expressed in mammary tissue only during lactation; it appears and disappears rapidly (2 to 4 hours) as a function of the sucking stimulus. The identity of this messenger RNA was confirmed by cloning the rat PTH-LP complementary DNA, which predicts a peptide with strong similarity to the human homolog. Moreover, extracts from lactating mammary tissue stimulated parathyroid hormone–dependent adenylate cyclase. These findings suggest that PTH-LP plays a physiological role in lactation, possibly as a hormone for the mobilization or transfer (or both) of calcium to the milk.

A PARATHYROID HORMONE–LIKE PEPTIDE produced by many tumors from patients with humoral hypercalcemia of malignancy (HHM) (*1*) was recently characterized. Amino-terminal sequence analysis (*2–4*) and subsequent cDNA cloning (*5–7*) revealed a peptide of approximately 16 kD, with amino-terminal homology to PTH. A synthetic peptide containing amino acids 1 to 34 of the human PTH-LP produces the same effects on renal and osteoblast membranes in vitro and on calcium and inorganic phosphate fluxes in vivo (*8, 9*) as the peptide 1–34 of human PTH, thus explaining the resemblance of the HHM syndrome to hyperparathyroidism. In addition to tumors, the only other cells reported to produce PTH-LP are human keratinocytes in culture (*10*); however, the physiological function of the peptide is unknown.

To investigate further the expression and function of PTH-LP, we screened RNAs from adult and embryonic rat tissues for hybridization to a human PTH-LP cDNA (Fig. 1). Among all the normal tissues examined, relatively high levels of a 1.5-kb PTH-LP mRNA were detected only in RNA from lactating mammary tissue. However, similar levels of this mRNA were seen in RNA from rat Leydig cell tumor, which secretes a PTH-LP and causes hypercalcemia in rats (*11*). Skin and spleen gave weak hybridization signals; however, the actual production and possible function of the peptide in these tissues remain to be elucidated. No PTH-LP mRNA was detected in RNA from nonlactating mammary gland, in RNAs from brain, testis, intestine, kidney, liver, lung, parathyroid-thyroid gland, rat osteosarcoma (ROS 17/2.8), and heart of nonlactating animals, or in RNAs from these tissues in lactating animals. Moreover, this mRNA was not detected in rat embryos, in placenta, or in kidney and skin of newborn rats. Thus, we have no evidence to support a role for this peptide in development, although we cannot exclude the possibility that small amounts of this mRNA may be expressed in specific embryonic tissues, since whole embryo RNA was used for this analysis.

Further study showed that the expression of PTH-LP mRNA in mammary tissue was

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