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Human placental syncytiotrophoblastic M_r 75,000 polypeptide defined by antibodies to a synthetic peptide based on a cloned human endogenous retroviral DNA sequence

(chloriocarcinoma/*gag* gene/immunoblotting/immunohistochemistry)

JUKKA SUNI*†, ALE NÄRVÄNEN*, TORSTEN WAHLSTRÖM‡, MARTINA AHO*, RAIMO PAKKANEN*, ANTTI VAHERI*, TERRY COPELAND§, MAURICE COHEN§, AND STEPHEN OROSZLAN§

Departments of *Virology and ‡Pathology, University of Helsinki, 00290 Helsinki, Finland; †Virus Laboratory, Aurora Hospital, 00250 Helsinki, Finland; and §Biological Carcinogenesis Program, National Cancer Institute, Frederick, MD 21701

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ABSTRACT **Antibodies to a synthetic undecapeptide (NH_2-Cys-Glu-Asn-Pro-Ser-Gln-Phe-Tyr-Glu-Arg-Leu-COOH), the sequence (except cysteine) of which was deduced from a previously reported cloned human retroviral *gag*-gene-related DNA sequence *erv*-1, were raised in rabbits. In immunohistochemical staining these antibodies reacted with normal human first-trimester placentas and with blighted ova and benign and malignant trophoblastic tumors (hydatidiform and destructive moles, choriocarcinomas) but not with any other normal embryonic or adult tissues tested. In all tissues the reactivity was mainly confined to cells with trophoblastic morphology. In immunoblotting the antibody detected an M_r 75,000 polypeptide in syncytiotrophoblasts isolated from first-trimester placentas and in three different lines of cultured choriocarcinoma cells. The undecapeptide blocked the reactivity of the antibody.**

Type C endogenous retroviruses are widespread in birds and mammals. They have also been recovered from several primates, including the Old World monkeys, baboon, stump-tail macaque, rhesus, and colobus, and the New World owl monkeys. The major source for isolation of endogenous retrovirus from baboons is the placenta (see ref. 1 for review). In humans, particles resembling type C or type D retroviruses have been seen budding from the syncytiotrophoblast layer of placentas (2, 3) and from cultured teratocarcinoma cells (4). In addition, small retrovirus-like particles have been seen in some human oocytes (5). Human endogenous retroviruses have not been isolated in an infectious form and producer cell lines have not been established.

Evidence has been presented that human DNA contains retrovirus-related nucleotide sequences. Nucleic acid reassociation studies first revealed that humans and other primates contain multiple baboon endogenous virus (BaEV)-related sequences in their chromosomal DNA (6). A defective, endogenous provirus was recently isolated from a human recombinant DNA library by using as probe an endogenous chimpanzee retroviral *pol* fragment highly related to that of BaEV (7). This genome, termed HC-20 (or endogenous retrovirus-1; *erv*-1), has been assigned to human chromosome 18 (8). The *erv*-1 provirus contains *gag* and *pol* genes, which are significantly related to those of both Moloney murine leukemia virus (Mo-MuLV) and BaEV, *env*-related sequences of the expected length, and a 3′ long terminal repeat (LTR), but no 5′ LTR (ref. 7; unpublished). Other reports have described the isolation of retroviral clones from a human library that are related to MuLV (9, 10), BaEV LTR (11), or mouse mammary tumor virus (MMTV) (12).

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In a previous study (13) using the feline endogenous retrovirus RD114 and antibodies to its major structural protein p30 as reagents, we demonstrated p30-related antigen in human placental syncytiotrophoblasts. Further, a study (13) of 1540 human cord blood sera revealed the presence of RD114 p30-reactive antibodies in 118 (7.7%) sera. Antibodies to synthetic peptides, which were based on MuLV p30 amino acid sequences or sequences of *env* genes, have been found to be reactive with the native structural proteins of murine retroviruses (14). In the present study we used antibodies to an undecapeptide, which has partial sequence homology to MuLV and BaEV p30 proteins. The undecapeptide sequence was inferred from the nucleotide sequence of the human proviral locus, *erv*-1 (7). This antibody detects a polypeptide antigen in syncytiotrophoblastic cells of human placentas both *in vivo* and in culture.

MATERIALS AND METHODS

Synthetic Peptides and their Antibodies. An undecapeptide (NH_2-Cys-Glu-Asn-Pro-Ser-Gln-Phe-Tyr-Glu-Arg-Leu-COOH) was synthesized by using the solid-phase method (15). Residues 2–11 of the peptide sequence were deduced from the nucleotide *gag* sequence of the human proviral locus, *erv*-1 (7). The sequence of this synthetic peptide (Sp23) fits 6 of 10 with the BaEV p30 and 7 of 10 with the Mo-MuLV p30 sequence (Table 1). An unrelated synthetic undecapeptide (Sp49) was prepared by using the same method. In addition, an unrelated synthetic dodecapeptide (LSp2) (Universal Biochemicals, Cambridge, U.K.) was used.

The undecapeptides were conjugated to keyhole limpet hemocyanin by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as the coupling reagent (20) and were used to immunize rabbits from which preimmune sera had been collected by using three subcutaneous injections of 100 μg of the peptide in Freund's incomplete adjuvant at 2-wk intervals. The rabbits were bled 10 days later.

Cultured and Isolated Trophoblastic Cells. Human choriocarcinoma JAr (gift from R. A. Pattillo; ref. 21), BeWo (ATCC CCL98, American Type Culture Collection), and JEG-3 cells (ATCC HTB 36), all known to secrete human chorionic gonadotropin, were grown as monolayer cultures in Eagle's minimal essential medium (ME medium) supplemented with 10% fetal bovine serum. Under these conditions, 1–2% of the cells had a syncytiotrophoblastic morphology; the rest of the cells were cytotrophoblast-like. Adult

Abbreviations: AMV, avian myeloblastosis virus; BaEV, baboon endogenous virus; HTLV, human T-cell leukemia virus; Mo, Moloney; MMTV, mouse mammary tumor virus; MuLV, murine leukemia virus; SSAV, simian sarcoma-associated virus; LTR, long terminal repeat.

Table 1. Sequence homology of Sp23 undecapeptide with defined retrovirus p24–p30 proteins

Peptide		Ref.
Sp23 (HC-20, human DNA)	Cys-*Glu-Asn-Pro-Ser-Gln-Phe-Tyr-Glu-Arg-Leu*	7
MuLV (murine)	-*Glu*-Ser-*Pro-Ser*-Ala-*Phe*-Leu-*Glu-Arg-Leu*	16
BaEv (baboon)	-*Glu*-Ser-*Pro*-Ala-Ala-*Phe*-Met-*Glu-Arg-Leu*	17
SSAV (woolly monkey)	-*Glu*-Pro-*Pro-Ser*-Val-*Phe*-Leu-*Glu-Arg-Leu*	18
HTLV (human)	-*Glu*-Pro-Tyr-His-Ala-*Phe*-Val-*Glu-Arg-Leu*	19

Homologies with Sp23 are italicized. In the original publication (7), leucine and tyrosine residues in the seventh positions were inadvertedly switched between the human and MuLV sequence. SSAV, simian sarcoma-associated virus; HTLV, human T-cell leukemia virus.

human skin fibroblasts of a strain established in our laboratory (22) were grown in the same medium.

Syncytiotrophoblastic cells were isolated from the first-trimester placentas received from elective abortions (based on socioeconomic grounds). The trophoblast isolation procedure described in detail by Saksela *et al.* (23) was briefly as follows. The placenta was washed with Dulbecco's phosphate-buffered saline supplemented with antibiotics followed by sectioning with scissors and treatment with a mixture of collagenase/hyaluronidase/soybean trypsin inhibitor. The separation of the loosened trophoblastic cells from placental matrix was done by successive filtration through iron mesh (1×1 mm) and cotton mesh (30 μm) in which the large syncytiotrophoblastic cells were entrapped. The cells were rinsed from the mesh with ME medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The isolated cells were sedimented with low-speed centrifugation and the pellet was frozen at −20°C until use.

Tissue Sections and Immunoperoxidase Staining. Immunoperoxidase staining of 38 normal placentas, the gestation time of which varied from 5 to 41 wk, 10 blighted ova, 10 hydatidiform moles, 10 destructive moles, and 10 choriocarcinomas was performed on 4-μm-thick tissue sections in which endogenous peroxidase activity had been destroyed by incubation at room temperature for 30 min in methanol containing 0.3% hydrogen peroxide. The antisera were diluted 1:100 in phosphate-buffered saline, and the avidin-biotin-peroxidase method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), as recommended by the manufacturer, was employed using 3-amino-9-carbazole as the chromogenic enzyme substrate. The cover glasses were mounted with Aquamount (Gurr, Hopkin & Williams, Chadwell Heath, England).

A large variety of other normal tissues, of both adult and fetal origin, including skin, brain, parotic gland, lung, heart, spleen, liver, muscle, adrenal gland, ovary, Fallopian tube, uterus, urinary bladder, prostate, and testis, was also studied.

Immunoblotting. For immunoblotting, the isolated syncytiotrophoblastic cells from placentas and cultured cells or purified retroviruses [BaEV, MuLV, SSAV, and avian myeloblastosis virus (AMV); the latter purchased from Life Sciences, St. Petersburg, FL] were treated as follows. The cells were directly dissolved in Laemmli sample buffer. The proteins were separated by $NaDodSO_4$/polyacrylamide gradient (5–16%) slab gel electrophoresis ($NaDodSO_4$/PAGE) according to Laemmli (24) with reducing conditions. After separation the proteins were transferred electrophoretically to a nitrocellulose sheet and immunoblotted according to the procedure by Towbin *et al.* (25), modified as described (26). For the immunological staining of the transferred proteins the specific and control antibodies were diluted 1:500 in TEN-Tx buffer (50 mM Tris·HCl, pH 7.0/5 mM NaCl/0.05% Triton X-100). The immunoreactive polypeptide bands were visualized by using the Vectastain ABC kit as described above. In some experiments immunological detection of bound antibodies was with ^{125}I-labeled protein A followed by autoradiography (25).

In control experiments, anti-Sp23 diluted 1:100 was mixed with disrupted (0.01% Triton X-100 and ultrasonic treatment) purified BaEV or AMV, 100 μg of protein per ml, incubated for 1 hr at room temperature in an end-over mixer, then incubated at +4°C overnight, and then used for immunoblotting at a final 1:500 dilution of anti-Sp23. In a second series of similar control experiments, anti-Sp23 diluted 1:500 was mixed with 2 or 0.2 μg of Sp23 or of an unrelated dodecapeptide (LSp2) per ml incubated as above and used for immunoblotting.

RESULTS

Immunoblotting of Cultured Choriocarcinoma Cells. Immunoblotting of JEG-3 choriocarcinoma cells with anti-Sp23 detected a major polypeptide at M_r 75,000 from proteins separated by $NaDodSO_4$/PAGE under reducing or nonreducing conditions and transferred onto a nitrocellulose sheet. In addition, a doublet of polypeptides at M_r 100,000–110,000 reacted with anti-Sp23. The relative intensity of the doublet to that of the M_r 75,000 polypeptide varied but was always lower (Fig. 1, lane 1). Similar results were obtained with proteins of JAr and BeWo choriocarcinoma cells but not with human fibroblasts or several other control cells. Under the same experimental conditions anti-Sp23 detected from proteins of isolated placental syncytiotrophoblastic cells a similar M_r 75,000 polypeptide (Fig. 1, lane 4). The double seen in immunoblots of choriocarcinoma cells was not obtained from

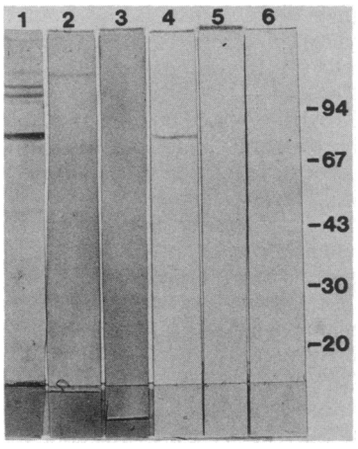

FIG. 1. Immunoblotting of cell extracts. The polypeptides were first electrophoresed in a $NaDodSO_4$/polyacrylamide gel and then transferred to a nitrocellulose sheet and stained with the antibodies by using the immunoperoxidase technique. Lane 1, JEG-3 choriocarcinoma cells, stained with the anti-Sp23 rabbit serum; lane 2, JEG-3 cells, stained with a control rabbit antiserum raised against an unrelated undecapeptide; lane 3, JEG-3 cells, stained with a preimmune rabbit serum (of anti-Sp23); lane 4, placental syncytiotrophoblasts, anti-Sp23; lane 5, placental syncytiotrophoblasts, control rabbit antiserum; lane 6, placental syncytiotrophoblasts, preimmune rabbit antiserum. Molecular weights of the marker proteins are indicated in $M_r \times 10^{-3}$ on the right.

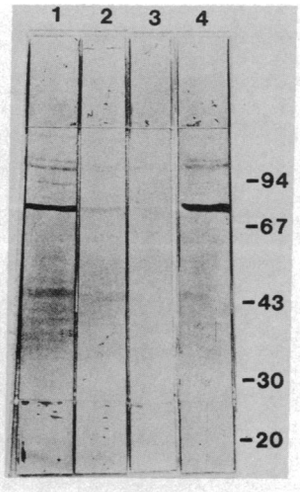

FIG. 2. Blocking of anti-Sp23 reactivity with the undecapeptide Sp23. Anti-Sp23 diluted 1:500 was mixed with 2 μg of Sp23 per ml (lane 2), with 200 ng of Sp23 per ml (lane 3), with 2 μg of an unrelated dodecapeptide per ml (lane 4), or with buffer control (lane 1) and subsequently used in immunoblotting of JEG-3 choriocarcinoma cells. Molecular weights of the marker proteins are indicated in $M_r \times 10^{-3}$ on the right.

the proteins of syncytiotrophoblasts. These findings were obtained regularly by using choriocarcinoma cells grown to different passage levels and by using syncytiotrophoblasts isolated from individual human first-trimester placentas. Sera from two anti-Sp23 rabbits gave similar results. A preimmune serum and a control antiserum raised against an unrelated undecapeptide gave constantly negative results. Anti-Sp23 pretreated with the synthetic peptide Sp23 did not react with the M_r 75,000 polypeptide; pretreatment with an unrelated synthetic dodecapeptide (Fig. 2), with disrupted BaEV or with disrupted AMV, had no effect on the reactivity of anti-Sp23.

Reactivity of Anti-Sp23 with Normal and Malignant Human Placental Tissues. Immunoperoxidase staining of tissue sections with anti-Sp23 gave a strong reaction for the syncytiotrophoblastic cells of all early (<15 wk) placentas examined but was negative for older placentas and for all other normal tissues studied (Table 2). The typical pattern of staining was scattered and localized predominantly to large multinuclear syncytiotrophoblasts (Fig. 3). In addition, occasional cells with cytotrophoblastic morphology were positive. The staining was, in both types of cells, diffuse and confined to the cytoplasm with no apparent enrichment to the cell surface. When in the staining procedure, anti-Sp23 was replaced with the preimmune rabbit serum, the antibody to an unrelated synthetic undecapeptide, or a buffer control, consistently negative results were obtained.

In benign (hydatidiform mole) and malignant placental tumors (destructive mole, choriocarcinoma) cells with trophoblastic morphology reacted with anti-Sp23 (Table 2, Figs. 4 and 5) but not with the controls.

Table 2. Immunoperoxidase staining of tissue sections with the anti-Sp23 serum

Tissue	No. of specimens tested	No. positive
Human placental syncytiotrophoblast		
5–15 wk	17	17
16–41 wk	21	0
Blighted ova	10	10
Hydatidiform mole	10	10
Destructive mole	10	10
Choriocarcinoma	10	10
Normal embryonal (16–24 wk)	*	0
Normal adult	*	0

*See *Materials and Methods*.

DISCUSSION

The search for retroviruses in human neoplastic disease and in human tissues has only recently been successful in the isolation of the HTLV (27, 28), which has been associated with certain T-cell leukemias (29). According to nucleic acid hybridization data, this virus is not endogenous in normal human DNA. Thus far, no isolations of a human endogenous retrovirus have been made.

Evidence has been provided that human DNA contains sequences related to the replication-competent type C retroviruses isolated from other mammals (see Introduction). On the other hand, electron microscopical and indirect immunological evidence of retroviral gene products in human placentas has been reported (2, 3, 13). In the present study we used antibodies to a synthetic peptide, based on one of the cloned endogenous human retrovirus-related cloned DNA sequences (7), to search for a human endogenous

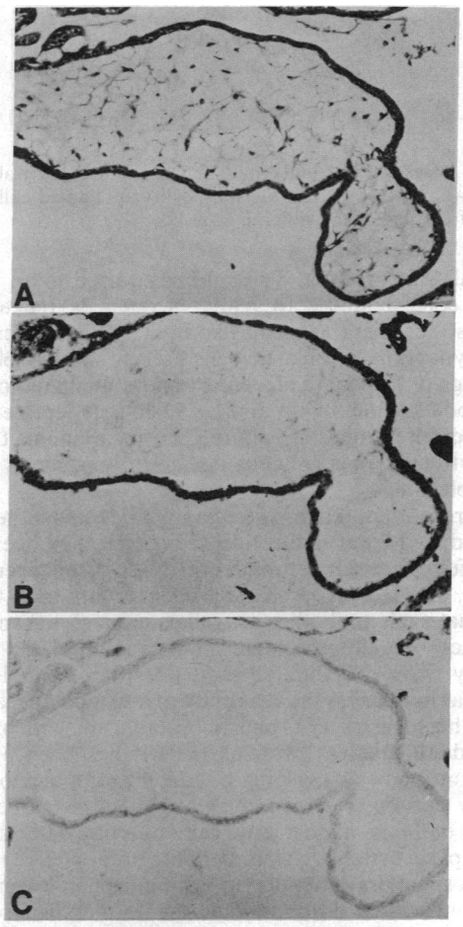

FIG. 3. Normal placenta (gestation 7 wk). (*A*) Hematoxylin/eosin staining. (*B*) Immunoperoxidase staining with the anti-Sp23 rabbit serum. (*C*) Immunoperoxidase staining, with the control rabbit antiserum. Note the strong positive staining of syncytiotrophoblasts in *B*. (×200.)

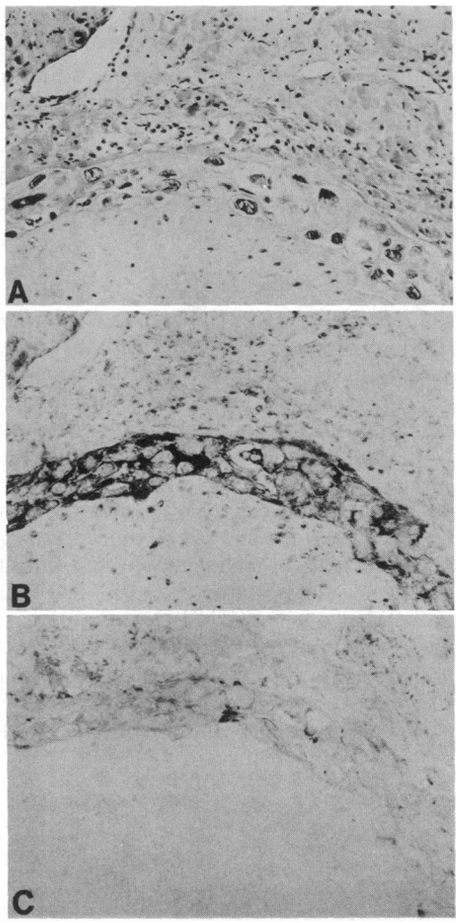

FIG. 4. Destructive mole invading the muscular wall of the uterus. *A–C*, as in Fig. 3. Note the positively stained cells on the surface of the malignant villus. (×200.)

retroviral gene product. The antibody raised to an undecapeptide corresponding to $p30^{gag}$ sequences reacted with antigen(s) confined, according to immunohistological staining, to syncytiotrophoblastic cells present in early placentas and benign or malignant placental tumors. In immunoblotting the antibody reacted with an M_r 75,000 polypeptide present in three different lines of cultured choriocarcinoma cells and in syncytiotrophoblastic cells isolated from human first-trimester placentas.

The present approach, use of antibodies to synthetic peptides deduced from nucleotide sequences, has been highly successful, for example, in identification of oncogene products (30, 31). However, the approach suffers from the limitation that such antibodies as well as monoclonal antibodies may react with antigenic determinants in unrelated proteins that may share only limited sequences. An example of this molecular mimicry is the reactivity of a monoclonal antibody both with a measles virus phosphoprotein and with one of the intermediate filament proteins (32). It has been estimated that an antibody recognizing a sequence of 4 amino acids is virtually certain to encounter an identical sequence in another protein in almost any cell, but the odds against a heptapeptide match are already of the order of 250:1 (33, 34). In this respect the antibodies to synthetic peptides and monoclonal antibodies differ in that the former can recognize several epitopes within the peptide sequence—in the present case, 11 amino acids long.

The human *erv*-1 retroviral locus from which the present sequence is derived contains *gag*, *pol*, and *env* genes and a 3′ LTR, as shown by DNA sequencing and alignment with

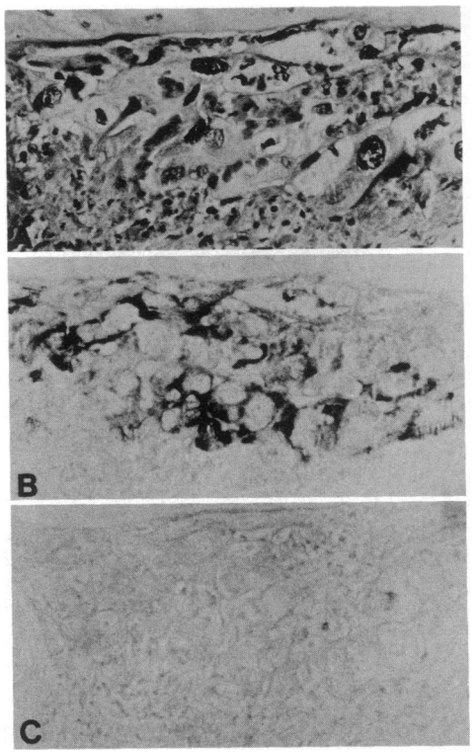

FIG. 5. Poorly differentiated choriocarcinoma invading the muscular wall of the uterus. *A–C*, as in Fig. 3. Note the positive stain in the large atypical syncytiotrophoblastic cells in *B*. (×200.)

those regions of Mo-MuLV or BaEV (ref. 7; unpublished results). The Sp23 undecapeptide sequence is within the *gag* p30 gene region of *erv*-1 on the basis of the extensive homology (Table 1) with the p24–p30 proteins of known retroviruses. With the information available we cannot conclude that the M_r 75,000 protein is indeed a product of the *erv*-1 p30 gene. The human genome contains multiple retrovirus-related DNA sequences (7–12) and the Sp23 sequence may be shared by several of them. A second complication is that *erv*-1 is a defective proviral sequence because it does not contain a 5′ LTR (7). Thus, for *erv*-1 to be expressed as mRNA and protein, it may have to be under control of a cellular promoter. This phenomenon has been noted for the avian *ev*-6 locus, the endogenous retroviral locus of chickens that lacks a 5′ LTR and *gag* sequence but possesses a normal *env* gene that can be expressed as mRN (35, 36). In chickens homozygous for *ev*-6, expression of the *ev*-6 *env* gene product is heightened in lymphoblasts relative to resting and immature lymphocytes (37). Thus, regulation of endogenous retroviral gene expression by cellular promoters may not be an uncommon method for the cell to utilize defective proviruses.

The observation that two rabbits immunized with Sp23 produced antibodies reacting with M_r 75,000 and that the undecapeptide blocked the reactivity confirms that this or a closely related sequence is within the M_r 75,000 polypeptide. However, the evidence that this polypeptide is retrovirus-related remains indirect: (*i*) although anti-Sp23 did not detect MuLV and BaEV p30s, it reacted with MuLV p68, and (*ii*) anti-Sp23 gave in immunohistochemistry a staining pattern similar to that previously obtained with antibodies to RD114 p30 (13). If the M_r 75,000 polypeptide is indeed a product of human endogenous retroviral sequences it could represent a gag–precursor or a gag–pol fusion polypeptide. Studies on

the transcription products of human *erv*-1 sequences and of related DNA clones (7) and biochemical characterization of the M_r 75,000 polypeptide are necessary.

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