Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 6501–6505, October 1984
Medical Sciences

Detection and immunochemical characterization of a primate type C retrovirus-related p30 protein in normal human placentas

(retroviral core protein/two-dimensional electrophoresis/immunoblot analysis)

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Communicated by Lloyd J. Old, May 29, 1984

ABSTRACT **We find that 12 of 14 specimens of normal human term placentas analyzed by one- or two-dimensional electrophoresis and immunoblotting contain a protein or polypeptide of ≈30,000 daltons that is antigenically cross-reactive with p30 core protein of the simian sarcoma-associated virus/gibbon ape leukemia virus primate retrovirus group and is physicochemically similar to reference murine and primate type C retrovirus p30s. This finding may lead to an understanding of endogenous type C retrovirus gene expression in humans.**

Endogenous type C retroviruses are present in many mammalian species, including subhuman primates (1–4) among which the baboon (1, 5) has the closest evolutionary relation to man. These endogenous viruses exist as stable DNA proviruses that are integrated into the chromosomal DNA and are transmitted along with cellular genes in a Mendelian manner.

There is as yet no established endogenous human retrovirus. Electron microscopic studies reveal the presence of budding type C retrovirus-like particles in virtually every human normal term placenta examined (6–9). This finding may have a parallel to the presence and location of endogenous retrovirus particles in the placentas of sub-human primate and other mammalian species (10–12). Nevertheless, in the absence of an established human placental retrovirus isolate, the biological nature of the retrovirus-like particles in human placentas remains unknown. Recent investigations in molecular biology (13–16) indicate that normal human chromosomal DNA contains nucleotide sequences that are homologous to the genes of retroviruses previously isolated from mice and primates and have the structure of an integrated, but incomplete, type C retrovirus (14).

A distinguishing characteristic of the proteins associated with primate and other mammalian type C retroviruses is the presence of a major structural core protein of 27,000–30,000 daltons (termed p30) that has group-specific (17) and cross-reacting interspecies-specific (18) determinants (epitopes) on the same molecule (19). The NH_2-terminal amino acid sequences of mammalian type C viral p30s are highly conserved (20). The p30 proteins of mammalian type B, type C, and type D retroviruses share cross-reacting interspecies determinants in keeping with an evolutionary relationship of these three major retrovirus genera (21).

A protein antigen that cross-reacts with p30 of the simian sarcoma-associated virus (SSAV)/gibbon ape leukemia virus (GaLV) primate type C retrovirus group was recently detected in every normal human term placenta examined by immunofluorescence microscopy (22). This antigen was located in the syncytiotrophoblast, which is the site where budding type C virus-like particles are seen in the human placenta by electron microscopic study.

We now report that normal human term placentas contain a protein antigen of ≈30,000 daltons (under dissociating/reducing conditions) that is physicochemically similar to reference murine and primate type C retrovirus p30s and antigenically cross-reactive with p30 of the SSAV/GaLV primate retrovirus group. A preliminary report of some aspects of this work has been presented elsewhere (23).

MATERIALS AND METHODS

Human Tissues. Fourteen fresh specimens of normal human placentas, including five from a related study (22), were obtained immediately after normal term delivery and promptly chilled at 4°C. Mainly trophoblast tissue was grossly dissected from the placental surface facing the decidua, quickly frozen, and stored at −70°C. Control normal adult human tissues were fresh autopsy specimens.

Viruses. Density gradient–purified virus concentrates were obtained from the Division of Cancer Cause and Prevention, National Cancer Institute. Simian sarcoma virus/SSAV, GaLV, and baboon endogenous virus (BEV) were grown in human lymphoid NC-37 cell line. Feline endogenous virus RD-114 was harvested from human rhabdomyosarcoma-derived RD cell line. N-tropic murine leukemia virus (N-tropic MuLV) was grown in wild mouse embryo SC-1 cell line. Viruses were disrupted by Tween 80/ether treatment (25).

Antiserums. Goat antiserums produced against retroviral p30 proteins purified to apparent homogeneity were obtained from the Division of Cancer Cause and Prevention, National Cancer Institute: anti-SSAV p28 (two separate serums: 2S-696 and 2S-673), anti-RD-114 p28 (2S-781), anti-BEV p28 (5S-463), anti-GaLV p29 (2S-279), anti-feline leukemia virus (FeLV) p27 (F26601), anti-Rauscher murine leukemia virus (Ra-MuLV) p30 (5S-123), and anti-avian myeloblastosis virus p27 (2S-753).

Sucrose Density Gradient Fractionation. Frozen human tissue (100 g) was homogenized in a low-salt extraction buffer (0.005 M KCl/0.025 M Tris·HCl, pH 7.5/0.001 M EDTA) containing Trasylol (Mobay, New York) at 100 kallikrein-inactivation units/ml. Subcellular ("microsomal") fractions were separated on a linear gradient of 20–60% sucrose (wt/vol) by equilibrium ultracentrifugation for 14 hr at 110,000 × *g* and 4°C as described (25).

Antibody Affinity Chromatography. The IgG fraction of goat anti-SSAV p28 serum was covalently bound to cyanogen bromide-activated aminohexyl-Sepharose 4B (Pharmacia) as described (26). Sucrose gradient fractions of human placenta in sample buffer [0.15 M NaCl/0.01 M phos-

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Abbreviations: SSAV, simian sarcoma-associated virus; GaLV, gibbon ape leukemia virus; MuLV, murine leukemia virus; Ra-MuLV, Raucher MuLV; FeLV, feline leukemia virus; BEV, baboon endogenous virus.

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phate buffer, pH 7.4 (P_i/NaCl)/0.01% sodium azide/0.1 mM phenylmethylsulfonyl fluoride/2 mM EDTA/0.1% Tween 20/50 kallikrein-inactivation units of Trasylol per ml) were applied to the columns in an upward direction. Unbound proteins (>99% of sample) were washed through the columns with P_i/NaCl and with 0.5 M NaCl. Bound proteins (approximately microgram quantities) were eluted from the columns in a downward direction by 0.1 M glycine·HCl, pH 2.8/0.1 M NaCl, neutralized by 1.0 M K_2HPO_4, dialyzed against P_i/NaCl, and concentrated by vacuum. Samples also were applied to columns of normal goat IgG covalently coupled to Sepharose 4B and eluted under the same conditions.

CM-Cellulose Chromatography. Starting samples (30 g) of human placental tissue were homogenized and extracted in buffer (5 mM Tris·HCl, pH 9.2/1 mM EDTA/400 mM KCl) containing 0.1% Triton X-100 as described (27) but also containing 100 kallikrein-inactivation units of Trasylol per ml and 0.2 mM phenylmethylsulfonyl fluoride. The placental extract was dialyzed against 0.15 M citrate phosphate buffer (pH 4.7). CM-cellulose 52 (Whatman) was equilibrated in the same buffer, and the placental extract was applied to the column. The bound proteins were eluted from the column by applying a pH gradient with equal volumes of 0.015 M citrate phosphate buffer and 0.02 M Na_2HPO_4 as described (28).

Two-Dimensional Gel Electrophoresis. Proteins were separated by the method of O'Farrell (29). In brief, samples were incubated in lysis buffer and applied to 5% polyacrylamide tube gels containing 2% (wt/vol) Bio-Lyte (Bio-Rad). Proteins were focused in the first dimension with 0.02 M NaOH as catholyte and 0.01 M H_3PO_4 as anolyte. The gels were applied in the second dimension to a 5–17.5% gradient $NaDodSO_4$/polyacrylamide gel using the discontinuous system of Laemmli (30). Molecular weight markers were placed at the end of the slab gel. x and y coordinates were measured from cathodal reference points (first and second dimension).

Electrophoretic Transfer and Immunoblotting. Placental or reference virus samples were solubilized in an equal volume of 2% $NaDodSO_4$/8 M urea/0.16 M dithiothreitol and resolved on a 5–17.5% gradient $NaDodSO_4$/polyacrylamide gel as described above. Proteins were electrophoretically transferred from gel to nitrocellulose paper (Schleicher and Schuell) (31, 32) and were detected as antigens by the immunoperoxidase method (32) using 3,3′-diaminobenzidine hydrochloride as enzyme substrate. Goat anti-retroviral p30 serums (and normal serum and buffer controls) were used as the first antibody, and rabbit anti-goat IgG avidin–biotin–peroxidase complex (33) (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) was used as the second antibody as described by the manufacturer. Blots were presoaked for 60 min at 37°C in buffer containing 3% bovine serum albumin and 5% normal rabbit serum. Incubation with the first antibody at 1:100–1:400 dilution in 1% normal rabbit serum was for 1–2 hr at 37°C on a rocking platform.

Enzymoimmunoassays. The antibody dilution titers of anti-retrovirus p30 serums were determined by indirect enzymoimmunoassay as described (34). Competition enzymoimmunoassays were developed as described (35) for SSAV/GaLV p28 by using GaLV target and goat IgG anti-SSAV p28 and for BEV/RD-114 p28 by using BEV target and goat IgG anti-RD-114 p28.

RESULTS

Characterization of Anti-Retrovirus p30 Antiserums. The antibody dilution titers (log_{10}) of two separate but high-titered and broadly cross-reactive anti-SSAV p28 serums were 6.2 against SSAV p28 and against GaLV p28, 4.5 against Ra-MuLV p30, 4.3 against FeLV p27, and 4.8 against RD-114 p28. The titer of anti-RD-114 p28 serum was 4.8 against RD-114 p28 and against BEV p28, 4.0 against Ra-MuLV p30 and against FeLV p27, and 4.2 against SSAV p28. The titers of goat anti-Ra-MuLV p30 and anti-FeLV p27 serums were similar to those described (34).

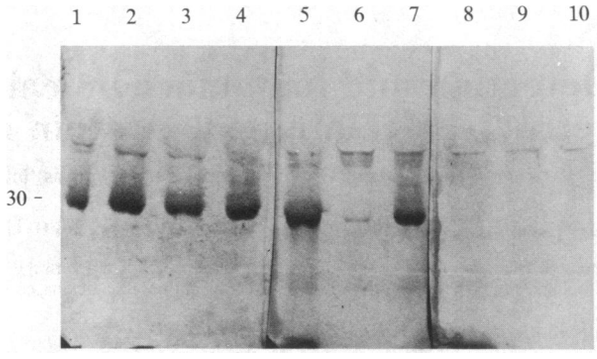

FIG. 1. Immunoblot of viral proteins developed with goat anti-SSAV p28 serum (lanes 1–4), anti-RD-114 p28 serum (lanes 5–7), and normal goat serum (lanes 8–10) against 50 ng of SSAV (lanes 1, 6, and 8), 50 ng of GaLV (lane 2), 50 ng of RD-114 (lanes 3, 5, and 9), and 200 ng of MuLV (lanes 4, 7, and 10). Molecular size marker is 30 kilodaltons. The dye marker is at the bottom of the gel (lanes 5 and 8).

The reactivities of goat anti-SSAV p28 and anti-RD-114 p28 serums were determined by immunoblot analysis. A 1:400 dilution of anti-SSAV p28 serum reacted strongly with estimated 5-ng amounts of SSAV p28, GaLV p28, and RD-114 p28 and 20 ng of MuLV p30 (Fig. 1, lanes 1–4). The antigenic specificity of the reaction was confirmed by removal of antibody activity by absorption with disrupted SSAV (100 μg/ml of antiserum). A 1:200 dilution of anti-RD-114 p28 serum (Fig. 1, lanes 5–7) reacted strongly with 5 ng of RD-114 p28 and 20 ng of MuLV p30 and weakly with 5 ng of SSAV p28. A 1:200 dilution of normal goat serum (Fig. 1, lanes 8–10) did not react with viral p30s. Nonspecific reactivity in the 60,000-dalton region of the blot was a feature common to both anti-p30 and normal control serums (Fig. 1).

Characterization of Anti-SSAV p28-Reactive Human Placental p30 Protein. Immunoblots of proteins separated by one-dimensional electrophoresis from 100- to 200-μl gradient fractions (1–10 mg of total protein) of human placentas indicated the presence of an anti-SSAV p28-reactive placental p30 protein (or polypeptide) with an average molecular size of 30,000 daltons and an overall range of 27,000–33,000 daltons. This placental p30 was detected by immunoperoxidase reaction with a 1:400 dilution of anti-SSAV p28 serum as a single narrow protein band (Fig. 2, lanes 5 and 6) located at a migration distance close to that of reference MuLV p30 (Fig. 2, lane 1). The placental p30 was detected by two separate goat anti-SSAV p28 serums but not by goat anti-RD-114 p28, anti-BEV p28, and anti-FeLV p27 serums (not shown). The placental p30 was not detected with control normal goat serum (Fig. 3, lane 5) nor with goat anti-avian myeloblastosis virus p27 serum (not shown). An anti-SSAV p28-reactive protein similar to that of human placentas was not detected by immunoblot analysis of gradient fractions of normal adult human spleen, kidney, or liver prepared and analyzed under the same conditions (not shown).

The antigenic specificity of the anti-SSAV p28 serum reaction with human placental p30 and with reference MuLV p30 in the same immunoblot was confirmed by absorbing a working dilution of anti-SSAV p28 serum with disrupted SSAV (100 μg/ml of antiserum), which removed the antibody activity against both placental p30 and MuLV p30. In contrast, the antibody activity against both p30s was retained after absorption with the same amount of negative control

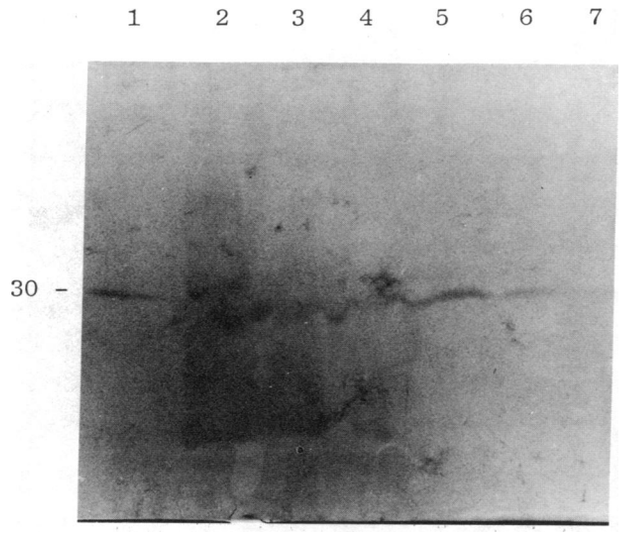

FIG. 2. Immunoblot of reference MuLV (lane 1) and sucrose density gradient fractions (lanes 2–7) of human placental proteins developed with anti-SSAV p28 serum. Sucrose densities were 1.11, 1.13, 1.14, 1.16, 1.18, and 1.19 g/ml for samples in lanes 2–7, respectively.

cells (sonicate of uninfected human lymphoid NC-37 cells) in which SSAV was grown.

The anti-SSAV p28-reactive placental p30 was detected by immunoblot analysis of density gradient (1.14–1.18 g/ml) fractions of 12 of 14 separate anatomical specimens of human placentas.

Antibody Affinity Purification of Human Placental p30 Protein. Placental proteins were purified from a pool of immunoblot-positive density gradient (1.15–1.16 g/ml) fractions of a human placenta by anti-SSAV p28 affinity chromatography. The affinity-purified placental proteins reacted in immunoblots with anti-SSAV p28 serum as a single protein band of ≈32,000 daltons (Fig. 4, lane 2) and did not react with normal goat serum (Fig. 4, lane 4). A similar anti-SSAV p28-reactive p30 protein was also subsequently purified from homogenates of this same placenta by CM-cellulose chromatography, as described below (see Fig. 6).

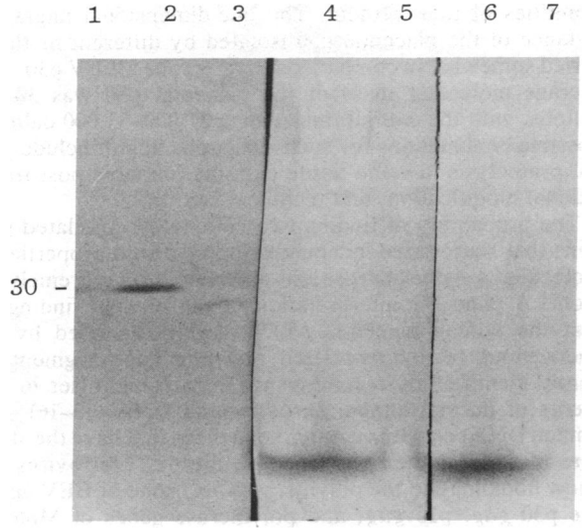

FIG. 3. Immunoblot of sucrose density (1.16–1.18 g/ml) gradient fraction of human placental proteins developed with anti-SSAV p28 serum (lane 3), normal goat serum (lane 5), and buffer control (lane 7) compared to reference MuLV (lane 1, 120 ng; lanes 2, 4, and 6, 600 ng).

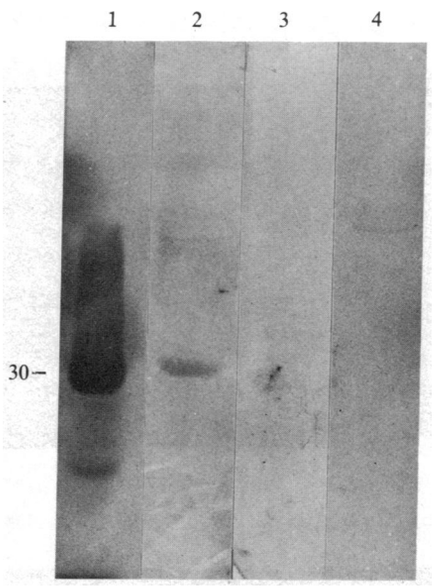

FIG. 4. Immunoblot of affinity-purified human placental proteins developed with anti-SSAV p28 serum (lane 2) and normal goat serum (lane 4) compared to reference MuLV (lanes 1 and 3, respectively).

An anti-SSAV p28-reactive placental p30 was also purified by affinity chromatography from individual density gradient (1.16 g/ml, 1.18 g/ml) fractions of another human placenta, whereas eluate from a Sepharose-normal goat IgG column did not contain an anti-SSAV p28-reactive placental protein. The specificity of the immunoblotting reaction given by affinity-purified placental p30 was shown by removal of anti-SSAV p28 activity against both placental and MuLV p30s by absorption with disrupted SSAV (100 μg/ml of antiserum) but not by absorption with the same amount of negative control human NC-37 cells. This affinity-purified placental protein also was subsequently examined by two-dimensional electrophoresis and immunoblot analysis.

Two-Dimensional Analysis of Proteins. Two-dimensional separation of the placental protein isolated by affinity chromatography and developed by immunoblotting with the anti-SSAV p28 serum revealed three dots with coordinates of x = 4.7, 5.3, and 5.8 and y = 4.2, 4.2, and 4.2 cm (Fig. 5*A*). The coordinates for MuLV p30, run under identical conditions in both dimensions, were x = 5.3 and 5.7 cm and y = 4.2 and 4.2 cm (Fig. 5*B*). SSAV p28, run in parallel with the above two preparations in the first dimension, produced at least five dots with x coordinates of 3.5, 3.9, 4.7, 5.3, and 5.7 cm (Fig. 5*C*). Since the most acidic band for SSAV p28 had an isoelectric point (pI) of 5.8 and the most basic band for MuLV p30 had a pI of 6.4 when analyzed by agarose gel isoelectric focusing and immunoblotting (36) (not shown), we infer that the pI values for the placental protein lie between 5.8 and 6.4. Control experiments with gradient fractions of adult human spleen, kidney, and liver and affinity-column eluate of human lymphoid NC-37 cell extract did not show dots with similar coordinates (not shown).

CM-Cellulose Chromatography. Immunoblots of placental proteins that were eluted from CM-cellulose column between pHs 5.4 and 6.4 (pool 2) revealed that two major protein bands (33,000 and 16,000 daltons) and two minor bands (40,000 and 15,000 daltons) reacted with anti-SSAV p28 (Fig. 6, lane 2). Three of these proteins, but not the 33,000-dalton protein, also reacted with normal goat serum (Fig. 6, lane 4). Proteins that were eluted at a pH lower than 5.4 (pool 1) gave one band (16,000 daltons) of reaction with anti-SSAV p28 serum (Fig. 6, lane 3) that was also seen with normal goat serum (Fig. 6, lane 5). The elution of an anti-SSAV p28-

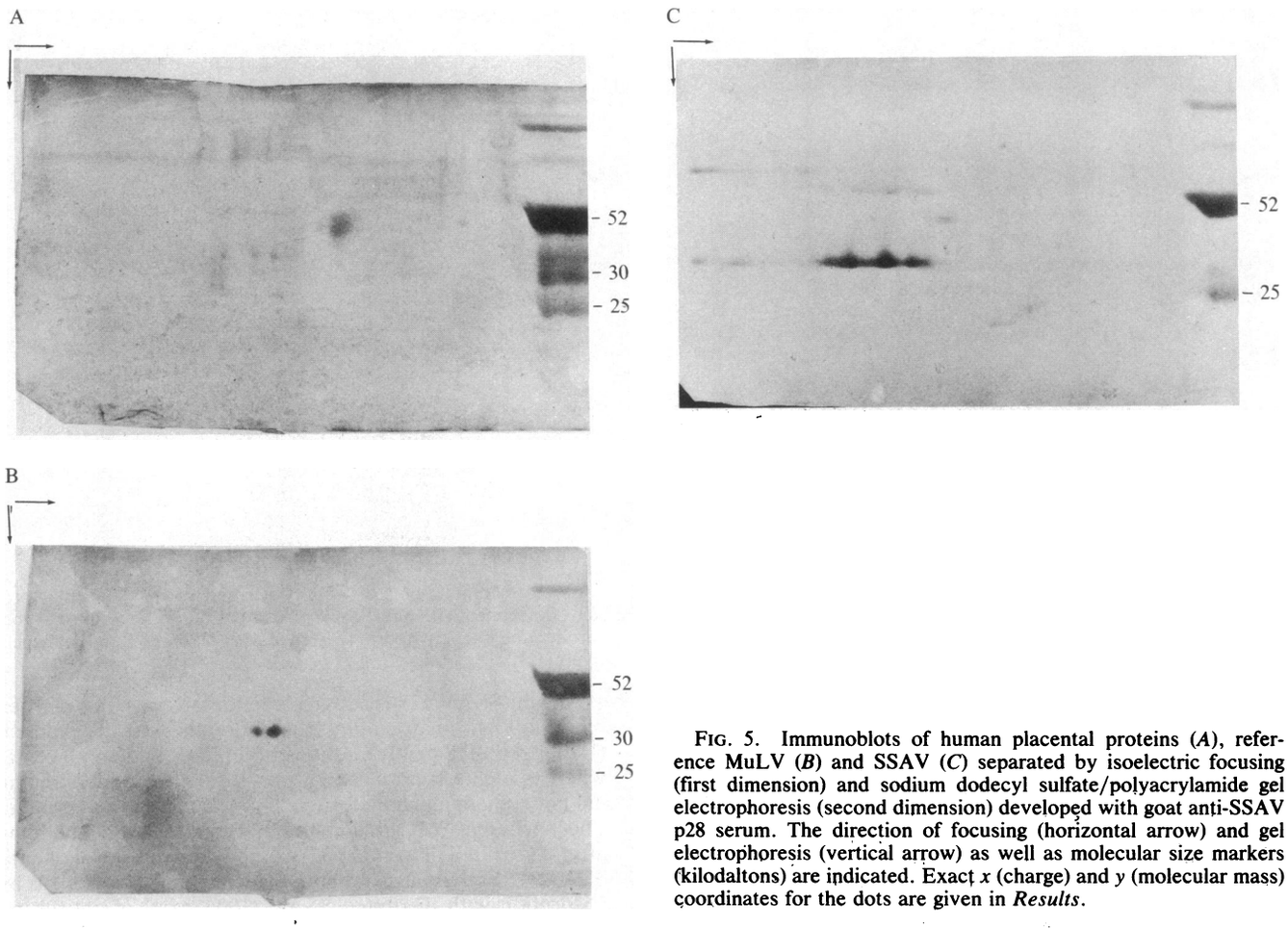

FIG. 5. Immunoblots of human placental proteins (*A*), reference MuLV (*B*) and SSAV (*C*) separated by isoelectric focusing (first dimension) and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (second dimension) developed with goat anti-SSAV p28 serum. The direction of focusing (horizontal arrow) and gel electrophoresis (vertical arrow) as well as molecular size markers (kilodaltons) are indicated. Exact *x* (charge) and *y* (molecular mass) coordinates for the dots are given in *Results*.

reactive placental protein of ≈30,000 daltons from the CM-cellulose column at pH 5.4–6.4 (but not at a pH lower than 5.4) supports the finding by two-dimensional electrophoresis of a placental protein with molecular mass and charge characteristics similar to the p28 protein of SSAV (Fig. 5 *A* and *C*) and GaLV (not shown).

Competition Enzymoimmunoassays. Placental proteins (3 mg) purified by CM-cellulose chromatography (Fig. 6) gave 50% competition against GaLV for anti-SSAV p28 antibodies (13 ng of GaLV gave 50% competition) and also 25% competition against BEV for anti-RD-114 p28 antibodies (640 ng of GaLV gave 16% competition). Placental proteins (<1 μg) purified by affinity chromatography (Fig. 4) gave 25% competition against GaLV for anti-SSAV p28 antibodies (5 ng of GaLV gave 25% competition).

DISCUSSION

The chief conclusions of this work are that 12 of 14 anatomical specimens of normal human term placentas analyzed by one- or two-dimensional electrophoresis and immunoblotting contain a protein or polypeptide of ≈30,000 daltons that is antigenically cross-reactive with p30 of the SSAV/GaLV retrovirus group and is physicochemically similar to the p30s of reference murine and primate type C retroviruses.

Several findings in this study require additional comment. The placental p30 protein was detected in gels by immunoblotting only with anti-SSAV p28 serums. This apparent restriction in reactivity might be attributed to properties of the anti-SSAV p28 serums, which were both high-titered and broadly cross-reactive (Fig. 1); or, alternatively, the placental p30 might possess cross-reacting determinants that are limited in number or irreversibly denatured by $NaDodSO_4$. When isolated by chromatography under nondenaturing conditions, the placental p30 competed strongly against GaLV for anti-SSAV p28 antibodies, as expected, and also competed to a lesser extent, as did reference GaLV, against BEV for anti-RD-114 p28 antibodies. The placental p30 derived from 30–100 g of tissue was detected in gradient- and chromatography-purified samples containing 1–10 mg of total protein and in affinity eluates containing at most microgram quantities of total protein. The one-dimensional migration distance of the placental p30 isolated by different methods varied somewhat in comparison to reference MuLV p30. The average molecular mass of the placental p30 was 30,000 daltons, with the overall range being 27,000–33,000 daltons. Possible explanations for such variations might include partial proteolysis in some tissue extracts, variable post-translational modification, and technical factors.

The probability of finding two completely unrelated proteins that share three independently measured properties of molecular weight, charge, and antigenicity is extremely remote. A plausible interpretation of the present findings is that the human placental p30 protein is encoded by the endogenous retrovirus-related p30 *gag* gene segment recently identified by recombinant DNA technologies in segments of normal human chromosomal DNA (13–16). The human DNA segments contain sequences that have the structure of an integrated but incomplete type C retrovirus and show homology to the polymerase (*pol*) gene of BEV and to the p30 *gag*, p15 *gag*, and polymerase genes of Moloney MuLV (14, 15). The human DNA segment termed *ERV-1* (endogenous retrovirus-1) has been mapped to human chromosome 18 (16). The human p30 amino-acid sequence has been predicted from DNA sequence determinations of the endogenous retrovirus-related p30 *gag* gene (14, 15).

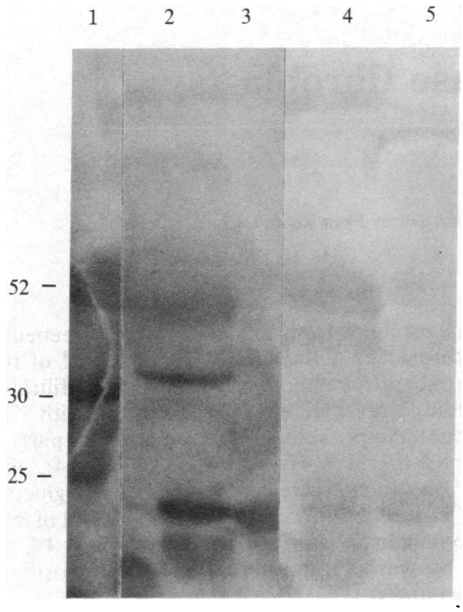

FIG. 6. Immunoblots developed with anti-SSAV p28 serum (lanes 1–3) and normal goat serum (lanes 4 and 5) showing reactivity of human placental proteins eluted from CM-cellulose column in pool 2 (lanes 2 and 4) and pool 1 (lanes 3 and 5). Lane 1 contains reference MuLV plus heavy and light chains of goat IgG.

Reverse transcriptase activity similar to that of retroviruses but not inhibited by antiserums to established retroviral polymerases is demonstrable in density gradient-purified fractions of normal human placentas (25). Immunohistologic studies indicate the presence of an anti-SSAV p28-reactive protein (22) or other type C (37, 38) or type B (39) retrovirus-related proteins in human placentas. It is known that distinct classes of cross-reacting p30 determinants are shared by mammalian type C retroviruses (18, 19, 40, 41) and by mammalian type B, C, and D retroviruses (21) and that certain retrovirus p30 determinants are strongly shared by retroviruses that have little sequence homology between their genomes (42).

A comparison should be made between the retrovirus-related human placental p30 protein and the predicted protein product of the human endogenous retrovirus p30 *gag* gene (14, 15), the SSAV p28 cross-reactive protein found in some patients with leukemia (35, 45, 46) or system lupus erythematosus (27, 34, 43), and the p24 core protein of the exogenous human T-cell leukemia virus (44).

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