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Human Endogenous Retrovirus K10: Expression of Gag Protein and Detection of Antibodies in Patients with Seminomas

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The human endogenous retrovirus K10 (HERV-K10) has been identified in the human genome by its homology to retroviruses of other vertebrates (M. Ono, T. Yasunaga, T. Miyata, and H. Ushikubo, J. Virol. 60:589–598, 1986). Using PCR amplification, DNA cloning, sequencing, and procaryotic expression, we were able to demonstrate that HERV-K10 encodes a 73-kDa protein which was processed by a HERV-K10-encoded protease to yield proteins p22/p26, p30, and p15/16. Analysis of the teratocarcinoma cell line Tera 1 or tumor tissues by immunoblotting demonstrated that the 80-kDa polyprotein of HERV-K10 *gag* and a processed protein of 39 kDa were expressed. In addition, a major protein of 39 kDa and additional species of 30, 22, 19, and 17 kDa could be detected in the supernatant of Tera 1 cells, suggesting that HERV-K10 Gag proteins are either secreted or processed to probably incomplete viral particles. In addition, the *gag* gene of HERV-K10 was expressed in the baculovirus system. Using this recombinant system to test antisera from patients with different diseases and healthy individuals, we were able to detect antibodies against the N-terminal part of HERV-K10 Gag in 2 to 4% of groups of tumor patients with titers ranging between 1:80 and 1:640, while approximately 0.1 to 0.5% of healthy individuals exhibited antibodies with lower titers. In contrast, patients with seminoma had antibody titers in the range of 1:2,560 at the time when the tumor was detected. Immunohistochemistry using specific rabbit sera or monoclonal antibodies against HERV-K10 Gag revealed that the Gag protein is expressed in the cytoplasm of the tumor cells. Furthermore, an 80-kDa protein corresponding to the HERV-K10 Gag polyprotein could be detected in tumor biopsies. For the first time, these data indicate that HERV-K10 Gag proteins are synthesized in seminoma cells and that patients with those tumors exhibit relatively high antibody titers against Gag. So far, no information on which role HERV-K10 plays in the development of this tumor exists.

Human endogenous retroviruses (HERVs) or human endogenous proviral DNAs have been identified in human genomic DNA by their homology to retroviruses of other vertebrates (for reviews, see references 9, 11, and 20). The human genome probably contains numerous copies of proviral DNAs. However, nothing is known about their function. Because of different degrees of homology and other features, these proviral DNAs are divided into distinct families. Recently, it was shown that the proviral element K10 contains an open *pol* reading frame (15) as well as open *gag* and *env* reading frames (10, 13) while all other proviral elements exhibit reading frames with multiple stop codons (9). The semiquantitative Southern blot analysis of genome copy numbers revealed that the HERV-K10 element is present in 25 to 30 copies or more within the genome of human individuals (13, 15). Procaryotic expression of HERV-K10 *gag* and *prt* region demonstrated that this element encodes a full-length *gag* homologous 73-kDa protein and a functional protease which is located in a −1 position relative to the reading frame of *gag* (13).

Expression of proviral DNA, especially of HERV-K, has been observed in placental tissue (3), teratocarcinoma cell lines, and breast cancer cell lines by detection of distinctly sized transcripts (3, 5, 10, 14). Recently, the expression of HERV-K10 Gag polyprotein and processed protein in a teratocarcinoma-derived cell line (GH) has been described previously (10). Furthermore, the same authors found evidence that particles related to HERV-K were released from GH cells in the supernatant (1). Up until now, nothing was known about the function of HERV-K genes or a possible association between HERVs and human diseases, although it was speculated that HERV might play a role in autoimmunity (16).

In this study, we were interested in the characterization of the HERV-K10 *gag*-encoded proteins and in a possible association of this retroviral element with a human disease. Using various polyclonal sera and monoclonal antibodies against different regions of the HERV-K10 Gag polyprotein, we were able to delineate the processed Gag proteins. Furthermore, by using the recombinant expression of HERV-K10 *gag* in the baculovirus system, antibodies against this protein could be detected in human individuals.

While healthy donors exhibited reactivity to a very low percentage (approximately 0.1 to 0.5%), in tumor patients, depending on the disease, varying percentages of antibody reactivity in the range between 1 and 4% could be observed. An exception was patients with testicular tumors, in particular with seminoma.

A total of 70 to 80% of patients with those tumors exhibited

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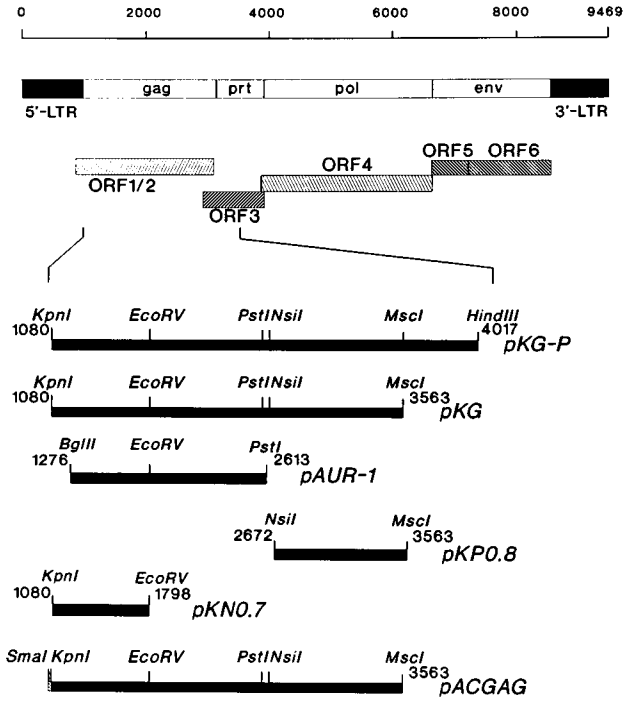

FIG. 1. Restriction map according to references 13 and 15 and open reading frames of the HERV genome (HERV-K10). Shown is a schematic representation of the plasmids which were used for procaryotic expression (pKG-P, pKG), for eucaryotic expression (pACGAG), for raising polyclonal rabbit sera (pKG, pAUR-1, pKN0.7, pKP0.8), and for generating monoclonal antibodies (pAUR-1, pKP0.8). LTR, long terminal repeat; ORF, open reading frame.

high antibody titers to HERV-K10 proteins at the time of the detection of the tumor. In addition, HERV-K10 Gag proteins could be identified in seminoma tumor biopsies as well as in a teratocarcinoma-derived cell line, Tera 1. Furthermore, this cell line was found to secrete detectable amounts of HERV-K10 Gag proteins in the supernatant.

MATERIALS AND METHODS

Cells. The cell lines Tera 1 (2) and T47D (6) were obtained from the American Type Culture Collection. The B95-8 cell line was originally established by infecting marmoset B lymphocytes with Epstein-Barr virus (12). Tera 1 cells were maintained in McCoy's 5a medium supplemented with 10% fetal calf serum, 40 U of penicillin per ml, and 50 μg of streptomycin per ml and were subcultured once a week. Tera 1 and T47D cells were grown in plastic tissue culture flasks. Transfer of Tera 1 cells was performed by shaking off the loosely adhering cells from the culture flasks. For subculturing of T47D cells, a standard trypsin treatment was used. B95-8 and T47D cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 40 U of penicillin per ml, 50 μg of streptomycin per ml, 10 U of moronal per ml, and 10 μg of neomycin sulfate per ml and were subcultured once or twice a week.

SF158 is a continuous insect cell line derived from *Spodoptera frugiperda* (23). Cells were maintained as described previously (4). Wild-type baculovirus *Autographa californica* nuclear polyhedrosis virus was amplified by infection of SF158 cells. Extracellular virions and viral genomic DNA were prepared as described previously (23). *Escherichia coli* BL21-DE-3 has been described previously (22).

Supernatant of cell cultures. Three to four days after subculturing, the culture supernatants were removed from cells and debris by low-speed centrifugation (1,500 × *g* for 10 min), and the supernatants were filtered through a 0.45-μm-pore-size disposable filter. The supernatant was centrifuged in a Beckman SW28 rotor at 28,000 rpm for 3 h (100,000 × *g*). Subsequently, the pellets were suspended in sample buffer (125 mM Tris-HCl [pH 6.8], 6%-sodium dodecyl sulfate [SDS], 10% glycerol, 10% β-mercaptoethanol).

Construction and induction of recombinant procaryotic expression vector and production of polyclonal rabbit antibodies. The location of all constructs on the HERV-K10 genome is shown in Fig. 1. The plasmids pKG-P, pKG, and pAUR-1 have been described recently (13). To generate pKP0.8, the *Eco*RI-*Msc*I fragment excised from pKG-P was cloned into pATH11 (7) digested with *Eco*RI and *Sma*I. To generate pKN0.7, the *Eco*RI-*Eco*RV fragment excised from pKG was cloned into pATH11 digested with *Eco*RI and *Sma*I. The plasmids pKP0.8 and pKN0.7 encode fusion proteins containing 37 kDa of the amino terminus of the anthranilate synthetase (TrpE) of *E. coli* and the C-terminal (pKP0.8) or the N-terminal (pKN0.7) part of the HERV-K10 Gag protein.

E. coli BL21-DE-3 was transformed with the plasmids, and induction of the fusion proteins was carried out as described previously (7). The fusion proteins were used to produce polyclonal antisera. In this purpose, they were purified by electrophoresis in SDS–10% polyacrylamide gels and used to immunize rabbits as described previously (4, 13). The rabbit sera raised against the fusion proteins encoded by the plasmids pKG, pKN0.7, and pKP0.8 are referred to as serum no. 6897 (anti-*gag* serum), serum no. 266 (anti-N-terminal part serum), and serum no. 8037 (anti C-terminal part serum).

Construction of baculovirus transfer vector and generation of recombinant baculoviruses. An *Aat*II-*Hin*dIII fragment excised from the vector pUC19 was cloned into pKG digested with *Aat*II and *Hin*dIII. The resulting plasmid, pUCGAG, was digested with *Kpn*I. After isolation of a *Kpn*I-resistant *gag*-specific fragment, it was cloned in a *Kpn*I-digested pUC19 vector. From this plasmid, designated pUC-GAG*, a *Sma*I-*Msc*I fragment was isolated and cloned into *Sma*I-digested baculovirus transfer vector pAC409. The resulting plasmid, called pACGAG, was used to generate the recombinant baculovirus. Furthermore, the insect cell line SF158 was cotransfected with *Autographa californica* nuclear polyhedrosis virus genomic DNA and the recombinant transfer vector pACGAG as described recently (4).

Immunofluorescence. Indirect immunofluorescence for the determination of antibody titers against HERV-K10 Gag was carried out as follows. SF158 cells in the logarithmic growth phase were infected with pACGAG. Wild-type baculovirus-infected cells served as a control. Cells were harvested 42 h postinfection and mixed with uninfected cells at a ratio of 1:40. Cover slides were coated with the cell mixture and fixed at −20°C for 10 min in acetone. The cells were incubated for 45 min with human or rabbit sera diluted in phosphate-buffered saline (PBS) and stained by indirect immunofluorescence with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (IgG) or goat anti-rabbit IgG diluted 1:50, respectively.

Immunoblots. Proteins were separated on SDS-polyacrylamide gels (SDS-polyacrylamide gel electrophoresis [PAGE]) and electrophoretically transferred to Immobilon membranes (Millipore Corp.) (24). The blots were incubated with rabbit antisera at a dilution of 1:200 and then stained indirectly by using peroxidase-conjugated goat anti-rabbit antibodies. When the staining was performed by the enhanced chemiluminescence technique (Amersham), nitrocellulose membrane was used for the electrophoretic transfer.

Immunoblotting analysis of tumor biopsies. Tissue samples stored at −70°C were homogenized on ice in sample buffer. After incubation at 95°C for 10 min and centrifugation for 10 min at 15,000 × *g*, the supernatant was analyzed by SDS-PAGE and immunoblotting as reported recently (18).

Competition assays. In competition fluorescence assays, the antisera used for staining were preincubated with 2 μg of isolated fusion protein or anthranilate synthetase protein per ml overnight at 4°C. In competition immunoblot assays, the preincubation was carried out in 10% nonfat milk.

Production of monoclonal antibodies. The plasmids pAUR-1 and pKP0.8 encoding the central and the N-terminal parts of the HERV-K10 Gag protein, respectively (Fig. 1), were used to produce monoclonal antibodies. The fusion proteins were isolated as described above. Approximately 20 μg of SDS-PAGE-purified fusion protein dissolved in 200 μl of PBS and emulsified with 200 μl of Freund's complete adjuvant was injected intraperitoneally and subcutaneously into Lou/c rats (E20, Bazin JIM 112:53). On day 21, the procedure was repeated with Freund's incomplete adjuvant. Three days before fusion, a final boost without adjuvant was given intraperitoneally. Fusion of the myeloma P3X63Ag8.653 with the rat immune spleen cells was performed according to the general procedure described in reference 8. Hybridoma supernatants were tested in a solid-phase immunoassay with bacterial extract from either *E. coli* expressing the parental nonfusion TrpE protein or HERV-K10 Gag fusion protein.

Polystyrene microtiter plates (Greiner, Frickenhausen, Germany) were coated with the crude *E. coli* extracts diluted 1:200 in carbonate-bicarbonate buffer (50 mM, pH 9.5), as determined with anti-TrpE monoclonal antibody (M1). The wells were blocked with PBS containing 1% nonfat milk (Fink, Herrenburg, Germany). Culture supernatants were incubated for 1 h, and bound rat monoclonal antibodies were detected with goat anti-rat IgG coupled with horseradish peroxidase (Dianova, Hamburg, Germany), with *O*-phenylenediamine (Sigma, Deisenhofen, Germany) as substrate. Antibody-producing hybridomas which reacted positively with the HERV-K10 Gag fusion protein and negatively with nonfusion TrpE protein were tested against fusion protein of the C-terminal or middle part of the HERV-K10 Gag and cloned at least twice by limiting dilution. The immunoglobulin type was determined in a solid-phase enzyme-linked immunosorbent assay with mouse anti-rat antibodies as capture and biotinylated monoclonal mouse anti-rat Ig class (anti-IgM, Zymed) and anti-IgG subclass antibodies as indicators (21). The positive clones were confirmed by immunofluorescence with full-length Gag protein expressed in SF158 insect cells, as described above. Unspecific binding was blocked by PBS containing 5% fetal calf serum. Hybridoma supernatant was incubated for 1 h, and bound rat monoclonal antibodies were detected by goat anti-rat IgG-fluorescein isothiocyanate.

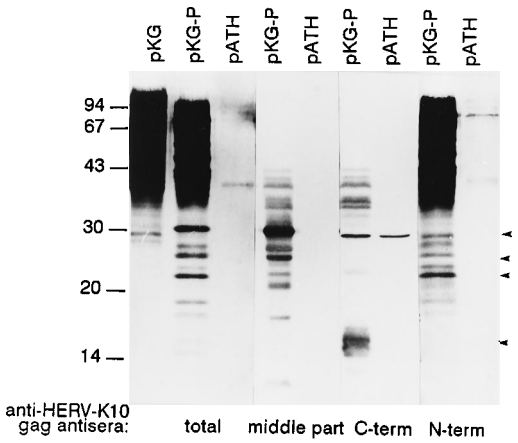

FIG. 2. Procaryotic expression and immunoblot analysis of protein products by the construct pKG containing the *gag*-homologous region of HERV-K10, pKG-P containing the putative protease gene in addition to the *gag*-homologous region, or the vector pATH alone as described in Materials and Methods. Total bacterial protein (5 μg) was subjected to SDS–15% PAGE. After blotting, the filter membranes were probed with an anti-Gag polyclonal rabbit serum, a polyclonal rabbit serum raised against the N-terminal part (pKN0.7) of the Gag protein, and monoclonal antibodies generated against the middle part (pAUR-1) or against the C-terminal part (pKP0.8) of the Gag protein. The apparent molecular masses were calculated from comigrating molecular mass standards and are given in kilodaltons.

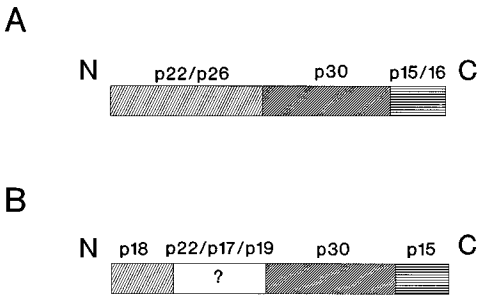

FIG. 3. Tentative location of the processed HERV-K10 Gag proteins: procaryotic expressed Gag protein in *E. coli* (A) and Gag protein expressed in Tera 1 cells (B).

Immunohistochemistry. Formalin-fixed, paraffin-embedded testicular tumor tissue or, as a control, Tera 1 cells were sectioned at 4 μm, deparaffinized, rinsed in PBS, and incubated for 30 min in hydrogen peroxide (0.6% in methanol) and rinsed again in PBS. The tissue sections were pretreated in citrate buffer (0.01 M; pH 6.0) in a microwave oven for 5 min at 600 W and 5 min at 450 W. After being cooled to room temperature, the sections were incubated with swine serum for 20 min. For immunostaining, the slides were incubated at room temperature with the rabbit anti HERV-K10 Gag serum or, as a control, preimmune serum diluted 1:40 for 1 to 2 h and then incubated with biotin-conjugated swine anti-rabbit antibodies, diluted 1:200 (DAKO), for 30 min and with avidin-biotin complex, diluted 1:100 (DAKO), for 30 min and developed with diaminobenzidine. After each incubation step, the slides were rinsed in PBS for 5 min.

RESULTS

Characterization of the HERV-K10 Gag proteins. Recently, we reported that HERV-K10 encodes a full-length *gag* homologous 73-kDa protein and a functional protease. Furthermore, we were able to demonstrate that HERV-K10 Gag polyprotein was specifically processed by the HERV-K10-encoded protease which exhibits features of an aspartate-type protease (13, 19).

In order to distinguish the processed HERV-K10 Gag proteins from the 73-kDa Gag polyprotein, a DNA fragment containing the entire *gag* reading frame and the adjacent protease region construct pKG-P (Fig. 1) was inserted into the expression vector pATH11 and expressed in *E. coli* BL21-DE-3 as described recently (13).

Protein extracts were analyzed by protein immunoblot with monoclonal antibodies or polyclonal rabbit sera directed against different parts of the HERV-K10 Gag polyprotein as shown in Fig. 2.

With the polyclonal rabbit serum, three dominant protein bands with molecular masses of 30, 26, and 22 kDa could be observed while a monoclonal antibody directed against the C-terminal part of HERV-K10 Gag reacted predominantly with two proteins of 15 and 16 kDa. A polyclonal rabbit serum directed against 228 amino acids of the N-terminal part exhibited the strongest reaction against the 22-kDa protein. In the reaction of a monoclonal antibody directed against the central part of HERV-K10 Gag, the 30-kDa protein was found to exhibit strong activity while a protein band of 26 kDa reacted to a smaller extent. Additional protein bands on the upper part of the gel with extremely strong reactions presumably represent the partially processed HERV-K10 Gag polyprotein. Several protein bands in the lower part of the gel might represent degradation products of HERV-K10 Gag due to the presence of bacterial proteases. The data obtained from the experiments whose results are shown in Fig. 2 clearly locate the 15- and 16-kDa proteins at the C terminus. Located next to these proteins is the 30-kDa protein, while good evidence exists for the location of the 22-kDa protein at the N terminus. Finally, the 26-kDa protein seems to be encoded by a central region between the 22- and the 30-kDa proteins. A tentative model for the processing of the HERV-K10 Gag protein is shown in Fig. 3A.

Eucaryotic expression of HERV-K10 Gag. Recently, the expression of HERV-K10 Gag protein in the human teratocarcinoma cell line GH was reported (1). In order to study the expression and the processing of the Gag polyprotein in more detail, protein extracts from the teratocarcinoma cell line Tera 1 and the 100,000 × *g* pellet of the supernatant of this cell line were analyzed by immunoblotting with different specific rabbit anti-HERV-K10 Gag sera or monoclonal antibodies. Representative results are shown in Fig. 4. The investigation of Tera 1 cell extracts with a serum against the entire Gag polyprotein (serum no. 6897) revealed the existence of an 80-kDa protein which seems to correspond to the full-length Gag polyprotein. Further protein bands of approximately 70, 53, and 39 kDa could be identified, representing partially processed Gag proteins. By analyzing the 100,000 × *g* pellet of the supernatant from Tera 1 cells by the same method, additional protein bands of 30, 22, 19, and 17 kDa could be observed. The corresponding material from HERV-K10-negative B95-8 cells (New World monkey) or T47D cells served as a control.

By analyzing the 100,000 × *g* pellet by using rabbit antisera against different parts of the Gag polyprotein, it was possible to construct a tentative order of processed HERV-K10 Gag proteins expressed in the cell line Tera 1 (Fig. 3B). For example, a 15-kDa protein was recognized only by the serum against the C terminus while a 30-kDa protein reacted most dominantly with the serum directed against the middle part of HERV-K10 Gag. In addition, an 18-kDa protein was identified by the serum against the N terminus.

At the present time, we have no definite information about in which order the 22-, 19-, and 17-kDa proteins are located on the HERV-K10 *gag* gene. These proteins are detected by the antiserum against the middle part of *gag*. Only the 22-kDa protein exhibits a weak reaction with the N-terminally directed serum. Concerning the C-terminal part of HERV-K10 *gag*, the

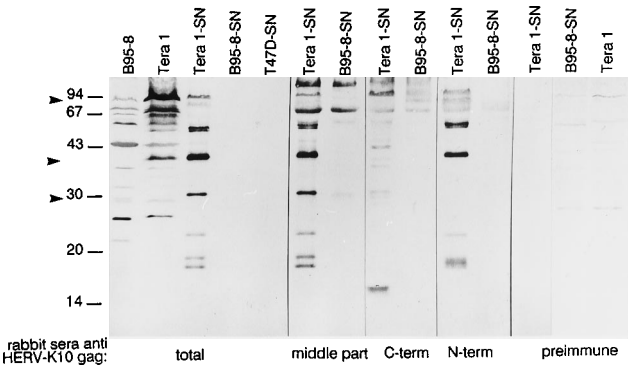

FIG. 4. Immunoblot analysis of cellular extracts and supernatants (SN), as described in Materials and Methods, of the cell line Tera 1 with polyclonal antisera against HERV-K10 Gag. The cell lines Tera 1, a teratocarcinoma cell line, and T47D, a mammocarcinoma cell line, were obtained from the American Type Culture Collection. B95-8 is a HERV-K10-negative New World monkey cell line. To yield the supernatant extracts (SN), the culture supernatants were removed from cells and debris by low-speed centrifugation and were filtered through a 0.45-μm-pore-size disposable filter. Subsequently, the supernatant was centrifuged in a Beckman SW28 rotor at 28,000 rpm for 3 h (100,000 × *g*). The resulting pellets were resuspended in sample buffer, and 1 μg was subjected to SDS–15% PAGE. After blotting, the filter membranes were probed either with rabbit sera directed against the middle part (pAUR-1), the C-terminal part (pKP0.8), or the N-terminal part (pKN0.7) or with the entire Gag protein (total, pKG) as indicated. Controls were performed by using preimmune serum. The apparent molecular masses were calculated from comigrating molecular mass standards and are given in kilodaltons.

procaryotic expression in *E. coli* and the expression of HERV-K10 *gag* in Tera 1 cells revealed good correspondence.

Analysis of human antibodies reacting with recombinant HERV-K10 Gag protein. To investigate a possible role of HERV-K10 expression in the development of pathogenic events in humans, we expressed HERV-K10 Gag in the baculovirus system and established a system for testing human antibodies by immunofluorescence as described in Materials and Methods.

For analyzing human sera, insect cells expressing HERV-K10 *gag* were diluted 1:40 with uninfected cells to reach a concentration of 5 to 15 Gag-positive cells per visual field in order to avoid an overrepresentation of Gag antigen. Human sera were screened at an initial concentration of 1:40 and titrated in twofold dilutions. A variety of healthy individuals as well as patients with different diseases as indicated in Tables 1 and 2 were investigated. While approximately 0.4% of healthy individuals exhibit low antibody titers, patients with autoimmune diseases, human immunodeficiency virus infection, or different tumors exhibited antibodies in 2 to 4% of the cases.

In contrast, patients with seminoma, a particular form of testicular tumor, had high antibody titers to Gag proteins. A total of 10 of 14 serum samples from seminoma patients with primary tumors tested within 6 weeks after the beginning of therapy had titers against Gag of up to 1:2,560 and in one case up to 1:20,480 (Table 3). In addition, 4 seminoma patients with recidivous tumors exhibited antibodies against Gag while 4 patients with fresh primary tumors and 17 seminoma patients after a longer time of therapy showed no antibody reactivity against HERV-K10 Gag. Of a total of 31 seminoma patients, 45% had HERV-K10 Gag antibodies regardless of whether they had freshly detected tumors or not. In one case, three consecutive serum samples of one patient were available. When these sera were tested, the serum taken 36 months before tumor diagnosis was HERV-K10 Gag antibody negative while the serum taken at the time of tumor detection showed a titer of 1:2,560. Six months after onset of therapy, a titer of 1:40 could be determined.

The specificity of the immunofluorescence reaction was

TABLE 1. Determination of antibodies against HERV-K10 Gag in sera from healthy individuals or patients with nontumor diseases

Diagnosis	No. of serum samples tested	No. of positive serum samples	Titer	%
Autoimmune disease[a]	153	2	1:40 1:40	1.3
Immunosuppression	53			0
HIV[b] positive	113	3	1:160 1:160 1:80	2.7
HIV negative	106	1	1:320	0.9
Controls (students and hospital staff)	233	1	1:40	0.4
Total	658	7		1.0

[a] Lupus erythematosus, multiple sclerosis, mixed connective tissue disease, scleroderma, Sharp syndrome, Sjögren's syndrome, Cogan syndrome, arthritis.
[b] HIV, human immunodeficiency virus.

TABLE 2. Determination of antibodies against HERV-K10 Gag in sera from patients with tumor diseases

Diagnosis[a]	No. of serum samples tested	No. of positive serum samples	Titer	%
Mammary carcinoma	103	3	1:80 1:80 1:160	2.9
Brain tumor	128	4	1:40 1:80 1:320 1:320	3.6
AML	59			
Leukemia	7		1:80	
ALL	81	3	1:40	3.7
CML	8		1:80	
CLL	14			
Non-Hodgkin lymphoma	63	3	1:2,560 1:320 1:640	4.8
Hodgkin's disease	164	3	1:80 1:40 1:80	1.8
Burkitt's lymphoma	8			0
Nasopharyngeal carcinoma	6			0
Hairy leukoplakia	1			0
Colon carcinoma	3			0
Lymphoma	88	2	1:40 1:640	2.3
Tumor disease	341	10	1:320 1:320 1:160 1:80 1:40 1:160 1:320 1:40 1:160 1:40	2.9
Testicular tumors[b]	17			
Teratoma	9	1	1:160	11
Seminoma	31	14	—[c]	45
Total	1,131	43		3.9

[a] AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia.
[b] Mixed tumors, Leyding cell tumors, embryonal carcinoma, urothelial carcinoma.
[c] See Table 3.

TABLE 3. Determination of antibodies against HERV-K10 Gag in sera from patients with seminoma

Diagnosis	No. of positive serum samples/no. of serum samples tested	Titer	%
Primary seminoma	10/14[a]	1:2,560	
		1:160	
		1:640	
		1:640	
		1:40	
		1:20,480	
		1:160	78
		1:2,560	
		1:2,560	
		1:320	
Seminoma relapse	4/4[a]	1:80	
		1:160	
		1:160	
		1:640	
Seminoma	0/17[b]		
Total	14/31		45

[a] Sera tested within 6 weeks after start of therapy (half-life of IgG = 20 to 23 days).
[b] Sera tested months or years after therapy.

proven by competition experiments using different recombinant fusion proteins of HERV-K10 Gag (pKG, pAUR-1, pKP0.8, pKN0.7) as demonstrated in Fig. 1. The results in Table 4 clearly indicate that the Gag fusion protein covering the N-terminal part or the entire *gag* region of HERV-K10 *gag* completely inhibited binding of the serum antibodies to the Gag protein while fusion proteins from the middle or C-terminal part as well as the bacterial part of the fusion protein (TrpE) achieved no or only minor effect. These data provide evidence that the major epitopes recognized by positive human sera are located probably within the 54 N-terminal amino acids of Gag while no reactivity against the central part of Gag was detectable.

Expression of HERV-K10 Gag protein in tumor biopsies. The presence of antibodies against HERV-K10 Gag predominantly in patients with seminoma raised the possibility that this protein might be expressed in the tumor itself. In order to prove this hypothesis, tumor biopsies from patients with antibody titers against HERV-K10 Gag were tested by immunohistochemistry and immunoblotting analysis for the expression of HERV-K10 Gag.

Sections of formalin-fixed paraffin wax blocks from a seminoma mixed with embryonal carcinoma cells or a pure seminoma were stained by using a rabbit antiserum (serum no. 6897) directed against the entire HERV-K10 Gag as described in Materials and Methods. The results as shown in Fig. 5B and C indicate that Gag-related proteins could be detected in the form of clusters concentrated within the cytoplasm of the tumor cells. In contrast, in the surrounding tissue or in tissue of healthy testes no positive reaction could be found. Used as positive controls were acetone-fixed insect cells expressing recombinant HERV-K10 *gag* (data not shown) or formalin-fixed and paraffin wax-embedded Tera 1 cells in which positive reactive material was found predominantly concentrated in the cytoplasm (Fig. 5A).

From a few biopsies, frozen material was available. These tissues were processed and analyzed by immunoblotting with rabbit anti-HERV-K10 Gag serum (serum no. 6897) or monoclonal antibodies to Gag as described in Materials and Methods. Representative results are shown in Fig. 6. The rabbit anti-Gag serum specifically recognizes a protein band of 80 kDa which presumably represents the Gag polyprotein which was predominantly found in biopsy 1, while in biopsies 3, 4, 8, and 5 only a weak reaction could be observed (Fig. 6A). By using a monoclonal antibody directed against the middle part of HERV-K10 Gag, the 80-kDa protein band was clearly detected in biopsies 1, 3, 4, and 8 (Fig. 6B).

Several background bands with a molecular mass of about 50 kDa were found by using both antisera or monoclonal antibodies. At the present time, nothing is known about the nature of those 50-kDa proteins. In Table 5, the biopsies analyzed in Fig. 6 are summarized. The numbering of biopsies corresponds to the numbers given in Table 5. In competition experiments using full-length recombinant fusion protein of HERV-K10 Gag (pKG) for preincubation of the specific rabbit serum (no. 6897) or monoclonal antibodies, the binding of specific antibodies to the 80-kDa protein from biopsies 1 and 8 could be abolished (17).

It should be mentioned that in the *E. coli* expression system the monoclonal antibody directed against the middle part of HERV-K10 Gag as well as the monoclonal antibody against the C-terminal part recognized the processed Gag proteins only but did not react reproducibly with the 80-kDa Gag polyprotein (Fig. 2). One possible explanation for this different reaction pattern could be that the relevant epitopes are not accessible for the described monoclonal antibodies on the Gag-TrpE fusion protein because of an overload by other bacterial proteins.

TABLE 4. Inhibition of HERV-K10 immunofluorescence[a]

Serum	Inhibition by:					
	pATH 11 (vector)	pKG (total)	pAUR-1 (middle)	pKP0.8 (C terminus)	pKN0.7 (N terminus)	IF without competition
1. Human	+ (+)	0	+	+	0	+
2. Human	++	0	(+)	+	0	++
3. Human	+	0	(+)	(+)	0	++
4. Human	++	0	++	++	0	++
5. Human	+ (+)	0	+	+	0	+
6. Human	+	0	(+)	(+)	0	+
7. Human	+	0	+	+	0	+
8. Human	+	0	(+)	(+)	0	+
Rabbit anti-HERV-K10 Gag	++	0	++	++	+++	+++

[a] Prior to immunofluorescence (IF), sera were preincubated with isolated fusion proteins encoded by constructs as indicated and described in Materials and Methods. (+), a weak positive reaction.

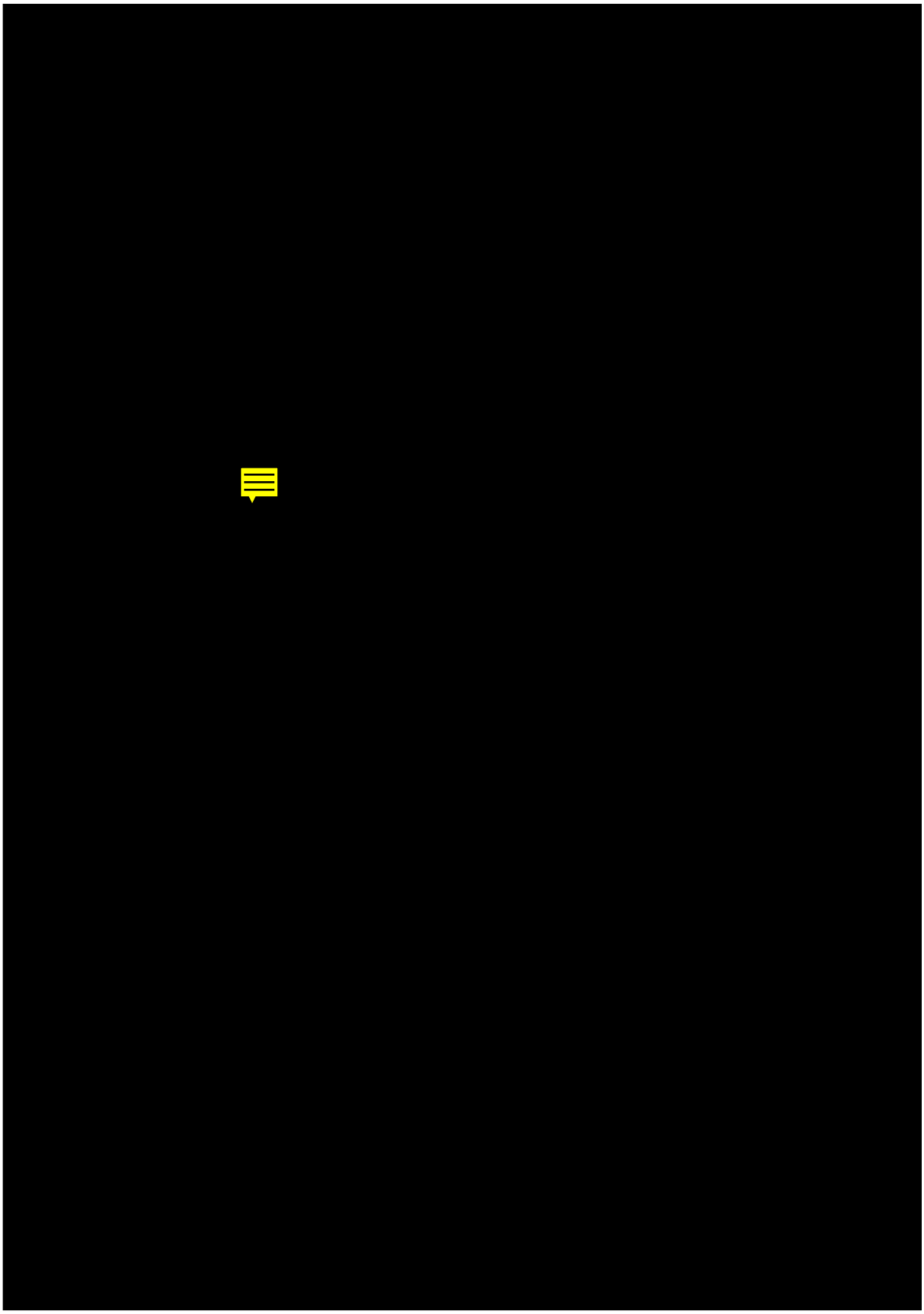

FIG. 5. Immunohistochemistry analysis of Tera 1 cells (A) or of biopsies from patients with a seminoma and a combined embryo carcinoma (B) or with seminoma (C). Sections of formalin-fixed paraffin-embedded tumor tissue or Tera 1 cells were incubated with rabbit anti-HERV-K10 Gag serum no. 6897 followed by biotin-conjugated swine anti-rabbit serum and incubation with an avidin-biotin complex. The immunostaining was performed with diaminobenzidine.

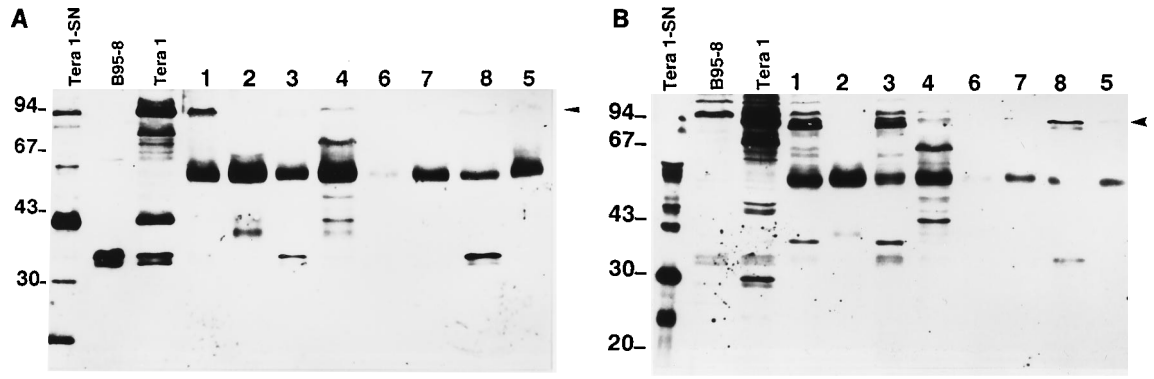

FIG. 6. Immunoblot analysis of testicular tumor biopsies. Tissue samples were homogenized on ice in sample buffer. After incubation at 95°C for 10 min and centrifugation for 10 min at 15,000 × *g*, the supernatant was analyzed (lanes 1 to 8). From every material, 15 μg of protein was subjected to SDS–12.5% PAGE. After blotting, the membranes were probed with rabbit serum no. 6897 directed against the entire Gag protein (pKG) (A) or with monoclonal antibodies directed against the middle part of the Gag protein (pAUR-1) (B). Tera 1 cells served as positive controls, and B95-8 cells served as negative controls. Numbers at left of panels, molecular masses in kilodaltons. Lane numbers correspond to numbering of biopsies as given in Table 5.

DISCUSSION

Recently, the expression of HERV-K10 *gag*-encoded protein in *E. coli* or in a human teratocarcinoma cell line has been reported (1, 10, 13). In addition, it was shown that HERV-K10 encodes a functional protease of the aspartate type (13).

In order to study the processing of HERV-K10 Gag polyprotein in more detail, antisera or monoclonal antibodies specifically directed against the N or C terminus or against the central part of HERV-K10 Gag were generated. By using these antibodies to analyze procaryotic expression or expression in Tera 1 cells, it was possible to determine the tentative sequence of the processed Gag proteins. Both procaryotic and eucaryotic expression revealed that at the C-terminal end of the Gag polyprotein is an approximately 15-kDa protein followed by a 30-kDa protein. By analyzing the HERV-K10 expression in Tera 1 cells, an 18-kDa protein could clearly be determined to be located at the N-terminal end of HERV-K10 Gag, while the exact localization of three proteins of 22, 19, and 17 kDa derived from the central part of Gag could not definitively be assigned. Similarly, in *E. coli* the exact order of the 26- and 22-kDa proteins derived from the N terminus cannot be determined yet by the methods used for these experiments.

The detection of processed HERV-K10 Gag proteins in the 100,000 × *g* pellet of the supernatant from Tera 1 cells suggests that those proteins are secreted. These data are supported by recent reports indicating particle formation from human teratocarcinoma cells (1). At the present time, no information is available as to whether those particles contain HERV-K10 RNA and whether they constitute infectious particles.

In order to search for a potential pathophysiological role of HERV-K10, the *gag* region was expressed in the baculovirus system. The recombinant protein was utilized to screen human sera for antibody reactivity by employing the immunofluorescence technique. While the expression of *gag*-specific antibodies in healthy individuals is a rare event (only 1 case of 233 tested serum samples with a titer of 1:40), 2 to 4% of patients with various diseases or tumors exhibited antibody titers in the range between 1:40 and 1:640. One exception was found in a patient with a non-Hodgkin lymphoma with a titer of 1:2,560. In contrast, 45% of patients with seminoma revealed antibody titers against HERV-K10 Gag ranging between 1:40 and 1:20,480. An even higher percentage (78%) of patients with seminoma exhibited antibody titers at the time of diagnosis or when recidivous tumors occurred. This observation was confirmed by the analysis of three follow-up serum samples from one patient which indeed only contained a high amount of antibodies at the time of tumor detection and before therapy.

The specific epitopes reacting in the immunofluorescence test were narrowed down to 54 amino acids at the N-terminal part of HERV-K10 *gag*. By computer analysis using the PALIGN program of PC/GENE (IntelliGenetics, Inc., Moun-

TABLE 5. Characterization of patients by immunoblotting, immunohistochemistry, and antibody reaction against HERV-K10 Gag

Registration no.	Clinical diagnosis	Protein immunoblot	Immunohistochemistry	Antibody titer
1	Seminoma	Positive	Positive in compartments (++)	1:160
2	Teratoma	Negative	Negative	ND[a]
3	Seminoma	Positive (MAb[b])	Negative	ND
4	Seminoma	Positive	Positive (++)	ND
5	Mixed seminoma	Positive	Negative (material in bad condition)	ND
6	Surrounding tissue	Negative	Negative	
7	Surrounding tissue	Negative	Negative	ND
8	Seminoma	Positive	Frozen section positive (+) Fixed tissue negative	ND
9, patient P	Mixed seminoma	ND	Positive (+++)	1:2,560
10, patient Z	Seminoma	ND	Positive (++)	1:2,560
11, patient B	Mixed seminoma	ND	Positive (++)	ND

[a] ND, not determined.
[b] MAb, monoclonal antibody.

tain View, Calif.), these amino acids showed a minor homology of 5 amino acids to the *gag* genes of Mason-Pfizer monkey virus, Jaagsiekte sheep retrovirus, and squirrel monkey retrovirus but no homology to other known viruses or cellular genes. Therefore, we believe that the antibodies found among healthy individuals and patients are specifically induced by the expression of HERV-K10 Gag.

In order to test the hypothesis that the Gag-specific antibodies were indeed due to the presence of antigen, the expression of HERV-K10 *Gag* protein in biopsy material of patients with seminoma and related tumors was analyzed either by immunohistochemistry or by immunoblotting. The use of both methods demonstrates that in seminoma tumor biopsies from seropositive patients as well as for seminoma patients from whom no antisera were available, protein with HERV-K10 Gag reactivity could be reproducibly identified. To our knowledge, this is the first demonstration that HERV-K10 Gag proteins are synthesized in seminoma tumor cells and that a high percentage of patients with those tumors exhibit elevated antibody titers against Gag. Furthermore, the data obtained with the follow-up sera of one patient might suggest that antibodies in seminoma tumor patients have diagnostic and prognostic value. At the present time, no information exists on which role HERV-K10 plays in the development of this tumor. Furthermore, we have no indication whether HERV-K10 *gag* or other proviral genes are expressed in other tumors of germ line origin. These questions remain to be elucidated in further investigations.

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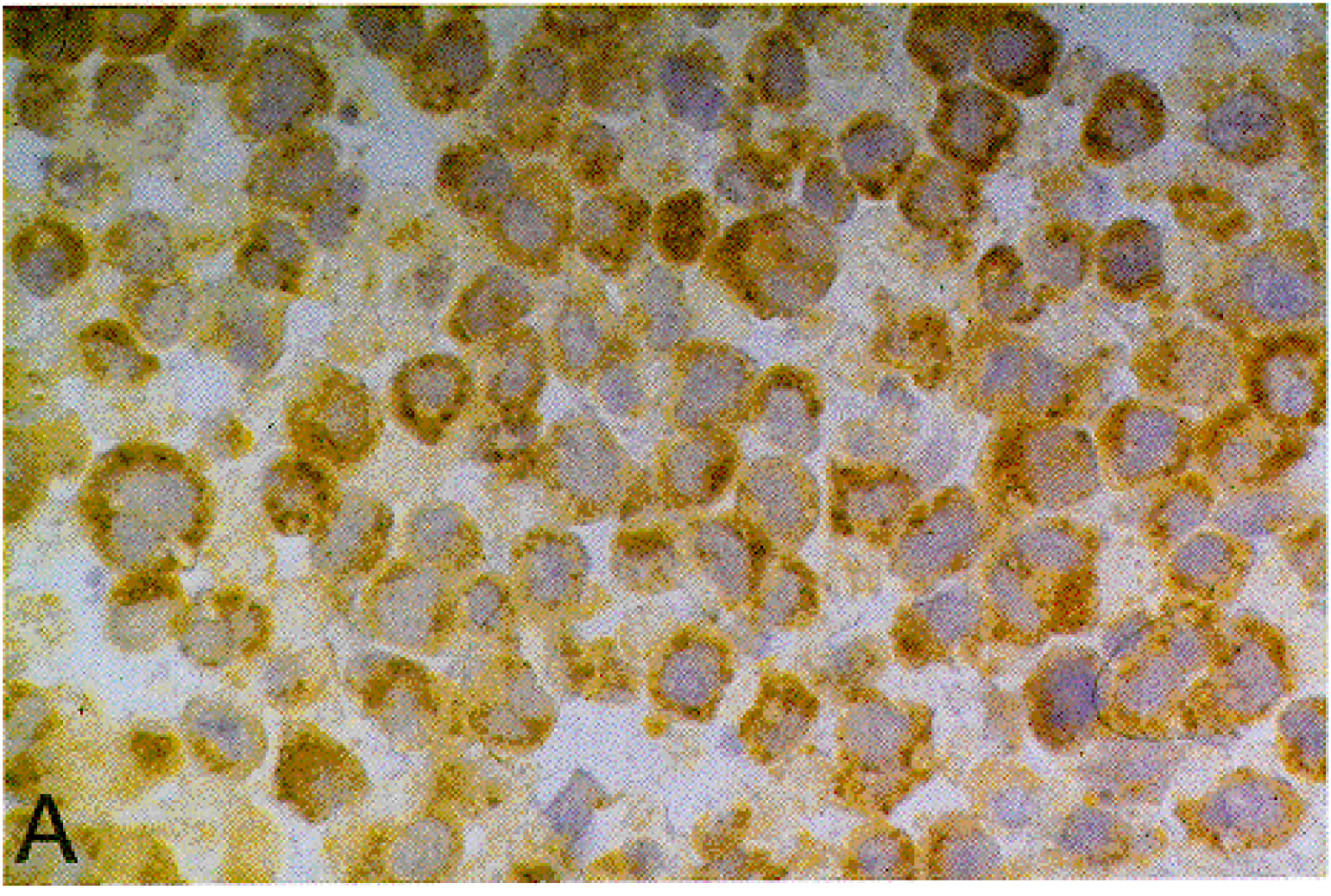
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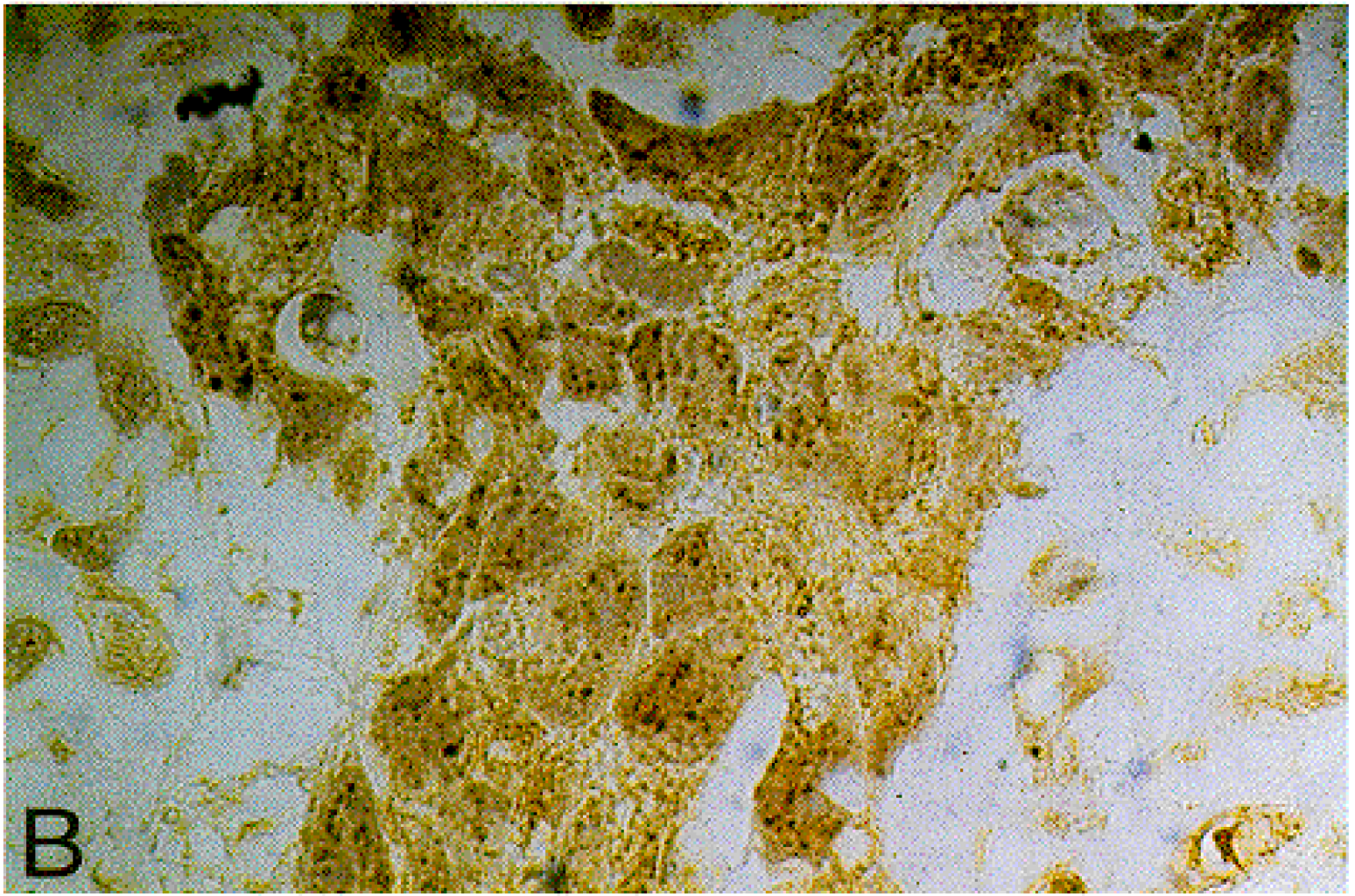
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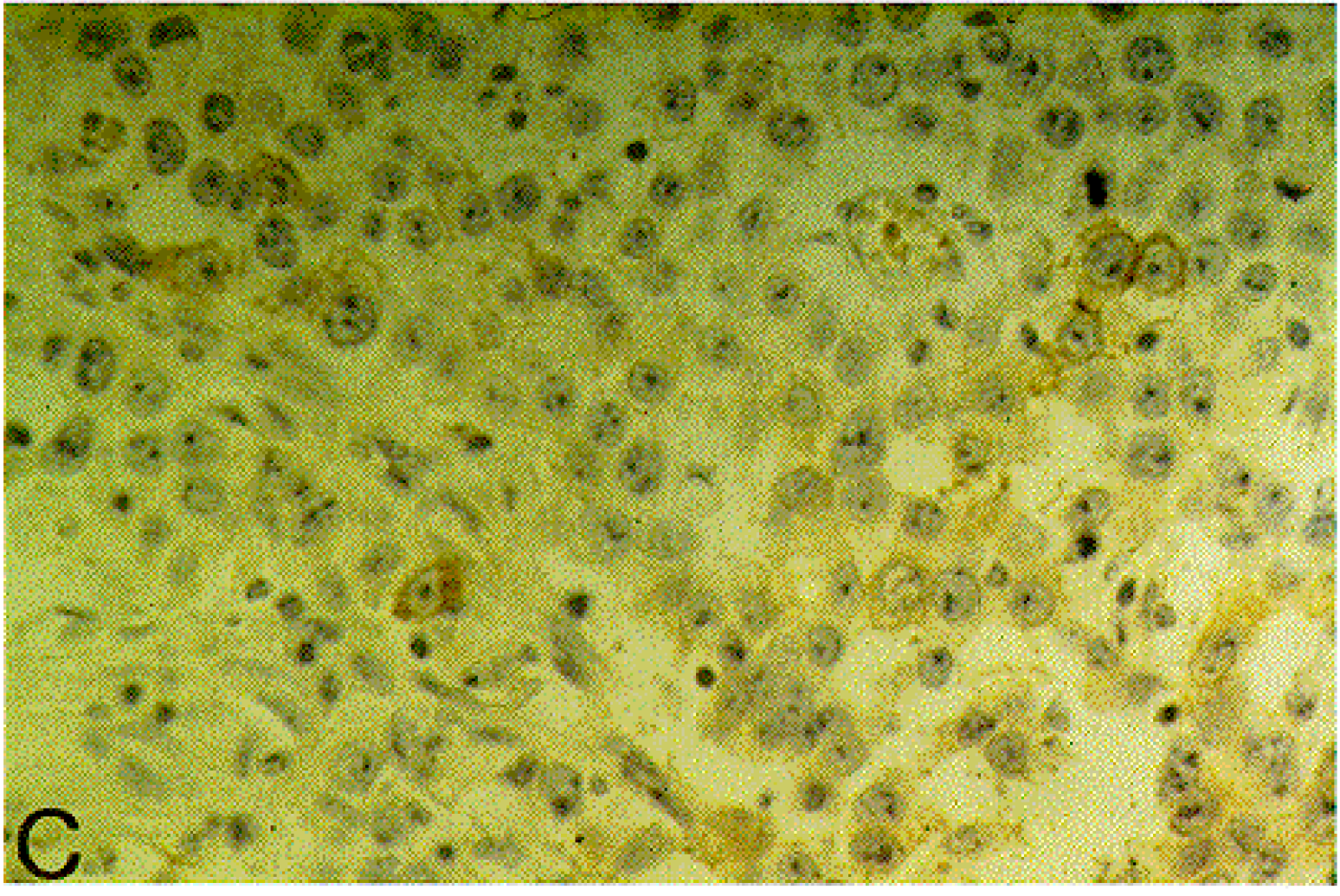
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