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Coexpression of Nestin in Neural and Glial Cells in the Developing Human CNS Defined by a Human-Specific Anti-nestin Antibody

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The presence of the intermediate filament protein nestin has been the predominant marker used to describe stem and progenitor cells in the mammalian CNS. In this study, a 998-bp fragment in the 3′ region of the nestin mRNA was cloned from human fetal brain cells (HFBC). The nucleotide sequence of the cloned cDNA revealed 21 differences with the previously published human nestin sequence, resulting in 17 amino acid changes. A 150-amino-acid fragment derived from the cloned nestin cDNA was coupled to glutathione *S*-transferase and used as an immunogen to generate a rabbit polyclonal antiserum that selectively detects human nestin. HFBC that proliferated in response to basic fibroblast growth factor incorporated 5-bromo-2′-deoxyuridine into their nuclei and immunostained for nestin, indicating nestin expression in proliferating CNS progenitor cells. In all cell cultures, nestin costained with the neuroepithelial cell marker vimentin. A small subset of nestin-stained cells (1–2%) immunostained with neuronal marker MAP-2 during the first week and after 4 weeks in culture. However, during the first week in culture, approximately 10–30% of the total cell population of HFBC stained for the glial cell marker GFAP, and nearly all coimmunostained for nestin. After 4 weeks in culture, a subset of GFAP-positive cells emerged that no longer costained with nestin. These results describe nestin expression not only in CNS progenitor cells but also in the cells which were in transition from a progenitor stage to glial differentiation. Collectively, these data suggest a differential temporal regulation of nestin expression during glial and neuronal cell differentiation. © 2000 Academic Press

Key Words: **stem and progenitor cells; intermediate filament; glia; human CNS**

INTRODUCTION

The sequential expression of intermediate filament proteins has been used to define the developmental progression of cells in the mammalian CNS (9, 30, 32, 35, 44). Nestin, a class VI intermediate filament protein, was originally found to be expressed early in the developing CNS (16). Its expression correlated with proliferating progenitor cells in the rodent brain (6, 12, 21). In the rat brain, nestin expression appears at the time of neuronal migration from E11 and diminishes by P6 in the spinal cord and by P21 in the cerebellum (16).

Although most of the work examining nestin expression has been done on rodent CNS cells, there are few studies that have addressed nestin expression in the human brain. In the human CNS, immunohistochemical staining revealed nestin in 6- to 40-week gestation brains, in similar regions to those in the rodent CNS (38). In this study, nestin was found in neuroepithelial cells lining the central canal of the spinal cord and in the germinal matrix and radial glia cells of the cortex (38). In addition to nestin expression in the normal developing human CNS, nestin was also detected in immature neuroepithelial tumor cell lines and a variety of CNS tumors, predominantly in gliomas (5, 38).

Despite the previous work describing nestin expression, there is little information characterizing developmental progression of nestin-expressing cells in the rodent or human CNS. The majority of studies examining nestin-expressing cells has focused on expression in stem and progenitor cells, with almost no studies exploring the progression of these cells as they differentiate into neurons and glial cells. Although nestin has been associated with stem and progenitor cells in the CNS, there is evidence that nestin is also associated with cells that are not progenitors and that the presence of nestin may not identify all CNS progenitor cells (6, 19, 37). Furthermore, there have been no studies to examine the specific cell types expressing nestin during the development of the human CNS. In this regard, a more specific phenotypic characterization of nestin-expressing cells is important in order to determine the progression of cells expressing nestin in the developing human CNS.

The ability to further characterize nestin-expressing cells in the developing human CNS has been complicated by the availability of an antibody that selectively and specifically detects the human nestin protein. The

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antibodies used in the majority of previous studies to investigate nestin in human cells were generated against the rat nestin protein (5, 38). Since the amino acid sequence of the rat nestin protein is only about 50% identical to that of the human protein, there is a potential for low specificity of the anti-rat nestin antibodies to human nestin protein. Additionally, there may be important protein epitopes of the human nestin protein that may not be detected using the anti-rat nestin antibody.

The goal of this study was to generate an antibody that was specifically designed against the human nestin protein to characterize nestin-expressing cells in the developing human CNS. In addition, this study sought to phenotypically characterize nestin-expressing cells to determine their lineage progression. This report describes sequence differences in a nestin cDNA obtained from human fetal brain cells compared to the previously published human nestin sequence (7). A rabbit polyclonal antiserum was generated against a 150-amino-acid segment derived from the cloned nestin cDNA. Using this antiserum, nestin was detected in progenitor cells and in a small subset of neurons. In addition, nestin was also detected in cells that were in transition from a progenitor stage to glial differentiation in a temporal pattern different from that in neurons. These data suggest a differential regulation of nestin expression during glial and neuronal cell differentiation.

METHODS

Cell Culture

Human fetal brains were obtained and processed as previously described (10) in accordance with guidelines at the NIH. Briefly, brain tissues were washed, separated from meninges and blood vessels, and then mechanically dissociated. Dissociated cells were cultured in poly-D-lysine (Sigma, St. Louis, MO) coated 75-cm^2 flasks and four-well slide chambers. Cells were grown in EMEM supplemented with 10% fetal calf serum, glutamine (2 mM), amphotericin B (0.5 μg/ml), gentamicin (50 μg/ml), and penicillin/streptomycin (100 IU/ml) at 37°C with 5% CO_2. U373 and U251 glioma cell lines and the immortalized human neuroglial cell line, SVG (26), were cultured in EMEM supplemented with 10% fetal calf serum, glutamine, and gentamicin at 37°C with 5% CO_2. Cell culture products were obtained from Mediatech (Herndon, VA) and Quality Biologicals (Gaithersburg, MD).

RT-PCR and Cloning of the 3′ Region of the Human Nestin mRNA

Total RNA was isolated from human fetal brain cells (HFBC) and cell lines using the RNeasy total RNA isolation system (Qiagen, Valencia, CA). RNA was then treated with DNase 1 (10 U/μg RNA, Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C. First-strand cDNA synthesis was performed on 1 μg of total RNA using oligo(dT) primers and Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD). Each sample was run in duplicate, one with reverse transcriptase and the other without, as a control for reverse transcription of genomic DNA.

Each cDNA sample was then amplified by PCR using the following primers: nestin, (5′-GAGGACCAGGACTCTCTATC-3′) and (5′-AGCGAGGAGGATGAGCTCGG-3′); and GAPDH, (5′-GAGCTTGACAAAGTGGTCGT-3′) and (5′-CCACAGTCCATGCCATCACT-3′). PCR amplification consisted of 30 cycles using the following cycling conditions: 30 s at 95°C; 30 s at 55°C; and 1 min at 72°C using the Perkin–Elmer 2400 thermal cycler. PCR products (30 μl) were electrophoresed through a 1% agarose gel and photographed. The amplified 998-bp region of human nestin mRNA corresponds to nucleotides 3853 to 4844 in the 3′ region of the human nestin gene (7, GenBank Accession No. X65964).

For cloning, RT-PCR was performed as above from total RNA on cells from two human fetal brains at 8 and 16 weeks of gestation and from the immortalized human neuroglial cell line, SVG. PCR products were ligated into the pCR2.1 vector using the TOPO TA cloning system (Invitrogen, Carlsbad, CA) and sequenced in both directions.

Generating Anti-human Nestin Antiserum

Subcloning of the nestin cDNA fragment, bacterial expression of the nestin fragment, and inoculation of rabbits were conducted using protocols developed and used by Veritas Laboratories (Bethesda, MD). Primers (5′-TGTGCATATGGAGACCCTTCCAGACTCACATCC-3′ and 5′-TGTGCATATGTCATGGCCCAGGTGAACAGGAG-3′) were used to amplify the 3′ end region of the cloned 998-bp nestin fragment. The PCR product was cut at *Nde*I restriction endonuclease sites located within the primers, resulting in the 450-bp fragment. The 450-bp fragment from the cloned human nestin cDNA was then ligated into pGEX 4T-1 bacterial expression vector containing a glutathione *S*-transferase (GST) tag at the C-terminus. The resulting vector was transformed into BL21 (DE3) competent cells and protein expression was induced with 1 mM IPTG. The 150 amino acids of the human nestin which were produced correspond to positions 1464 to 1614 of the human nestin protein sequence (7). The GST fusion protein was purified over a GST column and was then used to inoculate two rabbits, resulting in nestin-331A and nestin-331B antisera. Similar immunostaining results were obtained with both the 331A and the 331B antiserum; however, all experiments in this study were conducted using the nestin-331B antiserum. The nestin-

331A antiserum was not used for experiments described in this study due to a lower apparent sensitivity.

SDS–PAGE Immunoblotting

Cultured cells or brain tissue were resuspended in 50–200 µl Laemmli sample buffer containing β-mercaptoethanol, sonicated, and boiled at 95°C for 15 min. Total cell lysates (20 µg) were electrophoresed on a 4–15% gradient SDS–polyacrylamide gel (Bio-Rad, Hercules, CA) as previously described (20) and electrophoretically transferred (40) onto Opti-tran nitrocellulose membrane (Schleicher and Schuell, Keene, NH) for 16 h at 15 V at 4°C. Nonspecific protein binding to the nitrocellulose membrane was blocked with PBS containing 5% nonfat milk and 0.05% Tween (PBS/milk/Tween). The membranes were incubated in a 1:5000 dilution of the nestin-331B antiserum in PBS/milk/Tween for 1 h at room temperature. Blots were then incubated for 1 h with peroxidase-conjugated anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1:5000, followed by chemiluminescence detection of peroxidase activity (NEN, Boston, MA).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.02% Triton. The fixed cells were then incubated with mouse monoclonal antibodies against vimentin (1:100 dilution; Dako, Carpinteria, CA), glial fibrillary acidic protein (GFAP 1:50 dilution; Boehringer Mannheim), or microtubule-associated protein-2 (MAP-2 1:200 dilution; Calbiochem, San Diego, CA) at room temperature for 60 min followed by fluorescein-conjugated goat anti-mouse secondary antibody (1:100 dilution; Jackson ImmunoResearch) for 60 min. A second primary antibody (rabbit anti-nestin-331B at 1:200 dilution) was then added for 60 min followed by rhodamine-conjugated donkey anti-rabbit secondary antibody (1:100 dilution) for 60 min. Cells were incubated with bisbenzimide (5 ng/ml) for 10 min to label nuclei of all cells in the culture. Immunopositive cells in four fields of approximately 60–100 cells were counted for each antibody combination that was done in duplicate from each brain sample cultured. Four brain samples examined were cultured for 1 week, and three brain samples examined were cultured for 4 weeks.

For 5-bromo-2′-deoxy-uridine (BrdU) labeling experiments, primary human fetal brain tissue was dissociated and cultured directly in neural basal medium (Life Technologies, Grand Island, NY) containing N2 components (Life Technologies), antibiotics, and 30 ng/ml basic firoblast growth factor (bFGF) (Sigma). Cells were grown in the serum-free medium for 72 h before replacing medium with serum-free medium containing 10 µmol/L BrdU for 48 h. Cells were then fixed with 70% ethanol in 50 mM glycine buffer, pH 2.0, followed by detection of BrdU using a fluorescein-conjugated mouse anti-BrdU antibody (1:20 dilution; Boehringer Mannheim). Immunofluorescent labeled cells were examined on a Zeiss axiovert inverted microscope with appropriate fluorescence filters. Images were captured with a CCD camera and digitized using a frame grabber and Scion image program.

RESULTS

Sequence Analysis of the 3′ Region of Human Nestin mRNA

Figure 1A shows a schematic of the human nestin protein with the corresponding region of the mRNA that was cloned and sequenced. Figure 1A also shows the region of the nestin protein used to generate anti-nestin antiserum. RT-PCR cloning and sequencing of 998 nucleotides in the 3′ region of the nestin mRNA was generated from cells of two human fetal brains at 8 and 16 weeks of gestation and from the immortalized human neuroglial cell line, SVG. All three samples yielded the same sequence with 21 nucleotide differences (data not shown) from the previously published human nestin sequence (7). The two nucleotide sequences are 98% identical, with all of the differences resulting from insertions, deletions, or replacement of one to three nucleotides. Restriction digest of the 998-bp cloned cDNA demonstrated that nucleotide changes in the new sequence resulted in the loss of the *Hha*I and *Hin*PI restriction sites, both located at position 4693 in the published nestin sequence (data not shown).

The cloned 998-bp cDNA resulted in an in-frame predicted 332-amino-acid sequence that is 95% identical to the corresponding region of the published human nestin protein sequence (7). There were 17 amino acid differences between the 332 amino acids derived from the cloned cDNA compared to the published human nestin sequence (Fig. 1B). The amino acid differences were clustered in two regions. At amino acid positions 1312–1322, 8 of 11 amino acids were changed from Thr-Pro-Leu-Glu-Ser-Arg-Gly-His-Pro-Leu-Lys to Asp-Pro-Thr-Gly-Glu-Gln-Arg-Pro-Pro-Pro-Gln-Gly. Similarly, at positions 1580–1585, 5 of 6 amino acids were changed from Ser-Gly-Ala-Arg-Asn-Ala to Val-Gly-Gln-Gly-Met-Pro. Subsequent protein sequencing of the nestin protein fragment used to generate the nestin antiserum confirmed the amino acid sequence described in this report.

The 450 nucleotides at the 3′ end of the cloned 998-bp region of the human nestin cDNA were selected for use in generating the nestin antiserum (see Methods). The predicted 150-amino-acid sequence generated from the 450-bp region was chosen as the immunogen for gener-

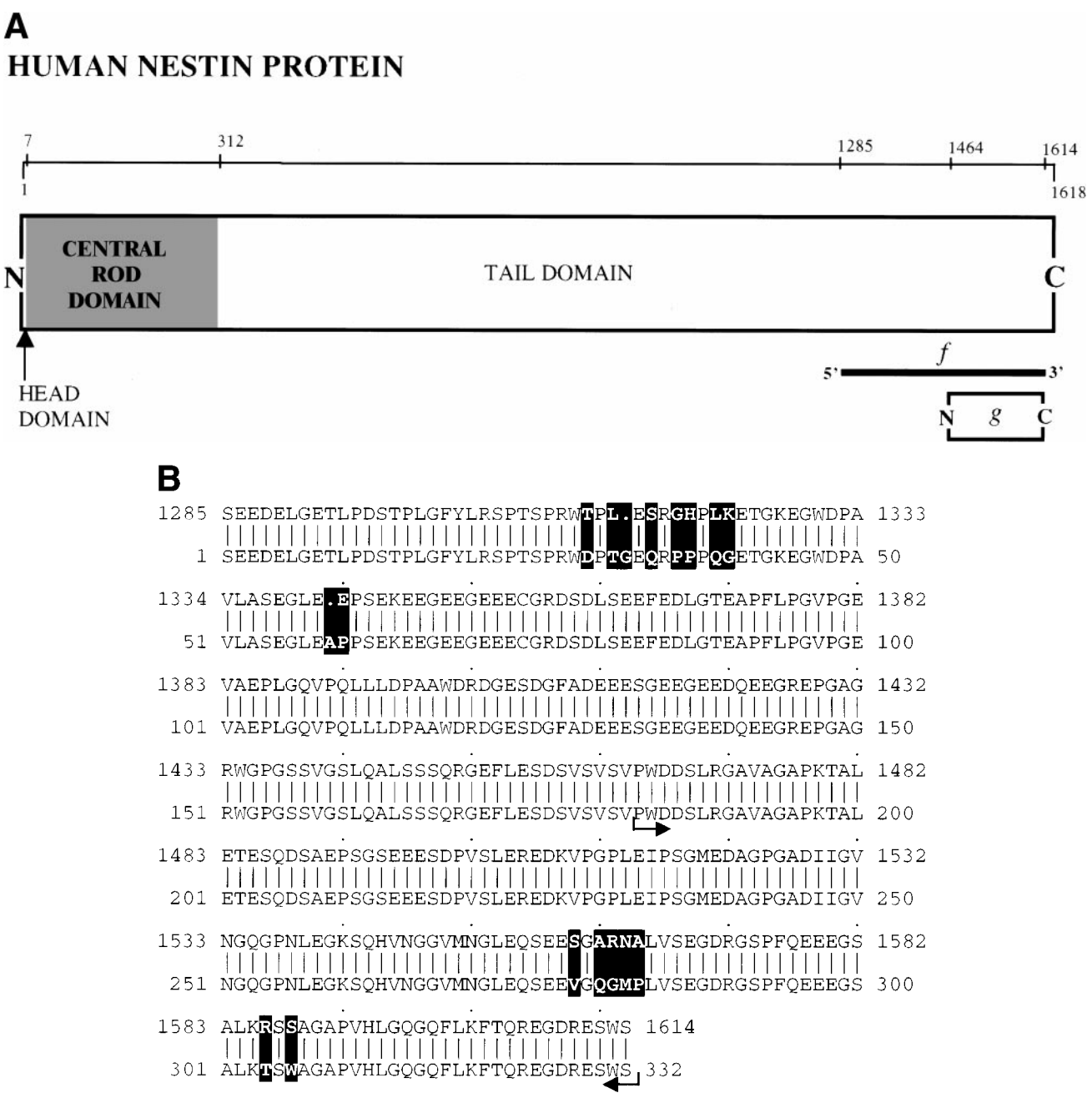


FIG. 1. Sequence of the human nestin protein and sequence comparison of the C-terminal region of human nestin. (A) Schematic of the human nestin protein showing the three protein domain regions: the head domain, the central rod domain common to the intermediate filament protein family, and the tail domain. Shown is the corresponding location of the 998-bp fragment of the human nestin mRNA that was PCR amplified and sequenced (f). Also shown is the location of the 150-amino-acid region of the cloned nestin cDNA used to generate nestin antiserum, nestin-331B (g). Numbers derived from the published human nestin protein sequence (7). (B) Comparison of the predicted 332-amino-acid sequence from the cloned 998-bp cDNA (bottom sequence) and the corresponding region of the previously published human nestin sequence (7) (top sequence). Amino acid differences are highlighted in black. The 150 amino acids used to generate the anti-nestin antiserum, nestin-331B, used in most of this study, are located between the arrows.

ating nestin antiserum since it had no significant homology to any other intermediate filament proteins based on BLAST database searches (1). Furthermore, that region is located outside the central rod domain common to intermediate filament proteins and is only 48% identical to the similar region of the rat nestin protein sequence. The antiserum generated against the 150-amino-acid region, named nestin-331B, was used for the experiments in this study to detect nestin in human CNS cells.

Expression of Nestin in Human Glioma Cell Lines

RT-PCR demonstrated nestin expression in U251 and U373 human glioma cell lines, as previously reported (21, 36, 38), and in SVG cells (Fig. 2A). The antiserum generated in this study, nestin-331B, detected nestin from total cell protein extracts of both glioma cell lines on Western blots (Fig. 2B). The doublet protein bands of nestin detected with nestin-331B antiserum migrated to 220–240 kDa on denaturing SDS gels, as previously reported (5, 13, 38). A very low amount of nestin protein was also detected from total cell extracts of SVG cells, consistent with RT-PCR data (Fig. 2B). There was no observed cross-reactivity of the nestin-331B antiserum with any other human proteins

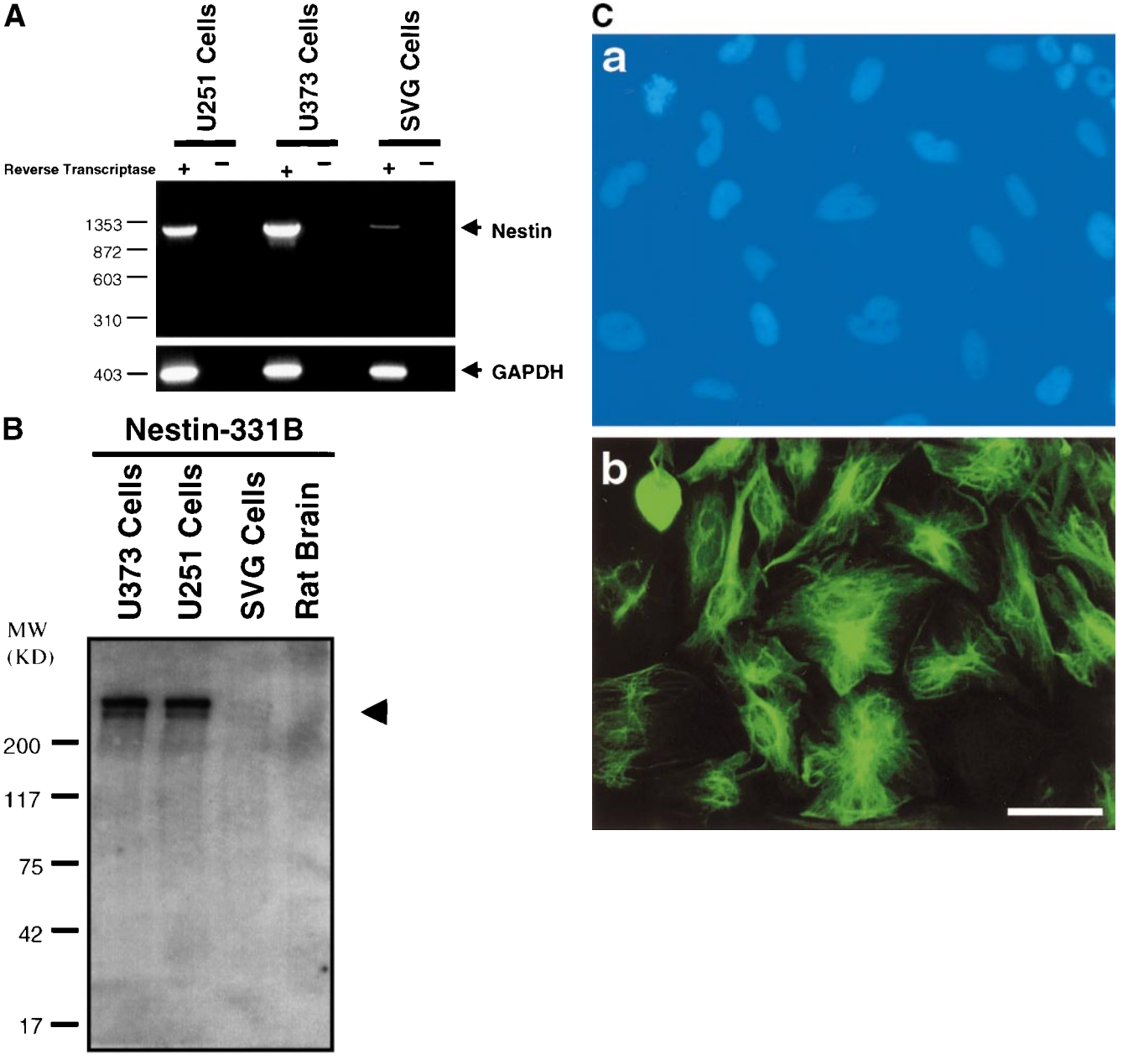


FIG. 2. Expression of nestin in U251, U373, and SVG cell lines. (A) Agarose gel electrophoresis of RT-PCR product of nestin using GAPDH as a control. The 998-bp nestin product found in all three cell lines is indicated by an arrow. (B) Western blot of total cell extracts from U251, U373, and SVG cell lines as well as from newborn rat brain immunoblotted using the nestin-331B antiserum. The arrowhead indicates the electrophoretic mobility of the nestin protein bands located at 220–240 kDa. (C) Immunofluorescence staining of the human glioma cell line, U251. Shown are nuclei stained with bisbenzimide (a) and cells stained by nestin-331B antiserum (b, 1:200 dilution). Note the filamentous network staining within the cells. Bar, 50 μm.

on Western blot, and no detection of nestin protein from newborn rat brain (Fig. 2B). In comparison, the antibody used in previous studies to detect nestin in human cells (5, 38), nestin-130, detected similar protein bands on denaturing SDS gels at 220–240 kDa from protein extracts of U251 and U373 glioma cell lines, as well as from newborn rat brain cells (data not shown). The nestin-331B antiserum immunostained U251 (Fig. 2C) and U373 (data not shown) cells, showing filamentous staining as previously reported (21, 36, 38).

Expression of Nestin in HFBC

Nestin expression was detected by RT-PCR in all 8- to 16-week gestation HFBC samples tested in this study (Fig. 3). Nestin expression was observed in noncultured brain tissue and in HFBC cultured for up to 4 weeks in serum-containing medium (Fig. 3). The nestin-331B antiserum detected nestin protein in total cell extracts of all 8- to 16-week gestation HFBC (Fig. 4). The doublet protein bands of nestin detected by the nestin-331B antiserum migrated to 220–240 kDa on denaturing SDS gels (Fig. 4), as was found in the glioma cell lines (Fig. 2B). Nestin protein was detected in noncultured brain tissue and in HFBC cultured for up to 4 weeks, consistent with RT-PCR data.

This study further examined nestin expression in progenitor cells in the developing human brain. Dissociated human fetal brain tissue was cultured directly in serum-free medium containing 30 ng/ml bFGF. Cells that divided in response to bFGF incorporated BrdU into their nuclei and coimmunostained for nestin (Fig. 5), indicating nestin expression in human CNS progenitor cells. Double immunocytochemical staining was performed to further phenotypically characterize nestin-expressing cells from the human brain. Nestin was immunostained along with GFAP that identified astrocytes, MAP-2 that identified neurons, and vimentin that identified cells of mesenchymal origin. In cultured HFBC, nestin-positive cells composed 30–60% of the total cell population. In all HFBC cultured in serum-containing medium, nestin costained with vimentin (Figs. 6A, 6D, and 6G). However, not all vimentin-stained cells coimmunostained for nestin, suggesting that there is no cross-reactivity of the nestin antiserum with vimentin (Figs. 6A, 6D, and 6G). A small subset of nestin-stained cells (1–2%) also immunostained with neuronal marker MAP-2 (Figs. 6B, 6E, and 6H) during the first week of culture and after 4 weeks in culture. For HFBC in the first week of culture, approximately 10–30% of the total cell population stained for GFAP and nearly all coimmunostained for nestin (Figs. 6C, 6F, and 6I). Nestin-positive cells were also found in cells cultured for up to 4 weeks (Fig. 7), consistent with RT-PCR and Western blot data. After 4 weeks in culture there was the emergence of a subset of GFAP-positive

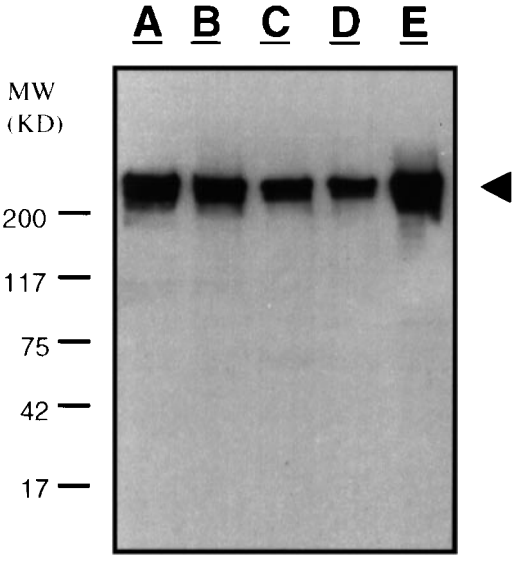


FIG. 4. Western blot of total cell extracts from HFBC immunoblotted using nestin-331B antiserum. Shown are proteins from (A) a noncultured 8-week gestation brain tissue; (B) a 9-week gestation brain cultured for 4 weeks; (C) a noncultured 10-week gestation brain; (D) a 14-week gestation brain cultured for 2 weeks; and (E) a 16-week gestation brain cultured for 2 weeks. Arrowhead indicates the electrophoretic mobility of the nestin protein bands at 220–240 kDa in all samples.

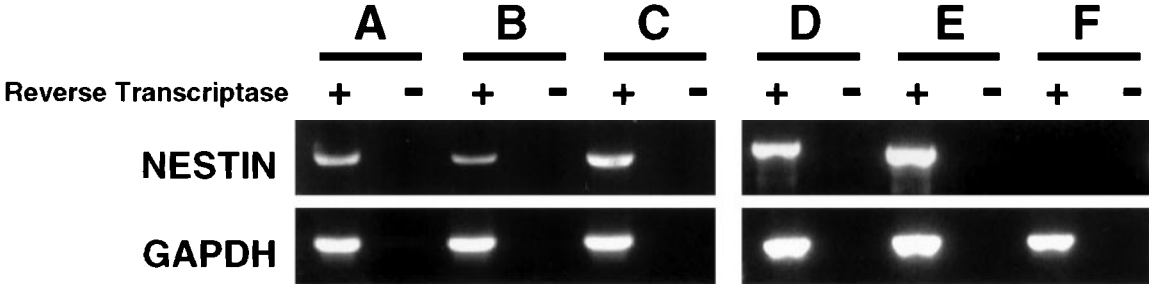


FIG. 3. Expression of nestin along with GAPDH in HFBC determined by RT-PCR. Total RNA was examined from (A) a 9-week gestation brain cultured for 4 weeks; (B) a 14-week gestation brain cultured for 2 weeks; (C) a noncultured 10-week gestation brain; (D) an 8-week gestation brain cultured for 1 week; (E) a 16-week gestation brain cultured for 1 week; and (F) peripheral blood mononucleated cells used as a control.

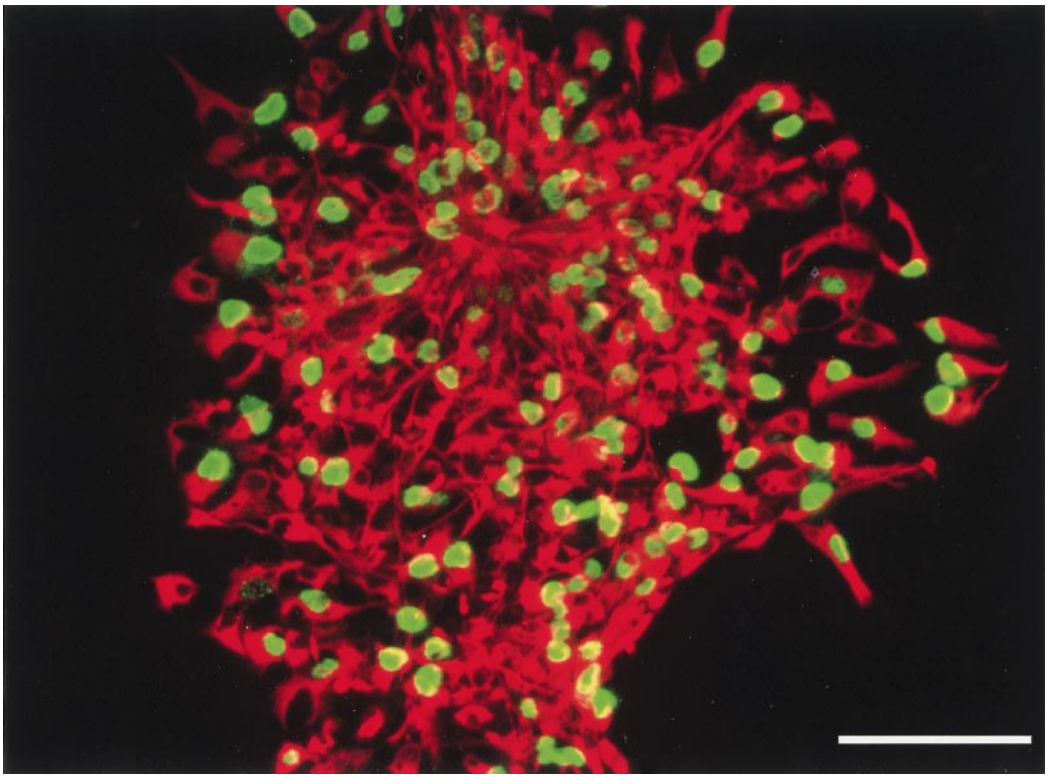


FIG. 5. Double immunofluorescence staining for nestin and BrdU. Cells from a 10-week gestation human fetal brain were cultured in serum-free medium containing 30 ng/ml bFGF for 3 days followed by the addition of BrdU for an additional 2 days. Cells were immunostained for nestin using the nestin-331B antiserum (red, 1:200 dilution) and for BrdU (green, 1:20 dilution). The overlay of the BrdU and the nestin staining is shown. Bar, 100 μm.

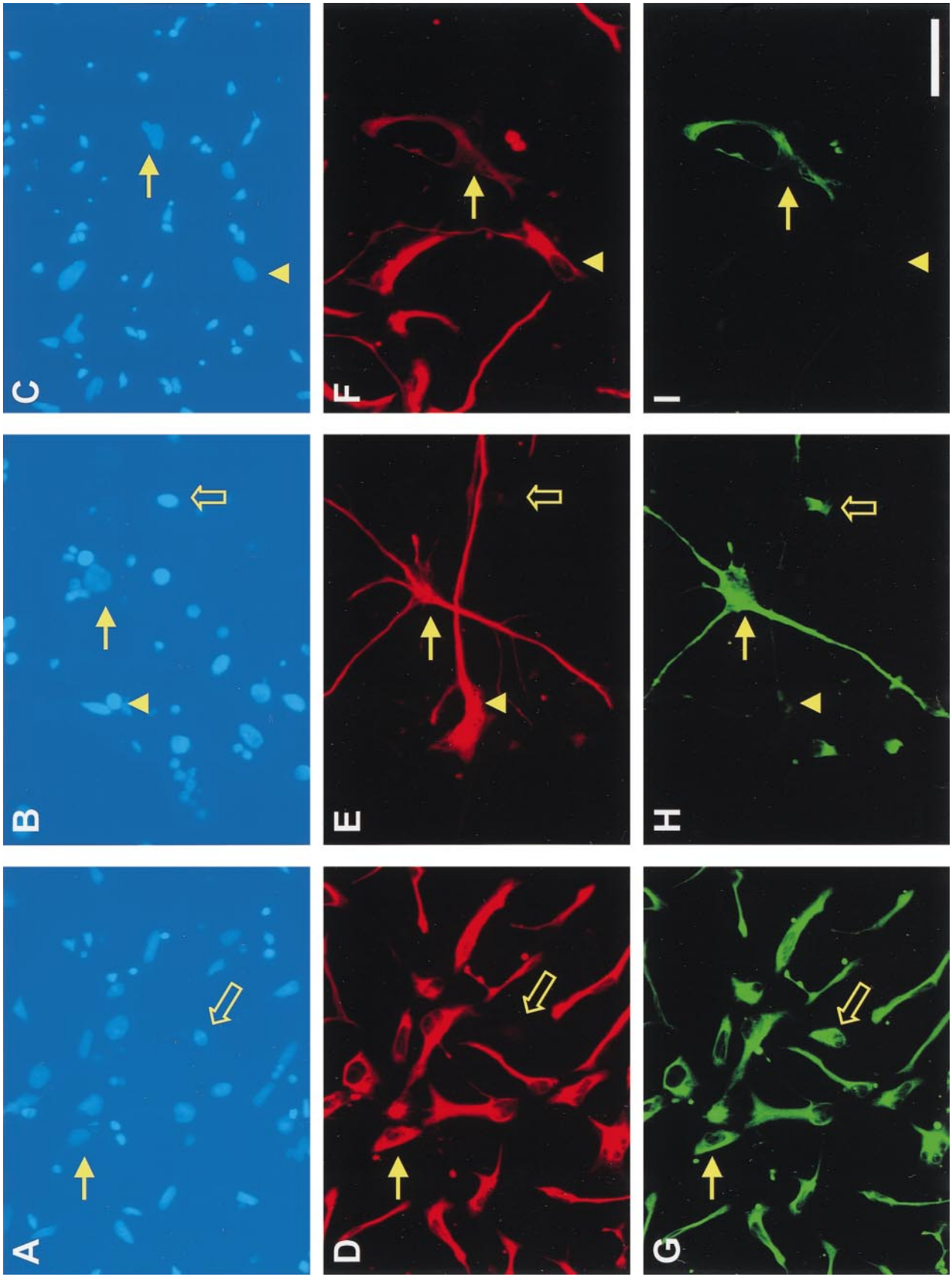
cells that no longer costained with nestin (Fig. 7). After 4 weeks in culture nestin still coimmunostained with vimentin (data not shown). The presence of GFAP-stained cells that did not coimmunostain with nestin again suggested that there is no cross-reactivity of the nestin antiserum with GFAP (Fig. 7).

DISCUSSION

Sequence Analysis of the Cloned 3′ Region of Human Nestin cDNA

The cloned 998-bp segment in the 3′ region of the human nestin mRNA was derived from two independent human fetal brains and a brain-derived cell line. There were 21 nucleotide differences between the cloned nestin fragment and the previously published human nestin sequence (7). The sequence obtained in this study was confirmed by sequencing in both directions from all three samples, thus minimizing the likelihood that the nucleotide differences were a result of PCR error. Furthermore, the absence of the *Hha*I and *Hin*PI restriction sites aid in confirming nucleotide changes in the new sequence. In addition, a sequence from the EST database (Accession No. T28212) from human embryo contained 12 of the nucleotide differences found in this study. Together these data provide strong support for the validity of the sequence obtained in this study. The 998-nucleotide sequence resulted in an in-frame 332-amino-acid sequence for the entire sequence (Fig. 1B). The 332 amino acids are 95% identical to the published human nestin amino acid sequence (7). Since the sequence obtained from this study contained differences with the previously published nestin sequence, we cannot rule out the possibility that we have identified a different isoform of the nestin protein. It is also possible that there are polygenic regions in the nestin gene that are responsible for the nucleotide differences described in this study. Existing EST sequences contain a variety of nucleotide differences from both the sequence obtained in this study and the sequences previously published (Accession No. AA181605, AI810042, and C15784). In addition, Northern blots revealed only one mRNA band (data not shown). Cloning studies would be required to determine if there are indeed multiple forms of the nestin mRNA. Such studies are presently being pursued.

The 150 amino acids used to generate the nestin antiserum were derived from the cloned nestin cDNA. This 150-amino-acid sequence is only 48% identical to the rat nestin protein and has no significant homology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with vimentin, GFAP, or the neurofilament proteins. Protein sequencing of the 150-amino-acid peptide used to generate the nestin antiserum confirmed the predicted amino acid sequence obtained in this study. The 150 amino acids include seven differences with the previously published human nestin amino acid sequence (7). This includes a stretch of 5 of 6 amino acids changed from Ser-Gly-Ala-Arg-Asn-Ala to Val-Gly-Gln-Gly-Met-Pro at positions 1580–1585. In particular, a change from an alanine to a proline occurring at position 1585 may alter the conformation of the protein. These regions of amino acid differences may serve as important epitopes to determine if there are different expressed nestin proteins. Further study is required to determine the existence of and the functional significance of different protein isoforms of nestin in the developing human CNS.

Characterizing the Human Specific Nestin Antiserum

RT-PCR demonstrated nestin expression in 8- to 16-week gestation HFBC and in U373 and U251 glioma cell lines. The nestin-331B antiserum detected nestin protein of the appropriate molecular weight in HFBC (Fig. 4) and in the glioma cell lines (Fig. 2B). The discrepancy between the apparent molecular weight of human nestin on denaturing SDS gels and the predicted molecular weight of 200 kDa has been previously reported (5, 13, 36, 38). The nestin protein sequence has several consensus sequences for phosphorylation, myrstylation, and glycosylation that are likely responsible for the electrophoretic mobility of the nestin protein. Phosphorylated and glycosylated forms of nestin may affect intracellular localization or act as a means of functional regulation in specific cell types or brain regions. The neurofilament proteins of the class IV intermediate filament family exemplify posttranslational regulation in development and disease (15, 29, 31). Similar regulation may exist for nestin. The nestin-331B antiserum is specific to human nestin as it did not detect nestin from newborn (Fig. 2B) or E14 (data not shown) rat brain cells. The faint nestin protein bands observed in SVG cells (Fig. 2B) corresponded to a low-level nestin expression observed by RT-PCR (Fig. 2A). In addition the nestin-331B antiserum was able to detect nestin intermediate filaments in HFBC and in the glioma cell lines with no apparent cross-reactivity with GFAP or vimentin.

An antibody that specifically detects nestin in the human brain can aid in the characterization of subtypes of progenitor cells differentiating into distinct lineages. Also, the nestin-331B antiserum may have special utility in assessing survival and differentiation of human CNS progenitor cells transplanted into rodent brain in cellular grafting studies. To our knowledge, there is presently no other antibody available that detects human nestin without cross-reacting with rodent nestin. In addition, the antiserum may prove to be useful for identification of neuroepithelial tumor cells, particularly gliomas and glioblastomas as previously shown (5, 38). Efforts are currently being undertaken to generate monoclonal antibodies using the same 150-amino-acid fragment used to generate the nestin-331B antiserum.

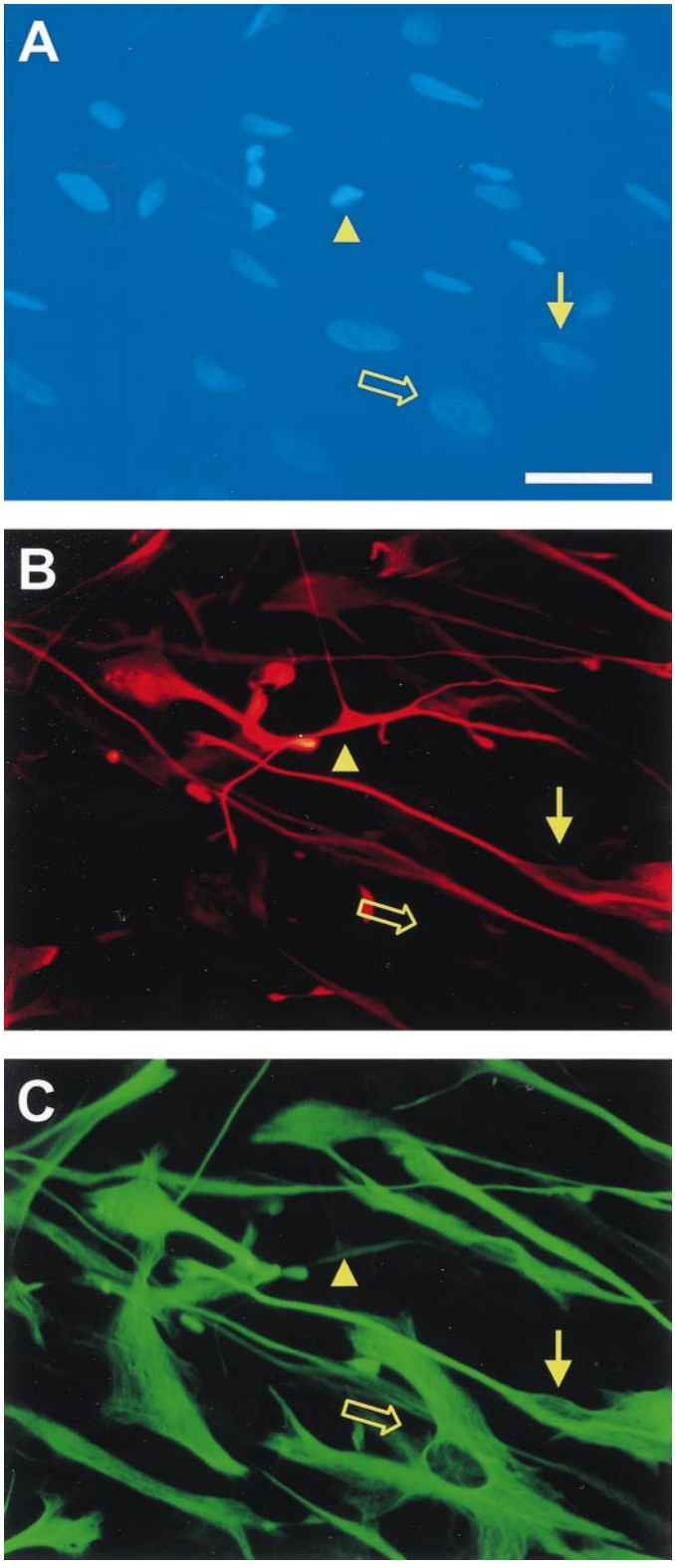
The antibodies used in previous studies (nestin-129 and nestin-130) to detect nestin in human tissue (2, 5, 37, 38) were generated against a fusion protein of the C-terminal 1197 amino acids of rat nestin protein coupled to the bacterial TrpE protein (38). The 1197 amino acids of the rat nestin are about 50% identical to the same region in the human nestin protein. It is not known if the previously generated anti-rat nestin antiserum identifies different protein forms of nestin or cell types in the human brain compared to those identified in this study. Further work could be conducted to address the specificity of the two antibodies for human cells. Other antibodies have recently been reported that detect human nestin (13, 36), but have not been extensively used to characterize nestin-expressing cells in the developing human CNS.

Phenotypic Characterization of Nestin-Expressing Cells in HFBC

All HFBC samples examined in this study contained multiple populations of cells that include neurons expressing neurofilaments and MAP-2, astrocytes expressing GFAP, and progenitor cells expressing vimentin and nestin. The presence of nestin in 8- to 16-weeks gestation HFBC is consistent with previous studies (38, 43); however, the differential temporal expression of nestin in developing neurons and astrocytes has not been reported.

To our knowledge, this is the first study to specifically examine cell-type-specific expression of nestin in developing human CNS cells. Proliferating CNS cells that

FIG. 6. Double immunocytochemical staining for nestin along with vimentin, GFAP, and MAP-2. All panels were from the immunostaining of a 8-week gestation HFBC cultured for 4 days. Each column represents cells in the same field (A, D, G), (B, E, H), and (C, F, I). The top panels (A, B, C) are cells stained with the nuclear dye, bisbenzimide. The middle panels (D, E, F) are cells stained with 1:200 dilution of nestin-331B antiserum. The lower panels are cells stained for vimentin (G, 1:100 dilution), MAP-2 (H, 1:200 dilution), and GFAP (I, 1:50 dilution). Arrows in A, D, and G indicate a cell costained for nestin and vimentin while the open arrows indicate a cell stained for vimentin but negative for nestin. Arrows in B, E, and H show a cell costained for MAP-2 and nestin, while the arrowheads show a cell stained for nestin but negative for MAP-2. The open arrows show a cell stained only for MAP-2. Arrows in C, F, and I indicate a cell costained for nestin and GFAP, while the arrowheads show a cell stained for nestin but negative for GFAP. Bar I, 50 μm.



incorporate BrdU in serum-free medium containing bFGF were previously demonstrated to be progenitor cells (3, 14, 28). The proliferating cells detected by the nestin-331B antiserum (Fig. 5) suggest nestin expression in human CNS progenitor cells. The progenitor cells in medium containing bFGF largely did not stain for GFAP or MAP-2, indicating that these cells had not committed to a lineage pathway.

The coexpression of nestin and vimentin shown in this study (Figs. 6A, 6D, and 6G) has been previously suggested and is also consistent with nestin expression in neuroepithelial progenitor cells (34, 38, 39). The coexpression of nestin and GFAP during the first week in culture (Figs. 6C, 6F, and 6I), along with the appearance of GFAP-expressing cells that no longer express nestin after 4 weeks in culture (Fig. 7), suggest cells in transition from the progenitor state to differentiation into astrocytes. Cells have subsequently been examined after 7 weeks in culture with a similar pattern of results. These cultures showed a greater number of GFAP-positive cells that were nestin negative as well as less intense nestin staining (data not shown). There have been recent reports in rodent CNS cells that describe coexpression of nestin and GFAP in reactive astrocytes following CNS injury (17, 22, 23, 27). However, the phenotypic identification of transition progenitor cells in normal human brain cells is novel. Based on previous studies, the astrocytes cultured in this study are essentially not reactive since they produce barely detectable levels of TNFα or prostaglandins, but can be activated by IL-1β, HIV tat, and PMA (4, 8, 42, and unpublished observations).

The small number of neurons coexpressing nestin is consistent with previous studies that suggest loss of nestin expression in rodent progenitor cells that differentiate into neurons (3, 11, 18, 33). However, the absence of a similar pattern of transitional neuronal progenitor cells expressing nestin suggests a differential temporal regulation of nestin in neurons and astrocytes. An antibody that detects another early neuronal differentiation marker, β-tubulin BIII (TUJ1), similarly showed small numbers of neurons costaining with nestin after 1 and 4 weeks in culture. It may also be possible that progenitor cells in serum-containing medium preferentially differentiate into glia, or that a subset of neuronal progenitor cells express nestin. Alternatively, other culture conditions not performed in

FIG. 7. Double immunofluorescence staining for nestin and GFAP. Staining was conducted on a 9-week gestation HFBC in culture for 4 weeks. Nuclei were stained with bisbenzimide (A), and cells were stained for nestin using nestin-331B antiserum (1:200 dilution (B) and for GFAP (1:50, C). Arrows indicate a cell costained for nestin and GFAP, while the open arrows indicate a cell stained for GFAP but negative for nestin. The arrowheads indicate a cell stained for nestin but negative for GFAP. Bar A, 50 μm.

this study may preferentially support neuronal progenitors (41).

This study has contributed information that suggests that there may be multiple levels of regulation of nestin in the developing CNS, as have been suggested (23, 25). Sequence differences in the nestin cDNA, multiple bands of nestin protein on Western blots, and the differential temporal protein expression of nestin in neuronal and glial cells supports the existence of multiple regulation of nestin expression. Recent studies have begun to describe enhancer elements that are involved in general and regional-specific expression of nestin in the CNS (24, 25, 45). Additional studies that determine the temporal expression of nestin in neuronal cells may prove important for the understanding of neuronal progenitors. In conclusion, studies in our laboratory have been conducted to further examine cellular and molecular changes in phenotypic cell markers in the developing human CNS, including intermediate filaments, to aid in the understanding of cellular differentiation (manuscript in preparation).

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