Basonuclins 1 and 2, whose genes share a common origin, are proteins with widely different properties and functions

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Communicated by Howard Green, Harvard Medical School, Boston, MA, June 22, 2006 (received for review May 7, 2006)

Basonuclin (bn) 1 possesses three separated pairs of zinc fingers and a nuclear localization signal. It is largely confined to the basal cells of stratified squamous epithelia and to reproductive germ cells. bn1 can shuttle between the nucleus and the cytoplasm, and its location is correlated with the proliferative potential of the cell. The recently discovered bn2 also possesses three separated pairs of zinc fingers and a nuclear localization signal. Conservation of the zinc fingers and the nuclear localization signal by bn1 and bn2 indicates a common origin. However, in contrast to bn1, bn2 is found in virtually every cell type and is confined to the nucleus. Bn2 but not bn1 colocalizes with SC35 in nuclear speckles and, therefore, is likely to have a function in nuclear processing of mRNA.

RNA processing | speckles | zinc fingers

Basonuclin (bn1) is a zinc finger protein with highly restricted tissue distribution: It is found mainly in basal keratinocytes of stratified squamous epithelium and in reproductive germ cells (1–5). bn1 possesses three separated pairs of zinc fingers, a nuclear localization signal (NLS) and a serine-rich region that has been called the serine stripe (1). Although the evidence is not conclusive, it seems that the function of bn1 is related to the potential for cell proliferation (6). The only known function of bn1 is that of a transcription factor in the synthesis of ribosomal RNA (7, 8), but it is possible that bn1 possesses a nucleoplasmic function in the regulation of expression of genes transcribed by RNA polymerase II (9).

A gene encoding a second basonuclin (bn 2) recently has been discovered (10, 11). Although the deduced amino acid sequences of bn1 and bn2 are only slightly >40% identical, bn2 possesses all of the characteristic features of bn1 described above. The bn2 mRNA is abundant in cell types that possess bn1 but is also found in tissues that lack bn1, such as kidney, intestine, and uterus. The genes for bn1 and bn2 differ greatly in size and are located on different chromosomes, but it is clear that they have a common evolutionary origin. The evolutionary conservation of bn2 is much greater than that of bn1. It has been postulated that the gene for bn2 is the older of the two. After its duplication, the gene for bn1 was free to evolve in other directions, whereas the gene for bn2 remained virtually invariant (9, 11).

Previous work on immunocytological detection of bn1 was carried out with polyclonal antisera raised against most or all of the bn1 protein (6, 12). In view of the similarities between bn1 and bn2, it was possible that these antisera did not distinguish between the two basonuclins. To detect each basonuclin independently, we generated antibodies to specific determinants not shared by bn1 and bn2. Using these antibodies, we show that, although the two basonuclins are of common origin, they possess widely different functions, because bn2 is likely to have a function in pre-mRNA processing.

Results

Evolutionary Conservation of bn2. We had reported that bn2 was extremely conserved in vertebrates, more so than bn1. Human and mouse bn2 showed 97.2% identity at the amino acid level, whereas the corresponding value for bn1 was 88% (11). This study suffered from two limitations: (*i*) it was not clear whether the bn2 sequences included the N-terminal part of the protein because the ORF of the cDNA from which the protein sequence was deduced extended up to the first nucleotide of the cDNA, and (*ii*) bn2 exists as a multitude of splicing isoforms, and it was possible that the mouse cDNA used in the comparison had been derived from one of the minor mRNA isoforms.

We have recently characterized the 5′ end of both human and mouse bn2 mRNA by 5′ RACE and determined that the main bn2 isoform consists of 1,099 residues encoded by seven exons (13). We determined the human/mouse identities of both bn1 and bn2 and compared these identities with those of 48 other randomly chosen C2H2 zinc finger proteins by using the ALIGNp program (Fig. 1*A*). It can be seen that bn2 was among the most conserved C2H2 zinc finger proteins: Eight proteins exceeded bn2 in their level of conservation, whereas 41 proteins showed less conservation. Even essential proteins, such as Sp1, GATA1, and TFIIIA, showed substantially less conservation than bn2. The unusual evolutionary stability of the bn2 sequence suggests that the protein possesses an essential function. In contrast, the level of evolutionary conservation of bn1 was below average (Fig. 1*A*), although the protein recently has been shown to be required for embryonic development (14).

The human sequence then was used as a guide in the identification of the bn2 exons present in chicken and *Xenopus* genomic sequences. From these exons, nearly complete bn2 sequences of the two species were assembled. We also assembled the previously unreported sequences of both chicken and *Xenopus* bn1 (see Table 1, which is published as supporting information on the PNAS web site). Alignment of bn1 and bn2 of the human, mouse, chicken, and *Xenopus* by using the ClustalX program allowed us to determine the parts of the two proteins that were most conserved in evolution (Fig. 1*B*). Conservation of bn2 was not evenly distributed over the length of the protein. A segment of 244 residues toward the N-terminal region of the protein (residues 102–345) was extremely conserved: 236 residues were identical in all four species examined. This segment cannot be related to any known function. In the rest of the molecule, conservation was strongest in the zinc fingers and, particularly, the first pair. The NLS and its C-terminal flanking region also were very conserved. The N-terminal region and the regions separating the pairs of zinc fingers showed less conservation, although there existed in these regions numerous blocks of conserved residues.

The most conserved regions of bn1 included a 141-residue N-terminal segment (residues 47–187), the first pair of zinc fingers, zinc fingers 4 and 6, the NLS, and the region surrounding it. All

Conflict of interest statement: No conflicts declared.

Abbreviations: bn, basonuclin; NLS, nuclear localization signal.

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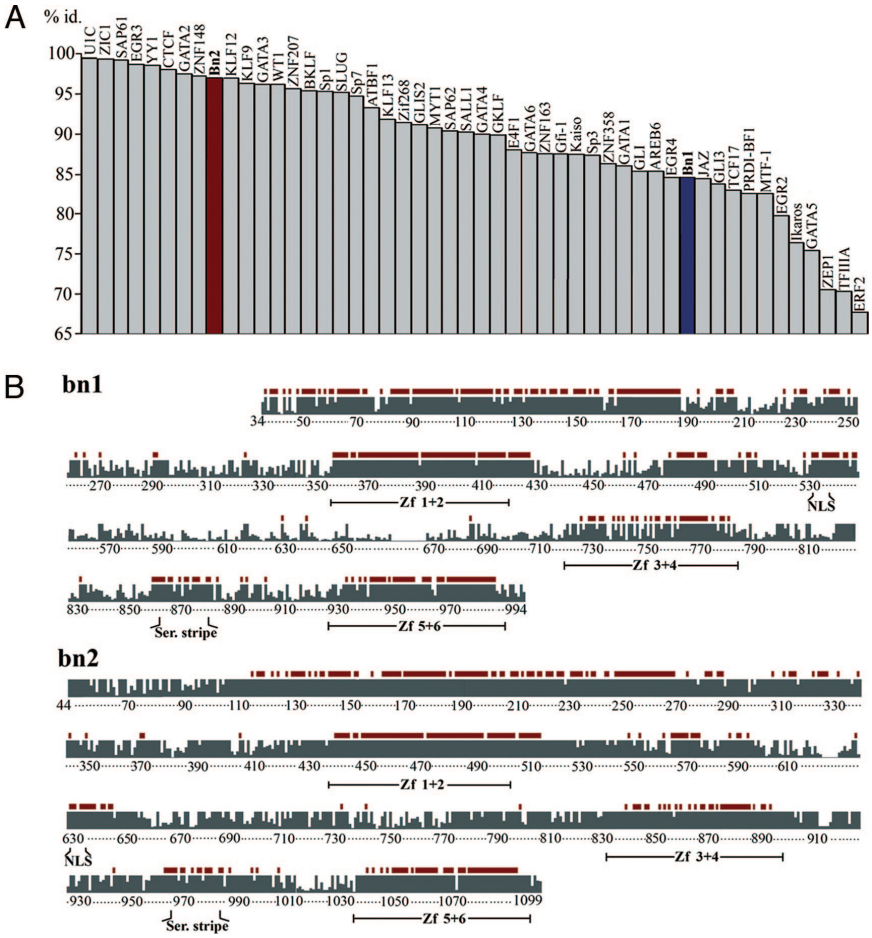

Fig. 1. Evolutionary conservation of bn1 and bn2. (*A*) Comparison of the human/mouse identities of 50 C2H2 zinc finger proteins, including bn1 (blue bar) and bn2 (red bar). bn2 is among the most conserved zinc finger proteins, whereas conservation of bn1 is below average. (*B*) Alignment of the deduced amino acid sequences of human bn1 and bn2 with the orthologous sequences of mouse, chicken, and *Xenopus*. The conservation score at each amino acid position is shown as a black rectangle. The highest score (tallest bars) indicates a residue that is conserved in all species, whereas low scores (shorter bars) indicate poorly conserved residues. Red rectangles above conservation scores indicate conserved residues that are shared by both bn1 and bn2. The ruler below the diagram shows amino acid positions in the human protein. The most conserved regions of bn2 include a stretch of ≈250 aa in the N-terminal region, the three pairs of zinc fingers, and the NLS. In these regions, numerous conserved residues of bn2 diverge from those at corresponding positions in bn1.

other regions, including zinc fingers 3 and 5, were less conserved (Fig. 1*B*).

In general, the regions that were conserved in bn1 also were conserved between bn1 and bn2. This observation is consistent with the identities between bn1 and bn2 being concentrated in the N-terminal region, the NLS, and the zinc fingers, particularly the first pair. Elsewhere, bn2 possessed numerous conserved residues that diverged from the corresponding residues of bn1 (Fig. 1*B*). This divergence implies that the functions of the two proteins are likely to be different.

Generation of Antibodies Specific to bn1 and bn2. The part of the two basonuclins that is most divergent is located between zinc fingers 2 and 3 (Fig. 1*B*). In this part, the two basonuclins show only 25% amino acid identity. The corresponding DNA fragments, encoding ≈250 residues, were amplified by PCR and subcloned into a bacterial expression vector, where they were fused in frame with the GST coding region. The fusion proteins were overproduced in bacteria, highly purified by the affinity of the GST for reduced glutathione and by SDS/PAGE, and injected into rabbits. The antisera were purified by affinity chromatography before use. A monoclonal anti-bn2 antibody was obtained by panning a human antibody phage display library with the GST-bn2 protein fragment used to produce the polyclonal antibody. Western blotting of bacterial homogenates containing the bn1 and bn2 fragments used as antigens showed that each of the three antibodies recognized specifically the basonuclin against which it was raised (Fig. 2).

bn2 Is Colocalized with SC35 in Nuclear Speckles of Human Keratinocytes and hES Cells. Because keratinocytes are the only cultivable cell type that contains both bn1 and bn2 (10, 11), they were used to compare the subcellular distribution of the two proteins. Keratinocytes were plated with 3T3 support on glass coverslips in Petri dishes at a density of 10^4 cells per cm^2. After 5 days, cells were fixed and stained with the antibodies specific for each basonuclin. Both bn1 and bn2 were found to be confined to the nucleus, where they were largely excluded from the nucleoli. Whereas bn1 appeared relatively uniformly in the nucleoplasm, bn2 was mostly concentrated in nuclear aggregates (Fig. 3*A*). Double staining with the monoclonal and the polyclonal anti-bn2 antibody showed that the aggregates stained by the two antibodies were identical. Neither bn1 nor bn2 staining was affected whether methanol or paraformalde-

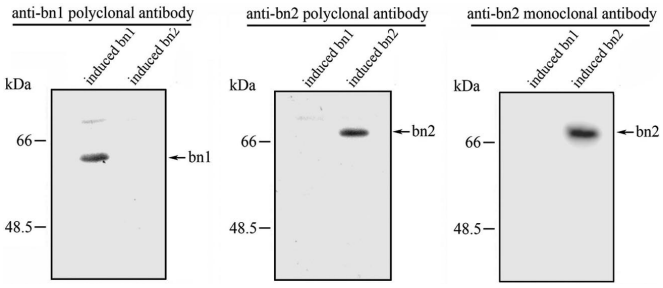

Fig. 2. Specificity of the anti-human basonuclin antibodies. Proteins prepared from bacteria containing the fusion proteins (10 μg) were analyzed by Western blotting with the polyclonal anti-bn1, the polyclonal anti-bn2, or the monoclonal anti-bn2. Each antibody recognized only the GST-basonuclin fusion protein against which it was raised and did not cross-react with the other basonuclin.

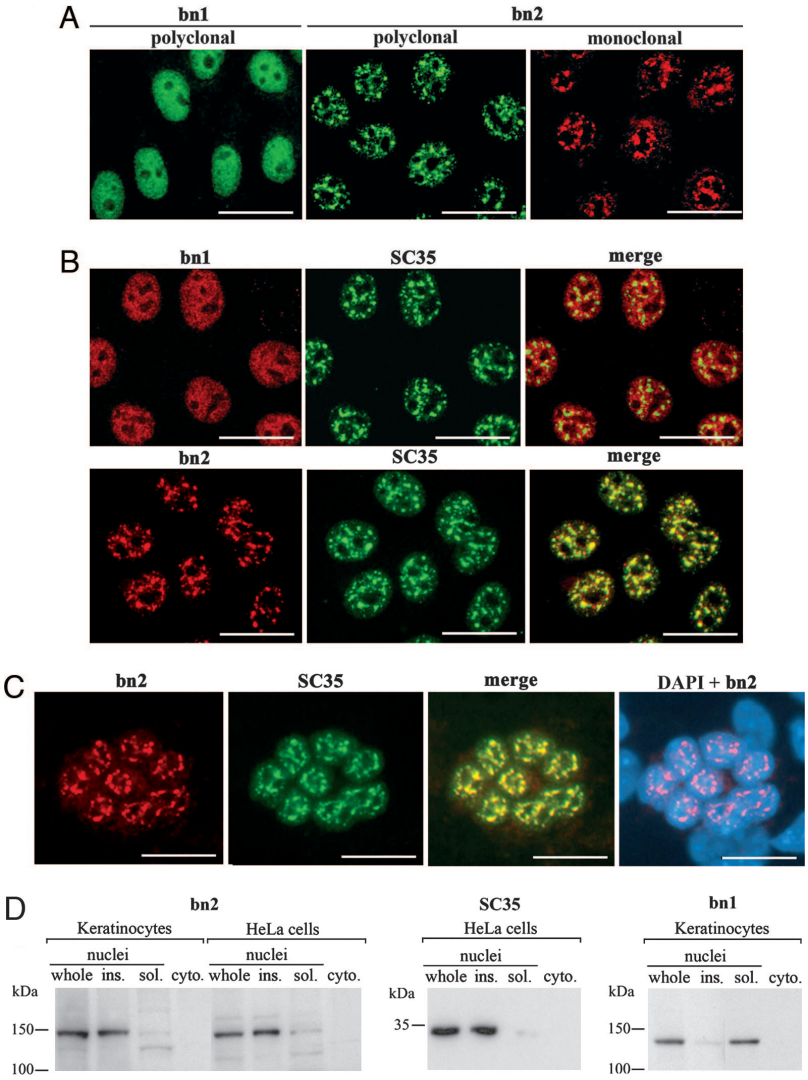

Fig. 3. bn2 in nuclear speckles and the insoluble nuclear fraction. (*A*) Rapidly multiplying human keratinocytes were fixed and stained with the polyclonal anti-bn1, the polyclonal anti-bn2, or the monoclonal anti-bn2. Both proteins are nuclear. bn1 shows a relatively homogeneous distribution throughout the nucleoplasm but is excluded from the nucleoli. As shown by two antibodies, bn2 is distributed in patches reminiscent of nuclear speckles and is likely excluded from nucleoli. (*B*) Human keratinocytes were double stained for either bn1 or bn2 and for SC35, a marker of nuclear speckles. bn2 is almost entirely colocalized with SC35 in nuclear speckles; no such colocalization is observed with bn1. (*C*) H9 human ES cells were stained for bn2 and SC35, and the DNA was counterstained with DAPI. Like human keratinocytes, hES cells show complete colocalization of bn2 and SC35. Supporting mouse fibroblasts with prominent heterochromatin are not stained by the anti-human bn2 antibody (photograph kindly provided by Shiro Iuchi, Harvard Medical School, Boston, MA). (Scale bars, 25 μm.) (*D*) Immunoblot showing insolubility of both bn2 and SC35 and solubility of bn1. bn1, bn2, and SC35 are all absent from the cytoplasm (cyto.) because they are entirely nuclear (whole nuclei), but their subnuclear distribution differs. bn1 is found in the high-salt soluble nuclear extract (sol.), whereas bn2 and SC35 both are predominant in the high-salt insoluble nuclear fraction (ins.), which contains the interchromatin granule clusters that form the speckles identified by immunofluorescence staining.

hyde was used in the fixation. The polyclonal anti-bn2 antibody produced stronger staining than the monoclonal antibody and, therefore, was used in all subsequent experiments.

The bn2-containing aggregates were reminiscent of the speckles in which splicing factors are concentrated (15–17). One splicing factor widely used for the detection of speckles is SC35 (16–18). Keratinocytes were double-stained with either the anti-bn1 or anti-bn2 antibody and with a monoclonal antibody to SC35. Virtually all of bn2 colocalized with SC35. No such colocalization was observed between bn1 and SC35 (Fig. 3*B*). The use of preimmune serum as primary antibody gave no appreciable staining of cultured keratinocytes. In hES cells also, bn2 was entirely colocalized with SC35 in nuclear speckles (Fig. 3*C*). The nearly complete colocalization of bn2 and SC35 implies associated functions of the two proteins.

When nuclei are incubated in high salt buffer, soluble nuclear proteins are released, but the interchromatin granule clusters, which are the main component of speckles, remain insoluble and can be separated from the soluble nuclear proteins by centrifugation (19). HeLa cells and cultured human keratinocytes were lysed in the presence of Nonidet P-40. Half of the homogenate was submitted to centrifugation, and both the pellet containing intact nuclei and the supernatant containing the cytosol were collected. The other half was centrifuged under the same conditions, and the nuclear pellet was resuspended in 0.4 M NaCl. After a 40-min incubation, the preparation was cleared by centrifugation. Both the supernatant consisting of the nuclear extract soluble in high salt buffer and the pellet containing the nuclear fraction insoluble in high salt buffer were collected. The same proportion of each of the four collected fractions was loaded on the gel. This proportion corresponded to the fraction of the soluble nuclear extract containing 100 μg of protein. Proteins were analyzed by Western blotting with the anti-bn1, anti-bn2, or anti-SC35 antibody (Fig. 3*D*).

The main human bn2 mRNA isoform is predicted to encode a 1,099-residue protein with a molecular mass of 122 kDa (13). The deduced bn1 protein contains 994 residues and its molecular mass is 111 kDa (1), but the electrophoretic mobility of bn1 corresponds to $\approx$125 kDa (12). The main protein detected by the anti-bn2 antibody in both keratinocytes and HeLa cells had a mobility corresponding to $\approx$145 kDa. The protein was entirely nuclear.

bn2 showed a subnuclear distribution very similar to that of SC35, because most bn2 was associated with the insoluble fraction. In contrast, bn1 was almost exclusively detected in the soluble nuclear fraction, where its electrophoretic mobility corresponded to $\approx$130 kDa. The Western blot analysis therefore confirmed the result of the immunofluorescence stainings. bn1, which showed a diffuse immunofluorescence staining typical of soluble proteins, was found in the soluble nuclear fraction by Western blot analysis, whereas bn2, which appeared aggregated in speckles by immunofluores-

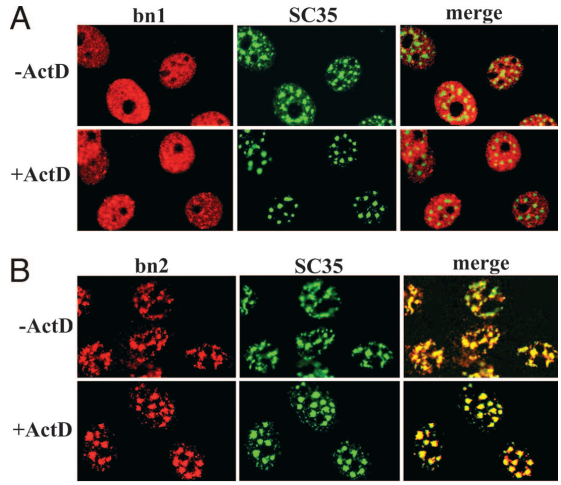

Fig. 4. Arrest of transcription affects bn2 but not bn1. Cultured keratinocytes (YF23) were treated with actinomycin D, fixed, and double stained for SC35 and either bn1 (*A*) or bn2 (*B*). Actinomycin D caused SC35 to become entirely confined to enlarged and rounded nuclear speckles. bn2 was redistributed exactly as SC35, whereas bn1 remained diffuse throughout the nucleoplasm.

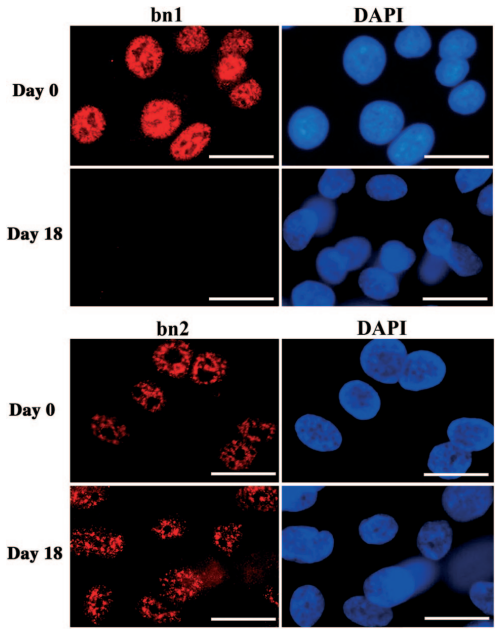

Fig. 5. Effect of cell multiplication on nuclear bn1 and bn2. Rapidly dividing human keratinocytes (day 0) and keratinocytes that had been confluent for 10 days (day 18) were stained for either bn1 or bn2. Nuclear bn1 was abundant in multiplying keratinocytes but became barely detectable in the confluent cells. In contrast, the level of nuclear bn2 was not affected by the confluent state. Supporting 3T3 cells were not stained by either basonuclin antibody. (Scale bars, 25 μm.)

cence, was detected in the insoluble nuclear fraction by Western blotting.

Inhibition of Transcription with Actinomycin D Causes Similar Nuclear Reorganization of SC35 and bn2 but Does Not Affect bn1. In cultured cells in which transcriptional level is high, speckles are irregularly shaped, connected in places, and set against a diffuse nuclear labeling. Upon inhibition of RNA pol II transcription, speckles become round and increase in size, whereas diffuse nuclear labeling disappears. These changes are caused presumably by the arrest of splicing resulting from transcriptional inhibition: All splicing factors then leave the nucleoplasm and accumulate in the speckles (20). To determine whether inhibition of transcription would cause a redistribution of bn2, multiplying keratinocytes were treated with actinomycin D for 2 h. Cells then were fixed and doubled-stained for SC35 and either bn1 or bn2. The drug did not appreciably affect the diffuse nuclear distribution of bn1 (Fig. 4*A*), whereas it caused redistribution of both SC35 and bn2 to enlarged and rounded speckles and a complete loss of diffuse nuclear staining (Fig. 4*B*). These results lend further support to the idea that bn2 participates in mRNA processing.

The Presence of bn2 Is Independent of the Growth Potential of the Cell. The synthesis of bn1 in keratinocytes is regulated. In cultured keratinocytes, the disappearance of bn1 mRNA is associated with the loss of colony-forming ability and the appearance of the mRNA for involucrin, a protein specific of terminally differentiated keratinocytes. These observations have lead to the conclusion that bn1 possesses a regulatory function in the maintenance of proliferative potential and the prevention of terminal differentiation (6).

To determine whether the synthesis of bn2 was similarly affected by the proliferative potential of the cell, we inoculated keratinocytes at a density of 10^4 cells per cm^2 in the presence of supporting 3T3 cells. Two days later, when the cells were multiplying rapidly, some cultures were fixed (day 0), whereas the others continued to be fed. These cultures reached confluence after 8 days and were allowed to remain in the confluent state for another 10 days (day 18). All cultures then were stained for either bn1 or bn2. In cultures of rapidly multiplying keratinocyte fixed at day 0, bn1 was found in the nucleus of virtually every cell, but by day 18, bn1 had become barely detectable, presumably because the cells were undergoing terminal differentiation and had lost their proliferative potential. In contrast, staining for bn2 remained as strong at day 18 as it was in the rapidly multiplying keratinocytes stained at day 0 (Fig. 5). We may conclude that the level of bn2 does not depend on the growth potential of the cell.

bn2 Does Not Translocate Between the Nucleus and the Cytoplasm. In growing colonies of human keratinocytes cultivated in the presence of supporting 3T3 cells, bn1 is entirely nuclear, but when 3T3 cells are removed, growth is reduced and bn1 becomes mainly cytoplasmic (5). To determine whether bn2 would similarly leave the nucleus after removal of 3T3 support, we cultivated human keratinocytes in the presence of 3T3 cells, and then removed 3T3 cells and resumed the cultivation for an additional 6 days. We did observe that after 6 days in the absence of 3T3 support, bn1 had largely translocated to the cytoplasm. Four hours after 3T3 cells were added back, all bn1 had left the cytoplasm to accumulate in the nucleus (Fig. 6*A Left*). In contrast, removal of 3T3 support did not affect the localization of bn2, which remained entirely nuclear at all times (Fig. 6*A Right*). These experiments showed that bn2 did not shuttle between the nucleus and the cytoplasm, depending on factors affecting cell growth.

It has been shown that nuclear localization of human bn1 depends mainly on the dephosphorylated state of serine 541, located immediately C-terminal to the NLS. Ser-537, located inside the NLS, has a similar but weaker effect (12). The region of the NLS of bn1 is conserved entirely between the fishes, the frog, the chicken, and the mammals. The region of the NLS of bn2 also is identical from fishes to mammals. The only difference between the regions of the NLS of the two proteins lies in the replacement of Ser-537 of bn1 by a proline in bn2. We suggest that bn2 does not translocate to the cytoplasm because the introduction of a helix-breaking proline at position 537 alters the relation of the serine at position 541 to the signal sequence and prevents its phosphorylation (Fig. 6*B*).

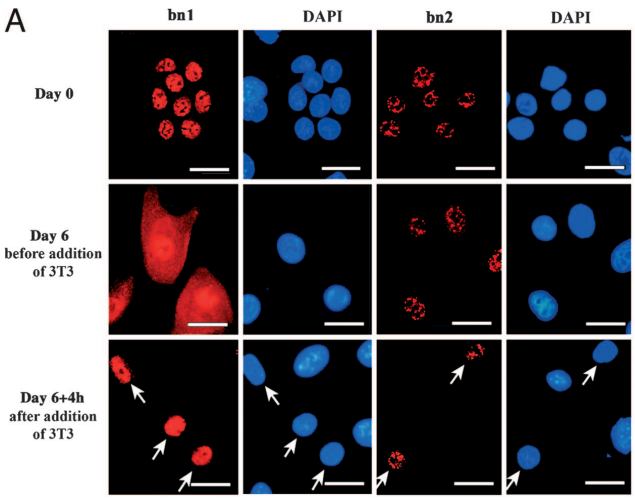

Fig. 6. Effect of removal of supporting 3T3 on nuclear localization of bn1 and bn2. (*A*) Human keratinocytes were plated with mitotically inactivated 3T3 cells; some cultures were fixed after 2 days (day 0). 3T3 cells were removed from the remaining dishes by a short incubation in the presence of trypsin/EDTA. The keratinocytes were then trypsinized and plated onto new dishes, where they were maintained in the absence of 3T3 support for 6 days (day 6). Half of these cultures were fixed. Mitotically inactivated 3T3 cells were added back to the remaining half of the cultures, whose cells were fixed 4 h after the addition of 3T3 cells. All fixed cultures were stained for bn1 and bn2. Much of the bn1 moved from the nucleus to the cytoplasm when 3T3 cells were removed and returned quickly to the nucleus when 3T3 cells were readded. In contrast, bn2 remained nuclear whether 3T3 cells were present. Arrows identify keratinocytes. (*B*) Alignment of the region of the NLS of bn1 and bn2 of the human (Hu), mouse (Mo), chicken (Ch), *Xenopus* (Xe), zebrafish (Zf), and *Fugu* (Fu). The seven residues composing the NLS are in bold. The region is entirely conserved between bn1 and bn2 of all species, except that Ser-537 of bn1 is replaced by a proline in bn2. (Scale bars, 25 μm.)

bn2 Is Present in All Living Layers of the Epidermis and at All Stages of Spermatogenesis. In human epidermis, bn1 is largely nuclear (6). When frozen sections of a foreskin of a human newborn were stained with the anti-bn1 antibody, bn1 was found to be largely confined to the nuclei of basal or immediately suprabasal cells (Fig. 7*A*). In contrast, bn2 was detected in all living layers of the epidermis, where it showed a speckled nuclear distribution. Double staining with the monoclonal anti-SC35 antibody showed virtually complete colocalization of bn2 and SC35 (Fig. 7*A*).

In the epidermis of rodents, the basal and immediately suprabasal cells contain bn1, mostly concentrated in the cytoplasm (5). Staining of mouse plantar epidermis with the anti-bn1 antibody confirmed the predominantly cytoplasmic and basal localization of bn1. Staining for bn2 showed that in mouse epidermis, as in human epidermis, bn2 was present in all of the living layers and was always nuclear. A few cells, mostly in the upper layers, were not stained by the anti-bn2 antibody, but these cells might not have been in the plane of section (Fig. 7*B*). These results confirmed those obtained with cultured keratinocytes: (*i*) bn2 is a nuclear protein that does not shuttle between the nucleus and the cytoplasm, and (*ii*) bn2 is present whether the cell is able to multiply, such as in the basal layer, or has lost its growth potential, such as in the suprabasal layers.

Because bn2 mRNA is particularly abundant in testis (11), we decided to stain frozen section of human testis for bn2. All bn2-positive cells showed speckled nuclear staining. Cells containing bn2 appeared to be distributed over the entire seminiferous epithelium. Although we could not clearly identify the different stages of spermatogenesis, the presence of bn2 in all layers of the epithelium suggests that spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids all contain nuclear bn2 (Fig. 7*C*). bn1 is also nuclear at all stages of spermatogenesis, except mature sperm cells (3).

Discussion

Many RNA processing factors, involved in either splicing or postsplicing processes such as mRNA export, nonsense-mediated decay, and polyadenylation, localize in speckles (16, 21–24) and a speckled staining pattern indicates with near certainty a role in RNA processing (24–26). The virtually complete colocalization of bn2 with SC35 in nuclear speckles strongly suggests that bn2 has a function in RNA processing, and its unusual evolutionary stability implies that this function is essential. Because RNA processing is needed by the cell at all times, a function in RNA processing would explain why bn2 never leaves the nucleus and is found in a large number, if not all cell types, including the cells of all of the epidermal layers. Therefore, the function of bn2 appears to be unrelated to that of bn1, a protein thought to regulate the maintenance of the proliferative potential of keratinocytes. In contrast to bn2, bn1 shuttles between the nucleus and the cytoplasm and disappears from cells that have lost their proliferative potential (6, 12).

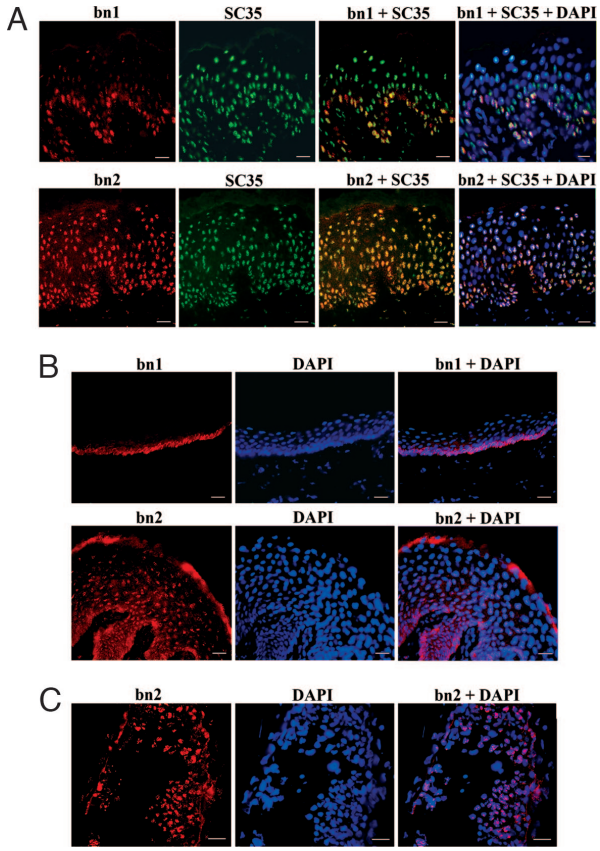

Fig. 7. bn2 in epidermis and testis. (*A*) Frozen sections of foreskin from human newborn were fixed and double stained for SC35 and either bn1 or bn2. DNA was counterstained with DAPI. bn1 is typically confined to basal nuclei, whereas bn2 is present in the nuclei of all cell layers, where it is colocalized with SC35. (*B*) Sections of mouse plantar epidermis. bn1 is restricted to the basal layer but appears concentrated in the cytoplasm, leaving nuclei unstained. In contrast, bn2 is found in all cell layers and is entirely nuclear because its staining largely overlaps with nuclear DAPI staining. (*C*) Sections of human testis. bn2 staining is entirely nuclear and speckled. bn2-containing cells appear to be distributed over the entire stratified seminiferous epithelium. (Scale bars, 25 μm.)

The *bn2* gene belongs to the *tyrosinase-related protein 1* (*Tyrp1* or *brown*) deletion complex. Deletion of *bn2* is likely to participate in the early embryonic lethality observed in *l4Rn3*, one of the *Tyrp1* deletion loci. A chromosomal inversion, *white-based brown* (B^w), also occurs within the *brown* locus. Overexpression of *bn2* placed under the control of the *Tyrp-1* promoter by the inversion almost certainly is responsible for the melanocyte cell death that is presumed to cause the inversion phenotype (27). It appears probable that these two phenotypes are related to alterations in RNA processing caused by the absence or overexpression of *bn2*.

Because it possesses multiple promoters and is subject to extensive alternative splicing, the gene for bn2 can generate many protein isoforms. The most abundant bn2 mRNA isoform encodes a protein with six zinc fingers, but other bn2 mRNA isoforms encode proteins with reduced numbers of zinc fingers and/or alterations in the putative nucleic acid binding domain of finger 4 (13). It will have to be determined why bn2 exists in so many other isoforms and whether some of these isoforms might have functions unrelated to RNA processing.

Materials and Methods

Antibodies. Fragments of human bn1 and bn2 were amplified by PCR from genomic DNA by using the following primers containing BamHI and EcoRI restriction sites: bn1, 5′-CGCGGATCCTCTGAGAACTACAAGTGCC and 5′-CCGGAATTCATGCATGCTGACCCACGTGC; bn 2, 5′-CGCGGATCCCCAATGGGTTTTACCACTCC and 5′-CCGGAATTCTGGGGTCTGTAAATTCTTCC. The PCR products were generated by 30 cycles of amplification (95°C for 1 min, 57°C for 1 min, and 72°C for 1 min). PCR fragments were cloned in the bacterial expression vector pGEX-2T (Amersham Pharmacia, Buckinghamshire, U.K.) as a fusion protein to GST. The soluble GST fusion proteins were purified on glutathione-agarose beads (Sigma, St. Louis, MO). The proteins were released from the beads with reduced glutathione.

For production of polyclonal anti-bn1 and anti-bn2 antibodies, affinity-purified GST-bn1 and GST-bn2 proteins were resolved by electrophoresis through an 8% denaturing polyacrylamide gel and stained with Coomassie Brilliant Blue R. Bands of interest were pulverized under liquid nitrogen. The powder was rinsed in PBS, and distributed into aliquots of 200 μg of protein, which then were injected into rabbits (eight injections). Antibodies were purified by affinity chromatography on cyanogen bromide-activated Sepharose 4B coupled to the fusion proteins. Antibody concentration was 0.14 mg/ml for bn1 and 0.32 mg/ml for bn2. Antibodies were generated for us by Primm (Milan, Italy).

For production of an anti-bn2 monoclonal antibody, a human phage display antibody library (28) was panned with the GST-bn2 fusion protein (Antibodies by Design, Martinsried, Germany).

Western Blotting. For assessing the specificity of the anti-bn1 and anti-bn2 antibodies, bacterial homogenates (10 μg of protein) containing GST-bn1 or GST-bn2 were resolved by electrophoresis through a 10% denaturing polyacrylamide gel and electroblotted onto nitrocellulose. The membranes were blocked for 3 h at room temperature in PBS containing 5% nonfat dry milk and 0.1% Tween 20 (PBST) and incubated overnight at 4°C in the presence of the polyclonal anti-bn1 (1:3,000), the polyclonal anti-bn2 (1:10,000), or the monoclonal anti-bn2 antibody (0.15 μg/ml). Membranes were washed with PBST before being incubated for 1 h at room temperature with either a donkey anti-rabbit IgG diluted 1:1,000 (Amersham Pharmacia) or an anti-human $F(ab')_2$ IgG fragment (Pierce, Rockford, IL) diluted 1:5,000. Both secondary antibodies were coupled to horseradish peroxidase. bn1 and bn2 were visualized by chemiluminescence (ECL+ kit, Amersham Pharmacia).

Western analysis of bn1, bn2, and SC35 in cultured cells was carried out as described above, except that antibody dilutions were 1:600 for bn2, 1:350 for SC35 (BD Biosciences), and 1:150 for bn1.

Indirect Immunofluorescent Staining. Human epidermal keratinocytes derived from foreskin of a newborn (strain YF23) were propagated on mitomycin-treated 3T3-J2F cells as described in ref. 29. Indirect immunofluorescence staining was carried out as described earlier in ref. 29.

For bn1 and bn2 staining, cells were incubated overnight in the presence of the polyclonal anti-bn1 (1:100), the polyclonal anti-bn2 (1:200), or the monoclonal anti-bn2 (1:100) antibody. Cells exposed to the polyclonal antibodies then were incubated in the presence of a goat anti-rabbit IgG linked to either Alexa Fluor 488 (1:100 dilution, Jackson ImmunoResearch, West Baltimore, PA) or cyanin 3 (1:200 dilution, Jackson ImmunoResearch). Cells probed with the monoclonal antibody subsequently were incubated with a cyanin 3-conjugated goat antibody specific for the $F(ab')_2$ IgG fragment (1:600 dilution, Jackson ImmunoResearch). Coverslips were mounted onto slides in mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). For double staining of either bn1 or bn2 with SC35, cells were incubated in the presence of either anti-bn1 or anti-bn2 polyclonal antibody as described. Cells then were incubated for 1 h at room temperature with a mouse anti-SC35 monoclonal antibody (1:500, BD Biosciences). Cells were incubated for 1 h at room temperature with both a goat anti-rabbit IgG antibody linked to cyanin 3 (1:200; Jackson ImmunoResearch) and a goat anti-mouse IgG antibody linked to FluoProbes 488 (1:100; Interchim, Montluçon, France).

We thank Fabrice Daubigney for technical assistance. This work was supported by the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer, and the Ligue contre le Cancer. A.V. was a recipient of fellowships from the Fondation pour la Recherche Médicale and the Fondation Bettencourt-Schueller.

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