

Gli Proteins Up-Regulate the Expression of Basонуclin in Basal Cell Carcinoma

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ABSTRACT

Tumorigenesis is frequently accompanied by enhanced rRNA transcription, but the signaling mechanisms responsible for such enhancement remain unclear. Here, we report evidence suggesting a novel link between deregulated Hedgehog signaling and the augmented rRNA transcription in cancer. Aberrant activation of the Hedgehog pathway in keratinocytes is a hallmark of basal cell carcinoma (BCC), the most common cancer in light-skinned individuals. We show that Gli proteins, downstream effectors of the Hedgehog pathway, increase expression of a novel rRNA gene (rDNA) transcription factor, basонуclin, whose expression is markedly elevated in BCCs. The promoter of the human basонуclin gene contains a Gli-binding site, which is required for Gli protein binding and transcriptional activation. We show also that the level of 47S pre-rRNA is much higher in BCCs than in normal epidermis, suggesting an accelerated rRNA transcription in the neoplastic cells. Within BCC, those cells expressing the highest level of basонуclin also exhibit the greatest increase in 47S pre-rRNA, consistent with a role for basонуclin in increasing rRNA transcription in these cells. Our data suggest that Hedgehog-Gli pathway enhances rRNA transcription in BCC by increasing basонуclin gene expression.

INTRODUCTION

The association of augmented ribosomal biogenesis and cancer has been noted for many decades. Augmented ribosome biogenesis may play an important role in tumor pathogenesis because of the increased proliferation and metabolic activities in most neoplastic cells. Recent works have shown that several proto-oncogenes and tumor suppressors can influence the regulation of rRNA synthesis (1). However, very little is known regarding the role of components of signaling pathways in increasing ribosomal biogenesis in cancer (2).

Constitutive activation of the Hedgehog pathway is associated with basal cell carcinoma (BCC) and several other types of tumors (3–9). The Hedgehog pathway, highly conserved from *Drosophila* to human, plays an essential role in organogenesis during development. An important function of Hedgehog signaling is to trigger its target cell types to proliferate at a given developmental stage (10–16). This Hedgehog function is mediated by the Gli family of transcription factors, a group of zinc-finger proteins, first discovered in glioblastoma (17). In BCC, Gli proteins serve as a key link between aberrant Hedgehog signaling and BCC formation, because the expression of GLIs is elevated in human BCC and overexpression of GLI1 or GLI2 in epidermal keratinocytes of transgenic mice is sufficient to induce the tumor (18, 19). However, the mechanism by which Hedgehog/Gli pathway induces formation of BCC remains to be elucidated.

Basонуclin is a zinc-finger protein with a highly restricted tissue

distribution (20). Thus far, basонуclin has been detected mainly in the basal keratinocytes of stratified squamous epithelia (skin, oral epithelium, esophagus, vagina, and cornea) and in the gametogenic cells of the testis and ovary. In human and mouse skin, the highest level of basонуclin is found in the hair follicular keratinocytes of the outer root sheath and the bulb (21, 22). There is now considerable evidence that basонуclin is a cell-type-specific transcription factor for rRNA genes (rDNA). The zinc fingers of basонуclin interact with three evolutionarily conserved sites within the rDNA promoter (23–25). Basонуclin remains associated with the rDNA loci during mitosis, a behavior typical of transcription factors of RNA polymerase I (24). In a cotransfection assay, basонуclin can increase transcription from an rDNA promoter and an isolated zinc-finger domain of basонуclin can inhibit polymerase I transcription in developing mouse oocytes (25). Here, we show that basонуclin expression is markedly elevated in BCCs, and this enhanced expression correlates with augmented rRNA transcription in BCC cells. Our data strongly suggest that basонуclin is regulated directly by the Gli proteins through a Gli-binding site in the promoter of the basонуclin gene.

MATERIALS AND METHODS

Immunocytochemistry. Immunocytochemistry on cryosectioned skin biopsies was performed as described previously (26). In brief, surgical specimens were frozen in OCT (Miles, Inc.), and cryosections (5 μ m) were fixed for 5 min each in methanol and acetone at -20°C and stained with an affinity-purified rabbit antihuman-basонуclin antiserum (ah34; Ref. 20). The primary antibody was visualized by a Texas-Red-conjugated antirabbit antibody (1:400; Molecular Probes, Inc.). When indicated, the DNA was stained by Hoechst 33258 (1 ng/ml) for 2 min at room temperature.

Reverse Transcription-Polymerase Chain Reaction. RNAs were prepared from tissue samples or cultured cells by a TRIzol kit (Life Technologies). Single-stranded cDNA was synthesized with SuperScript Pre-amplification System (Life Technologies). Primers and conditions for amplifying human basонуclin fragments and β -actin (26); primers and conditions for amplifying mouse basонуclin were also described previously (25). Primers for amplifying GLI1 were 5'-ACTGAAGACCTCTCCAGC and 5'-GCTGACAGTATAGCAGA (nucleotides 1501–1746; the beginning of cDNA, +1), 5'-GAGGTGGCAGGCTAGGAGG, and 5'-CTGGGGCATCTTGTGCATGG (nucleotides 3121–3500); and for GLI2, 5'-CAAGGATTCCTGCTCATGGG and 5'-AGTGGCTGCCGCTACTT (nucleotides 1087–2279), 5'-CAGTACCCGGGCTACAGTCC, and 5'-GAAGTGGTTGAGGGCCCCAC (nucleotides 3131–3385). Cycle parameters were 95°C for 5 min, 35 cycles of 95°C for 1 min, 62°C for 1 min, 72°C for 30 s, followed by a 7-min incubation at 72°C .

DNase I Footprinting. cDNA of the GLI zinc-finger domains (amino acids 220–408 for GLI1 and amino acids 98–277 for GLI2) were isolated by PCR (from a cDNA clone and from HeLa cell cDNA, respectively) and expressed in *Escherichia coli*, and the resulting recombinant proteins purified to 90% homogeneity by chromatography on a BioRex 70 column. Human basонуclin promoter fragment was prepared by PCR with one of the primers end-labeled. DNase I footprinting was done as described previously (24).

Transfection. A chloramphenicol acetyl transferase (CAT) gene reporter plasmid (pCAT-hBP) was constructed, with basонуclin promoter as the transcriptional control, and deletions from the distal end of the promoter were generated. As a control, the fragment containing δ -crystallin basal promoter and eight Gli-binding sites was isolated from $8 \times 3'$ Gli-BS and $8 \times 3'$ mGli-BS (27) and inserted into *HindIII-SmaI* sites of

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pCAT3basic (Promega, Inc.). To express the Gli proteins, the cDNAs of human GLI-1 and GLI-3 (28) and mouse Gli2 (29) were placed behind the CMV promoter in the pCDNA 3.1 vector (pGli-1, -2, and -3, respectively). All cDNA constructs were checked by *in vitro* translation and by reverse transcription-PCR for expression. No significant variation in expression level was detected among the different constructs. The pCAT-hBP plasmid was cotransfected with the individual pGli plasmid into the COS-7 or Swiss 3T3 cells using Lipofectamine (BRL-Life Technologies, Inc.). Either a green fluorescent protein (GFP)-expression (Stratagene, Inc.) or a β -galactosidase-expression plasmid (Promega, Inc.) was included as a control for transfection efficiency. CAT, GFP, or β -galactosidase activities were measured 48-h post-transfection by scintillation, fluorescent microscopy, and spectrophotometry, respectively. The CAT activity, which is expressed as cpm increase per minute (cpm/min), was normalized according to transfection efficiency.

In Situ Hybridization. The BCCs specimen were confirmed by J. T. S., a dermatopathologist. The [35 S]UTP-labeled probes (sense and antisense) were synthesized by *in vitro* transcription with T3 and T7 RNA polymerase, respec-

tively, from a template of the leader sequence of human 47S pre-rRNA (nucleotides 1–310). *In situ* hybridization was done as described previously (30).

RESULTS

Basonuclin Expression Is Increased in Basal Cell Carcinoma.

We examined basonuclin expression in BCC by immunocytochemistry and reverse transcription-PCR. Surgical specimens of infiltrative (Fig. 1, A–D, $n = 4$) and nodular (Fig. 1, E and F, $n = 5$) types of BCC were cryosectioned and stained with an affinity-purified, rabbit-antihuman-basonuclin antibody (α hB34; Ref. 24). The amount of basonuclin was much greater in tumor cells than in the unaffected basal keratinocytes of the epidermis (Fig. 1, A, G, and G', arrowheads) and the outer root sheath of the hair follicles (Fig. 1, D and H, arrowheads), the normal sites of basonuclin expression. This increase of basonuclin content is within individual tumor cells (Fig. 1, G' and

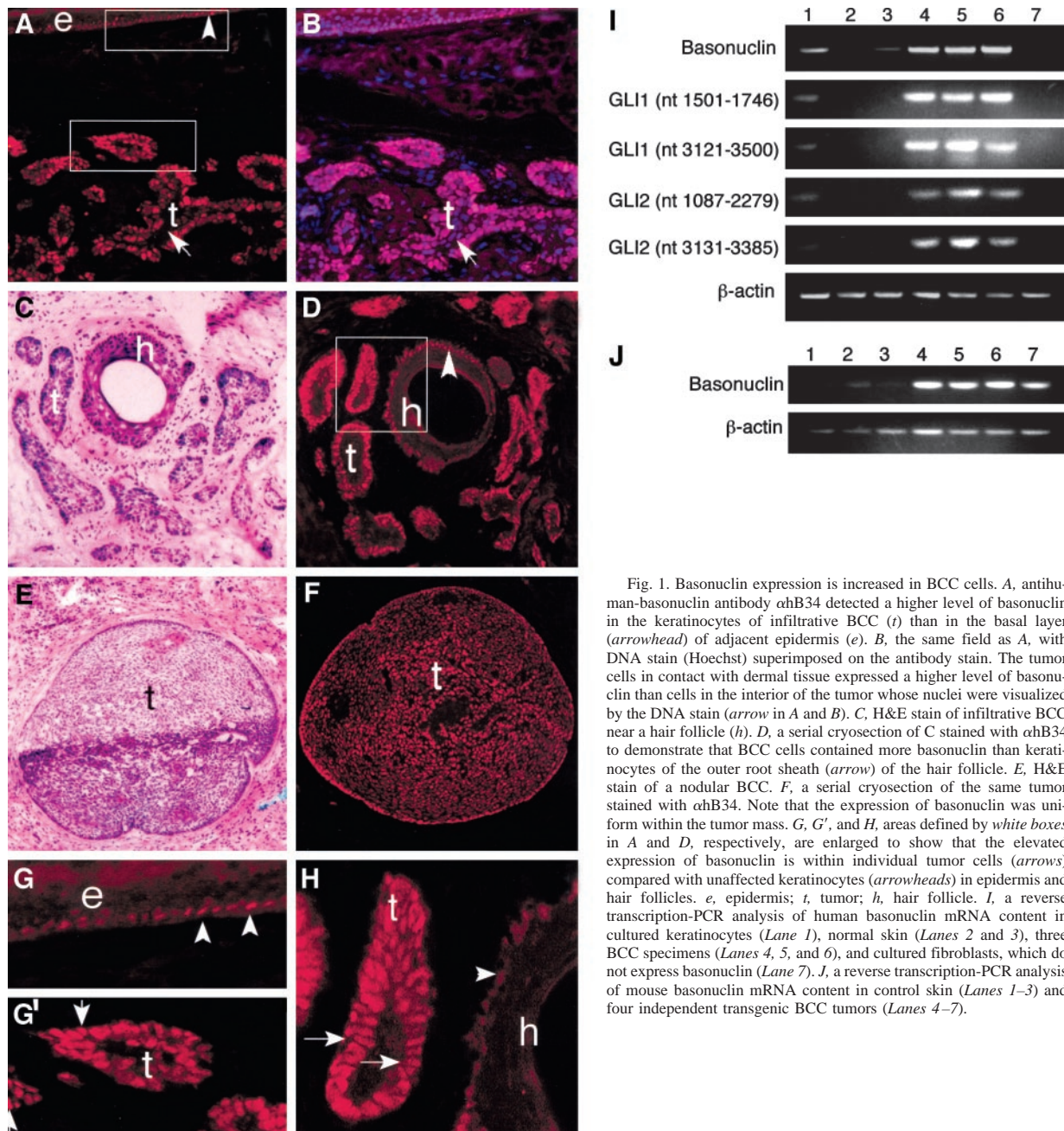


Fig. 1. Basonuclin expression is increased in BCC cells. A, antihuman-basonuclin antibody α hB34 detected a higher level of basonuclin in the keratinocytes of infiltrative BCC (t) than in the basal layer (arrowhead) of adjacent epidermis (e). B, the same field as A, with DNA stain (Hoechst) superimposed on the antibody stain. The tumor cells in contact with dermal tissue expressed a higher level of basonuclin than cells in the interior of the tumor whose nuclei were visualized by the DNA stain (arrow in A and B). C, H&E stain of infiltrative BCC near a hair follicle (h). D, a serial cryosection of C stained with α hB34 to demonstrate that BCC cells contained more basonuclin than keratinocytes of the outer root sheath (arrow) of the hair follicle. E, H&E stain of a nodular BCC. F, a serial cryosection of the same tumor stained with α hB34. Note that the expression of basonuclin was uniform within the tumor mass. G, G', and H, areas defined by white boxes in A and D, respectively, are enlarged to show that the elevated expression of basonuclin is within individual tumor cells (arrows) compared with unaffected keratinocytes (arrowheads) in epidermis and hair follicles. e, epidermis; t, tumor; h, hair follicle. I, a reverse transcription-PCR analysis of human basonuclin mRNA content in cultured keratinocytes (Lane 1), normal skin (Lanes 2 and 3), three BCC specimens (Lanes 4, 5, and 6), and cultured fibroblasts, which do not express basonuclin (Lane 7). J, a reverse transcription-PCR analysis of mouse basonuclin mRNA content in control skin (Lanes 1–3) and four independent transgenic BCC tumors (Lanes 4–7).

H, arrows) and not an illusion of the higher cell density within the tumor. In infiltrative lesions, which are clinically more aggressive than nodular BCCs and contain small irregular cords or islands of cells scattered throughout the dermis, virtually all peripheral cells in contact with the surrounding dermis contained a high level of baso-nuclin, whereas only a small fraction of the cells in the interior of the tumor islands expressed baso-nuclin (Fig. 1, *B* and *D*, arrows in *A* and *B* show an example of a baso-nuclin-negative cell in the interior of the tumor). In contrast, in nodular BCCs, baso-nuclin staining was more uniform within the tumor with a slight increase in fluorescence intensity at the periphery, which is probably due to higher cell density (Fig. 1*F*).

The enhanced baso-nuclin content in human BCC was verified at the mRNA level. By reverse transcription-PCR, we detected a higher baso-nuclin mRNA level in BCC-affected human skin (Fig. 1*I*). In these BCC tumors, as expected, Gli expression was also elevated. Using two primer pairs for each Gli, we amplified separate regions of Gli1 (nucleotides 1501–1746 and 3121–3500) and Gli2 (nucleotides 1807–2279 and 3131–3385; Fig. 1*I*) from the BCC specimen but not that of normal skin. As a control, we amplified Gli mRNA in human keratinocytes cultured according to Rheinwald and Green (Ref. 31; Fig. 1*I*, Lane 1). Furthermore, consistent with our finding in human BCC, reverse transcription-PCR analysis detected a similar increase of baso-nuclin mRNA in four independent BCC tumors derived from transgenic mice overexpressing mGli2 in cutaneous keratinocytes (Ref. 18; Fig. 1*J*).

The Promoter of the Human Baso-nuclin Gene Contains a Gli-Binding Site. Because of the central role of the Gli proteins in BCC formation, we searched for evidence that Gli proteins could directly

regulate baso-nuclin transcription. The human baso-nuclin gene promoter has not been fully characterized. However, we know that the 5' end of the human gene contains no TATA box and is extremely GC rich (Fig. 2*A*; Ref. 32). By RNase I protection, four distinctive baso-nuclin transcripts were detected (mRNA I–IV; I was the longest), the 5' ends of which were mapped within a region of 400 bases of the genomic sequence (Fig. 2*A*; Ref. 33). Based on this information, we located a single copy of the Gli consensus binding site 5'-GACCAC-CCA (27) at approximately 280 bp upstream from the 5' end of baso-nuclin mRNA I (Fig. 2, *A* and *B*). A notable feature of the location of this Gli-binding site is that it is 3' adjacent to a long TG repeat (16 units; Fig. 2*B*). No other sequence feature is apparent surrounding this binding site.

To investigate whether Gli proteins interact with this binding site, we purified recombinant proteins that contained the zinc-finger domains of human Gli1 and Gli2. In a DNase I footprinting assay, both recombinant Gli1 and Gli2 zinc-finger domains protected the Gli consensus and the flanking sequences from DNase I digestion (Fig. 3*A*). The protected region was not centered on the consensus sequence but skewed toward the 3' side. For both Gli proteins, a complete protection of the consensus sequence was achieved at the lowest protein concentration on the coding strand but not on the noncoding strand even at the highest protein concentration, suggesting an asymmetry of the protein/DNA interaction along the axis of DNA. No other binding site was detected by sequence comparison or by gel shift. The consensus Gli-binding site had been deduced earlier using Gli1 (28). At a low protein concentration, the DNase I footprint of Gli1 zinc finger on baso-nuclin promoter was very similar to that reported previously (28), but at a higher concentration; some additional se-

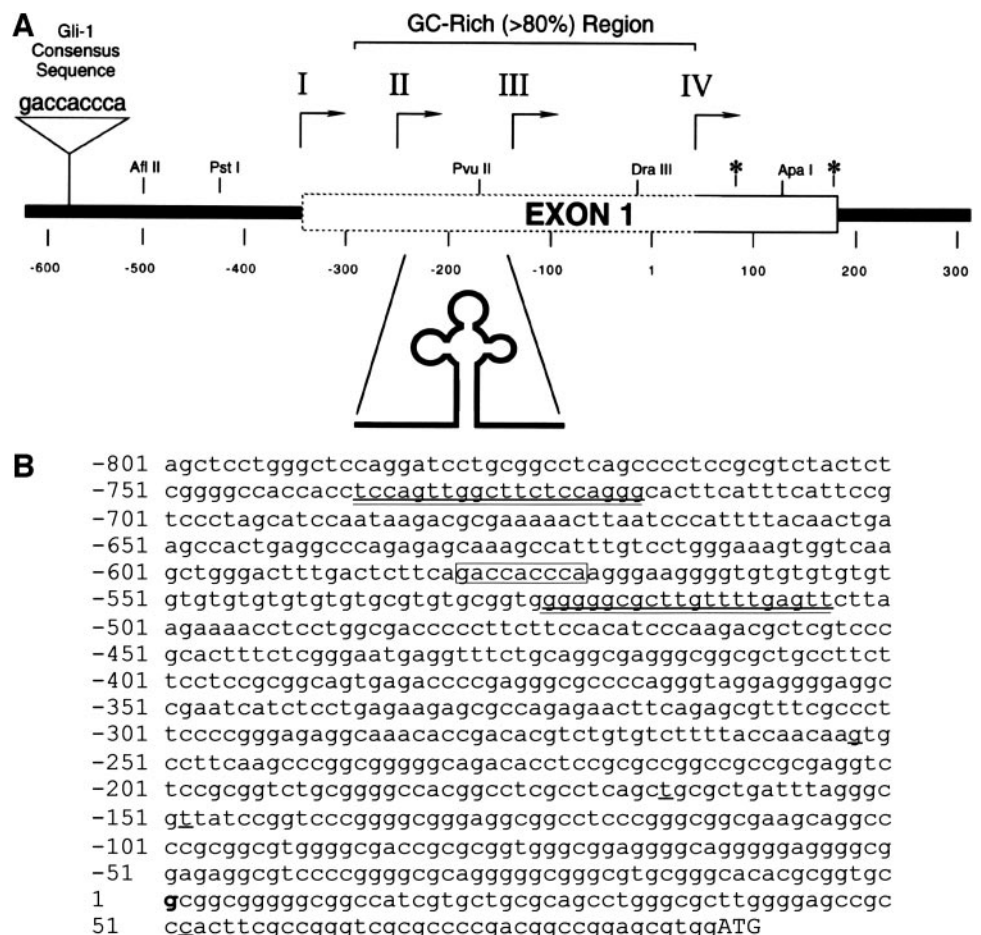


Fig. 2. The promoter of the human baso-nuclin gene contains a consensus Gli-binding site. *A*, diagram of the 5' region of the human baso-nuclin gene. Genomic DNA is depicted by a thick line and the first exon by an open box. The GC-rich region is indicated by a bracket above the genomic DNA. The positions of the four 5' ends of baso-nuclin mRNA (I–IV) are shown by bent arrows. The location of the Gli consensus sequence is indicated on the left. Also shown are several restriction sites (above the genomic DNA); a coordinate, in which the beginning of the baso-nuclin cDNA is +1 (below the genomic DNA); the position of the first two AUGs (*); and the position of a putative secondary structure within the 5'-untranslated region. *B*, the DNA sequence surrounding the Gli-binding site, which is indicated by a box. The position of the 5' ends of baso-nuclin mRNAs are underlined. The first nucleotide (G) of the cDNA sequence is shown in bold. The first translation initiation codon is shown in capital letters. Double underlines indicate the sequences of PCR primers used to isolate the promoter fragment for DNA-binding studies by DNase I footprinting or gel shift (Fig. 3*A*; Fig. 5*B*).

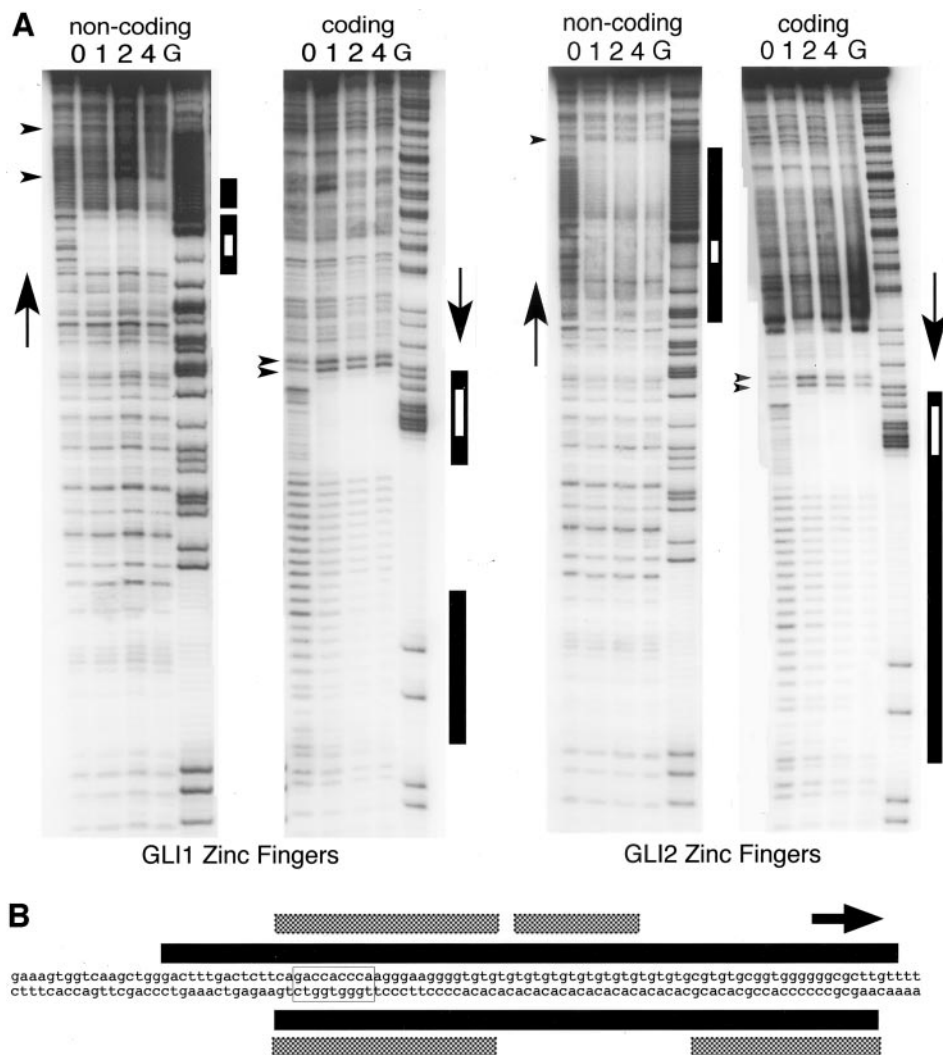


Fig. 3. A, DNase I footprints of the zinc-finger domains of GLI1 (left) and GLI2 (right) on the promoter region of the basonuclin gene. Footprints of the Gli zinc fingers on both strands are shown, the number on top of each lane indicates the relative amount of the Gli zinc-finger domain. Black bars, footprints; white bar within black bar, position of the Gli-binding consensus sequence. Arrowheads show sites of enhanced DNase I cleavage. B, diagrammatic representation of the DNase I footprints in A. Solid bars, the sequence protected by GLI2 zinc fingers; shaded bars, that by GLI1 zinc fingers. The Gli consensus binding site is framed in a box and the direction of transcription is indicated by an arrow.

quence at the 3' side of the consensus was also protected. Because the sequence of the GLI1 zinc fingers differs from that of GLI2 by 19 amino acids, the binding property and sequence requirement of the two proteins need not be exactly the same. Indeed, on the promoter of the basonuclin gene, the GLI2 zinc fingers protected a region larger than that of GLI1 at the same protein concentration, and this difference was more pronounced on the noncoding strand (Fig. 3, A and B).

Gli Proteins Enhance Transcription from Basonuclin Promoter. To determine transcriptional activity of basonuclin promoter, a 4.5-kb genomic fragment, which encompasses the 5' ends of the four basonuclin transcripts and approximately 4 kb of upstream sequence (33), was cloned in front of a CAT reporter gene (pCAT-hBP), and then serial deletions from the distal end of the genomic clone were made (Fig. 4A). The pCAT-hBP and deletion mutants were tested for their ability to promote reporter gene expression in the COS-7 cell, which is commonly used for assaying transcriptional effect of Gli proteins. The promoter activity of the 4 kb upstream sequence was the strongest, resulting in a more than 20-fold increase of the CAT expression over the control, which was the CAT plasmid without the basonuclin promoter. When the distal one-half of the 4 kb sequence was removed (pCAT-hBPxba), the promoter activity was reduced to 50%. But no significant reduction of promoter activity was seen until additional deletions removed the 5' sequence of the first exon (pCAT-hBPpvu; Fig. 4A).

To test the effect of the Gli proteins on basonuclin gene transcription, we used as a reporter pCAT-hBPstu (Fig. 4A), which had approximately 40% of the intrinsic promoter activity as pCAT-hBP but the same magnitude of transcriptional response to Gli. We co-transfected COS-7 cells with the reporter and the Gli expression plasmids. CAT activity was significantly increased in the presence of all three wild-type Gli proteins (Fig. 4B). Gli2 appeared to be at least twice as effective in increasing CAT gene expression than did GLI1 and GLI3, which showed a similar effect on transcription. A mutant Gli2 (C4), which lacks the COOH-terminal activator domain (29), lost its ability to stimulate transcription. We found no significant difference of the effect of Gli proteins on pCAT-hBPstu and pCAT-hBP (data not shown), suggesting that no other Gli-responsive sequence is present upstream from the *StuI* site (Fig. 4A). The transcriptional stimulation effects of GLI1 and Gli2 were dosage dependent (Fig. 4C, only data on Gli2 shown). To ensure that our experimental system is consistent with that of other investigators, we used the δ -crystalline promoter linked with eight synthetic wild-type or mutant Gli-binding sites (27, 29) as a control. We found that GLI1 was far more effective in stimulating transcription from this promoter than Gli2 (Fig. 4D), which is in good agreement with the previous report.

The Gli-Binding Site Is Required for the Transcription-Enhancing Effect of the Gli Proteins on the Basonuclin Promoter. To demonstrate that the Gli-binding site in basonuclin promoter mediates the Gli transcription effect, we constructed three mutations

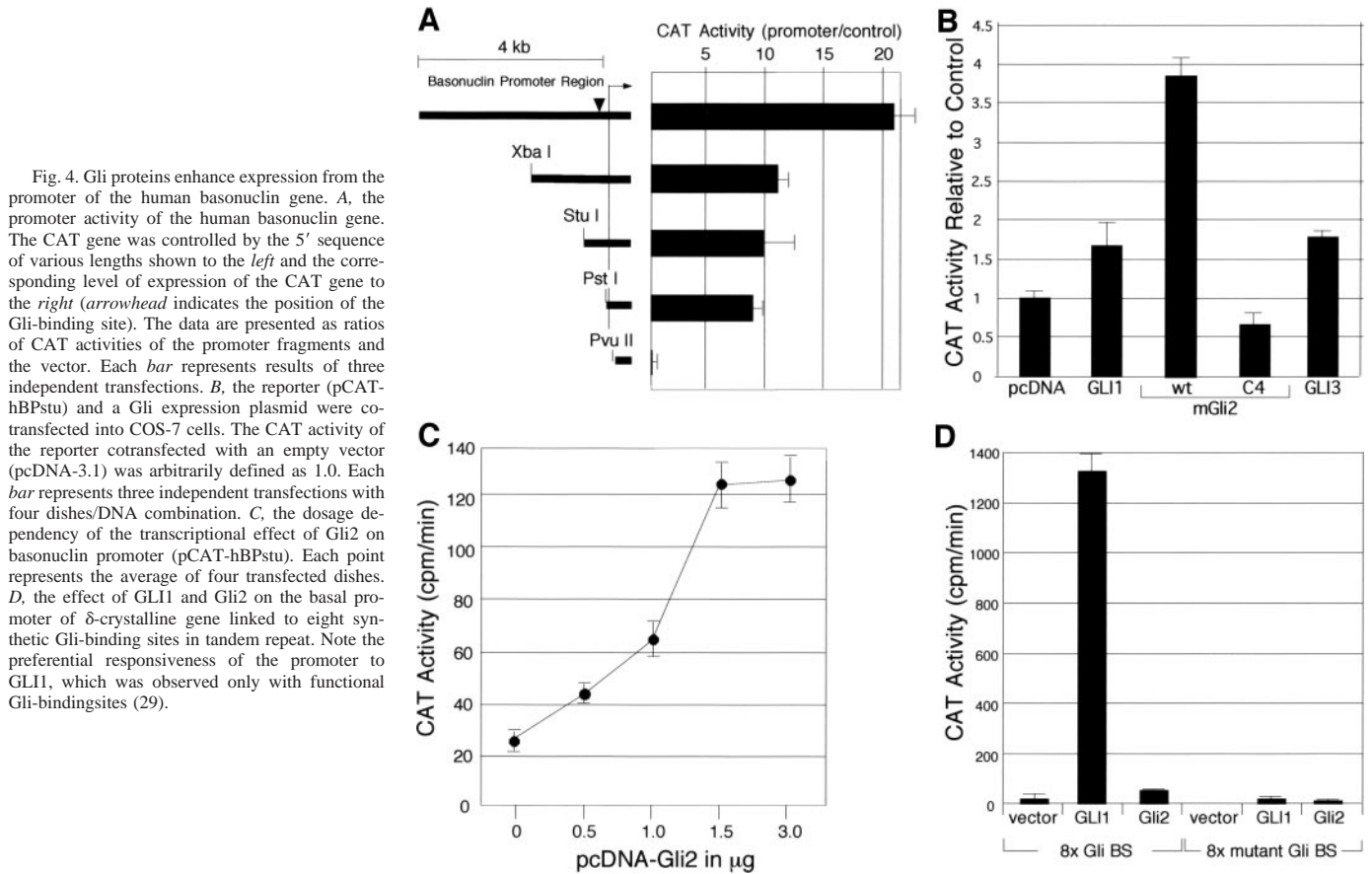


Fig. 4. Gli proteins enhance expression from the promoter of the human basонуclin gene. *A*, the promoter activity of the human basонуclin gene. The CAT gene was controlled by the 5' sequence of various lengths shown to the left and the corresponding level of expression of the CAT gene to the right (arrowhead indicates the position of the Gli-binding site). The data are presented as ratios of CAT activities of the promoter fragments and the vector. Each bar represents results of three independent transfections. *B*, the reporter (pCAT-hBPst) and a Gli expression plasmid were co-transfected into COS-7 cells. The CAT activity of the reporter cotransfected with an empty vector (pcDNA-3.1) was arbitrarily defined as 1.0. Each bar represents three independent transfections with four dishes/DNA combination. *C*, the dosage dependency of the transcriptional effect of Gli2 on basонуclin promoter (pCAT-hBPst). Each point represents the average of four transfected dishes. *D*, the effect of GLI1 and Gli2 on the basal promoter of δ -crystalline gene linked to eight synthetic Gli-binding sites in tandem repeat. Note the preferential responsiveness of the promoter to GLI1, which was observed only with functional Gli-binding sites (29).

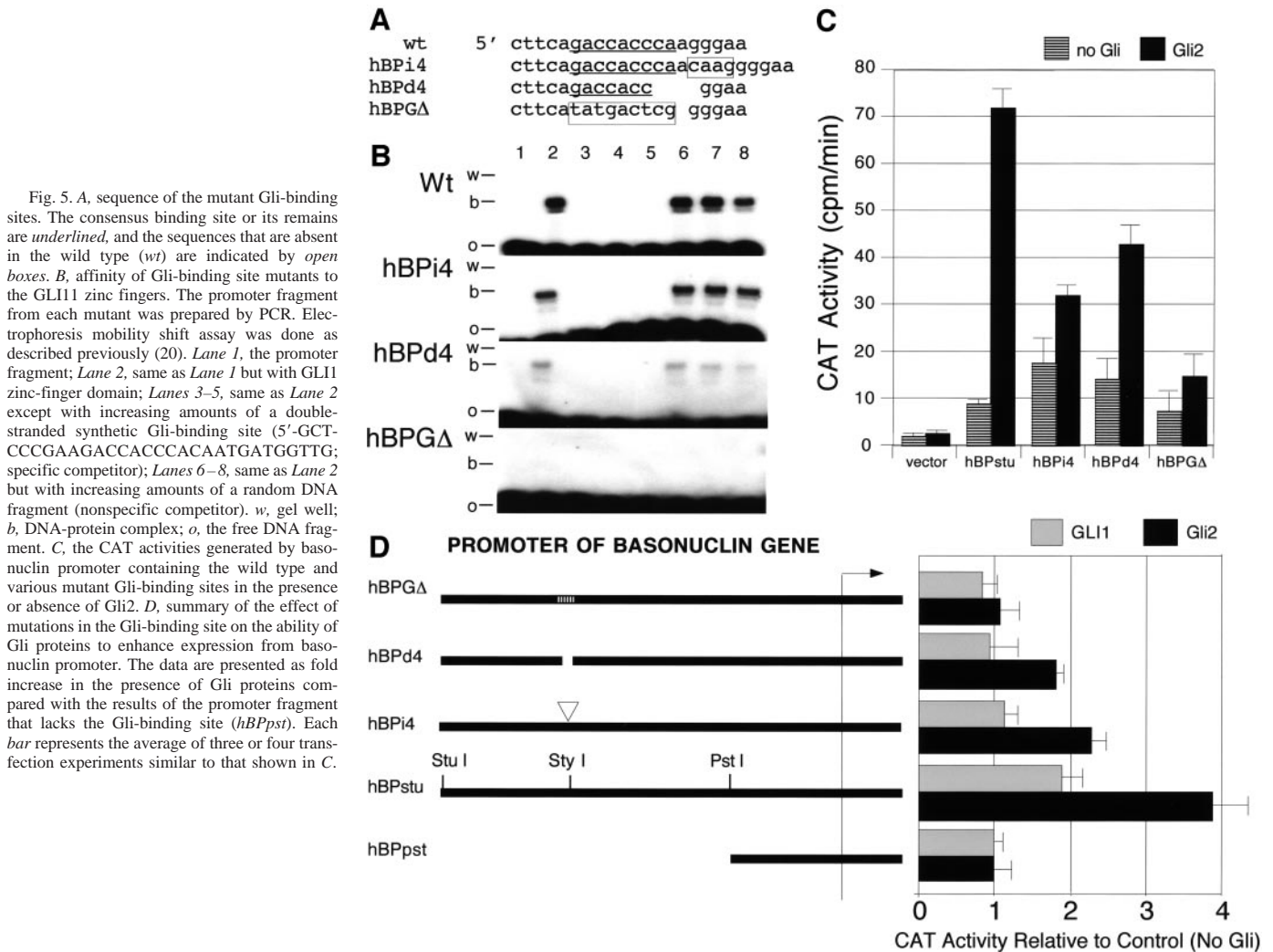
that altered the binding site (Fig. 5A). Mutant phBPd4 is a 4-base deletion at the 3' end of the binding site, which reduced the GLI zinc finger binding (Fig. 5B) and the ability of Gli to increase CAT expression (Fig. 5C). Mutant phBPi4 is a 4-base insertion that retains the binding site but increases its distance from the transcription initiation site as well as rotates the site by approximately 144 degrees (assuming B DNA). The GLI zinc fingers still could bind to BPi4 (Fig. 5B), but the ability of the Gli proteins to stimulate the expression of the reporter gene was reduced to 50% of the wild-type promoter (Fig. 5, C and D). In the mutant phBPG Δ , the 9-base Gli-binding site was replaced by an irrelevant sequence of the same length (Fig. 5A). This mutant lost its ability to interact with the Gli zinc fingers as well as most of its response to at least two Gli proteins (GLI1 and Gli2; Fig. 5, B–D). From these analyses, we conclude that the integrity of the sequence and the spatial arrangement of the Gli-binding site are crucial for the effect of Gli proteins on transcription of basонуclin gene.

The High Level of Basонуclin Correlates with a High Level of 47S Pre-rRNA. Because basонуclin enhances rRNA transcription, we investigated the rate of rRNA transcription in BCC. We showed recently that the quantity of the nascent rRNA transcript (47S pre-rRNA) correlates positively with the rate of rRNA transcription. We adopted this method as an *in situ* hybridization procedure and demonstrated its validity *in vivo* (30). Using this method, we found that BCC cells contained a higher level of 47S pre-rRNA than keratinocytes in the epidermis and hair follicles (Fig. 6A). Using serial tissue sections, we demonstrated a tight correlation between high basонуclin content and high level of the 47S pre-rRNA. In the infiltrative type BCC, the peripheral tumor cells in contact with dermis had more basонуclin and 47S pre-rRNA than the cells in the interior of the tumor (Fig. 6, B and C), whereas in the nodular type, the contents of

basонуclin and the 47S pre-rRNA were uniform among the cells in the interior of tumor mass (Fig. 6, D and E), and the higher level of signal in the periphery is likely due to a higher cell density. In contrast, cells with high level of 47S pre-rRNA were only a subset of the population containing Ki67, a cell cycle marker (not shown), suggesting that only cells cycling at higher rate would increase their rRNA synthesis. In conjunction with previous observations (23–25), the tight correlation between basонуclin and 47S pre-rRNA suggests that basонуclin is at least in part responsible for the active synthesis of rRNA in the BCC cells.

DISCUSSION

Our results strongly suggest that in BCC, the Hedgehog/Gli pathway can up-regulate rRNA synthesis via increasing basонуclin expression. We present here three pieces of critical evidence to support this hypothesis: (a) basонуclin level is markedly increased in BCC cells; (b) Gli protein can up-regulate the expression of basонуclin gene; and (c) rRNA transcription is highly active in BCC cells and this increased activity correlates with a higher level of basонуclin. We demonstrate, for the first time, that basонуclin expression is elevated in both human and mouse BCCs. This increase in basонуclin expression is likely a result of enhanced Gli activity in BCC because the promoter of basонуclin gene contains a single copy of the Gli-binding site, which can mediate Gli binding and is required for the transcription-enhancing effect of the Gli proteins on basонуclin promoter. Equally important, by using our recently developed *in situ* hybridization technique (30), we show here, that indeed, the rRNA transcription rate is markedly increased in BCC cells, and this increase correlates tightly with the elevated level of basонуclin in BCC. These observations, in con-



junction with the demonstrated function of basonuclin as an rDNA transcription factor (25), are consistent with the novel notion that basonuclin is a target gene of the Shh/Gli pathway, which up-regulates rRNA synthesis by increasing basonuclin function through transcription activation. Our data, however, do not show, whether other means are also used to increase rRNA synthesis in BCC, *e.g.*, by mutated tumor suppressors (p53 and retinoblastoma) or by phosphorylation of UBF (34, 35). Although the p53 mutation status in our BCC samples is not known, approximately 50% of human BCCs may contain p53 mutations (36, 37). Additional study is necessary to clarify the contribution of other factors on enhancing rRNA synthesis in BCC. Because no BCC cell culture system exists, we cannot demonstrate at this time that the increased basonuclin and rRNA expression play a functional role in the biology of BCC tumor cells. These issues must be addressed in the future by other experimental approaches, such as transgenic animals.

The notion that the Hedgehog/Gli pathway can up-regulate rRNA synthesis is novel, yet such capability is consistent with the physiological role of the Hedgehog/Gli pathway in development. We know that one of the key roles of the Hedgehog protein (Sonic, Indian, and Desert Hedgehog) in development is to induce cell proliferation, which requires accelerated rRNA synthesis (38). Furthermore, there is an emerging link between the function of the Hedgehog pathway and the up-regulation of basonuclin expression in normal development.

Sonic hedgehog (Shh) is essential for hair follicle development (39, 40). Perhaps not coincidentally, the expression of basonuclin is among the early genes to be up-regulated during hair follicle morphogenesis (22) and is more abundant in the mature hair follicular keratinocytes than that in the epidermis (21, 22). In the absence of Shh signal, Gli1 and Gli2 are down-regulated in the nascent follicular keratinocytes, whose proliferative ability is thus impaired but can still differentiate (39, 41). In another example, Desert hedgehog is required for the proliferation of testicular spermatogenic cells (41), in which Gli and basonuclin are also coexpressed (26, 42, 43). Therefore, our data here also suggest that basonuclin may be a target of the Hedgehog/Gli pathways in both of these developmental systems. However, this notion implies neither that Gli is necessary for basal level basonuclin expression, as in basal epidermal keratinocytes, in which Gli expression has not been detected; nor that Shh pathway is the only signal that can enhance the expression of basonuclin gene, such as in developing oocytes (25, 26), in which basonuclin is highly expressed but the role of Shh pathway has not been established.

Our results suggest that Gli2 is a more potent regulator of basonuclin gene transcription than GLI1 and GLI3. Because Gli1 and Gli2 have overlapping functions (44) and because the levels of both GLI1 (45, 46) and GLI2 (47) are elevated in BCC, both may play a role in increasing basonuclin expression in this tumor. To distinguish the individual role of Gli1 and Gli2 in enhancing basonuclin expression requires more detailed molecular study and is beyond the purpose and

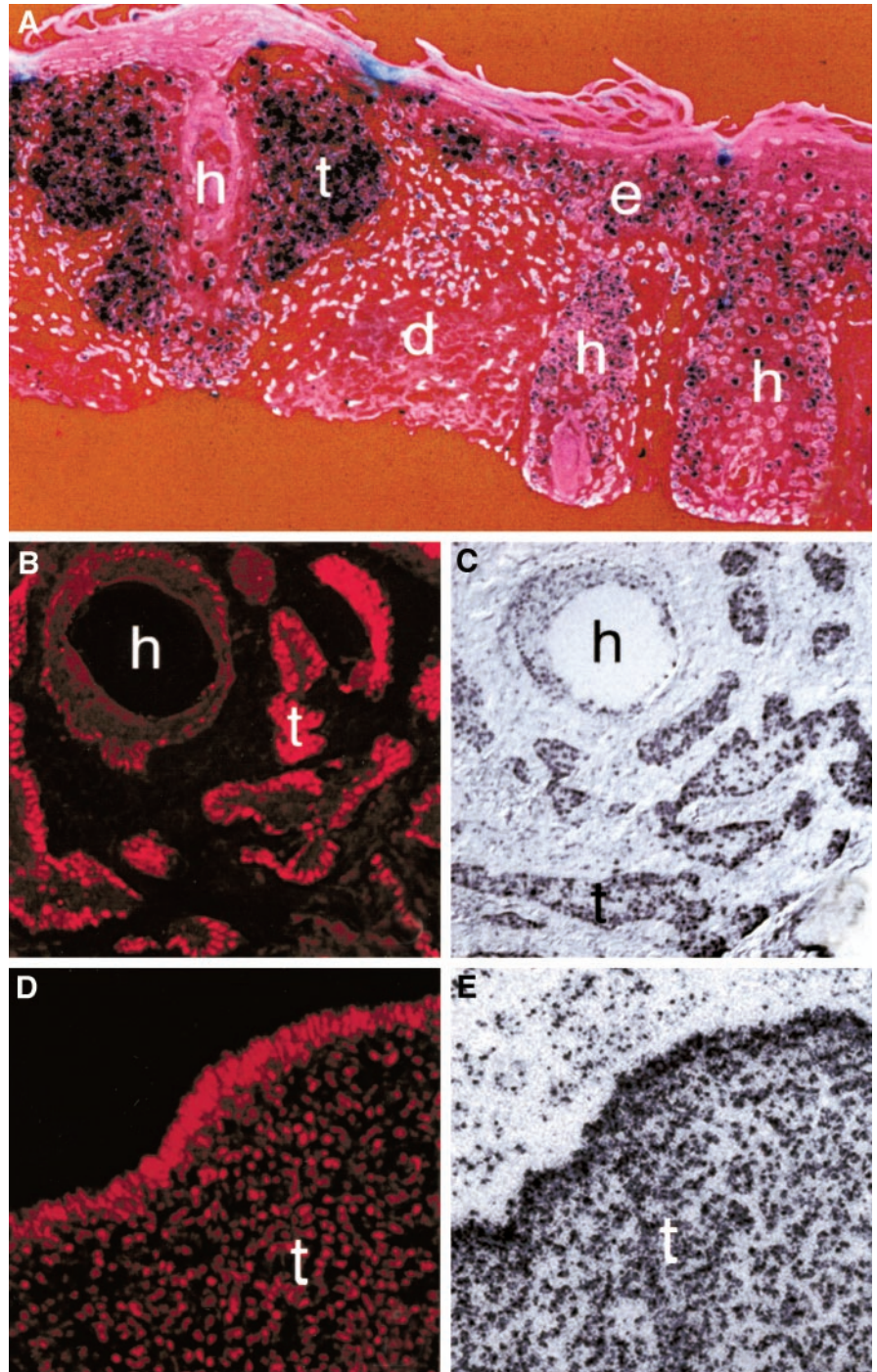


Fig. 6. Correlation of basonuclin expression and the content of 47S pre-rRNA. A, the level of 47S pre-rRNA in cells of BCC-affected human skin was detected by *in situ* hybridization with a probe for the leader of the 5'-external transcribed spacer (ETS) of the pre-rRNA. The specimen was illuminated simultaneously by transmitted light (for *in situ* hybridization signal) and by epi-fluorescence (for DNA staining). Unlike that of the mRNA, the hybridization signal of the pre-rRNA (black dots) is localized within the nucleus (light blue). In the infiltrative BCC (B and C), the high contents of basonuclin (B) and the 47S pre-rRNA (C) were more evident in the peripheral cells. In the nodular BCC (D and E), basonuclin (D) and 47S pre-rRNA (E) could also be found in the interior of the tumor. e, epidermis; d, dermis; h, hair follicle; t, BCC.

scope of this work. The role of GLI3 in basonuclin expression in BCC is not clear, because its expression is not appreciably altered relative to normal keratinocytes (18, 45, 46).

rRNA synthesis is increased during tumorigenesis, presumably due to a higher demand for protein synthesis in tumor cells (2, 48). It was demonstrated more than 30 years ago that infection of tumor virus SV40 stimulated rRNA synthesis in a number of cell types. A recent study showed that this stimulatory effect is brought about by large T-antigen, which activates rRNA transcription by binding to the TBP-TAF1, a ubiquitous coactivator of polymerase I (49). Tumor repressor proteins Rb and p53 also regulate rRNA synthesis. Rb protein can down-regulate rRNA production by interacting with several components of the polymerase I transcriptional machinery (50).

Despite these advances, much is to be learned regarding the molecular mechanism by which a signaling pathway could directly regulate polymerase I activity and augment rRNA synthesis (2). Our observations suggest such a novel mechanism, whereby aberrant Shh signaling, operating through Gli, increases rRNA synthesis by enhancing basonuclin expression during tumorigenesis.

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