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# Proliferation Marker pKi-67 Occurs in Different Isoforms With Various Cellular Effects

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**Abstract** The Ki-67 antigen, pKi-67, is a commonly used proliferation marker in research and pathology. It has been recognized that the protein exists in two different splice variants that differ in one exon. In the current work, we present three new splice variants of human pKi-67 consisting of two naturally occurring isoforms and one atypical version. Additionally, data is presented indicating that alternative splicing of the pKi-67 N-terminus is common in tumor cell lines. Analyzing 93 tissues mainly consisting of brain tumor specimens, we found evidence that long and short isoform can be expressed independently of each other. Induction of mitosis in human peripheral blood mononuclear cells revealed that short pKi-67 appears earlier in the cell cycle than the long isoform and reaches its expression maximum when transcription of the latter sets in. Finally, transfection of mammalian culture cells with exon 7 (specific for the long pKi-67 isoform and not present in the short isoform) in a tetracycline regulated expression system decreased the rate of cell proliferation without affecting the cell cycle. In summary, we present evidence that the pKi-67 N-terminus is differentially spliced resulting in at least five different isoforms with different functions. J. Cell. Biochem. 91: 1280–1292, 2004. 

**Key words:** alternative splicing; exon 7; MIB-1; MIB-7; pKi-67

The nuclear protein pKi-67 is a frequently used cell proliferation marker and was originally described as associated with the active phases of the cell cycle ($G_1$, S, $G_2$, and M phase) [Gerdes et al., 1983]. Further investigations revealed that pKi-67 was undetectable in quiescent cells ($G_0$ phase) [Verheijen et al., 1989]. During the cell cycle, its localization in the nucleus alters. In early $G_1$ phase, it is distributed as small foci over the nucleoplasma, then associates with the dense fibrillar component of reforming nucleoli where it remains until middle $G_2$ phase [Kill, 1996]. While the nucleoli disintegrate pKi-67 spreads throughout the nucleoplasm and forms a mesh-like structure that is weakly associated with the condensing chromosomes in prophase [Verheijen et al., 1989; Ross and Hall, 1995]. In metaphase, it forms the perichromosomal scaffold around the chromosomes [Gautier et al., 1992; Kill, 1996], which disappears at the end of mitosis when the chromosomes decondense.

Two isoforms of pKi-67 have been described with predicted molecular weights of 359 and 320 kDa encoded by the same gene. The shorter variant results from an alternative splicing event of exon 7 during mRNA processing. Cloning of the pKi-67 cDNA revealed a very large mRNA (approximately 12,500 bp) with 15 exons [Schlüter et al., 1993; Duchrow et al., 1996]. Exon 13 encodes the central part of the protein and contains 16 tandem repeats. The protein is capable of binding DNA [MacCallum and Hall, 2000], contains two nuclear localization signals, a potential ATP/GTP-bind-

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ing site at the C-terminus and 10 PEST sites [Schlüter et al., 1993; Duchrow et al., 1994; Duchrow et al., 1995], which direct the protein to degradation via the ubiquitin-pathway. Additionally, it encloses a large number of phosphorylation sites [Heidebrecht et al., 1996], which are targeted to yield a high phosphorylation status during the cell cycle [MacCallum and Hall, 1999; Endl and Gerdes, 2000]. Dephosphorylation releases pKi-67 from condensed chromosomes and impacts formation of the perichromosomal scaffold.

Functionally, it is known that expression of pKi-67 anti-sense oligonucleotides [Schlüter et al., 1993; Kausch et al., 2003], microinjection of anti-pKi-67 antibodies [Starborg et al., 1996], or over-expression of recombinant pKi-67 tandem repeats [Duchrow et al., 2001] hampers cell division and attenuates progression of the cell cycle. Recently, we were able to demonstrate that pKi-67 is involved in the regulation of cell division by organizing the lifecycle of nucleoli dependent on cyclin B and ran [Schmidt et al., 2003], which effects were self-regulated [Schmidt et al., 2002a].

To further analyze the function of pKi-67, we tried to understand the meaning of different pKi-67 isoforms. For that reason, we generated expression profiles in different cell types and tissues. In this process, we detected new splicing variants of pKi-67 and found a divergence in the expression of long and short splicing variant. Further analysis revealed a profound difference in their functional impact on cell proliferation.

## METHODS

### Amplification, Sequencing, and Constructs

Specific oligonucleotides against different regions of pKi-67 cDNA were prepared based on GenBank sequence file NM_002417 (Fig. 1A and Table I). Total RNA of cell lines and fresh frozen brain tumor tissues was carefully isolated under permanent cooling using the QIAmp RNA Blood Mini Kit (QIAGEN, Hilden, Germany). DNA-free mRNA was reversely transcribed by the ThermoScript kit (Invitrogen, Carlsbad, CA) using an oligo-$(dT)_{20}$ primer. For qualitative PCR analysis, cDNA was amplified by Platinum-Taq polymerase (Invitrogen) using a standard protocol. In brief, 30 s of DNA-denaturation at 95°C were followed by 30 s of annealing of primers to DNA at 52°C, and 1 min elongation of DNA at 95°C. The N-terminal fragment was elongated for 3 min due to the maximum size of 3 kbp.

For sequence analysis, DNA-fragments were randomly cloned into the pCR2.1-TOPO vector of the TOPO-TA cloning kit (Invitrogen), and cycle sequencing was performed with dye labeled M13 forward (−20 and −40) or M13 reverse primers. Subsequently, DNA-sequences were determined in either a DNA-sequencer model 4200L (LI-COR, Lincoln, NE) or a Long-Read Tower (Visible Genetics, Suwanee, GA). DDBJ/EMBL/GenBank accession numbers of new identified pKi-67 isoforms: pKi-67 γ (lacks exons 3, 4, and 7): AJ567756; pKi-67 δ (lacks exons 3 to 12): AJ567757; pKi-67 ε (lacks a part of exon 7): AJ567755.

For transient expression in a tetracycline regulated expression system, pKi-67 cDNA fragments (Fig. 1A) were amplified from HeLa cDNA with *Pfu* proofreading DNA polymerase and cloned into the expression vector pUHD10/3 of the Tet-system (Clontech, Heidelberg, Germany). Constructs were modified by addition of a Kozak sequence at the 5′ end as well as a STOP codon at the 3′ end with or without an additional nuclear localization sequence (NLS). Constructs were sequenced as a control. Correct protein expression was checked by RT-PCR and immunoblotting in HT-OFF cells (HeLa cells stably transfected with the tetracycline/doxycycline regulated repressor used in the TET-system (Clontech, Heidelberg, Germany)). Transcription was inhibited by addition of 5 ng/ml doxycycline.

### Cells and Cell Lines

All cell lines used were cultured under standard conditions in DMEM supplemented with antibiotics and 10% fetal calf serum (FCS). Peripheral blood mononuclear cells (PBMC) were isolated from blood using Ficoll density gradient centrifugation and cultured in RPMI medium plus antibiotics and 10% FCS. Cultured cells were transfected using Polyfect reagent (Qiagen, Hilden, Germany) and harvested 48 h after transfection.

### Immunocytochemistry and Immunoblotting

PBMC were grown on slides over night. The next day mitosis was induced by 10 μg/ml phytohemagglutinin (PHA) for different time-periods and cells were subsequently fixed in 75% ethanol at −20°C for at least 1 h. Endogen-

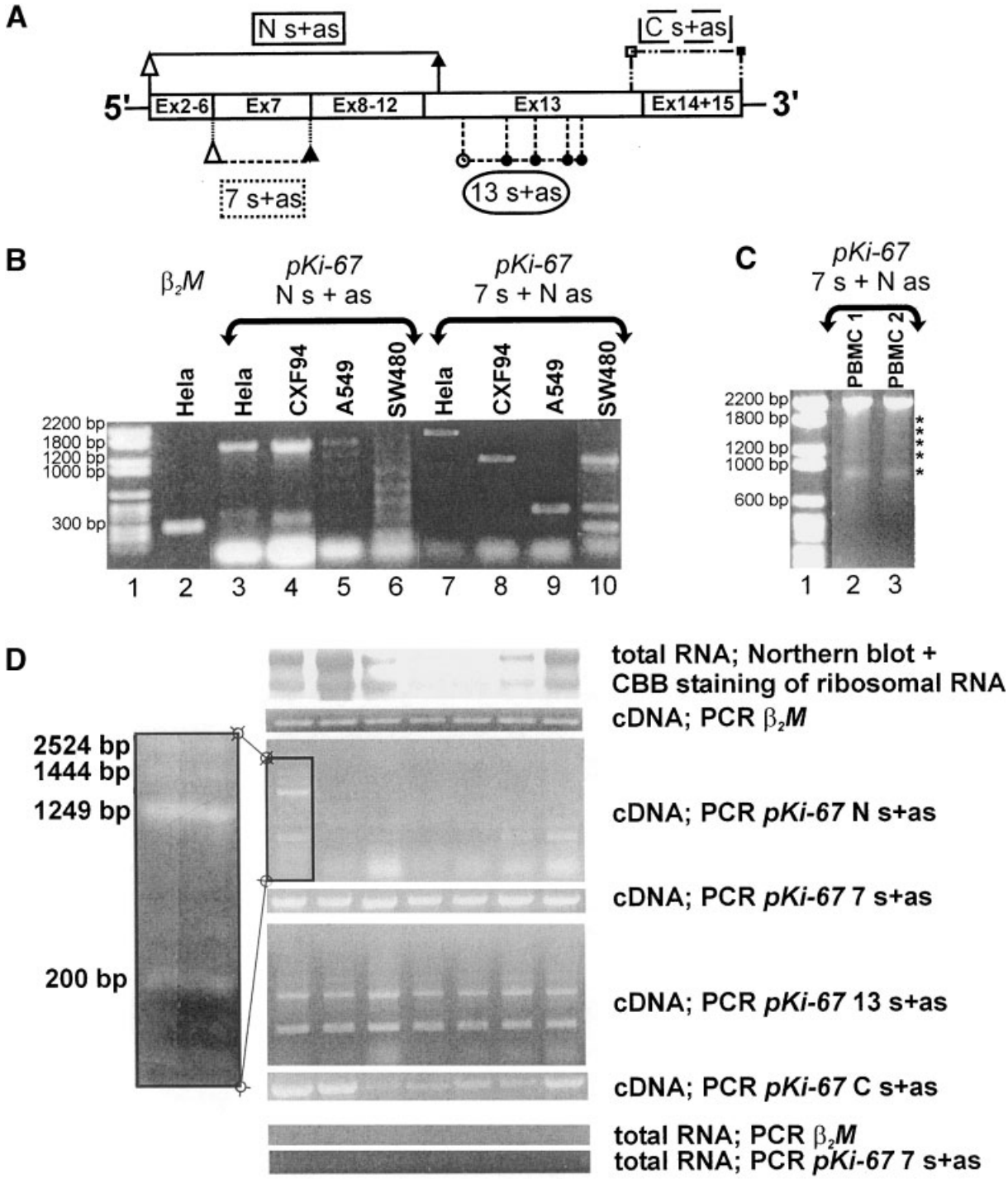


**Fig. 1.** **A**: Schematic diagram of pKi-67 cDNA specific primer sets used in this work (based on GenBank sequence file NM_002417 and Table I). **B**: Amplification of pKi-67 N-terminus in cervix (HeLa) and colon carcinoma (CXF94, A549, SW480) implicated unknown splicing variants. **C**: Stimulation of PBMC with PHA for 50 h resulted in multiple bands after amplification of the pKi-67 N-terminus. **D**: RNA was isolated from fresh frozen tumor tissues and the concentration of isolated RNA was checked by Northern blot and subsequent staining with Coomassie brilliant blue (CBB) solution in order to visualize ribosomal RNA. Domains N, 7, 13, and C of pKi-67 as well as $\beta_2 M$ as a control were amplified by PCR. Total RNA underwent PCR with indicated primer sets to guarantee DNA free RNA. The magnified window shows four bands that appeared after PCR with N-terminal primers of meningioma cDNA. These were identified as pKi-67 isoforms by DNA sequencing.

**TABLE I. Sequences of Oligonucleotides Used in This Work**

| Primer | Sequence | Position in cDNA (bp) |
|---|---|---|
| PKi-67 N s | 5′-TAT ACA TAT GAT GTG GCC CAC GAG ACG-3′ | 197–213 |
| PKi-67 N as | 5′-TAT AGG ATC CTA CGT TTC CAT TTT CTC TAA TAC AC-3′ | 2720–2695 |
| PKi-67 7 s | 5′-ATA TGA ATT CCA CCA TGG AGA AAG CTC AAG ATT CCA AGG-3′ | 599–620 |
| PKi-67 7 as | 5′-ATA TGG ATC CTC AAA TGG AAT CAC CAA AGT TGT TGA-3′ | 1675–1653 |
| PKi-67 13 s | 5′-TAT AGA ATT CCA CCA TGG AGC CCG TGT AAC TGG AAT GA-3′ | 3433–3452 |
| PKi-67 13 as | 5′-ATA TGG ATC CTC AGT CTT CTA GGG GTT GGG CCT T-3′ | 4594–4574, 6776–6758, 8230–8210, 8947–8927 |
| PKi-67 C s | 5′-TAT AGA ATT CGC TGC TGA TAG CTT AAG CG-3′ | 9008–9021 |
| PKi-67 C as | 5′-TAT AGG ATC CAT ATC TTC ACT GTC CCT ATG ACT TCT GGT TG-3′ | 9963–9933 |
| $\beta_2$M as | 5′-ATC CAG CGT ACT CCA AAG ATT-3′ | 861–881 |
| $\beta_2$M as | 5′-CAT GTC TCG ATC CCA CTT AAC TAT-3′ | 1157–1134 |

ous pKi-67 was immunocytochemically stained with either 1 μg/ml MIB-1, which detects the long and short isoform of the protein or 40 μg/ml MIB-7 that is specific for the long variant only. StreptAB-complex/HRP-duett kit (Dako, Hamburg, Germany) was used as a secondary reagent. Immunoblotting of pKi-67 following standard SDS–PAGE was performed with 8 μg/ml MIB-1 as a primary and alkaline phosphatase conjugated goat anti-mouse IgG (A-2429, Sigma, Deisenhofen, Germany) as a secondary antibody. Lanes were visualized with BCIP/NBT as a chromogen.

### Cell Cycle Analysis and Proliferation Assay

DNA staining of transfected cells was carried out using the Cycle Test Plus DNA Reagent Kit (BD Biosciences, Heidelberg, Germany). In brief, nuclei of transfected cells ($5 \times 10^5$/500 μl) were isolated by disruption of the cell membrane and the cytoskeleton with a combination of trypsin and spermine tetra-hydrochloride. DNA was stained with propidium iodide (PI) at a final concentration of at least 1.25 μg/ml. Nuclei were analyzed with a FACScan device (BD Biosciences), fitted with an Argon ion laser adjusted to emit 500 mW at 480 nm and a doublet discriminator. Red fluorescence (PI) was measured through an Lp 650 nm filter. Fluorescence distributions were analyzed with the computer programs Cellquest and ModFit (supplied by BD Biosciences).

Cell proliferation was quantified with the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega, Mannheim, Germany) that detects changes in absorbance at 490 nm caused by the conversion of a tetrazolium compound into a formazan product in cycling cells.

## RESULTS

In order to analyze expression of different pKi-67 isoforms in cultured cells, we prepared cDNA from HeLa cervix carcinoma and CXF94, A549, and SW480 colon carcinoma cell lines. Subsequent PCR with primer sets illustrated in Figure 1A revealed no obvious differences in the expression of exon 7 alone (7 s + as), exon 13 (13 s + as), or the C-terminus (C s + as) of pKi-67 (data not shown). However, analysis of the N-terminus (N s + as) showed at least four different bands (Fig. 1B, lanes 3–6) while only the signal at 1,444 bp was expected. This band corresponds to the short isoform of pKi-67. Sequencing of the major bands identified them as pKi-67 cDNA. However, DNA concentration of faint bands was too low in order to successfully clone them and therefore might be artificial. The long variant is approximately 1,080 bp longer but is often not detected due to the thermodynamically preferred amplification of the shorter fragment. To overcome this shortcoming, we combined the sense primer of exon 7 (7 s) with the anti-sense primer of the N-terminus (N as). The second oligonucleotide corresponds to a region in exon 13 immediately upstream of the first "Ki-67 repeat." Unexpectedly, we detected bands of different size for every cell line analyzed (Fig. 1B, lanes 7–10). Only HeLa cell cDNA yielded the expected fragment of 2,122 bp, while colon carcinoma cDNA contained either different sized or multiple bands. To analyze if this result was caused by a cell culture artifact we collected PBMC from two different persons and induced cell proliferation by incubation with 10 μg/ml PHA for 50 h. Subsequently, RT-PCR using the same primer set as above (7 s + N as) revealed that although the expected fragment of 2,122 bp

was the prevalent band amplified at least five more variants could be detected (Fig. 1C). Furthermore, amplification of the pKi-67 N-terminus of cDNA from human meningioma also revealed at least four major bands (Fig. 1D; magnified window). Sequencing of these four marked lanes identified them as pKi-67, while the faint bands turned out to be unspecific PCR products. Together these results, derived from different tissues and primary and cultured cells, suggest that the N-terminus of pKi-67 exists in more than two isoforms.

Different sized N-terminal pKi-67 fragments were subsequently detected in HEK 293 human embryonic kidney cells as well as U87, LNZ308, LN18, and A172 glioma cell lines. Cloning into the TOPO vector pCR2.1 and subsequent DNA sequence analysis identified the amplified fragments as pKi-67 variants. In addition to the already described long (pKi-67 α) and short (pKi-67 β) isoform of pKi-67, we were able to identify three new forms and refer to them as pKi-67 γ (lacking exon 3, 4, and 7), pKi-67 δ (lacking exons 3 to 12) as well as pKi-67 ε (lacking parts of exon 7; Fig. 2A,B). pKi-67 α to δ where isolated from HEK 293, U87, LNZ308, LN18, and A172 cell lines as well as meningioma tumor samples, while pKi-67 ε was exclusively isolated from activated PBMC. In Figure 2A, pKi-67 ε appears smaller than β or γ since it was amplified using primers flanking exon 7 (7 s + as) only, while the other fragments were cloned using primers covering the complete N-terminus (N s + as). The transition of exon 2 to 5 or 13, respectively, in pKi-67 γ and δ corresponds to an exon–intron branch site within the pKi-67 gene. It is likely that both isoforms occur as natural variants of pKi-67. Splicing of exon 7 in pKi-67 ε on the other hand is not based on an alternative recombination site. Different spliced versions of this exon have been described in mouse tissues and are considered atypical [Scholzen and Gerdes, 2000].

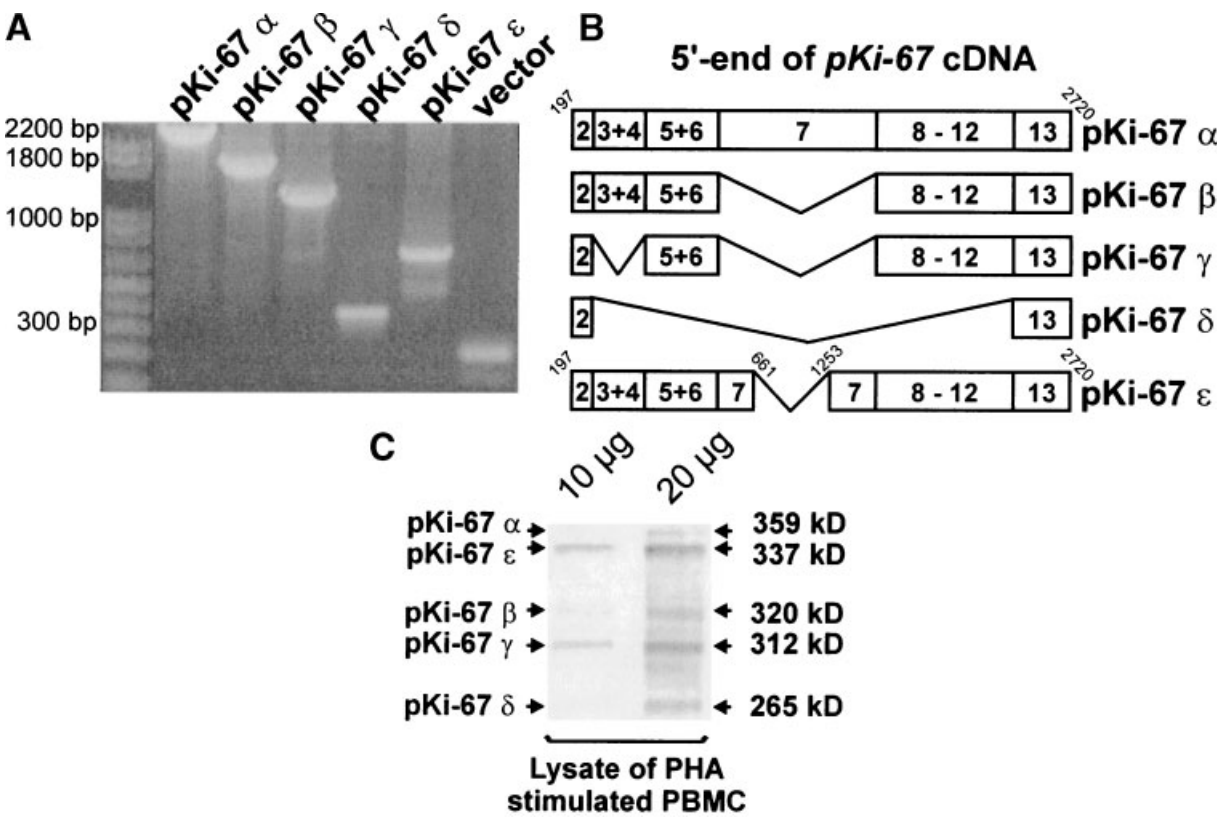


**Fig. 2.** **A**: Five pKi-67 N-terminal isoforms were detected in several tumor tissues and cultured cell lines. The agarose gel illustrates these variants that were cloned into pCR2.1-TOPO for sequencing and underwent PCR with a plasmid specific primer set. Long (pKi-67 α) and short (pKi-67 β) isoforms have already been described and differ in the presence of exon 7. pKi-67 γ, δ as well as ε are described for the first time. The former two lack exons 3, 4, and 7 or 3 to 12, respectively, while ε lacks a part of exon 7. **B**: Illustration of various N-termini described in the text. **C**: MIB-1 immunoblot of lysates of PBMC triggered with PHA for 50 h showing various isoforms of pKi-67 on the protein level.

The protein products of pKi-67 γ and ε use the same start codon as the long and short isoforms α and β, and theoretically result in proteins of 312 or 337 kDa, respectively. However, δ uses a start codon at the beginning of exon 13 and yields a cDNA that is missing the first 12 exons compared to pKi-67 α and has a calculated molecular weight of 265 kDa. All isoforms have similar isoelectric points (pI) ranging from 9.4 to 9.6 and contain seven to eight potential bipartite nuclear localization sequences. Despite different N-termini, all five pKi-67 variants contain identical central and C-terminal regions starting with exon 13 that carries the 'Ki-67 repeats' with the FKELF motifs, which is the epitope for the MIB-1 monoclonal antibody (mAb). This offered the possibility to perform an immunoblot with protein lysates of PBMC that have been triggered with PHA for 50 h (Fig. 2C). We chose this model since pKi-67 ε was exclusively isolated from PBMC and we intended to detect all five isoforms at once. The blot showed multiple bands that, based on the highest molecular weight standard, likely correspond to the predicted gene products. It has to be noted that there are no adequate markers in the size of pKi-67, since standard markers cover proteins of up to 250 kDa. Additionally, separation within the gel is not linear but represents the upper third of a standard mini gel due to the massive size of full-length pKi-67.

In order to analyze human tumors on preferential expression of certain pKi-67 isoforms, we analyzed 93 tissues mainly consisting of brain tumor specimens. Fresh frozen tissue samples were crushed under liquid nitrogen and total RNA was isolated, mRNA reverse transcribed and PCR performed as described above. Special attention was paid to ensure that no genomic DNA contaminated the isolated RNA to avoid false positive results if primer pairs spanning only one exon were used. To detect potential DNA contamination of RNA samples, we amplified a region within the *β2-microglobulin* (*β2M*) gene, which is present and expressed in every human cell, as well as exon 7 of pKi-67 with pure isolated RNA as a template. Only if no signal could be detected in both the cases, the RNA was used for further analysis. If DNA contamination was observed, the RNA was incubated with DNase I for 1 h and re-isolated and re-tested. Following reverse transcription, four different regions of pKi-67, and *β2M* as a positive and quality control, were amplified from every cDNA by specific primer sets (Fig. 1A). Primer pair 13 sense and anti-sense resulted in multiple bands due to four binding sites of the anti-sense primer. Figure 1D illustrates the results of every single step. PCR of the pKi-67 N-terminus, which promised insights into the expression profile of the new isoforms, was not reliable, due to the low RNA yield of tissue samples compared to cultured cells, which resulted in a signal that was most often too weak to be detected. Only few tissue specimens yielded strong bands that displayed different isoforms in a tumor type specific manner. However, exon 7, exon 13, and the C-terminus could be amplified in a reproducible manner and were used to analyze if different tumor tissues discriminate between long and short isoforms of pKi-67. Despite, due to multiple binding sites of the 13 anti-sense primer PCR results of exon 13 varied more than those of exon 7 and the C-terminus. The latter was therefore most reliable in estimating pKi-67 concentration without being quantitative. For that reason, focus of this study was the prevalent expression of pKi-67 isoforms in certain tumor types. Results are summarized in Table II. Overall, most samples expressed pKi-67 but the MIB-1 staining index as measured by the pathologists after excision of the tumor did not correlate strongly with the PCR results. Tissues with little MIB-1 index were still able to yield strong pKi-67 signals, independent of the domain amplified. Among the 93 samples, we detected five that were pKi-67 positive but did not yield a positive signal after amplification of exon 7. Usually, the presence of minimal amounts of exon 7 resulted in a strong signal using our PCR method. Therefore, cDNA of these five samples was freshly prepared two times and results of PCR were reproduced each time. In our opinion, this indicates that expression of pKi-67 alone is not sufficient for the expression of its long isoform.

The splice variants could have different functions and the loss of the long version seems to occur in some tumors. To test this hypothesis, PBMC were isolated from five patients, pooled and after 24 h under cell culture conditions, mitosis was initiated with 10 μg/ml PHA for the indicated time-periods (Fig. 3). Subsequently, RNA was isolated, cDNA synthesized, and PCR was performed with primer sets specific for β2*M*

**TABLE II. Expression Profile of Different pKi-67 cDNA Domains in Various Brain Tumor and Normal Tissues**

| No. | Tumor type | Age | Gender | MIB-1 index (%) total tumor | PCR 7 | PCR 13 | PCR C | PCR β2*M* | No. | Tumor type | Age | Gender | MIB-1 index (%) total tumor | PCR 7 | PCR 13 | PCR C | PCR β2*M* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 26 | A II | 62 | F | 2.6 | ++ | + | +++ | +++ | 286 | N | 58 | M | n.a. | 0 | 0 | 0 | + |
| 1407 | A II | 58 | F | 2.5 | +++ | +++ | +++ | +++ | 286 | N | 58 | M | n.a. | 0 | 0 | 0 | + |
| 1032 | A III | 41 | M | 3 | ++ | +++ | ++ | ++ | 521 | N | 22 | M | n.a. | 0 | 0 | + | ++ |
| 1232 | A III | 51 | F | 23 | +++ | +++ | +++ | ++ | 533 | N | 11 | F | n.a. | 0 | + | + | +++ |
| 1258 | AA | 55 | F | 15 | 0 | + | + | +++ | 1266 | N | 44 | M | n.a. | ++ | + | ++ | +++ |
| 1295 | AA | 58 | F | n.a. | + | + | + | +++ | 1493 | N | 73 | F | n.a. | + | + | + | +++ |
| 1344 | AA | 59 | M | 14 | ++ | ++ | +++ | +++ | 1514 | N | 69 | M | n.a. | +++ | + | + | +++ |
| 200 | AMNG | 50 | M | n.a. | +++ | ++ | +++ | +++ | 87 | O | 65 | F | 6 | +++ | ++ | +++ | ++ |
| 200 | AMNG | 50 | M | n.a. | +++ | ++ | +++ | +++ | 95 | O | 35 | M | 0.9 | + | + | ++ | ++ |
| 509 | AMNG | 74 | M | 2.5 | ++ | ++ | +++ | +++ | 285 | O | 60 | M | 10 | + | + | +++ | +++ |
| 1417 | AMNG | 62 | M | 1.5 | ++ | +++ | +++ | +++ | 327 | O | 72 | M | 7.5 | +++ | +++ | +++ | +++ |
| 117 | AO | 66 | M | 20 | ++ | + | ++ | ++ | 329 | O | 32 | F | 4.5 | ++ | + | + | ++ |
| 251 | AO | 53 | M | 17.5 | +++ | ++ | +++ | ++ | 332 | O | 33 | M | 9 | ++ | + | + | ++ |
| 966 | AO | 55 | M | 10 | +++ | + | +++ | +++ | 434 | O | 64 | M | 2.5 | + | ++ | + | + |
| 1126 | AO | 48 | M | 20 | +++ | +++ | +++ | +++ | 453 | O | 78 | F | n.a. | + | ++ | + | ++ |
| 1334 | AO | 27 | F | 6 | +++ | +++ | +++ | +++ | 471 | O | 35 | M | 10 | ++ | + | +++ | +++ |
| 1493 | AO | 73 | F | 50 | ++ | + | +++ | +++ | 604 | O | 43 | M | 4 | +++ | +++ | +++ | +++ |
| 1036 | EPN | 68 | M | 0.5 | 0 | 0 | 0 | +++ | 868 | O | 34 | M | 40 | +++ | +++ | +++ | +++ |
| 1054 | EPN | 17 | M | n.a. | ++ | ++ | +++ | +++ | 899 | O | 55 | M | 9 | +++ | +++ | +++ | +++ |
| 608 | GA | 52 | M | 6 | +++ | +++ | + | +++ | 910 | O | 39 | M | 20 | +++ | +++ | +++ | +++ |
| 953 | GA | 38 | M | 0.9 | ++ | +++ | +++ | +++ | 914 | O | 41 | M | 4 | ++ | ++ | ++ | +++ |
| 894 | GBM | 55 | M | 11 | 0 | + | + | 0 | 931 | O | 37 | F | 8 | +++ | + | +++ | +++ |
| 1057 | GBM | 61 | F | 50 | +++ | +++ | + | ++ | 962 | O | 32 | M | 1.5 | +++ | +++ | +++ | +++ |
| 1078 | GBM | 47 | M | 51 | +++ | +++ | +++ | +++ | 1150 | O | 69 | M | 33 | +++ | +++ | +++ | +++ |
| 1242 | GBM | 46 | M | 19 | ++ | ++ | ++ | +++ | 1325 | O | 35 | F | 3 | ++ | + | ++ | +++ |
| 1254 | GBM | 63 | F | 40 | ++ | + | + | + | 1381 | O | 31 | M | 2 | ++ | +++ | + | +++ |
| 1280 | GBM | 42 | F | 55 | +++ | ++ | ++ | +++ | 1489 | O | 52 | F | 1 | +++ | + | +++ | +++ |
| 1326 | GBM | 52 | M | 30 | ++ | ++ | + | ++ | 1613 | O | 34 | M | 5 | ++ | + | +++ | +++ |
| 1331 | GBM | 41 | F | n.a. | +++ | ++ | + | ++ | 22 | OA | 24 | M | 3.5 | ++ | +++ | ++ | ++ |
| 1338 | GBM | 51 | M | 12 | +++ | +++ | + | +++ | 1049 | OA | 34 | M | 2.75 | + | + | + | +++ |
| 1397 | GBM | 65 | F | n.a. | +++ | +++ | + | +++ | 1297 | OA | 59 | F | 22 | +++ | +++ | + | +++ |
| 1429 | GBM | 77 | F | 40 | ++ | ++ | ++ | +++ | 1433 | OII | 44 | F | 1.5 | 0 | + | + | +++ |
| 106 | GNGL | 44 | F | n.a. | + | +++ | + | +++ | 1502 | OII | 38 | F | 3 | 0 | + | + | +++ |
| 1027 | GNGL | 25 | F | 0.9 | ++ | ++ | +++ | +++ | 1551 | OIII | 30 | F | 13 | ++ | + | +++ | +++ |
| 631 | JPA | 5 | M | n.a. | +++ | ++ | +++ | +++ | 1155 | PA | 22 | M | 0.9 | ++ | ++ | ++ | +++ |
| 1300 | JPA | 4 | M | 0.9 | +++ | + | +++ | +++ | 1313 | PA | 33 | F | 1 | ++ | + | +++ | +++ |
| 1224 | MED | 33 | M | 20 | +++ | + | +++ | +++ | 449 | PIT | 60 | F | n.a. | 0 | ++ | ++ | +++ |
| 101 | MED | 42 | F | n.a. | +++ | +++ | ++ | +++ | 1261 | PIT | 44 | M | n.a. | +++ | ++ | ++ | +++ |
| 603 | MED | 32 | M | 30 | +++ | ++ | +++ | +++ | 1310 | PIT | 77 | F | n.a. | +++ | +++ | +++ | +++ |
| 1258 | MG | 55 | F | 18 | + | +++ | +++ | +++ | 207 | PNET | 52 | M | n.a. | +++ | + | +++ | +++ |
| 192 | MNG | 66 | F | n.a. | +++ | ++ | +++ | +++ | 493 | PNET | 70 | M | n.a. | ++ | + | +++ | +++ |
| 288 | MNG | 77 | M | n.a. | + | +++ | + | +++ | 585 | PNET | 11 d | F | 31 | ++ | ++ | +++ | +++ |
| 441 | MNG | 81 | F | n.a. | ++ | +++ | +++ | +++ | 887 | PNET | 25 | F | 70 | +++ | +++ | +++ | +++ |
| 994 | MNG | 50 | F | n.a. | +++ | +++ | +++ | ++ | 372 | SCHW | 18 | F | n.a. | +++ | +++ | +++ | +++ |
| 1248 | MNG | 67 | M | n.a. | +++ | +++ | ++ | +++ | 641 | SCHW | 51 | M | n.a. | ++ | ++ | +++ | +++ |
| 1361 | MNG | 79 | F | n.a. | ++ | ++ | +++ | +++ | 690 | SCHW | 54 | F | 0.9 | +++ | ++ | +++ | +++ |
| 1438 | MNG | 56 | M | n.a. | ++ | +++ | + | +++ | | | | | | | | | |

0: no PCR signal; +: weak PCR signal; ++: strong PCR signal; +++: very strong PCR signal; n.a.: not available.
A, astrocytoma; AA, anaplastic astrocytoma; AMNG, atypical meningioma; AO, anaplastic oligodendroglioma; EPN, ependymoma; GA, gemistocytic astrocytoma; GBM, glioblastoma multiforme; GNGL, ganglioglioma; JPA, juvenile pilocytic astrocytoma; MED, medullablastoma; MG, malignant glioma; MNG, meningioma; N, normal tissue; O, oligodendroglioma; OA, oligoastrocytoma; PA, pilocytic astrocytoma; PIT, pituitary adenoma; PNET, primitive neuroectodermal tumor; SCHW, schwannoma.

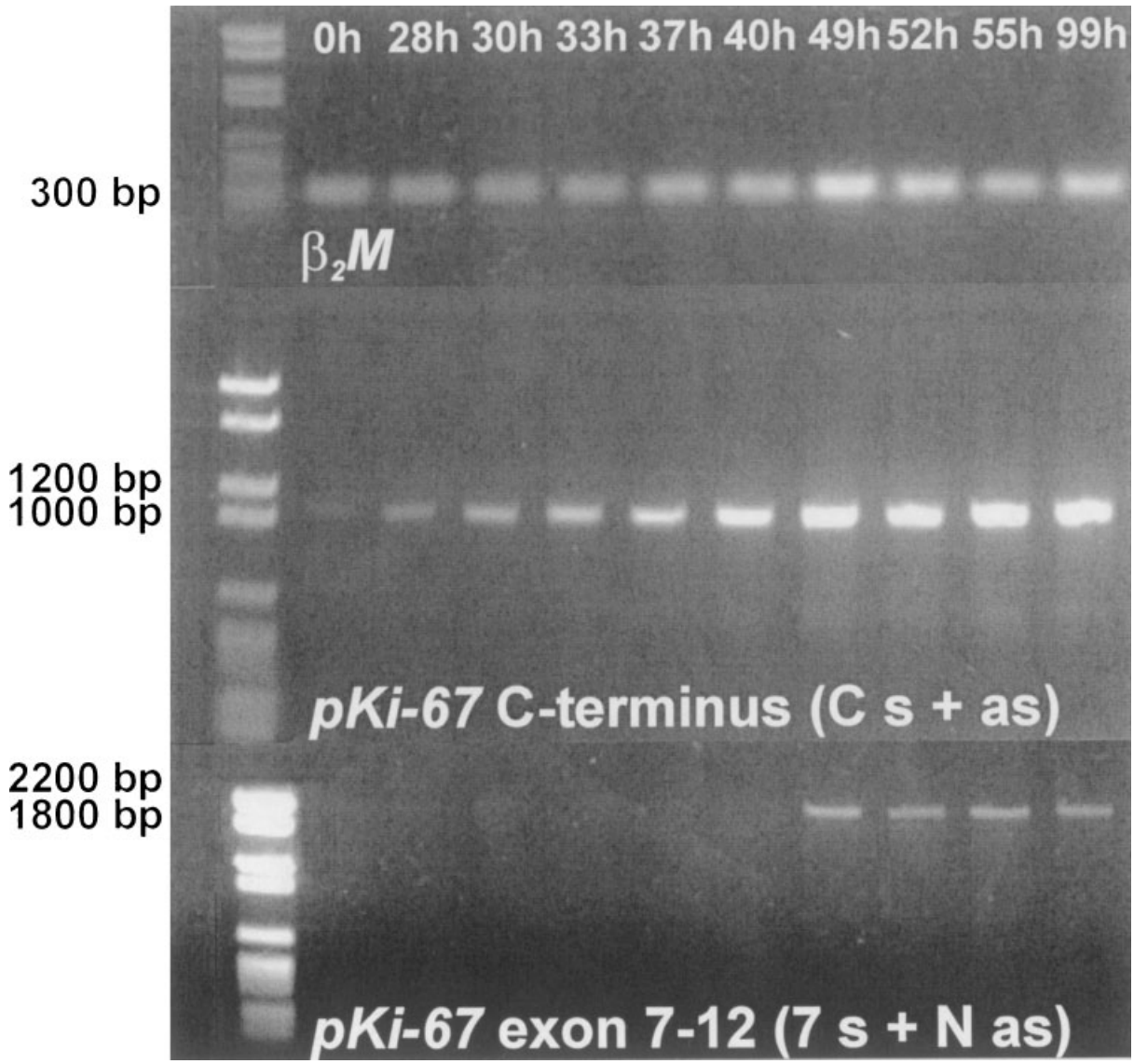


**Fig. 3.** PBMC were stimulated with PHA for the indicated time-periods. RNA was isolated at each time-point, cDNA synthesized, and PCR performed with primers against pKi-67 N- and C-terminus or $\beta_2M$ as a positive control, respectively. A C-terminal signal was detectable from 28 h after stimulation on with its maximum of expression at 49 h. At this time-point, a signal specific for the long pKi-67 isoform started to appear with a delay of about 20 h.

as a positive control, the pKi-67 C-terminus (covering all isoforms) and exon 7 sense primer (7 s) together with the N-terminal anti-sense primer (N as; specific for the long isoform). Agarose gel electrophoresis revealed that from 28 h after PHA stimulation on the amount of pKi-67 mRNA increased with its peak at 49 h. After that the amount remained constant. The long isoform on the other hand could be detected from 49 h after PHA stimulation on, which correlates with the peak of total pKi-67 expression. The $\beta_2M$ signal is overall equal (except 49 h lane) indicating a similar amount of cDNA used in individual PCR. Control-PCR using HeLa cDNA revealed similar efficiency in amplification of all three fragments (data not shown). Both controls indicate that shifted expression of pKi-67 $\alpha$ and $\beta$ is not a PCR artifact caused by different sizes of amplified fragments. The experiment was repeated and PBMC were seeded on slides, fixed and stained with MIB-1 and MIB-7 mAb. The former antibody detects several epitopes in exon 13 that is present in all described isoforms, while the latter is specific

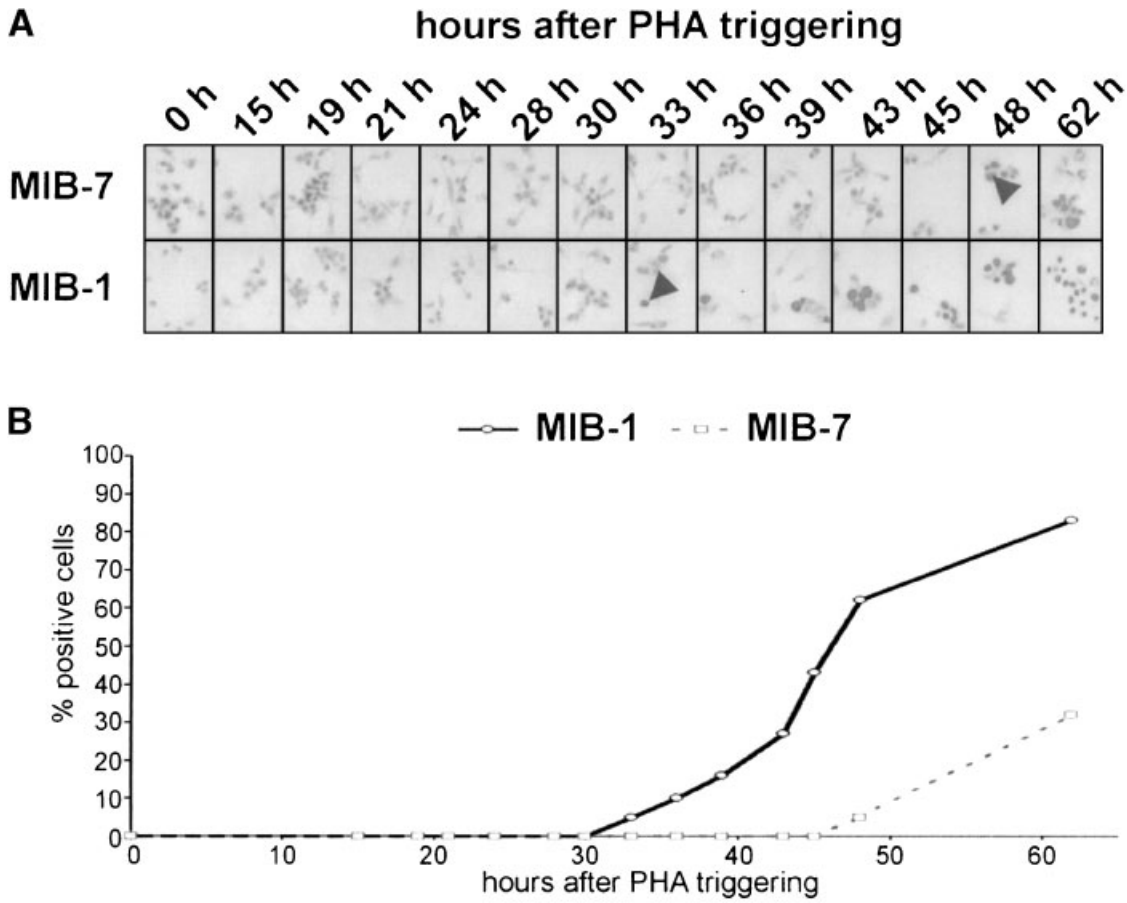


**Fig. 4.** **A**: PBMC were stimulated with PHA for the indicated time-periods, fixed, and stained with MIB-1 (α and β pKi-67 isoform) or MIB-7 (α isoform only). Positive nuclei are marked with arrow-heads. MIB-1 stained cells appeared about 33 h after stimulation, while MIB-7 positive cells were detectable after 48 h with a delay of 25 h. **B**: Quantitative blot of (A) with MIB-1 or MIB-7 positive nuclei counted.

for one epitope in exon 7 of the long isoform. Figure 4A shows that MIB-1 positive nuclei appeared 33 h after stimulation with PHA, while MIB-7 positive cells were detected about 48 h after stimulation. Positive cells were counted and blotted against PHA incubation time (Fig. 4B). A sensitivity problem of antibodies was excluded by staining with a C-terminus specific antibody detecting a single epitope (MIB-21) similar to MIB-7. pKi-67 signal was detectable from about 30 h on (data not shown). Both experiments indicate that the long isoform (pKi-67 α) is expressed relatively late following stimulation. Again this offers a potential difference in the function of the two splice variants, since in PBMC the long isoform seems not to be expressed until completion of the first cell cycle.

In order to analyze, cellular effects of different pKi-67 cDNA regions illustrated in Figure 1A were cloned into a tetracycline regulated mammalian expression system. In each instance, the cDNA was modified by addition of a 5′ terminal Kozak sequence as well as either a 3′ terminal STOP codon (Ns, 7s, 13s) or a nuclear localization sequence followed by a STOP codon (Nn, 7n, 13n). Constructs were transiently transfected into HeLa TET-OFF cells. As a reference, expression was terminated by addition of 5 ng/ml doxycycline.

PI staining of exon 7 transfected cells was performed followed by flowcytometric cell cycle analysis (Fig. 5). We found that expression of exon 7 barely affected the cell cycle. Due to late expression of the long compared to the short pKi-67 isoform in PBMC, some effect of exon 7 on cell proliferation was expected. HeLa TET-OFF cells were transiently transfected with constructs described above and a cell cycle proliferation assay was performed. N-terminus

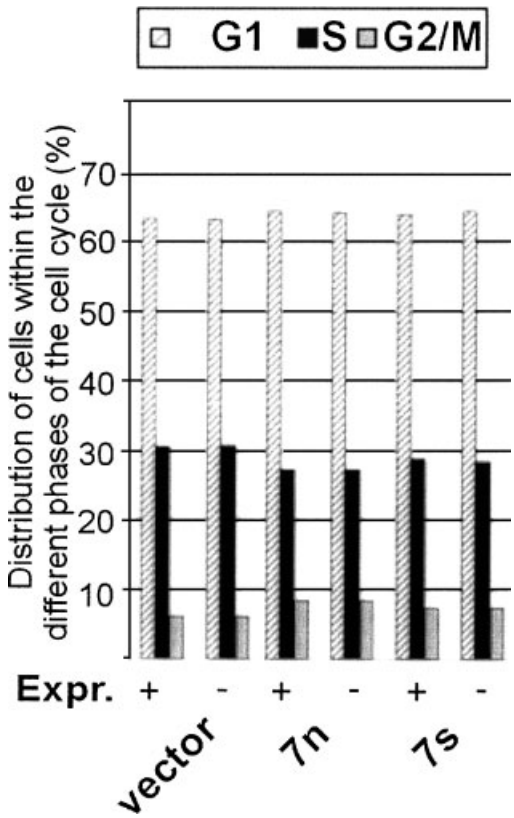


**Fig. 5.** No major influence of recombinant exon 7 on the cell cycle of transiently transfected HeLa cells was detectable comparing plasmid transcribing (+) and non-transcribing (–) cells. 7: exon 7; n: 3′-terminal NLS plus STOP codon; s: 3′-terminal STOP codon.

and repeat region of pKi-67 increased cell proliferation but exon 7 had the opposite effect and slightly diminished the rate of cell division (Fig. 6). This effect is surprising, since we did not detect an influence on cell cycling. Trypan blue staining of exon 7 transfected HeLa TET-OFF cells before and after activation of the Tet promotor with doxycycline revealed about 12% dead cells in either sample, excluding the possibility of a toxic effect mediated by exon 7 measured in the cell proliferation assay. Therefore, cell viability was not affected. Due to the late appearance of exon 7 in the cell cycle, we speculate that it has an impact on transition from $G_1$ to $G_0$ phase. No difference was seen in cell cycle analysis, since the amount of chromosomes, which is measured in this kind of assay, is equal in both phases. As the cultured cells used do not differentiate, they do not stay in $G_0$ phase and potentially reenter active phases of the cell cycle, which shows up as a brief delay in cell proliferation.

## DISCUSSION

This work set out to analyze the function of pKi-67 by characterizing its different isoforms. In the process of studying long and short splice variants of this gene, we found three new derivates. These were isolated from various cell lines and tissues and are therefore not a cellular artifact of one specific cell type. One of the new isoforms, pKi-67 ε, contains an unusual short form of exon 7. In agreement with this data, alternative splicing of this exon has been reported for human pKi-67 (Dr. Thomas Scholzen, Research Center Borstel, Borstel, Germany, unpublished communication) and its murine ortholog [Scholzen and Gerdes, 2000]. The new isoforms of human pKi-67 (γ, δ, and ε) we report here have not been previously described to our knowledge. In contrast to the α, β, and ε form, γ and δ lack a forkhead-associated domain (FHA) [Durocher and Jackson, 2002] at the N-terminus. The function of this nuclear signaling domain is not yet well characterized in pKi-67, and therefore the implications for its absence are not clear at present, but are likely to be significant. The FHA domain has been reported to interact with the human kinesin-like protein 2 (Hklp2) [Sueishi et al., 2000] and with the nucleolar protein NIFK [Takagi et al., 1999]. It has been suggested that pKi-67 is involved in the progression of mitosis by its interaction with Hklp2. Additionally, it has been speculated that pKi-67 has a regulatory effect on the rRNA and rDNA metabolism by the interaction with NIFK. Isoforms lacking the FHA domain would almost certainly be impaired in fulfilling these functions.

Despite varying N-termini, all five splice variants are identical downstream from exon 13 on and therefore contain pKi-67s tandem repeats as well as its C-terminus with the DNA-binding domain and the potential ATP/GTP loop. Especially, the tandem repeats have been reported to be of significant importance for interactions with other proteins [Schmidt et al., 2003]. pKi-67 has been shown to undergo a significant amount of interactions, which could be mostly linked to the tandem repeat region of the protein. The C-terminus was implicated in DNA binding [MacCallum and Hall, 2000] and mediates the interaction with heterochromatin protein 1 (HP1) [Scholzen et al., 2002; Schmidt et al., 2003]. In this context, pKi-67 was implicated to have a role in chromatin

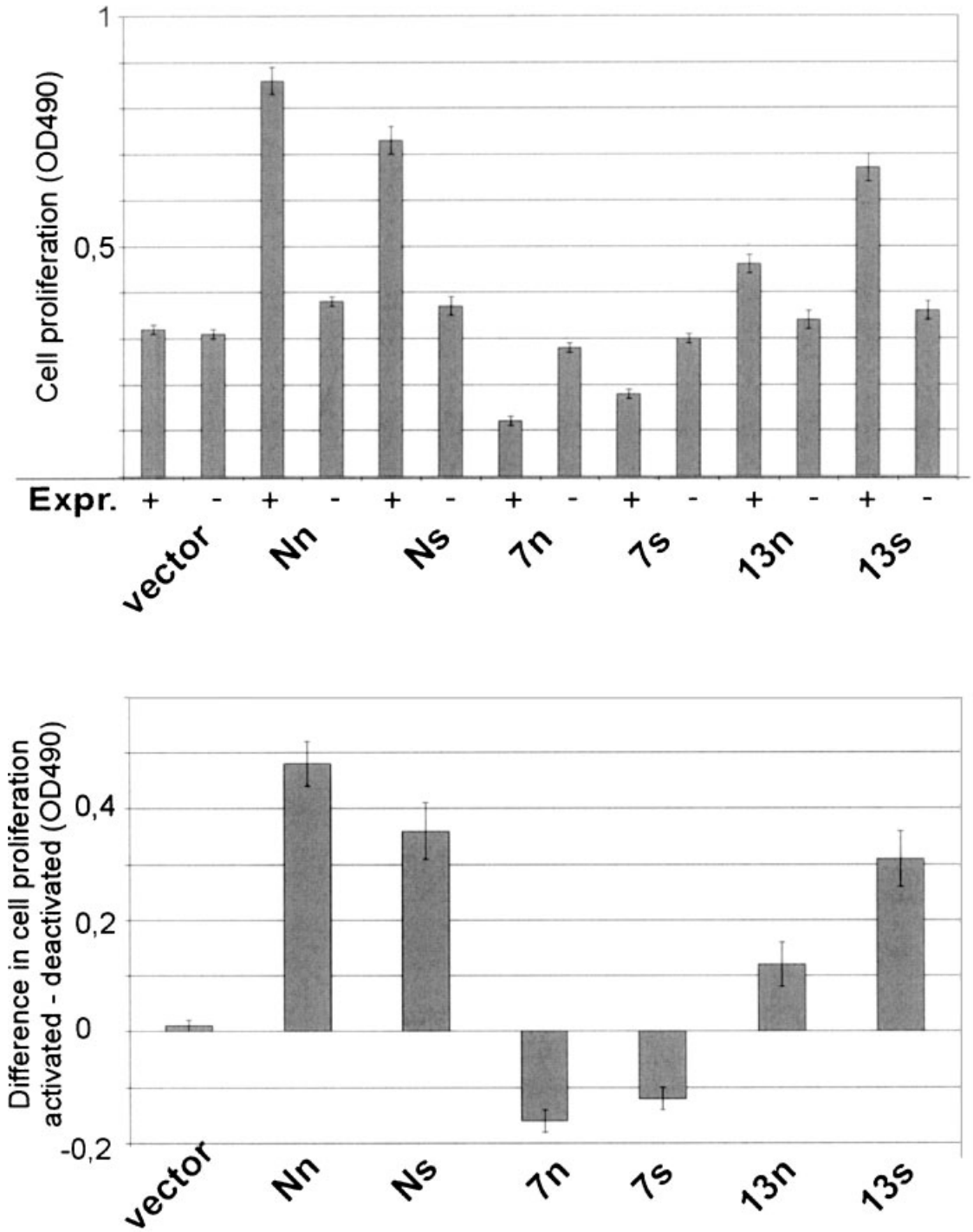


**Fig. 6.** Influence of recombinant pKi-67 domains on cell proliferation of transiently transfected HeLa cells. The upper diagram shows total cell proliferation. The lower diagram illustrates differences between plasmid transcribing (+) and non-transcribing (−) cells. N: N-terminus; 7: exon 7; 13: repeats one to three of exon 13; n: 3′-terminal NLS plus STOP codon; s: 3′-terminal STOP codon.

structure and to mediate attachment of chromatin to the inner nuclear lamina. All isoforms probably show a similar spectrum of interactions but could be alternatively localized due to different N-termini. Since pKi-67 forms mesh-like structures that are mediated by self-association [Schmidt et al., 2003] of the protein's N-terminus one is tempted to speculate that

isoforms carrying differentially spliced N-terminal structures could act in a dominant negative way and interrupt the network of interactions of full length pKi-67.The concentration of new splice variants compared to the already known ones within the cell was low on the mRNA level. This could explain why these forms have not yet been described and allows that they are not produced throughout the cell cycle. Alternatively, these variants could reflect alterations in the accuracy of the splicing machinery during cell division, which results in alternate splicing patterns.

In order to determine if pKi-67 isoforms fulfill different functions, we analyzed 93 tissue specimens. On the quantitative level, we found no correlation of PCR results with MIB-1 staining indices of tumors as determined by pathologists after surgery. One explanation is that massive pKi-67 entities are not detected by MIB-1 as described before [Schmidt et al., 2002b]. Another possibility is that RT-PCR studies using tumor specimens are to unspecific and do not give evidence about real pKi-67 concentration in tumor cells. We cannot speculate how much of the signal was caused by tumor-surrounding astrocytes or infiltrating lymphocytes. Due to this quantitative uncertainties, we did not go deeper in correlation studies but focused on the prevalent expression of pKi-67 $\alpha$ and $\beta$ splice variants in tumor specimens. In most tissues, both isoforms were detected but five specimens expressed only the $\beta$ variant. It is unclear why only approximately 5% of tissues expressed a single isoform, but this may indicate that most cancers are not clonal with respect to pKi-67 expression or that long and short splicing variation occur independently of each other. Additionally, expression of either isoform could depend on the current cell cycle phase, which was supported by the fact that the large pKi-67 isoform was expressed later in a PHA triggered PBMC model. In this context, it has been reported that only one isoform of pKi-67 is expressed during each cell cycle phase [Scholzen and Gerdes, 2000], which could explain why long and short isoform are not expressed at the same time in this model.

It remains questionable what advantage a tumor could get from loosing specific splice variants of pKi-67. Some indication was derived from cell cycle and cell proliferation assays. Exon 7 had no detectable effect on the distribution of cells within the different phases of the cell cycle but contrary to other pKi-67 domains it had an anti-proliferative effect. We concluded that this data implies a role of exon 7 in differentiation of cells, leading to a $G_1$ to $G_0$ transition that was undetectable by standard cell cycle analysis. Loosing this region could thereby contribute to the permanent cell cycling of some tumor cells.

Summarizing, the data presented in this work indicate that pKi-67 exists in more than two isoforms and that alternative splicing of the N-terminus is common in cultured cells and tumor tissues. Furthermore, different splicing variants potentially have alternative or even opposite functions, which allows pKi-67 to broadly regulate the cell cycle. One can speculate that the short isoform keeps the cell cycle going, the long isoform drives the cell towards differentiation and the intermediates are used to break up the rigid pKi-67 structures that build up during different phases of the cycle. If this model is correct, switching the balance of isoforms towards the long structure could induce differentiation of cycling cells.

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## REFERENCES

Duchrow M, Gerdes J, Schlüter C. 1994. The proliferation-associated Ki-67 protein: Definition in molecular terms. Cell Prolif 27:235–242.

Duchrow M, Schlüter C, Key G, Kubbutat MH, Wohlenberg C, Flad HD, Gerdes J. 1995. Cell proliferation-associated nuclear antigen defined by antibody Ki-67: A new kind of cell cycle-maintaining proteins. Arch Immunol Ther Exp (Warsz) 43:117–121.

Duchrow M, Schlüter C, Wohlenberg C, Flad HD, Gerdes J. 1996. Molecular characterization of the gene locus of the human cell proliferation-associated nuclear protein defined by monoclonal antibody Ki-67. Cell Prolif 29: 1–12.

Duchrow M, Schmidt MHH, Zingler M, Anemüller S, Bruch HP, Broll R. 2001. Suppression of cell division by pKi-67 antisense-RNA and recombinant protein. Cell Physiol Biochem 11:331–338.

Durocher D, Jackson SP. 2002. The FHA domain. FEBS Lett 513:58–66.

Endl E, Gerdes J. 2000. Posttranslational modifications of the KI-67 protein coincide with two major checkpoints during mitosis. J Cell Physiol 182:371–380.

Gautier T, Dauphin-Villemant C, Andre C, Masson C, Arnoult J, Hernandez-Verdun D. 1992. Identification and

characterization of a new set of nucleolar ribonucleoproteins which line the chromosomes during mitosis. Exp Cell Res 200:5–15.

Gerdes J, Schwab U, Lemke H, Stein H. 1983. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13–20.

Heidebrecht HJ, Buck F, Haas K, Wacker HH, Parwaresch R. 1996. Monoclonal antibodies Ki-S3 and Ki-S5 yield new data on the 'Ki-67' proteins. Cell Prolif 29:413–425.

Kausch I, Lingnau A, Endl E, Sellmann K, Deinert I, Ratliff TL, Jocham D, Sczakiel G, Gerdes J, Bohle A. 2003. Antisense treatment against Ki-67 mRNA inhibits proliferation and tumor growth in vitro and in vivo. Int J Cancer 105:710–716.

Kill IR. 1996. Localisation of the Ki-67 antigen within the nucleolus. Evidence for a fibrillarin-deficient region of the dense fibrillar component. J Cell Sci 109:1253–1263.

MacCallum DE, Hall PA. 1999. Biochemical characterization of pKi67 with the identification of a mitotic-specific form associated with hyperphosphorylation and altered DNA binding. Exp Cell Res 252:186–198.

MacCallum DE, Hall PA. 2000. The biochemical characterization of the DNA binding activity of pKi67. J Pathol 191:286–298.

Ross W, Hall PA. 1995. Ki67: From antibody to molecule to understanding? J Clin Pathol Mol Pathol 48:M113–M117.

Schlüter C, Duchrow M, Wohlenberg C, Becker MH, Key G, Flad HD, Gerdes J. 1993. The cell proliferation-associated antigen of antibody Ki-67: A very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. J Cell Biol 123:513–522.

Schmidt MHH, Broll R, Bruch HP, Duchrow M. 2002a. Proliferation marker pKi-67 affects the cell cycle in a self-regulated manner. J Cell Biochem 87:334–341.

Schmidt MHH, Broll R, Bruch HP, Duchrow M. 2002b. The proliferation marker pKi-67 becomes masked to MIB-1 staining after expression of its tandem repeats. Histochem Cell Biol 118:415–422.

Schmidt MHH, Broll R, Bruch HP, Bögler O, Duchrow M. 2003. The proliferation marker pKi-67 organizes the nucleolus during the cell cycle depending on Ran and cyclin B. J Pathol 199:18–27.

Scholzen T, Gerdes J. 2000. The Ki-67 protein: From the known and the unknown. J Cell Physiol 182:311–322.

Scholzen T, Endl E, Wohlenberg C, van der Sar S, Cowell IG, Gerdes J, Singh PB. 2002. The Ki-67 protein interacts with members of the heterochromatin protein 1 (HP1) family: A potential role in the regulation of higher-order chromatin structure. J Pathol 196:135–144.

Starborg M, Gell K, Brundell E, Hoog C. 1996. The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression. J Cell Sci 109:143–153.

Sueishi M, Takagi M, Yoneda Y. 2000. The forkhead-associated domain of Ki-67 antigen interacts with the novel kinesin-like protein Hklp2. J Biol Chem 275: 28888–28892.

Takagi M, Matsuoka Y, Kurihara T, Yoneda Y. 1999. Chmadrin: A novel Ki-67 antigen-related perichromosomal protein possibly implicated in higher order chromatin structure. J Cell Sci 112:2463–2472.

Verheijen R, Kuijpers HJ, Schlingemann RO, Boehmer AL, van Driel R, Brakenhoff GJ, Ramaekers FC. 1989. Ki-67 detects a nuclear matrix-associated proliferation-related antigen. I. Intracellular localization during interphase. J Cell Sci 92:123–130.