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

Carsten Schlüter, Michael Duchrow, Claudia Wohlenberg, Michael H. G. Becker, Göran Key, Hans-D. Flad, and Johannes Gerdes

Department of Immunology and Cell Biology, Division of Molecular Immunology, Forschungsinstitut Borstel, D-23845 Borstel, Germany

Abstract. The antigen defined by mAb Ki-67 is a human nuclear protein the expression of which is strictly associated with cell proliferation and which is widely used in routine pathology as a "proliferation marker" to measure the growth fraction of cells in human tumors. Ki-67 detects a double band with apparent molecular weights of 395 and 345 kD in immunoblots of proteins from proliferating cells. We cloned and sequenced the full length cDNA, identified two differentially spliced isoforms of mRNA with open reading frames of 9,768 and 8,688 bp encoding for this cell proliferation-associated protein with calculated molecular weights of 358,761 D and 319,508 D, respectively. New mAbs against a bacterially expressed part and a synthetic polypeptide deduced from the isolated cDNA react with the native Ki-67 antigen, thus providing a circle of evidence that we have cloned the authentic Ki-67 antigen cDNA. The central part of the Ki-67 antigen cDNA contains a large 6,845-bp exon with 16 tandemly repeated 366-bp elements, the "Ki-67 repeats", each including a highly conserved new motif of 66 bp, the "Ki-67 motif", which encodes for the epitope detected by Ki-67. Computer analysis of the nucleic acid and the deduced amino acid sequence of the Ki-67 antigen confirmed that the cDNA encodes for a nuclear and short-lived protein without any significant homology to known sequences. Ki-67 antigen-specific antisense oligonucleotides inhibit the proliferation of IM-9 cell line cells, indicating that the Ki-67 antigen may be an absolute requirement for maintaining cell proliferation. We conclude that the Ki-67 antigen defines a new category of cell cycle-associated nuclear nonhistone proteins.

C ELL proliferation is a biological process of fundamental importance controlled by highly coordinated mechanisms. Complex regulatory networks mediate the embryonic and normal development and are responsible for the systemic response to wounding and infection, whereas their dysregulation may result in tumor formation. Substantial progress in the understanding of the mechanisms and the regulation of the cell cycle has been made in recent years, and it has been found that numerous structures, among them key regulatory molecules such as p34^{cdc2} or p53, are cell cycle associated and undergo cell cycle-related modulations (e.g., reviewed by Norbury and Nurse, 1992, and Kirschner, 1992). Many cell cycle–associated proteins occur in considerable amounts only transiently in parts of the cycle, and the presence of many of them is not always strictly correlated with cycling cells. For example, the "proliferating cell nuclear antigen" (PCNA)[1] is also detectable in almost all quiescent cells adjacent to some tumors (Hall et al., 1990) and, presumably due to its long biological half-life, is also noticed in many quiescent tumor cells of different entities (McCormick and Hall, 1992). A possible reason for the occurrence of PCNA in quiescent cells is its involvement, together with enzymes of the DNA replication machinery, in nucleotide excision repair mechanisms, as demonstrated in quiescent cells after ultra-violet irradiation in vitro (Celis and Madsen, 1986; Toschi and Bravo, 1988) and in vivo (Hall et al., 1993). In 1983 a mAb, designated Ki-67, was described which selectively reacts with the nuclei of proliferating cells in all human tissues tested (Gerdes et al., 1983). Detailed cell cycle analyses have revealed that this proliferation-associated antigen is expressed in all active parts of the

---

Drs. Schlüter and Duchrow contributed equally to this publication.

cell cycle (G_1, S, G_2, and mitosis), but is absent in resting cells (G_0) (Gerdes et al., 1984; Schrape et al., 1987; Braun et al., 1988; Tazzari et al., 1990; Bruno and Darzynkiewicz, 1992), indicating that the Ki-67 antigen could be used as a marker for cells of the "growth fraction". This has been confirmed in a tumor xenograft model by comparing the Ki-67 labeling index with the growth fraction as determined by the fraction of labeled mitoses method (Scott et al., 1991). Consequently, the determination of the growth fraction with the antibody Ki-67 has been widely used in histopathology (reviewed by Brown and Gatter, 1990, and Gerdes, 1990), particularly in numerous studies on the prognostic value of assessing cell proliferation in clinical samples of human neoplasms (Gerdes et al., 1987; Grogan et al., 1988; Hall et al., 1988; Bouzubar et al., 1989; Holte et al., 1989; Nicholson et al., 1991; Tungekar et al., 1991; Wintzer et al., 1991; Fontana et al., 1992; Locker et al., 1992). All these studies have indicated that the Ki-67 labeling index is an independent prognosticator for assessing the clinical outcome of individual patients.

Several groups have demonstrated that the topographical distribution of the Ki-67 antigen is cell cycle dependent (Braun et al., 1988; Guillaud et al., 1989; du Manoir et al., 1991): in G_1 phase the Ki-67 antigen is predominantly localized in the perinucleolar region, in the later phases of the cell cycle the antigen is also detected throughout the nuclear interior, being predominantly localized in the nuclear matrix (Verheyen et al., 1989a). In mitosis, the Ki-67 antigen is present on all chromosomes (Gerdes et al., 1983, 1984) and appears in a reticulate structure surrounding the metaphase chromosomes (Verheyen et al., 1989b). Because Ki-67 immunostaining rapidly decreases during anaphase and telophase, it has been concluded that the antigen is degraded with a biological half-life of the detectable antigen of less than one hour, as estimated in postmitotic cells during stathmokinesis induced by vinblastine (Bruno and Darzynkiewicz, 1992). In contrast to many other cell cycle-associated proteins (see above), the Ki-67 antigen is consistently absent in quiescent cells and is not detectable during DNA repair processes (Hall et al., 1993). Thus, the presence of the Ki-67 antigen is strictly associated with the cell cycle and confined to the nucleus, suggesting an important role of this structure in the maintenance and/or regulation of the cell division cycle.

However, functional analysis of the antigen detected by Ki-67 has been hampered by the fact that although it was first described a decade ago, it has not been characterized in molecular terms. Using a very rapid protein preparation method, we could recently demonstrate that Ki-67 detects a double band of polypeptides (345 and 395 kD) in immunoblots of gel electrophoretically separated proteins from lysates of proliferating cells, which was absent in lysates of quiescent cells (Gerdes et al., 1991). The solubilization protocol, however, is not appropriate for further biochemical purification of the protein, as this method involves boiling in SDS-containing buffers. Furthermore, our study showed that the antigen is highly susceptible to proteases. On the other hand, the fact that Ki-67 detects its antigen in Western blots indicated that it could be used for immunoscreening of cDNA expression libraries. By screening a λgt11 cDNA library of the human cell line IM-9, we could isolate two partial clones of the Ki-67 antigen cDNA (Gerdes et al., 1991).

In this study we present the complete amino acid sequence of this exceptionally large protein, designated "Ki-67 protein" in the following, which displays unusual molecular properties and obviously represents a novel kind of nuclear protein.

Materials and Methods

Cell Lines and Antibodies

The human multiple myeloma cell line IM-9 was obtained from Dr. Harald Stein (Institut für Pathologie, Klinikum Steglitz, Berlin, Germany). IM-9 cell line cells were cultured under standard conditions in RPMI medium supplemented with antibiotics and 10% FCS. Peripheral blood mononuclear cells were obtained from healthy donors by standard methods. mAb Ki-67 (IgG1) was prepared as described (Gerdes et al., 1983). The mAbs MIB 5 and MIB W 21 were prepared by using either a bacterially expressed part of the Ki-67 antigen cDNA (Key et al., 1993) or a 30 amino acid synthetic peptide (MIB W21) as immunogens. Somatic cell fusion, cloning, and screening were performed as described (Key et al., 1993).

Gel Electrophoresis and Immunoblotting

Gel electrophoresis and immunoblotting were carried out as described by Key et al. (1992, 1993).

Immunohistochemistry

The APAAP technique was performed according to Cordell et al. (1984).
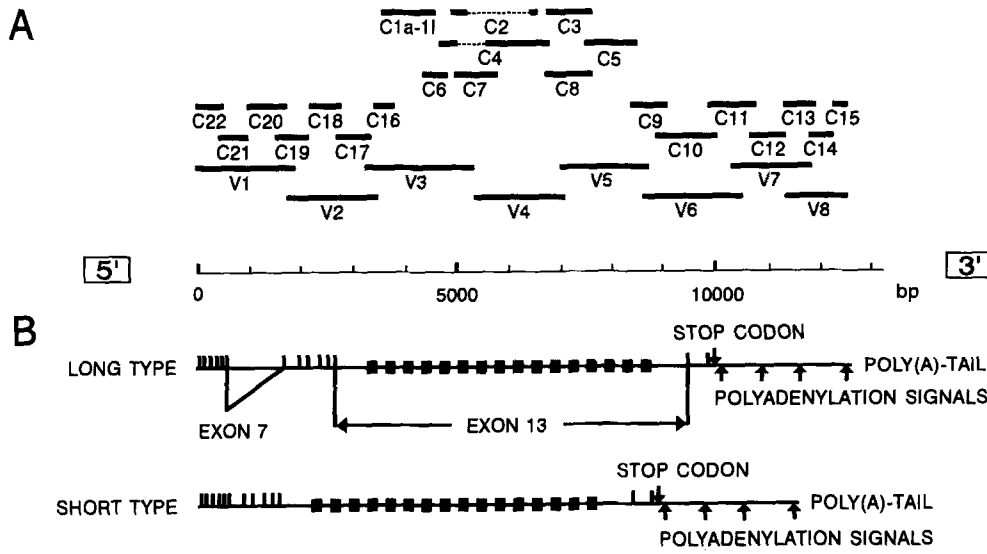
cDNA Cloning

Immunoscreening of a λgt11 library of IM-9 cell line cDNA, preparation of λgt11 DNA, and isolation of the cDNA inserts were performed as described earlier (Gerdes et al., 1991). The resulting cDNA probes were used to isolate overlapping cDNA clones from the λgt11 library by plaque hybridization according to the guidelines of the manufacturer (Clontech Laboratories Inc., Palo Alto, CA). The resulting clones were orientated and aligned with PCR, using different primer combinations and IM-9 cell line cDNA as template. Since the isolated cDNA clones did not contain the complete coding sequence, "RACE"-PCR techniques (rapid amplification of cDNA ends) (Frohmann et al., 1988) were applied to obtain the 5' end and 3' end of the cDNA. Transitions between exons and introns were determined by "genomic walking" with inverse PCR (Triglia et al., 1988) and intron spanning PCR, using genomic DNA from IM-9 cell line cells as template.

DNA and RNA Isolation and Analysis

Human genomic DNA, total cellular RNA and poly(A)-RNA were prepared from IM-9 cell line cells and peripheral blood lymphocytes according to standard protocols (Sambrook et al., 1989). Northern blot analysis was carried out as described previously (Gerdes et al., 1991), except that due to the large size of the Ki-67 antigen mRNA total RNA and poly(A)-RNA were electrophoretically separated with the "clamped homogeneous electric field" (CHEF) technique (Carle et al., 1986). For the synthesis of cDNA, 1 μg up to 3 μg total RNA were reversely transcribed with oligo(dT) or sequence-specific oligonucleotides, using the Superscript kit (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's guidelines. The complete reverse transcriptase reactions were used for "RACE" protocols (Frohmann et al., 1988) or diluted up to 500 μl for subsequent PCR reactions.
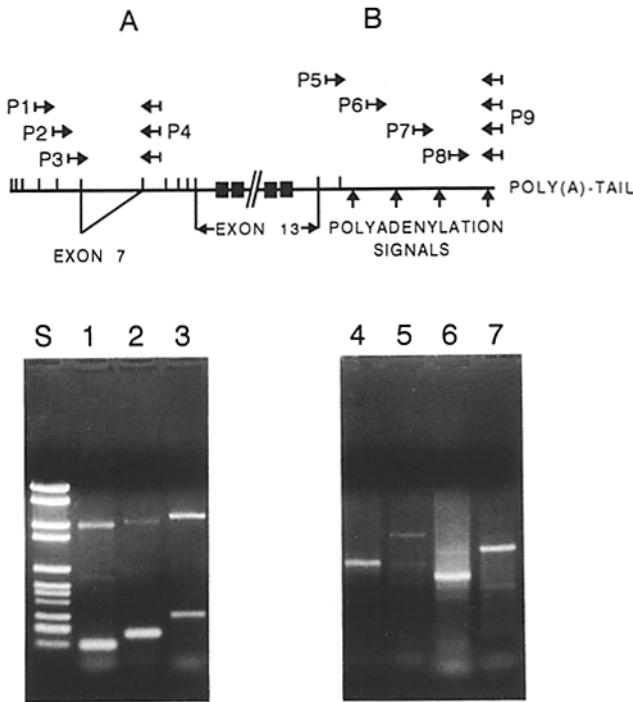
PCR Amplification

About 100 ng genomic DNA or 50 ng reversely transcribed total RNA were subjected to 25–35 cycles of PCR, each consisting of denaturation at 94°C for 2 min, primer annealing at various temperatures (45–60°C) for 2 min, and primer extension at 72°C for 3 min. PCR products were electrophoresed through a 1% agarose gel and stained with ethidium bromide. For sequence determination, the PCR products were subcloned into the multiple cloning site of M13mp18/mp19.

A

C1a-1l C2 C3
C4 C5
C6 C7 C8
C22 C20 C18 C16 C9 C11 C13 C15
C21 C19 C17 C10 C12 C14
V1 V3 V5 V7
V2 V4 V6 V8

5' 3'

0   5000   10000   bp

B

STOP CODON

LONG TYPE POLY(A)-TAIL
POLYADENYLATION SIGNALS

EXON 7 EXON 13

STOP CODON

SHORT TYPE POLY(A)-TAIL
POLYADENYLATION SIGNALS

Figure 1. The human Ki-67 protein cDNA. (*A*) Cloning strategy. The overlapping cDNA clones that were sequenced are shown above the scale. The λgt11 clones isolated by immunoscreening are designated C1a–C3, those isolated by plaque hybridization C4 and C5; all other clones (*C6–C22*) were isolated by PCR techniques (Frohmann et al., 1988; Triglia et al., 1988). The fragments resulting from PCR for the purpose of resequencing the entire cDNA are designated as V1–V8. (*B*) Structure of the human Ki-67 protein cDNAs. The horizontal lines depict Ki-67 protein (*LONG TYPE*) and Ki-67 protein (*SHORT TYPE*) mRNA, with the direction of transcription from 5' ends (*left*) to 3' ends (*right*). Positions of introns as determined by comparing PCR fragments from chromosomal and cDNA are indicated by vertical lines. The positions of the sixteen homologous "Ki-67 repeats" in the large exon 13 are demonstrated by filled boxes. The exon 7 (1,080 bp) containing mRNA of Ki-67 protein is indicated as long type. In the short type mRNA Ki-67 protein exon 7 is missing. The positions of the stop codon, the four putative polyadenylation signals, and the poly(A)-tail are indicated. The first exon is nontranslated and contains 14 CpGs (CpG-rich island). The start codon is located in exon 2.

Nucleotide Sequence Determination

DNA fragments were subcloned in M13mp18/mp19 in both orientations. ssDNA was isolated according to standard protocols (Sambrook et al., 1989), and nucleotide sequences were determined with ALF (Automated Laser Fluorescent DNA Sequencer; Pharmacia, Uppsala, Sweden) using the chain termination method (Sanger et al., 1977) with T7 DNA polymerase and fluorescent primers according to the manufacturer's protocol (Pharmacia). All oligonucleotides were synthesized using an automated DNA synthesizer (model DNA SM; Beckman Instruments, München, Germany). DNA and amino acid sequences were aligned and analyzed using the following computer programs: MICROGENIE (Rel. 7.1; Beckman Instruments), SITESCAN (Rel. 1.2; M. Duchrow, Forschungsinstitut Borstel, Germany), and HUSAR (Rel. 3.0; German Cancer Research Center, Heidelberg, Germany).

Antisense Oligonucleotide Inhibition Assay

1.0×10^5 IM-9 cell line cells/150 μl were seeded in microtiter plates. After preincubation for 48 h, different concentrations of a specific synthetic oligonucleotide (5'ATGTGGCCCACGAGACGCCTG) deduced from the Ki-67 antigen cDNA (at the position 197-218) orientated in the sense or antisense direction were added. After further 3 h, cells were incubated with [³H]thymidine (0.5 μCi) for 5 h. Subsequently cells were harvested, and the [³H]thymidine incorporation was measured. All assays were performed in triplicate, and the mean values were used to determine the specific inhibition of the antisense oligonucleotide in comparison to the sense oligonucleotide.

Figure 2. Verification PCR. (*A*) Exon 7 spanning PCR. To verify the existence of two differentially spliced forms of Ki-67 protein cDNA, three different primers (*P1–P3*) were selected located within exon 4, 5, and 6 and combined in PCR reactions with a reverse orientated primer (*P4*) located within exon 8. All PCR reactions were separated electrophoretically (lanes *1–3*), and each resulted in two different cDNA fragments. Sequence analysis of these fragments revealed the absence of exon 7 in the small PCR fragment (data not shown). (*B*) 3'-"RACE" PCR. To demonstrate that three different poly(A)-tail forms could be isolated, we combined the

Frohmann Adapter (*P9*) (Frohmann et al., 1988) with four different primers (*P5–P8*) 200–500-bp upstream of the four potential polyadenylation signals. As demonstrated, the PCR reactions resulted in several distinct cDNA fragments (lanes *4–7*). Sequence analysis of three of these fragments (lanes *4*, *6*, and *7*) revealed the predicted poly(A)-tails 10–20-bp upstream of the first, third and fourth potential polyadenylation signals, but the poly(A)-tail of the fragment in lane *5* was identical with the fragment in lane *6*.
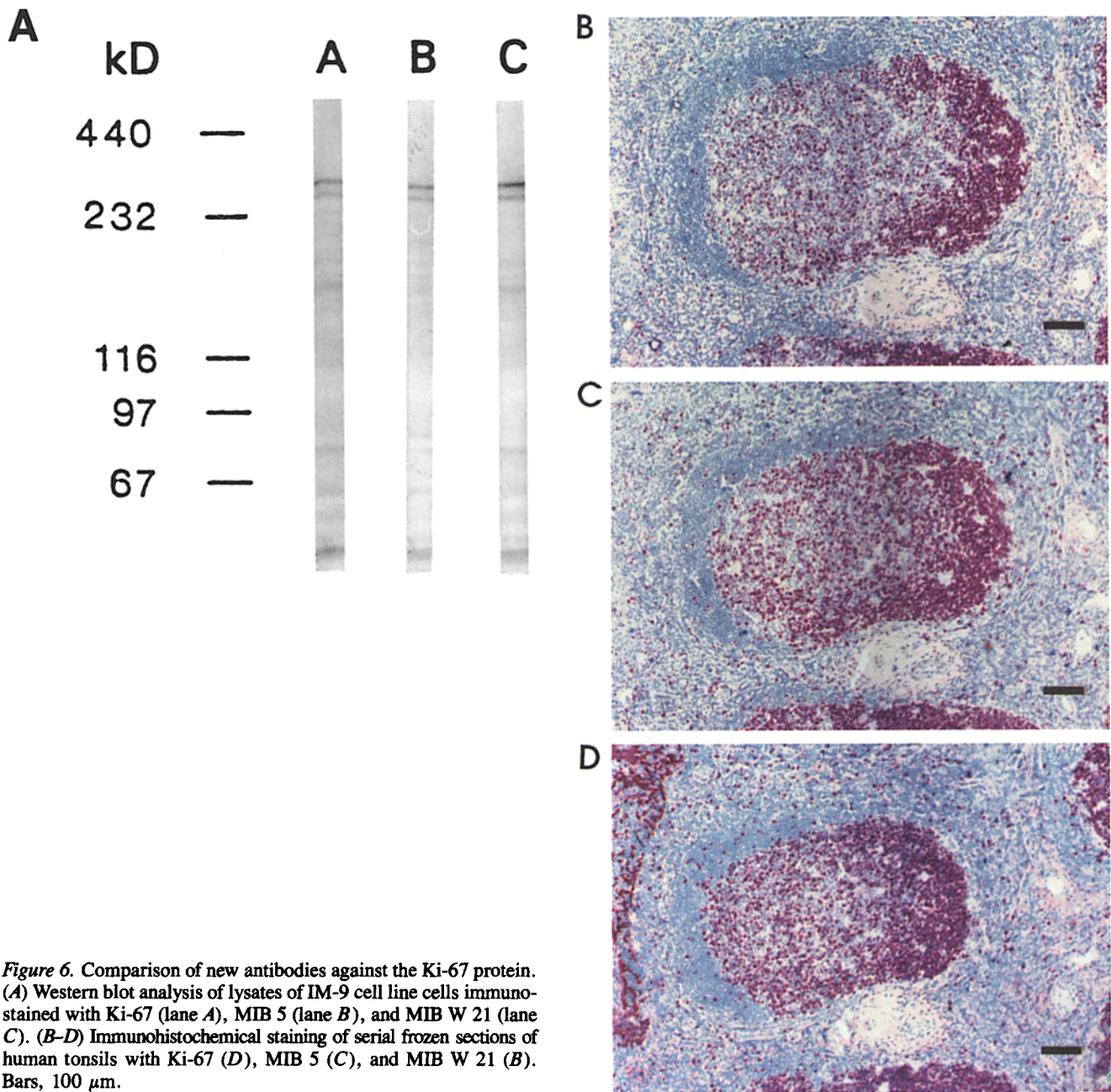
Figure 3. Complete nucleotide sequence of the cDNA encoding the human antigen of mAb Ki-67. The deduced amino acid sequence of the Ki-67 protein cDNA is shown beneath the nucleotide sequence. Coordinates for the nucleotide and amino acid sequences are shown on the right of each line. Upstream of the poly(A)-tail of the cDNA there are four consensus polyadenylation signals (AAUAAA; underlined at positions 9,994, 10,529, and 12,468 bp and CAUAAA; underlined at position 11,237). Three signals are actively used, since we found three different poly(A)-tails immediately downstream of each signal at positions 10,013, 11,262, and 12,494 bp. The 24-bp-long direct repeated boxes (positions 130-153, 154-177) are indicated below the nucleic acid sequence. Introns have been located at positions 107, 208, 367, 483, 550, 596, 1676, 1852, 2165, 2284, 2456, 2612, 9457, and 9901; exons 7 and 13 are indicated. A potential " ATP/GTP binding site motif A" (P-loop) (Saraste et al., 1990) was found between amino acid positions 3034–3041, two putative nuclear targeting sequences (Chelsky et al., 1989; Silver, 1991) were found between positions 502-505 and 687-690, and eight potential "bipartite nuclear targeting signals" (Dingwall and Laskey, 1991) between amino acid positions 536-550, 1516-1530, 2244-2258, 2365-2379, 2651-2665, 2890-2904, 2997-3011, and 3141-3155, as indicated below the amino acid sequence. Furthermore, the positions of all 16 "Ki-67 repeats" and of the "Ki-67 motifs" containing the epitope recognized by mAb Ki-67 are indicated. CpG dinucleotides in the CpG-rich island (1-130 bp) are underlined. These sequence data are available from the EMBL database under the accession numbers X65550 and X65551.

Results

Cloning

To determine the primary structure of the Ki-67 protein, we used mAb Ki-67 to screen an expression library of IM-9 cell line cDNAs constructed in λgt11. From 2 × 10⁶ plaques screened, 14 independent clones were isolated based on their reactivity with the antibody. By sequencing, 12 of these clones exhibited the identical 1095-bp sequence previously described (Gerdes et al., 1991; Fig. 1 *A, Cla-Cll*), and two clones contained different cDNA fragments (Fig. 1 *A, C2* and *C3*). Thus, immunoscreening yielded three separate cDNA fragments which were subsequently used to rescreen the λgt11 IM-9 cell line cDNA library by plaque hybridization technique, revealing two additional cDNA fragments (Fig. 1, *C4* and *C5*). To verify and align these cDNA clones, mRNA of IM-9 cell line cells was reversely transcribed and amplified by PCR using primer combinations deduced from these cDNA clones resulting in the overlapping clones C6-C8. Using 5'- and 3'-"RACE" PCR techniques (Frohmann et al., 1988), 14 additional overlapping cDNA fragments (Fig. 1, *C9-C22*) could be cloned and sequenced. From a total of 22 overlapping cDNA fragments (Fig. 1, *C1-C22*) we constructed the full-length cDNA.

This arrangement of cDNA was verified by PCR, using overlapping primer combinations and by subsequent sequencing of the PCR products (Fig. 1 *A*, clones *VI-V8*). As demonstrated in Fig. 2 *A*, these PCR products indicated the existence of two cDNA types differing only in the presence or absence of a 1,080-bp element. Chromosomal walking in both directions of the gene locus by inverse PCR (Triglia et al., 1988) and intron spanning PCR, using genomic DNA from IM-9 cell line cells as template, revealed that this gene consists of 14 introns and 15 exons (Fig. 1 *B*) and that the 1,080-bp element mentioned above represents one single exon, i.e., exon 7.

Figure 3.

To confirm that we have cloned the complete 5' end of the cDNA, multiple independent 5'-"RACE" PCR experiments were performed using three different primers adjacent to the putative 5' end (positions 644–663, 347–365, and 118–138), and the resulting PCR products were cloned and sequenced. 6 out of 9 (primer 1), 4 out of 7 (primer 2), and 2 out of 4 (primer 3) isolated clones exhibited identical 5' ends, whereas the remaining clones were randomly truncated resulting from presumably damaged or incompletely transcribed mRNA. From these results we concluded that the 5' end, as shown in Fig. 3, represented the authentic start of transcription. Besides the putative polyadenylation signal within the 3' portion of the cDNAs preceding the poly(A)-tail shown in Fig. 3 we found two additional conservative putative polyadenylation sites (AAUAAA) and one site with a single base difference (CAUAAA). Since we could demonstrate three of these poly(A)-tail forms by 3'-"RACE" PCR (Fig. 2 B), we concluded that maximally only three polyade-

nylation sites are biologically active. Thus, in combination with the two different isoforms at least six different forms of Ki-67 protein mRNAs have to be postulated.

The complete nucleotide sequence and the predicted amino acid sequence are shown in Fig. 3. The polypeptides deduced from the two open reading frames of 9,768 and 8,688 bp, respectively, between the first possible start codon at nucleotide position 197 and the stop codon at position 9,965 have calculated molecular weights of 358,761 and 319,508 D and calculated isoelectric points of pI 9.87 and pI 9.94, respectively.

The central part of both isoforms of the cDNAs is encoded by the large (6,845 bp) exon 13, which contains 16 concatenated repeats, the consensus sequence of which is a 366-bp long element, the "Ki-67 repeat" (Fig. 4). The predicted amino acid sequences of these "Ki-67 repeats" exhibit a high degree of identical amino acids, from 43 to 62%, with the consensus sequence shown in Fig. 4. Within these "Ki-67

```
                                                                            Ki-67 MOTIF
Consensus   *KTTKI*CKSPQPE*VDTPTSTK*RPK*SL*K*DV*EE*LA**KLT*TSGKTTHT*K*P*G**K*IKAF*ETPKQKLD*A**LTGSKR*PRTPKEKAQALEDLAGFKELFQTPGHTEES*T*
                 ↑                        ↑                ↑                                        ↑                         ↑↑

1  (1000)   G-I--MP-Q-L---PIN---H--QQL-A--G-VG-K--L--VG-F-R---E----HRE-A-DG-S-RT-K-S---I--P-ARV--M-KW------E--S---------------PS---M-D
2  (1122)   E-----A----P--S--------QW--R--R-A--E--F--LR---PSA--AML-P-PAG-DE-D----MG--V----L-GT-P----QLQ--------------------------LVAA
3  (1244)   G-----P-D---SDP-------Q---R-IR-A--EG-L--CRN-MPSA--AM--P-PSV-EE-D-II-VG--V----LTEN------R-Q----E-------T--------------AVAA
4  (1366)   G----MP-E-SP--SA------RRQ--TP-E-R--QK-LS-LK---Q--E----D-V-G-ED-S-N--R--A-----P-ASV-----H-K- ----P------W-------VC-DKPT-H
5  (1487)   E-----A-R---DP------S-PQS-R--R-V--E--FF-LR-R-PSA--AM--P-PAVSGE-N-Y--MG--V----LTEN------RLQ--------------------R------M-N
6  (1608)   D--A-VA---S--DLDKN-A-S-R-L-T--G-VG-K--L--VG---Q---E----HTE-T-DG-SM---M-S---I--S-AS------QL----G-SEVP------I------S--K--M-N
7  (1730)   E----VSYRAS--DL-------S-PQ---R--R-A-TE--F--FR-Q-PSA--AM--P-PAV-EE-D-NT-LG--V----QPGN-P--N-RLQ-R--------E-T--R------C -DNPTAD
8  (1851)   EKT-K-IL----SDPA----N--Q---R--K-A--E--F--FR---PSA--AM--P-AAV-EE-D-NT-VG--VE---LLGN-P-----R-Q------K-------------------M-D
9  (1974)   D-I-EVS------DP-K----S-Q-L-I--G-VG-K--V-PVG---Q------Q-HRETA-DG-S----K-SA--M--P-NYG--ME-W------E--S---------------D-----T-D
10 (2096)   D-----A----P--SM------RR---TP-G-R-IV--LS-LKQ--Q    ----D-V-GDED-G-NV-R--A-----P-ASV-----Q-----G---P------L-------VC-DKPT-H
11 (2214)   E-----A-R----DP-G---IF-PQS-R--R-A--E--S--LR-R-PSV--AMD-P-PAG-DE-DM---MG--V----LPGN-P----W-Q--------------------- -DKPT-D
12 (2336)   E-----A------DP----A--Q---RN-R-A--E--F--LR-R-PSA--AMD-P-PAVSDE-N-NT-V---V----LLGN-P----Q-Q------E-----V-------------M-D
13 (2457)   D-I-EVS------SFK-SR-S-Q-L-IP-V-V-MK--P--VS---R---E--Q-HTE-T-DS-S----K-S---I--P-ASV---R-QL--R----R-----VD-----SA-------M-I
14 (2579)   D-N---P----P--LT--A----RC--TRPR-E -K--LS-VER---Q---QS---H-E-ASGDEG--VLKQRA-K-PNPVEEEP -R-R--A------P-------T--SE-S---Q--L-A
15 (2699)   G-A---P-E--PL-V---TA---RHLRTRVQ-VQ-K--PS-V -F-Q---E--DAD-E-A-ED-G---LK-SA--TPAP-ASV---R-R--A-R-S---I-------DPA A ------M-D
16 (2818)   D-----P---SP -LE--A--S-R--RTRAQ-VE-K--L--VG---Q---E----D-E-V-EG-GT---KQPA-RNV- -EDVI--R-Q--A------P-----S-Q--S--------LA N
```

Figure 4. Comparison of the sixteen "Ki-67 repeats". All repeats belong to exon 13 of the Ki-67 protein cDNA and are direct repeats. Conserved amino acids shared by more than eight repeats are written as a consensus sequence in the top line, using the standard single letter code of the predicted amino acid sequence. Asterisks denote positions with less than eight identical amino acid residues, open spaces indicate gaps modified for optimal alignment. The numbers in parentheses indicate the amino acid positions. Dashes represent identical residues. The highly conserved sequence of the "Ki-67 motifs" and the strong PEST sequences are underlined. The highly conserved amino acid residues are indicated with arrows.

repeats" there is a highly conserved 22 amino acid element (72 to 100% identity with the consensus sequence), the "Ki-67 motif". As all clones obtained by immunoscreening of the λgt11 IM-9 cell line cDNA library contained a 100% identical "Ki-67 motif", it was tempting to assume that this repetitive 66-bp element encodes for the epitope detected by Ki-67. To examine this hypothesis, we cloned and expressed different parts of the Ki-67 protein cDNA, and it turned out that Ki-67 only detected fusion proteins including this "Ki-67 motif" (see also Key et al., 1992, 1993).

Another feature of the central exon was the presence of several highly conserved amino acid residues within the sixteen repetitive elements (Fig. 4), e.g., a cysteine at position 8, glutamic acids at positions 38 and 101, glycines at positions 50 and 85, and leucine at position 103 of the consensus sequence.

Northern Blot Analysis

Earlier Northern blot analysis using the 1,095-bp fragment (clones Cla-Cll), now known to be a part of exon 13, had shown hybridization signals with mRNA from proliferating cells over a range of more than 2 kb, whereas no hybridization was observed with mRNA of quiescent cells (Gerdes et al., 1991). In this study these findings could be confirmed and extended by using three isoform-specific cDNA fragments and the 1,095-bp fragment of the Ki-67 protein cDNA as hybridization probes. It was predicted above that six different mRNA species (Fig. 5, 12.5, 11.4, 11.2, 10.2, 10, and 8.9 kb) should exist. As demonstrated in Fig. 5, mRNA prepared from proliferating cells showed two distinct bands with a probe specific for the 3' end of the mRNA (Fig. 5, lane 1), indicating the existence of two Ki-67 protein mRNA iso-

Figure 5. Northern blot analysis. Northern blots of poly(A)-RNA from IM-9 cell line cells (lanes 1-4) and PBLs (lane 5). Lane 1, Hybridization with a probe specific for the 3' end of the mRNA (positions 11738-12302); lane 2, hybridization with a probe specific for exon 7 (positions 681-1401 of the long cDNA type); lane 3, hybridization with a probe specific for the mRNA without exon 7 (positions 505-688 of the short cDNA type); lanes 4 and 5, hybridization with the 1095-bp cDNA fragment (clone Cla).

Figure 6. Comparison of new antibodies against the Ki-67 protein. (A) Western blot analysis of lysates of IM-9 cell line cells immunostained with Ki-67 (lane A), MIB 5 (lane B), and MIB W 21 (lane C). (B–D) Immunohistochemical staining of serial frozen sections of human tonsils with Ki-67 (D), MIB 5 (C), and MIB W 21 (B). Bars, 100 μm.

forms. Using an exon 7–specific cDNA probe (Fig. 5, lane 2) and a probe specific for the "short type" cDNA (Fig. 5, lane 3), the six different mRNA species predicted above could be demonstrated. This was further substantiated by hybridization with the 1,095-bp cDNA fragment (clone *1a*, i.e., part of exon 13) as shown in lane 4: here four strong hybridization bands are seen, two of which presumably represent a double-band since four out of the six predicted mRNA species only differ by 200 bp which could not be resolved efficiently by gel electrophoresis. It is important to note that none of the probes used hybridized with mRNA prepared from quiescent PBL (for an example of the 1,095-bp probe see Fig. 5, lane 5).

Antibody Production

To substantiate that the cDNAs isolated encode for the authentic Ki-67 protein, new mAbs were prepared against a bacterially expressed part of exon 13 and a synthetic peptide representing a region near the 3' end from the Ki-67 protein cDNA sequence. Fig. 6 *A* shows that all new antibodies specifically react with the native Ki-67 protein in immunoblot analysis, and Fig. 6, *B–D* shows the immunostaining on cryostat sections through human tonsils. Essentially identical staining patterns were seen with mAb Ki-67 (Fig 6 *D*), MIB 5, an antibody obtained using bacterially expressed parts of exon 13 (Fig 6 *C*), and MIB W 21, an antibody obtained against the synthetic peptide W 21 deduced from the Ki-67 protein sequence at amino acid position 3095–3125 (Fig. 6 *B*). The specificity of MIB 5 and MIB W 21 was further confirmed by the fact that the immunostaining of both antibodies could be completely inhibited by the corresponding synthetic peptides. Ki-67 and MIB 5 staining was com-

pletely abrogated by the synthetic peptide GAP deduced from the "Ki-67 motif" of the second "Ki-67 repeat" of exon 13 (Fig. 3, positions 3830–3889), but remained unaffected by synthetic peptide W 2. Vice versa, staining with MIB W 21 was inhibited by W 2 (the synthetic peptide used for the preparation of this antibody), while preincubation of MIB W 21 with GAP had no effect. Furthermore, the epitopes recognized by the original antibody Ki-67 (see also Key et al., 1992) and the new mAb MIB 5 are localized on the "Ki-67 motif", whereas the epitope recognized by MIB W 21 is localized on a 30 amino acid region near the COOH terminus (see above).

Sequence Analysis

The cDNA-derived amino acid sequence of the Ki-67 protein seems to be unique, as it did not reveal any significant homology to any of the known sequences in databases (EMBL Nucleotide Sequence, Rel. 34; GenBank, Rel. 75; PIR, Rel. 37; Swiss prot, Rel. 37). Computer analysis of the cDNAs and of the amino acid sequences derived thereof indicated that the first exon is untranslated and contains a CpG-rich island and two 24-bp direct repeated boxes (Fig. 3, positions 130–153, 154–177 bp). Two potential "nuclear targeting signals" (Chelsky et al., 1989; Silver, 1991), at amino acid positions 502–505 and 687–690, and eight potential "bipartite nuclear targeting signals" (Dingwall and Laskey, 1991), at amino acid positions 536–550, 1516–1530, 2244–2258, 2365–2379, 2651–2665, 2890–2904, 2997–3011, and 3141–3155, are well in line with the concept that both cDNA isoforms encode for nuclear proteins. At the COOH terminus (amino acid positions 3034–3041) we found sequence homologies with the ATP/GTP binding site motif A ("P loop") (Saraste et al., 1990). Furthermore, the proteins seem to comprise more than 200 potential phosphorylation sites (143 PKC, 89 casein kinase II, two tyrosine kinase sites), 19 N-myristoylation sites, and three amidation sites.

The amino acid sequence also contains several regions rich in proline, glutamic acid, serine, and threonine: designated as PEST sites (Rogers et al., 1986). 40 weak and 10 strong PEST sites have been determined, the latter exclusively in exon 13, flanking the highly conserved cysteine residue at position 8 of the "Ki-67 repeat" (Fig. 4). As the deduced amino acid sequence is rich in proline, conformation analysis (Chou and Fasman, 1978) predicts a high number of turns and only a few putative α-helical regions and β-sheets (not shown).

Inhibition of Thymidine Incorporation by Antisense Oligonucleotides

To examine the possible involvement of the Ki-67 protein in the maintenance of cell proliferation, we studied the effect of a synthetic deoxy-oligonucleotide complementary to the start of translation of the Ki-67 protein, using the corresponding sense oligonucleotide as controls. Human IM-9 cell line cells were treated with different concentrations of Ki-67 protein antisense oligonucleotides, and proliferation was determined by measurement of [³H]thymidine uptake. As shown in Fig. 7, [³H]thymidine incorporation in IM-9 cell line cells was inhibited by antisense oligonucleotides in a dose-dependent manner.
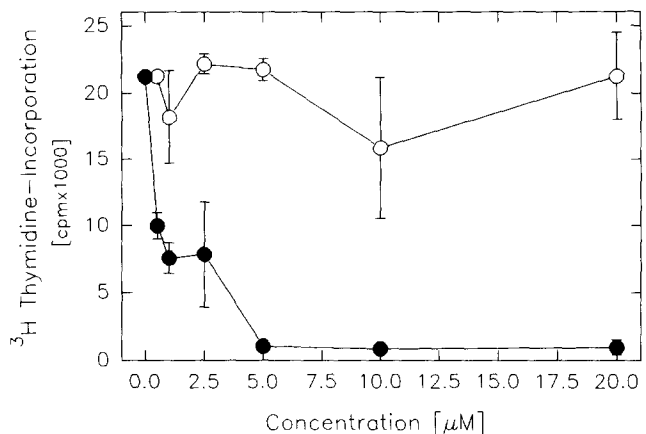

Figure 7. Antisense oligonucleotide inhibition assay. IM-9 cell line cells were incubated with various concentrations of Ki-67 protein mRNA-specific sense and antisense oligonucleotides, and [³H]thymidine incorporation was measured.

Discussion

In the present study we provide evidences demonstrating that we have indeed cloned the gene that encodes the Ki-67 protein. The clones identified by an immunoscreening strategy (Gerdes et al., 1991) have been used to isolate a series of overlapping clones that encompass a cDNA with a unique highly repetitive central portion. When expressed in prokaryotes, the resulting protein has been used to prepare antibodies which have been shown to have identical operational properties to the prototypical Ki-67 antibody in immunoblot and immunohistochemical assays. Furthermore, these antibodies recognize similar epitopes, and their immunostaining could be specifically inhibited by synthetic peptides deduced from the Ki-67 protein cDNA. This defines the epitopes of both the prototypical mAb Ki-67 and the new antibody MIB 5 to an identical 20 amino acid sequence, whereas the epitope recognized by MIB W 21 resides within a different stretch of 30 amino acids. Coupled with the results obtained by Northern blot analysis, these data lead to the conclusion that the reported cDNA encodes for the Ki-67 protein.

Analysis of the primary structure of the Ki-67 protein cDNA does not show any significant homologies with any known sequence. Indeed the structure shows a number of unusual features. The center of the Ki-67 protein cDNA is formed by a large 6,845-bp exon harboring 16 concatenated direct repeats (the "Ki-67 repeats") which themselves contain a highly conserved 66-bp element (the "Ki-67 motif") now known to encode the epitope recognized by Ki-67. This repetitive and highly basic structure may have important functional properties and may, as has been discussed for other genes containing repeats, have arisen from some ancestral gene segment by internal duplication (e.g., McLean et al., 1987; Wasenius et al., 1989; Krueger et al., 1990).

We identified two cDNAs which exhibited identical sequences except that one exon (exon 7) is missing in the shorter form. It is, therefore, likely that those two cDNAs represent different mRNAs formed from the same gene by alternative mRNA splicing. The molecular weights of the proteins deduced from the Ki-67 protein cDNAs correspond well to the sizes of the polypeptides detected as a double

band in immunoblots of proteins from proliferating cells (Gerdes et al., 1991). Thus, we assume that each of the two polypeptide chains is encoded by one of these two mRNAs and is not a result of posttranslational modification or proteolytic degradation. It is likely that the Ki-67 protein is efficiently transported into the nucleus, because we found ten potential "nuclear targeting signals," eight of which are bipartite, in both isoforms, which are essential for the transport of proteins into the nucleus (Chelsky et al., 1989; Silver, 1991; Dingwall and Laskey, 1991).

The amino acid residues highly conserved within the "Ki-67 repeat," e.g., cysteine, glycine, and glutamic acid, may be involved in structure formation or functional aspects. The highly conserved cysteine residue at the eighth position is particularly conspicuous, but in view of the reducing intracellular environment (e.g., Ball et al., 1992; Hwang et al., 1992) probably does not form intra- and intermolecular disulfide bonds. PEST sequences (Rogers et al., 1986) are presumed to represent signals for rapid proteolytic degradation (for review see Rechsteiner, 1989, 1990). According to their key roles in cellular events, all known "PEST proteins" are strictly regulated. The presence of multiple PEST sites in the Ki-67 antigen would be in line with previous observations, indicating that this antigen is highly susceptible to proteases (Gerdes et al., 1991) and that its biological half-life is very short (Bruno and Darzynkiewicz, 1992).

Recent studies have shown that the introduction of antisense oligonucleotides (e.g., antisense c-*myc* or antisense MZF 1) in eukaryotic cells can result in a specific inhibition of mRNA translation (Harel-Bellan et al., 1988; Bavisotto et al., 1991). Using Ki-67 protein antisense oligonucleotides [^{3}H]thymidine incorporation in IM-9 cell line cells could be inhibited in a dose-dependent manner. This finding indicates that the expression of Ki-67 protein might be an absolute requirement for cell proliferation. In conclusion, we assume that the Ki-67 protein represents a new kind of cell cycle maintaining proteins.

References

Ball, T., K., Y. Suh, and M. J. Benedik. 1992. Disulfide bonds are required for Serratia marcescens nuclease activity. *Nucleic Acids Res.* 20:4971–4974.

Bavisotto, L., K. Kaushansky, N. Lin, and R. Hromas. 1991. Antisense oligonucleotides from the stage-specific myeloid zinc finger gene MZF-1 inhibit granulopoiesis in vitro. *J. Exp. Med.* 174:1097–1101.

Bouzubar, N., K. J. Walker, K. Griffiths, I. O. Ellis, C. W. Elston, J. F. R. Robertson, R. W. Blamey, and R. I. Nicholson. 1989. Ki-67 immunostaining in primary breast cancer: pathological clinical associations. *Br. J. Cancer.* 59:943–947.

Braun, N., T. Papadopoulos, and H. K. Müller-Hermelink. 1988. Cell cycle dependent distribution of the proliferation associated Ki-67 antigen in human embryonic lung cells. *Virchows Arch. B.* 56:25–33.

Brown, D.C., and K. C. Gatter. 1990. Monoclonal antibody Ki-67: its use in histopathology. *Histopathology.* 17:489–503.

Bruno, S., and Z. Darzynkiewicz. 1992. Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. *Cell Prolif.* 25:31–40.

Carle, G. F., M. Frank, and M. V. Olson. 1986. Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science (Wash. DC).* 232:65–72.

Celis, J. E., and P. Madsen. 1986. Increased nuclear cyclin/PCNA antigen staining of non S-phase transformed human amnion cells engaged in nucleotide excision DNA repair. *FEBS (Fed. Exp. Biochem. Soc.) Lett.* 209:277–283.

Chelsky, D., R. Ralph, and G. Jonak. 1989. Sequence requirements for synthetic peptide-mediated translocation to the nucleus. *Mol. Cell. Biol.* 9:2487–2492.

Chou, P. Y., and G. D. Fasman. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* 47:45–147.

Cordell J. L., B. Falini, W. N. Erber, A. K. Ghosh, Z. Abulaziz, S. MacDonald, K. A. F. Pulford, H. Stein, and D. Y. Mason. 1984. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem.* 32:219–229.

Dingwall, C., and R. A. Laskey. 1991. Nuclear targeting sequences—a consensus? *Trends Biochem. Soc.* 16:478–481.

du Manoir, S., P. Guillaud, E. Camus, D. Seigneurin, and G. Brugal. 1991. Ki-67 labeling in postmitotic cells defines different Ki-67 pathways within the 2c compartment. *Cytometry.* 12:455–463.

Fontana, D., M. Bellina, L. Gubetta, G. Fasolis, L. Rolle, C. Scoffone, F. Porpiglia, M. Colombo, R. Tarabuzzi, and E. Leonardo. 1992. Monoclonal antibody Ki-67 in the study of the proliferative activity of bladder carcinoma. *J. Urology.* 148:1149–1151.

Frohmann, M. A., M. K. Dush, and G. R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA.* 85:8998–9002.

Gerdes, J. 1990. Ki-67 and other proliferation markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies. *Sem. Cancer Biol.* 1:99–206.

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

Gerdes, J., H. Lemke, H. Baisch, H.-H. Wacker, U. Schwab, and H. Stein. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.* 133:1710–1715.

Gerdes, J., H. Stein, S. Pileri, M. T. Rivano, M. Gobbi, E. Ralfkiaer, K. M. Nielsen, G. Pallesen, H. Bartels, G. Palestro, and G. Delsol. 1987. Prognostic relevance of tumour-cell growth fraction in malignant non-Hodgkin's lymphomas. *Lancet.* II:448–449.

Gerdes, J., L. Li, C. Schlüter, M. Duchrow, C. Wohlenberg, C. Gerlach, I. Stahmer, S. Kloth, E. Brandt, and H. D. Flad. 1991. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. *Am. J. Pathol.* 138:867–873.

Grogan, T. M., S. M. Lippman, C. M. Spier, D. J. Slymen, J. A. Rybski, C. S. Rangel, L. C. Richter, and T. P. Miller. 1988. Independent prognostic significance of a nuclear proliferation antigen in diffuse large cell lymphomas as determined by the monoclonal antibody Ki-67. *Blood.* 71:1157–1160.

Guillaud, P., S. du Manoir, and D. Seigneurin. 1989. Quantification and topographical description of Ki-67 antibody labelling during the cell cycle of normal fibroblastic (MRC-5) and mammary tumour cell lines (MCF-7). *Anal. Cell. Pathol.* 1:25–39.

Hall, P. A., M. A. Richards, W. M. Gregory, A. J. d'Ardenne, T. A. Lister, and A. G. Stansfeld. 1988. The prognostic value of Ki-67 immunostaining in Non-Hodgkin's Lymphoma. *J. Pathol.* 154:223–235.

Hall, P. A., D. A. Levison, A. L. Woods, C. C.-W. Yu, D. B. Kellock, J. A. Watkins, D. M. Barnes, C. E. Gillett, R. Camplejohn, R. Dover, N. H. Waseem, and D. P. Lane. 1990. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J. Pathol.* 162:285–294.

Hall, P. A., P. H. McKee, H. du P. Menage, R. Dover, and D. P. Lane. 1993. High levels of p53 protein in UV irradiated normal human skin. *Oncogene.* 8:203–207.

Harel-Bellan, A., D. K. Ferris, M. Vinocour, J. T. Holt, and W. F. Farrar. 1988. Specific inhibition of c-myc protein biosynthesis using an antisense synthetic deoxy-oligonucleotide in human T-lymphocytes. *J. Immunol.* 140:2431–2435.

Holte, H., C. De Lange Davies, K. Beiske, T. Stokke, P. F. Marton, E. B. Smeland, J. Hoie, and S. Kvaloy. 1989. Ki-67 and 4F2 antigen expression as well as DNA synthesis predict survival at relapse/tumour progression in low-grade B-cell lymphoma. *Int. J. Cancer.* 44:975–980.

Hwang, C., A. J. Sinskey, and H. F. Lodish. 1992. Oxidized redox state of glutathione in the endoplasmatic reticulum. *Science (Wash. DC).* 257:1496–1502.

Key, G., F. Meggetto, H. G. Becker, T. Al Saati, C. Schlüter, M. Duchrow, G. Delsol, and J. Gerdes. 1992. Immunobiochemical characterization of the antigen detected by monoclonal antibody IND.64. *Virchows Archiv B Cell. Pathol.* 62:259–262.

Key, G., M. H. G. Becker, B. Baron, M. Duchrow, C. Schlüter, H. D. Flad, and J. Gerdes. 1993. New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 66 bp repetitive elements encoding for the Ki-67 epitope.

Lab. Invest. 68:629–635.

Kirschner, M. 1992. The cell cycle then and now. *Trends Biochem. Sci.* 17:281–285.

Krueger, N. X., M. Streuli, and H. Saito. 1990. Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3241–3252.

Locker, A. P., K. Birrell, J. A. Bell, I. Nicholson, C. W. Elston, R. W. Blamey, and I. O. Ellis. 1992. Ki-67 immunoreactivity in breast carcinoma: relationships to prognostic variables and short term survival. *Eur. J. Surgical Oncology.* 18:224–229.

McCormick, D., and P. A. Hall. 1992. The complexities of proliferating cell nuclear antigen. *Histopathology.* 21:591–594.

McLean, J. W., J. E. Tomlinson, W. J. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein (a) is homologous to plasminogen. *Nature (Lond.).* 300: 132–137.

Nicholson, R. I., N. Bouzubar, K. J. Walker, R. McClelland, A. R. Dixon, J. F. R. Robertson, I. O. Ellis, and R. W. Blamey. 1991. Hormone sensitivity in breast cancer: influence of heterogeneity of oestrogen receptor expression and cell proliferation. *Eur. J. Cancer.* 27:908–913.

Norbury, C., and P. Nurse. 1992. Animal cell cycles and their control. *Annu. Rev. Biochem.* 61:441–470.

Rechsteiner, M. 1989. PEST regions, proteolysis and cell cycle progression. *Revis. Biol. Cellular.* 20:235–253.

Rechsteiner, M. 1990. PEST sequences are signals for rapid intracellular proteolysis. *Semin. Cell. Biol.* 1:433–440.

Rogers, S., R. Wells, and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science (Wash. DC).* 234:364–368.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 42 pp.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci USA.* 74:5463–5467.

Saraste, M., P. R. Sibbald, and A. Wittinghofer. 1990. The P-loop, a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* 15:430–434.

Schrape, S., D. B. Jones, and D. H. Wright. 1987. A comparison of three methods for the determination of the growth fraction in Non-Hodgkin's Lymphoma. *British J. Cancer.* 55:283–286.

Scott, R. J., P. A. Hall, J. S. Haldane, S. van Noorden, Y. Price, D. P. Lane, and N. A. Wright. 1991. A comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction. *J. Path.* 165:173–178.

Silver, P. A. 1991. How proteins enter the nucleus. *Cell.* 64:489–497.

Tazzari, P. L., A. Bontadini, M. Gobbi, C. Tassi, A. Dinota, G. Visani, M. G. Michieli, S. Pileri, M. Baccarani, and S. Tura. 1990. Comparison of the DNA content, bromodeoxyuridine incorporation and Ki-67 antigen expression in human acute myeloid leukemia. *Leuk. & Lymphoma.* 3:45–51.

Toschi, L., and R. Bravo. 1988. Changes in Cyclin/proliferating cell nuclear antigen (PCNA) distribution during DNA repair synthesis. *J. Cell Biol.* 107:1623–1628.

Triglia, T., M. G. Peterson, and D. J. Kemp. 1988. A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. *Nucleic Acids Res.* 16:8186.

Tungekar, M. F., K. C. Gatter, M. S. Dunnill, and D. J. Mason. 1991. Ki-67 immunostaining and survival in operable lung cancer. *Histopathology (Oxf.).* 19:545–550.

Verheijen, R., H. J. H. Kuijpers, R. O. Schlingemann, A. L. M. Boehmer, R. van Driel, G. J. Brakenhoff, and F. C. S. Ramaekers. 1989a. Ki-67 detects a nuclear matrix-associated proliferation-related antigen I. Intracellular localization during interphase. *J. Cell Sci.* 92:123–130.

Verheijen, R., H. J. H. Kuijpers, R. van Driel, J. L. M. Beck, J. van Dierendonck, G. J. Brakenhoff, and F. C. S. Ramaekers. 1989b. Ki-67 detects a nuclear matrix-associated proliferation-related antigen II. Localization in mitotic cells and association with chromosomes. *J. Cell Sci.* 92:531–540.

Wasenius, V.-M., M. Saraste, P. Salven, M. Erämaa, L. Holm, and V.-P. Lehto. 1989. Primary structure of the brain α-Spectrin. *J. Cell Biol.* 108:79–93.

Wintzer, H. O., I. Zipfel, J. Schulte-Mönting, U. Hellerich, and S. von Kleist. 1991. Ki-67 immunostaining in human breast tumors and its relationship to prognosis. *Cancer.* 67:421–428.