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REVIEW ARTICLES

The Ki-67 Protein: From the Known and the Unknown

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The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from resting cells (G_0), makes it an excellent marker for determining the so-called growth fraction of a given cell population. In the first part of this study, the term *proliferation marker* is discussed and examples of the applications of anti-Ki-67 protein antibodies in diagnostics of human tumors are given. The fraction of Ki-67-positive tumor cells (the Ki-67 labeling index) is often correlated with the clinical course of the disease. The best-studied examples in this context are carcinomas of the prostate and the breast. For these types of tumors, the prognostic value for survival and tumor recurrence has repeatedly been proven in uni- and multivariate analysis. The preparation of new monoclonal antibodies that react with the Ki-67 equivalent protein from rodents now extends the use of the Ki-67 protein as a proliferation marker to laboratory animals that are routinely used in basic research. The second part of this review focuses on the biology of the Ki-67 protein. Our current knowledge of the Ki-67 gene and protein structure, mRNA splicing, expression, and cellular localization during the cell-division cycle is summarized and discussed. Although the Ki-67 protein is well characterized on the molecular level and extensively used as a proliferation marker, the functional significance still remains unclear. There are indications, however, that Ki-67 protein expression is an absolute requirement for progression through the cell-division cycle. J. Cell. Physiol. 182:311–322, 2000.

With regard to the Ki-67 protein, the scientific community can be divided into three groups. The first group, that is, for those working in pathology, the Ki-67 protein and the corresponding antibodies are well known, whereas a second group of researchers working in other fields often tend to ignore this protein. The third, and by far the smallest group, consists of those who are actually interested in the biology and function of the Ki-67 protein itself. The aim of this review is to give information about the properties of the Ki-67 protein and its use as a proliferation marker in research and diagnostics. Moreover, the authors hope that this study will also help to raise more interest in the biology and the unique features of the Ki-67 protein.

The Ki-67 protein was originally defined by the prototype monoclonal antibody Ki-67 (Gerdes et al., 1983), which was generated by immunizing mice with nuclei of the Hodgkin lymphoma cell line L428. The name is derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate. Because the antigen was not initially characterized, it was referred to mainly as the *Ki-67 antigen.* When the antigen was found to be a protein and the primary structure could be deduced from the corresponding cDNA, it revealed no homology to any known polypeptide (see later discussion). For this reason and because the function of the protein remained indistinct, the initial name Ki-67 was kept. To prevent confusion, we refer to the prototype antibody and the antigen as the Ki-67 antibody and Ki-67 protein, respectively, throughout this study.

Characterization of the Ki-67 antibody revealed an interesting staining pattern. The antibody was reactive with a nuclear structure present exclusively in proliferating cells. A detailed cell cycle analysis revealed that the antigen was present in the nuclei of cells in the G_1, S, and G_2 phases of the cell division cycle as well as in mitosis. Quiescent or resting cells in the G_0 phase did not express the Ki-67 antigen (Gerdes et al., 1984). Because the Ki-67 antigen was present in all proliferating cells (normal and tumor cells), it soon became evident that the presence of this structure is an excellent operational marker to determine the growth fraction of a given cell population. For this reason, antibodies against the Ki-67 protein were increasingly used as diagnostic tools in different types of neoplasms. Despite this broad application, the nature of the antigen

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remained unclear until it was identified as a protein by screening a cDNA expression library (Gerdes et al., 1991). The complete primary structure was published two years later after cloning and sequencing the entire cDNA (Schlüter et al., 1993). Two protein isoforms were originally described, generated by alternative splicing of an mRNA precursor transcribed from a single gene. Both isoforms with calculated molecular masses of 320 and 359 kD contain a large central region with 16 repetitive elements (Ki-67 repeats) encoded by a single exon of 6845 base pairs. Meanwhile, the entire gene locus of the Ki-67 protein has been sequenced as well, comprising almost 30,000 base pairs (Duchrow et al., 1996).

In 1983 when the first monoclonal antibody against the Ki-67 protein was described, a picture of the molecular mechanisms of the eukaryotic cell cycle control was just beginning to develop. In the same year, the term *cyclin* was proposed for proteins that are destroyed at certain points in the cell cycle (Evans et al., 1983). In the following years, rapid progress was made in the elucidation of the intricate network of cyclins, cyclin-dependent kinases, and their inhibitors that govern the meticulously coordinated cell cycle progression (for review see Graña and Reddy, 1995; Pines, 1995; Lew and Kornbluth, 1996; Arellano and Moreno, 1997). Advancement has also been made in understanding the factors that control key events in the cell division cycle, for example, the transition from one phase to the next, DNA replication, and chromosome condensation and segregation. The tumor supressor genes p53 and pRb have been shown to be major targets in the control of the transition from G_1 to S phase (reviewed in Paggi et al., 1996; Levine, 1997; Mayol and Graña, 1997; Agarwal et al., 1998; Graña et al., 1998; Stiegler et al., 1998). Furthermore, the role of the ORC (origin recognition complex) and Mcm (mini chromosome maintenance) proteins in the control of DNA replication have been described (for review see Kearsey and Labib, 1998; Tada and Blow, 1998). Although our knowledge of the molecular regulation of the cell division cycle is far from being complete, a vast body of data has been accumulated and is beginning to form into a detailed picture of a complex network that is regulated by expression, modification (e.g., phosphorylation, ubiquitination), degradation, and translocation of a number of key proteins. Despite this extensive knowledge, none of these key regulators has yet been functionally linked to the Ki-67 protein. This is striking, because as mentioned earlier, Ki-67 protein expression is strictly correlated to cell proliferation and to the active phases of the cell cycle. To our knowledge no other protein shows an expression pattern that is equally tightly regulated in dependence to the proliferative status of the cell. One would therefore expect that the role of the Ki-67 protein in cell proliferation would be fundamental and therefore easy to reveal. If the former expectation may still hold to be true, the latter has definitely been proven wrong during the last 16 years.

Ki-67 EXPRESSION AS A MARKER FOR PROLIFERATING CELLS

Requirements for a proliferation marker

Before discussing the use of the Ki-67 protein in tumor diagnostics, it may be worth having a more theoretical look at the term *proliferation marker* in this context. The expression of the Ki-67 protein is thought to be an indicator for cells within the growth fraction of a given population, that is, the fraction of cells born into the proliferative category (Alison, 1995). For this kind of marker two requirements have been postulated (van Dierendonck et al., 1989): (1) the antigen should be continuously present during the cell cycle of all cell types and (2) the transition to whatever type of nonproliferative state from any part of the cell cycle should be followed by a rapid disappearance of the antigen. Although it has been reported that Ki-67 antigen staining may be faint or even undetectable at the onset of DNA synthesis (see later discussion), it is generally accepted that the Ki-67 protein is expressed during all active phases of the cell cycle. Furthermore, all tissues tested showed Ki-67 staining in cell populations known to proliferate (Gerdes et al., 1983) and no convincing evidence has been presented that the Ki-67 protein may not be expressed by any proliferating human cell type.

The second requirement is more difficult to fulfill. One problem is the definition of the term *nonproliferative state.* This expression presupposes that a cell can be found in only two clearly defined states: the proliferative or the nonproliferative. In practice, cells tend to have a rather broad range of intermitotic times. Intermitotic times can vary greatly, especially in vivo and under suboptimal growth conditions in vitro. Because there is no unequivocal feature that can distinguish between "proliferative" and "nonproliferative" cells, it is always a matter of definition which cells are termed nonproliferative. Different techniques have been developed to access cell proliferation (for review, see Alison, 1995). Standard procedures to determine the growth fraction are generally based on experimental protocols, in which newly synthesized DNA is labeled during the S phase. Samples are taken at different time points and the fraction of labeled nuclei (or labeled mitoses) is estimated. The data obtained in such experiments, together with assumptions about cell proliferation kinetics, are subsequently used to estimate a theoretical value for the growth fraction of the population. One has to consider that these approaches will underestimate the growth fraction if a considerable fraction of cells has intermitotic times that exceed the labeling interval. In contrast, Ki-67 protein expression directly reflects a certain physiological state of the cell. Although the functional role of the Ki-67 protein during cell proliferation is unknown, it is unquestionable that Ki-67 protein expression and cell proliferation are closely linked.

Even if an "ideal" proliferation marker could be found, there are theoretical limitations for its use in estimating the growth fraction. As mentioned previously, such an ideal proliferation marker must label "proliferative" cells during all (active) phases of the cell cycle. A cell should be positive as long as it is going to divide. The problem is that the term *going to divide* refers not to the actual state of the cell but to an event in the future. The cell must make the final decision whether to divide at some time point during the cell cycle. Let us assume, for example, that the decision is made in mid-G_1 phase. As stated earlier, for an ideal proliferation marker, one would postulate that during

all cell cycle phases, including early G_1 phase, the cell should be positive if the cell is going to divide, but negative if the cell is going to become quiescent or terminally differentiated. This leads to the contradiction that the proliferation marker must indicate the decision of the cell *before* it has actually been made. A correct indication of the proliferative state can be made only if the decision whether to continue proliferation is made before or at least at the time of the previous cell division. This assumption, however, is in disagreement with experimental data proving that the decision can be made in later phases of the cell cycle. Some authors postulate that cells can enter a quiescent state even after completing DNA synthesis (Darzynkiewicz and Traganos, 1982; Drewinko et al., 1984; Lazebnik et al., 1991; Wei et al., 1993). This leads to the conclusion that a proliferation marker can be used only to indicate the potential of a certain cell to divide, but not to predict the actual division of this cell.

This is especially true if the growth kinetics of a cell population is disturbed, for example, by administration of drugs. Van Oijen et al. (1998) showed that cells remain Ki-67 positive when DNA synthesis is blocked by hydroxyurea; the cells are arrested in mitosis by nocodazole or blocked in the cell cycle progression by the heterologous expression of the cyclin-dependent kinase inhibitor p21. They further demonstrated Ki-67 positive cells 24 h after inflicting DNA damage by the application of camptothecin. Under these conditions, p21 and p53 were upregulated and progression through the cell division cycle arrested as measured by incorporation of the thymidine analogue bromodeoxyuridine (BrdU). From these observations, the authors conclude that Ki-67 expression is not necessarily linked to actively proliferating cells and advise against the use of Ki-67 in studies of cells that overexpress p53 or p21. These statements result from the oversimplified model that cell proliferation can be assessed by monitoring some kind of master switch that, in any case, precisely reflects the proliferative state of the cell. In contrast to this model, all evidence supports the idea that cell proliferation is governed by a complex and intricate regulatory network. Artificially interfering with one part of the proliferative process will not necessarily lead to the corresponding adjustment of all involved parameters. In other words, it cannot be expected that a cell downregulates its Ki-67 protein expression just because DNA synthesis is blocked by the application of a drug. A cell that continues to express the Ki-67 protein is likely to maintain the potential to proliferate and may eventually do so after the block is released. In the case of experimentally inflicted DNA damage, Ki-67 antigen expression may also correspond to the ability of the cell to resume the cell division cycle after the DNA damage is repaired. The advice not to use Ki-67 in studies of cells that naturally overexpress p53 or p21 should be given only if it can be proven that such cells remain Ki-67 positive without proliferative activity for a prolonged time span. Up to now, convincing evidence for this is missing.

In conclusion, although the Ki-67 protein expression may not correspond in every case to the theoretically defined term of *growth fraction*, in general one finds a close correlation between these parameters. However, it should be kept in mind that positivity for the Ki-67 antigen does not necessarily mean that a cell is unquestionably going to divide. Such a cell may still decide to leave the active cell cycle and enter a quiescent state. It is also possible to arrest cells in the cell cycle without immediately influencing Ki-67 expression. In this context it has to be considered that positivity for the Ki-67 antigen may reflect the ability of a cell to continue to proliferate after the block is removed. In practice, these considerations should not be regarded as drawbacks. Because the Ki-67 index (percentage of cells staining positive for the Ki-67 antigen) is directly based on a physiological parameter involved in cell proliferation, it may give an even better insight into the growth characteristics of a tumor, its susceptibility to certain drugs, and to the outcome of a patient than the estimation of the growth fraction that precisely follows a theoretical definition. The usefulness of a marker in tumor diagnostics has to be tested for each tumor type and application. Only those markers that have been proven to be useful in practice should be considered. The prognostic value of the Ki-67 index has been established in numerous publications (see next section). But it is also evident that estimating the growth fraction alone is not sufficient to describe tumor growth. For example, the growth fraction (and the Ki-67 labeling index) relates only to the number (or fraction) of proliferative cells but not to the time needed for the completion of an intermitotic cycle. In other words, the estimation of the growth fraction gives information only about the state but not about the rate of proliferation; therefore, an additional marker would be helpful to assess this parameter. In the future, multiparameter analysis may provide a better means of analyzing cell proliferation and tumor growth. This may not only improve the prognostic value, but may also be a prerequisite for choosing the appropriate type of therapy for each individual case.

Antibodies against the human Ki-67 protein: operational markers to estimate the growth fraction in malignant neoplasia

Since Gerdes et al. (1984) first suggested that the growth fraction as estimated by the Ki-67 labeling index could by of prognostic value in non-Hodgkin's lymphomas, numerous studies have been performed to examine the usefulness of this marker in various types of malignant neoplasms. It is out of the scope of this review to give a comprehensive overview about the use of antibodies against the Ki-67 protein in tumor diagnostics. To date, the *Medline database* lists nearly 4000 entries referring to the Ki-67 protein or anti-Ki-67 protein antibodies, and most of these publications deal with the prognostic value of the Ki-67 antigen (PubMed database August 1999, National Library of Medicine, Bethesda, MD 20894, USA). After it was shown that the Ki-67 protein could be detected during all active phases of the cell cycle but not in resting cells (Gerdes et al., 1983, 1984), it became evident that the Ki-67 antibody could be used as a tool to estimate the growth fraction of any human cell population. This is of special interest in tumor diagnostics, where the proliferative activity of a given tumor is often difficult to assess. Methods that are widely used in experimental research such as the determination of the cellular DNA content by flow cytometry or the in vitro or in vivo labeling of

newly synthesized DNA, are for practical and sometimes also ethical reasons difficult to apply to human tumors. In contrast, Ki-67 immunostaining can easily be performed on various types of cytological and histological preparations, such as smear, squash, cytocentrifuge preparations, and histological sections. In comparison to the counting of mitotic figures, the Ki-67 labeling index is more sensitive, because cells in all active phases of the cell cycle are recognized. Furthermore, the reliable assessment of mitotic figures needs more experience and is more time-consuming than the counting of immunohistologically stained nuclei. One major drawback of the original Ki-67 antibody was the fact that it could not be used in formalin-fixed paraffin sections, which are routinely used in histopathology. This disadvantage could be overcome by the preparation of the Ki-67 equivalent monoclonal antibody MIB-1, which can be used on paraffin sections after antigen reassessment by microwave-processing (Cattoretti et al., 1992).

Although the usefulness of the Ki-67 labeling index in tumor diagnostics has often been questioned, in recent years a growing number of studies have proven its prognostic value. This discussion has repeatedly led to the publication of editorials in reputed journals, arguing for the use of the Ki-67 labeling index in histopathology (Sawhney and Hall, 1992; Schwarting, 1993; Lloyd, 1998). One reason for the reluctance of many pathologists to use the Ki-67 labeling index was the competition with another "proliferation marker," the proliferating cell nuclear antigen (PCNA). Before the MIB-1 antibody was available, the prototype PCNA antibody PC10 had the advantage that it could be used in formalin-fixed paraffin sections. However, it could be shown that PC10 staining is very sensitive to fixation and that the antigen reassessment by microwave treatment led to the staining of quiescent cells (Schwarting, 1993; Kubbutat et al., 1994). This was confirmed by the finding that PCNA not only has a role in DNA replication, but is also involved in DNA repair. These multiple roles of PCNA (reviewed by Prosperi, 1997) strongly argue against the use of PCNA expression as a reliable marker for proliferating cells.

Meanwhile, the usefulness of the Ki-67 labeling index has been well established for various types of malignant neoplasms. In the case of multiple myeloma it could be shown that Ki-67 expression correlates with the course of the disease and, furthermore, is a useful marker in distinguishing multiple myeloma from monoclonal gammopathy of unknown significance (Drach et al., 1992; Miguel-Garcia et al., 1995). For soft-tissue sarcoma, the Ki-67 index is a significant predictor for overall survival of the patients as well as for the occurrence of distant metastasis (Ueda et al., 1989; Rudolph et al., 1997; Heslin et al., 1998; Huuhtanen et al., 1999).

The prognostic value of the Ki-67 index can be of particular importance in those types of cancers in which the clinical course is difficult to predict by histological criteria alone. A good example is prostate cancer, in which the predictive value of the Ki-67 labeling index has been studied in numerous publications. In multivariate analysis, it was found that the Ki-67 labeling index is an independent and significant prognostic factor for disease-specific survival if all stage and grade categories are included (Aaltomaa et al., 1997; Borre et al., 1998). Furthermore, for patients treated with radical prostatectomy Ki-67 expression is an independent predictor of disease recurrence and progression (Bettencourt et al., 1996; Bubendorf et al., 1996; Moul et al., 1996; Keshgegian et al., 1998; Bai et al., 1999). Similar results were obtained for patients treated with transurethral resection (Stattin et al., 1997). Moreover, the Ki-67 labeling index is also a significant predictive marker for the postradiation recurrence in patients with adenocarcinomas (Scalzo et al., 1998). The estimation of the Ki-67 labeling index may also be useful in lymphatic metastases from prostate cancer. In a study with patients who underwent pelvic lymphadenoectomy and ^{125}I implantation, a correlation was found between the fraction of Ki-67-positive malignant cells and the survival of patients (Cher et al., 1996). In the future, the pretherapeutic assessment of Ki-67 expression may become of increasing importance in the evaluation of tumor aggressiveness and the selection of adequate treatment. In a recent study, Bubendorf et al. (1998) assessed the p53, Bcl-2, and Ki-67 expression of prostate tumors in core needle biopsies. It could be shown that in multiparametric analysis, the Ki-67 labeling index was the only independent predictor of tumor-specific survival.

Another type of cancer in which the Ki-67 labeling index has been extensively studied is the breast carcinoma. During recent years a vast body of evidence has accumulated, which supports the concept that the Ki-67 labeling index is an independent prognostic factor for survival and tumor recurrence. Some of the more recent publications are summarized in Table 1. Together, these studies cover more than 4600 cases proving that the Ki-67 labeling index is a significant prognostic factor. This was found not only in univarite analysis but (when performed) most studies showed that Ki-67 is also of independent prognostic value in multivariate analysis.

The data summarized previously clearly argue for the assessment of the Ki-67 labeling index in tumor diagnostics. In this context it is worth mentioning that the diagnostic value of the Ki-67 index depends on the tumor type and the availability of protocols for treatment. For certain tumor types, no correlation could be demonstrated between prognosis and the Ki-67 labeling index. This does not necessarily mean that Ki-67 expression is not a good marker for proliferative activity; it merely indicates that there is no variability in the course of the disease in relation to the proliferative activity. It is obvious that the proliferative activity, as assessed by the Ki-67 labeling index, may be of importance in those types of treatment protocols that are aimed at dividing cells. Here the Ki-67 labeling index may be of great value in predicting how a tumor responds to a certain type of therapy. Consequently, the assessment of Ki-67 protein expression may become one of the crucial parameters for deciding which therapy is best for an individual tumor. We are now at the brink of the introduction of the Ki-67 labeling index into routine clinical diagnostics. For this purpose, however, there is an urgent need for an international standardization of the staining procedures and a clinical-pathological validation by randomized, multicenter prospective studies.

TABLE 1. Prognostic value of the Ki-67 labeling index in breast cancer

Reference	Prognostic significance[1]	Characteristics of studied group[1]	Antibody	Number of cases
Archer et al., 1995	OS (UA)	Grades II & III	Ki-67	92
Molino et al., 1997	OS, DFS (UA, MA)		Ki-67	322
Weikel et al., 1995	OS, DFS (UA)		Ki-67	549
Rudolph et al., 1999	OS, DFS (UA, MA)	N_0	Ki-S5	356
Clahsen et al., 1998	DFS (UA, MA)	pm, N_0	MIB-1	441
Dettmar et al., 1997	DFS (UA)	N_0	MIB-1	90
Domagala et al., 1996	OS (UA, MA)		MIB-1	186
Haerslev et al., 1996	OS (UA)		MIB-1	487
Jansen et al., 1998	OS, DFS (UA, MA)		MIB-1	341
Jensen et al., 1995	OS (UA, MA)	T_{1-2}, N_0, M_0	MIB-1	118
Keshgegian & Cnaan, 1995	DFS (UA)		MIB-1[2]	135
Pinder et al., 1995	OS (UA, MA)	tumor $\leq$ 5 cm	MIB-1	177
Rozan et al., 1998	DFS (UA); OS (UA, MA)		MIB-1	326
Seshadri et al., 1996	OS, DFS (UA, MA)	N_0, N_+	MIB-1	740
Veronese et al., 1996	OS, DFS (UA)		MIB-1[2]	246

[1]OS, overall survival; DFS, disease-free survival; UA, univariate analysis; MA, multivariate analysis; N_0, tumors from patients with negative axillary lymph nodes; N_+, tumors from patients with positive axillary lymph nodes; M_0, tumors from patients with no histological evidence of invasion of skin or deep fascia; T_{1-2}, tumor diameter $\leq$ 50 mm; pm, tumors from premenopausal patients.
[2]In addition, the labeling index was determined using the Ki-67 prototype antibody.

New applications for antibodies against the Ki-67 protein

A drawback to the application of Ki-67 antibodies in basic research is the fact that monoclonal antibodies raised against the human Ki-67 protein have often a rather limited cross-species reactivity. The prototype Ki-67 antibody detects the human protein as well as the Ki-67 equivalents from other primate species. A panel of new monoclonal antibodies against the Ki-67 protein was raised and designated *MIB* (for Molecular Immunology Borstel). Additionally, MIB-1 can be used to stain the Ki-67 equivalent proteins from various mammals (e.g., cattle, dog, horse, and sheep). Until recently, MIB-5 was the only monoclonal antibody reactive with the Ki-67 equivalents from rodents. Gerlach et al. (1997a,b) showed that MIB-5 is useful for the assessment of proliferating cells in rats. Employing immunohistochemical methods on tissue sections, the authors could demonstrate that it is possible to distinguish proliferative from nonproliferative cells during embryonic development. Moreover, the MIB-5 antibody was also found to be useful in monitoring cell proliferation in regenerative processes in tissues of adult animals.

The fact that MIB-5 is a mouse monoclonal antibody makes its application on murine tissue sections troublesome, because background staining due to endogenous immunoglobulins is difficult to avoid. To overcome this problem we first raised a rabbit antiserum against the murine Ki-67 protein equivalent (Kosco-Vilbois et al., 1997) and recently a rat monoclonal antibody was prepared in a collaboration between the laboratory of Hanswalter Zentgraf at the German Cancer Research Center (Heidelberg), our own group, and Dianova GmbH (Hamburg). This antibody, designated as TEC-3, was raised by immunizing rats with a recombinantly expressed part of the murine Ki-67 equivalent protein. Figure 1a shows an example for the staining obtained with this antibody on a murine tissue section. In general, the TEC-3 staining pattern is very similar to the Ki-67 staining pattern on the corresponding human tissues.

With the development of MIB-5 and TEC-3, a gap was closed. It is now possible to assess cell proliferation by the means of monoclonal Ki-67-equivalent antibodies in most mammals that are routinely used as experimental animals. This should lead to an increasing utilization of these antibodies in basic research. The possibility of identifying proliferative cells in mammalian tissues and cell cultures with little effort by immunological methods should be beneficial in various fields of basic and applied research. These include such different areas as drug screening, the study of animal models of various human diseases, wound healing and other regenerative processes, aging, and tumor biology as well as developmental biology. An interesting example of the latter is the recent paper of Lee et al. (1999). The authors demonstrated that the expression of the transcription factor whn is principally associated with terminally differentiated cells. Nevertheless, a small subclass of cells expresses whn in combination with the Ki-67 protein, suggesting that whn expression may be involved in the transition from the proliferative to the postproliferative state.

The utilization of the Ki-67 protein as a proliferation marker could be further extended if Ki-67 equivalent proteins in nonmammalian species could be identified. If these proteins exist, the characterization of homologues in *Xenopus*, *Drosophila*, and *Caenorhabditis* would be of special interest. The identification of these proteins may be valuable not only in assessing cell proliferation in these well-studied animal models but could even provide us with a clue to understanding the function of the mammalian Ki-67 protein.

THE BIOLOGY OF THE Ki-67 PROTEIN

From structure to function?

In 1993, 10 years after the initial description of the antigen, when the entire cDNA sequence coding for the Ki-67 protein was published by Schlüter et al., it could be expected that the identification of sequence motifs of potential functional significance and homology searches would lead to rapid progress in the elucidation of the function of the molecule; indeed, the primary structure revealed numerous interesting features. Two differentially spliced mRNA isoforms were described

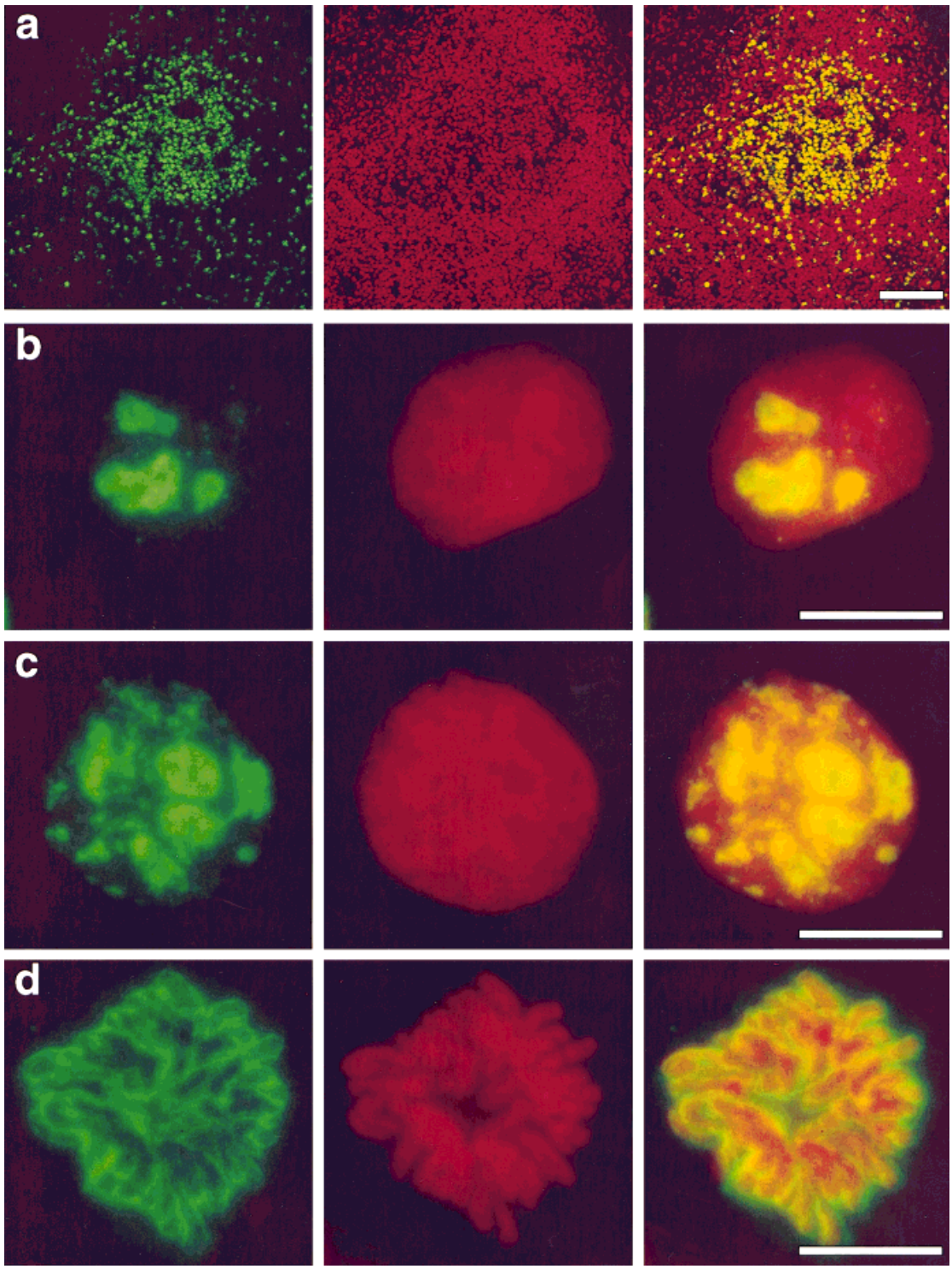

Fig. 1. Detection of the Ki-67 protein by immunofluorescence. Left-hand column, staining for the Ki-67 protein (green); center column, staining of DNA (red); right-hand column, merged images. (**a**) Murine lymph node stained with the rat monoclonal antibody TEC-3. Indirect immunofluorescence was performed using a fluorescein isothiocyanate-conjugated goat anti-rat IgG. Nuclei positive for the murine Ki-67 antigen appear in yellow. In the germinal centers, the nuclei of nearly all centroblasts and some centrocytes react with TEC-3. In contrast, most cells in the follicular mantle are negative for the Ki-67 antigen. Bar, 100 μm. (**b–d**) Cytospin preparations of human PHA stimulated peripheral mononuclear blood leukocytes stained with the monoclonal antibody MIB-1. Indirect immunofluorescence was performed using a fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Bar, 10 μm. Note the different staining patterns during interphase (b, c) and mitosis (d).

with sizes of 8688 and 9768 base pairs. These two forms differ by the absence or presence of the region encoded by exon 7 of the Ki-67 gene. The molecular mass of the small and the large protein variant derived from these isoforms was calculated to be 320 and 359 kD, respectively. A potential "ATP/GTP binding site motif A" (P-loop) was predicted in the carboxy terminal region of the molecule (Saraste et al., 1990). Two putative nuclear targeting signals (Chelsky et al., 1989; Silver 1991) and eight potential "bipartite nuclear targeting signals" (Dingwall and Laskey, 1991) were in accordance with the finding that during interphase the antigen is exclusively detectable in the nuclei of the cells. Furthermore, the protein contains 143 protein kinase C, 89 casein kinase II, and 2 tyrosine kinase consensus sites.

Although some of the features predicted by the sequence analysis have been verified and characterized in later studies, for example, phosphorylation and nuclear transport (see later discussion), the function of the Ki-67 protein still remains unclear. In part, the difficulties in the determination of the functional role can be attributed to the absence of obvious homology with other proteins.

Although conserved domains, shared with proteins of characterized function would be an excellent guide in the initial steps of functional characterization, until recently (see later discussion), no protein—with the exception of some Ki-67 equivalents in other mammals—exhibits clear similarity with the human Ki-67 protein.

The failure to detect regions of similarity between Ki-67 and other proteins may be partially due to the fact that conventional search algorithms are too insensitive to detect only weakly conserved regions of homology. Using an advanced search method for sequence profiles, Hofmann and Bucher (1995) located a so-called forkhead-associated (FHA) domain in the amino terminal region of the Ki-67 protein. Interestingly, the authors could further show that this type of sequence motif is a common feature in a number of proteins known to be involved in cell-cycle regulation (e.g., DUN1 and RAD53 protein kinases from *S. cerevisiae* that link the S phase checkpoint to DNA-damage and the *S. pombe* cds1 kinase that is also involved in S phase regulation). Unfortunately, the function of the FHA domain is far from clear. Recently, it has been suggested that one of the two FHA domains of the Rad 53 protein kinase in *S. cerevisiae* is a protein binding domain that can interact with phosphorylated Rad9, thereby integrating DNA damage signals (Sun et al., 1998). Further progress in the understanding of the function of FHA domains and the identification of additional proteins that interact with these domains will lead to a better understanding of the cell cycle regulatory network and may also give some insight into the functional role of the Ki-67 protein.

A unique feature found in the Ki-67 primary structure is the occurrence of 16 repeated elements, each with a size of approximately 122 amino acid residues. These "Ki-67" repeats share between 43 and 62% identical amino acid residues. Within these repetitive elements there is a highly conserved region, also called the *Ki-67 motif*, exhibiting 72 to 100% identity with the consensus sequence. Moreover, the Ki-67 motif also includes the epitope (F K E L) that is recognized by the prototype Ki-67 antibody. The entire repetitive region is encoded by a single exon (13) of the Ki-67 gene. With a length of 6845 base pairs, Ki-67 exon 13 is one of the largest mammalian exons known.

Until recently, the Ki-67 repeats seemed to be a unique feature of the human Ki-67 protein and its equivalents in other mammals. This may have changed with the discovery of a new protein in cells from the rat kangaroo designated as *chmadrin* (Takagi et al., 1999). Like the Ki-67 protein, chmadrin contains a central repetitive domain. Although this domain is much smaller, the chmadrin repeats exhibit clear sequence similarity to the repeats of the Ki-67 protein. Similarity was also found between other limited regions of the proteins. Interestingly, like the Ki-67 protein, chmadrin contains an FHA domain close to its amino terminal end. Currently, it is not possible to decide whether chmadrin is the equivalent of the Ki-67 protein in the rat kangaroo or if it belongs to a new family of Ki-67-related proteins. In any case, the analysis of regions that are conserved in both proteins may be fruitful for the identification of functionally important domains and sequence motifs.

Ki-67 protein expression during the cell cycle

After preparation of the prototype Ki-67 antibody, it soon became evident that the recognized antigen could be exclusively detected in those cell populations that were known to proliferate (Gerdes et al., 1983). A detailed analysis of phytohemagglutinin (PHA) stimulated peripheral mononuclear blood leukocytes (PBL) showed that unstimulated (i.e., G_0 cells) were consistently negative for the Ki-67 antigen. In contrast, after PHA stimulation the antigen was expressed in S, G_2, and M phase cells (Gerdes et al., 1984). The expression in the first G_1 phase is controversially discussed. Whereas Lopez et al. (1991) claimed cells in the initial G_1 phase to be entirely negative for the Ki-67 antigen, Gerdes et al. (1984) reported the onset of Ki-67 expression already in the late G_1 phase (termed G_{1B}). However, both studies agree that G_1 cells in subsequent division cycles are positive for the Ki-67 protein, although there is controversy about the relative expression during the G_1 phase. Whereas some authors report an increase of Ki-67 antigen staining already starting in late G_1 phase (Braun et al., 1988; Starborg et al., 1996), others found a decrease until the onset of DNA synthesis (Lopez et al., 1991; Bruno and Darzynkiewicz, 1992). Van Dierendonck et al. (1989) even reported the striking observation that the Ki-67 antigen was undetectable in cells entering S phase, a fact that was disputed by others (du Manoir et al., 1991; Kill, 1996).

These inconsistencies can be explained, in part, by the hypothesis of du Manoir et al. (1991). The authors propose three different pathways during the G_1 phase. The *Ki-67 decrease pathway* is characterized by a declining Ki-67 staining and leads eventually to the exit from the active cell cycle (G_0). If cells on this pathway get stimulated by growth factors, they can enter the *Ki-67 increase pathway* that brings the cells back into S phase. Cells following the *Ki-67 stable pathway* exhibit a constant intensity of Ki-67 staining during the G_1 phase. This pathway is thought to correspond to optimal local growth conditions.

It is generally accepted that the Ki-67 antigen staining increases during S phase (du Manoir, 1991; Bruno and Darzynkiewicz, 1992). Moreover, it was shown that this even exceeds the increase in DNA content (Sasaki et al., 1987; Bruno et al., 1991). During G_2 phase a further increase in Ki-67 staining intensity was reported (du Manoir et al., 1991). The highest staining intensity of the Ki-67 antigen is found in metaphase (Braun et al., 1988; Verheijen et al., 1989b; du Manoir et al., 1991; Starborg et al., 1996), whereas during ana- and telophase the Ki-67 staining begins to decrease (Braun et al., 1988; Starborg et al., 1996).

These observations lead to the question in which way the changing staining intensity is linked to the biological function of the Ki-67 protein. The increase in Ki-67 staining intensity observed from the onset of S phase until metaphase cannot merely be explained by accumulation of the protein synthesized during this time period, because the biological half-life of the Ki-67 protein was estimated to be rather short. By radioactive pulse labeling of L428 cells, the half-life was determined to be approximately 90 min (Heidebrecht et al., 1996). This is in good agreement with the observation of Bruno and Darzynkiewicz (1992), who determined the decrease in Ki-67 staining intensity of HL60 after the inhibition of protein synthesis by cycloheximide. From these experiments the half-life of the Ki-67 protein was estimated to be approximately 1 h. Interestingly, the authors found no variance between cells in different cell cycle phases. The Ki-67 protein expression must therefore be the result of an exactly regulated de novo synthesis in association with effective degradation processes. This suggests that the precise regulation of the Ki-67 protein expression during the cell cycle may be crucial for its biological activity. As discussed in the next section, the cellular localization during the cell cycle seems to be of importance as well.

The cellular localization of the Ki-67 protein is cell cycle phase dependent

It is now well established that the cellular distribution of the Ki-67 protein is not constant but is subjected to dramatic changes during the cell division cycle (Fig. 1b,c,d). During the early G_1 phase, the Ki-67 staining is restricted to numerous foci throughout the nucleoplasm. It has been suggested that these foci correspond predominantly to the sites of the reforming nucleoli (van Dierendonck et al., 1989; du Manoir, 1991). In contrast, Kill (1996) found that although some of these foci are associated with sites containing nucleolar antigens, they are not necessarily co-localized. This was confirmed by a recent study showing that the Ki-67 protein is integrated into the reforming nucleoli at a relatively late time point, when fibrillarin, nucleolin, Nop52, and hPop1 are already present (Savino et al., 1999). In a detailed study, Bridger et al. (1998) showed that the localization of the Ki-67 protein in the very early G_1 phase coincides with regions of satellite DNA (centromeric alpha satellite, telomeric minisatellite, and the satellite III of heterochromatic blocks). During the progression of the G_1 phase, this co-localization declines. After integration of the Ki-67 protein into the nucleoli, only a few satellite regions remain associated with the Ki-67 staining. After reassembly of the nucleoli in the mid-G_1 phase, the Ki-67 protein is localized mainly in these structures (Braun et al., 1988; van Dierendonck et al., 1989; Verheijen, 1989a; Kill, 1996). For the murine Ki-67 equivalent, Starborg et al. (1996) showed that in addition to the nucleolar localization, the antigen could also be detected in the heterochromatic regions. Immunoelectron microscopy revealed that the Ki-67 protein is absent from the granular components and the fibrillar centers within the nucleoli (Verheijen, 1989a). It was suggested that the Ki-67 protein is confined to a newly defined compartment designated the "fibrillarin-deficient region of the dense fibrillar component" (Kill, 1996).

The distribution of the Ki-67 antigen during the S phase is a matter of discussion. According to Kill (1996), the Ki-67 staining during the S phase is restricted to the nucleoli, whereas other authors report an additional staining of the nucleoplasm (Braun et al., 1988; van Dierendonck et al., 1989; du Manoir et al., 1991). There are also controversial reports about the antigen distribution during the G_2 phase. Although some authors found the Ki-67 protein to be localized throughout the nucleus (du Manoir et al., 1991), others reported brightly stained foci in addition to a diffuse nucleoplasmic distribution (Braun et al., 1988). These foci, which only partially correspond to the nucleoli, have also been reported for the murine Ki-67 equivalent protein (Starborg et al., 1996).

A prominent redistribution of the Ki-67 protein occurs during mitosis. In prophase, the Ki-67 protein is reorganized and becomes detectable as a fine meshwork associated with the condensing chromatin (Verheijen et al., 1989b). In metaphase, a bright Ki-67 antigen staining is visible covering the surface of the individual chromosomes (Braun et al., 1988; Verheijen et al., 1989b; du Manoir et al., 1991; Starborg et al., 1996) (Fig. 1d). The entire Ki-67 protein, however, is not associated with the chromatin. After the breakdown of the nuclear membrane, part of the Ki-67 protein can also be detected distributed diffusely in the cytoplasm (Braun et al., 1988). Some authors reported that, toward the end of mitosis (ana- and telophase), the Ki-67 antigen exhibited a granular staining pattern (Braun et al., 1988; Starborg et al., 1996). In contrast, du Manoir et al. (1991) found a homogeneous staining during these phases.

The discrepancies described previously with regard to the localization of the Ki-67 protein during the cell cycle may be attributed, in part, to the fact that the studies were performed with different types of cells (even from different species), using different protocols for fixation and staining. An inherent problem with the visualization of the Ki-67 protein is the complete dependence on immunological methods for detection; however, it has been shown that the binding of antibodies to the Ki-67 protein depends on such factors as ionic strength (Bruno et al., 1992) or the binding of the Ki-67 protein to DNA (Lopez et al., 1994). On the one hand, this makes the visualization of the Ki-67 protein sensitive to the conditions used for fixation and staining of the samples. On the other hand, this leads to the intriguing speculation that the entire Ki-67 protein of the cell is not accessible to immunological detection with the antibodies available. It has been proposed that the newly synthesized Ki-67 in the cytoplasm cannot be detected, because the antigenic determinant is

masked (Braun et al., 1988). This may also be true in a more general way. The redistribution of the Ki-67 antigen staining described earlier may only in part be due to a genuine topological redistribution of the Ki-67 protein. The changing staining patterns may also reflect changes in the accessibility of the antigen, caused by alterations in the biological properties of the Ki-67 protein. Conformational changes, the forming of supramolecular complexes with other proteins, or the binding to nucleic acids may influence the interaction between antibody and antigen. It could be shown that in the presence of calcium ions, the Ki-67 protein structure is changed in such a way that the immunohistochemical detection with the monoclonal antibody MIB-1 was greatly reduced (Shi et al., 1999).

In this context, an alternative approach for the detection of the Ki-67 protein may prove to be useful. The expression of chimeric molecules consisting of the green fluorescent protein (GFP) and the Ki-67 protein (or parts thereof) by recombinant DNA technology may help to gain insight into the distribution of the Ki-67 protein during the cell cycle. A major advantage of this method is that these fusion proteins can be visualized in living cells without the need for fixation and subsequent antibody staining. Furthermore, by the expression of single protein domains or short fragments, it may be possible to identify the topogenic structures of the Ki-67 protein. A more detailed analysis of the cell-cycle-dependent localization, especially with respect to other nuclear macromolecules, may prove to be of crucial importance for insight into the functional role of the Ki-67 protein.

The biological significance of the Ki-67 protein

It is astounding that 16 years after its initial description, the functional role of the Ki-67 protein is still unknown. This is even more striking considering the enormous progress that has been made in the field of cell cycle research. So why has the Ki-67 protein never been linked to the network of proteins responsible for cell cycle control and progression? Could it be, that the Ki-67 protein, despite the strict correlation, is of no significance for cell proliferation? This is rather unlikely. It was shown that the incubation of cultured cells with oligodeoxynucleotides complementary to the Ki-67 mRNA inhibits DNA synthesis (Schlüter et al., 1993). Moreover, the microinjection of antibodies directed against the murine Ki-67 equivalent into the nuclei of Swiss-3T3 cells resulted in a decreased rate of cell division (Starborg et al., 1996). Similar results were obtained by microinjecting antibodies against the human Ki-67 protein (Heyden, 1997). In combination, these data strongly suggest that the Ki-67 protein has an essential role in cell proliferation.

As already mentioned, one reason for the paucity of knowledge regarding the Ki-67 protein function is the absence of apparent homology with other proteins of known function; other reasons are the size and the high susceptibility to protease cleavage. This makes the Ki-67 protein difficult to handle in biochemical assays, and with over 300 kD the molecule may easily be missed on standard gels for protein separation. Nevertheless, profound progress has recently been made in the characterization of the biological and biochemical properties. As described in the previous section, the cell-cycle-dependent reorganization of the Ki-67 protein has extensively been studied. It could been shown that some of these redistributions are accompanied by posttranslational modifications. For example, it was found that, during mitosis, the Ki-67 protein shifts to forms of higher apparent molecular mass and that these changes depend on protein phosphorylation (Elmer Endl, personal communication). Now it is necessary to determine which of the numerous theoretical predicted phosphorylation sites are of biological importance and which kinases and phosphatases are responsible for the modification of these sites.

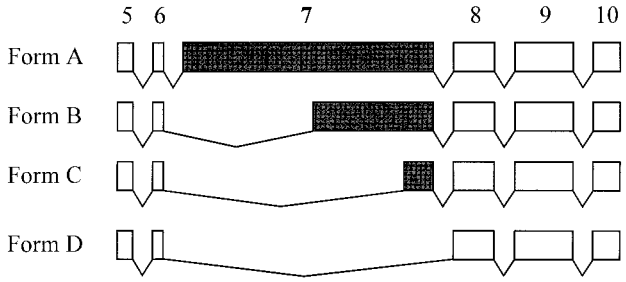

Fig. 2. Schematic representation of alternative splice forms of the Ki-67 mRNA found in murine tumor cell lines. Form A and Form D correspond to the "large" and the "small" isoform previously identified in human cells. Form B and Form C utilize internal splice acceptor sites located within the exon 7 equivalent. Exons and introns are indicated by boxes and lines, respectively.

Progress has also been made in the characterization of the signals that are responsible for the nuclear import of the Ki-67 protein. Although 10 putative nuclear targeting signals were predicted from the amino acid sequence (see previous discussion), one seems to be sufficient to accomplish nuclear import. When a carboxy terminal fragment of the Ki-67 protein, which contains only one putative "bipartite" nuclear targeting signal, is expressed in mammalian cells, this fragment alone, or as a GFP fusion protein, is targeted into the nucleus. In contrast, the predicted "bipartite" nuclear targeting signal located in the Ki-67 repeat 14 is not able to achieve nuclear localization (unpublished observation).

Another interesting finding is the presence of more than the two Ki-67 isoforms that were originally described. Analysis of the region encoded by exons 5–10 using reverse transcriptase PCR revealed multiple splice forms in mRNA preparations from human and murine cell lines. Figure 2 shows a schematic representation of the variants found in murine cells. In addition to the "large" and "small" forms, which correspond to the isoforms previously described in human cells (with or without exon 7), there are additional splice products that use acceptor sites located within the murine exon 7 equivalent. In all forms analyzed so far, the reading frame is kept intact, allowing for the generation of protein (Scholzen et al., 1997). In this context, it is tempting to speculate that the different splice variants are associated with certain "physiological states" of the cell, such as the positioning within the cell division cycle. This idea is supported by the observation that, in general, only one or two of these splice forms are detectable in single cells (Christiane Dimmler, personal communication).

As summarized in this study, the growing interest in the Ki-67 protein has led to an increasing number of publications dealing with different aspects of the biological properties of this molecule. In the near future these findings, like pieces of a jigsaw puzzle, may help to build a complete picture, providing an understanding of the biological significance of the Ki-67 protein, although at the moment, some of the central pieces are missing. How can these pieces be found? There are different strategies that may prove to be fruitful. The identification of binding partners, especially proteins, could be very helpful. In an initial step, co-localization studies may be useful in identifying candidates for the interaction with the Ki-67 protein. More direct approaches, like the two-hybrid-screening assay may also be productive.

Alternatively, the function of the Ki-67 protein could be inhibited. The functional role may then be deduced from the resulting phenotype. For example, functional inactivation could be achieved by the application of antibodies (see earlier discussion) or antisense molecules (DNA or RNA). Another promising approach is the generation of dominant-negative mutants (Herskowitz, 1987). The expression of such mutants inhibits the biological activity of the endogenous wild-type protein. A problem in the generation of these mutants lies in the fact that, without a detailed knowledge of protein function, it is difficult to predict which kind of mutant will exhibit a dominant-negative phenotype. Therefore, in the case of the Ki-67 protein, the generation of a dominant-negative mutant would be more or less a chance hit. Another way to inhibit protein function is the generation of null mutations in the mouse by gene targeting. The fact that the Ki-67 protein is probably essential for cell cycle progression makes it likely that the resulting phenotype is lethal. Therefore, a conditional approach, using the cre/loxP system (Gu et al., 1994; Rajewsky et al., 1996) may be the appropriate choice. In this system, a gene can be deleted only in certain cell populations of the organism (e.g., T cells or hepatocytes). This makes it possible to study null mutations, even when a complete "knock out" proves to be lethal for the organism, as it is to be expected for the Ki-67 protein.

Sixteen years after the initial description, we may now be on the verge of resolving the physiological role of the Ki-67 protein. It is to be hoped that the efforts to elucidate the biological function of this protein will not only contribute to improving our understanding of the complex regulatory network that governs cell proliferation, but also support the acceptance of the Ki-67 protein as a general proliferation marker.

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