

The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression

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SUMMARY

We have isolated the murine homologue of the human Ki-67 antigen. The Ki-67 antigen is used as a marker to assess the proliferative capacity of tumour cells; however, its cellular function is not known. The murine Ki-67 cDNA sequence (TSG126) was found to contain 13 tandem repeats, making up more than half of the total protein size. A comparison of this repetitive sequence block to its human counterpart, which contains 16 consecutive repeat units, revealed several conserved sequence motifs, including one motif frequently observed in proteins interacting with DNA. An antiserum developed against the product of the TSG126 cDNA clone identified a protein with an apparent molecular mass of 360 kDa, mainly expressed in proliferating cells. The TSG126 protein begins to accumulate during the late G₁ stage of the cell cycle and is first seen as numerous small granules evenly distributed throughout the nucleus. During the S and the G₂ phases, larger foci that overlap with the nucleoli and

the heterochromatic regions are formed. At the onset of mitosis the TSG126 protein undergoes a dramatic redistribution process and becomes associated with the surface of the condensed chromosomes. The relative absence of the TSG126 protein from G₁ interphase cells strongly argues against a model where the association of the TSG126 protein with mitotic chromosomes merely reflects a mechanism for the symmetrical distribution of nucleolar proteins between daughter cells. Instead, the intracellular distribution of the TSG126 protein during the cell cycle suggests that it could have a chromatin-associated function in both interphase and mitotic cells. Microinjection of anti-TSG126 antibodies into proliferating Swiss-3T3 fibroblasts was found to delay cell cycle progression, indicating that the TSG126 protein has an essential nuclear function.

Key words: Cell cycle, Mitosis, Cell proliferation marker, Chromosome scaffold, Nucleolar protein, Heterochromatin

INTRODUCTION

Eukaryotic cells are divided into separate functional domains. The different organelles found in the cytoplasm of cells and their roles in the cellular machinery have been thoroughly analyzed. However, it is becoming increasingly clear that similar functional compartmentalization also exists within the nucleus. DNA replication, RNA transcription and pre-mRNA processing have been shown to take place in defined nuclear domains (reviewed by Spector, 1993). This nuclear architecture undergoes a major reorganization as cells enter mitosis, when the degradation of the nuclear membrane acts as a starting signal for a dramatic redistribution of nuclear proteins. A large majority of these proteins distributes evenly in the cytoplasm, whereas a smaller group becomes associated with the condensing chromosomes of mitotic cells. Among the proteins that associate with the chromosomes, two different types have been recognized: those that only transiently become bound to the chromosomes and those that remain associated with the chromo-

somes throughout mitosis (reviewed by Earnshaw and Mackay, 1994; Rattner, 1992).

One subgroup of proteins, known to be associated with mitotic chromosomes throughout mitosis, is localized in the nucleolar regions during interphase (Rattner, 1992). This group of proteins includes perichromonucleolin (Luji et al., 1987), NO38 (Schmidt-Zachmann et al., 1987), three proteins having molecular masses of 53, 66 and 103 kDa (Gautier et al., 1992a,b), and the Ki-67 antigen (Gerdes et al., 1983; Verheijen et al., 1989a,b). The functions of these proteins in interphase cells are not clear; however, three possible roles for these proteins during mitosis have been suggested: they could be involved in the condensation and decondensation of chromosomes, in the protection and stabilization of chromosomes, or the localization of these proteins along the periphery of the chromosomes could be a mechanism for the symmetrical distribution of nucleolar proteins between daughter cells.

The Ki-67 antigen was originally identified by Gerdes and coworkers, using a mouse monoclonal antibody against a nuclear antigen from a Hodgkin's lymphoma-derived cell line

and shown to be expressed exclusively in proliferating cells (Gerdes et al., 1983, 1984). The usefulness of this antibody was immediately recognized and today it is a commonly used clinical marker to diagnose tumour development and for prognosis (Gerdes, 1990). The Ki-67 protein is predominantly localized in the nucleolar cortex and in the dense fibrillar components of the nucleolus during interphase, whereas during mitosis it becomes associated with the periphery of the condensed chromosomes (Isola et al., 1990; Verheijen et al., 1989a,b). It has been suggested that the Ki-67 protein is associated with the nuclear matrix (Verheijen et al., 1989a), with preribosomes (Isola et al., 1990) or with the DNA of interphase cells (Lopez et al., 1994; Sasaki et al., 1987) and with the chromosome scaffold of mitotic cells (Verheijen et al., 1989b). The human Ki-67 mRNA sequence encoding the Ki-67 antigen was recently isolated and characterized, and as the result of an alternative splicing process it was shown to encode two different proteins with calculated molecular masses of 359 kDa and 320 kDa (Gerdes et al., 1991; Schluter et al., 1993).

We have isolated and characterized a cDNA clone (TSG126) that most likely represents the murine homologue of the human gene encoding the Ki-67 antigen. An antiserum developed against the product of the TSG126 mRNA sequence identified a protein having a molecular mass of approximately 360 kDa, which is predominantly expressed in proliferating cells. An analysis of the temporal expression and the spatial localization of the TSG126 protein during interphase shows that this protein begins to accumulate in late G₁ cells and that during S phase it forms increasingly larger nuclear foci. These foci overlap with the nucleolar and heterochromatic regions in these cells. At the onset of mitosis the foci dissolve and the TSG126 protein instead becomes localized at the periphery of the mitotic chromosomes. Microinjection of anti-TSG126 antibodies into proliferating Swiss-3T3 cells was found to delay cell cycle progression, indicating that the TSG126 protein takes part in an essential cell cycle-regulated process.

MATERIALS AND METHODS

RNA and cDNA analysis

Total RNA and poly(A)⁺ RNA from different mouse (CBA) tissues were isolated as described (Sambrook et al., 1989). RNA filter hybridization was done as described previously (Starborg et al., 1992). cDNA was synthesized from poly(A)⁺ RNA isolated from testis of 6- to 12-day-old mice and cloned into the λ uni-ZAP vector from Stratagene (Höög, 1991). Positive λ ZAP plaques were purified and converted into plasmids according to the manufacturer's protocol (Stratagene). Plasmid DNA preparations and manipulations were carried out by standard methods (Sambrook et al., 1989).

Genomic DNA analysis

Genomic filter hybridization was performed using commercial filters (Clontech). The filter was hybridized according to standard procedures (Sambrook et al., 1989). The probe consisted of a PCR amplified fragment (Saiki et al., 1988), extending from position 7475 to position 9471 of the TSG126 cDNA sequence. After hybridization, the filters were washed twice with 0.3 M NaCl, 3 mM sodium citrate, pH 7.0, and 0.05% SDS, for 10 minutes at 50°C, and exposed to X-ray film at -70°C, with an intensifying screen, for three days.

Isolation and assembly of a composite TSG126 cDNA clone

A cDNA clone, TSG126.1, was isolated from an oligo(dT)-primed

cDNA library as part of a screen for cDNA clones predominantly expressed in juvenile mouse testis (Höög, 1991; Starborg et al., 1992). TSG126 contained a 2.0 kb long insert representing the 3' end of the TSG126 mRNA, followed by a poly(A)⁺ tail. A full-length cDNA clone was assembled from 4 different cDNA clones (TSG126.1, TSG12626, TSG12620, TSG12646), after three consecutive rescreenings of two different juvenile mouse testis cDNA libraries using standard methods (Sambrook et al., 1989).

DNA sequencing

cDNA clones TSG126.1, TSG12626, TSG12620 and TSG12646 were sequenced on both strands using subclones generated by an exonuclease III/S1 kit (Promega Biotec). Automated DNA sequencing was performed as described previously (Höög, 1991). Assembled sequence data were analyzed using software from the Genetic Computer Group (Devereux et al., 1984).

Preparation of antibodies

A 897 bp fragment (position 7513-8410) from the TSG126 cDNA insert ligated in-frame with the initiator-methionine of the bacterial expression vector pQE-9 (Quiagen), was transformed into M15pREP4 *Escherichia coli* cells. After induction by IPTG, the recombinant protein was purified in one step by Ni²⁺-chelate affinity chromatography, as described by the supplier (Quiagen). Polyclonal antibodies were prepared and affinity purified as described (Starborg et al., 1995).

Immunoblotting

Mouse Swiss-3T3 fibroblasts were washed with PBS and rapidly lysed with SDS-reducing buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 10 mM DTT and 4 M urea) as described by Schluter et al. (1993). Extracts were separated on 5% SDS-PAGE and proteins were transferred to a Immobilon-P membrane in transfer buffer (41 mM Tris-HCl, 192 mM glycine, 0.02% SDS, pH 8.3; Laemmli, 1970). The filters were incubated with anti-TSG126 antiserum (diluted 1:200), or a pre-immune serum affinity purified in a similar way (diluted 1:200), after which washing and detection were done as described previously (Starborg et al., 1995).

Cell culture, cell synchronization and immunofluorescence microscopy

Mouse Swiss-3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DME), from Gibco, containing 10% fetal calf serum (Sigma). Cells were plated at low density and grown at 37°C in a humidified atmosphere containing 5% CO₂, 95% air. To synchronize cells, 3T3 cells were cultured in DME medium, including 0.1% fetal calf serum (Sigma), for 48 hours (Björklund et al., 1992; Bravo and Macdonald-Bravo, 1987; Fox et al., 1991). An equal number of cells (1 × 10⁶) was then processed at 2 hour intervals following addition of DME medium, including 10% fetal calf serum, as described by Starborg et al. (1995). Cells were fixed in ice-cold methanol:acetone (50:50, v/v) for 5 minutes and pre-incubated with 3% BSA prior to addition of the first antibody. The affinity-purified murine anti-TSG126 antiserum was diluted 1:20 (v/v), the monoclonal anti-fibrillar antibody 1:500 (a gift from Dr Joan Steitz, Yale University) and the monoclonal anti-PCNA antibody 1:200 (Boehringer Mannheim). The secondary antibodies were a fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (diluted 1:50, Boehringer Mannheim), and a rhodamine-conjugated goat anti-mouse IgG (diluted 1:80, Boehringer Mannheim). The cells were stained with 1 μ g/ml of Hoechst 33258 for 1 minute. The slides were mounted in a 78% glycerol mounting medium, containing 1 mg/ml para-phenylene diamine, examined in a Zeiss Axioscope microscope and photographed with Kodak T-MAX 400 film.

Microinjection

Affinity-purified anti-TSG126 antibodies were concentrated using

Centricon 10 (Amicon) columns to a concentration of 10 mg/ml and filtered through a 0.2 μm filter. A commercial rabbit IgG antibody fraction (Dakopatts) was diluted to 10 mg/ml and filtered in the same way. The antibodies were microinjected into the nuclei of synchronized Swiss-3T3 mouse fibroblasts 12-14 hours after serum stimulation using an automated injection system (AIS, Zeiss) as described previously (Harlow and Lane, 1988). The cells were fixed using methanol:acetone (50:50, v/v) 36 hours (and 20 hours) after serum stimulation and a secondary FITC-conjugated anti-rabbit antibody was applied to label the injected cells. The cells were simultaneously stained with Hoechst 33258.

RESULTS

Isolation of a murine cDNA clone related to the human Ki-67 gene

A cDNA clone (TSG126.1) was isolated from a murine testis cDNA library as part of a large-scale cDNA sequencing project (Höög, 1991; Starborg et al., 1992). When the 2.0 kb insert from this clone was labelled and used to hybridize poly(A)⁺ RNA isolated from different tissues, an approximately 10 kb large band was detected in prepubertal testis, whereas no band was seen in adult testis, heart, kidney or brain (Fig. 1A). In contrast to the adult tissues investigated, a large fraction of the cells found in prepubertal testis are undergoing continuous proliferation, suggesting that the TSG126.1 cDNA clone labels an RNA transcript that is predominantly expressed in the proliferating cells of prepubertal testis. In situ hybridization of sectioned adult mouse testis, using an anti-sense TSG126.1 oligonucleotide, revealed a strong signal in clusters of small cells located just inside the basal lamina of the seminiferous tubules, supporting the idea that the *TSG126* gene is predominantly expressed in proliferating spermatogonial cells (not shown). To determine whether additional TSG126-related sequences exist in the murine genome, the TSG126.1 insert was used to hybridize murine genomic DNA digested with *EcoRI* and hybridized at reduced stringency conditions (Fig. 1B). Only one band was seen in this blot, suggesting that no additional genes closely related to *TSG126* exist in the murine genome.

Comparison of the sequence of the TSG126.1 cDNA insert with release 37 of the EMBL Data Library revealed that the murine TSG126.1 sequence is related to the human Ki-67 cDNA sequence encoding a cell proliferation-associated antigen (Schluter et al., 1993). No additional sequence similarities were found. Taken together, the above results suggest that the TSG126 cDNA clone represents the murine homologue of the human *Ki-67* gene. To verify this, we set out to characterize the complete TSG126 transcript and its encoded protein.

Characterization of the murine *Ki-67* related gene

A composite cDNA clone (TSG126) with a total length of 9471 bp was assembled from overlapping cDNA clones isolated from two different mouse testicular cDNA libraries (see Materials and Methods). The TSG126 cDNA sequence contains an open reading frame of 2938 amino acids, which is preceded by several in-frame stop codons and followed by a 311 base long 3'-untranslated region. Within the 3'-untranslated region a consensus poly(A)⁺ addition signal (AATAAA)

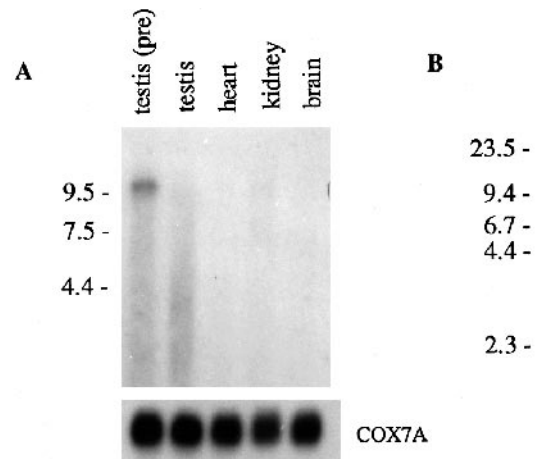


Fig. 1. RNA and genomic blot analysis of the *TSG126* gene. (A) One microgram of poly(A)⁺ RNA, purified from the indicated tissues, was resolved by formaldehyde denaturing gel electrophoresis and transferred onto a nitrocellulose filter. The insert from the TSG126.1 cDNA clone was labelled with [³²P]dATP and used as a probe. The X-ray film was exposed for five days. After autoradiography, the filter was stripped and rehybridized with a cytochrome oxidase VIIa probe as a control (Starborg et al., 1992). (B) A filter containing Balb/c mouse kidney genomic DNA digested with *EcoRI*, was hybridized with a 2.0 kb DNA fragment from the TSG126.1 cDNA sequence. After hybridization, the filter was washed as described in Materials and Methods, and exposed to X-ray film for three days. Size markers in kilobases are indicated to the left.

is found 18 nucleotides upstream of the first A in the poly(A) tail of this cDNA sequence (accession number X82786 in the EMBL Data Library). The amino acid sequence deduced from this nucleotide sequence is presented in Fig. 2. The open reading frame encodes a putative protein with a calculated relative molecular mass of 324,294 Da and an isoelectric point (pI) of 10.53. A comparison of this amino acid sequence with the amino acid sequences stored in the PROSITE protein motif data base revealed a large number of consensus sites for protein kinase C (125) and casein kinase II (54), as well as two putative nuclear localization signals (KRRR and KRQR) at positions 463-466 and 628-631, respectively. The most unique feature found within this open reading frame, however, was the occurrence of 13 tandem repeats located in the middle of the putative TSG126 protein, each repeat unit being approximately 121 amino acids in length. This repetitive sequence domain makes up a total of 1536 amino acids, i.e. more than half the length of the protein.

The TSG126 amino acid sequence was compared with the amino acid sequence of the human Ki-67 protein (Schluter et al., 1993), the result of which is displayed as a dot plot in Fig. 3A. It is apparent from this plot that the amino acid sequences of TSG126 and Ki-67 are similar throughout their entire length. The sequence comparison can, however, be divided into three parts: an N-terminal non-repetitive domain, a central repetitive region and a C-terminal non-repetitive domain, each domain being 57%, 40% and 42% identical, respectively, between these two proteins. As indicated by the non-contiguous line seen in the dot plot for the N-terminal domain, this region contains several shorter regions displaying different levels of sequence conservation. Three regions within the N-terminal

K R S G D D D G A E F P L E L S E C L F G R E I B C 35
D I R I Q L P V V S Q R K C P I V V Q H Q E A I L 60
Y N F E I T H P T Q V N G C V T I D E P V L R B G 85
D I T T I I D R S F A Y E D G W H E D G S K P T E 110
F R C K S L G K E P S R R A S R D I F C A D D D G 135
H G Q D T X A S K M T A S R R S E V Y A X G L S A 160
D S P A S D G S E K N S V S Q D S S O V V E H Q H T 185
R N Y I V E P T S G G S L L R S P G L Q G A V I G W 210
R S L L P T Q S L E H S W E K K S P F E K L Y Q S 235
M K E E L D V X S G K S C R X I H P Q P D R A A E 260
E S R R T O L L V S G R A R A K A S E G S T P V T A 285
A S S E K V G K I M T E R N R G G H V P V Q T Y T 310
E T A K K R T P V R X S O Q L K D E D S R V T O R 335
R B S V N L D E G C I A Q A V H K T V T P G K L A 360
I R N Q I P V E A G D V G S P A D I E H E S I P 385
O R S I P A K V E A P I S A N T Q N R L S L T O R L 410
V P G E R K K T P K C I F S K P E K L A T A A K D T 435
C G G L P G L S E V D I S N F G D S I N K S S G M 460
P M K R R R V S P G G M L R P L E L F D E N L P R M 485
T P L K R G E T P T K R X L L G T H S P A V L K T 510
I I K E R P Q S V G K Q E S P G I T P P R T W D D 535
R R R S G R T S S O S N F L C H T D I P K K A G A 560
K S G N L P A K R A S I S A S G H G I L Q M I C S 585
K R R S G A S E A N L I V A K S W A D V V K L G V 610
K Q T Q T R V A K H V P P K Q T S K R O R R P S T 635
P M X P T S N L H N Q P T T G H A N S F C T I V V 660
C R A G I E E K V I V P A R P E F K K L M L K L W R 685
K V D F S E H L S G L T E M F K T P V K E K Q Q O 710
M S D T G S V L S N S A N L S E R Q L O V T N S G 735
D I Z P E P I T T E I L G E K V L S E T R N A A K Q 760
Q S D R Y S A S E T L A R R S I K H E N T V Q T F 785
K N V H R I T D L E K R T P V S E T E P L R T A S 810
S V S K L R R S R E L A H I T L V K T M H E R T E A 835
V L A R N I T A R S L A G T E R S E G V D Q Q V Q 860
D N E N A P Q R C K E S G E L S E S E K T S A R 885
R S S A R K Q E P T E D L L G S Q W V T Q I A D Y 910
A E E L L S Q G G G I O M L E E N H N Q N T S 935
I S E D Q G I T E K E V N I I V Y A T K R H S P 960
K T P P G K A Q P L E G P A G L K S H P E T P N P 985
K D E P I F E D R T R V L C K S P G V T T E M I T 1010
T M T K P Q T S T S G K K V D N K E S A L T X 1035
R I E H P G E S R H N P K I L K L R C E D I K A L 1060
K Q S E M E M L T A T V N G S E R T L G K S E K K 1085
A G P L E D L T C F G S L F I S E V P T N I I K K 1110
I P S K S P H T Q E V R T P A S T K R L S K T G L 1135
S K V D Y R Q E P S T L G R R T M S P G R A P G T 1160
P A R V Q E E N D C T A X M E T P K Q K L E S I E 1185
M L T G L R K O R A T P K D I T G E Q D S F Q I P 1210
D R A N G E L V V V T R K K H F S P Q P E S A 1235
I T R K S R E R Q S R A S I S K I D V K H E L L E 1260
S E E B L Q L G E G V D T F Q V S T W K V I A S S 1285
R K P A K R K L D S T A G M P N S X R N R C S S E 1310
D N T P C L E D L H G F Q E L F O M P F G Y A N D S 1335
L T T G I S T N L A R S P O L G P V R T Q I N X R 1360
S L P K I I L R X M D V T E E I S G L W K Q S L G 1385
R V H T T Q E Q R D N A I K A I N H I P K E T L Q 1410
T A A D G T R L T R Q P Q T P K E R V D P L F D E 1435
S V F Q E L F O T S R Y C S D P L I G M K Q T A H 1460
S L R E P Q R G P V R T P R T S K R L A K T E V G 1485

M I A V R E K I S P V S L P Q C A T G E V V X I P 1510
I G P E D D T E H K Q V K R I T P Q T L D S S A S 1535
R T V S K R Q Q G A R E R H P Q P S G D L P F P Q 1560
R L F Q T P A S G K D P V T V D E T T X I A L Q S 1585
P Q P S E I I M P A H M K R G E H M I L R K D M R 1610
E F S I L E K Q T Q S R Q R D A G T P A F H Q E E 1635
W G T T A I M E T P R Q K L D F I E H I T G H R R 1660
R P R T P K M R A Q P L E D L D G F G Q H L P Q T F 1685
A G A E D P V S V H E S A X I S L A S I G A H F V 1710
R T P A S T K R R S K T G L E K V D V R Q H R S U 1735
L G K R M E S L G R A P Q T P A F V Q H E N D S T 1760
A F M H T P K Q E L L D E T G H S S G H K R R P Q T 1785
P K I R A Q P L H D L D G F G Q E L F Q I P A G A N 1810
D S V T V S E S V K M S L E S S Q A S E V K T P A 1835
E T E R L E K T G L S E V D V N E D P S I L E K K 1860
T R S P C T P A P V Q S E W D C T A F M H T P K Q 1885
X L D P T G N S S G E X R A R P R T P X I R A Q P L 1910
E D L D G F Q E L P Q T P A G A S D E V T V E S E 1935
A K N S L E S S Q A E P V R T P A S T X R L S E F 1960
G L S E V D V R E D P S T L G K K T E K I P G R A P 1985
G T P A P V Q E R W D E T A F M H T P K Q E L D F 2010
A H H S S D S E K R A S R T E K X S O P L E D L D 2035
G F Q E L P D T P A G A E N V S E E S A K I E 2060
L E E I G A N P V T R A S T X R L S E K T G L M X 2085
H D V R E G H S P L S K S E C A S C R V M Q T L T 2110
L G E D H G R E T K D O R V L L A K L E P A I Y 2135
V A R G E R Q G R E C K K R S G S P H D L S G V O 2160
E V F O T A G H K E D S V I T V D H L A K L E S S S 2185
E P L E P S T D T S V T E R R Q A R T G L R A K V H V 2210
K H E L S G G I N H P Q I S G E I V D L P R H E F 2235
C H G X V I K T A K Q S V K R K L D T E V N V P R 2260
S K R O R I T R A E K T L E D L E G F Q E L C Q A 2285
P E L V M D S V I V H E T P K M P D X S P E P V D 2310
T S E T G A R R L R L L V V T H E P I F Q R K 2335
T I R V T Q T A N T Q H E P I R D M O G H R E F 2360
K H S E V Q E Q D E P V S L Y G R R M Q P R T V K 2385
E K T Q P L E E L I F Q E E T A K R I S S K E P 2410
Q P E K E T L A G L K R Q L R I O L I N D G V K 2435
S E P T A Q R K Q P S R E T R M T L K E P V G D S 2460
I M V E E V K S T K Q K I D P V A S P V V S K R 2485
P R R V P K H X A Q A L E L L A G L G P I Q T L C 2510
E T D E R A S D K G P T O M P C H S L Q P E Q V D 2535
E F O S E P R P R T R R G R K V E A D E H E P S A V 2560
R X T V T S A Q T M R S R K V P E I G N H T Q 2585
V S K A S I K Q T L D T V A K V T G E R R Q L R T 2610
H X G N S T L L K L L G D S K E I T Q I S D H S 2635
K L L A H D T S I L N E T Q Q Q E P D S V K P L R 2660
T C R R V L R A S X H V P K E V L V D R D S A T 2685
L O E K S M P L L E P K K S A R D G S I V R Y R 2710
A L R E L A P K Q W A T D E E K P V P E K K R A A S 2735
E K R Y V S F E P V K N H L I V S H K L E S V 2760
H Q V S T V M X I E E N E A K R E N H P V T P D Q 2785
H S R Y R K K T H V R Q P R P K F D A S A E N V G 2810
I X X M R T N K I A S G H T E L O M P D D G A K 2835
K T S E R G Q V S G K R T C L R S R G T T E N P Q 2860
P C B A E E X E X P A A B I L I X P Q E E X G V 2885
S G S E D V R C L R Y R K K T R V A L D S H P K P R 2910
V E R G T X X D A R T L X E D E D I V C I K E L R 2935
T R S * 2960

Fig. 2. Sequence of the composite TSG126 cDNA clone. The amino acid sequence of the composite mouse TSG126 cDNA clone is shown. A termination codon at the end of the sequence is indicated by an asterisk (*). The single letter amino acid code is used. The internal repeat units are indicated by vertical lines. The nucleotide sequence data are available from the EMBL Data Library under accession number X82786.

domain, including amino acids 1-100, 460-565 and 630-765, are more than 75% identical between the two proteins, whereas the intervening amino acid sequences only are approximately 40% identical, strongly suggesting that the three highly conserved regions represent important functional domains.

A comparison of each repeat unit within the central region of the TSG126 sequence against a consensus sequence prepared from all thirteen repeat units revealed a large sequence variation between the individual units, e.g. whereas some of the repeat units are 65% identical to the consensus

consensus sequence) were found to be conserved between the murine and the human repeat units. Similar clusters of threonine-proline-X-X amino acid sequence motifs have been observed in many gene regulatory proteins and suggested to contribute to DNA binding (Suzuki, 1989).

The *TSG126* gene encodes a 360 kDa nuclear protein

An antiserum was prepared against a bacterially expressed domain of the TSG126 protein and used to analyze protein extracts prepared from proliferating Swiss-3T3 cells. A large protein with a molecular mass of approximately 360 kDa could be identified (Fig. 4). The observed molecular mass of this protein agrees well with the size of the TSG126 protein predicted from the open reading frame of the TSG126 cDNA clone.

The same antiserum was next used to investigate the expression and subcellular localization of the TSG126 protein in proliferating Swiss-3T3 cells using indirect immunofluorescence microscopy. To visualize all cells, Hoechst 33258, a DNA binding dye that preferentially stains the intranuclear heterochromatic regions of murine cells was used in parallel (see e.g. Nakayasu and Berezney, 1989). A strong nuclear labelling was seen in 60-70% of the cells with the TSG126 anti-serum, whereas the remaining cells only displayed a very weak and diffuse nuclear signal (Fig. 5A and C). No immunofluorescence signal was detected using the preimmune serum (not

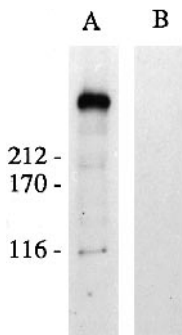


Fig. 4. Identification of a 360 kDa TSG126 protein in proliferating Swiss-3T3 cells. Protein extracts were prepared from asynchronously growing Swiss-3T3 cells (A and B) and the proteins were separated using SDS-polyacrylamide gel electrophoresis. After immunoblotting, the filters were analyzed using affinity-purified anti-TSG126 antiserum (A), or a pre-immune serum (B). Molecular sizes are given in kilodaltons.

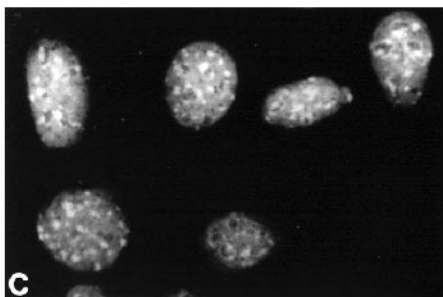
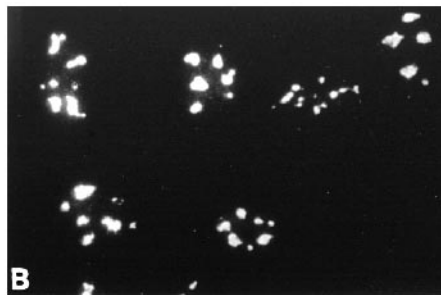
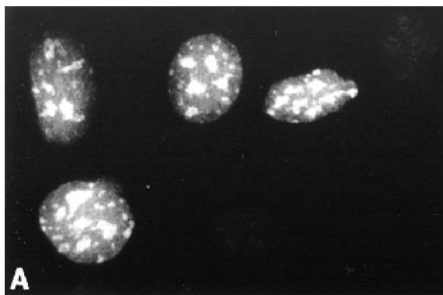


Fig. 5. The TSG126 gene encodes a nucleolar protein that is differentially expressed in asynchronously growing Swiss-3T3 cells. Asynchronously growing Swiss-3T3 cells were stained in parallel with affinity-purified anti-TSG126 antiserum (A), the anti-fibrillarin antibody (B) and Hoechst 33258 (C). The secondary antibodies used were: a FITC-conjugated swine anti-rabbit IgG and a rhodamine-conjugated goat-anti-mouse IgG, after which the cells were analyzed using indirect immunofluorescence microscopy.

shown). In the strongly labelled cells, both smaller and larger foci were seen, foci that appeared to overlap with both the nucleolar and the heterochromatic regions found in these cells (compare Fig. 5A and C). To confirm this, an antibody directed against fibrillarin, a constitutively expressed nucleolar protein (Ochs et al., 1985), was used to label the same cells (Fig. 5B). A comparison between the anti-TSG126, the anti-fibrillarin and the Hoechst labelling patterns reveal that the anti-TSG126 antibody identifies two different groups of foci, one group that overlaps with nucleolar regions stained by the anti-fibrillarin antibody and a second group that colocalizes with the heterochromatic regions as defined by Hoechst staining. In contrast to the TSG126 protein, the fibrillarin protein was found to be expressed in all the interphase cells investigated.

The expression of the TSG126 protein is both temporally and spatially regulated during interphase of the mammalian cell cycle

To investigate if the expression level and the intranuclear location of the TSG126 protein could be linked to particular stages of the cell cycle, the expression of the protein in synchronized cells was analyzed. Swiss-3T3 cells were forced to enter G₀ by serum starvation and were then given medium containing 10% serum to permit the cells to re-enter the cell cycle. The majority of the cells synchronized in this manner reached early S phase 12-14 hours after addition of serum, whereas they entered the G₂ phase 20-22 hours after serum addition (see Materials and Methods). The synchronized cells were analyzed in parallel using the affinity-purified anti-TSG126 antiserum, the anti-fibrillarin antibody, a monoclonal antibody directed against PCNA (the proliferating cell nuclear antigen) and by staining with Hoechst 33258 (Figs 6 and 7).

A nuclear signal was seen with the anti-TSG126 antiserum irrespective of the time point after serum addition at which the cells were analyzed (Fig. 6). The intensity and the intranuclear distribution of the antigen, however, varied dramatically. A very weak and evenly distributed nuclear TSG126 signal was seen in serum-starved cells prior to addition of serum (Fig. 6A), as well as in cells 4 hours after the addition of serum (not

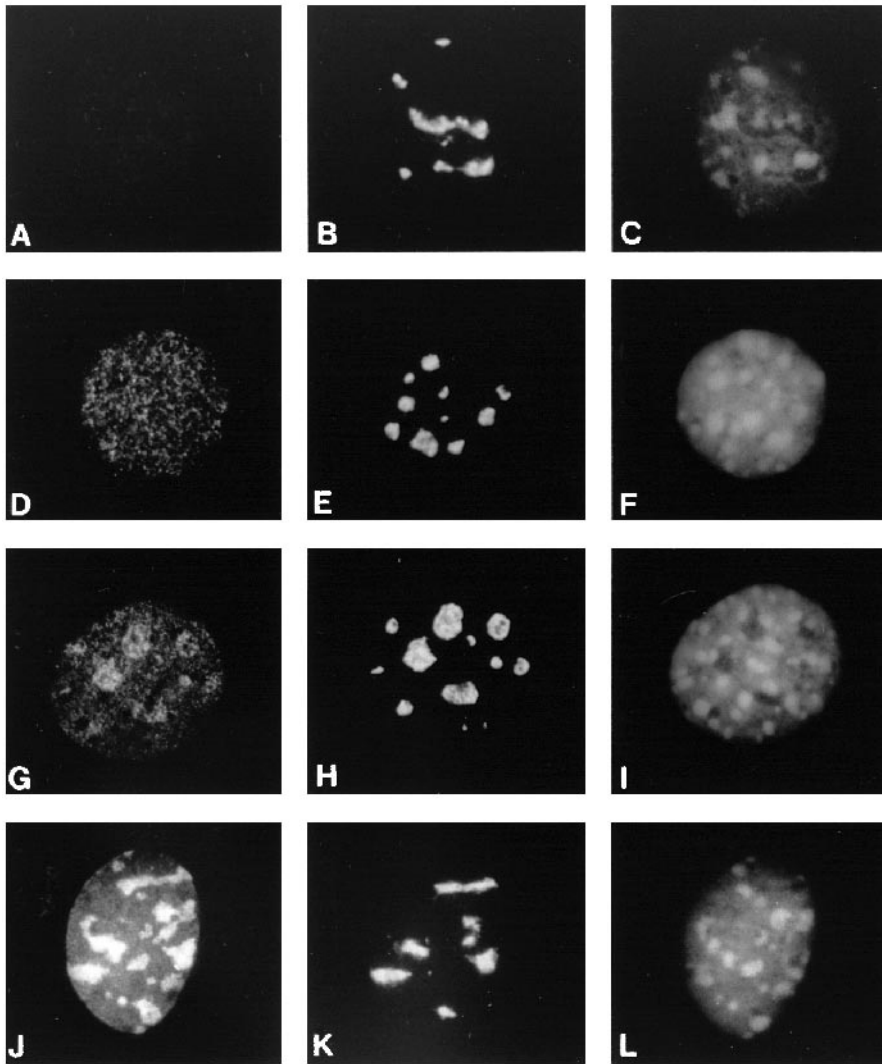


Fig. 6. Comparison of the cell cycle expression and intranuclear localization of the TSG126 protein with a nucleolar protein, fibrillarin, in synchronized mouse Swiss-3T3 cells. Cells were synchronized as described in Materials and Methods, and analyzed at different time points after addition of 10% serum to the cells: 0 hours (A-C), 10 hours (D-F), 12 hours (G-I), 18 hours (J-L). Cells at different time points were stained in parallel with affinity-purified anti-TSG126 antibodies (A,D,G,J), a monoclonal anti-fibrillarin antibody (B,E,H,K), and Hoechst 33258 (C,F,I,L). The secondary antibodies used were a FITC-conjugated swine anti-rabbit IgG and a rhodamine-conjugated goat anti-mouse IgG, after which the cells were analyzed using indirect immunofluorescence microscopy.

shown). Ten hours after serum addition several hundred small and evenly distributed, brightly stained granules became visible (Fig. 6D). After two additional hours large foci labelled by the anti-TSG126 antibody appeared (Fig. 6G). In cells 18 hours after serum addition, the labelled nuclear foci became more distinct (Fig. 6J), whereas the pattern did not change further in cells 22 hours after serum addition (not shown).

The same cells were also analyzed using the anti-fibrillarin antibody (Fig. 6B, E, H, K). In contrast to what was seen with the anti-TSG126 antibody, the anti-fibrillarin antibody gave rise to distinct foci in cells irrespective of the time point after serum addition. A comparison of the intranuclear localization of the foci labelled by the anti-TSG126 antibodies and the anti-fibrillarin antibodies reveals that only a subset of the TSG126-positive foci colocalizes with the nucleolar regions labelled by the anti-fibrillarin antibody (Fig. 6J and K). As was seen in the asynchronously growing Swiss-3T3 cells (Fig. 5), the remaining TSG126-positive foci instead overlap with the heterochromatic regions labelled by Hoechst 33258 (Fig. 6J and L).

To be able to correlate the intranuclear patterns seen with the anti-TSG126 antiserum in synchronized cells to a well-characterized event taking place during the mammalian cell

cycle, we used an antibody directed against PCNA (proliferating cell nuclear antigen, a factor associated with DNA polymerase δ). Euchromatic and heterochromatic DNA are being replicated at different stages during S phase, giving rise to different intranuclear patterns, a process that can be monitored using an anti-PCNA antibody (Bravo and Macdonald-Bravo, 1987; Celis and Celis, 1985; Fox et al., 1991; Humbert et al., 1992; Nakayasu and Berezney, 1989). Replication granules, displayed by the anti-PCNA antibody, were first seen in synchronized cells 12 hours after serum addition (Fig. 7E), indicating that these cells had reached early S phase and that euchromatic DNA in these cells is being replicated at this time point (Bravo and Macdonald-Bravo, 1987; Celis and Celis, 1985; Fox et al., 1991; Humbert et al., 1992; Nakayasu and Berezney, 1989). Six hours later, the anti-PCNA antibody gave rise to large intranuclear foci (Fig. 7H), indicating that the heterochromatic regions are undergoing DNA replication (Bravo and Macdonald-Bravo, 1987; Celis and Celis, 1985; Fox et al., 1991; Humbert et al., 1992; Nakayasu and Berezney, 1989). No PCNA-positive granules or foci are seen in cells 10 hours after serum addition (Fig. 6B), or in cells 0 or 22 hours after serum addition (not shown), suggesting that these cells represent G₁, G₀ and G₂ cells, respectively. By comparing the

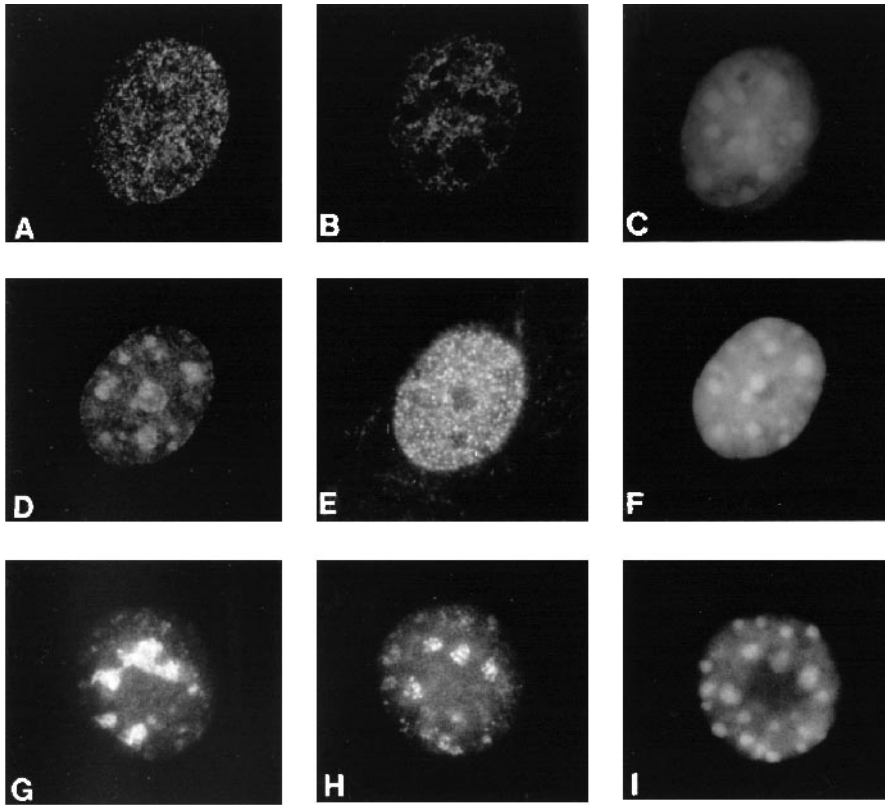


Fig. 7. Comparison of the cell cycle expression and the intranuclear localization of the TSG126 protein with a replication protein, PCNA, in synchronized mouse Swiss-3T3 cells. Cells were synchronized as described in Materials and Methods, and analyzed at different time points after addition of 10% serum to the cells: 10 hours (D-F), 12 hours (G-I), 18 hours (J-L). Cells at different time points were stained in parallel with affinity-purified anti-TSG126 antibodies (A,D,G), a monoclonal anti-PCNA antibody (B,E,H), and Hoechst 33258 (C,F,I). The secondary antibodies used were a FITC-conjugated swine anti-rabbit IgG and a rhodamine-conjugated goat anti-mouse IgG, after which the cells were analyzed using indirect immunofluorescence microscopy.

accumulation pattern of the TSG126 protein with the accumulation of the PCNA protein during interphase of the cell cycle, it is clear that the TSG126 protein begins to accumulate prior to PCNA, i.e. at the end of G₁ (Fig. 7A,B). Furthermore, the TSG126-positive foci begin to form during early S phase (Fig. 7D,E), and both the TSG126 protein and the PCNA protein accumulate in the heterochromatic regions during late S phase (Fig. 7G,H,I).

This experiment therefore shows that the expression of the TSG126 protein during interphase deviates in two distinct ways from the expression seen normally for nucleolar proteins, i.e. the level of the TSG126 protein varies during the cell cycle and it accumulates in regions outside the nucleoli, i.e. in the heterochromatic regions. We find that the TSG126 protein begins to accumulate at the end of the G₁ phase (10 hours after serum addition), when a large number of small and evenly distributed nuclear granules first appear. During S phase the TSG126 protein assembles into two types of foci, one that colocalizes with the heterochromatic regions and a second that colocalizes with the nucleolar regions.

The TSG126 protein associates with the condensed chromosomes in mitotic cells

In order to analyze the subcellular distribution of the TSG126 protein at different stages of mitosis an indirect immunofluorescence experiment was performed. The TSG126 protein was found to become associated with the condensing chromosomes of prophase cells (Fig. 8A,E). This association is most evident in prometaphase and metaphase cells, where the TSG126 protein is localized at the periphery of the condensed chromosomes (Fig. 8B,F and C,G). TSG126 remains bound to the surface of the chromosomes also during anaphase of the

mitotic cell cycle; however, the signal intensity displayed by the anti-TSG126 serum is reduced (Fig. 8D,H).

Microinjection of anti-TSG126 antibodies into synchronized 3T3 cells interferes with cell cycle progression

To examine the function of the TSG126 protein during cell proliferation, affinity-purified anti-TSG126 antibodies were injected into the nuclei of Swiss-3T3 cells. The cells had first been synchronized by serum-starvation after which the cell cycle had been reinitiated by the addition of serum. Anti-TSG126 antibodies or control antibodies were injected 12-14 hours after serum stimulation and the cells were fixed 36 hours after serum stimulation. Cells were then labelled by using a FITC-conjugated anti-rabbit IgG serum and with Hoechst 33258, and the number of FITC-positive cells was counted. The majority of the synchronized cells will divide 28-30 hours after serum stimulation; therefore, the number of injected cells should have approximately doubled when assayed 36 hours after serum stimulation. We found that 80% of the cells injected with control antibodies had divided, whereas only 64% of the cells injected with the anti-TSG126 antibodies had undergone cell division (Table 1). This result can be interpreted in two ways: that the anti-TSG126 antibody delays the cell cycle or that a greater number of cells die after injection with this antibody. To differentiate between these two possibilities, cells were injected as before, but were instead fixed 20 hours after serum stimulation, i.e. prior to cell division. We injected 397 cells with the anti-TSG126 antibody and 357 cells with the control antibody, and after fixation 6-8 hours later 409 cells injected with the anti-TSG126 antibody and 361 cells injected with the control antibody were detected. This result

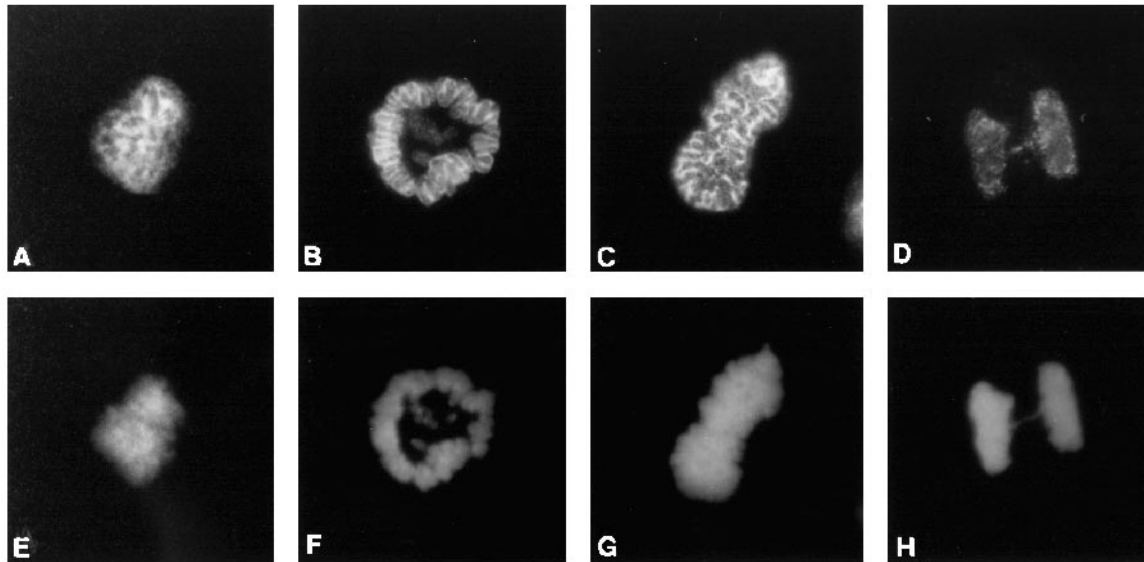


Fig. 8. The TSG126 protein becomes associated with the periphery of the condensed chromosomes during mitosis. Cells at different stages of mitosis: (A,E) prophase, (B,F) prometaphase, (C,G) metaphase (D,H) anaphase were analyzed using the anti-TSG126 antibodies (A,B,C,D) and in parallel stained with Hoechst 33258 (E,F,G,H). The secondary antibody used was a FITC-conjugated swine anti-rabbit IgG, after which the cells were analyzed using indirect immunofluorescence microscopy.

Table 1. Effects of microinjected antibodies against TSG126 on cell proliferation in synchronized Swiss 3T3 cells

Experiment	No. of injected cells		No. of positive cells after 36 hours		No. of mitotic cells		Total no. of cells expected after 36 hours		Percentage divided cells*	
	TSG126	Control	TSG126	Control	TSG126	Control	TSG126	Control	TSG126	Control
1	100	100	120	161	0	0				
2	100	100	125	144	6	1				
3	100	100	149	128	11	1				
4	100	100	128	191	9	3				
5	101	100	123	174	7	0				
6	102	100	120	164	6	0				
7	80	100	103	157	7	0				
Total	683	700	868	1,119	46 (5.3%)†	5 (0.4%)†	1,366	1,400	64%	80%

Synchronized Swiss-3T3 cells were injected with anti-TSG126 or control Ig and evaluated as described.

*The percentage of injected cells that have divided was calculated as the total number of positively stained cells after 36 hours divided by the expected number of cells after 36 hours for both anti-TSG126 and the control.

†The percentage of mitotic cells was calculated as the number of mitotic cells divided by the total number of positively stained cells after 36 hours.

strongly suggests that neither of the two antibodies has a toxic effect on the cells.

We also found that of the cells injected with the anti-TSG126 antibody, a much larger fraction of the analyzed cells were found at different stages of mitosis 36 hours after injection, suggesting an effect on the mitotic process (Table 1). The intranuclear distribution of the anti-TSG126 antibody seen in interphase and mitotic cells after injection was identical with the pattern seen using the anti-TSG126 antibody in immunofluorescence experiments (Figs 5 and 8), strongly suggesting that the microinjected antibodies bind to the TSG126 protein *in vivo*. Together, these results indicate that the anti-TSG126 antibodies interfere with the function of the TSG126 protein and that this protein is essential for cell cycle progression.

DISCUSSION

We have isolated and characterized a gene (*TSG126*) that is pre-

dominantly expressed in proliferating cells and for which no closely related gene exists in the murine genome. Sequence comparisons reveal that this mouse gene is strongly related to the human *Ki-67* gene, encoding a proliferation-associated antigen (Schluter et al., 1993). A comparison of the nonrepetitive N- and C-terminal regions of the TSG126 and the Ki-67 proteins reveals that the N-terminal regions are more conserved than the C-terminal regions (Fig. 2). This is mainly due to the occurrence of three 100-150 amino acid long sequence blocks within the N-terminal regions of the two proteins, each being approximately 75% identical. Furthermore, a comparison of the 13 tandem repeat units found within the TSG126 protein with the 16 repeat units located in the Ki-67 protein, revealed amino acid sequences strongly conserved between the two proteins (Fig. 3), e.g. the Ki-67 motif, the 'ETPKQKLD' motif and four pairs of threonine-proline amino acid residues. The strong conservation of repetitive and non-repetitive amino acid sequences in these two proteins implies that these regions are of particular importance.

We have analyzed the expression and the subcellular distribution of the TSG126 protein in proliferating and quiescent Swiss-3T3 cells and compared them with the known expression of the human Ki-67 antigen. The Ki-67 antigen was originally identified as a nuclear antigen present in proliferating cells but not in quiescent cells, and on the basis of this criterion it was defined as a proliferation marker (Gerdes et al., 1983, 1984). It is predominantly localized in the nucleolar cortex and in the dense fibrillar compartment of the nucleoli in interphase cells (Isola et al., 1990; Verheijen et al., 1989a). We found that in 30-40% of the asynchronously growing Swiss-3T3 only a very weak anti-TSG126 signal is seen. A similar weak signal is also seen in Swiss-3T3 cells after 48 hours of serum starvation (Fig. 6A), as well as in serum-starved cells four hours after the addition of serum (not shown). This implies that the weakly labelled, asynchronously growing cells represent early G₁ cells, an assumption that is further supported by the fact that none of the synchronized cells that are weakly labelled by the TSG126 antiserum simultaneously displays a strong PCNA signal.

During the S and the G₂ phases of the cell cycle, the TSG126 protein accumulates in large intranuclear foci, some of which overlap with the nucleolar regions (Figs 6 and 7). We have compared the cell cycle expression and the intranuclear distribution of the TSG126 protein with the expression of a known nucleolar protein, fibrillarin. Fibrillarin is associated with small nucleolar RNAs (Tyc and Steitz, 1989) and localized in the nucleolus of interphase cells (Ochs et al., 1985). We found that, in contrast to fibrillarin, as well as most other characterized nucleolar proteins (reviewed by Hernandez-Verdun, 1991), the accumulation of the TSG126 protein in the nucleoli is regulated during the interphase of the cell cycle.

Another interesting aspect of the expression of the TSG126 protein is the dramatic redistribution of this protein that occurs at the onset of mitosis and the association of this protein with the periphery of mitotic chromosomes (Fig. 8). The human Ki-67 antigen has been shown to associate with the surface of mitotic chromosomes, forming a chromosome scaffold (Verheijen et al., 1989b). Several other nucleolar proteins have previously been shown to become associated with the chromosomes during mitosis, including perichromonucleolin (Luji et al., 1987), NO38 (Schmidt-Zachmann et al., 1987) and three proteins of 52, 66 and 103 kDa (Gautier et al., 1992a,b). Two main models have been put forward to explain these observations. In one model the proteins have dual functions, acting both as nucleolar and mitotic proteins, whereas in the second model the association of these proteins with the periphery of the mitotic chromosomes merely acts as a mechanism for the symmetrical distribution of nucleolar proteins between daughter cells. In contrast to the above discussed nucleolar proteins, the low level of the TSG126 protein in G₁ cells strongly favours the first model described above, i.e. that the TSG126 protein has a dual function in interphase and mitotic cells.

An important observation made from the analysis of the localization of the TSG126 protein in interphase nuclei is its accumulation in heterochromatic regions (Figs 5, 6, 7). This observation has not been previously reported for the human Ki-67 protein, most likely because the heterochromatic regions in murine cells are more strongly stained by Hoechst 33258 than the corresponding regions in the human cells (see e.g.

Nakayasu and Berezney, 1989). Interestingly, however, a nucleoplasmic reactivity represented by a variable number of small discrete structures, not overlapping with the nucleoli, has also been observed in human cells using the anti-Ki67 antibody (Isola et al., 1990; Verheijen et al., 1989a), suggesting also that the human Ki-67 antigen could be accumulating in the heterochromatic regions. Biochemical experiments have also suggested that a fraction of the human Ki-67 protein is associated with chromatin (Lopez et al., 1994; Sasaki et al., 1987). We therefore postulate that the TSG126 protein and the Ki-67 protein associate with both interphase and mitotic chromosomes, having a chromatin-associated function. The nature of this chromatin association is not clear as it could involve either a direct DNA contact or an association with other protein components of chromatin. The conserved threonine-proline-X-X motifs found within the repeat units of the TSG126 and the Ki-67 proteins could be involved in binding to chromatin/DNA, as has been suggested for other proteins containing this motif (Suzuki, 1989).

In order to begin an analysis of the cellular function of the TSG126 protein, anti-TSG126 antibodies were microinjected into the nuclei of proliferating Swiss-3T3 cells 12-14 hours after serum stimulation and the cells were then analyzed 36 hours after serum stimulation. We found that whereas 80% of the cells injected with the control antibodies had divided, only 64% of the cells injected with the anti-TSG126 antibodies had divided. Furthermore, 46 cells injected with the anti-TSG126 antibodies were in the mitotic stage of the cell cycle, whereas only four cells injected with the control antibodies were found to display a mitotic staining pattern with Hoechst 33258. The distribution of the TSG126 antibodies in interphase and mitotic cells 36 hours after injection was indistinguishable from the pattern seen in Figs 5 and 8, strongly suggesting that the microinjected anti-TSG126 antibodies interact with the TSG126 protein *in vivo*. These results suggest that the anti-TSG126 antibodies interfere with the progression of the cell cycle and that the TSG126 protein is essential for cell proliferation. Transformation of human IM-9 cells with an anti-sense oligonucleotide complementary to the translational start site of the KI-67 transcript has previously been shown to inhibit cell proliferation (Schluter et al., 1993). The pleiotropic expression of this protein in interphase and mitotic cells suggests that it carries out multiple nuclear functions. The isolation of its murine homologue now sets the stage for further genetic analysis.

We thank Dr Bertil Daneholt for valuable suggestions, Dr Hans Mehlén for computer support and Dr Joan Steitz for generously supplying us with the anti-fibrillarin antibody. We are grateful to Dr Henrik Garoff and the members of his laboratory for instructing us how to carry out the automated microinjection method. This work was supported by the Swedish Natural Science Research Council, Kjell och Märta Beijers stiftelse, Axel and Margareta Ax:son Johnsons Stiftelse, The Swedish Cancer Society, the Swedish Medical Research Council, Alex and Eva Wallströms Stiftelse and the Karolinska Institutet.

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