Abnormal A-type lamin organization in a human lung carcinoma cell line

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Received January 16, 1995
Accepted April 6, 1995

Lung cancer — nuclear lamina — A-type lamin — intermediate filaments

We have studied the expression of lamins A and C (A-type lamins) in a lung carcinoma cell line using type-specific monoclonal antibodies. Using immunofluorescence and immunoblotting studies it was noted that several irregularities in lamin expression exist in the cell line GLC-A1, derived from an adenocarcinoma. First, the expression of the A-type lamin was lower than in other adenocarcinoma cell lines of the lung. Also the ratio between lamins A and C proteins was 1:8 instead of the 1:1 ratio seen in the other cell lines. Northern blotting confirmed the altered level of A-type lamin expression. Secondly, an abnormal localization of lamin A was observed. Intensely fluorescing lamin A aggregates were observed in the nucleus, rather than the typical perinuclear staining pattern. Confocal scanning laser microscopy revealed that the lamin A aggregates were indeed present throughout the internal nucleus. When these cells were extracted with Triton X-100, the nucleoplasmic aggregates disappeared, which indicates that the A-type lamins are not properly incorporated into the lamina. The A-type lamins in other cell lines derived from adenocarcinomas remained present in the nuclear periphery after extraction with the non-ionic detergent. Immunoblotting studies of the Triton X-100 soluble and insoluble fractions showed that lamin A and an apparently truncated product, which was detected with the lamin A antibody, were present in the insoluble fraction of GLC-A1. This truncated product is partly Triton X-100 soluble since it was also detected in the detergent soluble fraction. Thirdly, using an antibody to A-type lamins sporadic GLC-A1 cells showed a filamentous cytoplasmic staining pattern, which was Triton X-100 resistant. Double labeling immunofluorescence studies revealed that these cytoplasmic lamins colocalized with the vimentin cytoskeleton in this cell line.

Introduction

Lamins are nuclear proteins, which belong to the family of intermediate filament proteins and consist of A-type lamins and B-type lamins. In mammalian cells, A-type lamins are represented by lamins A and C, which are different transcripts arising from the same gene [11, 29]. B-type lamins are represented by lamin B1 and B2 in mammalian cells and are encoded by different genes [17], while recently a third B-type lamin, denoted lamin B3, has been detected in mouse spermatocytes [13]. Lamins may have a significant impact on the expression of genes by interacting with the heterochromatin, directly or indirectly, by serving as a matrix for the attachment of nuclear matrix-associated proteins, which are thought to play important roles in gene regulation. In contrast to B-type lamins, the expression of A-type lamins appears to be related to the state of cellular differentiation: well-differentiated cells do in general express A-type lamins, whereas relatively undifferentiated cells or cells at early embryonic development do not express detectable levels of A-type lamins (for reviews, see [16, 22, 31]). In addition, it was shown that A-type lamins were not expressed in proliferating cells of some adult human tissues such as the basal keratinocytes of the skin [5] or certain cell lineages of the hematopoietic system [35]. Studies on the expression of nuclear lamins in neoplasms are relatively rare. In parallel to studies in embryonic cells, Lebel et al. [24] showed the absence of A-type lamins in undifferentiated teratocarcinoma cell cultures. In addition, Kaufmann et al. [21] have shown that A-type lamins were decreased by more than 80 % in small cell lung cancer (SCLC) cell lines compared to non-SCLC cell lines. The findings were confirmed in our recent study on the expression of lamins in solid SCLC and non-SCLC [3], which showed the absence or reduction of A-type lamins in SCLC as compared to non-SCLC. In addition, striking aberrations in the organization patterns of A-type lamins were observed in some non-SCLC. In particular adenocarcinomas, which showed cytoplasmic rather than nuclear lamin staining patterns in a part of the tumor cells. In this study we have examined an adenocarcinoma cell line of the lung, which showed irregularities in A-type lamin expression.

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Materials and methods

Cell lines
Three adenocarcinoma cell lines, i.e., NCI-H125 [6, 14], GLC-A1, GLC-A2 [9] were used. The characteristics and growth conditions of these cell lines have been described previously. All cell lines were maintained in a humidified incubator at 37°C in 5% CO₂ and were grown in Roswell Park Memorial Institute Medium 1640 (RPMI-1640, ICN Flow, Irvine, UK) containing 4.5 g/l NaHCO₃, supplemented with 10% newborn calf serum (ICN Flow). Cells were grown to almost confluency before harvesting.

Antibodies
The following mouse monoclonal antibodies were used in this study: LN43 (IgG1), directed against lamin B2 and not cross-reacting with lamin B1 [17], kindly provided by Dr. E. B. Lane (Dundee, UK); XX23 (IgG1), directed against lamin B2 and not cross-reacting with lamin B1 [17], kindly provided by Dr. G. Krohne (Würzburg/Germany); 119D5 (IgG1), directed against lamin B1 and not cross-reacting with lamin B2 (unpublished); 41C4 (IgM) [4], kindly provided by Dr. G. Warren (London/United Kingdom). This antibody recognizes human lamin A and C in immunoblotting; R27 (IgM) was a gift from Dr. G. Krohne. This antibody recognizes both lamin A and C and does not react with human B-type laminas [40]; 133A2 (IgG3), raised against the carboxy terminus of 98 amino acids present in lamin A and absent from lamin C. This antibody recognizes lamin A but not lamin C. Epitope mapping using several deletion mutants of lamin A showed that the amino acids 598-611 form the epitope recognized by 133A2 [20]; RV202 (IgG1), directed against vimentin [34]; RCK102 (IgG1), directed against keratin 5 and 8 [34]; pNF160, a rabbit polyclonal antibody, directed to neurofilaments, kindly provided by Dr. Y. Nakazato [30].

In immunofluorescence and immunoblotting studies LN43, X223, 41C4, R27, RV202 and RCK102 were used as undiluted culturing supernatant, while 119D5 (mouse ascites) was used 1:750 in immunofluorescence and 1:250 in immunoblotting and 133A2 (mouse ascites) 1:2500 in immunofluorescence and 1:1000 in immunoblotting, pNF160 (rabbit serum) was used 1:600 in immunofluorescence.

The antibodies 41C4 and R27 both recognize lamin A and C and will be referred to as lamin A/C antibodies unless otherwise specified. Since the antibodies LN43 and XX23 both recognize lamin B2, they will be referred to as lamin B2 antibodies unless otherwise specified. 133A2 will be referred to as lamin A antibody and 119D5 will be referred to as lamin B1 antibody.

Indirect immunofluorescence technique
The indirect immunofluorescence technique that was applied to the cell lines has been previously described [2]. Briefly, cells were grown on glass coverslips for 1 to 2 days. After washing in phosphate buffered saline (PBS, containing 8.5 g/l NaCl, 0.2 g/l KCl, 1.5 g/l Na₂HPO₄, and 0.2 g/l KH₂PO₄ at pH 7.4), the cells were fixed in methanol (−20°C for 5 sec) followed by acetone (room temperature, 3 times 5 seconds) and air dried. For single labeling immunofluorescence, cells were then incubated with the primary antibody for 30 to 45 min, and after several washes with PBS, incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig (1:100, DAKO A/S, Glostrup/Denmark) for 30 to 45 min. For double labeling immunofluorescence, the cells were incubated with a lamin A/C antibody of the IgM subclass for 30 to 45 min and after several washes with PBS incubated with FITC-conjugated goat anti-mouse IgM (diluted 1:80, Southern Biotechnology Associates (SBA), Birmingham, AL, USA). Then after several washes with PBS, the antibody RV202 was applied for 5 to 10 min and after several washes with PBS the cells were incubated with Texas Red-conjugated goat anti-mouse IgG (diluted 1:80, SBA). Thereafter, cells were washed again in PBS, mounted in 90% glycerol containing 0.02 M Tris-HCl, pH 8.0, 0.002% NaN₃, 2% 1,4 diazabicyclo (2,2,2)-octane (DABCO; Merck, Darmstadt/Germany) and 0.5 g/l MgCl₂ 4·6 diamino-2-phenylindole (DAPI; Merck) for DNA staining, and photographs were taken with an automatic camera using 400 ASA Tri-X pan film (Kodak/UK).

Triton X-100 extraction of cells for immunofluorescence studies
Prior to Triton X-100 treatment the cells on the coverslips were rinsed with PBS at room temperature (RT). Then the coverslips were placed on ice for Triton X-100 extraction. Precooled (4°C) PBS containing 1 mM EDTA (Tritrix III; Merck), 1 mM ethylene glycol-bis[(β-aminoethyl ether) N,N,N’,N’-tetraacetic acid (EGTA; Tritrix VL, Merck), 0.4 mM phenylsulfonylfluoride (PMSF; Aldrich, Steinheim/Germany) and 0.1% Triton X-100 (BDH, Poole/UK) was used for a 5-min extraction. The coverslips were then washed three times with cold PBS containing 1 mM EDTA, 1 mM EGTA, 0.4 mM PMSF and the cells were fixed and immunostained as described above.

Sample preparation for one-dimensional gel electrophoresis
Samples of non-extracted cells. The cells were rinsed with PBS at RT and the culture flask was placed on ice. Then 10 ml PBS containing 1 mM EDTA, 1 mM EGTA, 0.4 mM PMSF was added and the cells were collected by scraping with a rubber policeman from the bottom of the culture flask. The cell suspension was centrifuged at 4000 g, and the pellet and the supernatant were separated. The proteins in the supernatant were precipitated with trichloroacetic acid (TCA; Merck), which was added to a final concentration of 10% (w/v) and allowed to precipitate on ice for 30 to 60 min. The precipitated proteins were pelleted by centrifugation at 2500 g for 10 min, washed twice with ice-cold acetone (−20°C) and air dried. The 410 g cell pellet and the TCA-pellet of the PBS-soluble fractions were resuspended in 200 µl sodium dodecyl sulfate (SDS)-sample buffer and boiled for 5 min.

Samples of Triton X-100 extracted cells. The cells were washed twice with PBS at RT. Then the flasks were placed on ice, and the cells were extracted for 5 min with cold (4°C) PBS containing 1 mM EDTA, 1 mM EGTA, 0.4 mM PMSF and 0.5% Triton X-100 (BDH). The supernatant was discarded, and the cell remainders were rinsed twice with cold PBS containing 1 mM EDTA, 1 mM EGTA and 0.4 mM PMSF. The cell remainders were collected by scraping in cold PBS containing 1 mM EDTA, 1 mM EGTA and 0.4 mM PMSF, the culture flasks were rinsed with the same buffer, and the two fractions were pooled. These pooled fractions were centrifuged at 4100 g and the pellet and supernatant were separated. The proteins in the supernatant were TCA-precipitated as described above. All samples were dissolved by boiling for 5 min in 200 µl SDS-sample buffer.

As a result of this approach equal sample volumes of different cellular fractions correspond with equal cell numbers.

Sample preparation for two-dimensional gel electrophoresis
Cells were washed with cold PBS (4°C) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Merck) and collected by scraping in cold PBS containing 0.5 mM PMSF. They were centrifuged for 5 min at 1500 g and the pellet was Triton X-100 extracted for 10 min by stirring the pellet in Tris-buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSF and 0.5% Triton X-100. The extracted cells were centrifuged at 1500 g and the pellet was treated with RNase/DNase mix (1 mg/ml RNase A, 0.5 mM PMSF, 0.05 mg/ml RNase A in 10 mM Tris-HCl, pH 7.4, 110 mM NaCl and 1.5 mM MgCl₂) for 20 min at RT. Then cold Tris-buffer was added, and the cells were centrifuged at 1500 g. The supernatant was discarded and the pellet was boiled in SDS-sample buffer for 5 min. These samples were stored at −20°C. Before loading onto an isoelectric focusing (IEF) gel an equal volume of buffer containing 2% Nonidet P-40 (Fluka, Buchs/ Switzerland), 5% β-mercaptoethanol (Merck), 5% Bio-Lyte 3/10 carrier ampholytes (Bio-Rad Laboratories, Hercules, CA/USA) and 9 M urea (Merck) was added followed by addition of 1/5 volume 1 M (3-cholamidopropyl)dimethylammonio)propanesulphonate (CHAPS, Sigma, St. Louis, MO/USA) followed by addition of urea (Merck) until saturation.
Gel electrophoresis and immunoblotting
One-dimensional SDS-gel electrophoresis in polyacrylamide was performed according to Laemmli [23] using gels containing 10% polyacrylamide (Bio-Rad Laboratories) and 0.1% SDS (Merck). Gels were run on the Mini-Protean II system (Bio-Rad Laboratories) for approximately 45 min at 200 V.

Two-dimensional gels were run essentially as described by O'Farrell [32], with the following modifications. Isoelectrofocusing (IEF) was performed in 4% polyacrylamide gels containing 8% urea, 14% Biolyte 3/10 carrier ampholytes (Bio-Rad) and 25 mM CHAPS (Bio-Rad Laboratories). Gels were run on the Mini-Protean II 2-D system (Bio-Rad Laboratories) and for IEF no preelectrophoresis was performed. Gels were run at increasing voltages starting at 500 V for 10 min, followed by 1000 V for 20 min and 1500 V for 100 min.

In the second dimension, 10% polyacrylamide SDS-gels were run similar to the one-dimensional gel electrophoresis as described above.

Western blotting was performed essentially according to Towbin [38] using the Mini Trans-Blot Cell (Bio-Rad) at 100 V for 1 h. Proteins were blotted onto nitrocellulose (BAAS; Schleicher and Schuell, Dassel, Germany). After blotting, nitrocellulose sheets were air-dried and stored until immunological detection.

Prior to application of the primary antibodies, the Western blots were blocked for 1 h at RT in buffer containing 10 mM Tris-HCl, pH 7.6, 0.35 mM NaCl and 3% bovine serum albumin (BSA; Sigma). Thereafter, the blots were incubated with the primary antibodies for 30 to 60 min at RT in appropriate dilutions in buffer containing 10 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 0.3% BSA, 1% Triton X-100, 0.5% deoxycholate (Merck) and 0.1% SDS (Merck). Then the blots were washed three times with PBS containing 0.5% Triton X-100 (BDH). The secondary peroxidase-conjugated rabbit anti-mouse Ig antibody (DAKO A/S) was diluted 1:1000 in PBS containing 0.5% Triton X-100 and 0.5% BSA and the blots were incubated with this antibody for 30 to 60 min at RT. Then the blots were extensively washed with PBS containing 0.5% Triton X-100, and peroxidase activity was detected by enhanced chemiluminescence (ECL-kit; Amersham, Amersham, Bucks, UK). RX Fuji medical X-ray films (Fuji, Tokyo, Japan) were used for the visualization of the luminescent signals.

Messenger (m)RNA isolation and Northern blotting analysis
Total RNA was isolated from the cell lines by the guanidine isothiocyanate procedure as described previously [7, 8]. The RNA was dissolved in 200 to 300 µl diethyl pyrocarbonate (DEPC; Sigma)-treated mQ water, two volumes of ethanol (p.a., Merck) were added and the samples were stored at −70°C.

Twenty µg of total RNA, as determined at OD 260, was size-fractionated on a formaldehyde agarose gel [23] and blotted onto Hybond-N+ membrane (Amersham Life Science, Little Chalfont, United Kingdom). The blots were air-dried for several hours and then the RNA was crosslinked with 120 Joules/cm² in an U.V. Stratalinker 1800 (Stratagene, La Jolla, CA/USA). The blots were prehybridized for 3 h at 65°C in a hybridization mix containing 7% SDS, 1% BSA, 2 mM EDTA, 0.5 M NaHPO4, at pH 7.0, and an excess of single-stranded sheared salmon sperm DNA. Then the 32P-radioactive labeled probe (see below) was added to this mixture and hybridized overnight at 65°C. After hybridization, the blots were washed with sodium citrate buffer (SSC, 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS. Stringency conditions were optimized for each DNA probe. Autoradiography was done on RX Fuji medical X-ray films (Fuji) at −70°C using an intensifying screen. Densitometry of the autoradiograms was performed using a Computing Densitometer 300 (Molecular Dynamics, Sunnyvale, CA/USA) with the

Fig. 1. Immunofluorescence micrographs of cell lines GLC-A2 (a–c) and GLC-A1 (d–f), stained with the lamin B1 antibody 1H10S (a), the lamin B2 antibody L5-N3 (d), the lamin A antibody 133A2 (b, e), the lamin A/C antibody 41CC4 (c) and the lamin A/C antibody R27 (f). – Bar 12 µm.
Quantity one (PDJ, Protein Databases Inc., Huntington Station, NY USA) software on a Sun Sparc 1+ station.

Probes
Hybridization was performed with 50 to 200 ng probe DNA. The lamin A/C probe, kindly provided by Dr. F. McKeon [29], was labeled with \( \left[ ^{32}P \right]dATP \) by random priming labeling [19] and purified over a Sephadex G50 fine (Pharmacia, Uppsala/Sweden) column. A GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe [12] was used as a control for the amount mRNA loaded onto the gel.

Results
Subcellular lamin localization as detected by immunofluorescence
Representative reactivity patterns of perinuclear lamin expression in the lung adenocarcinoma cell lines with the different lamin antibodies are shown in Figures 1a to c.

The antibody to lamin A showed strong decoration of the nuclear lamina in the cell lines, but in GLC-A1 (Fig. 1e) an aberrant A-type lamin protein distribution pattern was observed in the immunofluorescence assay, in that intensely stained aggregates were detected in the nucleus. Confocal scanning microscopy revealed that the nuclear aggregates were localized throughout the whole nucleus and not only on the perinuclear lamina (Fig. 2). In addition to the clustered appearance, a normal lamina was stained in these cells with lamin A/C antibodies (Fig. 1f), but the fluorescence intensity was only very weak compared to the signal obtained in the other cell lines. Also when compared to the reaction with B-type lamin antibodies in GLC-A1 (Fig. 1d), the perinuclear A-type lamin staining had to be interpreted as weak. The nuclear aggregates were dissolved when the cells were treated with 0.1% Triton X-100 prior to fixation (Fig. 3). However, the normal lamina staining with A-type and B-type lamin antibodies persisted treatment with Triton X-100 and was still detectable in the cells that had lost the aggregates.

Furthermore, in GLC-A1 another aberrant lamin distribution pattern was detected when stained with the lamin A/C antibody R27. In most cells the lamina was only very weakly stained. However, in some cells an apparent normal intensity of A-type lamin perinuclear staining was observed (Fig. 1f). Additionally, in a small fraction of the cells strongly staining extranuclear aggregates were observed with the antibody R27 (not shown), next to a faint filamentous staining. These A-type lamins appeared to be stably incorporated in cytoskeletal filaments, since they persisted treatment with 0.1% Triton X-100. Double-labeling immunofluorescence studies with antibodies to cytoskeletal proteins revealed colocalization of the cytoplasmic A-type lamins with vimentin. Colocalization of A-type lamins with neurofilaments and cytokeratins did not seem to occur.

Solubility properties of lamins as detected by immunoblotting
In order to further characterize the Triton X-100 soluble and Triton X-100 insoluble lamin fractions in the cell lines, one-
Blots of the same samples were also incubated with antibody R27, reacting with lamin A and C. The results of the cell lines NCI-H125 and GLC-A2 were comparable (illustrated for NCI-H125 in Fig. 4, lanes 5–8), in that the Triton X-100 cell pellets and PBS cell pellets of the cell lines showed both lamin A and lamin C as two strong bands in an approximate 1/1 ratio. The lamin A band is actually a doublet, which is seen as a single band after prolonged exposure in chemiluminescence.

In GLC-A1 the intensity of lamin A and lamin C bands of the Triton X-100 cell pellet and PBS cell pellet was completely different from those seen in the other cell lines, while also the ratio of lamin A over lamin C was aberrant (Fig. 4, lanes 9–12). The lamin A band was hardly detectable in the GLC-A1 Triton X-100 cell pellet and PBS cell pellet, while the lamin C band was clearly visible, but weaker when compared to the other cell lines. In addition, when examined closer, this lamin C band appeared to be a doublet. The overall weaker reaction of lamin A/C antibodies on blots of GLC-A1 samples matched with the results of the immunofluorescence studies, in which the lamin staining with these antibodies was also weak and aberrant. In the soluble fractions of the cell lines no reactivity was detected with the lamin A/C antibodies (Fig. 4, odd numbered lanes).

When stained with lamin A antibody 133A2 the blots of the soluble and insoluble fractions of the cell lines showed distinct differences between the cell lines (Fig. 5).

The blotting results for the cell lines NCI-H125 and GLC-A2 were identical (Fig. 5, lanes 1–8). In the Triton X-100 cell pellet and PBS cell pellet of these two cell lines a lamin A doublet of approximately 72 kDa was detected (Fig. 5, lanes 2, 4, 6, 8). Furthermore, a relative faint additional band with a lower molecular mass of approximately 63 kDa was detected with the antibody to lamin A. In the Triton X-100 supernatant of NCI-H125 and GLC-A2 four bands were detected (Fig. 5, lanes 1, 5). The upper two bands were at the same molecular weight levels as the bands detected in the Triton X-100 cell pellets (Fig. 5, lanes 2, 6), while the two additional bands showed a lower molecular weight. In the PBS-soluble fractions no bands were detected (Fig. 5, lanes 3, 7).

The immunoblots with the GLC-A1 samples showed again that the lamin A expression level was much lower as compared to the other cell lines (Fig. 5, lanes 9–12). In the Triton X-100 cell pellet and PBS cell pellet of GLC-A1 identical bands were detected as in NCI-H125 and GLC-A2, but the intensity ratio between the two bands was changed (Fig. 5, lanes 2, 4, 6, 8, 10, 12). Furthermore, in the Triton X-100 supernatant of GLC-A1 cells only the three lower molecular weight bands, indicated as A*, were detected, while the band at the normal lamin A level was missing in this sample. Again no bands were detected in the PBS soluble fractions (Fig. 5, lane 11). It is remarkable that with the antibodies to lamin A and

![Fig. 3. Immunofluorescence staining patterns of GLC-A1 cells with the lamin A antibody 133A2 before (a) and after (b) treatment with 0.1% Triton X-100. Bar 10 μm.](image)

![Fig. 4. One-dimensional immunoblotting of lamin B2 and lamin A/C in the Triton X-100 supernatants (lanes 1, 5, 9), Triton X-100 cell pellets (lanes 2, 6, 10), PBS supernatants (lanes 3, 7, 11), and PBS cell pellets (lanes 4, 8, 12). Blots of these fractions of GLC-A1 (lanes 1–4) cells were incubated with the lamin B2 antibody LN43, blots of these fractions of NCI-H125 (lanes 5–8) and GLC-A1 (lanes 9–12) cells were incubated with the lamin A/C antibody R27.](image)
C (R27 and 41CC4) never any band was detected at the lamin A level in the Triton X-100 soluble fractions.

Immunostaining of two-dimensional blots of the insoluble fraction of GLC-A1 showed that after applying the lamin antibody 133A2 and using an exposure time as used for the other cell lines and antibodies, only one very small spot was visible for lamin A. After relatively long exposure it became evident that two arrays of several isoforms of lamin A could be detected in GLC-A1 (Fig. 6a). However, the protein with the lower molecular weight, as detected in one-dimensional blotting (Fig. 5, lane 10, indicated as lamin A'), was not visible in this two-dimensional blotting. When the two-dimensional immunoblot of GLC-A1 was stained with the lamin A/C antibody R27, a strong reaction was seen with lamin C and only a faint spot was detected for lamin A under these exposure conditions (Fig. 6b). The signal for lamin C was a doublet of arrays with little difference in molecular masses and distinct phosphorylation isoforms. When the different lamins were detected simultaneously in one immunoblot, the relative abundance of each component became apparent. The relatively high abundance of B-type lamins is obvious from Figure 6c, while also the extremely low concentration of A-type lamins is evident as compared to identical blots of the other cell lines (Fig. 6d).

The lamin A signals were seen as doublets, with only small differences in molecular masses. The isoelectric mobilities of both arrays of lamin A isoforms were identical. Several spots with lower molecular masses were consistently detected by the lamin antibodies. The most prominent of these have molecular masses of approximately 46 kDa, most likely representing degradation products of the lamins [33].

**Messenger RNA levels of lamins as detected by Northern blotting**

RNA isolated from the three cell lines was subjected to Northern blotting analysis (Fig. 7). Hybridization with an A-type lamin probe showed major differences in lamin A and C mRNA expression between the cell lines. In the cell lines NCI-H125 and GLC-A2 both lamin A and lamin C mRNA were detected. The lamin A over lamin C mRNA ratio varied from 1:1.5 to 1:2 as measured by densitometry. However, in the mRNA samples isolated from GLC-A1, the total A-type lamin mRNA expression was 5 to 10-fold decreased compared to the A-type lamin mRNA expression in the other cell lines. Moreover, the expression ratio of lamin A over lamin C was changed to 1:8. Consequently, the lamin A mRNA was hardly detectable. No abnormally sized mRNA was detected in the cell lines. The decreased A-type lamin mRNA level and altered ratio of lamin A over lamin C in GLC-A1 was in concordance with the immunoblotting results. The Northern blot was rehybridized with a GAPDH probe as a control for the amount of RNA blotted onto the membrane. This confirmed that the amount of mRNA from GLC-A1 loaded onto the gel was comparable to that of the other cell lines.

**Discussion**

In this study the molecular basis of an aberrant expression pattern of lamins in a human lung carcinoma cell line has been investigated. Previous studies on lamin expression in normal lung tissues and lung tumors showed that B-type lamins seem to be ubiquitously expressed in cells of normal tissues and
most lung cancers [36, 39], but that in adenocarcinomas of the lung they could not be detected in certain areas of these malignancies. Furthermore, cytoplasmic instead of nuclear localization of A-type laminas in adenocarcinomas has been reported [3]. In the study presented here, the A-type lamin expression showed aberrancies at the protein and mRNA levels in an adenocarcinoma cell line.

In immunofluorescence studies with antibody 133A2, directed to the carboxy-terminal tail of lamin A, which is lacking in lamin C [20], an aberrant lamin A localization was seen in GLC-A1 cells. The perinuclear lamina was only slightly stained, while heavily stained aggregates were detected throughout the nucleoplasm. Our experiments showed that the majority of the nuclear aggregates were removed upon Triton X-100 treatment. These results indicate that a soluble form of lamin A is present in these aggregates. It has been described that non-precipitated prelamin A accumulates as nucleoplasmic inclusions in the nuclei of cells that have been blocked in prenylation [28]. However, the possibility of accumulation of prelamin A in the nuclei of GLC-A1 could be excluded by using an antibody specific for prelamin A (kindly provided by Dr. M. Sienkiewicz [37]), which did not show any nuclear reactivity in GLC-A1 (data not shown).

In order to identify the protein that was responsible for the aggregate-like staining pattern, immunoblotting studies were performed. In one-dimensional immunoblots of GLC-A1, that were incubated with the lamin A antibody 133A2, a protein band with a lower molecular weight than mature lamin A was detected. This lamin A’ appeared to be preferentially extracted, as compared to lamin A. Probably this lamin A’ is responsible for the Triton X-100 soluble nuclear aggregates that were detected in GLC-A1 cells. However, lamin A’ was also present in the other cell lines, but the ratio of normal lamin A over the smaller lamin A’ protein was completely different in these cell lines, in that the lamin A’ was expressed at relatively very low levels. Therefore, the relative excess of normal lamin A might obscure the presence of the lamin A’ protein in these cell lines.

Loewinger and McKeon [27] have described abnormal nuclear aggregation of lamin A lacking the carboxy-terminal part distal to amino acid 444, indicating that this part of lamin A is necessary for correct incorporation into the lamina. Additional studies [18] have shown that not only the carboxy-terminal CAAX motif is responsible for the incorporation, but that additional sequences of lamin A are required for proper incorporation. The fact that lamin A’ is recognized by the antibody 133A2 indicates that at least part of the carboxy-terminal region is intact, since the epitope for this antibody is localized between amino acids 598 and 611. However, the decreased molecular weight might predict that deletions within the molecule occur.

The observation that lamin A’ is not detectable under our standard isoelectric focusing conditions, encompassing a pH range of 4.4-8, might indicate that such a deletion causes an extreme shift in the isoelectric point. The exact nature of lamin A’ remains, however, to be established.

The absence of the typical aggregate-like reactivity with the lamin A/C antibodies may be explained by the observation that in two-dimensional blottings the lamin A/C antibodies have a stronger reactivity with lamin C than with lamin A, while the lamin A antibody showed a very strong reactivity with lamin A. Therefore, preferential staining of lamin C with the lamin A/C antibodies might also occur in immunofluorescence assays, through which nucleoplasmic lamin A aggregates remain invisible.

Furthermore, total A-type lamin expression levels were decreased in GLC-A1, and the ratio of lamin A over lamin C was altered, as detected in immunoblotting and Northern blotting. Since laminas A and C arise from the same gene by alternative splicing [26], an explanation for the altered ratio of lamin A over lamin C may be an altered relative preference for alternative splicing. However, the mechanism behind the regulation of the lamin A to C mRNA ratio is still unclear [26].

The shift in lamin A over lamin C protein ratio in the GLC-A1 cell line probably has consequences for lamina assembly, in particular for the incorporation of lamin C into the lamina, since it has been reported that the absence of lamin A disturbs proper assembly of lamin C into the lamina [19]. Most likely, in some cells the relative excess of lamin C becomes stably integrated in newly formed cytoplasmic filaments or into the preexisting vimentin cytoskeleton, which leads to a Triton X-100 insoluble cytoplasmic localization. This would explain why the cytoplasmic filaments that were seen in some GLC-A1 cells, were detected with an antibody directed to both lamin A and C and not with the antibody that recognized only lamin A. Unincorporated lamin C will not remain for long in the cytoplasm, since it has been found that unassembled laminas have a short half-life [15].

In summary, we have described an abnormal nuclear and cytoplasmic localization of A-type laminas in the adenocarcinoma cell line GLC-A1, and by additional immunoblotting and Northern blotting studies we have tried to elucidate the nature of these aberrancies. To further validate and confirm our present results, additional one-dimensional immunoblotting and molecular biology studies have been initiated.

Acknowledgements. The authors thank Dr. M. Sienkiewicz, Dr. E. B. Lane, Dr. G. Krohn, Dr. G. Warren, and Dr. Y. Nakazato for providing the antibodies, and Dr. F. McKeon for providing the lamin A/C probe.
References


