3 Methods and materials

Chemicals were obtained from Sigma (Deisenhofen, Germany), Merck (Darmstad, Germany), Boehringer (Mannheim, Germany) and Roth (Karlsruhe, Germany). Materials, such as tubes (10 ml or 50 ml), culture flasks and culture dishes were from Falcon (Becton Dickinson, Franklin Lakes, N.J., U.S.A.). Micro-tubes (1.5 ml) and pipettes (5, 10, 25 ml) were from Sarsted (Nürnbrecht, Germany).

PBS : In most of the assays calcium-magnesium free PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) was used.

3.1 Cell lines

The work described here was carried out using cell lines which were routinely checked to be free of mycoplasma. Medium: Dulbecco's Modified Eagle Medium plus 110 mg/l sodium pyruvate and 1000 mg/l glucose or RPMI-1640 plus 110 mg/l sodium pyruvate and 1000 mg/l glucose were obtained from Gibco (Karlsruhe, Germany). Fetal bovine serum, penicillin/streptomycin (10000 IU/ml), L-glutamine (200 mM in 0.85% NaCl) and EDTA-trypsin (10 x) were also from Gibco (Karlsruhe, Germany).

Small cell lung cancer cell lines

H69-WT (HTB 119, American Type, Culture Collection, Rockville, MD, U.S.A.) was derived from a patient with small cell carcinoma of the lung (Gazdar et al, 1980). H69-VP is a subclone of NCI-H69 carrying a homozygous NLS mutation of the topoisomerase IIα gene. The establishment of H69-VP is described in Jensen et al, 1993.

Cells were grown in suspension as floating aggregates and maintained in RPMI-1640 containing 10 g/l penicillin/streptomycin, 1% L-glutamine supplemented with 10% (normal conditions) or 20% fetal bovine serum (accelerated growth conditions) in a humidified atmosphere containing

7.5% (vol/vol) CO_2. Under normal growth conditions the mutant subclone H69-VP had a similar growth rate as the parental cell line H69-WT (Jensen et al, 1993; Brock et al, 1995). To avoid a drift of the geno and phenotype the mutant subclone was re-established from frozen stock every 20 passages.

Human epidermoid carcinoma

Human A431 epidermoid cells (CRL 1555, American Type, Culture Collection, Rockville, MD, U.S.A). The cells were established from solid tumour cells (Giard et al, 1973). Cells were grown in monolayer and cultured in DMEM containing 10 g/l penicillin and streptomycin, 1% L-glutamine supplemented with 10 % fetal bovine serum in a humidified atmosphere containing 5% (vol/vol) CO_2.

Lymphocytes

Primary lymphocytes from peripheral human blood were grown in RPMI containing 10 g/l penicillin/streptomycin, 1% L-glutamine supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% (vol/vol) CO_2.

3.2 Lymphocytes isolation

The lymphocytes were isolated from human blood, kindly provided by the Red Cross. The *buffy coats* were diluted 1:1 with haemaccel (4°C) and subsequently 1% heparin (liquemin^R N 25000, Roche, Grenzach-Wyhlen, Germany) was added. After 20 min at 20°C two phases have been generated. The erythrocytes sediment to the bottom of the culture flask, whereas the upper phase contains the lymphocytes and other blood cells. Following sedimentation of the upper phase (1000 x g, 4°C,10 min) the pellet was resuspended in 5 ml medium (RPMI-1640 containing 10 g/l penicillin/streptomycin, 1% L-glutamine,10% fetal bovine serum) and additionally 1% heparin was added. Cells were laid on the top of a Ficoll

gradient, which was created by adding 25 ml of Ficoll-Paque (Pharmacia, Freiburg, Germany) to a 50 ml tube. After sedimentation across a single step gradient of Ficoll (1600 x g, 4°C, 10 min) a white ring was obtained, which contained lymphocytes, monocytes and thrombocytes, whereas granulocytes and the rest of the erythrocytes were present in the cell pellet. The ring was transferred to a new tube, and subsequently resuspended in the medium, following sedimentation by centrifugation (900 x g, 15 min, 4°C). The last centrifugation step was repeated until none or only a small amount of thrombocytes was present. The content of the various cell types was measured using a cell counter (K1000, Digitana AG, Germany).

Separation of the lymphocytes from the monocytes

Cells resuspended in medium and incubated for 2 h in 37°C. Monocytes would be sedimented to the bottom of the culture dish, whereas lymphocytes were floating in suspension.

3.3 Cell counting

The suspension cells could be directly transferred to a 50 ml tube, and sedimented by centrifugation: for small cell lung cancer (1200 x g, 10 min, 4°C) and for the lymphocytes (1600 x g, 10 min, 4°C). The A431 cells were detached by trypsinization with 10 ml EDTA-trypsin for 10 min at 37°C. A431 cells were sendimented by centrifugation (800 x g, 5 min, 4°C). The cell pellet was washed twice in the medium (for RNA isolation) or in PBS (for other assays), and resuspended either in 1 ml of either medium (for RNA isolation) or in 1 ml PBS (for other assays). *Cells per ml* : In 900 µl 0.5% trypan blue 100 µl from the cell-suspension was added. After 5 min at 20°C the cells were counted in a microscope. The colour of the viable cells was clear, whereas the colour of the dead cells was blue. Using a Neubauer chamber, the number of cells per ml

was found: In a given area in the chamber, the number of cells were counted and multiplied with the dilution factor (10 x), and the number 15625 to give cells per ml in the cell suspension.

3.4 Cell cycle analysis

Analysis of cell cyle phases was performed on cells fixed with 70% ethanol (20 min, -20°C). The fixed cells were centrifuged (5000 x g, 5 min, 4°C) and the fixative was removed completely. Cells were permeabilized with solution A (Partec, Münster, Germany) for 15 min at 4°C by gentle shaking. DNA was stained with DAPI by adding 4 volumes of solution B (Partec, Münster, Germany) and cellular DNA-content was analysed using a PAS-2 flow cytometer (Partec, Münster, Germany) equipped with a Hg-lamp and appropriate filters. Trout erythrocytes served as a DNA-standard. Cells were arrested in *mitosis* or *S-phase* by incubation with demecolcine (0.26 µM, 24 h) or aphidicoline (0.36 µM, 36 h) respectively (both Sigma, Deisenhofen, Germany).

3.5 Drugs

Etoposide (VP16) and teniposide (VM26) were obtained from Bristol, München, Germany.

3.6 Antibodies

Topoisomerase IIα.

The mouse monoclonal antibody directed against the proliferation-associated nuclear antigen Ki-S1 (indirect immunofluorescence microscopy) is specific for a COOH-terminal epitope of human topoisomerase IIα and does not cross-react with topoisomerase IIβ (Boege et al, 1995). For immunoblotting topoisomerase IIα was probed with peptide antibodies generated against a unique COOH-terminal

peptide (residues 1513-1531) of human topoisomerase IIα (CIC, Genosys Biotechnologies, Cambridge, UK) and against the unique NH_2-terminal peptide (residues 1-12) of human topoisomerase II (named 676). The antibodies did not cross-react with topoisomerase IIβ (Meyer et al,1996).

Topoisomerase IIβ

Topoisomerase IIβ was probed with the mouse monoclonal antibody 3H10 (indirect immunofluorescence microscopy) (Kimura et al,1996) or rabbit antibodies 779 (immunoblotting) both raised against peptides corresponding to unique COOH-terminal sequences (residues 1611-1621) of the β-isoenzyme (Boege et al,1995). The antibodies do not cross-react with topoisomerase IIα.

Tubulin antibodies

Centrosomes were labelled with the mouse monoclonal anti-γ-tubulin (immunoblotting), which is a peptide antibody generated against a highly conserved NH_2-terminal peptide (residues 38-53) (Sigma, Deisenhofen, Germany). For indirect immunofluorescence microscopy centrosomes were recognized with a rabbit antibody raised against peptides corresponding to the NH_2-terminal region (residues 38-53) (Sigma, Deisenhofen, Germany). Both tubulin antibodies do not cross-react with other tubulin isoforms (α and β).

3.7 Topoisomerase IIα standard

Recombinant human DNA-topoisomerase IIα was expressed in *Saccharomyces cerevisiae* and purified according to the procedure described in Boege et al, 1995.

3.8 Indirect immunofluorescence microscopy

Slides were siliconized : The slides were washed in 97% acetone (5 min) followed by siliconation for 5 min in 2% triethoxysilylpropylamin in 97%

acetone. After washing (2 x 1 min) with ddH_2O, the slides were dried at 20°C. Before use, the siliconized slides were sterilized either by autoclaving or in 96% EtOH for 20 min.

Different methods were used to obtain optimal results for each cell lines.

Attachement of the cells to the slides: The small lung cancer cells and the lymphocytes (approximately 4 x 10^2 cells per slides) had to be sedimented onto microscopic slides by cytospinning (15 min, 500 x g, 20°C) using centrifuge Cytospin 2 (Shandon, Frankfurt, Germany). The A431 cells are grown as a monolayer and can therefore be grown directly on the slide. Approximately 1 x 10^2 A341 cells were added onto the slide. After 1 h at 37°C cells were sufficiently attached to the slides, and could be covered with the medium (DMEM containing 10 g/l penicillin and streptomycin, 1% L-glutamine, 10 % fetal bovine serum). After 1-2 days in a humidified atmosphere containing 5% (vol/vol) CO_2 the now confluent cells could be used for immunofluorescence.

Immunofluorescence using the small cell lung cancer cell lines:

For analysis of *whole cells*, cells were sedimented (500 x g, min, 20 °C) onto the microscopic slides.

For analysis of *chromosomal metaphase* spreads, cells were blocked by demecolcine (0.26 µM, 24 h, 37°C) and swollen with 75 mM KCl at 4° C for 10 min before sedimentation (2000 x g, 15 min, 20°C) onto microscopic slides.

Cells or chromosomal spreads were fixed with formaldehyde (3.7% in PBS, 10 min, 5°C) and permeabilized (Triton X-100, 0.1 % in PBS, 5 min, 4° C). After washing with PBS, cells were blocked (PBS containing 5% standard goat serum, 1 h, 20° C) and subsequently incubated for 30 min at 20° C with primary antibodies diluted in PBS containing 1% bovine serum albumin and 1% standard goat serum. After washing (4 x 5 min), bound antibodies were visualized by incubation for 1 h at 20° C

with goat-anti-mouse or -rabbit Fab_2-fragments labelled with CY3 or CY2 (Dianova, Hamburg, Germany) and diluted 1:1000 in PBS containing 1% bovine serum albumin and 1% standard goat serum. In the first PBS washing, DNA was counterstained with DAPI (0.2 µg/ml in PBS, 5 min, 20° C). After washing (3 x 5 min) with PBS and subsequently a few seconds in ddH_2O, the slides were mounted in an antifade solution (PBS containing 1.5% N-propyl-gallate and 60% glycerol) and examined at a 630 x or 1000 x magnification, using a Zeiss Axioplan epifluorescence microscope coupled to a cooled CCD camera (SensiCam, photometrics, München, Germany). Camera control and image acquisition was done with an Apple Macintosh computer equipped with imaging software from IPLab (IPLabSpectrum). Fluorophores were selectively imaged with filters especially prepared for a reliable distinction of signals from DAPI, CY2, and CY3.

Immunofluorescence using the lymphocytes

Cells were fixed with formaldehyde (2% in PBS, 15 min, 5°C) before sedimentation on the slide (500 x g, 15 min, 20°C) and then permeabilized (0.5% Triton X-100, 1% formaldehyde in PBS, 5 min, 4° C). After washing the cells were blocked as described above.

Immunofluorescence using A431 cells

Cells were fixed with formaldehyde (2% formaldehyde in PBS, 15 min, 5° C) and permeabilized (0.5% Triton X-100, 1% formaldehyde in PBS, 5 min, 4° C). After washing the cells were blocked as described in the above (Meyer et al, 1996).

Primary antibodies: 1:600 (3H10; mouse anti-topoisomerase IIβ); 1:1000 (KiS1; mouse anti-topoisomerase IIα); 1:500 (CIC; rabbit anti-topoisomerase IIα); 1:100 (676; rabbit anti-topoisomerase IIα) and 1:250 (γ-tubulin; rabbit anti-tubulin).

Secondary antibodies 1:1000 (CY2 or CY3, Dianova,Germany).

3.9 Pre-absorption of antibodies in immunofluoresence

Specificity of immunostaining was routinely controlled by pre-absorption with purified recombinant topoisomerases produced in yeast. The purified topoisomerase (1µg) was heat-inactivated (60°C, 5 min) and subsequently incubated for 30 min at 20° C with primary topoisomerase antibodies (Ki-S1 or γ-tubulin) diluted in PBS containing 1% bovine serum albumin and 1% standard goat serum in a volume of 300 µl. After centrifugation (10000 x g, 30 min, 4°C), 200 µl of the supernatant was taken out for staining in immunofluorescence.

3.10 Decatenation

Topoisomerase II-specific activity was determined by decatenation of catenated network DNA (200 ng) from *crithidia fascicutata,* kinetoplast TopoGen Inc. Columbus, Ohio, U.S.A.) (Marini et al, 1980; methods reviewed in Boege, 1996). Serial dilutions of whole cell extract, cytosolic fraction, centrosome extract, or pure human recombinant DNA-topoisomerase IIα were incubated for 45 min at 37°C in 30 µl reaction buffer (10 mM BTP, pH 7.9, l0 mM $MgCl_2$, 100 mM KCl, 0.5 mM dithiothreitol, 0.03 mg/ml bovine serum albumin) in the presence of 1 mM ATP. Controls were without extract. The reaction was terminated by adding 7.5 µl loading buffer (10 mM TrisHCl pH 8.0, 1.46 M sucrose, 100 mM EDTA,1.5 mM bromophenol blue). The samples were subjected to a neutral agarose (1%) gelelectrophoresis.

3.11 Agarose gelelectrophoresis

Agarose gelelectrophoresis was used for separation of DNA or RNA fragments or for identifying different conformational forms of DNA (Maniatis, 1982). Agarose was completely dissolved in Tris/Boric/EDTA buffer (89 mM TrisHCl pH=7.5, 89 mM Boric acid, 2.5 mM EDTA).

After electrophoresis (1 V/cm, 24 h) the gel was stained with 0.5 µg/ml ethidium bromide. Fluorescence of ethidium bromide in the gels (excitation 302 nm, emission > 600 nm) was documented by digital photography (EDAS 120, Kodak, Germany).

3.12 Extract for decatenations assay

Small cell lung cancer: Preparation of whole cell extracts. Exponentially growing cells were treated for 10 min at 4°C with 10 mM TrisHCl pH 7.5, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, 5 mM pefabloc and subsequently extracted by addition of 0.5 M NaCl. Debris was removed by centrifugation (10 min, 10000 x g, 4° C). *Whole cell lysate* (supernatant) of the two cell lines were used for decatenations assay.

Lymphocytes: Preparation of the *centrosome extracts* which were made from the lymphocytes. The cytosolic fraction obtained from lysis of the lymphocytes was sedimented (20000 x g, 30 min, 4°C). The sediments containing the centrosomes were extracted for 30 min at 4°C with 500 mM NaCl (in a lysis buffer) and the supernatant obtained from a subsequent second centrifugation (20000 x g, 30 min, 4°C). The centrosome extract (supernatant) was used for the decatenation assay.

3.13 Immuno-band depletion assay

Exponentially growing cells were incubated with and without topoisomerase II interacting drugs for 1 h at 37° C, followed by sedimentation (1000 x g, 5 min, 4° C), and subsequently lysed in a sample buffer (1x Lämmli, 0.1% glycerol, 20 mM EDTA pH 7.8, 50 µM pefabloc and 200 µM dithiothreitol). After 5 min at 90° C and mechanical DNA-shearing with a syringe the samples equivalent to 5 x 10^5 cells were subjected to SDS-polyacrylamide (5 or 10 %) gelelectrophoresis.

3.14 Biochemical methods for revealing subcellular organization of topoisomerase II

Cells were lysed in an appropriate buffer with a non-ionic detergent, e.g. Triton X-100. Cells (98%) were effectively permeabilized, as determined by a subsequent addition of 0.5% trypan blue and a microscopic score of stained nuclei. DNA-topoisomerase would be selectively extracted with 350 mM NaCl, whereas other structural proteins, e.g. histones, remained bound to the genomic DNA.

For small cell lung cancer cells: Cells (1x 10^6) were disrupted at 4°C in a hypotonic lysis buffer containing 100 mM KCl, 10 mM TrisHCl pH 7.8, 5 mM $MgCl_2$, 1 mM PMSF by 10 passages through a 29 gauge needle (U-40 insulin, Omnican^R40, Braun, Melsungen, Germany). *The whole cells lysate* was either supplemented with a sample buffer (1x Lämmli, 0.1% glycerol, 20 mM EDTA pH 7.8, 50 µM pefabloc, 200 µM dithiothreitol) and directly applied to immunoblotting, or centrifuged (10000 x g, 10 min, 4° C) using a swing-out rotor (mikrorapid/k, Hettic, Tuttlingen, Germany) to obtain *cytosolic fraction* (supernatant) and *nuclei* (pellet). The cytosolic fraction was either precipitated with trichloroacetic acid or used for further assays. The nuclei, which were extracted with 200 mM NaCl in a buffer (50 mM HEPES pH 7.5, 1 mM dithiothreitol, 1 mM phenyl methyl sulfonyl fluoride, 10 µg/µl aprotinin, 50 µg/ml leupeptin, 50 µg/ml pepstatin, 10% glycerol, 1 mM benzamidine, 1 mM pefabloc) and followed by a second centrifugation (13000 x g, 15 min, 4°C), were either precipitated with trichloroacetic acid or were used for further assays.

For A431 cells and lymphocytes:

A431 cells or lymphocytes (2 x 10^6 cells/ml) were treated for 10 min at 4°C with a lysis buffer (15 mM HEPES pH 7.5, 60 mM KCl, 15 mM NaCl, 0.5 mM EGTA, 1 mM dithiothreitol, 1 mM phenyl methyl sulfonyl

fluoride, 30 µg/ml spermine, 7.5 µg/ml spermidine, 1 µg/µl aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 0.5 % Triton X-100). *The whole cells lysate* was lysed in a sample buffer (1x Lämmli, 0.1% glycerol, 20 mM EDTA pH 7.8, 50 µM pefabloc and 200 µM dithiothreitol) or centrifuged (3000 x g, 10 min, 4° C), using a swing-out rotor (microrapid/k, Hettic, Tuttlingen, Germany) to obtain *cytosolic fraction* (supernatant) and *nuclei* (pellet). The cytosolic fraction was either lysed in a sample buffer or was used for further assays. The nuclei (3.3×10^6 nuclei/ml) were extracted with 500 mM NaCl in a buffer (50 mM HEPES pH 7.5, 1 mM dithiothreitol, 1 mM phenyl methyl sulfonyl fluoride, 10 µg/µl aprotinin, 50 µg/ml leupeptin, 50 µg/ml pepstatin, 10% glycerol, 1 mM benzamidine, 1 mM pefabloc), followed by a second centrifugation (13000 x g, 15 min, 4°C). Nuclear extracts were either lysed in a sample buffer or used for further assays. Samples or salt-insoluble chromatin remnants dissolved with a sample buffer were subjected to SDS-polyacrylamide (5% or 10%) gelelectrophoresis.

3.15 Salt-extraction of chromatin bound topoisomerase II

The nuclei (from cell lysis of small cell lung cancer cells) were sequentially extracted with 200, 400, 600, and 800 mM KCl in a buffer (50 mM HEPES pH 7.5, 1 mM dithiothreitol, 1 mM phenyl methyl sulfonyl fluoride, 10 µg/µl aprotinin, 50 µg/ml leupeptin, 50 µg/ml pepstatin, 10% glycerol, 1 mM benzamidine, 1 mM pefabloc). Followed by a second centrifugation (13000 x g, 15 min, 4°C) the proteins in the various fractions and extracts were precipitated by trichloroacetic acid and salt-insoluble chromatin remnants were dissolved with a sample buffer (1 x Lämmli, 8.7% glycerol, 25 mM dithothritol, 5 M UREA) and subjected to SDS-polyacrylamide (5%) gelelectrophoresis.

3.16 Centrosome isolation

The centrosomes were isolated from A431 cells by isopyknic sucrose gradient centrifugation (Bornens et al, 1987; Komesli et al, 1989). Cells (1×10^8) were lysed for 10 min at 4°C with 5 ml lysis buffer (1 mM TrisHCl pH 8.0, 0.5 mM EGTA, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenyl methyl sulfonyl fluoride, 30 µg/ml spermine, 7.5 µg/ml spermidine, 1 µg/µl aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 0.5 % Triton X-100). Nuclei were sedimented (3000 x g, 10 min, 4°C) and the supernatant was layered on the top of a sucrose gradient. The gradient was created by stacking 1 ml portions of the lysis buffer supplemented with 70, 60, 50, 30, 20 or 10 % (w/v) sucrose inside a 14 x 95 mm polycarbonate ultracentrifugation tube, followed by an equilibration for 48 h at 4°C. After ultracentrifugation (200000 x g, 24 h, 4°C, SW 40 TI, Beckman Instruments Inc., Palo Alto, U.S.A.), the gradient was collected in 10 portions of 1.2 ml each from the bottom of the tube. The actual sucrose concentration of the fraction was measured by optical diffraction. The fraction was diluted 20-fold with the lysis buffer and sedimented (20000 x g, 30 min, 4°C). The sediments were subjected to SDS-polyacrylamide (5% or 10%) gelelectrophoresis.

3.17 Phosphatase treatment

Topoisomerase can be reversibly denatured and precipitated by amonium sulphate. The cytosolic or nuclei fractions were precipitated with 3 M ammonium sulfate (2 h, 4°C) and sedimented (10000 x g, 4°C, 15 min). The precipitates were renatured with an appropriate reaction buffer and treated for 24 h at 37°C with 50 U calf intestine alkaline phosphatase or 90 U potato acid phosphatase (both obtained from Boehringer, Mannheim, Germany) (Kazuhiro & Mitsuhiro, 1992; Meyer et al, 1996). The reaction buffer for acid phosphatase was 40 mM PIPES pH 6.0, 1

mM dithiothreitol, 20 μg/ml aprotinin and 20 μM leupeptin. The reaction buffer for alkaline phosphatase was 200 mM diethanolamine pH 9.9, 2 mM $MgCl_2$, 1 mM PMSF. Controls included treatment with an equal amount of heat inactivated (90°C, 10 min) phosphatases and untreated samples. The samples were subjected to SDS-polyacrylamide (5%) gelelectrophoresis.

3.18 TCA- precipitation

A sample containing the diluted protein for SDS-PAGE was precipitated with trichloroacetic acid (TCA) 100% W/V to a final concentration of 15%. The sampel was incubated for 10 min at 37° C and subsequently the pellet was sedimented by centrifugation (10000 x g, 10 min, 4° C). The rest of the TCA was removed by washing the pellet with 300 μl ice cold 97% acetone, following centrifugation (10000 x g, 10 min, 4° C). The pellet was dried for 2 min at 20°C and dissolved in a 50–100 μl sample buffer (1 x Lämmli, 8.7% glycerol, 25 mM dithothritol, 5 M UREA).

3.19 Samples for SDS-PAGE gel

Before loading the samples on the SDS-PAGE gel, they were boiled for 3–5 min on the heating block (96°C). Subsequently dithiothreitol was added to the sampels solution (1 μl 1 M dithiothreitol per 100 μl of samples solution).

3.20 SDS-Polyacrylamid gel electrophoresis

SDS-polyacrylamide gel electrophoresis was used to separate proteins based on their molecular weights (Lämmli, 1970). The gel chamber and the casting casettes (1 mm) are both from Novex (San Diego, U.S.A.). A better result for *immunoband depletion assay* was obtained by using 1.5

mm gel casettes. The ready made SDS-PAGE running buffer (10 x) is provided from Roth (Karlsruhe, Germany).

For chasting a gel: Seperations gel containing 5% or 10% polyacrylamid (30.8% T, 2.7 bis), 8% glycerin, 1 x seperation buffer (1.5 M Tris-HCl, 0.4% SDS pH 8.8) and temed and ammoniumpersulphate (each to a final concentration of 0.06%.). The stacking gel containing 4.5 % polyacrylamid (30.8%T, 2.7 bis), 1 x stacking buffer (0.5 M Tris-HCl, 0.4% SDS pH 6.8), and temed, and ammoniumpersulphate (each to a final concentration of 0.06%). Sampels were run into the stacking gel at 10 mA/gel, and were separated with 20 mA when the samples have entered separation gel.

Marker: Migration distance of immunostained protein bands was compared to those of rabbit muscle myosin (212 kD), α2-macroglobulin from bovine plasma (170 kD), β-galactosidase from *Escherichia coli* (116 kD), human transferrin (76 kD) and bovine liver glutamic dehydrogenase (53 kD).

3.21 Western blot

A semidry system between two horizontal graphite plates was used to transfer the proteins, which had migrated in the SDS-PAGE gel to a membrane (Kyhse-Andersen,1984; Tovey & Baldo,1987). The filters (3 mm) were from Schleicher & Schuell (Dassel, Germany) and the membrane was from Immobilon P (Millipore Corp., Bedford MA, U.S.A)

A discontinuous system was used: Anode buffer solution 1 (0.3 M Tris, 10% methanol pH 10.4), anode buffer solution 2 (25 mM Tris, 10% methanol pH 10.4), and the cathode buffer for topoisomerase IIα (40 mM 6-amino-n-caprioic acid, 20 % methanol) and for 205 kDa (40 mM 6-amino-n-caprioic acid, 20 mM Tris, 0.01% SDS). The transfer time was between 2-4 hours and the current density is 0.8 mA/cm^2.

3.22 Immunodetection for Western blot

Immuno-staining:. Immobilized proteins were incubated with primary antibodies (1 h at 20°C) followed by with a peroxidase-labelled secondary antibodies from Dianova (Hamburg, Germany), or from Amersham (Freiburg, Germany). The antibody-labelled protein bands were visualized with the enhanced chemiluminescence (ECL) detection methods according to the manifacturer's protocol (Amersham, Freiburg, Germany). Signals of immunoblots were quantified by densitometry using a transilluminating flatbed scanner linked to a Power Macintosh 4400-200 computer equipped with the NIH Image 1.61 software.

Primary antibodies: 1:8000 (CIC; rabbit anti-topoisomerase IIα); 1: 1000 (676; rabbit anti-topoisomerase IIα) and 1:1000 (779; rabbit anti-topoisomerase IIβ) and 1:5000 (γ-tubulin; mouse anti-tubulin).

Secondary antibodies: 1:25000 (goat anti secondary antibodies, Dianova Hamburg, Germany) and 1:7500 (anti rabbit Ig, horseradish peroxidase from Donkey, Amersham, Freibrug, Germany).

3.23 RNA isolation and reverse transcription

To avoid degradation of RNA, hand gloves were used, chemicals and materials were either autoclaved or sterilized.

RNA- isolation

Total RNA from A431 cells or lymphocytes were isolated by using Trizol Reagent kit (Life Technologies, Karlsruhe, Germany). A431 cells and lymphocytes were lysed with Trizol (1 ml per 10 cm^2 of the culture dish for A431 and 1 ml per 5 x 10^6 for lymphocytes cells (according to the protocol). Adding of chloroform (0.2 ml chloroform per 1 ml Trizol reagent) followed by centrifugation (12000 x g, 15 min, 4°C) separated the solution into an aqueous phase and an organic phase. The RNA remained in the upper aqueous phase. The RNA was recovered by

precipitation with isopropyl alcohol (0.5 ml isopropyl alcohol per 1 ml Trizol reagent) followed by centrifugation (12000 x g, 10 min, 4°C). The RNA pellet was washed with ethanol (1 ml 75 % ethanol per 1 ml Trizol reagent). The RNA was dissolved in RNa'se free water and stored at – 20°C. The isolated RNA had a yield of 8-15 µg for the cells and the $A_{260/280}$ ratio was 1.8 measured in a spectrophotometer (LKB, Biochrom, Freiburg, Germany). The isolated RNA was subjected to 1% agarose (1g/100ml ddH2O) gelelectrophoresis (Tris/acetate/EDTA buffer, 1 V/cm, 4 h) with 0.5 µg/ml ethidium bromide. Two predominant ribosomal RNA bands at 5 kb (28S) and at 2 kb (18S) were observed indicating an intact RNA.

cDNA synthesis

To obtain cDNA from RNA reverse transcriptase was performed. The first strand of cDNA was synthesized using 5 µg total RNA and 500 µg/ml Oligo $(dt)_{12-18}$ in a volume of 12 µl. The mixture was heated (10 min, 70°C), then chilled on ice. Addition of 4 µl 5 x first strand buffer, 2 µl 0.1 M dithiothreitol, 1 µl 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, dTTP) followed by incubation (2 min, 42°C). Subsequently 1 µl 200 U Superscript II was added and incubated (50 min, 42°C). The reverse transcriptase was inactivated by heating (15 min, 70°C). Before cDNA can be used as a template for amplification in PCR, RNA was completely removed by adding 1 µl (2U) of *E.coli* RNa'se H and incubate (37°C, 20 min).

PCR

For poly chain reaction (PCR) 10% of the cDNA (from above) was admixed in a total volume of 100 µl containing 20 mM TrisHCl pH 8.4; 50 mM KCl, 1.5 mM Mg_2Cl, 0.2 mM dNTP mix, 10 µM amplification primer 1, 10 µM amplification primer 2, 5 U/µl *Tag* DNA polymerase.

Over the reaction add 100 µl silicone oil, and, for denaturation, the samples were heated for 3 min at 94°C.

PCR was carried out using the Expand Long Template PCR system (Roche, Mannheim, Germany) with an initial denaturation for 5 min at 94°C, followed by 36 cycles of 94°C for 1 min, annealing at 52°C for 45 sec and 90 sec polymerisation at 72°C. Final extension 5 min at 72°C.

The nucleotides based on intron-exon studies (Lang et al, 1998)

Exon 1-9:

Ex 1. fw 5‘ AGA-AGC-GGC-TTG-GTC-GG 3‘

Ex 9. rv 5‘ GCT-TTT-ACT-GCT-ACA-CCA-CCC 3‘

Exon 9-22:

Ex 9.fw 5‘ GTG-GCA-GAC-ATG-TTG-ATT-ATG-TAG-C 3‘

Ex 22. rv 5‘ TGG-GTC-CAT-GTT-CTG-ACG-G 3‘

Exon 22-33

Ex 22. fw 5‘ TTC-CAA-GTT-ACA-AGA-ACT-TCA-AGG-G 3‘

Ex 33.rv 5‘ TGT-CAC-ATT-CTT-TTT-AGG-AAC-TGG-G 3‘

Exon 33-35

Ex 33.fw 5‘ TAC-CAC-TGT-CTT-CAA-GCC-CTC-C 3‘

Ex 35.rv 5‘ CCC-CAA-ACT-AAA-TTC-AGA-GGG-G 3‘

Nucleotide primers based on the epitopes from the forwards primer of NH_2- and the reversed primer of COOH- terminal sequence of topoisomerase IIα:

N-term.fw 5‘ GGA-AGT-GTC-ACC-ATT-GCA-GCC-TG 3‘

C-term.rv 5‘ AGA-TTT -TGC -CCG -AGG –AGC- CAC-AG 3‘

Nucleotide primers based on the epitopes from the reversed primer (rv) of NH_2- and the forwards primer (fw) of COOH- terminal sequence of topoisomerase IIα:

N-term.rv 5‘ TGT-ACG-GGC-AAA-GAA-ACC-TAT-AAA-GTA-CCT- 3‘

C-term.fw 5‘ TCA-TTT-ACA-GGC-TGC-AAT-GGT-GAG A 3‘

Marker : Migration distance of PCR products was compared to the 1 kb DNA ladder (12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 506 bp) obtained from Gibco (Karlsruhe, Germany).

3.24 Statistics

Since quantitative statistics could not be applied to most of the data, representative examples of experimental results are shown. Unless otherwise stated, fluorescent images of single cells are representative of the whole cell population inspected in at least 10 separate fields of view. For all data shown (indirect immunofluorescence microscopy, catalytic assays, immunoblotting, and cell cycle analysis) similar results were obtained in at least three independent experiments done on different days and with a different sets of cells. When quantitative evaluations of immunoblots are stated in the text, they result from densitometry of the X-ray films and represent mean values of at least three independent experiments. The standard errors of the mean are not mentioned, since they were less than 20% in all cases.