# Characterization of DNA Topoisomerase IIα/β Heterodimers in HeLa Cells†

Irina Gromova,‡,§ Harald Biersack,§,|| Sanne Jensen, Ole Frederik Nielsen, Ole Westergaard, and Anni H. Andersen*

*Department of Molecular and Structural Biology, University of Aarhus, C. F. Møllers Allé, Building 130, 8000 Århus C, Denmark*

*Received June 12, 1998; Revised Manuscript Received August 24, 1998*

ABSTRACT: In mammalian cells, DNA topoisomerase II is the product of two distinct genes encoding the α and β isoforms of the enzyme. Besides homodimeric topoisomerase IIα and IIβ, we have recently shown that α/β heterodimers constitute a third population of topoisomerase II in HeLa cells. We found that topoisomerase II heterodimers are not restricted to HeLa cells but exist in different mammalian cell types, and up to 25% of the total topoisomerase IIβ population is involved in heterodimer formation. Studies of topoisomerase II phosphorylation in HeLa cells show that heterodimers are phosphorylated in vivo to a significantly lower level compared to homodimeric α enzymes, but in contrast to the latter neither heterodimers nor topoisomerase IIβ homodimers coprecipitate together with a kinase activity that is able to mediate their phosphorylation. However, both enzymes can still be phosphorylated by exogenously added casein kinase II. The differential phosphorylation of topoisomerase II heterodimers suggests an alternative regulation of this topoisomerase II subclass compared to the homodimeric topoisomerase IIα counterparts.

Eukaryotic DNA topoisomerase II is an abundant nuclear enzyme essential for DNA metabolic processes such as DNA replication, chromosome segregation, and chromosome condensation (*40, 41*). It maintains these functions by catalyzing topological conversions of DNA in a reaction involving transient cleavage of the DNA strands by the two subunits of the dimeric enzyme (*3, 25, 28*). Besides its physiological functions, much attention has been given to topoisomerase II, as it appears to be a major cellular target for numerous clinically employed antitumor drugs (*16, 38*). In higher eukaryotic cells, two isoforms exist of topoisomerase II, the α and β forms, which are encoded by separate genes (*37*), but at present it is not clear how the multiple functions of topoisomerase II are assigned to the two isoforms. Biochemical and pharmacological differences have been observed between the two enzymes although their amino acid sequences show a very high degree of structural similarity (*4, 15, 19*). In addition, differences in cell cycle expression profiles and nuclear localization have been observed for the two isoforms. Whereas topoisomerase IIα shows proliferation and cell cycle-dependent expression, topoisomerase IIβ is mainly expressed in stationary cells and at a constant level during all phases of the cell cycle (*15, 21, 29, 45*). Contradictory results have been reported on the nuclear localization of the two topoisomerase II isoforms. Several studies have shown that topoisomerase IIα is ubiquitously located in the nucleoplasm. The β isoform, however, has been claimed to be only located in the nucleoli (*27, 46*), but a recent study suggests that this isoform is exclusively present in the extranucleolar nucleoplasm (*26*). The discrepancy concerning topoisomerase II localization possibly reflects differences in the specificities of the antibodies used in these studies.

Topoisomerase IIα and IIβ are phosphoproteins (*35*), and it has been suggested that phosphorylation of the enzymes serves as a regulatory mechanism throughout the cell cycle (*10, 32*). Both isoforms are hyperphosphorylated during the $G_2$/M phase, and the modification gives rise to a changed electrophoretic mobility which is most pronounced for topoisomerase IIβ (*8, 9, 20, 21*). Previous studies have indicated that phosphorylation correlates with an increased enzymatic activity (*1, 13, 43*), but a more recent study has questioned this role of phosphorylation (*22*). As an alternative, phosphorylation may direct the formation of higher order complexes. Vassetzky et al. (1994) thus reported a stimulation of multimerization of yeast topoisomerase II in vitro upon phosphorylation, which could be reversed by dephosphorylation of the enzyme (*39*).

Several phosphorylation sites have been located to the extreme N- and C-terminal domains of eukaryotic topoisomerase IIα (*10, 18, 42, 43*) where mainly serine and to a lower extent threonine residues are phosphorylated (*8*). These amino acids are also labeled in topoisomerase IIβ, but so far specific sites have not been determined in this enzyme. Much effort has been paid to identify the kinases responsible for topoisomerase II phosphorylation. In vitro studies have shown that casein kinase II (CKII) (*1, 12, 13, 42*), protein kinase C (*14, 30*), and cdc2 kinase (*12*) are able

† This work was supported by the Danish Cancer Society (95-100-40, 97-100-32), the Danish Center for Human Genome Research, the Danish Natural Science Research Council, and the Danish Center for Molecular Gerontology. H.B. was supported by the Deutsche Forschungsgemeinschaft (Bi 541/1-1).

* Corresponding author. Tel: +45 89 422600. Fax: +45 89 422612. E-mail: aha@mbio.aau.dk.

‡ Present address: Department of Medical Biochemistry, University of Aarhus, Ole Worms Allé, Bldg. 170, 8000 Århus C, Denmark.

§ These authors contributed equally to the work.

|| Present address: Division of Internal Medicine, Department of Hematology/Oncology, Hannover Medical School, Carl-Neuberg-Str. 1, 30623 Hannover, Germany.

10.1021/bi981391l CCC: $15.00 
Published on Web 11/05/1998

to phosphorylate topoisomerase II. Two-dimensional tryptic phosphopeptide mapping performed in vivo and in vitro has, however, pointed to CKII as the most likely candidate for in vivo phosphorylation of topoisomerase IIα, although kinases other than CKII are involved in the specific phosphorylation of topoisomerase IIα as well as II$\beta$ in mitosis (*9, 20, 43*).

In a previous study, we have demonstrated that topoisomerase IIα/$\beta$ heterodimers are present in HeLa cells where the two forms naturally coexist and in a yeast model system where the two human topoisomerase II isoforms are coexpressed. Furthermore, purification of human topoisomerase II heterodimers showed that this new population of topoisomerase II is catalytically active (*5*). In the present paper, we have further characterized human topoisomerase IIα/$\beta$ heterodimers concerning their abundance and phosphorylation status. Heterodimers are not restricted to HeLa cells but are present in different mammalian cell lines, where up to 25% of the entire $\beta$ population is involved in heterodimer formation. The α subunits in heterodimers are phosphorylated to a much lower extent compared to homodimeric α, and in contrast to the latter enzyme neither heterodimers nor homodimeric $\beta$ can be immunoprecipitated from HeLa cells together with an active kinase. The difference in phosphorylation of α/$\beta$ heterodimers compared to homodimeric α enzymes suggests that these topoisomerase II subclasses are differentially regulated in the cell. However, such a differential regulation cannot be explained in terms of phosphorylation-mediated changes in heterodimer stability since the stability of the topoisomerase II heterodimers was shown not to be affected by their phosphorylation status.

## EXPERIMENTAL PROCEDURES

*Cell Cultures and in Vivo Labeling of HeLa Cells.* HeLa, HL60, and NIH3T3 cells were maintained as subconfluent monolayer cultures grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 3 mM stabilized L-glutamine, and antibiotics at 37 °C under a humidified 5% $CO_2$ atmosphere. For metabolic labeling, medium was replaced by phosphate-free DMEM containing dialyzed 10% FCS. After 2 h, [$\gamma$-$^{32}$P]orthophosphate was added at 100 $\mu$Ci/mL, and cells were incubated for an additional 6 h at 37 °C.

*Cell Extract Preparation and Immunoprecipitation.* Cell extract was prepared from logarithmically growing cells. Cells were washed twice in phosphate-buffered saline (PBS) (140 mM NaCl/2.5 mM KCl/1.5 mM $KP_i$, pH 7.4) followed by resuspension in 10 mM Tris·HCl,[1] pH 7.5/4 mM $MgCl_2$/5 mM KCl/0.2% Nonidet P-40/10 mM *p*-nitrophenyl phosphate/10 mM PMSF. Immunoprecipitation was performed by incubation of the extract with the respective antibodies in immunoprecipitation (IP) buffer (100 mM Tris·HCl, pH 8.0/1 M NaCl/0.75% Nonidet P-40/2 mM EDTA/10 mM *p*-nitrophenyl phosphate/1 mM PMSF). A 6% solution of Protein A-Sepharose beads in IP buffer was subsequently added, and after further incubation for 2 h, the immunocomplexed beads were washed several times in IP buffer suplemented with 0.25% Nonidet P-40 and 0.5% Triton X-100 and once in 10 mM Tris·HCl, pH 8.0/1 mM EDTA. The resulting immunocomplexes adsorbed to the beads were extracted with SDS/Laemmli buffer, heated to 100 °C for 3 min, and analyzed by 7.5% SDS−PAGE followed by silver staining, immunostaining, or autoradiography.

*Antibodies and Immunostaining.* DNA topoisomerase IIα from human and mouse cells was recognized by the polyclonal rabbit anti-topoisomerase IIα antibody raised against a C-terminal peptide (R-A-K-K-P-I-K-Y-L-E-E-S-D-E-D-D-L-F) of human topoisomerase IIα (diluted 1:10.000) (Genosys). The C-terminal sequence is conserved between human and mouse topoisomerase IIα. The polyclonal rabbit antibody recognizing topoisomerase II$\beta$ was raised against a C-terminal peptide (E-E-D-D-V-D-F-A-M-F-N) of the human enzyme (diluted 1:10.000) (*6*). The specificity of antibodies during immunostaining and immunoprecipitation was verified by cross-incubation of antigens and antibodies in both procedures and by immunoprecipitation carried out with rabbit preimmune serum (*5*). For immunostaining, cell extracts or immunoprecipitated materials were submitted to SDS−PAGE, and proteins were transferred to nitrocellulose (Schleicher and Schüll; 0.45 $\mu$m) using a semidry blotting method. The filter was subsequently incubated with the appropriate topoisomerase II-specific antibodies. Gold-particle-labeled anti-rabbit antibodies were used as secondary antibodies and finally visualized by silver enhancement (Amersham).

*Alkaline Phosphatase Treatment.* Logarithmically growing HeLa cells were harvested, and cell extracts were prepared as described (*5*); 10X alkaline phosphatase buffer (500 mM Tris·HCl, pH 9.0/10 mM $MgCl_2$) and 100 units of alkaline phosphatase from calf intestine (Sigma) were added to 200 $\mu$L of cell extract. The sample was incubated with gentle agitation at 30 °C for 1 h and afterward transferred to 4 °C. Immunoprecipitation was performed as described above. In control experiments, cell extracts were incubated under similar conditions but without alkaline phosphatase. Alkaline phosphatase treatment of immunocomplexed topoisomerase II was performed as follows. Protein A-Sepharose beads containing topoisomerase II precipitated by either anti-topoisomerase IIα or II$\beta$ antibodies were washed several times with modified IP buffer (100 mM Tris·HCl, pH 8.0/700 mM NaCl/0.75% Nonidet P-40/1% Triton X-100/10 mM *p*-nitrophenyl phosphate/10 mM PMSF) and four times with alkaline phosphatase buffer. The samples were subsequently treated with 5−10 units of alkaline phosphatase for 1 h at 30 °C in a 50-$\mu$L reaction volume. In control experiments the samples were treated as above but without alkaline phosphatase.

*Topoisomerase II Phosphorylation by a Copurified Kinase or Casein Kinase II.* Immunoprecipitated material was resuspended in kinase buffer (50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/150 mM KCl/0.1 mM EDTA) supplemented with 10 $\mu$M [$\gamma$-$^{32}$P]ATP. The phosphorylation reaction was carried out for 10−30 min at 30 °C and stopped by addition of 10 mM EDTA. The samples were washed several times in IP buffer supplemented with 0.1% SDS and once with 10 mM Tris·HCl (pH 8.2). Phosphorylation by casein kinase II was perfomed as described above except that the immunoprecipitated material was incubated with 0.05 mU of CKII (Boehringer) for 30 min at 30 °C and stopped by addition

[1] Abbreviations: CKII, casein kinase II; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

of 10 mM EDTA.

*Densitometric Scanning.* The ratio of topoisomerase IIα and IIβ subunits in immunoprecipitated topoisomerase II material was determined by densitometric scanning of silver stainings using a Shimadzu model CS930 dual-wavelength thin-layer chromatoscanner. Linearity of quantitation of silver-stained gels was ensured by loading different amounts of the samples on the gel.

## RESULTS AND DISCUSSION

*Detection of DNA Topoisomerase IIα/β Heterodimers in Mammalian Cells.* In a previous study, we demonstrated that HeLa cells contain topoisomerase IIα/β heterodimers as well as the corresponding topoisomerase II homodimeric forms (*5*). To determine the abundance of heterodimers, topoisomerase II was precipitated from logarithmically growing HeLa cells using anti-topoisomerase IIα or IIβ antibodies. The precipitated material was analyzed by immunostaining with the anti-topoisomerase IIα and IIβ antibodies to verify the presence of the two subunits (Figure 1A) and by silver staining (Figure 1B). The relative amounts of topoisomerase II heterodimers and homodimers were quantified by densitometric scanning of the bands representing the topoisomerase IIα and IIβ subunits in the silver staining (Figure 1B). The material precipitated by the anti-topoisomerase IIβ antibody (IPβ; Figure 1B, lanes 2 and 3) displays a ratio of 1:4 between the topoisomerase IIα and IIβ subunits, and no significant variability has been discovered in the relative levels of topoisomerase IIα and IIβ subunits when results from different experiments were compared. The α subunits present in the IPβ material represent solely heterodimeric α molecules, and since one β subunit is required per heterodimer, the obtained α:β ratio implies that 25% of the entire topoisomerase IIβ population is present in a heterodimeric form. In other words, for every three molecules of topoisomerase IIβ homodimers, two heterodimers exist in HeLa cells. As evident from the material precipitated by the anti-topoisomerase IIα antibody (IPα; Figure 1B, lane 1), the ratio of heterodimeric to homodimeric α enzyme is much lower than 1:4 due to the significantly higher expression level of topoisomerase IIα compared to the β form in human cells (*23, 44*). On the basis of an earlier suggested difference of 10:1 in expression of topoisomerase IIα and IIβ in human cells (*23*), the fraction of α subunits taking part in heterodimerization may be less than 5%.

To demonstrate that topoisomerase IIα/β heterodimers are not restricted to HeLa cells, topoisomerase II was precipitated from different mammalian cell types and the presence of heterodimers was verified by immunostain analysis as described above. The results obtained from the use of human amnion cells (AMA), human promyelocytic leukemia cells (HL60), and mouse fibroblasts (NIH3T3) are presented in Figure 1C. As shown all three cell types allow precipitation of topoisomerase IIα when the β antibody is used for immunoprecipitation (Figure 1C, upper panel, lanes 1, 3, and 5) and of topoisomerase IIβ when the α antibody is used (Figure 1C, lower panel, lanes 2, 4, and 6) demonstrating an existence of topoisomerase II heterodimers. Moreover, no significant difference in the level of topoisomerase II heterodimers exists in the analyzed cell lines as seen from

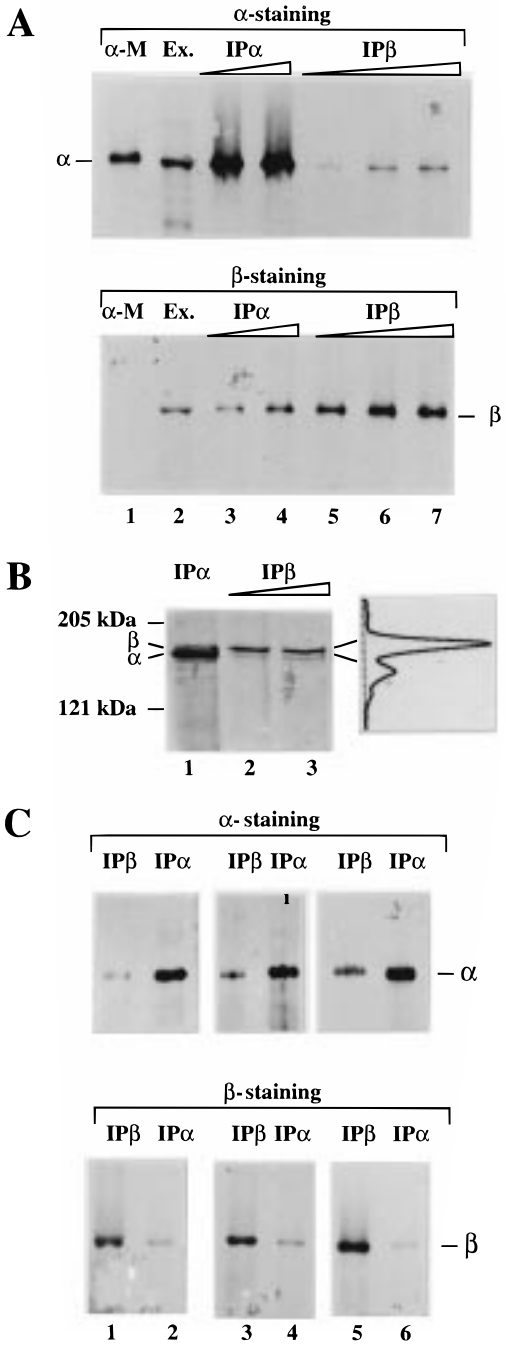


FIGURE 1: Abundance of topoisomerase IIα/β heterodimers in mammalian cells. A: Extract from logarithmically growing HeLa cells was immunoprecipitated by either anti-topoisomerase IIα or anti-topoisomerase IIβ antibodies (IPα and IPβ, respectively). The precipitated material was analyzed by 7.5% SDS−PAGE followed by immunostaining with the anti-topoisomerase IIα (upper panel) or the anti-topoisomerase IIβ (lower panel) antibody. Lane 1, marker where C-terminally tagged human topoisomerase IIα was expressed and purified from yeast; lane 2, crude HeLa cell extract prior to immunoprecipitation; lanes 3 and 4, material precipitated by different amounts of the anti-topoisomerase IIα antibody; lanes 5−7, material precipitated by an increasing amount of the anti-topoisomerase IIβ antibody. B: Samples from lanes 4−6 in panel A were analyzed by 7.5% SDS−PAGE, and proteins were visualized by silver staining. A densitometric scanning of lane 3 is shown to the right. C: As in panel A except that extract was obtained from logarithmically growing human amnion cells (AMA) (lanes 1 and 2), human HL60 promyelocytic leukemia cells (lanes 3 and 4), and mouse NIH3T3 fibroblasts (lanes 5 and 6), and topoisomerase II was visualized by α staining (upper panel) or β staining (lower panel) after SDS−PAGE.

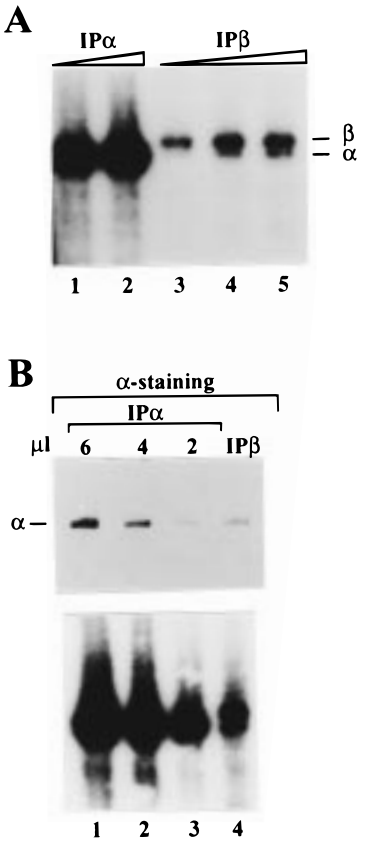


FIGURE 2: Phosphorylation of topoisomerase IIα/*β* heterodimers in vivo. A: Autoradiogram demonstrating the phosphorylation level of material precipitated from HeLa cell extracts by increasing amounts of anti-topoisomerase IIα (IPα; lanes 1 and 2) or anti-topoisomerase II*β* (IP*β*; lanes 3−5) antibodies. Extracts were obtained from $^{32}$P-labeled, logarithmically growing HeLa cells and analyzed by SDS−PAGE and immunoblotting before autoradiography. The film was exposed for 20 h. B: Immunoprecipitation with anti-topoisomerase II antibodies was carried out on extracts from $^{32}$P-labeled HeLa cells. Different amounts of material precipitated by the anti-topoisomerase IIα antibody (lanes 1−3, upper panel) are compared to a given amount of heterodimeric α obtained after immunoprecipitation with the anti-topoisomerase II*β* antibody (IP*β*; lane 4, upper panel). Lower panel, autoradiogram of the immunoblot presented in the upper panel.

comparison of the levels of heterodimeric *β* in Figure 1A, lower panel, lane 3, and Figure 1C, lower panel, lanes 2, 4, and 6. The results strongly indicate that the heterodimeric subpopulation of topoisomerase II has a ubiquitous nature in human cells and in mammalian cells in general.

*Phosphorylation of DNA Topoisomerase IIα/β Heterodimers in Vivo.* Phosphorylation is an important post-translational modification by which various protein functions and enzymatic activities are regulated (*17, 41*). Previous studies have shown that both isoforms of human topoisomerase II are phosphoproteins, where their phosphorylation level varies throughout the cell cycle (*9, 20, 21, 23, 42*). To investigate whether topoisomerase IIα/*β* heterodimers are phosphorylated in vivo, logarithmically growing HeLa cells were labeled with $^{32}$P and used for immunoprecipitation analysis. The labeled precipitate was resolved by SDS−PAGE, and the phosphorylation level of topoisomerase II was visualized by autoradiography (Figure 2A). In the material precipitated by the anti-topoisomerase II*β* antibody (IP*β*; lanes 3−5), two bands migrating as the topoisomerase IIα and II*β* subunits are detected. The identity of the bands were verified by immunostaining (data not shown). The lower band, which represents solely heterodimeric topoisomerase IIα subunits, demonstrates that at least the α subunit of heterodimers is phosphorylated in vivo. The upper band demonstrates that a mixture of hetero- and homodimeric topoisomerase II*β* is phosphorylated (Figure 2A, lanes 3−5). However, it is not possible to determine whether heterodimeric *β* subunits per se are phosphorylated since a potential phosphorylation of these subunits is masked by the strong phosphorylation of topoisomerase IIα as evident in the IPα experiment (Figure 2A, lanes 1 and 2).

To determine possible differences in the phosphorylation level of heterodimeric versus homodimeric α subunits, a comparative immunoprecipitation experiment was performed using extracts from $^{32}$P−labeled cells. In this analysis, a given quantity of heterodimeric α (IP*β*) was compared to different amounts of α subunits precipitated by the anti-topoisomerase IIα antibody (IPα) either by immunostaining (Figure 2B, upper panel) or by autoradiography (Figure 2B, lower panel). Interestingly, a difference in the phosphorylation level is observed when similar amounts of α precipitated with the two antibodies are compared (lanes 3 and 4). Thus, the phosphate incorporation in the heterodimeric α subunits (lane 4) is much lower than the incorporation in the predominantly homodimeric topoisomerase IIα population shown in lane 3. Disregarding the negligible amount of labeling derived from heterodimers in lane 3, we have estimated the difference in phosphorylation level of homodimeric versus heterodimeric α to be more than 20-fold. The results demonstrate that although heterodimers are phosphorylated in vivo the α subunit of heterodimers is phosphorylated to a significantly lower extent compared to their homodimeric topoisomerase IIα counterparts.

*DNA Topoisomerase IIα/β Heterodimers Are Not Associated with a Kinase Activity in Vivo That Is Able To Mediate Their Phosphorylation upon Immunoprecipitation.* It has previously been suggested that topoisomerase II from yeast is strongly associated with CKII in vivo (*7, 12*), and the kinase activity has been copurified with topoisomerase II from several sources (*31, 33*). To seek an explanation for the differential phosphorylation of heterodimeric versus homodimeric α enzymes, we investigated the interaction between human topoisomerase IIα/*β* heterodimers and a possible kinase activity, as the difference in phosphorylation might reflect a change in the affinity of the two topoisomerase II populations for a kinase. To investigate this possibility, topoisomerase II was recovered from HeLa cell extracts by immunoprecipitation under highly stringent conditions (1 M NaCl). The precipitated material was then submitted to immunostaining (Figure 3A, upper panel) and autoradiography (Figure 3A, lower panel) after an in vitro phosphorylation assay, where the precipitate was incubated in kinase buffer containing [*γ*-$^{32}$P]ATP (Figure 3A). As evident in Figure 3A (lower panel, lanes 1−3), the anti-topoisomerase IIα antibody precipitate (IPα) contained a kinase activity able to phosphorylate topoisomerase II. However, no incorporation of phosphate was detected after immunoprecipitation with the anti-topoisomerase II*β* antibody (IP*β*, lanes 4−6), demonstrating that neither topoisomerase II heterodimers nor homodimeric *β* associate with a kinase activity which is able to mediate their phosphorylation under the employed conditions. To eliminate the

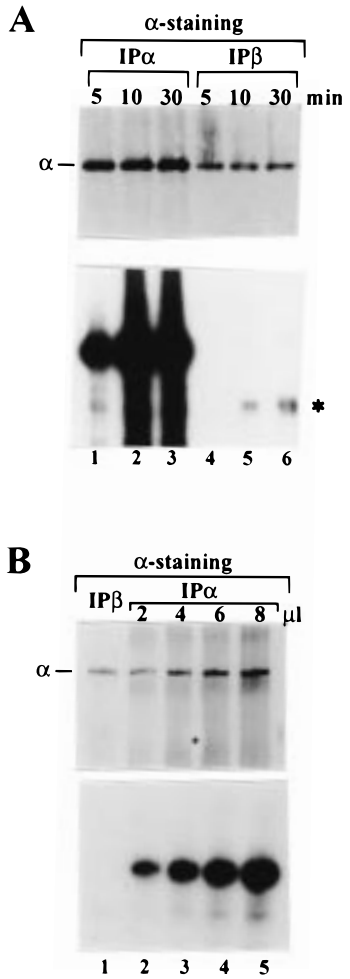


FIGURE 3: Topoisomerase IIα/β heterodimers and β homodimers are not associated with an active kinase. A: Material immunoprecipitated from HeLa cell extract by either anti-topoisomerase IIα (IPα; lanes 1−3) or anti-topoisomerase IIβ (IPβ; lanes 4−6) antibodies was phosphorylated in the presence of [γ-$^{32}$P]ATP for 5, 10, or 30 min as indicated. Samples were analyzed by 7.5% SDS−PAGE followed by immunostaining with the anti-topoisomerase IIα antibody (upper panel). Lower panel, autoradiogram of the immunoblot presented in the upper panel. The asterisk at the right margin denotes the position of a protein with a molecular mass of less than 100 kDa. B: Different amounts of material precipitated by the anti-topoisomerase IIα antibody (IPα; lanes 2−5) were analyzed by 7.5% SDS−PAGE followed by immunostaining with the anti-topoisomerase IIα antibody (upper panel). The samples were compared to a specific amount of material precipitated by the anti-topoisomerase IIβ antibody (IPβ; lane 1). Lower panel, autoradiogram of the immunoblot presented in the upper panel. The film was exposed for 3 h.

possibility that the result reflects use of different amounts of precipitate, a given quantity of IPβ was titrated against IPα and visualized by immunostaining using anti-topoisomerase IIα antibodies (Figure 3B, upper panel) and by autoradiography (Figure 3B, lower panel). Of the equivalent amounts of α subunits presented in Figure 3B, lanes 1 and 2, only the predominantly homodimeric topoisomerase IIα presented in lane 2 is phosphorylated. The α subunit from heterodimers (lane 1) is not phosphorylated to a detectable level. Overexposure of the autoradiograms in Figure 3 revealed a weak band with a molecular mass of less than 100 kDa (denoted by an asterisk). This band might result from autophosphorylation of a kinase which is associated with heterodimers but unable to carry out their phosphorylation.

Our results demonstrate that only human topoisomerase IIα homodimers coimmunoprecipitate with a kinase activity able to mediate their phosphorylation. The interaction between heterodimers and the kinase(s) responsible for their in vivo phosphorylation may be temporary or too weak to allow the kinase(s) to coimmunoprecipitate. The observed difference between topoisomerase II heterodimers and homodimeric α enzymes concerning their association with an active kinase might account for the differential phosphorylation observed with these topoisomerase II counterparts in vivo. However, it cannot be excluded that heterodimers as well as homodimeric β in fact are associated with a kinase but the immunocomplexes formed with the β antibody are resistant to phosphorylation by the associated kinase.

*Exogenous Casein Kinase II Is Able To Phosphorylate Topoisomerase IIα/β Heterodimers.* Several studies have demonstrated that human topoisomerase IIα in vitro can be phosphorylated by CKII (*1*, *12*, *13*, *42*), protein kinase C (*14*, *30*), and cdc2 kinase (*12*), although in vivo studies point to CKII as one of the major candidates for topoisomerase II phosphorylation in the cell (*10*). Furthermore, it has been speculated that this kinase also phosphorylates several sites in topoisomerase IIβ (*21*). To investigate whether immunoprecipitated topoisomerase II heterodimers can act as a substrate for exogenously added CKII, the material immunoprecipitated by anti-topoisomerase II antibodies was used in a CKII phosphorylation assay (Figure 4A,B). As expected, the material precipitated by anti-topoisomerase IIα antibodies (IPα), which consists mainly of topoisomerase IIα homodimers, was strongly phosphorylated in vitro (Figure 4B, lane 2), although some of the labeling is caused by the endogenous kinase present in the IPα precipitate (Figure 3A). Analysis of the topoisomerase IIβ antibody precipitate (IPβ), which is free of any endogenous kinase activity, reveals that topoisomerase IIβ subunits as well as heterodimeric α subunits also are phosphorylated by CKII (Figure 4B, lane 4). The result demonstrates that material precipitated by the anti-topoisomerase IIβ antibody is not resistant toward phosphorylation. As the β subunits in the IPβ material are a mixture of homo- and heterodimeric β, we cannot at present conclude to what extent heterodimeric β is phosphorylated. But since the α subunits in the IPβ material are purely of heterodimeric nature, our result demonstrates that at least the α subunit of heterodimers can act as a substrate for CKII in vitro. However, further investigations are required to clarify whether CKII plays any role for heterodimer phosphorylation in vivo. Also, it is so far unknown to what extent other kinases are involved in phosphorylation of heterodimers.

To estimate the relative levels of phosphorylation of homo- and heterodimeric α subunits, equal amounts of α subunits from IPα and IPβ material that had prior been submitted to a CKII phosphorylation assay were analyzed by immunostaining (Figure 4C) and autoradiography (Figure 4D). As mentioned above some of the homodimeric topoisomerase IIα labeling shown in Figure 4D, lane 2, is caused by the endogenous kinase being tightly associated with this enzyme. However, the contribution to overall phosphorylation by the endogenous kinase is relatively minor. This is seen from comparison of phosphorylation levels obtained from phos-

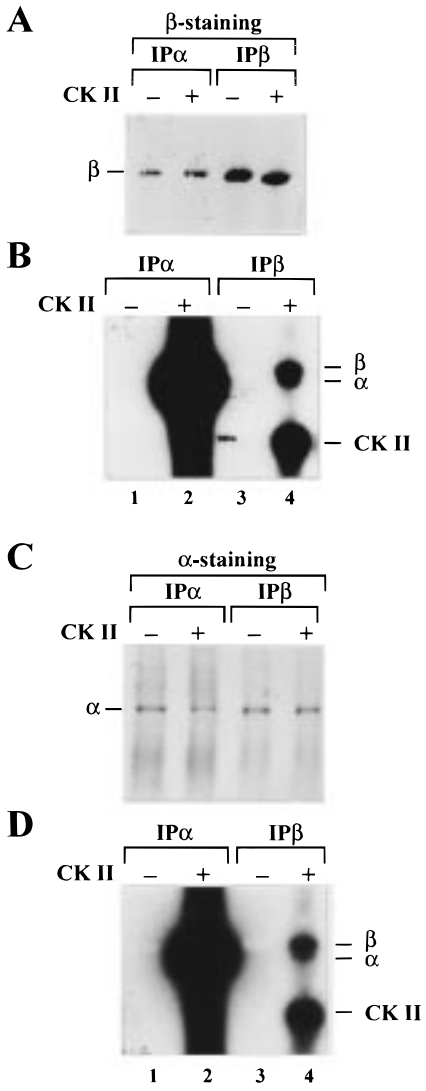


FIGURE 4: Human topoisomerase IIα/*β* heterodimers can be phosphorylated in vitro by CKII. Extracts from logarithmically growing HeLa cells were immunoprecipitated with anti-topoisomerase IIα (IPα; lanes 1 and 2) or anti-topoisomerase II*β* (IP*β*; lanes 3 and 4) antibodies. The precipitated material was phosphorylated by the addition of CKII (lanes 2 and 4) or incubated in kinase buffer without kinase enzyme and without [$\gamma-^{32}$P]ATP (lanes 1 and 3). A: Samples were analyzed by 7.5% SDS−PAGE followed by immunostaining with the anti-topoisomerase II*β* antibody. B: Autoradiogram of the immunoblot presented in panel A. The film was exposed for 3 h. The positions of topoisomerases IIα and II*β* and CKII are indicated to the right. C: Equal amounts of homo- and heterodimeric α subunits from IPα and IP*β* material used in A were analyzed by 7.5% SDS−PAGE followed by immunostaining with the anti-topoisomerase IIα antibody. D: Autoradiogram of the immunoblot presented in panel C. The film was exposed for 3 h.

phorylation of equal amounts of heterodimeric α by the endogenous kinase alone (Figure 3B, lane 3) and by a combination of CKII and the endogenous kinase (Figure 4D, lane 2). Thus, the difference in accessibility of homodimeric versus heterodimeric α subunits to CKII can roughly be estimated by comparing levels of homo- and heterodimeric α phosphorylation in Figure 4D, lanes 2 and 4, respectively. Here it is clearly seen that homodimeric topoisomerase IIα is highly preferred as a substrate for CKII as compared to heterodimeric α, but a quantitative estimation of the relative phosphorylation levels cannot be made due to the very strong phosphorylation of homodimeric topoisomerase IIα.

Earlier experiments have demonstrated that topoisomerase II*β* changes its electrophoretic mobility as a result of hyperphosphorylation in mitosis, although the kinase responsible for this modification has not been discovered yet (*9*, *21*). As visualized by immunostaining in Figure 4A, neither heterodimeric *β* subunits (lanes 1 and 2) nor *β* subunits precipitated by the anti-topoisomerase II*β* antibody (lanes 3 and 4) change their electrophoretic mobility after CKII-mediated phosphorylation. Thus, CKII is not the kinase responsible for the shift in electrophoretic mobility of topoisomerase II*β* observed in mitotic cells, or additional nuclear factors are required to obtain the mobility shift.

*Dephosphorylation of Topoisomerase II Does Not Influence the Stability of α/β Heterodimers.* The results described above show that topoisomerase IIα/*β* heterodimers can be phosphorylated in vivo as well as in vitro. Previously, it has been suggested that the major sites of phosphorylation are localized in the C terminal part of topoisomerase II (*10*, *34*, *42*). Phosphorylation at these sites can direct a multimeric nature of yeast topoisomerase II (*39*), which again can be reversed by dephosphorylation of the enzyme. To investigate the role of phosphorylation on subunit interactions in α/*β* heterodimeric enzymes, we analyzed whether the stability of heterodimers is affected by their dephosphorylation. For this purpose, cell extract from labeled HeLa cells was divided into two parts where one was treated with an excess of alkaline phosphatase before immunoprecipitation was carried out, and the other was left untreated. As demonstrated by immunostaining in Figure 5A, phosphatase-treated material and untreated material show the same level of heterodimers (compare lanes 2 and 4 in upper panel and lanes 1 and 3 in lower panel). The autoradiogram of the resolved immunoprecipitated material (Figure 5B) confirms that the dephosphorylation of topoisomerase II by alkaline phosphatase was complete. Thus, the data presented here demonstrate that the phosphorylation status of topoisomerase II does not influence the stability of α/*β* heterodimers. Identical results were obtained when dephosphorylation was performed after immunoprecipitation (data not shown). Furthermore, topoisomerase IIα/*β* heterodimers expressed in yeast cells and purified by metal-chelating chromatography as previously described (*5*) retained their heterodimeric structure after dephosphorylation (unpublished data).

In conclusion, we have demonstrated that human topoisomerase IIα/*β* heterodimers are phosphorylated in vivo, where the α part of the heterodimers is phosphorylated to a significantly lower extent compared to homodimeric α enzymes. In sharp contrast to topoisomerase IIα homodimers, immunoprecipitated heterodimers as well as topoisomerase II*β* homodimers are not associated with a kinase activity able to mediate their phosphorylation. Different possibilities may account for these observations. First, topoisomerase II heterodimers and *β* homodimers might be differently located in the nucleus compared to their homodimeric α counterparts. In this way, they might never be in contact with the kinase(s) which strongly phosphorylates topoisomerase IIα and precipitates together with this enzyme. Second, since heterodimers are more similar to *β* homodimers in their behavior concerning kinase interaction, acquisition of a *β* subunit in addition to the α subunit in heterodimers might lead to a structural change in the enzyme

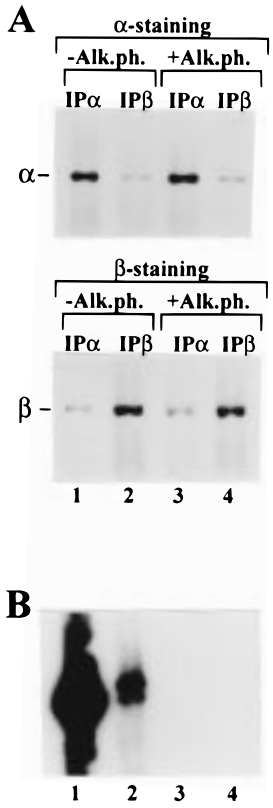


FIGURE 5: Stability of human topoisomerase IIα/β heterodimers is not affected by their phosphorylation status. A: Cell extract from logarithmically growing $^{32}$P-labeled HeLa cells was incubated in alkaline phosphatase buffer in the presence or absence of alkaline phosphatase as indicated, and immunoprecipitation was subsequently performed using either anti-topoisomerase IIα (IPα; lanes 1and 3) or anti-topoisomerase IIβ (IPβ; lanes 2 and 4) antibodies. Samples were analyzed by 7.5% SDS−PAGE followed by immunostaining with either anti-topoisomerase IIα (upper panel) or anti-topoisomerase IIβ (lower panel) antibodies. B: Autoradiogram of the immunoblots presented in panel A.

which could transiently or constantly disrupt the interaction between a kinase and the α subunit. Third, such a structural change might not disrupt the interaction to a kinase but prevent the kinase from reaching its normal recognition sites due to steric hindrance. Of the protein kinases known to phosphorylate topoisomerase II in vitro, much attention has been given to CKII as the sites recognized by this enzyme in vitro correspond to the major in vivo phosphorylation sites of topoisomerase II (*10, 11, 42*). Interestingly, exogenously added CKII can phosphorylate heterodimers, although it appears that heterodimeric α subunits are phosphorylated to a lower extent compared to homodimeric α in vitro (Figure 4B). The similarity in the phosphorylation pattern obtained in vivo and in vitro with homodimeric α being phosphorylated to a much higher level compared to heterodimeric α favors a hypothesis where CKII is the major kinase responsible for phosphorylation of topoisomerase II homodimers as well as heterodimers in vivo. According to this hypothesis, the difference in phosphorylation observed between the two topoisomerase II populations may reflect that some of the CKII-specific phosphorylation sites in the α subunit are not available in heterodimers or heterodimerization simply disturbs the interaction between CKII and the heterodimeric enzyme. A more detailed investigation of the subcellular localization of the human topoisomerase II populations is needed to clarify if differences in cellular compartmentalization of the enzymes also to some extent contribute to the diversities observed in phosphorylation. So far, data on topoisomerase II localization are highly controversial and hampered by the use of antibodies with differences in specificity. Unraveling the localization of heterodimers will probably await acquisition of antibodies specifically targeting this topoisomerase II subpopulation. An extensive in vitro characterization of purified topoisomerase II heterodimers will also be helpful in the further elucidation of the exact mechanisms responsible for the difference in phosphorylation observed in this study.

Several studies have so far been performed to identify the specific phospho-acceptor sites in eukaryotic topoisomerase II (*2, 9, 10, 42*). As evident from the results presented in this paper, attention is required when such studies are performed on topoisomerase IIβ as the obtained phosphorylation pattern may reflect a mixture of hetero- and homodimeric β enzymes. In the case of the more abundant topoisomerase IIα enzyme, such precautions are less important as heterodimeric α constitutes a minor fraction of the topoisomerase IIα population.

The exact role of topoisomerase II phosphorylation has not yet been clarified, although it has been suggested that phosphorylation regulates enzyme activity and mitotic functions of topoisomerase II as well as influences the higher order structure of the enzyme (*1, 39, 41*). The different mode of phosphorylation of topoisomerase IIα/β heterodimers compared to α homodimers as reported in this study may reflect an alternative regulation in the cell of heterodimers compared to the homodimeric counterparts. The effect of phosphorylation on heterodimer formation and activity is so far unknown. However, a total dephosphorylation of topoisomerase II does not influence the stability of heterodimers indicating that this modification is not a prerequisite for the essential contacts between the heterologous subunits in heterodimers.

Numerous studies have demonstrated that topoisomerase II is a primary cellular target for a wide variety of antitumor drugs (*16, 24, 38*). At this point, the drug susceptibility of topoisomerase II heterodimers remains to be determined. Previously, a correlation has been demonstrated between topoisomerase II phosphorylation and drug sensitivity both in vitro and in vivo, where hyperphosphorylation of topoisomerase II resulted in increased drug resistance (*14, 36*). The decreased phosphorylation level of heterodimers in relation to homodimeric α enzymes might indicate that heterodimers have an altered response to antitumor drugs compared to topoisomerase IIα. The natural balance between topoisomerase II hetero- and homodimers may therefore be important for the final cellular drug response.

## ACKNOWLEDGMENT

We thank Dr. F. Boege for kindly providing anti-topoisomerase IIβ antibodies.

## REFERENCES

1. Ackerman, P., Glover, C. V., and Osheroff, N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3164−3168.
2. Ackerman, P., Glover, C. V., and Osheroff, N. (1988) *J. Biol. Chem. 263*, 12653−12660.

3. Andersen, A. H., Bendixen, C., and Westergaard, O. (1996) *DNA Replication in Eukaryotic Cells*, pp 587−617, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
4. Austin, C. A., Marsh, K. L., Wasserman, R. A., Willmore, E., Sayer, P. J., Wang, J. C., and Fisher, L. M. (1995) *J. Biol. Chem. 270*, 15739−15746.
5. Biersack, H., Jensen, S., Gromova, I., Nielsen, I. S., Westergaard, O., and Andersen, A. H. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 8288−8293.
6. Boege, F., Andersen, A., Jensen, S., Zeidler, R., and Kreipe, H. (1995) *Am. J. Pathol. 146*, 1302−1308.
7. Bojanowski, K., Filhol, O., Cochet, C., Chambaz, E. M., and Larsen, A. K. (1993) *J. Biol. Chem. 268*, 22920−22926.
8. Burden, D. A., Goldsmith, L. J., and Sullivan, D. M. (1993) *Biochem. J. 293*, 297−304.
9. Burden, D. A., and Sullivan, D. M. (1994) *Biochemistry 33*, 14651−14655.
10. Cardenas, M. E., Dang, Q., Glover, C. V., and Gasser, S. M. (1992) *EMBO J. 11*, 1785−1796.
11. Cardenas, M. E., and Gasser, S. M. (1993) *J. Cell. Sci. 104*, 219−225.
12. Cardenas, M. E., Walter, R., Hanna, D., and Gasser, S. M. (1993) *J. Cell. Sci. 104*, 533−543.
13. Corbett, A. H., DeVore, R. F., and Osheroff, N. (1992) *J. Biol. Chem. 267*, 20513−20518.
14. DeVore, R. F., Corbett, A. H., and Osheroff, N. (1992) *Cancer Res. 52*, 2156−2161.
15. Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T., and Mirabelli, C. K. (1989) *Biochemistry 28*, 8154−8160.
16. Froelich Ammon, S. J., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 21429−21432.
17. Gasser, S. M., Walter, R., Dang, Q., and Cardenas, M. E. (1992) *Antonie Van Leeuwenhoek 62*, 15−24.
18. Ishida, R., Iwai, M., Marsh, K. L., Austin, C. A., Yano, T., Shibata, M., Nozaki, N., and Hara, A. (1996) *J. Biol. Chem. 271*, 30077−30082.
19. Jenkins, J. R., Ayton, P., Jones, T., Davies, S. L., Simmons, D. L., Harris, A. L., Sheer, D., and Hickson, I. D. (1992) *Nucleic Acids Res. 20*, 5587−5592.
20. Kimura, K., Nozaki, N., Enomoto, T., Tanaka, M., and Kikuchi, A. (1996) *J. Biol. Chem. 271*, 21439−21445.
21. Kimura, K., Nozaki, N., Saijo, M., Kikuchi, A., Ui, M., and Enomoto, T. (1994) *J. Biol. Chem. 269*, 24523−24526.
22. Kimura, K., Saijo, M., Tanaka, M., and Enomoto, T. (1996) *J. Biol. Chem. 271*, 10990−10995.
23. Kimura, K., Saijo, M., Ui, M., and Enomoto, T. (1994) *J. Biol. Chem. 269*, 1173−1176.
24. Liu, L. F. (1989) *Annu. Rev. Biochem. 58*, 351−375.
25. Maxwell, A., and Gellert, M. (1986) *Adv. Protein Chem. 38*, 69−107.
26. Meyer, K. N., Kjeldsen, E., Straub, T., Knudsen, B. R., Hickson, I. D., Kikuchi, A., Kreipe, H., and Boege, F. (1997) *J. Cell. Biol. 136*, 775−788.
27. Negri, C., Chiesa, R., Cerino, A., Bestagno, M., Sala, C., Zini, N., Maraldi, N. M., and Astaldi Ricotti, G. C. (1992) *Exp. Cell. Res. 200*, 452−459.
28. Osheroff, N., Zechiedrich, E. L., and Gale, K. C. (1991) *Bioessays 13*, 269−273.
29. Prosperi, E., Sala, E., Negri, C., Oliani, C., Supino, R., Astraldi Ricotti, G. B., and Bottiroli, G. (1992) *Anticancer Res. 12*, 2093−2099.
30. Sahyoun, N., Wolf, M., Besterman, J., Hsieh, T., Sander, M., LeVine, H. D., Chang, K. J., and Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1603−1607.
31. Saijo, M., Enomoto, T., Hanaoka, F., and Ui, M. (1990) *Biochemistry 29*, 583−590.
32. Saijo, M., Ui, M., and Enomoto, T. (1992) *Biochemistry 31*, 359−363.
33. Sander, M., Nolan, J. M., and Hsieh, T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6938−6942.
34. Shiozaki, K., and Yanagida, M. (1992) *J. Cell. Biol. 119*, 1023−1036.
35. Taagepera, S., Rao, P. N., Drake, F. H., and Gorbsky, G. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8407−8411.
36. Takano, H., Kohno, K., Ono, M., Uchida, Y., and Kuwano, M. (1991) *Cancer Res. 51*, 3951−3957.
37. Tsai Pflugfelder, M., Liu, L. F., Liu, A. A., Tewey, K. M., Whang Peng, J., Knutsen, T., Huebner, K., Croce, C. M., and Wang, J. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7177−7181.
38. Vassetzky, Y. S., Alghisi, G. C., and Gasser, S. M. (1995) *Bioessays 17*, 767−774.
39. Vassetzky, Y. S., Dang, Q., Benedetti, P., and Gasser, S. M. (1994) *Mol. Cell. Biol. 14*, 6962−6974.
40. Wang, J. C. (1996) *Annu. Rev. Biochem. 65*, 635−692.
41. Watt, P. M., and Hickson, I. D. (1994) *Biochem. J. 303*, 681−695.
42. Wells, N. J., Addison, C. M., Fry, A. M., Ganapathi, R., and Hickson, I. D. (1994) *J. Biol. Chem. 269*, 29746−29751.
43. Wells, N. J., Fry, A. M., Guano, F., Norbury, C., and Hickson, I. D. (1995) *J. Biol. Chem. 270*, 28357−28363.
44. Woessner, R. D., Chung, T. D., Hofmann, G. A., Mattern, M. R., Mirabelli, C. K., Drake, F. H., and Johnson, R. K. (1990) *Cancer Res. 50*, 2901−2908.
45. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) *Cell Growth Differ. 2*, 209−214.
46. Zini, N., Martelli, A. M., Sabatelli, P., Santi, S., Negri, C., Astaldi Ricotti, G. C., and Maraldi, N. M. (1992) *Exp. Cell. Res. 200*, 460−466.

BI981391L