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A protein kinase activity tightly associated with *Drosophila* type II DNA topoisomerase

(eukaryotic topoisomerase/protein phosphorylation/*Drosophila melanogaster*)

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ABSTRACT **A protein kinase activity has been identified that is tightly associated with the purified *Drosophila* type II DNA topoisomerase. The kinase and topoisomerase activities are not separated when the enzyme is subjected to analytical chromatography (phosphocellulose, single-strand DNA agarose, and Sephacryl S-300) and analytical glycerol gradient sedimentation. These two activities are also inactivated to the same extent by either heat or *N*-ethylmaleimide treatment. The evidence, however, does not rule out the possibility that the kinase activity resides in a polypeptide other than the topoisomerase polypeptide. The topoisomerase-associated protein kinase activity is not stimulated by Ca^{2+} or cyclic nucleotides. It shows a broad substrate range, including the DNA topoisomerase itself, casein, phosvitin, and histones. Phosphoamino acid analysis identified phosphoserine and phosphothreonine in polypeptides modified by the topoisomerase-associated protein kinase. No similar activity has been identified previously in *Drosophila melanogaster*.**

Topoisomerase activities, first identified in *Escherichia coli* cell extracts (1), have been found in many organisms and are considered to be ubiquitous in nature (for reviews, see refs. 2–4). These enzymes are of two types: type I activities make transient single-strand breaks in DNA and can change the linking number of DNA molecules by 1 unit at a time, whereas type II topoisomerases make transient double-strand breaks, changing DNA linking number by units of 2. ω Protein and DNA gyrase, two bacterial enzymes, are the prototypes of type I and type II topoisomerases, respectively.

In the bacterial system, some functional roles of the topoisomerases are well defined. DNA gyrase plays a role in maintaining chromosomal supercoiling and functions in both the initiation and elongation phases of DNA replication (5–7). Recently, Steck and Drlica (8) presented evidence that DNA gyrase is also involved in facilitating separation of daughter chromosomes. Variation in the level of superhelical tension of the bacterial chromosome causes pleiotropic effects (2, 9, 10) and the maintenance of a given level of chromosomal supercoiling requires the coordinate activities of type I and type II bacterial topoisomerases (11, 12). Only recent work has addressed the question of how topoisomerase activities might be regulated in the bacterial cell: Menzel and Gellert (10) provided evidence for the existence of a mechanism by which synthesis of gyrase is regulated by the level of DNA supercoiling.

Identification of functional roles for the eukaryotic enzymes has been hampered by the lack of specific inhibitors and the greater difficulties in applying genetic techniques in eukaryotic organisms. Recently, mutant yeast strains with altered topoisomerase I activity have been reported (13). Goto and Wang (14) report the cloning of the yeast topoisomerase II gene and its identification as a single-copy essential gene. The mechanism by which the eukaryotic topoisomerase activities are regulated is not yet clear. Since variations in chromatin structure are associated with the processes of transcription, replication, and cell division, it is likely that topoisomerase-mediated alteration in chromatin structure will be regulated in a complex manner.

Recently, a type I topoisomerase isolated from Novikoff hepatoma cells has been shown to be a phosphoprotein (15). In our studies on *Drosophila* type II topoisomerase we have established that the enzyme isolated from the Kc_0 *Drosophila* cell culture line is present in a phosphorylated form (unpublished data). A substantial amount of evidence exists indicating the importance of protein phosphorylation as a mechanism by which enzyme activity is regulated in higher cells (16, 17). Thus, it will be of interest to determine if the functions of eukaryotic topoisomerase polypeptides might be regulated by phosphorylation.

The purification (18) and physical characterization (19) of *Drosophila* type II topoisomerase was presented previously. We report here the identification of a cyclic nucleotide- and Ca^{2+}-independent protein kinase activity that catalyzes the phosphorylation of the *Drosophila* type II topoisomerase and that remains tightly associated with the topoisomerase throughout its purification. Other substrates of the activity are *Drosophila* histones H1, H3, H4, and H2a, *Drosophila* protein D1, casein, and phosvitin.

MATERIALS AND METHODS

Enzymes and DNAs. *Drosophila* topoisomerase II was purified to homogeneity according to the previously described procedure (18). In some cases ion-exchange chromatography on Bio-Rex 70 substituted for the phosphocellulose column step. P4 knotted DNA was prepared according to Liu *et al.* (20). Single-strand f1 phage DNA was the generous gift of Robert Webster.

Protein Substrates for Kinase Assay. Phosvitin and casein were from Sigma. Samples were dephosphorylated at alkaline pH and high temperature according to Reimann *et al.* (21). They were neutralized and stored at −20°C at a protein concentration of 50 mg/ml. Calf thymus histones were from Sigma. *Drosophila* core histones, histone H1, and protein D1 were purified from *Drosophila* early embryos, according to the methods of Alfageme *et al.* (22, 23).

Topoisomerase Assay. *Drosophila* type II topoisomerase was assayed for unknotting activity as described (19). The purified topoisomerase had a specific activity of 5×10^5 units/mg, in which 1 unit of activity is defined as the amount of enzyme required to unknot 0.3 μg of P4 knotted DNA under the standard assay conditions.

Kinase Assay. *Acid precipitation.* Assay solution contained 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1.0 mM dithiothreitol, and 1.0 μM [γ-^{32}P]ATP (0.1–0.25 Ci/mmol; 1 Ci = 37 GBq). Protein substrate was 1 mg of casein per ml or as indicated. The reaction was initiated by addition of enzyme (1–1.5 μl) to 20 μl of assay, and incubation proceeded at 30°C for 10–20 min. The reaction was terminated by addition of 1

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ml of 10% trichloroacetic acid/0.1 M sodium pyrophosphate. Precipitates were then collected on Whatman glass fiber filters (2.4 cm) by using an aspirator manifold. Each filter was washed with 25 ml of cold 10% trichloroacetic acid/0.1 M sodium pyrophosphate, rinsed with ethanol, dried, and assayed for radioactivity. In all cases, nonspecifically retained radioactivity was determined (no enzyme added), and this value was subtracted from all other samples. Filtration of the assay mixture prior to addition of substrate or enzyme was found to reduce background levels.

$NaDodSO_4$/polyacrylamide gel analysis. Assay solution and incubation was as above, except [γ-^{32}P]ATP was at a specific activity of 1.0–2.5 Ci/mmol. The reaction was terminated by the addition of 80 μl of phosphate-buffered saline (0.137 M NaCl/2.68 mM KCl/1.47 mM KH_2PO_4/8.09 mM Na_2HPO_4) containing 2.5 mM Na_3EDTA. Samples were then brought to 10% trichloroacetic acid. Precipitates were collected by centrifugation, washed once with acetone, and resuspended in gel loading buffer. $NaDodSO_4$/polyacrylamide gel analysis was carried out as described by Laemmli (24). Where histone substrates were used (acid precipitation or gel analysis), the trichloroacetic acid concentration for precipitation was 20%.

Densitometry. Photographs of ethidium-stained agarose gels and autoradiographs of dried $NaDodSO_4$/polyacrylamide gels were scanned by using a Zeineh soft laser scanning densitometer equipped with an integrator. In some cases, the relative area under appropriate peaks was determined by weighing tracing paper cut to the shape of the peak. For topoisomerase, some activity curves represent the average of more than one determination.

Phosphoamino Acid Analysis. Phosphorylated proteins were purified by $NaDodSO_4$/polyacrylamide gels, electroeluted, and precipitated with acid or precipitated directly from the kinase reaction. Precipitates were resuspended in H_2O, made 6 M in HCl, and hydrolyzed 1½–4 hr under vacuum. Hydrolysates were dried in a Savant Speed Vac concentrator and resuspended in a solution of 2 mg of phosphoserine, phosphothreonine, and phosphotyrosine per ml. Paper electrophoresis on Whatman 3MM paper was carried out at pH 3.5 (H_2O/acetic acid/pyridine, 945:50:5) at 1000 V for 3 hr, with cooling. Standards were visualized by ninhydrin staining.

Other. Sephacryl S-300 (Pharmacia), phosphocellulose (cellulose phosphate P-11, Whatman), and DNA-agarose (Bethesda Research Laboratories) were washed and equilibrated in buffer P (10% glycerol/15 mM $NaPO_4$, pH 7.2/0.1 mM Na_3EDTA/0.1 mM dithiothreitol/1.0 mM phenylmethylsulfonyl fluoride) or buffer P + 200 mM NaCl (Sephacryl S-300). Glycerol gradient sedimentation was carried out in buffer S (50 mM KPO_4, pH 7.2/200 mM NaCl/0.1 mM Na_3EDTA/0.1 mM dithiothreitol/1.0 mM phenylmethylsulfonyl fluoride). [γ-^{32}P]ATP synthesis was a modification of the procedure of Johnson and Walseth (25). [^{32}P]P_i was from New England Nuclear. Phosphoamino acid standards, heparin, and *N*-ethylmaleimide were from Sigma.

RESULTS

Purified *Drosophila* Topoisomerase II Catalyzes Autophosphorylation and Phosphorylation of Exogenous Protein Substrates. When purified *Drosophila* topoisomerase II was incubated in the presence of [γ-^{32}P]ATP, label was transferred to a protein species whose mobility in $NaDodSO_4$/polyacrylamide gels was identical to that of the intact topoisomerase subunit (Fig. 1, lanes 1 and 2). We detected no other protein species that was labeled to any significant extent, even upon overexposure of the autoradiogram. Since the ability to catalyze autophosphorylation is characteristic of all known kinase activities (17), it is possible that the phosphorylated

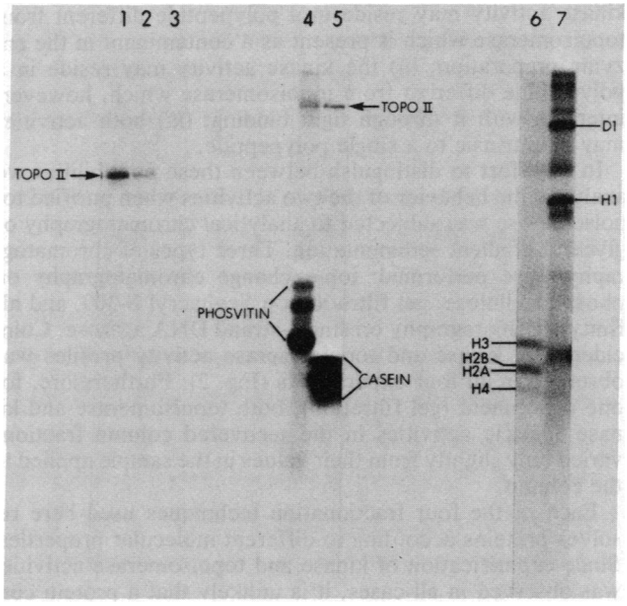

FIG. 1. Substrate specificity of topoisomerase-associated kinase. Kinase reactions were carried out in the presence of 0.50 μg (lane 1) or 0.25 μg (lanes 2 and 4–7) of *Drosophila* type II topoisomerase. In lane 3 no enzyme was added. Protein substrates were as follows: lane 4, phosvitin, 1 mg/ml; lane 5, casein, 1 mg/ml; lane 6, *Drosophila* core histone, 0.5 mg/ml; lane 7, *Drosophila* histone H1 and protein D1, 0.5 mg/ml. $NaDodSO_4$/polyacrylamide gels for analysis of the phosphoproteins were 8% in lanes 1–3, 10% in lanes 5 and 6, and 15% in lanes 6 and 7. Autoradiographs of the dried gels are shown. The ^{32}P-labeled topoisomerase polypeptide is indicated. All indicated polypeptides were identified by Coomassie blue staining.

species migrating with the topoisomerase subunit (M_r = 170,000) is a phosphorylated subunit of the species that is active as a kinase in our enzyme preparation.

As an initial step in characterizing this kinase activity, phosphate transfer to the following protein substrates was assayed: phosvitin, casein, *Drosophila* core histone, and *Drosophila* histone H1/protein D1 (Fig. 1, lanes 4, 5, 6, and 7, respectively). The results are shown in Fig. 1, in which the products of the phosphorylation reactions were resolved on $NaDodSO_4$/polyacrylamide gels that were subsequently dried and autoradiographed. All of these exogenous protein species served as substrates for the kinase activity. Of the *Drosophila* core histones, however, only histones H3 and H2a were phosphorylated efficiently. The autophosphorylation reaction was seen to proceed in the presence of each of the exogenous substrates (Fig. 1, arrow). In all cases, incubation of the substrate proteins in the complete kinase assay mixture showed them to be free of endogenous kinase activity (data not shown).

Using acid precipitation, we quantitated the amount of radioactivity incorporated into each protein substrate. The relative efficiency with which the substrates were phosphorylated, from greatest to least, was as follows: *Drosophila* histone H1/protein D1 > casein > *Drosophila* core histones > phosvitin (data not shown). Calf thymus core histones and histone H1 were also able to serve as phosphate acceptors.

Kinase and Topoisomerase Activities Copurify in Analytical Glycerol Gradient Sedimentation, Gel Filtration, Ion-Exchange, and Affinity Chromatography. The topoisomerase preparation obtained in our laboratory was >90% pure as estimated by densitometry of Coomassie-stained $NaDodSO_4$/polyacrylamide gels. Thus, three possible explanations for the presence of kinase activity associated with the purified *Drosophila* topoisomerase have been considered: (*i*) the

kinase activity may reside in a polypeptide different from topoisomerase which is present as a contaminant in the enzyme preparation; (*ii*) the kinase activity may reside in a polypeptide different from topoisomerase which, however, interacts with it through tight binding; (*iii*) both activities may be intrinsic to a single polypeptide.

In an effort to distinguish between these possibilities we analyzed the behavior of the two activities when purified topoisomerase was subjected to analytical chromatography or glycerol gradient sedimentation. Three types of chromatography were performed: ion-exchange chromatography on phosphocellulose, gel filtration on Sephacryl S-300, and affinity chromatography on single-strand DNA agarose. Coincidence of kinase and topoisomerase activity profiles was observed in all four experiments (Fig. 2). Furthermore, for one experiment (gel filtration), both topoisomerase and kinase specific activities in the recovered column fractions varied only slightly from their values in the sample applied to the column.

Each of the four fractionation techniques used here resolves proteins according to different molecular properties. Since copurification of kinase and topoisomerase activities was observed in all cases, it is unlikely that a protein contaminant present in the enzyme preparation contributed the kinase activity that was observed. If the two activities reside in separate protein species, they must then be interacting polypeptides.

Coinactivation of Kinase and Topoisomerase by *N*-Ethylmaleimide and Heat Treatments. Evidence suggestive of an intrinsic association of kinase and topoisomerase activities came from *N*-ethylmaleimide and heat inactivation curves for the two activities. As seen in Fig. 3*B*, the time course of inactivation by *N*-ethylmaleimide was coincident for topoisomerase and kinase. Similarly, for both activities the amount of residual activity after heating at each of four temperatures was equivalent (Fig. 3*A*).

The observation that topoisomerase and kinase activities were coinactivated by *N*-ethylmaleimide and heat treatments suggests that both activities might reside in a single polypeptide. Alternatively, two polypeptides may be similarly inactivated by these agents. Genetic analysis will be required to distinguish between these possibilities.

Determination of the Amino Acid Specificity of the Kinase Activity. Protein kinase activities can be distinguished by the identity of the amino acid that they convert to the corresponding phosphoamino acid. Serine/threonine and tyrosine specific kinase activities are known. The amino acids modified by the topoisomerase-associated kinase activity were determined in the following experiment. ^{32}P-labeled phosphoproteins were prepared by incubation of purified *Drosophila* topoisomerase II and [γ-^{32}P]ATP in the absence (autophosphorylation) or presence of casein as substrate. The protein then was purified either by polyacrylamide gel or by trichloroacetic acid precipitation and subjected to partial acid hydrolysis. High-voltage paper electrophoresis was used to resolve the hydrolysates. An autoradiograph of the electrophoretogram for each sample is shown in Fig. 4. The ^{32}P-labeled amino acids released by hydrolysis of casein comigrated with phosphoserine and phosphothreonine (Fig. 4, lane A). Hydrolysis of the autophosphorylated *Drosophila* type II topoisomerase yielded a major ^{32}P-labeled product comigrating with phosphoserine (Fig. 4, lane B). Upon further exposure of the autoradiogram, phosphothreonine could also be detected in this sample.

Characterization of the Topoisomerase-Associated Kinase Activity: Effectors and Inhibitors. The kinase activity was characterized further with respect to its divalent cation, ionic strength, and cyclic nucleotide requirements (Table 1). Mg^{2+} was the preferred divalent cation; Ca^{2+} and Mn^{2+} could substitute, although they were used less efficiently, resulting in 48% and 22% of maximal activity, respectively. The optimal Mg^{2+} concentration for activity was ≈10 mM

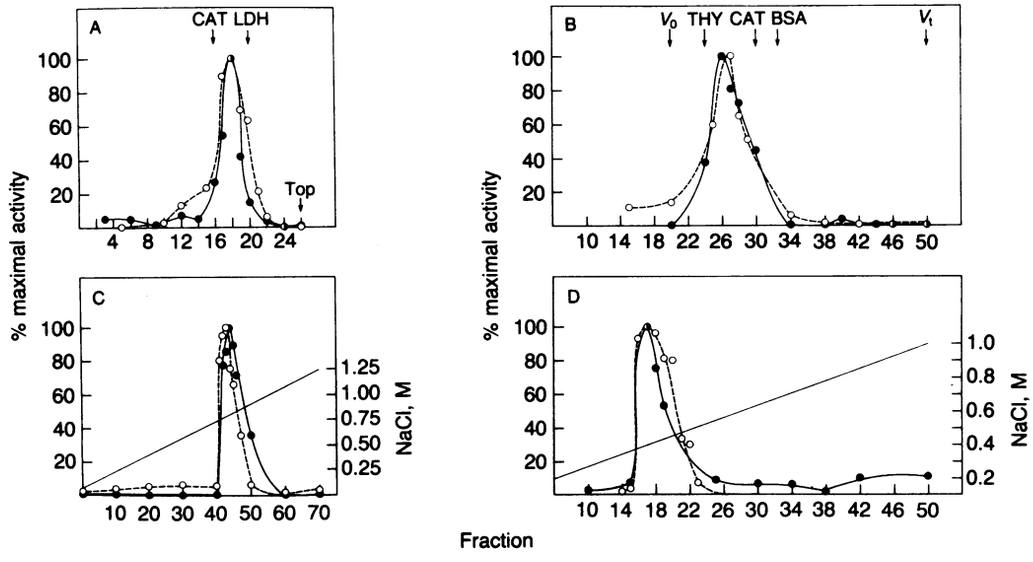

FIG. 2. Copurification of topoisomerase and kinase activities. ●—●, Kinase activity; ○---○, topoisomerase activity. (*A*) Glycerol gradient. Fifty micrograms of *Drosophila* topoisomerase II in 125 μl was layered onto a preformed 4.5-ml 30–60% glycerol gradient. Centrifugation was carried out at 50,000 rpm for 11 hr in a Sorvall TV-865 rotor. The tube was pierced at the bottom for collection of 125-μl fractions. The migration of marker enzymes was determined in a separate tube. (*B*) Sephacryl S-300. Three hundred micrograms of *Drosophila* topoisomerase II in 600 μl was underlaid onto a 16-ml SephaCryl S-300 column (0.7 × 42 cm). The column was operated at 4°C under gravity flow at 3 ml/hr, and 360-μl fractions were collected. Markers were chromatographed in a separate experiment. (*C*) Phosphocellulose. One hundred micrograms of *Drosophila* topoisomerase II in 200 μl was applied to a 0.5-ml phosphocellulose column (0.7 × 1.5 cm). The column was washed with 3 ml of buffer P + 0.1 M NaCl and eluted with a 10-ml gradient from 0.1 to 1.25 M NaCl in buffer P; 135-μl fractions were collected. (*D*) DNA agarose. Chromatography was as for the phosphocellulose column, except 150 μg of *Drosophila* topoisomerase II in 300 μl was applied, and the salt gradient for elution was from 0.1 to 1.0 M NaCl. CAT, THY, LDH, and BSA refer to catalase, thyroglobulin, lactate dehydrogenase, and bovine serum albumin, respectively. V_0 and V_t indicate void and included volume positions for gel filtration column.

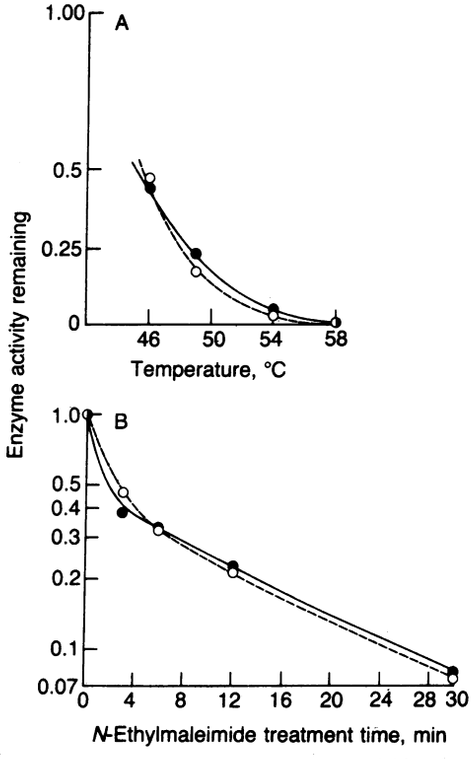

FIG. 3. Coinactivation of topoisomerase and kinase activities. (*A*) Heat inactivation of *Drosophila* type II topoisomerase was carried out by incubating 10-μl aliquots of the enzyme at 46, 49, 54, or 58°C for 5 min. The samples were then chilled on ice and topoisomerase and kinase activities were quantitated. (*B*) The time course of activity inactivation by *N*-ethylmaleimide is shown. *N*-Ethylmaleimide was added to topoisomerase to give a final concentration of 3.6 mM. Incubation was carried out at 15°C. Aliquots were taken at various time points, and dithiothreitol was added to a final concentration of 5 mM to quench the reaction. The enzyme was then assayed for kinase and topoisomerase activities.

(data not shown). Addition of increasing amounts of Na^+ gradually reduced activity, with 22% remaining at 200 mM

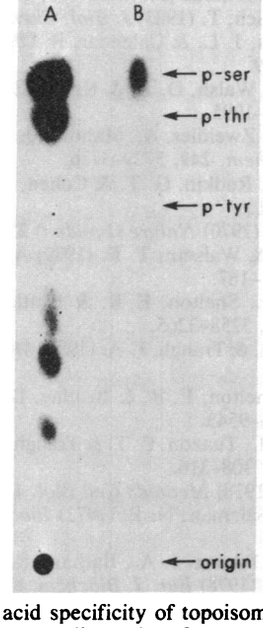

FIG. 4. Amino acid specificity of topoisomerase-associated kinase activity. The autoradiographs of paper electrophoretograms are shown. Lanes: A, phosphocasein hydrolysate; B, phosphotopoisomerase hydrolysate. p-ser, Phosphoserine; p-thr, phosphothreonine; p-tyr, phosphotyrosine.

Table 1. Kinase activity: effectors and inhibitors

Assay condition*	% maximal activity
Standard	100
No $MgCl_2$	2
+ 10 mM $CaCl_2$	48
+ 10 mM $MnCl_2$	22
5 mM $MgCl_2$ + 10.0 μM $CaCl_2$	100
1.0 μM cAMP	105
1.0 μM cGMP	106
NaCl	
50 mM	74
200 mM	22
ds DNA, 15 μg/ml	51
ss DNA, 15 μg/ml	30
Heparin	
20.0 μg/ml	9
2.0 μg/ml	42
0.2 μg/ml	61

Acid precipitation assays were carried out with casein as substrate. The values are the average of several determinations. Additions to assay are indicated. ds DNA and ss DNA, double-strand DNA and single-strand DNA, respectively. Maximal activity represents phosphate incorporation at the rate of 6.0 pmol/min per mg of protein.

*$MgCl_2$ concentration was 10 mM throughout, unless indicated otherwise.

NaCl. This is one of several parameters that distinguishes the kinase activity identified here from the previously identified *Drosophila* casein kinase II (ref. 26; see below for further discussion). In the presence of 5 mM $MgCl_2$, addition of Ca^{2+} in the concentration range 0.01–10.00 μM did not stimulate the kinase activity. Neither of the cyclic nucleotides cAMP or cGMP was found to be stimulatory (0.01–1.00 μM).

The activity described thus shares some properties with the casein kinases; these are Ca^{2+}- and cyclic nucleotide-independent kinases that have been isolated from numerous tissues and species, but, apart from the mammary gland enzyme, they have not been assigned an *in vivo* function. Unlike the *Drosophila* topoisomerase-associated kinase, the *Drosophila* casein kinase II identified by Glover *et al.* (26) is not reactive toward histones. Other casein kinases identified are also poorly reactive toward histone substrates (27). Inhibition by heparin, characteristic of type II casein kinases, was observed for the topoisomerase-associated kinase (Table 1); the concentration required for 50% inhibition is estimated at 1 μg/ml; for type II casein kinase, 50% inhibition is estimated to require 0.15 μg of heparin per ml (27).

Single-strand DNA is known to be a potent inhibitor of *Drosophila* topoisomerase II (28); at 0.3 μg/ml, 50% inhibition of relaxation activity results. It was found that single-strand DNA also inhibited the topoisomerase-associated kinase activity, although with lower efficiency than that demonstrated in inhibition of topoisomerase activity; 15 μg of single-strand DNA per ml reduced kinase activity to 30% of the value in the control reaction. Double-strand DNA also inhibited the topoisomerase-associated kinase, reducing activity to 51% of the control when present at 15 μg/ml.

The specific activity of the topoisomerase-associated kinase was, under the conditions of our assay, 6 pmol of phosphate incorporated per min/mg. This value is low in comparison with specific activities that have been reported for other kinase activities. We do not exclude the possibility that there exists some required effector of the activity that we have not as yet identified.

DISCUSSION

In this report, a kinase activity is identified that is tightly associated with *Drosophila* type II topoisomerase. The ac-

tivity is cyclic nucleotide- and Ca^{2+}-independent and catalyzes phosphorylation of the topoisomerase subunit itself as well as of *Drosophila* histones H1, H2a, H3, H4, *Drosophila* protein D1, casein, and phosvitin. Phosphorylation occurs on serine and threonine residues.

Several criteria differentiate this activity from the previously identified *Drosophila* casein kinase II, including ionic strength optimum, substrate preference, and degree of sensitivity to heparin. A type I casein kinase activity has not yet been identified in *Drosophila*. The topoisomerase-associated kinase cannot be considered a type I casein kinase, however, since it catalyzes the phosphorylation of both serine and threonine residues in casein and since it demonstrates sensitivity to heparin [casein kinase I modifies serine only and is insensitive to heparin (29)]. Glover *et al.* (26) report that the casein kinase activity in *Drosophila* behaves as a single homogeneous species throughout its purification. Since the activity described here is distinct from that previously identified activity, it is clear that we have identified a unique cAMP-independent protein kinase activity in *Drosophila*.

Nuclear kinase activities that have been purified generally belong to either the casein or the cAMP-dependent classes of protein kinases. However, a cAMP-independent chromatin-associated histone H1 kinase activity, not yet purified to homogeneity, has been identified in several mammalian tissue culture cell lines (30, 31). *Drosophila* histone kinase activity has not been characterized previously. In partially purified *Drosophila* topoisomerase preparations, we have identified two species of histone kinase activity, which can be separated into two activity peaks upon glycerol gradient sedimentation (unpublished data). The faster migrating species is the topoisomerase-associated kinase. The relationship between these two kinase activities has not yet been determined.

Although we have no evidence that the topoisomerase-associated kinase activity described here is active *in vivo* as a histone kinase, we suggest that this seems an attractive hypothesis for the following reasons. First, the activity shows its greatest reactivity toward *Drosophila* histone H1, of all the substrates tested. Second, the core histones modified by this activity are the same histone species that are modified *in vivo*. Since histone phosphorylation has been closely correlated with chromatin condensation (32), the tight association of this kinase activity with type II topoisomerase suggests that the two activities might be coordinately involved in modulation of chromatin structure. The topoisomerase-associated kinase activity may be involved in modifying topoisomerase function as well, in an as yet unidentified manner.

Although the kinase activity described here phosphorylates the topoisomerase subunit during *in vitro* incubation, we have not yet determined if this modification occurs at the same site(s) at which *Drosophila* topoisomerase II is modified *in vivo*. Topoisomerases modified both *in vivo* and *in vitro* contain phosphoserine; however, phosphothreonine, present in trace amounts in the *in vitro* phosphorylated polypeptide, has not been detected in the protein isolated from cell culture (unpublished data). The effect of autophosphorylation on both topoisomerase and kinase activities will be an important point for future study.

Copurification and coinactivation of protein kinase and type II topoisomerase activities has not been established in any other system as yet. However, the purified calf thymus type II topoisomerase does contain protein kinase activity (Brian Halligan and Leroy Liu, personal communication). Thus, the phenomenon described here may not turn out to be unique for the enzyme purified from *Drosophila* embryos.

As mentioned earlier, the application of genetic techniques will be necessary for further clarification of the relationship between the *Drosophila* type II topoisomerase and its associated kinase.

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