J. Biochem. **127**, 1109–1113 (2000)

Characterization of Recombinant Human DNA Topoisomerase IIIα Activity Expressed in Yeast[1]

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Received January 28, 2000; accepted April 3, 2000

Recombinant human DNA topoisomerase IIIα was expressed in mutant yeast cells devoid of both topoisomerases I and III, and the gene product was partially purified. The activity of the protein in supercoil removal was found to be limited and also biphasic: in the first phase it processively changed the linking-number of hypernegatively supercoiled DNA, but only to the superhelicity of a regular negative supercoil; in the second phase the enzyme relaxed the DNA further, but only slightly and slowly. The optimal solution conditions for the enzyme activity were found to be physiological. The assay results with a truncation mutant showed that the C-terminal 334 amino acids are unnecessary for the activity, suggesting that this region, and perhaps the entire protein, is involved in a function other than supercoil removal.

Key words: DNA supercoiling, DNA topoisomerase.

Mammalian DNA topoisomerase III (*TOP3* gene product), a type-IA topoisomerase, was first identified in man (*1*), and has been shown to be essential for embryonic development in the mouse (*2*). More recently, a second *TOP3* gene was identified (*3–5*), and the first gene has been renamed *TOP3α* and the second one designated as *TOP3β* (*2*). Although the functional interaction between human *TOP3α* and ataxia-telangiectasia (*6*), and testis-specific expression of the 3α and 3β genes (*5*, *7*) have been reported, the physiological roles of the gene products remain enigmatic, as contrasted by the weakness of the activity (*1*) and the severe consequence of *TOP3α* disruption (*2*). This conundrum was recognized in a study on *Saccharomyces cerevisiae* DNA topoisomerase III. While a mutation in the yeast *TOP3* gene leads to severe pleiotropic changes (*8–11*), the enzyme exhibits only very low activity as to the removal of negative supercoils (*12*), suggesting that the physiological role of the enzyme is probably other than supercoil removal (*12*). The finding of genetic interaction of *TOP3* with *SGS1* (*9*) has supported this premise, and suggested that a combination of Top3 and the Sgs1 protein, a DNA helicase (*9*, *13*), may exhibit reverse gyrase activity (*9*). Hence, the characterization of topoisomerase III activity is also important as a starting point for the understanding of the putative complex, possibly also existing in mammalian cells.

Another question, which is probably related to the question of the physiological function(s) of eukaryotic topoisomerases III, concerns the role of a C-terminal segment which is present in mammalian topoisomerases III and in other type-IA enzymes such as bacterial DNA topoisomerase I (for a review, *14*), but absent in yeast topoisomerase III. Although bacterial topoisomerase I can relax negatively supercoiled DNA nearly completely, eukaryotic type-IA topoisomerases studied so far (*1*, *4*, *12*), including the yeast enzyme, appear to remove negative supercoils only partially.

In our previous study (*1*), the presence of the endogenous yeast topoisomerase III hindered more detailed characterization of human topoisomerase III activity expressed in yeast. In the present study, the yeast topoisomerase III was eliminated by mutation, and the activity of the human topoisomerase IIIα was studied further.

MATERIALS AND METHODS

Yeast Strains and Plasmids—The *TOP3* gene in a *S. cerevisiae* strain, JEL1 *Δtop1* (*1*), was disrupted by the standard one-step method (*15*). Briefly, a *TOP3* DNA fragment interrupted by a *TRP1* fragment was used to transform JEL1 *Δtop1*. TRP^+ transformants were screened for *TOP3* disruption by PCR amplification of the *TOP3* locus, and the disruption in an isolate was further confirmed by blot-hybridization. The strain thus obtained was designated as JEL1 *Δtop1 Δtop3*.

Yeast expression vectors for human *TOP3α* and mutant derivatives of it were constructed from pG1TT (*13*). The *TOP3α* coding region, with cysteine at the 571st amino acid position, was fused to the influenza haemaglutinin (HA) epitope and a heart-muscle kinase site under the control of a gal1 promoter in the vector. The resulting polypeptide had the N-terminal amino acid sequence of MATEPVYPY-DVPDYARRASVRSM, where the first underlining indicates the HA epitope, and the second underlining the kinase site, and the last methionine corresponds to the putative initiation codon of human *TOP3α* (*1*). The 3′ end of the *TOP3α* coding region was fused to a DNA segment encoding a decahistidine sequence so that the carboxy terminal arginine of the human topoisomerase IIIα was followed by

[1] This work was supported by a research grant from Rikkyo University and a MESSC grant, 11780478, to RH.

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$CPQVH_{10}$. The plasmid thus constructed was designated as pG1TT-hTOP3α. A similar plasmid was constructed for a *TOP3α* mutant in which the active-site tyrosine at the 337th position was replaced with phenylalanine and the 571st amino acid was tyrosine, and was designated as pG1TT-hTOP3α-Y337F. A plasmid, pG1TT-hTOP3α-tailless, was constructed by ligating a unique *Afl*II site filled in with the Klenow enzyme and the decahistidine encoding sequence to give a fusion of the N-terminal 642 amino acids of the human *TOP3α*, ending with leucine, to $NQVH_{10}$. The plasmid was otherwise the same as pG1TT-hTOP3α.

Gene Expression and Protein Preparation—JEL1 Δtop1 Δtop3 was transformed with pG1TT-hTOP3α, pG1TT-hTOP3α-Y337F, or pG1TT-hTOP3α-tailless, and then grown in a synthetic medium lacking uracil but containing 2% lactic acid and 5% glycerol at 30°C. Expression of the plasmid-born gene was induced by the addition of solid galactose to 2% when the optical density at 600 nm of the culture was between 0.3 and 0.6, and was continued for 6 h. The culture was harvested by centrifugation, and the pellet was frozen in liquid nitrogen and stored at −70°C.

The frozen cells were thawed and two volumes of ice-cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris·HCl, pH 8.0) supplemented with 1 mM phenylmethylsulfonyl fluoride, 100 mg/ml benzamidine, 0.7 μg/ml pepstatin, and 0.5 μg/ml leupeptin was added. All operations were carried out at 4°C thereafter. The cells were disrupted by agitation with glass beads. The lysate and washes of the glass beads with the binding buffer were combined, and then centrifuged to remove undisrupted cells and cell debris. The lysate was loaded onto a 500 μl column of nitrilotriacetic acid (NTA) resin loaded with nickel according to the manufacturer's instructions (Novagen). The column was successively washed with 10 volumes of the binding buffer and 6 volumes of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris·HCl, pH 8.0). The bound protein was eluted twice with six volumes of elution buffer (0.8 M imidazole, 0.5 M NaCl, 20 mM Tris·HCl, pH 8.0), and the eluates were separately dialyzed first against 150 mM KCl, 0.1 mM EDTA, 20 mM Hepes, pH 7.5, 50% glycerol overnight, and then against the same buffer plus 5 mM DTT overnight. The dialyzed material was retrieved and stored at −20°C. The total protein concentration was determined by use of a commercial Bradford kit (Pierce) with bovine serum albumin as the standard. The concentrations of the expressed human topoisomerase IIIα and its derivatives were determined by blotting proteins separated by SDS polyacrylamide gel electrophoresis and by immunodetection using the 12CA5 monoclonal antibody (Boehringer-Mannheim) with serially diluted HA-tagged vaccinia topoiso-merase I as the standard. As the immunoblotting indicated the first eluates contained most of the expressed proteins, they were stored and used for assays.

Assaying of Topoisomerase Activity—Hypernegatively supercoiled pBluescript DNA (Stratagene) was prepared by relaxing DNA at 0.1 mg/ml with vaccinia topoisomerase in the presence of 10 μg/ml ethidium bromide (*1*, *16*). The standard assay mixture for human topoisomerase IIIα contained 0.4 μg of hypernegatively supercoiled DNA in 20 μl of 150 mM potassium chloride, 1.5 mM magnesium sulfate, 5 mM dithiothreitol, 20 mM Hepes·Na, pH 7.5, 0.1 mg/ml bovine serum albumin. The reaction was performed at 37°C for 1 h and terminated by phenol extraction. The samples were electrophoresed in 1% agarose containing 1 μg/ml ethidium bromide in TBE (50 mM Tris·borate, 1 mM EDTA), and the DNA was visualized as ethidium-bromide fluorescence after destaining. Two-dimensional gel electrophoresis was carried out in 1% agarose with 3 μg/ml chloroquin diphosphate for the first dimension and 0.5 μg/ml ethidium bromide for the second dimension in TBE (*16*). The DNA was visualized by blot-hybridization with a DIG-labeled pBluescript probe according to the manufacturer's instructions (Boehringer-Mannheim).

RESULTS

Expression and Partial Purification of a Recombinant Human Topoisomerase IIIα and Mutants of It—To eliminate background topoisomerase activity, a new *S. cerevisiae* strain was constructed by disrupting the *TOP3* gene in a strain, JEL1 *Δtop1* (see "MATERIALS AND METHODS"), which is suitable for galactose-induced gene expression and lacks the gene for topoisomerase I (*1*). A recombinant human topoisomerase IIIα was expressed in cells of the strain as a fusion with the HA epitope at the N-terminus and a decahisitidine at the C-terminus, and was partially purified by use of the histidine tag and NTA resin. The recombinant topoisomerase IIIα separated by 10% SDS polyacrylamide

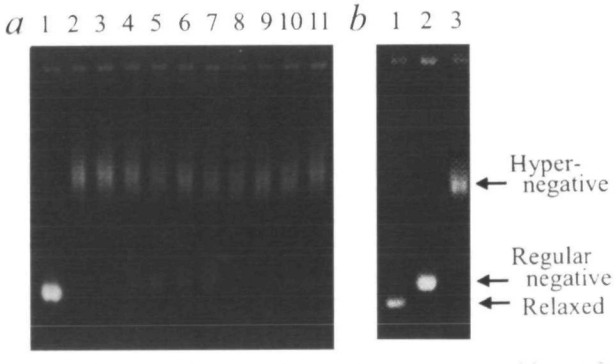

Fig. 1. **a: Supercoil-removal assaying of a recombinant human topoisomerase IIIα.** A recombinant human topoisomerase IIIα expressed in mutant yeast cells devoid of topoisomerases I and II was partially purified and assayed with hypernegatively supercoiled pBluescript DNA as a substrate. Each reaction mixture contained 0.4 μg of the substrate DNA and 1.6 ng enzyme in 20 μl of 150 mM potassium chloride, 1.5 mM magnesium sulfate, 20 mM Hepes·Na, pH 7.5, 5 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin, unless otherwise noted. The reaction was performed at 37°C for 1 h. The samples were analyzed by 1% agarose gel electrophoresis in the presence of 1 μg/ml ethidium bromide at a field strength of 2 V/cm and room temperature for 16 h. Lane 1 contained regular supercoiled DNA. Lane 2 contained hypernegatively supercoiled DNA incubated in the same buffer without the added enzyme. For lanes 3–9, the potassium chloride concentration was changed to 24, 60, 105, 150, 195, 240, and 285 mM, respectively. Lanes 10 and 11 contained reaction mixtures incubated at 37 and 65°C, respectively. **b: Separation of relaxed (lane 1), regular supercoiled (lane 2), and hypernegatively supercoiled (lane 3) pBluescript DNA by agarose gel electrophoresis under the same conditions as in a.** In a and b, relaxed DNA was prepared by treating DNA with vaccinia topoisomerase without an intercalator, regular supercoiled DNA was DNA prepared from *E. coli*, and hypernegatively supercoiled DNA was prepared by relaxing DNA with vaccinia topoisomerase in the presence of ethidium (see "MATERIALS AND METHODS").

gel electrophoresis, blotted, and detected with an anti-HA antibody gave a single band and had a molecular mass of 115 kDa, which is close to the 113 kDa calculated from the predicted amino acid sequence (data not shown). From the band intensity of the immunodetected topoisomerase IIIα against a HA-tagged control protein and from the total protein concentration determined by the Bradford method, the purity of the topoisomerase IIIα was estimated to be 1%. Purification by use of the NTA resin was calculated to be 420-fold from the protein concentration of the lysate loaded onto the resin. A mutant protein that had the active-site tyrosine replaced by phenylalanine was similarly prepared. A truncation mutant that contained only the N-terminal 642 amino acids plus the extraneous tag sequences was also prepared. The protein was detected with the anti-HA antibody as a single band corresponding to 84 kDa, which was similar to the predicted 77 kDa (data not shown).

Supercoil-Removal Activity of the Recombinant Human Topoisomerase IIIα—The prepared human topoisomerase IIIα was assayed as to its ability to remove supercoils from hypernegatively supercoiled circular DNA. The results are shown in Fig. 1. Under the electrophoretic conditions used, regular supercoiled DNA, which was prepared from *Escherichia coli* cells, was positively supercoiled because of intercalation of the ethidium present in the electrophoresis buffer. Hypernegatively supercoiled DNA was almost completely relaxed due to the binding of ethidium, and migrated more slowly than the regular supercoiled DNA (lanes 1 and 2, a, and lanes 2 and 3, b). Under the electrophoretic conditions, regular supercoiled DNA and relaxed DNA were also separated from each other (lanes 1 and 2, b). The human topoisomerase IIIα removed some supercoils, as indicated by the appearance of new DNA species that migrated slightly more slowly than the regular supercoiled DNA (lanes 4–7, a). Hypernegatively supercoiled DNA reacted with human topoisomerase IIIα in a separate experiment was subjected to two-dimensional agarose gel electrophoresis. Figure 2 clearly reveals topoisomer spots and confirms that the appearance of the new DNA species was indeed due to a linking-number change.

Next, the effects of the solution conditions on the enzyme activity were investigated. The optimal potassium chloride concentration for the activity appeared to be between 100–200 mM (lanes 3–9, Fig. 1a). The activity was lost at 65°C (lane 11), in contrast to those of yeast topoisomerase III and mouse topoisomerase IIIβ (*4*, *12*). The activity of the enzyme is dependent on magnesium, as inclusion of EDTA abrogated the topoisomerization (data not shown).

Under the reaction conditions in the experiments in Fig. 1, only part of the hypernegatively supercoiled DNA showed the linking-number change. This suggested that the enzyme activity is more or less processive with the optimal salt concentration; a completely distributive activity would have resulted in one topoisomer cluster shifting from the hypernegative to the regular supercoil position. In the experiment shown in Fig. 3, more protein was incubated with DNA in order to change the linking-number of all the hypernegatively supercoiled material and the time course was followed. At no time point was a significant population of DNA species seen between the two topoisomer clusters, confirming the above suggestion. The processivity is not unlimited, as blot-hybridization revealed the less-populated in-between spots with its high sensitivity (Fig. 2). Close examination of Figs. 2 and 3 also shows that the topoisomerization was biphasic. As seen in lane 7 of Fig. 3, the topoisomer distribution continued to shift very slowly toward the relaxed state after the initial major shift, corroborated by results in Fig. 2. The smeary appearance of the topoisomers in lane 7 of Fig. 3 also shows the second-phase activity was more distributive.

Activity of Truncated Human Topoisomerase IIIα—To assess the role of the C-terminal region of human topoisomerase IIIα, the enzyme activity of a truncation mutant containing only the N-terminal 642 amino acids was stud-

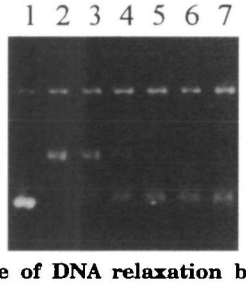

Fig. 3. **Time course of DNA relaxation by human topoisomerase IIIα.** Assays were carried out as in Fig. 2. The incubation times were 5, 10, 20, 40, and 60 min for lanes 3–7, respectively. Lane 1, regular supercoiled DNA; lane 2, hypernegatively supercoiled DNA.

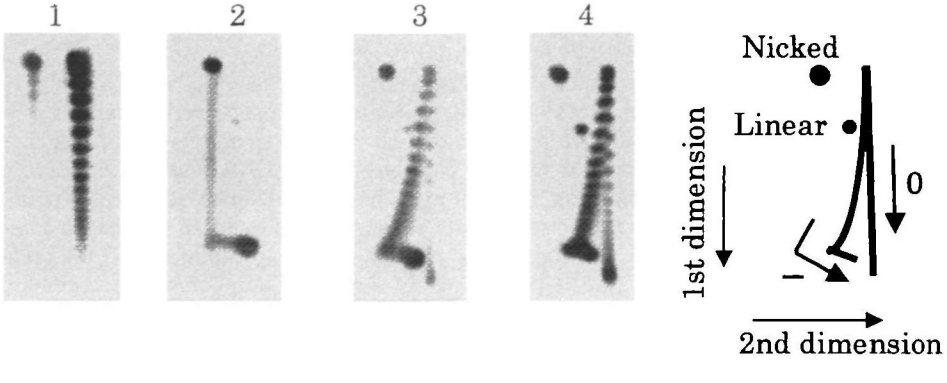

Fig. 2. **Two-dimensional agarose-gel electrophoresis of hypernegatively supercoiled DNA reacted with human topoisomerase IIIα.** A 1% agarose gel was used with 3 μg/ml chloroquine diphosphate in the electrophoresis buffer for the first dimension and 0.5 μg/ml ethidium bromide for the second dimension. Lane 1, regular supercoiled pBluescript DNA; lane 2, hypernegatively supercoiled DNA; lane 3, hypernegatively supercoiled DNA reacted as in Fig. 1, but with 8 ng of enzyme per 20 μl of reaction mixture for 10 min; lane 4, same as in lane 3 for 60 min. The assignment of DNA species is shown schematically at the right. The spots just beneath the nicked DNA in lane 1 presumably resulted from nicking during incubation in the ethidium-containing buffer between the two electrophoretic operations

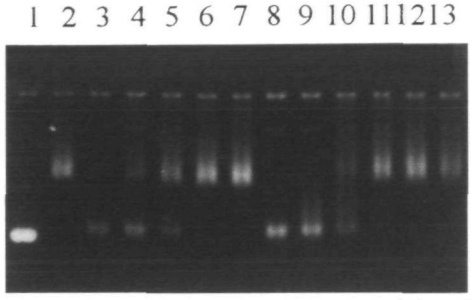

Fig. 4. **Comparison of supercoil-removal activities of the full-length human topoisomerase IIIα and its mutant derivatives.** Lanes 3–7 contained the full-length enzyme. The amounts per 20 μl standard reaction mixture were 8, 2.4, 0.8, 0.24, and 0.08 ng, respectively. Lanes 8–12 contained a truncation mutant comprising only the N-terminal 642 amino acids. The amounts per 20 μl standard reaction mixture were 8, 2.4, 0.8, 0.24, and 0.08 ng, respectively. Lane 13 contained 5 ng of a full-length mutant enzyme in which the active site tyrosine at the 337th position was replaced with phenylalanine. The incubation was performed at 37°C for 1 h.

ied together with the active-site mutant Y337F. The results of the assay with various amounts of the protein are shown in Fig. 4. The activities of the full-length human topoisomerase IIIα (lanes 3–7) and its truncated derivative (lanes 8–12) did not appear significantly different within the uncertainty in determining the protein concentration by immunoblotting. The assignment of the 337th tyrosine as the active site was also confirmed by the lack of activity of the Y337F mutant (lane 13).

DISCUSSION

In the present study, a recombinant human topoisomerase IIIα was expressed in cells of a newly constructed mutant yeast strain lacking both *TOP1* and *TOP3*, and was partially purified. The yeast strain constructed showed complete elimination of the background topoisomerase activity (lane 13, Fig. 4). This implies that *S. cerevisiae* has only two energy cofactor-independent DNA topoisomerases, at least as to those which are detectable with the current method. The limitation of the purity of the present preparation was due to a very low expression level of the introduced human gene in the yeast.

The supercoil-removal activity of the human topoisomerase IIIα was shown to be very weak. In the first phase of its biphasic activity, the enzyme only relaxed hypernegatively supercoiled DNA, and the extent of relaxation was only almost to the supercoil density of the regular negative supercoil in *E. coli*. Counting of topoisomers separated by 1D electrophoresis revealed the superhelicity of the regular supercoil was approximately –6%, in agreement with data reported previously (*17*). However, it has not been feasible to completely separate topoisomers in the negative to hypernegative range even by 2D electrophoresis. A guesstimate made from 1D and 2D electrophoresis results was –18% for the superhelicity of the hypernegatively supercoiled DNA prepared in the present study. Such weak activity, which relaxes supercoiled DNA only to the level of the regular negative supercoil, is common to the eukaryotic type-IA topoisomerases studied so far (*4*, *12*). The optimal potassium chloride concentration for the activity was found to be physiological (*18*). The activity in the first phase was found to be more processive than that in the second phase, as no significant population of intermediate topoisomers was detected during the course of the reaction. In the second phase of the supercoil-removal activity, the enzyme changed the linking-number further, but very slightly and slowly. The type-IA topoisomerases have been shown to require a single-stranded region for their ability to effect supercoil removal (*14*). The occurrence of the second phase of the human topoisomerase IIIα activity indicates that the enzyme has the ability to capture transient openings in regular supercoiled DNA, but it is very limited.

After completion of the present work, the characterization of human topoisomerase IIIα expressed in *E. coli* was reported (*19*). The relaxation activity of the protein was assayed with regular supercoiled DNA, and the reported activity appears to correspond to the second phase of DNA relaxation detected in the present study. The removal of supercoils from regular supercoiled DNA by the protein expressed in *E. coli* was shown to be very limited, in agreement with the present results. However, the effects of salt on the relaxation activity were different in the two studies. The removal of regular supercoils was reported to be inhibited in the presence of >100 mM monovalent ions (*19*), whereas in the present study the optimal salt concentration was found to be 100–200 mM. This discrepancy presumably reflects the difference in the reaction followed. Supercoil removal by topoisomerase III seems more relevant for hypernegatively supercoiled DNA, which has a stronger tendency to have permanent and transient single-stranded regions, since eukaryotic topoisomerases I and II only interact with double-stranded DNA (*14*), and therefore topoisomerization in single-stranded regions has to be dealt with by topoisomerase III. The epistatic relation of *sgs1* on *top3* in *S. cerevisiae* (*9*) appears to suggest that Top3 deals with DNA regions unwound by Sgs1 helicase. In addition, because eukaryotic topoisomerase I has very robust activity (*12*, *14*), the cell-biological relevance of the relaxation of regular supercoiled DNA by topoisomerase III under non-physiological conditions is not known.

The C-terminal region of the human topoisomerase IIIα was found to be unnecessary for the relaxation activity. The truncation mutant used in this study, which is the N-terminal 642 amino acids, covers the entire length of *S. cerevisiae* topoisomerase III and corresponds to the region conserved among type-IA DNA topoisomerases (*20*). In the case of *E. coli* topoisomerase I, also a member of the type-IA family, the C-terminal region has been shown to be necessary for supercoil relaxation (*21*). However, the truncation mutants used in the previous study have not been assayed with hypernegatively supercoiled DNA, and the conserved N-terminal region of *E. coli* topoisomerase I alone may be capable of changing the linking-number of DNA. The activity exhibited by the truncated human topoisomerase IIIα is reminiscent of the DNA relaxation activity of *E. coli* topoisomerase III (TopB), which lacks a C-terminal region (*22*). The enzyme can change the linking-number of supercoiled DNA only at an elevated temperature (*23*), where transient opening of the DNA duplex is presumably increased. In addition, the similarity in the phenotypes of *E. coli topB* and *S. cerevisiae top3*, *i.e.* that recombination between repeats is increased (*8*, *24*), implies that the two gene products are functionally similar beyond

in vitro topoisomerization activity.

The result also implies that the C-terminal region of mammalian topoisomerases III has a function other than changing the linking-number. It is possible that the C-terminal region may recognize a DNA structure(s) yet to be identified. However, the lack of the region in the yeast enzyme, and the functional similarities between the yeast and mammalian enzymes seem to suggest otherwise. *S. cerevisiae TOP3*'s interaction with the *SGS1* helicase gene (*9*) suggests that the mammalian topoisomerases III may interact with some protein(s). A search of the current sequence data-base revealed the presence of type-IA topoisomerases in such eukaryotes as *Caenohabditis elegans* and *Drosophila melanogaster*. The C-terminal region of the *D. melanogaster* topoisomerase III resembles more that of mammalian topoisomerase IIIβ; the C-terminal region of the *C. elegans* protein shows no apparent homology to either topoisomerase IIIα or IIIβ. This and the difference in the C-terminal region of mammalian topoisomerases IIIα and IIIβ may indicate that the region has undergone diversification to interact with different (sets of) proteins.

We wish to thank Dr. James C. Wang for the DNA materials and careful reading of the manuscript. We also thank Drs. Yoshinori Yamashita, Minoru Sugawara, and Toshiwo Ando for the materials and advice.

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