Identification of a DNA supercoiling activity in *Saccharomyces cerevisiae*

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ABSTRACT

A relaxed plasmid DNA is shown to become positively supercoiled in cell extracts from *top1* strains of *Saccharomyces cerevisiae*. This positive supercoiling activity is dependent on the presence of bacterial DNA topoisomerase I and ATP (or dATP), and the positive supercoils generated in this reaction are not constrained by protein(s). Non-hydrolyzable ATP analogs cannot substitute for ATP in this supercoiling reaction, and the supercoiling activity is not due to RNA synthesis. The presence of an ARS sequence in the DNA does not alter the activity. Furthermore, this activity is equally active against UV irradiated or intact DNA. Extracts prepared from *rad50* and *rad52* mutant cells exhibited the same activity. Partial purification of this activity suggests that a protein factor with a native molecular weight of approximately 150 kDa is primarily responsible for the activity. The possibility that this supercoiling activity may be due to tracking of a protein along the intact duplex DNA is discussed.

INTRODUCTION

Unidirectional movement of a protein along the DNA double-helix has been proposed to alter the local conformation of DNA in a manner analogous to that of an elongating RNA polymerase (1). Negative supercoils generated in the wake of the translocating proteins or protein complexes can be detected by using a DNA conformation specific probe, bacterial DNA topoisomerase I (2,3). The selective removal of negative supercoils results in apparent positive supercoiling of the DNA template (3). This simple assay was first applied to studies of RNA polymerases, where transcription from a single promoter on a closed-circular DNA template in the presence of *E.coli* topoisomerase I was demonstrated to induce rapid and extensive positive supercoiling of the DNA template (2). Although this positive supercoiling assay could potentially identify proteins that may supercoil DNA by mechanisms other than helix-tracking, so far only certain DNA

helicases such as SV40 T antigen (3) and the *E.coli* UvrAB complex (4) have been shown to be active in this assay. Using this positive supercoiling assay, a protein factor from *Xenopus laevis* has been partially purified and tentatively designated a DNA helix-tracking (HT) protein (5).

Because a closed circular DNA template is used in the positive supercoiling assay, these DNA helix-tracking proteins must be able to first enter intact duplex DNA and then translocate along the DNA. In the case of RNA polymerases, the entry sites are the promoters. In other cases, the entry sites have not been studied in detail. SV40 T antigen is expected to enter the DNA double-helix through its binding sites at the origin of DNA replication; however, non-specific entry has also been observed (6). The *E.coli* UvrAB complex selectively supercoils a UV-irradiated DNA template and may prefer to enter DNA at the damaged sites. However, it can also supercoil undamaged DNA, albeit at a reduced rate, and hence enter intact duplex DNA (4).

The DNA helix-tracking proteins identified using the positive supercoiling assay may overlap with DNA helicases identified by strand displacement assays (7). However, some DNA helicases identified by a strand displacement assay, such as Rep, DnaB and UvrD, fail to supercoil DNA in this supercoiling assay. The strand displacement assay may therefore reflect primarily the elongation step of these enzymes while the supercoiling assay may reflect both the initiation and elongation steps of these enzymes. It is possible that Rep, DnaB and UvrD may not be able to initiate on closed circular DNA in the absence of other protein factors and proper DNA sequences. Alternatively, helix-tracking may differ from strand displacement, since in principle, a DNA helix-tracking protein, unlike a DNA helicase, may track along the DNA helical grooves without unwinding, or melting, the DNA duplex.

The potential use of this supercoiling assay to identify enzymes involved in DNA functions has prompted us to direct our studies to yeast. We report our preliminary studies using this supercoiling assay on the identification of a protein factor in *top1* strains of *Saccharomyces cerevisiae*, which could be a DNA helix-tracking (HT) protein.

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MATERIALS AND METHODS

Enzymes, chemicals, DNAs, and yeast strains

E. coli DNA topoisomerase I was kindly provided by Dr. James C. Wang (Harvard University). ATP, adenosine 5'-[β,γ -imido] triphosphate (ATP-[β,γ -NH]), adenosine 5'-[β,γ -methylene] triphosphate (ATP-[β,γ -CH₂]), double stranded DNA cellulose, and Superose 12 gel filtration media were purchased from Pharmacia. BioRex 70 and hydroxylapatite (HAP) column media were from BioRad. VM-26 was generously provided by Bristol Myers-Squibb. Plasmid pUC.HSO is a derivative of pUC19 containing the origin bearing *Hind*III-*Sph*I fragment of SV40 DNA (6). pBM125, containing the *Gall* promoter inserted into YCP50, was kindly provided by Dr. Tao-Shih Hsieh (Duke University). Plasmid pAO-SLO has been described elsewhere (8). Monomeric and dimeric pAO-SLO DNAs were separately transformed into *E. coli* DH5 α and isolated by CsCl/ethidium banding. Irradiation of monomeric pAO-SLO DNA was via a dose of 720 J/m² with a germicidal lamp (254 nm) (4).

S. cerevisiae strains CH335 (*MATa his4-539 lys2-801 ura3-52*), TG205 (*MATa his4-539 lys2-801 URA3 top2^{ts} Δtop1*) and JCW1 (*MATa Δtop1 HIS4 lys2-801 ura3-52*) were provided by Dr. James C. Wang (Harvard University); *S. cerevisiae* AMR19 (*MATa ura3-52 leu2-3,112 top1-1 pep4::URA3*) was kindly provided by Dr. Rolf Sternglanz (SUNY at Stony Brook); *S. cerevisiae* strains JN394 (*MATa ISE2 ura3-52 leu2 his7 trp1 ade2 rad52::LEU2*), JN2-134 (*MATa ise1 ura3-52 leu2 his7 trp1 ade2-1 rad52::LEU2 top1-1*), R50 (*MATa ISE2 ura3-52 leu2 his7 trp1 ade2 rad50::URA3*), JN284 (*MATa ise1 ura3-52 leu2*), and JN392 (*MATa ise1 ura3-52 leu2 rad52::LEU2*) were generously provided by Dr. John Nitiss (Children's Hospital, Los Angeles).

Preparation of yeast extracts

A single yeast colony was inoculated into YPD media and grown to log phase (OD₆₀₀=2.3 to 3.0). Cultures were chilled on ice for 20 min, and pelleted at 6000×g for 10 min. Pellets were either frozen with dry ice and stored at -70°C or processed immediately. Pellets were resuspended and pelleted as above in 200 ml breaking buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, and 1 mM PMSF) per liter of original culture. Pellets were then resuspended in 112 ml of breaking buffer per liter of culture and placed in the pre-cooled (4°C) chamber of a Bead Beater containing approximately 200 ml of acid-washed glass beads. Breaking buffer was added to fill the chamber, and cells were disrupted by 15 cycles of 20 sec grinding and 40 sec pause. The supernatant (crude extract) was collected and pooled with 100 ml of breaking buffer used to wash the glass beads.

Partial purification of HT protein

NaCl was added to yeast crude extracts to a final concentration of 0.5 M, followed by a 10 min incubation on ice with occasional swirling. Polymyxin P was added to a final concentration of 0.5% (w/v), and incubation on ice was continued for 15 min. The supernatant was collected following 30 min centrifugation at 14,000×g. The supercoiling activity was then fractionated by ammonium sulfate precipitation and recovered in a 20–40% saturated ammonium sulfate fraction. The pellet was resuspended in 50 ml BioRex 70 column buffer (20 mM Hepes pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol, and 1 mM PMSF) and dialyzed against the same buffer for 6 hrs with 3 changes of buffer. The extract was then loaded onto

a pre-equilibrated BioRex 70 column and developed with a linear KCl gradient. Fractions were assayed, and those containing HT activity were pooled and loaded onto a HAP column pre-equilibrated with 0.1 M potassium phosphate (KP_i) pH 7.5 containing 10% glycerol and 1 mM PMSF. Fractions from a 0.1 to 1.0 M KP_i linear gradient were assayed, and those containing activity were pooled, dialyzed, and loaded onto a Mono S column pre-equilibrated with 20 mM Hepes pH 7.5, 0.1 M KCl, 0.5 mM DTT, 0.05 mM EDTA, 0.005% Triton X-100, and 1 mM PMSF. The column was developed with a 0.1 to 1.0 M KCl linear gradient, and fractions containing activity were pooled, diluted with dilution buffer (20 mM Tris pH 7.7, 0.05 mM EDTA, 0.005% Triton X-100, and 10% glycerol), and loaded onto a double stranded DNA cellulose column pre-equilibrated with

Figure 1. ATP-dependent supercoiling of a relaxed DNA template in yeast cell extracts supplemented with *E. coli* DNA topoisomerase I. Panel A shows a schematic representation of the positive supercoiling assay for the helix-tracking protein (HT protein). The movement of a HT protein along the right-handed DNA double helix drives the rotation of DNA about its helical axis, resulting in the generation of positive supercoils ahead of and negative supercoils behind the moving protein (left portion of panel). Positive and negative supercoils can be rapidly annihilated by rotational diffusion through the intervening DNA segment. The presence of bacterial DNA topoisomerase I preferentially removes the negative supercoils, resulting in accumulation of positively supercoils in the DNA (right portion of panel). Panel B demonstrates that both *E. coli* DNA topoisomerase I (to selectively remove negative supercoils) and ATP (to allow translocation along the DNA) are required for the positive supercoiling reaction. 2 μ l cell extract was present in each reaction. Lane 1, relaxed DNA control, no *E. coli* DNA topoisomerase I. Lane 2, with *E. coli* DNA topoisomerase I, but without ATP. Lane 3, with ATP, but without *E. coli* DNA topoisomerase I. Lane 4, complete reaction with both *E. coli* DNA topoisomerase I and ATP. II and ++ mark the positions of nicked circular and positively supercoiled plasmid DNAs, respectively.

dilution buffer containing 50 mM KCl. The column was stepwise eluted with buffer containing 0.2, 0.35, and 1.0 M KCl.

Positive supercoiling assay

Negatively supercoiled plasmid DNAs were relaxed by treatment with calf thymus DNA topoisomerase I and used as substrate for the positive supercoiling assay. 40 ng of relaxed plasmid was incubated with various amounts of crude yeast extracts or column fractions, and 80 ng *E. coli* DNA topoisomerase I in a buffer (20 μ l final volume) containing 20 mM Hepes, pH 7.5, 50 mM KCl, 8 mM $MgCl_2$, 4 mM ATP, 1 mM dithiothreitol, and 30 μ g/ml of bovine serum albumin. After incubation at 37°C for 1 hr, reactions were terminated by the addition of EDTA, SDS, and Proteinase K to final concentrations of 25 mM, 0.5%, and 300 μ g/ml, respectively. Incubation continued at 37°C for an additional 2 hrs. Electrophoresis was carried out in 1% agarose with 0.5 $\times$ TPE buffer. Two dimensional gel electrophoresis with 8 μ M chloroquine in the second dimension was done as previously described (9). Gels were dried and hybridized *in situ* as described (9).

RESULTS

ATP-dependent supercoiling of a relaxed DNA in yeast cell extracts supplemented with *E. coli* DNA topoisomerase I

To test whether yeast cell extracts contain a DNA helix-tracking activity, we prepared a cell extract from a *top1* mutant strain (AMR19) of *Saccharomyces cerevisiae* and assayed for DNA helix-tracking activity. A schematic representation of the assay used is shown in Fig. 1A. In the presence of *E. coli* DNA topoisomerase I and ATP, the relaxed pAO-SLO DNA (Fig. 1B, lane 4) became supercoiled in the cell extract. This apparent supercoiling activity can be detected in at least two other *top1* mutant strains (JCW1 and JN2-134) and a *top1 top2^{ts}* double mutant strain TG205. The supercoiling activity can be completely abolished by the addition of either excess calf thymus DNA topoisomerase I or calf thymus DNA topoisomerase II (data not shown), suggesting that the supercoils generated are not constrained by protein(s).

In order to prevent endogenous yeast topoisomerase II from interfering with the supercoiling reaction, we included 150 μ M VM-26, a topoisomerase II inhibitor, in the assay. However, the interference from yeast topoisomerase II was not significant; this may possibly be due to the low activity of this enzyme in the extract. When a cell extract prepared from a wild type TOP1 strain CH335 was used, no supercoiling activity was detectable using our assay. However, the same ATP-dependent supercoiling activity was demonstrable in Bio-Rex70 column fractions after partial purification of the extract (data not shown). Because of the strong interference of the supercoiling activity from endogenous topoisomerase I, we carried out all our studies in *top1* mutant strains.

The supercoiling reaction product is a positively supercoiled DNA

In order to characterize the supercoiling reaction product, we analyzed the product by chloroquine-containing two dimensional gel electrophoresis. The reaction product migrated in the gel at positions d and c, expected for relaxed and positively supercoiled topoisomers, respectively (Fig. 2, panel A). In order to confirm that the reaction product at position c is indeed positively

Figure 2. The reaction product is positively supercoiled. Panel A shows migration of the reaction product on a chloroquine-containing two dimensional gel. Panel B shows the same reaction product mixed with untreated, negatively supercoiled pUC.HSO DNA prior to electrophoresis. Panels C and D show the same mixed DNA (reaction product plus negatively supercoiled plasmid) incubated for 30 min with calf thymus DNA topoisomerase I (50 units) or *E. coli* DNA topoisomerase I (5 ng), respectively. Labels a, b, c, and d indicate the positions of negatively supercoiled plasmid DNA, nicked plasmid, positively supercoiled plasmid DNA, and relaxed plasmid, respectively. Both the reaction product at position c (the expected position for positively supercoiled topoisomers) and the internal control of negatively supercoiled pUC.HSO DNA at position a were completely relaxed by calf thymus DNA topoisomerase I (50 units) which is known to be indiscriminating between negative and positive supercoils (panel C). Treatment with *E. coli* DNA topoisomerase I (50 units), however, only led to relaxation of the internal control of negatively supercoiled pUC.HSO DNA, but not the reaction product at position c (panel D).

Figure 3. Yeast HT activity has no specificity for UV-irradiated DNA. All lanes contained undamaged relaxed dimeric pAO-SLO DNA as an internal control. Lanes 1, 3, and 5 contained undamaged relaxed monomeric plasmid DNA while lanes 2, 4, and 6 contained UV-irradiated relaxed pAO-SLO DNA. Lanes 1 and 2 were controls and contained no extract. Lanes 3 and 4 contained HT activity fractions from elution of a HAP column. Lanes 5 and 6 contained a mixture of Uvr A (20 ng) and UvrB (500 ng) proteins.

Figure 4. Partial purification of a DNA helix-tracking protein from *S. cerevisiae* AMR19. Panel A shows a partial purification scheme for the yeast HT protein. The preparation of yeast whole cell extract and the purification procedure were as described in MATERIALS AND METHODS. The approximate salt concentrations of column fractions containing HT activity are shown to the right of each arrow. Panels B and C show, respectively, the ammonium sulfate fractionation step and a sample column profile (BioRex70 column) of the HT activity. The majority of positive supercoiling activity precipitated at 40% ammonium sulfate and eluted at approximately 0.4 M KCl from the BioRex 70 column.

supercoiled pAO-SLO DNA, the reaction product was mixed with an internal control, negatively supercoiled pAO-SLO DNA (which migrates as a streak at position a) (Fig. 2, panel B), and treated with either HeLa DNA topoisomerase I (Fig. 2, panel C) or *E. coli* DNA topoisomerase I (Fig. 2, panel D). The reaction product was shown to be relaxed by HeLa DNA topoisomerase I but not by *E. coli* DNA topoisomerase I, while the internal control of negatively supercoiled pAO-SLO DNA was relaxed by either topoisomerase (Fig. 2, compare panels C and D). These results strongly suggest that the reaction product is positively supercoiled DNA.

Substrate specificity of the putative DNA helix-tracking activity

Since the assay was designed for DNA helix-tracking proteins, potential protein candidates for this activity are RNA polymerases. This possibility was ruled out by two experiments. First, RNaseA treatment had no effect on the supercoiling activity in our assay. Secondly, a number of plasmid DNAs (e.g. pUC19, pBR322, pBM125, pAO-SLO, and pUC.HSO), with or without yeast promoters, were tested and showed roughly the same activity. This activity is also nonspecific with respect to the yeast origin of DNA replication ARS1 found in pBM125 (data not shown).

The possibility that this activity is similar to the UvrAB complex was also tested using UV-irradiated DNA (Fig. 3, lane 2, pAO-SLO monomer is UV-irradiated). As shown in Fig. 3, the UvrAB complex preferentially supercoiled UV-irradiated monomeric pAO-SLO DNA (Fig. 3, compare lanes 5 and 6). Yeast extracts supercoiled both UV-irradiated and intact pAO-SLO DNA to the same extent (Fig. 3, compare lanes 3 and 4).

We have also tested the presence of this supercoiling activity in several yeast mutant strains deficient in DNA repair. However, the interference of yeast topoisomerase I limited our capacity to test many mutants. A wild type level of this supercoiling activity was detected in a cell extract of *S. cerevisiae* JN2-134 (*top1 rad52*). We have also tested the supercoiling activity in BioRex70 fractions of partially purified cell extracts of *S. cerevisiae* strains CH335 (TOP1) and R50 (*rad50*). In both cases, similar levels of the supercoiling activity were detected.

Partial purification of the putative DNA helix-tracking activity from yeast

In order to identify the protein(s) responsible for the DNA helix-tracking activity in yeast extract, we have carried out a partial purification. *S. cerevisiae* AMR19 total cell extract (from 10 liters) was fractionated according to the scheme shown in Fig. 4. The activity was followed by the supercoiling assay. Overall yield

Figure 5. Heterogeneity of the yeast HT activity. Panel A shows a glycerol gradient (10–30%) profile of the HT activity from pooled BioRex 70 column fractions. The majority of the HT activity sedimented just slightly slower than gamma-globulin (158 kDa). Assuming a similar shape and amino acid composition as gamma-globulin, the native molecular weight of the major yeast HT is about 150 kDa. There is also a minor activity peak sedimenting slightly slower than the thyroglobulin marker (670 kDa). Panel B shows a gel filtration column (Superose 12) profile of the same pooled fractions. The HT activity peak is again found to be split between two different molecular weights. The major activity eluted slightly slower than the gamma-globulin marker. The other activity peak eluted slightly ahead of the thyroglobulin marker.

Figure 6. Characterization of the partially purified HT protein fractions. Panel A shows the nucleoside triphosphate requirement of both the slow and fast sedimenting forms. The indicated nucleoside triphosphates were substituted for ATP in the standard positive supercoiling reaction. Both forms require either ATP or dATP for maximal activity, while UTP and CTP can weakly substitute. The ATP analogs, AMP-PNP and AMP-PCP, cannot support the supercoiling reaction. Panel B shows the salt dependence of both the slow sedimenting (approximately 150 kDa) and the fast sedimenting (approximately 600 kDa) form of the HT activity from the glycerol gradient. The positive supercoiling activity of the two forms were assayed with KCl concentrations ranging from 25 to 200 mM KCl.

of the purification was only 0.5%. Approximately 5 μ g of protein was recovered. Because of the non-quantitative nature of the assay, we were unable to accurately quantify the yield for each step.

The partially purified protein exhibits multiple bands in a silver-stained SDS-PAGE gel, and we have not been able to identify the protein(s) responsible for the activity. Activity assays for the ammonium sulfate fractionation step and the BioRex70 column step are shown in Fig. 4B and 4C respectively. The majority of

the activity was recovered in a 20–40% saturated ammonium sulfate step and at 0.4 M KCl in the gradient.

Heterogeneity of the putative DNA helix-tracking activity

Analysis of the DNA helix-tracking activity by glycerol gradient sedimentation indicated that majority of the activity sedimented as a single peak between ovalbumin (44 kDa) and gamma-globulin (158 kDa) as shown in Fig. 5A. A minor activity peak sedimented just slightly slower than the thyroglobulin marker (670

kDa). Similarly, gel filtration (on Superose 12) analysis revealed a broad band eluting between ovalbumin and gamma-globulin as shown in Fig. 5B. In addition to the main protein peak, there is an additional protein peak which was eluted slightly ahead of the thyroglobulin marker. If the faster sedimenting peak corresponds to the early peak on the gel filtration column, and the slower sedimenting peak corresponds to the late peak, the estimated native molecular weight for the small form of the HT protein is about 145 kDa and that of the large form about 600 kDa. Whether these two forms are related to each other in protein structure is not known. In order to address this question, we have tested the nucleoside triphosphates cofactor requirement and salt optimum for each form. As shown in Fig. 6A, the HT activity of both forms were identical in their nucleoside triphosphate requirement. The effectiveness of the nucleoside triphosphates in descending order is as follows: ATP = dATP > UTP > CTP. Other nucleoside triphosphates were inactive. Non-hydrolyzable ATP analogs such as AMP-PNP and AMP-PCP could not support the supercoiling reaction by either form. Also, Fig 6B shows that both forms were progressively inhibited by KCl. Above 200 mM KCl, very little activity was detectable.

DISCUSSION

The supercoiling activity we have detected in extracts of *top1* strains of *Saccharomyces cerevisiae* is not due to DNA helix-tracking by RNA polymerases; plasmid DNAs with or without yeast promoters were both supercoiled in our assay. Additionally, RNaseA treatment did not affect the supercoiling reactions. The supercoiling reaction by either crude cell extracts or partially purified fractions requires only ATP (or dATP), but not other ribo- or deoxyribo-nucleoside triphosphates. The DNA helix-tracking activities are not due to topoisomerases; no relaxation or supercoiling activities are detectable in the absence of bacterial DNA topoisomerase I. The observations that the positive supercoils generated in this assay are unconstrained by protein(s) and that hydrolysis of ATP is required for the supercoiling reaction suggest that a protein(s) capable of energy-dependent translocation on DNA double-helix could be responsible for the supercoiling activity. We tentatively refer to this activity as the DNA helix-tracking (HT) activity.

The use of *top1* mutant strains is necessary for the detection of this activity. Extracts prepared from wild type strains did not support the supercoiling reaction. This is most likely due to the presence of endogenous yeast topoisomerase I which is expected to rapidly relax both positive and negative supercoils generated by DNA helix-tracking protein(s). Indeed, the HT activity can also be detected in fractionated (BioRex70) wild type cell extract. The presence of topoisomerase II in the extract does not significantly affect the positive supercoiling reaction. This is probably due to the lower activity of topoisomerase II in cell extracts. The use of 150 μ M VM-26, a topoisomerase II inhibitor, in the supercoiling reaction is sufficient to suppress any topoisomerase II activity that might otherwise interfere with the supercoiling reaction. The absolute requirement for bacterial DNA topoisomerase I also indicates the lack of sufficient activity of topoisomerase III in these cell extracts (10).

The possible function(s) of this HT protein(s) has been studied in several preliminary experiments. The HT activity is detected in *rad50* and *rad52* mutant extracts and is therefore unlikely to be due to the activity of either RAD50 or RAD52. Since *E. coli* UvrAB preferentially supercoils an UV-irradiated DNA template

in this assay, we have also tested the possible preferential action of this HT activity on an UV-irradiated DNA template. No preference has been detected using either crude cell extracts or column fractions in the supercoiling assay. The yeast RAD3 protein has been shown to exhibit DNA helicase activity by a strand displacement assay and the unwinding reaction is unidirectional in the 5' to 3' direction (11). Our HT protein factor is probably not RAD3 ($M_r = 89,779$). First, no protein of 90 kDa was present in our partially purified fractions. Second, our HT protein factor is active above pH 7.5, a condition known to inactivate RAD3 ATPase and helicase activities (11). However, we have not tested for HT activity in a *rad3* mutant extract.

We have also tested the possible preferential action of this activity on an ARS-containing plasmid. Again, no preference was detected. However, the lack of ARS sequence specificity does not exclude the possibility that the HT protein may function in a manner similar to SV40 T antigen in initiating DNA replication, since SV40 T antigen exhibits little specificity toward SV40 origin-sequence in a similar assay (6). It is also possible that this HT protein(s) could be involved in functions other than replication or repair. The demonstration that a DNA helicase activity is associated with the transcription factor RPF30/74 (TFIIF) is interesting (12).

Purification of this activity has been difficult. This is partly due to the difficulty involved in quantitation of the assay and the rather time-consuming nature of the assay. The heterogeneity of the activity further complicates the purification. There are clearly at least two different protein fractions responsible for the observed supercoiling activity. The large protein factor is about 600 kDa in its native molecular weight while the small one is only about 150 kDa, and the two active factors may be related to each other. They exhibit identical salt dependence and nucleoside triphosphate requirements. There are probably stimulatory and inhibitory protein factors as well. We have not been able to sort out these problems. Further analysis is necessary to establish the identity of these two factors.

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