

Essential Roles of the RNA Polymerase I Largest Subunit and DNA Topoisomerases in the Formation of Fission Yeast Nucleolus

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Abstract. A temperature-sensitive lethal mutant *nucl-632* of *Schizosaccharomyces pombe* shows marked reduction in macromolecular synthesis and a defective nuclear phenotype with an aberrant nucleolus, indicating a structural role of the *nucl*⁺ gene product in nucleolar organization. We cloned the *nucl*⁺ gene by transformation and found that it appears to encode the largest subunit of RNA polymerase I. We raised antisera against *nucl*⁺ fusion polypeptides and detected a polypeptide (~190 kD and 2×10^4 copies/cell) in the *S. pombe* nuclear fraction. By immunofluorescence

microscopy, anti-*nucl*⁺ antibody revealed intense staining at a particular nuclear domain previously defined as the nucleolus. The nucleolar immunofluorescence by anti-*nucl*⁺ was faded in *nucl-632* at restrictive temperature and dramatically diminished in the absence of DNA topoisomerases I and II. Thus active RNA polymerase I appears to be required for the formation of the nucleolus as its major component, and DNA topoisomerases appear to be required for the folding of rDNA and RNA polymerase I molecules into the functional organization of nucleolar genes.

GENES implicated in higher order chromosome structure can be identified by distinct approaches. Isolation of temperature-sensitive (ts)¹ or cold-sensitive (cs) mutants defective in chromosome structures may lead to identification of the genes by cloning DNA sequences that complement mutant phenotypes. A successful example is identification of a human cell cycle gene that regulates the onset of chromosome condensation (Kai et al., 1986; Ohtsubo et al., 1987). Alternatively, mutants defective in chromosomal proteins that are known or presumed to be involved in the control of chromosome structures can be isolated by assaying their activities in extracts of mutagenized cells. Mutants of DNA topoisomerase (topo) I and II were thus isolated from budding and fission yeasts (DiNardo et al., 1984; Uemura and Yanagida, 1984). The other approach would be first to clone the gene (such as histone H1) with products possibly involved in the higher order chromosome structure but which are difficult to assay. Integration of the in vitro-mutagenized gene into a chromosome to replace the normal one may produce defective phenotypes in the chromosome structures.

We investigated genes implicated in the higher order chromosome structure by applying these approaches to the fission yeast *Schizosaccharomyces pombe* (e.g., Uemura et al., 1987a). *S. pombe* chromosomes are large (Smith et al., 1987) and easily amenable to light microscopy using 4',6-diamidino-2-phenylindole (DAPI) staining (Umesono et al.,

1983). We examined a large number of ts mutants and obtained a strain, *nucl-632*, which showed a nuclear cytological phenotype resembling that of the double mutant in topo I and II, *top1-top2* (Hirano et al., 1986). In the *top1-top2* double mutants of *S. pombe* and *S. cerevisiae* (Uemura and Yanagida, 1984, 1986; Goto and Wang, 1985; Brill et al., 1987; Uemura et al., 1987b), the topoisomerase activity to relax negative and positive supercoils is absent, and macromolecular synthesis is reduced, accompanied by an altered nuclear chromatin region (called "ring" phenotype) at restrictive temperature. Further analyses of the *S. cerevisiae* and *S. pombe* double mutants (Brill et al., 1987; Yamagishi and Nomura, 1988) indicated that DNA and ribosomal RNA syntheses are severely reduced, but mRNA and tRNA syntheses are relatively unaffected. Single *top1* mutants, however, grow normally and single *top2* mutants are specifically blocked in nuclear division (for review see Yanagida and Wang, 1987). Similarity in the cytological phenotypes suggested a possible relation between the *nucl*⁺ gene function and DNA topology. Therefore, we attempted to identify the gene product and function of the *nucl*⁺ gene.

Materials and Methods

Strains and Media

Schizosaccharomyces pombe haploid strains were used with the standard genetical procedures (Gutz et al., 1974). *nucl-632* was previously isolated as described in Hirano et al. (1986). The double mutants *top1-top2* in DNA topo I and II were described by Uemura and Yanagida (1984). For the gene disruption, two haploid strains TP4-5A (*h⁻ leul-32 ura4-d18 ade6-M210*) and TP4-1D (*h⁺ leul-32 ura4-d18 ade6-M216 his2*) were used. The diploid 5A/1D was made by a cross between TP4-5A and TP4-1D. TP6 is the diploid

1. **Abbreviations used in this paper:** cs, cold sensitive; DAPI, 4',6-diamidino-2-phenylindole; ts, temperature sensitive.

integrated with the 3.8-kb Nru I fragment of *pnucl::ura4⁺ (h⁻/h⁺ leu1-32/leu1-32 ura4-d18/ura4-d18 ade6-M210/ade6-M216 his2⁺ nucl::ura4⁺ +)*. TP6G1 is the Leu⁺ transformant of TP6 by pGK100, and TP6100 is a Leu⁺ Ura⁺ haploid segregant obtained from TP6G1. pGK100 is pDB248' (Beach et al., 1982) carrying 9.4-kb insert that complements *nucl-632*.

Escherichia coli HB101 and MC1061 were used for preparation of plasmids and BJ5183 for recovery of plasmids from *S. pombe*. For production of the fusion protein, BL21 and JM101 were used. For complete and minimal media for *S. pombe*, YPD and EMM were used. LB was used for *E. coli*.

Plasmids

The following plasmids were used: pDB248' for gene cloning (Beach et al., 1982); pUC18 for nucleotide sequence determination (Yanisch-Perron et al., 1985); and pAR3039 (Studier and Moffatt, 1986) and pUR291 (Rüther and Müller-Hill, 1983) for expression of fused-*nucl⁺* polypeptide in *E. coli*.

Gene Cloning by Transformation

The genomic DNA sequence that complemented *nucl* mutant was isolated by transformation from a genomic library that contained Sau IIIA partial digests in the vector pDB248' (Beach et al., 1982). Transformation was carried out by lithium method (Ito et al., 1983) using the host strain *h⁻ leu1 nucl*, and a Leu⁺ Ts⁺ transformant was obtained.

Nucleotide Sequence Determination, Southern Blotting, Northern Blotting

The dideoxy method (Sanger et al., 1977) was used with unidirectional progressive deletion (Yanisch-Perron et al., 1985) for nucleotide sequence determination of both strands. For Southern blotting, ³²P-labeled 3.2-kb Hind III fragment in pGK104 was used as the probe. *S. pombe* genomic DNA was isolated and digested with restriction enzymes. 5 μg DNA was run in agarose gel, and hybridization was carried out at 50°C (6X SSC, 0.5% SDS). For Northern blotting, the same probe as for Southern blotting was used. 10 μg polyA⁺ mRNA isolated from the wild-type *S. pombe* was run in agarose gel.

Integration of Cloned *nucl⁺* Sequence on Chromosome

The cloned sequence was integrated on the chromosome by homologous recombination (Botstein et al., 1979). pGK109 containing the insert that apparently did not have autonomously replicating sequence activity was digested by Bgl II which yielded a linear fragment with the termini in the *nucl⁺* gene region. The fragment was used for transformation of the host strain *h⁻ nucl-632 leu1*. Stable Leu⁺ Ts⁺ transformants obtained were crossed with *h⁺ his2 leu1 nucl*, and tetrads were dissected. Leu⁺ was tightly linked to *nucl* (PD:NPD: TT = 28:0:0). The transformant was also crossed with *adel (h⁺ his2 leu1 adel)*, which was previously known to be linked with *nucl*. Leu⁺ was closely linked with *adel* (PD:NPD: TT = 32:0:0).

Disruption of *nucl⁺* Gene

Plasmid pH3M was constructed by ligating the 3.2-kb Hind III insert in pGK104 with pUC18 (Yanisch-Perron et al., 1985) linearized with Hind III, and digested with Bgl II to remove the 567-bp-long internal sequence (corresponding to the residues from 300th to 489th codons) of the *nucl⁺* gene. Then, pURA4 (made by F. Lacroute, quoted in Russell and Nurse, 1986) was cleaved with Hind III. The resulting 1.8-kb fragment containing the *S. pombe ura4⁺* gene was associated at both ends with the 51-bp-long Eco RI/Hind III polylinker sequences derived from pUC18, followed by sub-cloning into the Eco RI site of pBR322 and cleaving with Bam HI. The 1.8-kb Bam HI fragment was ligated with pH3M digested with Bgl II. Thus plasmid *pnucl::ura4⁺* was constructed. The 3.8-kb Nru I fragment of *pnucl::ura4⁺* was used for disruption of the *nucl⁺* gene.

Ura⁻ homozygous diploid 5A/1D (Materials and Methods) was transformed with the 3.8-kb Nru I fragment. Stable Ura⁺ diploid transformants obtained were sporulated, and tetrads were analyzed. Two viable and two nonviable patterns were obtained in all the fifteen tetrads dissected. Furthermore, the thirty viable spores were all Ura⁻. These results indicate that the one-step replacement (Rothstein, 1983) of the *nucl⁺* gene by the Ura⁺ Nru

I fragment is the lethal event. To determine whether plasmid pGK100 carrying the *nucl⁺* gene can complement the disrupted Ura⁺ haploid, the diploid TP6 containing *nucl::ura4⁺* was transformed with pGK100 and resulting Leu⁺ Ura⁺ transformants were sporulated. Results of the Leu⁺ haploid segregants showed that Ura⁺ (*nucl⁻*) cells are viable if they contained Leu⁺ plasmids carrying the *nucl⁺* gene. This result showed that the gene disruption took place in the *nucl⁺* gene.

Plasmid Construction for Production of *nucl⁺* Protein in *E. coli*

We constructed two plasmids for the production of *nucl⁺* fusion polypeptides in *E. coli*. pURNUC1 is a derivative of pUR291 (Rüther and Müller-Hill, 1983) designed for the production of the *lacZ* product at its NH₂ end, which contains the multiple cloning sites for ligation of the other gene in the frame. pURNUC1 is inserted with Bgl II (at 1609)-Hind III (at 3637) fragment and encodes *lacZ* polypeptide (1,022 residues) fused with a 676-residues-long region derived from the *nucl⁺* gene. Plasmid was introduced into *E. coli* JM 101 and induced by isopropyl-β-D-thiogalactopyranoside (IPTG). The other plasmid pARNUC1 is a derivative of pAR3039 (Studier and Moffatt, 1986), which contains bacteriophage T7 gene 10 promoter and the 12-residues-long short coding region. pARNUC1 is inserted with Bgl II fragment (from 1038 to 1612) in a frame that can encode a 207-residues-long polypeptide. Plasmid was introduced into *E. coli* BL 21. By IPTG both T7 RNA polymerase (fused with lac UV5 promoter and integrated on host chromosome) and phage gene 10 promoter are induced.

Preparation of Antisera

Procedures described by Watt et al. (1985) were followed. One or two liter cultures of *E. coli* JM101 and BL21 carrying plasmids pURNUC1 and pARNUC1, respectively, were induced by IPTG (final 1 mM) and were incubated for 3 h. Cells were collected, freeze-thawed, and digested with lysozyme. Lysates were treated in a Waring blender, and the pellets were suspended in 0.05% sodium deoxycholate followed by 0.1% Triton X-100. Insoluble pellets containing overexpressed fused-*nucl⁺* polypeptide were dissolved in 8 M urea. Dissolved materials were run in SDS gel electrophoresis. Polyacrylamide gel containing the *nucl⁺* band was cut out. Polypeptide in gel was electroeluted, stored at -20°C and used as antigen. pURNUC1 and pARNUC1 produced 180- and 25-kD proteins in large quantity, respectively, and their molecular masses were consistent with the values predicted by nucleotide sequences. The amount of polypeptide obtained was more than 10 mg from one liter culture. Rabbit antisera against these polypeptides were prepared. For initial injection, ~100 μg of antigen was used with Freund's complete adjuvant after three injections (each 70 μg) at 2-wk intervals. Antisera were obtained 2 wk after each injection and contained antibodies strongly reacted with the antigen polypeptide made in *E. coli*. For *S. pombe* extracts, polypeptide of the same molecular mass was recognized by the two sera.

Western Blotting and Nuclear Isolation

Immunoblot (Western blotting) analyses were performed by transferring the proteins electrophoretically to nitrocellulose after SDS-PAGE (Towbin et al., 1979). The procedures for isolation of *S. pombe* nuclei by Percoll gradient centrifugation were described previously (Hirano et al., 1988).

Fluorescence and Immunofluorescence Microscopy

The procedure for DAPI staining (Toda et al., 1981) was followed. Immunofluorescence microscopy was performed by the method described (Kilmartin and Adams, 1984; Adams and Pringle, 1984) with modifications (Hagan and Hyams, 1988).

Measurements of DNA, RNA, and Protein

The procedures described by Nurse et al. (1976) were followed.

Results

Phenotype of *nucl-632*

We previously isolated a ts mutant, *nucl-632*, by examining

the nuclear morphological phenotypes among 600 ts strains (Hirano et al., 1986). At permissive temperature (26°C) the *nucl-632* cells grow more slowly (~50%) than the wild type. (The generation time of the wild type is ~2 h at 36°C and 3 h at 26°C, respectively, in rich media.) At restrictive temperature (36°C) the mutant cells are arrested after a single cell division (the cell number increases approximately two-fold). Its viability decreases to 40 and 10% after 1 and 4 h at 36°C, respectively. The mutant cells show a characteristic nuclear phenotype. By DAPI stain, the normally hemispherical nuclear chromosome domain at 26°C alters to a bowl-like chromosome domain (called ring phenotype) at 36°C (Fig. 1, a and b). The frequency of cells exhibiting the ring phenotype was 80% after 3 h. Interestingly, synchronous culture analysis at 36°C (Mitchison, 1970; Hirano et al., 1988) indicated that the viability of cells remained nearly 100% in the G2 phase but sharply decreased in mitosis (data not shown). At the same time, the number of cells showing the ring phenotype sharply increased. It should be noted that the nuclear division takes place once in the synchronous *nucl* mutant culture at 36°C. (The cell number also increased two-fold.) Therefore, the results described above indicated that the *nucl* mutation does not block mitosis but cells showing the ring phenotype die.

DNA, RNA, and protein synthesis (Materials and Methods) are reduced within one generation time and completely arrested after two generation times at 36°C (data not shown). Because the morphological phenotype of *nucl-632* is similar to that of *top1-top2* double mutant (Fig. 1 c), we measured the levels of DNA topoisomerase activities in the *nucl* mutant extracts and found that the extracts contain normal levels of the relaxing activities of topo I and II (Hirano et al., 1986; Konoha, G., unpublished results). Tetrad analysis indicated that *nucl-632* contains a single recessive ts mutation and that the ts allele cosegregated with the nuclear phenotype. Segregants containing both *nucl* and *top1* alleles showed a cytological phenotype identical to that of single *nucl-632*. Triple mutants *nucl-top1-top2* were constructed. Their growth at 26°C was the same as that of the single *nucl* mutant. Because only one ts allele of *nucl* was available, it was possible that the phenotype described here could be allele specific.

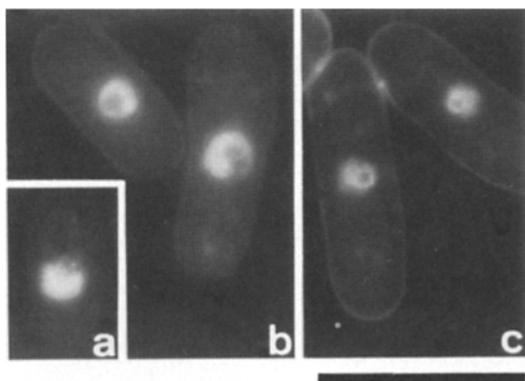


Figure 1. DAPI-stained fluorescence micrographs of an *S. pombe* ts mutant *nucl-632* at permissive (a) and nonpermissive temperature (b). Cells were first grown at 26°C, transferred to 36°C for 2 h, and stained by DAPI. For comparison, ts *top1-top2* double mutant cells incubated at 36°C for 2 h and stained by DAPI are shown in c. Bar, 10 µm.

Cloning of the *nucl*⁺ Gene

The *S. pombe* genomic DNA library made in pDB248' (Beach et al., 1982) was used for transformation of a Leu⁻ Ts⁻ haploid strain *leul nucl-632*, and Leu⁺ Ts⁺ transformants were obtained. A genomic DNA fragment inserted in plasmid pGK100 that was recovered from a transformant could complement the Ts⁻ phenotype of *nucl-632*. By subcloning, we estimated a 5.5-kb-long DNA fragment required for complementation (Fig. 2 a). To determine whether pGK100 was derived from the *nucl*⁺ locus, a subclone pGK109 was used to integrate the cloned sequence on a chromosome by homologous recombination (the marker gene used was the *S. cerevisiae* *LEU2* gene; see Materials and Methods). Tetrad dissection of resulting Leu⁺ integrants indicated that the Leu⁺ marker was tightly linked to *nucl* locus (PD:NPD:TT = 28:0:0, <1.7 cM) and also to *adel* (PD:NPD:TT = 32:0:0, <1.5 cM), which was previously shown to be linked to *nucl* (Hirano et al., 1986; Hirano, T., unpublished results). Therefore, the cloned genomic sequence is derived from the *nucl*⁺ gene.

The *nucl*⁺ gene was disrupted by the one-step replacement method (Rothstein, 1983) described in Materials and Methods. The disrupted haploid segregants were lethal, indicating that the *nucl*⁺ gene is essential. The disrupted spores germinated but did not divide. The disrupted haploid was rescued by plasmids carrying the *nucl*⁺ gene. These results were consistent with the ts lethal phenotype of *nucl-632*.

Genomic Southern hybridization probed with the 3.2-kb insert in pGK104 showed a single set of hybridizing bands consistent with the cloned *nucl*⁺ gene for three restriction enzymes (Fig. 2 b), and Northern hybridization showed an ~5.5-kb-long transcript (Fig. 2 c). Hence, the *nucl*⁺ gene appears to be unique in its genome and is transcribed to produce mRNA whose size is roughly equivalent to that of the minimal completable DNA.

Nucleotide Sequence Determination

We determined by the dideoxy method (Sanger et al., 1977) a 5,486-bp-long nucleotide sequence that can complement the *nucl* mutation. The nucleotide sequence is shown in Fig. 3 together with the predicted amino acid sequence. There is a 5,067-bp-long open reading frame that predicts the 1,689-amino acid residues polypeptide (189 kD). Intron is apparently not present. The cloned gene with only a 143-bp-long 5' upstream sequence (from the presumed first ATG codon) appears to be sufficient for complementation of the ts *nucl* mutation when the cloned sequence is introduced into multicopy plasmid.

By searching the NBRF protein data base, we found that the predicted *nucl*⁺ sequence reveals significant similarities to several domains of the largest subunits of *S. cerevisiae* RNA polymerase (pol) II and III (Allison et al., 1985) and also of *E. coli* pol subunit β' (Squires et al., 1981; Ovchinnikov et al., 1982). Furthermore, the sequence of *S. cerevisiae* pol I largest subunit recently reported by Memet et al. (1988) has the highest similarity to *nucl*⁺ polypeptide. No significant homology, however, is found in the upstream nucleotide sequences between the *nucl*⁺ and the *S. cerevisiae* largest subunit of pol I (Memet et al., 1988).

Results of dot matrix analyses between the amino acid sequences of *nucl*⁺ and the largest subunit of *S. cerevisiae* pol

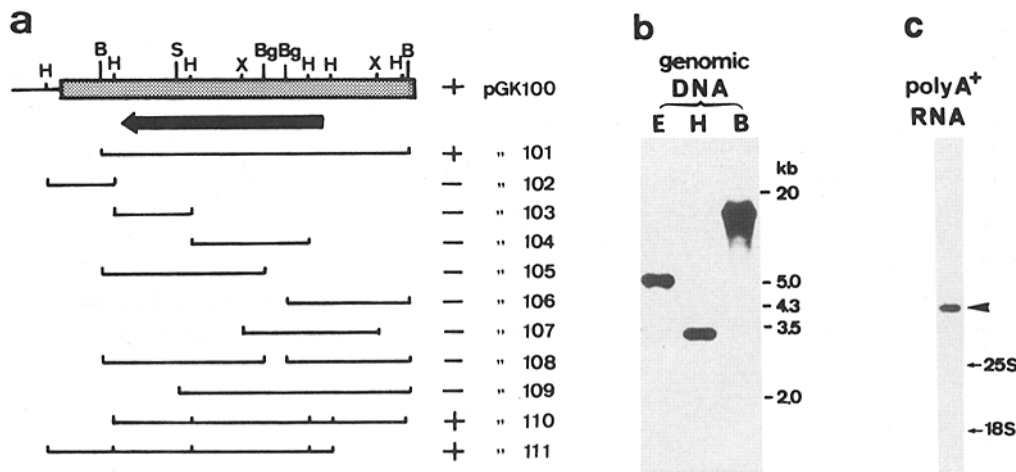


Figure 2. Cloning of the *nucl*⁺ gene. (a) A genomic DNA sequence (the shaded box in pGK100) that complemented *nucl*-632 mutant was isolated from an *S. pombe* gene library by transformation. Restriction map of pGK100 is shown. B, Bam HI; Bg, Bgl II; H, Hind III; S, Sal I; X, Xho I. Results of subcloning indicate that the minimal length for complementation is 5.5 kb. + indicates that the fragments can complement *ts nucl*-632. (b) Southern blotting of *S. pombe* genomic DNA probed with ³²P-labeled 3.2-kb fragment in pGK104. The numbers indicate the sizes of standard restriction fragments in kb. Genomic DNA was digested with Eco RI (E), Hind III (H), and Bam HI (B) and was run in 1% agarose gel. (c) Northern blotting of polyA⁺ mRNA probed with the same fragment in b. A 5.5-kb-long single hybridizing band was obtained (arrowhead). The positions of 25S and 18S RNA are also shown.

I, II, III, vaccinia virus pol (Broyles and Moss, 1986), or *E. coli* pol are shown in Fig. 4 a by identifying ≥8 matches over 20 contiguous amino acids. Similarity between the *nucl*⁺ and the pol I subunit is continuous except the two NH₂ and COOH regions rich in charged residues. These highly hydrophilic domains are found only in the pol I subunit (Memet et al., 1988). The *S. cerevisiae* pol I sequence contains 1,664/25 residues (186 kD), 19 residues less than the *nucl*⁺ sequence. The two sequences are aligned in Fig. 4 b. Overall similarity of the two sequences is ~49%.

Similarity to the largest subunit of the other RNA polymerases was found only in the restricted domains (Fig. 4 a). Domain A, present in all the eukaryotic sequences, also exists in the *nucl*⁺ sequence. Neither *nucl*⁺ nor pol I subunit (Memet et al., 1988) contain domain 1 (Allison et al., 1985). The other five domains found in all the largest subunits are also present in the *nucl*⁺ sequence although their sizes in β' are generally shorter.

Identification of the *nucl*⁺ Gene Product in *S. Pombe*

To identify the *nucl*⁺ protein in the cells of *S. pombe* by immunochemical methods, we raised antibodies against the *nucl*⁺ fusion polypeptides made in *E. coli*. Two plasmids pAR3039 (Studier and Moffatt, 1986) and pUR291 (Rüther and Müller-Hill, 1983) were used to express different parts of the *nucl*⁺ gene in *E. coli* (Materials and Methods). Fused *nucl*⁺ polypeptides obtained were purified and injected into rabbits as antigens. Antisera thus obtained strongly reacted with antigens made in *E. coli* (data not shown). Antibodies were affinity purified with the antigen made in *E. coli*.

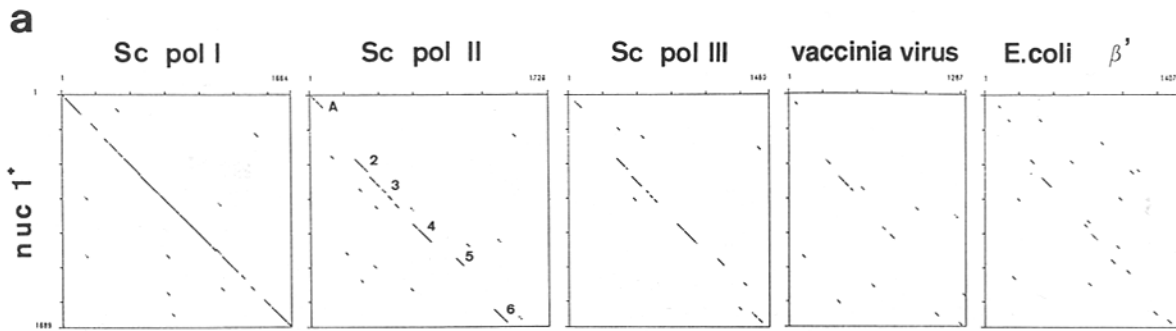
By Western blotting, we could detect a polypeptide (molecular mass, ~190 kD) in the extracts of *S. pombe* by antisera obtained 4 wk after injection (Fig. 5 a). Because two antisera raised against different parts of *nucl*⁺ produced a single band with a similar molecular mass in the extracts of *S. pombe* (data not shown), the protein band most likely

represents the same polypeptide; i.e., the product of *nucl*⁺ gene. Introduction of multicopy plasmid pGK100 containing the *nucl*⁺ gene into *S. pombe* did not increase the intensity of 190-kD protein band (data not shown), indicating that gene dosage did not affect the amount of *nucl*⁺ product.

To determine whether the *nucl*⁺ product exists in the nucleus, Percoll gradient centrifugation of *S. pombe* lysates was run to fractionate the nuclei (Hirano et al., 1988). Each fraction was run in SDS gel electrophoresis and analyzed by immunoblot using anti-*nucl*⁺ antisera. Results (Fig. 5 b) clearly demonstrated that the immunoblot band for *nucl*⁺ p190 is present exclusively in the nuclear fractions. Topo II band produced by anti-topo II (Shiozaki, K., and M. Yanagida, to be published) was obtained in the same nuclear fractions.

Localization of *nucl*⁺ Gene Product in Nucleolus

Using immunofluorescence microscopy with anti-*nucl*⁺ sera, we found intense immunofluorescence in the nucleus of the wild-type cells, specifically at the region of nucleolus, as shown in Fig. 6, a-c. The color micrograph (a) shows the *S. pombe* wild-type cells treated by anti-*nucl*⁺ antibody followed by rhodamine-conjugated second antibody. b shows the same cells stained by DAPI. c, obtained by double exposures for anti-*nucl*⁺ and DAPI staining of the same field, clearly shows that the *nucl*⁺ protein is associated with the nucleolar rods protruding from the chromosome domain. Previous work showed that the *S. pombe* interphase nucleus consists of two hemispherical domains, one chromosomal and the other a nonchromosomal domain rich in RNA. Nucleolar DNA exists at the boundary of the two hemispheres; the short rods were identified to be the rDNA clusters present in chromosome III (Toda et al., 1981, 1984; Umesono et al., 1983; Niwa and Yanagida, 1985; Smith et al., 1987; Matsumoto, T., unpublished results). Interestingly, the number of rods stained by anti-*nucl*⁺ is generally four for each nucleus in the interphase cells (mostly G2 cells) but two during or just after nuclear division (Fig. 7, right). The two protrusion



b

nuc1	HNIAQPVSSSEIKSVKFGIYDQDDVEKISVKQIVNPVLLDNLNHPITGGLYDLALGPVKNVSCATCFLAERYCPCGHGHI VLPSPAYHPLFFSOMYNLNR	100
pol I	MDISKPVGSEITSVDPGILTAKELRNLSAKQITNPTVLDNLGHPVSGGLYDLALGAFLRN-LCSTCGLDKFCPCGHGHIELPVPVYNPLFFNQLYIVLR	99
	STCLYCHNFKLSKVKVHLFFCRLKLLDYGLNES---EMVENVSLTEAIKKNNGTPLEDGSSEDSGLGHDDIAKDAATLHRI RDEFY--AKSIA DSRQNAHID	200
	ASCLFCNHRFLKSVSEVHRVYACKLRLLYGLIDESYKLDIETLGLSNSSMYTDDAEIETEDMDGEGSKQSKDISSLLNELKSKRSEYVDMAIAKALSDGRTER	205
	AQLTLLTHERKVKVRAFYHAISSRKOCDNCOFSFNFRRKGFAKIFEIPLSGKNLQFMEQTGKIRSDVLRDTSKXHHDEGYDGDSSNSESVEGIDL	300
	GSFTATVNDERKLVNFEHKKLLSRGKCDNCGHFSFRKRDGFTKIFETALNEKQITNRRVKGFIR---ODMIKKKQAKKLDG---SNEASANDEES	297
	FEEDPNPLKXKSPAIAGKAYMTSTEVRNHLRRLFKENVVLRLYAKR---GKPSADMFLLQNI AVPPTRFRPASKMGDEVHNIQNEULLTRIQSSIQ	400
	FQVGRNP---TTRPKTGSTYI LSTEYKXILDTVFRNEQCVL-QYVHRSRNLKRLVKADSPFMDVLVPPTRFRLPSKLOEEVHNSQNLKSKVLTSSL	395
	IASLSKSDSTVEVNPDEKLEERRSAPFELINAFVQLQNDVNSLDSNRNPSSGGQSRTPV-PCIKQILEKKEGLFRKHHMCKRVNYAARSVISPDPNIET	500
	IRDLNDD--LSKLDKQKVSLEDRRVIISRLMNAFVTIQNDVNAFIDS--TKAAGRTSQKVPVPGVQKALEKKEGLFRKHHMCKRVNYAARSVISPDPNIET	492
	NEIGVPPVFAVKLTYPEPVLYNFNEMRNVAVINGPKVPGASHIQNEEDGTLISLPLTI EQRTALANQLLTPQSNLISPPYSYSLINHXKVVYRNVKNG	600
	NEIGVPPVFAVKLTYPEPVLYNIAELRQAVIXGPDKVPGATIQNEEDGSLVSLIGMSVEQRKALANQLLTP----SSNVSTITL---HXKVVYRNIKNR	591
	DHLILNRQPTLHKPSMNAHKARILEPGEKTI RMHYANCSYNADFDGDEMMHHPDSTNARSEAFIARTDSQVLPVTSQDPLRGLIQDHVVHGVVLTCKD	700
	QVVLNHRQPTLRKASMHGKXVRLPNEKTLRLHYANTGAYNADFDGDEMMHHPDQENARAEALNANTDSQVLPVTSQDPLRGLIQDHISAGVVLTSKD	684
	TFYIRDEYQQLLFOALKPDETHGYR--IXTLPPAIQRPGIYWTGXQIISVLLNLKXSDRPLWLSKAKVPGKYVSPDSEEGSVLFDGCELLCGLDKSS	800
	SFFTREGYQYIYGCIRP-EDGHTSKSIYTLPPTIKPYPLTYGKQIITVLLNVTPDPHPIHLISKXIKNEYVVKGSLNEVLFKOGALLCGLDKSSQ	785
	FGASAFGLVNSVHELPGDIAGRLLSLSKLFATYAQRGFTCRMDRLDEQDGNVRRQLLENGKSFGLAAESEVGLSTOSP--IALLNANLEEVVRDDE	900
	YGASKYGI VNSLNEVYGPVAAKLSVLGRLFTNYITATAFTCGMDRLTAEGNKVRTDILKTSVDTGREAAAQVNTLKDQTPADDPPELLKRLQELRDN	887
	KLQGLDAAHKGNHGLTSSII NKCI PDGLLTKFPYHHQHTVSGAKGSHVYVSOISCLLQGOLEGRRVPVHVSQKSLPSPVVPYETSASGQFIASRFL	1000
	KSGILDVAITSSKVNIAISOVYKXCPVDPGTHKFPKNSHQAALSGAKGSHVYVSOIMCLLQGOALEGRRVPVHVSQKSLPSPVVPYETSASGQFIASRFL	987
	TGIAPOEYVFNCHAGREGLDITAVTKSRSGYLQRCLMKHLEGLCVQYDHTVROSDGSI VQFHYGEDSLDVTQKHLTOFEFSAKNYKSLIQKVKKSVLS	1100
	SGIKPQEYVFNCHAGREGLDITAVTKSRSGYLQRCLTKOLEGVHVSVDNSIROADGTLVQPHYGDAIDITKESHMTQEFCLDNYVALLKKNVPSALIE	1087
	AVDSETASSYAKKAL-----KPKYKYPVLDKYPSPRYLGSVSEKFORAVDEYTKNPKD-IASKKESKLDSD--LLNESKFXALNQLRYOQSLVDPGESVGLASQ	
	HLQVESALKYKSKTLKVRKXKSEPHYKOSVKYDPLAKYHPAKYLGVSSENFO-----DKLESFLDKNSKLFKSSDGVNEXFRALNQLKYNRSLINPGEAVGIIASQ	
	SI GEPSTQHTLNTFHFACFGAKNVTLCIPRLREIIMTASANIQTPMTLRLNDGVSQKASAPCKEVNKLVLSEVVRQVRYTE--KISGQGSDEQSKTYAIR	1300
	SVGEPSTQHTLNTFHFACGCAAVVTLGIPRLREIIMTASAAIKTPQHTLPVNDYSQEQADTFCKSIKVLVSEVIDKVI VTETTGTSNTAGCNAARSYVH	1293
	LDLYSRDEYQDEYGLQEEIESTFSRPLKILNRIIKSYLAKSKQRKSGKDDTYPEVGOALKPLEDIDEAPICEGAEALEDEQNDATNEXHVSRSKQK	1400
	HRFFDNNEYSEYDVSKEELONVINSQF-----IHLLEAAIVKEIK-KQKRTTGPDIQVA-VPRLOTQVA-NSSNSKRLE-EQND--EESHKKTRQA	1380
	ASVECPDEADKVALRQKGSNKVEDVNHDEEEDGCFKSDSESVDKFE--RKLLEKQNTVSI SERRELQKTAKEILSNCKHLDFQYVNGEATVLPVPI NT	1500
	VSYDEPDEDEIETHRAEKSSDEEGCISDXESD-SQSEDEY-DMNEQINKSIVEANNMKNVORDRQSAIISHRFITK-VNFDSESGKWCPEFKLEAADT	1480
	EKLLNVSLEKACSETVINEIPCI TRCFKSPDSALDTPVKVITEGVNLKAI VEFYNEISHNDIYTDIAAIDRIYGVQATRKAIVHEVSSVFCVGYGIAV	1600
	EKLLNVIIEEIKRKSIIROIPIHIDRCVHPPE--ENGRVLTGCVNFQAMVDQEAFTDVGITSNQVAAVLYTYGVEAARTIVNEINNVESRYAIVS	1577
	DPRNLSLIDAYNTFEGCYKAPNRMGIEYNTSPFAKNSFETTCHEALRGDQVDDLSNPSSRLVVGRCVGNFGTGSFDIFNPVVDSPAN	1689
	SFRNLDLIDMHTROGTLPANRQGMETSTSSFMKHSYETTCQPLTKAVLDNERQQLDPSARIIVYGKLNHVGTGSPVLAQV--PNA	1664

Figure 4. Sequence homology between *nuc1+* protein and the largest subunits of RNA polymerases. (a) The amino acid sequence of *nuc1+* polypeptide (*ordinate*) was compared with the sequences of the largest subunits of *S. cerevisiae* pol I, pol II, pol III, vaccinia virus, and *E. coli* RNA polymerases (*abscissa*) by looking for ≥ 8 identical residues over 20 contiguous amino acids in diagonal dot matrix analysis. Homology is highest to the pol I subunit. (b) The amino acid sequences of *nuc1+* polypeptide and the *S. cerevisiae* pol I subunit are aligned. The underscoring indicates the domains similar to the other RNA polymerases (broken line only for eukaryotic polymerases).

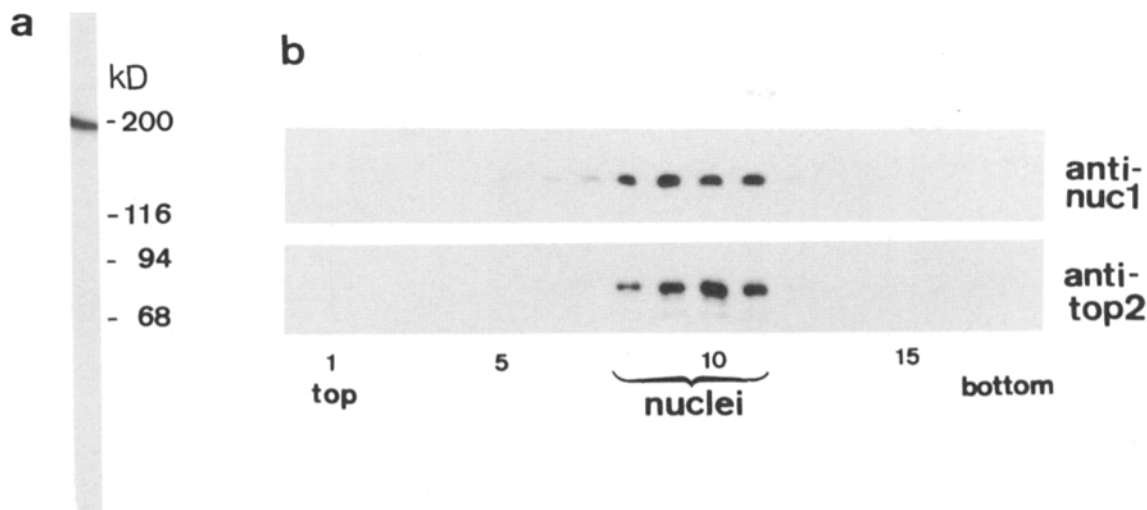


Figure 5. Immunoblotting identification of *nucl*⁺ gene product in *S. pombe* nucleus. (a) An antiserum raised against the *nucl*⁺ fusion polypeptides made in *E. coli* (Materials and Methods) gives a band at the position of ~190 kD. (b) Location of the *nucl*⁺ protein in the nuclear fraction. The wild-type *S. pombe* cells were grown at 30°C, and cells were digested with zymolyase followed by homogenization. Extracts were centrifuged on a 15–40% linear Percoll gradient. Fractions were observed by DAPI stain and run in SDS gel electrophoresis, followed by immunoblots using anti-*nucl*⁺ or anti-*topo* II. The *nucl*⁺ protein and *topo* II are present only in the nuclear fractions.

Immunofluorescence Microscopy of the Inactivated *nucl*⁺ Gene Product in the Mutant Cells

To investigate behavior of the *nucl* mutant protein, mutant cells were first grown at 26°C and then incubated at 36°C for different time intervals and stained by anti-*nucl*⁺ antibody. Nucleolar immunofluorescence was initially intense, though not as strong as wild type, and then slowly faded and disappeared after 3 h at 36°C (Fig. 8, right). (Such change did not occur in the wild-type cells transferred from 26 to 36°C.) Granular fluorescent materials were often seen in cytoplasm of the cells that had lost nucleolar immunofluorescence. Cells showing the altered chromosome domains (ring phenotype) have lost nucleolar immunofluorescence. Thus the aberrant nucleolus appeared to be the cause of the morphological phenotype of *nucl-632*. Western blotting of the *nucl* mutant extracts, however, indicated that the amount of *nucl*⁺ protein per cell (about one-third of the amount of wild type; see Discussion) remained nearly the same during the 3-h incubation at 36°C (Fig. 10). This apparent discrepancy between the time-dependent decay of immunofluorescence and the constant level of *nucl*⁺ protein in immunoblotting can be interpreted assuming that the *nucl*⁺ polypeptide highly densely present in the tiny rod was dispersed at restrictive temperature. This dispersion might be the result of the dissociation of *nucl*⁺ protein from nucleolar DNA, followed by dispersion to the cytoplasm and/or accumulation as cytoplasmic granular material.

Immunofluorescence Microscopy of *nucl*⁺ Product in DNA Topoisomerase Mutant

The ribosomal RNA synthesis is greatly diminished in the *S. cerevisiae* and *S. pombe top1-top2* double mutants whereas mRNAs and tRNA syntheses are relatively unaffected (Brill et al., 1987; Yamagishi and Nomura, 1988). Because the phenotype of *nucl* mutant was related to the nucleolar func-

tion, we investigated the fate of the *nucl*⁺ polypeptide in the absence of both DNA topoisomerases. In the *top1-top2* double mutant used in the present study, both *topo* I and II activities are inactivated at 36°C in vivo as well as in vitro (Uemura and Yanagida, 1984, 1986; Uemura et al., 1987b).

Surprisingly, nucleolar immunofluorescence by anti-*nucl*⁺ antibody rapidly diminished in the *top1-top2* mutant, as shown in Fig. 9 (right panels). 15 min after the shift to 36°C (Fig. 9 b) more than 80% of the cells lost nucleolar immunofluorescence. (By DAPI stain, 50% of the cells showed the ring phenotype; Uemura and Yanagida, 1984.) The initial stage of the decrease in immunofluorescence intensity appears to be the dispersion of nucleolar immunofluorescence. At 30 min (Fig. 9 c), the nucleolar fluorescence was completely absent, and the frequency of cells showing the ring phenotype reached 80%. Granular materials in cytoplasm were seen in these cells. Immunoblot analysis, however, showed that the level of *nucl*⁺ protein in the *top1-top2* double mutant extracts remained nearly the same during the 3-h incubation at 36°C (Fig. 10). These results indicated that the defect in DNA topology led to the rapid dispersion of *nucl*⁺ protein and nucleolar disorganization.

Discussion

S. pombe nucl⁺ Locus Encodes the Largest Subunit of RNA Polymerase I

In eukaryotic cells there are three forms of nuclear RNA polymerases, each with separate transcription functions. Pol I (A) is made for rDNA transcription, pol II (B) for mRNA, and pol III (C) for the small 5S and tRNA. Previous studies (Allison et al., 1985; Biggs et al., 1985; Memet et al., 1988) show that several domains in the largest subunits of eukaryotic pols I, II, and III are similar to each other and also to those in the largest subunit of prokaryotic RNA polymerase (Ov-

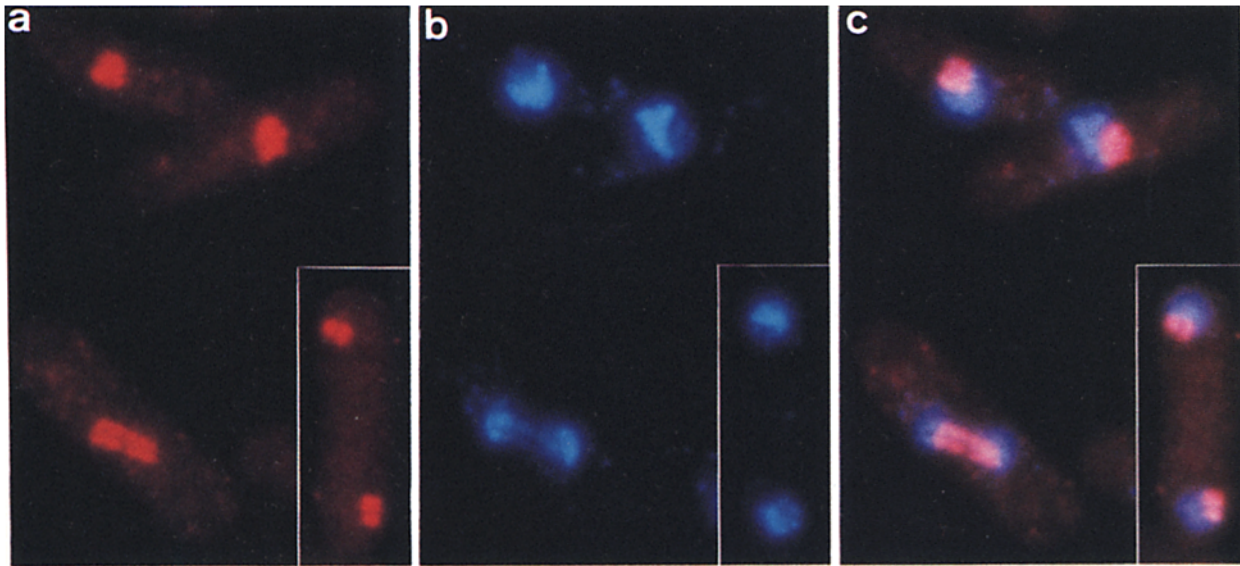


Figure 6. Immunofluorescence color photomicrographs of *S. pombe* wild-type cells stained with anti-*nucl*⁺ serum. (a) Anti-*nucl*⁺ serum stain followed by rhodamine-conjugated second antibody. (b) DAPI stain. (c) Double exposures of antibody and DAPI stain. Intense immunofluorescence is localized in nucleolus. Bar, 10 μ m.

chinnikov et al., 1982). We provide evidence in this paper that the *S. pombe nucl*⁺ gene encodes the largest subunit of RNA polymerase I.

We found that essential domains in the largest subunits of RNA polymerases (Allison et al., 1985; Memet et al., 1988) including a potential zinc-finger motif are highly conserved in *nucl*⁺ polypeptide. Therefore, the *nucl*⁺ gene most likely encodes the largest subunit for one of the three RNA polymerases. Comparison with the *S. cerevisiae* sequences (Allison et al., 1985; Memet et al., 1988) indicates that the *nucl*⁺ sequence shows the highest degree of similarity to that of the pol I subunit (overall homology is 49%). This value is identical to those obtained for topo I (47%) and II (49%) between two distantly related yeasts (Uemura et al., 1986, 1987b). Similarities of *nucl*⁺ protein to the largest subunits of pols II, III, and prokaryotic polymerase, however, are restricted in several domains.

Predicted *nucl*⁺ polypeptide has 1,689 residues, which is

the closest in number to the 1,664 residues of the *S. cerevisiae* pol I subunit (there are 1,726 and 1,460 residues for the pol II and III subunits, respectively). The COOH-terminal hexapeptide repeats found in the pol II subunits of different organisms (Allison et al., 1985; Corden et al., 1985) are not present in the *nucl*⁺ polypeptide. Domain 1 present in pols II and III (Allison et al., 1985) is not present in either pol I or *nucl*⁺ protein. The two highly hydrophilic regions near the NH₂ and COOH termini of pol I subunit (Memet et al., 1988) are also present in the *nucl*⁺ sequence. In the NH₂ region, several conserved stretches, such as EDXXDXEDS, ERKK, and FRKXGFXXKIFE, are present, suggesting that the region may have an ordered structure specific for pol I function.

Results of immunofluorescence microscopy are also consistent with the notion that the *nucl*⁺ polypeptide is a subunit of pol I. Anti-*nucl*⁺ antibodies intensely and specifically stain a nuclear region previously identified as nucleolus

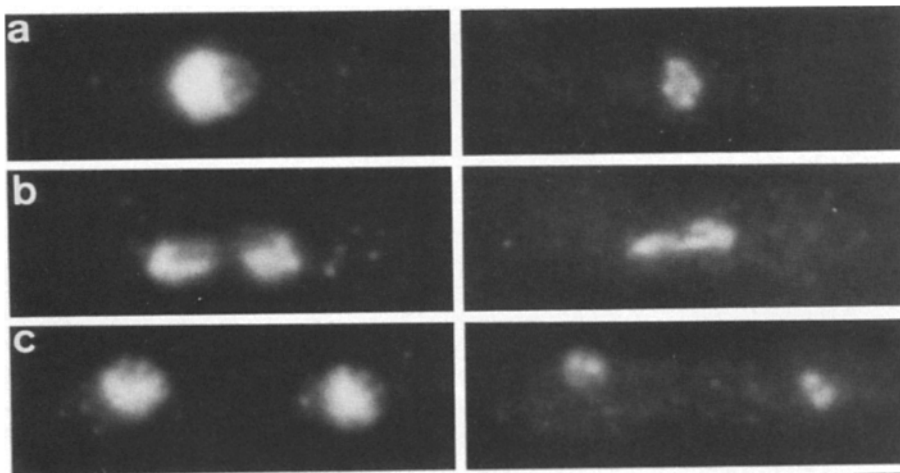


Figure 7. Nucleolar stain by anti-*nucl*⁺ serum. Interphase and mitotic cells of the wild type were stained by anti-*nucl*⁺ (right). DAPI stain (left). Note that the number of short protrusions is four in most interphase cells (a) but two for those cells during or just after nuclear division (b and c; see text). Bar, 10 μ m.

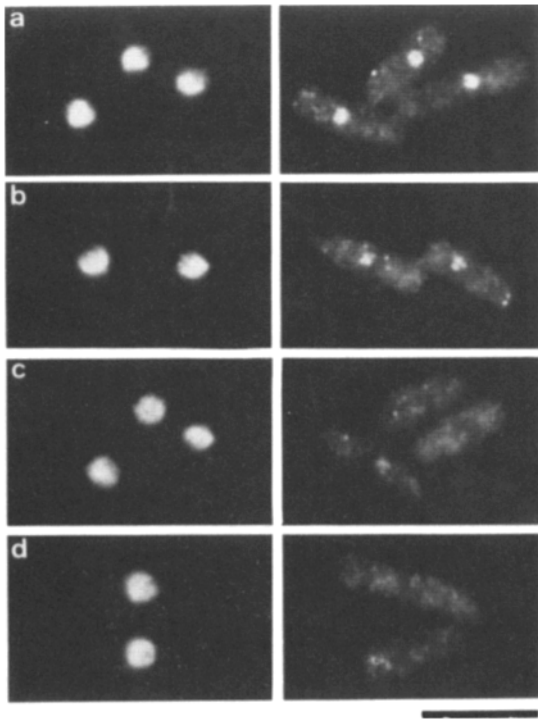


Figure 8. Nucleolar immunofluorescence diminished in *nucl* mutant cells at 36°C. Cells of *nucl-632* were first grown at 26°C and then transferred to 36°C. Cells were collected at intervals and were immediately fixed and stained by anti-*nucl*⁺ antibody. Nucleolar stain by anti-*nucl*⁺ (right) is initially strong but weakened at 36°C. DAPI stain (left). No fluorescence by anti-*nucl*⁺ is visible on nucleolus after 3 h at 36°C. a, 0 h; b, 1 h; c, 2 h; d, 3 h. Bar, 10 μm.

where rDNA repeats reside (Toda et al., 1981, 1984; Umesono et al., 1983; Niwa and Yanagida, 1985). In the *nucl* mutant cells incubated at 36°C the nucleolar fluorescence was faded. Although final proof may have to await direct determination of the amino acid sequence for the largest subunit of *S. pombe* pol I, we conclude that the *nucl*⁺ gene product is the largest subunit of pol I and interpret the mutant phenotype produced by its defect.

Role of *nucl*⁺ Protein in Nucleolar Organization

Little is known for certain about the function of the largest subunits of RNA polymerases. In the bacterial polymerase, the largest subunit may bind to DNA and play a role in promoter recognition (Fukuda and Ishihama, 1974; Glass et al., 1986). In the eukaryotic enzymes, the largest subunit may be involved in the RNA chain elongation and in the enzyme active site (Ruet et al., 1980; Greenleaf, 1983). The presence of one putative zinc-binding finger (Memet et al., 1988) also suggests that the subunit binds to DNA. A large part of rDNA may be occupied by *nucl*⁺ protein and the regular nucleosome structure might not be formed in rDNA. The short rods visualized by anti-*nucl*⁺ indicated higher order regularity of the packaged rDNA repeats; 100-μm long rDNA is condensed into these tiny (0.3 μm in length) rod-like structures. There seemed to be two classes of rDNA clusters in chromosome III (Chikashige, Y., unpublished results). The *nucl*⁺ subunit may be implicated in such higher order orga-

nization. A role of RNA polymerase I in nucleolar organization, recently shown by Benavente et al. (1987), demonstrated that microinjection of antibodies to RNA polymerase I into mitotic cells of PtK₂ inhibits the nucleolar reformation in the telophase.

Phenotypic Similarity between *nucl* and *top1-top2* Mutants

Similarity between the phenotypes of *nucl-632* and *top1-top2* is not pure coincidence (Fig. 11). In the former pol I mutant, the defect is expected in rRNA synthesis, which occurs in nucleolus. In the latter *top1-top2* double mutant, rRNA synthesis is known to be greatly depressed but mRNA synthesis is relatively unaffected (Brill et al., 1987; Yamagishi and Nomura, 1988). (The reason mRNA is synthesized in the absence of topoisomerase I and II is not understood.) A common feature in the cells of these two mutants is the arrest of rRNA synthesis. In the *nucl* mutant, the reduction of RNA synthesis is slower than that in *top1-top2* (Uemura et al., 1986; Yamagishi and Nomura, 1988; Hirano, T., unpublished results), suggesting that the time required for the inactivation of mutant *nucl* polypeptide at 36°C may be longer than those for mutant topoisomerase I and II.

We speculate that the hollow bowl-like structure (the ring phenotype) of the DAPI-stained chromosome region in *nucl* mutant is the result of inactivation of the large number of pol I enzyme molecules followed by their dispersion. That is, the destruction of nucleolus produces the ring phenotype. This hypothesis argues that pol I is required for the formation of nucleolus as its major component. In the *top1-top2* double mutant, on the other hand, nucleolus is damaged because of the defect in DNA topology that is discussed below.

Role of *nucl*⁺ Protein and DNA Topoisomerases for rRNA Synthesis

We raised antisera against *nucl*⁺ fusion polypeptide and could identify *nucl*⁺ polypeptide in *S. pombe* by Western blotting and immunofluorescence microscopy. Two antisera made for different parts of the *nucl*⁺ protein detected a nuclear protein of identical molecular mass (~190 kD). Neither sera, however, detected any protein in the *S. cerevisiae* extracts; they had few antibodies directed to the conserved domains of RNA polymerases (Hirano, T., unpublished results). The amount of *nucl*⁺ protein is significantly lower in the *nucl* mutant extracts obtained from the cultures grown at permissive temperature than that in the wild-type or *top1-top2* extracts. The ratios of two- to threefold decrease for the *nucl* mutant were obtained in several experiments. The reason for this is not understood but the mutant *nucl* protein might be defective in the folding for functional subunit or the assembly for mature RNA polymerase I. The low level of *nucl*⁺ protein might cause the retarded growth. The generation time of *nucl-632* at 26°C was ~50% longer than that of the wild type at 26°C.

Immunofluorescence by anti-*nucl*⁺ was initially intense in the nucleolus of *top1-top2* mutant but rapidly decayed when the mutant cells were incubated at 36°C. Within 15 min, most of immunofluorescence of the nucleolus had faded. This would not be proteolytic degradation because immunoblots showed nearly the same amount of *nucl*⁺ protein in the cells. Changes of antigenic properties or inaccessibility to antibodies are possible but unlikely. We suppose that

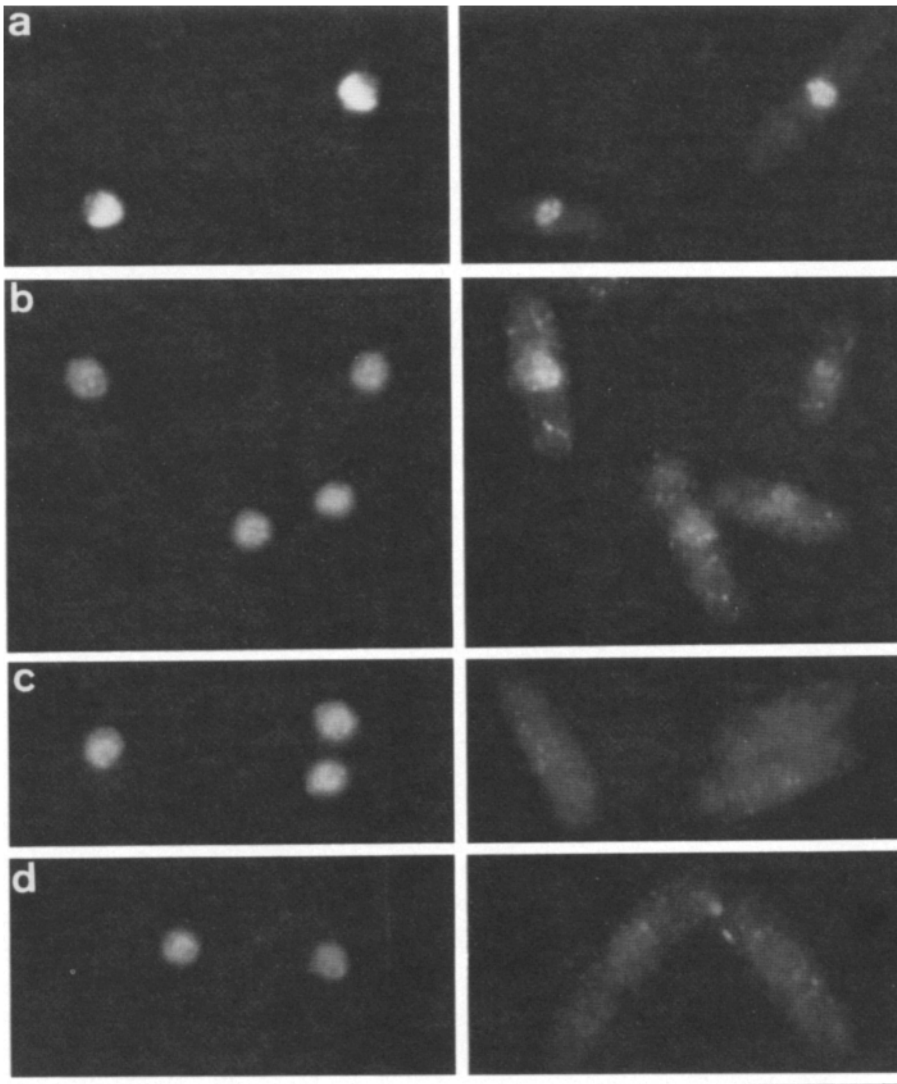


Figure 9. Rapid decay of immunofluorescence by anti-*nucl*⁺ in *S. pombe top1-top2* double mutant cells at 36°C. The double mutant cells were first grown at 26°C and then transferred to 36°C. Cells incubated at 36°C for 0 (a), 15 (b), 30 (c), and 120 (d) min were collected and immediately fixed, followed by anti-*nucl*⁺ antibody staining. Immunofluorescence (right). DAPI stain (left). Bar, 10 μm.

nucl⁺ molecules are dispersed in the absence of topo I and II. If so, the immediate question is how *nucl*⁺ protein is dispersed. It might be dissociated from nucleolar DNA and eventually diffuse away from the nucleus. Another possibil-

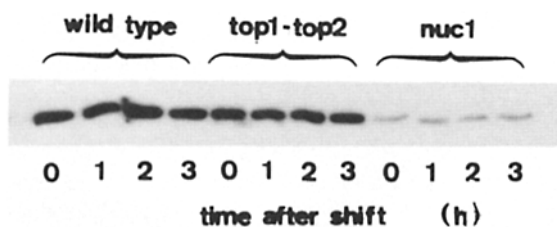


Figure 10. Immunoblots of wild-type, *top1-top2*, and *nucl-632* mutants using anti-*nucl*⁺ antibody. Cells of the wild type and the mutants were first grown at 26°C and then transferred to 36°C. Cells were collected at appropriate intervals, and extracts were prepared followed by SDS gel electrophoresis. Band intensities of immunoblots by anti-*nucl*⁺ did not significantly change within 3 h at 36°C. The amount of p190 protein in *nucl* cells appears to be lower than that in the wild-type and *top1-top2* cells, which is consistent with immunofluorescence microscopy.

ity is that the rDNA repeats are rapidly decondensed so that the bound *nucl*⁺ protein molecules are accompanied by the expanded rDNA (in this case, rDNA transcription is arrested in spite of pol I and rDNA association). Alternatively, the decondensation of rDNA may loosen the binding of pol I subunits, and concomitantly of enzyme molecules, to nucleolar DNA so that *nucl*⁺ protein is dissociated from nucleolar DNA. To determine the validity of these possibilities, we attempted to isolate the nuclei from the double mutant cells incubated at 36°C but failed because the nuclei seemed to become fragile.

In any case, a defect in the DNA superhelicity must be primarily responsible for the rapid nucleolar damage. This implies that proper superhelicity is required for the folding of nucleolar DNA and pol I enzyme molecules into the functional organization of the nucleolar genes. We are investigating the structural role of topoisomerases in nucleolus by

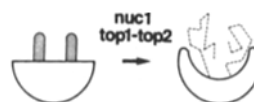


Figure 11. A speculative view on the alteration of fission yeast nucleolus in *nucl* or *top1-top2* mutants.

immunofluorescence microscopy using anti-topo I and anti-topo II prepared against fusion polypeptides.

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