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 × 10^6 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) 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 HI) 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 most likely involved in the higher order chromosome structure and usually unaffected. Single topo mutants, however, grow normally and single topo2 mutants are specifically blocked in nucleolar synthesis. 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 topo mutants, however, grow normally and single topo2 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 genetic 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- leu2-32 ura4-dl8 ade6-M210) and TP4-ID (h- leu2-32 ura4-dl8 ade6-M216 his2) were used. The diploid 5A/ID was made by a cross between TP4-5A and TP4-ID. 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' leu-32/leu-32 uri4+leu4-184D halo6-M210/ade6-M216 his2+/- nucl::ura4+/-). TP6G1 is the Leu* transformant of TP6 by pGK100, and TP6G1 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.

Eschercia coli HB101 and MC1061 were used for preparation of plasmids and B15183 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 (Rothstein and Müller-Hill, 1983) for expression of fused-nuc1+ 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., 1985) using the host strain h' leu 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 of pnucl was used as 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 poly(A)+ 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 (Bostein 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 leu 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 leu 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 E. 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 polynucleotide sequences derived from pUC18, followed by subcloning 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. pURA4 was used as pBR322-M216/Ade6-M216 his2+/- nucl::ura4+/- 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 (Rothstein 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 nM) and were incubated for 3 h. Cells were collected, freeze-thawed, and digested with lysosome. 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-nuc1+ polypeptide were dissolved in 8 M urea. Dissolved materials were run in SDS gel electrophoresis. Polyacrylamide gel containing the nucl+ band was cut out. Poly-peptide 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 top1 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.

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, <1.5 cM) and also to ade1 (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 complementable 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 θ (Squires et al., 1981; Ovchinikov 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 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.
I, II, III, vaccinia virus pol (Broyles and Moss, 1986), or E. coli pol are shown in Fig. 4a 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. 4b. 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. 4a). 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 I (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. Antiserum thus obtained was used to detect a polypeptide (molecular mass, ~190 kD) in the extracts of S. pombe as antigens. Antisera thus obtained was used to detect a polypeptide (molecular mass, ~190 kD) 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. 5b) clearly demonstrated that the immunoblot band for nucl+ pl190 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. 6a–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 protru-

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 the nucl-632. (b) Southern blotting of S. pombe genomic DNA probed with 32P-labeled 3.2-kb fragment in pGK104. The positions of 25S and 18S RNA are also shown. The two protru-
**Figure 3. Nucleotide sequence of the nuc1** gene. The 5,486-bp-long sequence containing a 5,067-bp single open reading frame is sufficient for complementation. Predicted amino acid sequence containing 1,689 residues is shown in single-letter designation.

We estimated the amount of nuc1 polypeptide in the wild-type *S. pombe* cells to be ~2 × 10² copies/cell. Estimation was based on a series of immunoblot analyses of *S. pombe* extracts using the fused nuc1 polypeptide as standard. If all the nuc1 molecules in *S. pombe* are associated with the tandemly repeated rDNA clusters, then one can calculate DNA length per bound molecule. Approximately 200 rDNA repeats (10.5 kb), each containing a 7-kb-long transcription unit, are supposed to be present per exponentially growing cell, so that the value of 70 (200 × 7 × 10²/10⁵) bp/bound molecule is obtained. Although not all of the polynucleic acids molecules might bind to rDNA, the polynucleic acid molecules are supposed to be present per exponentially growing cell to be visualized by electron microscopy (Miller and Beatty, 1969); ~120 bp/bound RNA polymerase unit was obtained for *Triturus* nucleolar genes.
Figure 4. Sequence homology between nucl\(^+\) protein and the largest subunits of RNA polymerases. (a) The amino acid sequence of nucl\(^+\) 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 $\geq$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 nucl\(^+\) 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).
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 cytoplasms 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 function, 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 on 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 μm.

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 I 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 FRKXGFXKIFE, 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 μm.
where rDNA repeats reside (Toda et al., 1981, 1984; Umeda et al., 1983; Niwa and Yanagida, 1985). In the mutant cells incubated at 36°C the nucleolar fluorescence was faded. Although final proof may have to await direct de-
nucleolus after 3 h at 36°C. a, 0 h; b, 1 h; c, 2 h; d, 3 h. Bar, 10 μm.

**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; Umeda 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 sub-
unit 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 pro-
moter 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 ac-
tive site (Ruet et al., 1980; Greenleaf, 1983). The presence of one putative zinc-binding finger (Mernet 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 struc-
tures. 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 organ-
ization. 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 PtK1 inhibits the nucleolar reforma-
tion in the telophase.

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

Similarity between the phenotypes of nucl-632 and topl-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 topl-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 topos I and II is not understood.) A common feature in the cells of these two mutants is the arrest of rRNA synthe-
sis. In the nucl mutant, the reduction of RNA synthesis is slower than that in topl-top2 (Uemura et al., 1986; Yamagishi and Nomura, 1988; Hirano, T., unpublished results) suggesting that the time required for the inactivation of mu-
tant nucl polypeptide at 36°C may be longer than those for mutant topos 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 topl-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 antiseras made for different parts of the nucl+ protein detected a nucl-632 and topl-top2 double mutant, rRNA syn-
thesis. In the former pol I mutant, the reduction of RNA synthesis is slower than that in topl-top2 (Uemura et al., 1986; Yamagishi and Nomura, 1988; 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 topl-
top2. 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 topl-
top2. The ratio of two- to threefold decrease for the nucl mutant were obtained in several experiments. The rea-
son 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 topl-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 immuno-
bots 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
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 possibility 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 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.

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 pI90 protein in nucl cells appears to be lower than that in the wild-type and top1-top2 cells, which is consistent with immunofluorescence microscopy.

Figure 11. A speculative view on the alteration of fission yeast nucleolus in nucl or top1-top2 mutants.
immunofluorescence microscopy using anti-α-tub I and anti-α-tub II prepared against fusion polypeptides.

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