Identification, molecular cloning, and mutagenesis of *Saccharomyces cerevisiae* RNA polymerase genes

(plaque hybridization/gene family/sequence conservation/gene disruption/Drosophila melanogaster)

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ABSTRACT Three different regions of *Saccharomyces cerevisiae* DNA were identified by using as a hybridization probe a fragment of *Drosophila melanogaster* DNA that encodes an RNA polymerase II (EC 2.7.7.6) polypeptide. Two of these regions have been molecularly cloned. Each contains a sequence related not only to the *D. melanogaster* DNA fragment that was used as a probe in its isolation but also to the immediately adjacent DNA fragment of the *D. melanogaster* RNA polymerase II gene. Two cloned *S. cerevisiae* DNA sequences are each the template for single transcripts in vivo, one of 5.9 kilobases and the other of 4.6 kilobases. *In vitro* translation of hybrid-selected cellular RNA indicated that the former locus encodes a protein of M, 220,000, equal in size to the largest polypeptide subunit of *S. cerevisiae* RNA polymerase II. Disruption of either gene by targeted integration of *URA3* DNA demonstrated that each is single-copy and essential in a haploid genome. We suggest that these *S. cerevisiae* loci are members of a family of related genes encoding the largest subunit polypeptides of RNA polymerases I, II, and III.

In *Drosophila melanogaster* a series of α-aminitin-resistant, lethal, and temperature-sensitive mutations affecting RNA polymerase II activity (EC 2.7.7.6) have all been mapped to a single genetic locus, *RP2II* (1, 2). By using the DNA of the transposable element *P* as probe, DNA sequences from a mutant strain of *D. melanogaster* bearing a lethal *P*-element insertion in this *RP2II* locus were cloned (3). Of the four different *in vitro* transcripts that originate from this *RP2II* region of *D. melanogaster* DNA, only one has a homolog in mammalian DNA (4). In interspecies DNA-mediated gene transfer experiments this conserved sequence was shown to encode the gene conferring sensitivity to inhibition of RNA polymerase II activity by α-aminitin (4). These latter studies (4) clearly identified the DNA of a conserved RNA polymerase II structural gene. This gene in *D. melanogaster* encodes the largest (*M* = 215,000) subunit of RNA polymerase II (5).

The conservation of eukaryotic RNA polymerase II subunit structure and antigenicity extends to fungal species such as *Saccharomyces cerevisiae* (6–11). We have therefore asked if yeast RNA polymerase II DNA could be detected by using the DNA of the *D. melanogaster* *RP2II* region as a probe. Unexpectedly, not one but three different loci were detected in the *S. cerevisiae* genome when the *D. melanogaster* RNA polymerase II DNA was used as a probe. These yeast DNA sequences thus appear to be three members of a family of related genes. We suggest that they encode subunit polypeptides of RNA polymerases I, II, and III.

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**MATERIALS AND METHODS**

*Escherichia coli* K-12 strains JF1754 (hsdR, lac, gal, metB, leuB, hisB) and HB101 (hsdS20, recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44) were used as hosts for plasmid propagation. Yeast DNA for Southern blots was purified as described (12). *S. cerevisiae* diploid strain JH101 (MATa/MATa, ade2-1/ADE2*, leu2-3,112/LEU2*, ura3-52/ura3-52, HIS4*/his4-912) was constructed for this study. pBR325 (13) was used as a cloning vehicle. pJH104 is a derivative of pBR325 with a 1.1-kilobase (kb) HindIII fragment insert carrying the *S. cerevisiae* *URA3* gene. Bacteriophage λ libraries of *S. cerevisiae* DNA were constructed by us, using *AgtWES* (14), or by M. Olson. using λMG14 [a derivative of λI059 (ref. 15) and λ Charon 30 (ref. 16)]. Transformation of *E. coli* and *S. cerevisiae*, preparation of plasmid and bacteriophage DNA, DNA blotting, RNA blotting, nick-translation, nucleic acid hybridization (4), and *in vitro* translation of hybrid-selected RNA (17) have been described previously and were carried out according to conventional methods (see, for example, refs. 18 and 19).

**RESULTS**

Cloning Yeast DNA Homologous to a *D. melanogaster* RNA Polymerase II Gene. Two subclones of *D. melanogaster* DNA, p4.1 and p4.2, together contain most of the structural gene information for the largest subunit of *D. melanogaster* RNA polymerase II (4, 5). We determined whether related sequences could be detected by hybridization to *S. cerevisiae* genomic DNA by using either of these *D. melanogaster* DNA species as probe. Yeast DNA (20 μg) was digested with the restriction endonuclease *EcoRI* and fractionated by electrophoresis in agarose gels; a nitrocellulose blot of this was probed under various degrees of stringency with nick-translated p4.2, a plasmid in which the *D. melanogaster* DNA is carried on pBR325. A number of hybridizing fragments were seen (Fig. 1A, lanes a and b) when the hybridization solutions contained either 30% or 50% (vol/vol) formamide. Most of these appeared to be vector-DNA related since they were also detected when the same DNA was probed with the labeled vector pBR325 DNA (Fig. 1A, lanes c and d). In 30% formamide, however, two additional yeast *EcoRI* fragments, 7.5 and 2.6 kb in size, were detected by the p4.2 probe, in addition to those detected by the vector DNA. These are the same conditions that allowed cross-hybridization of p4.2 with mammalian RNA polymerase II DNA (4). Probing the *EcoRI*-digested yeast DNA at this reduced stringency but with the gel-purified insert of p4.2 revealed, after a longer exposure, the 7.5- and 2.6-kb bands, as

*Abbreviations: kb, kilobase(s); Ura*” and *Ura*, uracil-independent and dependent.*

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Seven of these contained a 7.5-kb EcoRI DNA fragment that hybridized to the *D. melanogaster* p4.2 DNA (Fig. 1C, lane a). The remaining 23 contained a 2.6-kb EcoRI DNA fragment that hybridized with this same probe (Fig. 1C, lane d). None of the isolates contained both a 7.5-kb and a 2.6-kb EcoRI fragment.

Since the *S. cerevisiae* inserts in this *λgtWES* library were both relatively small and had been generated from genomic DNA by EcoRI digestion, we used nick-translated EcoRI fragments from two of these initial λ isolates to screen a second genomic library of *S. cerevisiae* DNA. This second library, constructed by M. Olson in the vector λMGI4 (a X1099-L Charon 30 hybrid bacteriophage), contains yeast DNA inserts generated by partial *Sau3A* digestion in the size range 15–20 kb. Over 100 positive plaques were detected upon screening 17,000 bacteriophage. The DNA from a number of these bacteriophage was in turn characterized by restriction enzyme mapping and hybridization experiments (data not shown, but see Fig. 2). The bacteriophage that we isolated from this second library also clearly represented two distinctly different loci. One set contained the 7.5-kb EcoRI fragment of *S. cerevisiae* DNA that was detected by p4.2 DNA in the whole genome Southern blots of EcoRI-digested yeast DNA. The other set of bacteriophage contained the 2.6-kb EcoRI fragment of *S. cerevisiae* that was detected by this same *D. melanogaster* probe. We have not yet succeeded in isolating a bacteriophage carrying the locus that includes the more weakly hybridizing 1.8-kb EcoRI fragment.

The likelihood that both of these cloned sequences represented *bona fide* RNA polymerase-related yeast genes was strengthened by the following observation. Both loci contained sequences that hybridized not only with the *D. melanogaster* RNA polymerase II DNA p4.2, the fragment that was originally used to detect these isolates, but also with a second *D. melanogaster* RNA polymerase II probe, p4.1. Probe p4.1 contains the DNA that lies immediately adjacent to the p4.2 DNA on the *D. melanogaster* chromosome (3, 4); together these two *D. melanogaster* DNA fragments and their flanking sequences make up the gene encoding the M<sub>r</sub> 215,000 subunit of RNA polymerase II (5). Both p4.1 and p4.2 DNA hybridized to the 7.5-kb EcoRI yeast DNA fragment (Fig. 1C, lanes a and d), p4.1 DNA did not hybridize to the 2.6-kb fragment detected by p4.2, but it did hybridize with the adjacent 2.3-kb EcoRI fragment (Fig. 1C, lanes c and d) in the same recombinant phage. More detailed mapping and cross-hybridization studies of the DNA of a number of the recombinant bacteriophage with *D. melanogaster* p4.1 and p4.2 DNA are summarized in Fig. 2, in which homology...
EcoRI in gels containing 20 µg RNA probes, (lane b) (not sizes the undertaken in DNA phoresis. S. cerevisiae RNA was from cus, polypeptides that cant and the Genes. translation approach spectively respectively pBR325-selected RNA (Fig. 3). Hybridization Analysis of Yeast RNA. Experiments were undertaken to determine the size and number of in vivo transcript(s) from each locus. Total RNA was isolated from cells in the logarithmic phase of growth, fractionated by electrophoresis in agarose under denaturing conditions, transferred to nitrocellulose, and probed with DNAs of each of the cloned S. cerevisiae RNA polymerase-related loci. When the 7.5-kb EcoRI fragment was used as a probe, a single large transcript, approximately 5.9 kb in length, was detected. When either the 2.6-kb or the 2.3-kb EcoRI fragment, which together make up the second RNA polymerase-related locus, was used, a single RNA species of approximately 4.6 kb was detected (Fig. 3). Thus each of these yeast RNA polymerase-related loci is expressed as a single RNA species, and the sizes of the two transcripts are different. It is significant that these two transcripts are sufficiently large to encode polypeptides of about M, 220,000 and M, 160,000, respectively (see Discussion).

Identification of a Polypeptide Encoded by One of the S. cerevisiae Genes. We have employed a direct biochemical approach to characterize the polypeptide encoded by one of these genes. DNA from the 7.5-kb EcoRI locus, subcloned in pBR325, was used for hybrid-selection of a specific messenger RNA species from total yeast RNA. The selected filter-bound RNA species were eluted and translated in vitro in a rabbit reticulocyte system. Fig. 4 shows the fluorograph of the [35S]methionine-labeled proteins synthesized in vitro, and separated by sodium dodecyl sulfate gel electrophoresis. A single protein of about M, 220,000, was the only protein band unique to the 7.5-kb EcoRI-selected RNA (Fig. 4, lane b). Control samples, representing the translation products of pBR325-selected RNA (Fig. 4, lane a) or H2O only (not shown), showed only the presence of reticulocyte-derived lower molecular weight proteins, which were also present in the translation products of the 7.5-kb DNA-selected RNA. This M, 220,000 polypeptide is the same size as the largest subunit polypeptide of the yeast RNA polymerase II enzyme (10, 20).

Using both total yeast RNA and oligo(dT)-cellulose-selected poly(A)+ RNA, we have as yet been unsuccessful in identifying a polypeptide encoded by RNA selected with DNA of the second (2.6-kb) yeast RNA polymerase-related locus.

Mutagenesis of the Yeast RNA Polymerase-Related Loci by Homologous Recombination. We have used insertional inactivation (21–23) to determine if each locus encodes a unique function essential for yeast growth. To carry out this experiment with the locus containing the 7.5-kb EcoRI fragment we used an internal region of the locus, itself flanked on both sides by sequences that hybridize to p4.2 or p4.1 D. melanogaster DNA (see Fig. 2). A 2.0-kb Cla I fragment was subcloned on a nonreplicating plasmid that also carries the S. cerevisiae URA3+ gene (pH104). A diploid ura3+/ura3- strain was transformed with this plasmid DNA, which had been digested with BgI II in order to target integration into the polymerase-related site (21). Ura-independent (Ura+) recombinants were selected on agar lacking uracil (Fig. 5A summarizes this procedure). As indicated by the DNA blots shown in Fig. 5B, one of two 7.5-kb EcoRI segments in the DNA of diploid recombinants was disrupted by the recombination event leading to Ura+ transformation. Two new EcoRI fragments of the expected sizes 10.6 and 6.0 kb were revealed in addition to the original 7.5-kb fragment in DNA blots of EcoRI-digested genomic DNA of ura3+/URA3+, diploid transformants. About one-third of these Ura+ recombinants contained an additional 9.1-kb EcoRI fragment (Fig. 5B, lane e), the result of tandem integration (24) of the 9.1-kb URA3+ plasmid at this same chromosomal site. The ura3+/URA3+ transformants shown in lanes b and c were then sporulated; dissected tetrads were scored both for viability and for uracil auxotrophy. Of 20 tetrads that were analyzed, 17 contained two viable Ura+ spores and two nonviable spores. The remaining three contained one viable Ura- spore and three nonviable spores, possibly the result of a gene conversion event or incomplete tetrads. All Ura- haploids contained only an intact 7.5-kb EcoRI segment (two examples are shown in Fig. 5B). The integration of the URA3+ gene, pBR325, and 2.0 kb of yeast RNA polymerase-related DNA in this S. cerevisiae locus was clearly a lethal event. This locus must therefore encode a single-copy essential gene.

A similar strategy was used to examine the second yeast RNA polymerase-related locus. A 640-base-pair EcoRI/Hae III fragment, also determined on the basis of the hybridization studies with D. melanogaster DNA to be internal to this gene (see Fig. 2), was cloned in the same nonreplicating.
URA3+ plasmid (pJH104). Integration of this plasmid in the homologous chromosomal locus of ura3"/ura3" diploids was targeted with a Bgl II cut. Recombination of the plasmid into the homologous site was confirmed by analysis of DNA blots (Fig. 5C). Digestion of the DNA of diploid Ura+ recombinants with EcoRI showed the presence of a normal 2.3-kb fragment hybridizing with the 2.3-kb EcoRI probe and a new fragment of the expected size, 7.7 kb. The copy number of this latter fragment varied, again due to tandem insertions (24) of the URA3+ plasmid. Sporulation and analysis of the diploid shown in lane b of Fig. 5C indicated, as for the 7.5-kb locus, that this second locus was also single-copy and essential. Eighteen tetrads contained two viable and two nonviable spores; the remaining two contained three viable spores. These viable Ura+ haploids contained only the 2.3-kb EcoRI fragment (Fig. 5C, lanes f and g).

These loci, which both contain DNA homologous to a single D. melanogaster RNA polymerase II structural gene, must represent different structural genes. Both appear to encode essential functions required for the growth of yeast strains. This evidence is consistent with the proposition that each of them encodes a different RNA polymerase polypeptide.

**DISCUSSION**

Success in using the D. melanogaster RNA polymerase II gene as a probe to identify the analogous gene in mammalian cells (4) prompted us to use a parallel approach to identify related genes in yeast. We have discovered and isolated two such genes from the S. cerevisiae genome; a possible third gene has been identified but not yet isolated. Analysis of cellular RNA indicated that the two cloned genes are templates for the transcription of RNA species that correspond in size, 5.9 and 4.6 kb, to messenger RNA species that could encode proteins of molecular weight approximately 220,000 and 160,000, respectively. *In vitro* translation of the RNA isolated by hybrid-selection with the DNA of one of these genes yielded a protein of Mr 220,000. Both cloned loci were shown by inactivation encoding to single-copy essential genes.

The *in vitro* translation experiment strongly suggests that the gene corresponding to the 7.5-kb EcoRI fragment encodes the largest subunit of RNA polymerase II. No other subunit of any of the three yeast RNA polymerases is as large as Mr 220,000 (8, 10, 20). The cloned *D. melanogaster* RpII DNA used as probe to isolate these *S. cerevisiae* genes has been shown to be homologous to the DNA that encodes the subunit of RNA polymerase II determining sensitivity to inhibition by α-amaminin in higher eukaryotes (4). In *D. melanogaster* this DNA is also the site of mutations to α-amaminin resistance (3) and it encodes the Mr 215,000 subunit of the RNA polymerase II enzyme (5). This is the gene we expected to isolate by using cross-species hybridization. We propose to call this *S. cerevisiae* gene RPO21.

Further evidence that the locus containing this 7.5-kb EcoRI fragment encodes the largest subunit of the S. cerevisiae RNA polymerases has been provided by R. A. Young (Stanford University). Using the entirely different approach of immunological screening (25) with an antibody raised against RNA polymerase II large subunits, DNA with identical restriction sites for EcoRI, HindIII, Bgl II, Pst I,
Genomic DNA termination of which has been 220,000 ing of RNA loci has been most asse-related I and III of polymerases share extents naming and served polymerase-related). Evidence also detected RPO1 common in loci to the 2.6-kb RNA the 2.6-kb EcoRI band might encode the largest subunit of S. cerevisiae RNA polymerase II gene. The identification of the second cloned gene (corresponding to the 2.6-kb EcoRI fragment) is not as clear. From the size of the transcript (4.6 kb) one can infer a maximum polypeptide product of about M, 165,000. This corresponds most closely to the largest subunit of RNA polymerase III [M, 160,000 (ref. 20)]. Alternatively, it could encode the second-largest subunit of RNA polymerase II [M, 150,000 (ref. 20)], the second-largest subunit of RNA polymerase I [M, 135,000 (ref. 20)] or some other as yet undescribed polymerase-related protein. We believe, however, that this second locus most likely is the gene for the polymerase III M, 160,000 polypeptide because not only do immunological studies indicate that there is a conservation of antigenic determinants among the three largest subunits of the different RNA polymerases in S. cerevisiae (11) but also the RNA polymerases I and III of different eukaryotic species are to various extents sensitive to inhibition by α-amanitin and may therefore share with RNA polymerase II a closely related α-amanitin-binding domain. Thus it is likely that there are conserved nucleotide sequences among the genes encoding the largest subunits of S. cerevisiae RNA polymerase I, II, and III. If this is indeed the RNA polymerase III gene, then we propose naming this second locus RPO31, although until further evidence is available, we retain the name RPR3 (RNA polymerase-related). We are suggesting that S. cerevisiae genes encoding subunits unique to RNA polymerase I, II, and III be named RPO11 through RPO19, RPO21 through RPO29, and RPO31 through RPO39, respectively. Subunits in common between RNA polymerases I, II, and III could be designated RPO1 through RPO9. A third, 1.8-kb fragment was also detected in the whole genome blot by using D. melanogaster p4.2 DNA as probe (Fig. 1B), and there are additional p4.1-related DNA sequences in genomic blots of S. cerevisiae DNA apart from those of the RPO21 and RPR3 loci (data not shown). However, we have not yet been able to isolate this third gene. The hybridization is relatively weak and it has been difficult to detect positive signals above the background in the plaque assays. On the basis of the foregoing discussion we speculate that the locus represented by the 1.8-kb EcoRI band might encode the largest subunit of RNA polymerase I (hence RPO11). In this case the large subunits of the three RNA polymerases in S. cerevisiae might be members of a gene family that have evolved from a single primordial ancestor (11, 20). Detailed sequence comparison of genes from both yeast and other organisms will be required to establish the validity of this idea. The demonstration that both of the genes that we have cloned are essential for cell viability was made possible by the fact that they are also single-copy and is, of course, to be expected of RNA polymerase functions. In vitro mutagenesis of the cloned genes, followed by gene replacement (24) and screening to obtain other classes of RNA polymerase mutants, not only will verify the nature of the functions that these genes encode but also will be extremely useful in physiological and biochemical studies of transcription. The clon-