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Yeast RNA Polymerase II Genes: Isolation with Antibody Probes

Richard A. Young and Ronald W. Davis

Summary. Genes encoding yeast RNA polymerase II subunits were cloned. Efficient isolation of these genes was accomplished by probing a phage λgt11 recombinant DNA expression library with polyvalent antibodies directed against purified yeast RNA polymerase II. The identity of genes that specify the largest RNA polymerase II subunits, the 220,000- and 150,000-dalton polypeptides, was confirmed by competitive radioimmune assay. Both of these genes exist in single copy in the yeast *Saccharomyces cerevisiae*.

Three distinct classes of RNA polymerase are responsible for the transcription of DNA into RNA in eukaryotes (*1, 2*). RNA polymerase I synthesizes ribosomal RNA; RNA polymerase II is responsible for the transcription of messenger RNA (mRNA); and RNA polymerase III synthesizes small RNA's such as transfer RNA and 5*S* ribosomal RNA. The RNA polymerase within each class is composed of 8 to 12 subunits, some of which belong only to that class and some of which are shared by polymerase from the other classes (*3*). Attempts to confirm and extend these observations have been hampered by the structural and functional complexities of these enzymes, by the limitations of current in vitro biochemical assays, and by the paucity of RNA polymerase mutants and difficulties in their isolation.

A thorough understanding of the processes controlling transcription and thus gene expression requires a detailed understanding of the components of the transcription apparatus. RNA polymerase subunits have been defined empirically as the smallest number of protein components that copurify and retain DNA-dependent RNA synthesis activity in vitro. However, this approach does not distinguish between proteins that are required for activity and those that simply copurify. Moreover, the assays used to define RNA polymerase subunits have not been useful in identifying proteins required for transcription initiation and termination activities, nor do they reveal other possible functions in which RNA polymerases might participate (for example, RNA processing).

Several factors make a systematic investigation of RNA polymerase subunit structure and function compelling in yeast. Yeast RNA polymerases have undergone careful biochemical scrutiny at the subunit level (*4, 5*). In addition, yeast RNA polymerases appear structurally and functionally very similar to those of higher eukaryotes; by immunological criteria, the two large RNA polymerase II subunits (220,000 and 150,000 daltons in yeast) are particularly well conserved (*6*). Finally, yeasts are amenable to study with a combination of biochemical and genetic tools. Thus, the isolation of genes encoding yeast RNA polymerase subunits should facilitate a genetic and biochemical definition of the enzyme's structure and function in eukaryotes.

As a means of cloning gene sequences efficiently when antibodies are used as probes of their polypeptide products, a method has been developed that permits rapid screening of large libraries of recombinant DNA in the phage expression vector λgt11 (*7*). This method was used with two modifications to isolate RNA polymerase II subunit genes (Fig. 1). Antigen produced in λ phage plaques rather than in λ lysogen colonies was immobilized on nitrocellulose filters. Host cells carrying multiple copies of the lac repressor gene, *lac*I, were used to conditionally regulate the potentially deleterious expression of the foreign genes controlled by the *lac*Z promoter. Only after taking this latter precaution was it possible to isolate some of the genes of interest.

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Rabbit antiserum directed against purified yeast RNA polymerase II was used to probe a λgt11 library of yeast genomic DNA containing 2×10^6 recombinant phage (Fig. 2). In a screen of 10^6 recombinants, 60 independent clones that produced strong signals were isolated. Thirty of these recombinant DNA clones were further screened for their ability to produce antigens in common with each of the two large yeast RNA polymerase II subunits (220,000 and 150,000 daltons). Arrays of phage plaques were probed with rabbit immunoglobulin G (IgG) directed against each of the two subunits, which had been purified by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Fig. 3). Four of the clones (Y3001, Y3002, Y3007, and Y3023) produced strong signals with the antibody to the 220,000-dalton subunit, and two others (Y3015 and Y3024) gave

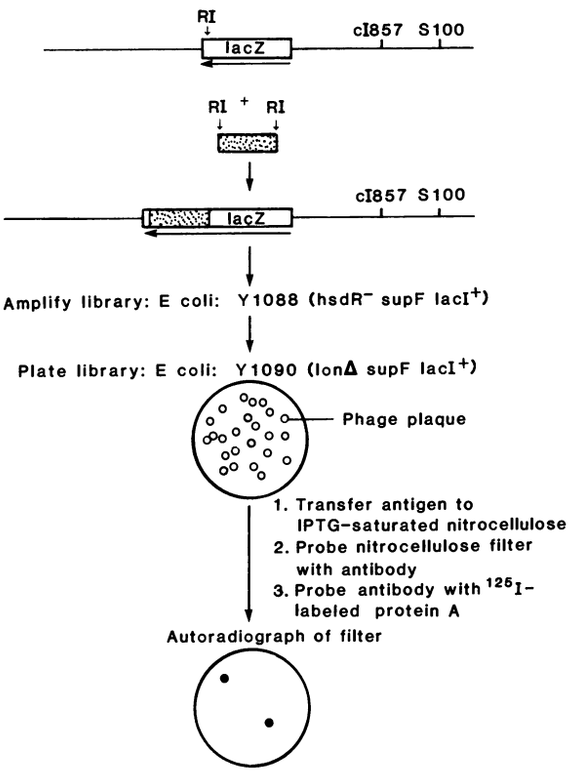


Fig. 1. Screening λgt11 phage plaques for specific antigens. This cartoon depicts construction of the recombinant library and the screening procedure: foreign DNA (genomic DNA or cDNA) is inserted into the unique Eco RI restriction site of λgt11 to produce a library of recombinant phage. The library is amplified by producing plate stocks at 42°C on *E. coli* Y1088 [*sup*E *sup*F *met*B *trp*R *hsd*R⁻ *hsd*M⁺ *ton*A21 *str*A *lac*U169 *pro*C::Tn5 (pMC9)](*15*). Important features of this strain include *sup*F (required for suppression of the phage amber mutation in the S gene), *hsd*R⁻ *hsd*M⁺ (necessary to prevent restriction of foreign DNA prior to host modification), *lac*U169 (a deletion of the lac operon reduces host-phage recombination and is necessary to distinguish between λgt11 recombinants (generally little or no β-galactosidase activity) from nonrecombinants (β-galactosidase activity), and pMC9 (*16*) (a pBR322 plasmid carrying *lac*I to repress expression of foreign genes that might be detrimental to host cell and phage growth). To screen the library for specific antigen-producing clones, λgt11 recombinant phage are plated on a lawn of *E. coli* Y1090 [Δ*lac*U169 *pro*A⁺ Δ*lon* *ara*D139 *str*A *sup*F [*trp*C22::Tn*10*] (pMC9)] (*15*) and incubated at 42°C for 3 to 4 hours. This host is deficient in the *lon* protease, thereby reducing the degradation of expressed antigens (*7*). A dry nitrocellulose filter, previously saturated with 10 m*M* isopropyl thio-β-D-galactopyranoside (IPTG), is overlaid; the plates are then removed to 37°C for 2 to 8 hours. IPTG is a gratuitous inducer of *lac*Z transcription that in turn directs the expression of foreign DNA inserts in λgt11. The position of the filter is marked with a needle, the filter is removed, washed in TBS buffer (50 m*M* tris, *p*H 8.0, and 150 m*M* NaCl), and incubated in TBS plus fetal calf serum (20 percent) for 10 minutes at room temperature. The filter is then incubated in TBS plus 20 percent fetal calf serum and antibody [serum or IgG (10 mg/ml) is diluted approximately 1:100] for 1 hour at room temperature. This solution can be reused several times. The filter is subjected to three 5-minute washings in TBS, then incubated with ^{125}I-labeled protein A (10^6 count/min per 90-mm filter; specific activity, 30 μCi/μg) in TBS plus 20 percent fetal calf serum. Finally, the filter is washed for 5 minutes in TBS, 5 minutes in TBS plus 0.1 percent NP-40, and 5 minutes in TBS before drying and autoradiography. Good signals are obtained after 4 to 8 hours of exposure at −70°C when Kodak X-Omat AR film is used with a Cronex Lightning Plus intensifying screen.

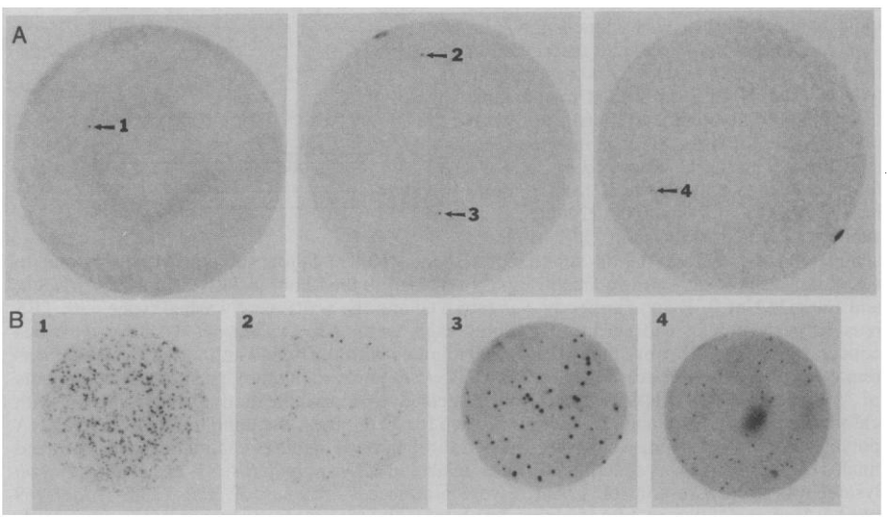


Fig. 2. Screening a λgt11 recombinant yeast genomic DNA library for RNA polymerase II subunit clones. A library containing 2×10^6 individual recombinants was constructed by Snyder (*17*) as follows. *Saccharomyces cerevisiae* X2180 DNA was mechanically sheared to an average length of approximately 5 kilobases (kb); this DNA was treated with T4 DNA polymerase and Eco RI methylase before ligation with synthetic Eco RI linkers and digestion with Eco RI. The DNA fragments were subjected to electrophoresis in an agarose gel, and fragments of 2.5 to 10 kb were eluted and inserted into Eco RI–cleaved and phosphatase-treated λgt11 DNA with T4 DNA ligase. Phage were packaged and amplified on *E. coli* Y1088. (A) Plaques (5×10^4) on 150-mm plates were screened as described in the legend to Fig. 1 with the use of a 1:100 dilution of rabbit serum produced against purified yeast RNA polymerase II. This serum and the IgG directed against individual subunits required removal of significant levels of antibody reactive to *E. coli* antigens. Removing the antibodies to the *E. coli* was accomplished most efficiently by mixing 2 ml of serum with 5 ml of Sepharose 4B resin to which was bound approximately 3 mg of a BNN 97 [C600 (λgt11)] (*7*) lysate, incubating the mixture overnight at 4°C, and loading it onto a column from which serum was eluted with an equal volume of fetal calf serum. The *E. coli* affinity column was constructed with CNBr-activated Sepharose 4B according to the manufacturer's (Pharmacia Fine Chemicals AB) protocol. (B) A 4-mm-diameter agar plug at the position of each of the signals in (A) was removed from the plates and incubated in 10 m*M* tris-HCl, *p*H 7.5, and 10 m*M* $MgSO_4$ for at least 1 hour. Phage in this solution were replated for plaques on 90-mm plates at a density of approximately 10^3 plaque-forming units (PFU) and rescreened. This replating and screening process was repeated until all plaques on the plate produced a signal.

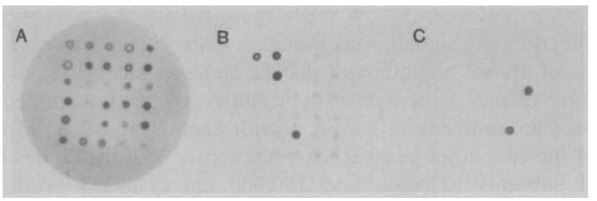


Fig. 3. Probing candidates for RNA polymerase II recombinant DNA clones for 220,000- and 150,000-dalton subunit antigens. Thirty λgt11 recombinant DNA clones were plated in drops of 10^2 PFU on *E. coli* Y1090. The arrays were probed as described in Fig. 1 for antigen contained in the two largest yeast RNA polymerase II subunits, using purified IgG directed against the proteins purified by SDS–polyacrylamide gel electrophoresis (*5*, *6*). Signals produced when the array was probed with serum directed against whole purified RNA polymerase II, those obtained with the antibody to the 220,000-dalton subunit, and those obtained with the antibody to the 150,000-dalton subunit are shown in (A), (B), and (C), respectively. Nonrecombinant λgt11 phage produced the plaque signal in the extreme lower right of each array.

strong signals with the antibody to the 150,000-dalton subunit. Twenty-four of the recombinant clones produced no signals with the respective antibodies to the two subunits; among these were clones that contain DNA for other RNA polymerase II subunits (*8*).

Recombinant DNA λgt11 clones that produced strong signals with the two subunit antibodies were examined in more detail by mapping their insert DNA's with restriction enzymes. Of the recombinant DNA phage that produced signals with the antibody to the 220,000-dalton subunit, two appeared identical (Y3002 and Y3023), and these shared DNA sequences with the other two (Y3001 and Y3007) (Fig. 4A). In each case, the inserted DNA was in the same orientation with respect to the λgt11 *lac*Z transcription unit. Recombinant DNA clones that were identified with the antibody to the 150,000 subunit likewise shared some DNA which, in both cases, was inserted in the same orientation with respect to *lac*Z (Fig. 4B).

Confirmation of gene identity. It is possible to misidentify a gene with this procedure, either because the protein it encodes shares an epitope with the protein of interest, or because some component of the antibody probe is directed against a contaminant in the original antigen preparation (the RNA polymerase). Thus, it was necessary to confirm the identity of these recombinant clones with an additional experiment. A radioimmune assay was designed to measure the ability of antigen expressed from different recombinant clones to prevent binding of native RNA polymerase II to multivalent subunit IgG. Antibodies to the subunits were bound to microtiter wells; crude *Escherichia coli* lysate from cells induced for recombinant antigen production was bound to the antibody; and the remaining available antigen-binding sites were assayed by adding ^{125}I-labeled yeast RNA polymerase II. Figure 5A shows the results of this assay, in which the antibody to the 220,000-dalton subunit was used to examine antigens expressed by clones Y3001, Y3002, and Y3007 in *E. coli*. Antigen in Y3002 and Y3007 lysates was nearly as proficient in binding RNA polymerase II combining sites in the multivalent subunit antibody as was pure yeast RNA polymerase II itself. The lysate produced by Y3001 was able to remove approximately 50 percent of the binding sites available to the labeled yeast enzyme. In contrast, a lysate of λgt11 in the same host was unable to compete for any RNA polymerase combining sites in the subunit antibody. Therefore, Y3002 and Y3007 appear to contain most of the RNA polymerase II epitopes recognized by the antibody to

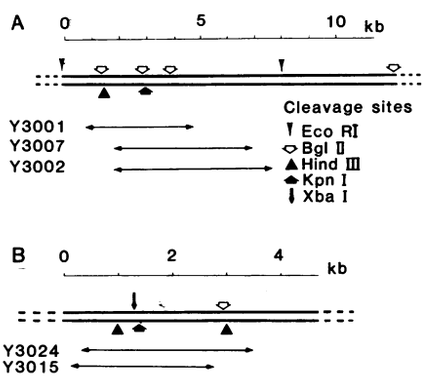
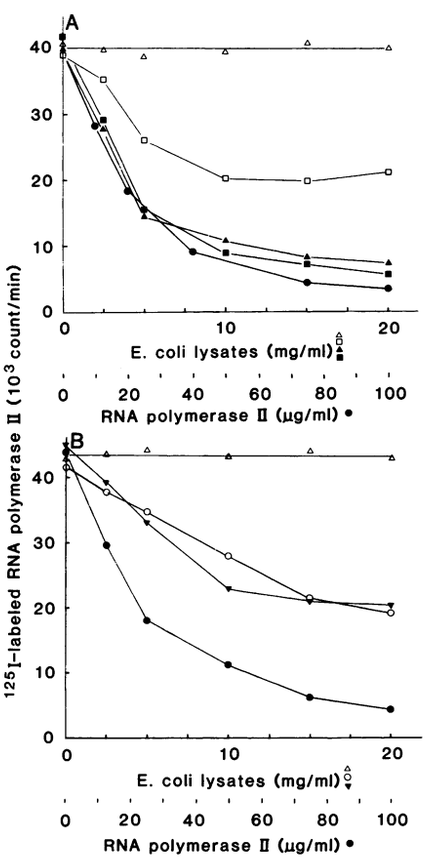


Fig. 4 (left). DNA restriction maps of cloned genomic loci were deduced by restriction analysis of the yeast DNA inserts in λgt11 clones Y3001, Y3002, Y3007, Y3015, and Y3024 and confirmed by direct mapping of those sites in the genome (*9*). (A) The RPO1 locus; (B) the RPO2 locus. Horizontal arrows indicate the extent of each clone's DNA insert. *Lac*Z promoter–directed transcription of the DNA inserts in λgt11 occurs from right to left. RPO1 DNA is transcribed in the same direction (from right to left) in yeast (*8*). Fig. 5 (right). Ability of antigens specified by specific λgt11 clones to exclude native yeast RNA polymerase II from combining sites for the antibody to the 220,000-dalton subunit. Purified polyvalent IgG directed against (A) the 220,000-dalton subunit or (B) the 150,000-dalton subunit (*5*) was bound to microtiter wells at saturating concentrations (0.07 mg/ml) for 1 hour in TBS (50 m*M* tris, *p*H 8.0, and 150 m*M* NaCl). Excess antibody was removed and the wells were washed twice with TBS plus 10 percent fetal calf serum (5 minutes each time). *Escherichia coli* lysates at various concentrations in TBS were incubated for 30 minutes with the bound antibody. The lysate was removed, the wells were washed twice with TBS (5 minutes each time), and saturating amounts of ^{125}I-labeled yeast RNA polymerase II [labeled to a specific activity 10^7 cpm/μg with chloramine T (*18*)] were added. After incubation for 30 minutes, the unbound ^{125}I-labeled RNA polymerase was removed, wells were washed three times (5 minutes each time) with TBS, and finally the ^{125}I count was determined. Pure unlabeled RNA polymerase II, rather than *E. coli* lysate, was used as a control. Lysates were produced as follows: *E. coli* Y1089 (Δ*lac*U169 *pro*A$^+$ Δ*lon ara*D139 *str*A *hfl*A150 [chr::Tn10] (pMC9) (*15*) cells were lysogenized with Y3001, Y3002, Y3007, Y3015, Y3024, and λgt11. A 50-ml culture of lysogen was grown at 32°C to an absorbance of 0.5 at 550 nm and induced for phage production by temperature shift to 44°C for 20 minutes. IPTG was added to 5 m*M* to induce antigen production, and the temperature was reduced to 37°C for 1 hour. Cells were harvested by centrifugation, and the pellet was suspended in 1.25 ml of TBS plus 0.2 m*M* phenylmethylsulfenyl fluoride at 0°C. Cells were disrupted by freezing in liquid nitrogen, thawing, then freezing once again. (●) Purified yeast RNA polymerase II; (□) Y3001 lysate; (▲) Y3002 lysate; (■) Y3007 lysate; (○) Y3015 lysate; (▼) Y3024 lysate; and (△) λgt11 lysate.



the 220,000-dalton subunit. The Y3001 lysate probably expresses fewer epitopes since the insertion breakpoint of the genomic DNA in the Y3001 clone is transcriptionally downstream from those in Y3002 and Y3007 (Fig. 4A). A similar experiment was performed with IgG directed against the 150,000-dalton subunit (Fig. 5B). *Escherichia coli* lysates containing antigen produced by Y3015 and Y3024 were both able to titrate more than 50 percent of the available RNA polymerase II combining sites. These results establish that the isolated clones encode most of the epitopes recognized by the two multivalent IgG preparations and thus provide compelling evidence that these recombinants specify at least a portion of the two large RNA polymerase II subunits.

Gene copy number. To ascertain whether the cloned DNA sequences are unique in the haploid genome, we examined the copy number of yeast genomic DNA sequences homologous to Y3002 and Y3015 insert DNA by quantitative hybridization analysis (*9, 10*). Known quantities of yeast genomic DNA were digested with restriction endonucleases; DNA fragments were separated electrophoretically on an agarose gel from which they were transferred to nitrocellulose, immobilized, and then hybridized with labeled insert DNA from recombinant clones. Various known amounts of cloned DNA, cleaved with the same restriction enzyme, were included as titration standards. The results indicated that DNA for the 220,000-dalton subunit of RNA polymerase II exists in single copy in the haploid yeast genome (Fig. 6). A similar experiment with cloned DNA encoding the 150,000-dalton subunit indicated that this subunit gene also occurs in single copy in the yeast genome. Thus, the cloned DNA sequences represent the genes that are expressed by the cell. The single-copy genes for the 220,000- and 150,000-dalton subunits of yeast RNA polymerase II have been designated RPO1 and RPO2, respectively.

Future prospects for RNA polymerase. The isolation of genes for the two large subunits of yeast RNA polymerase II and their presence in single copy in the genome will facilitate the construction of mutants at these loci. Conditionally lethal mutations with readily identifiable defects in transcription will help to identify the putative subunits as authentic components of the RNA polymerase. Moreover, the analysis of second-site suppressors (*11*) of these conditionally lethal mutations may reveal previously uncharacterized gene products with important roles in transcription (*12*).

Gene Isolation with Antibody Probes: Other Applications

The power of the gene isolation technique described here lies in the ability to screen large numbers ($> 10^6$) of antigen-producing recombinant DNA's efficiently. Thus, it provides an effective strategy for the isolation of genes whose protein products are easily purified and makes feasible new approaches to complex antigenic problems that are amenable to a systematic clonal analysis. For example, one application involves identifying the protein components of human pathogens that are targets of the immune system: malarial blood-stage antigens that are candidates for protective vaccines have been isolated from a λgt11 complementary DNA library of *Plasmodium falciparum* with the use of human serum antibodies (*13*).

Further considerations for expression libraries. The factors that influence the successful use of antibody probes to isolate and identify clones from recombinant DNA expression libraries merit further comment. As with most cloning techniques, success usually depends on the kind and quality of the probe. Polyvalent serum antibodies from which components active against *E. coli* have been removed have proved to be satisfactory probes. Monoclonal antibodies can also be used to isolate antigen-producing clones (*14*). Monoclonal antibodies alone, however, may recognize more than the unique DNA expression products of interest since single epitopes can be shared by different proteins in the same cell or organism. Hence, a single monoclonal antibody cannot be used to identify definitively the cloned gene.

The ideal recombinant DNA expression library would have the potential to produce all single polypeptide antigenic determinants encoded in the genome of interest. A recombinant library of genomic DNA with randomly generated insert breakpoints is most suitable since DNA is expressed in all possible orientations and translation frames irrespective

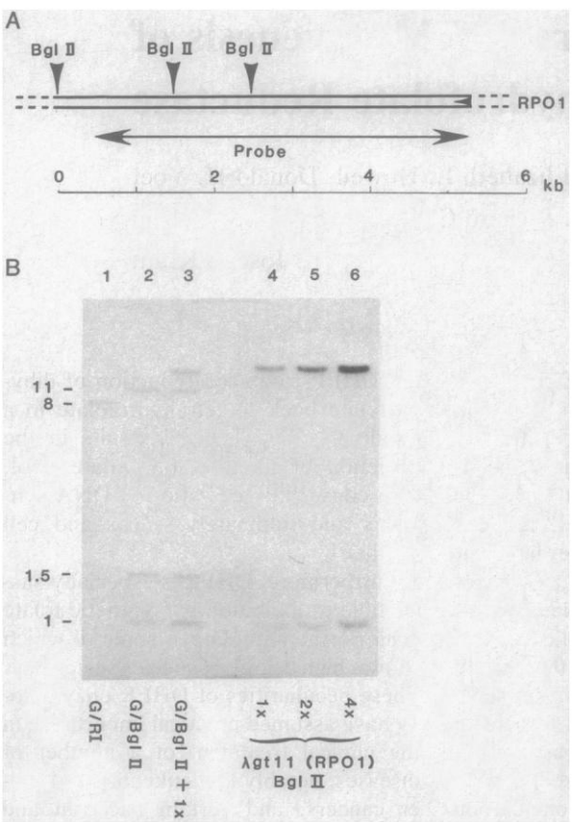


Fig. 6. Quantitation of genomic DNA sequences homologous to RPO1 DNA. RPO1 sequences were titrated by immobilizing yeast strain 2180 genomic DNA restriction fragments on a nitrocellulose filter (*9*) and hybridizing with the ^{32}P-labeled 5.5-kb insert of Y3002 [shown in (A)]. (B) Yeast genomic (G) DNA (2.2 μg) was digested to completion with Eco RI (lane 1) or Bgl II (lanes 2 and 3). Lanes 3, 4, 5, and 6 contain 8, 8, 16, and 32 ng of Y3002 phage DNA digested with BglII, representing one (*1X*), one, two, and four gene equivalents of DNA, respectively [assuming 13,800 kb per haploid genome (*19*)]. Before digestion, 2.2 μg of carrier DNA (*Bacillus amyloliquefaciens*) was added to Y3002 DNA destined for lanes 3 to 6. Hybridization (*20*) was carried out in probe excess (2 μg). The signal obtained with a known amount of genomic DNA could be compared to that obtained with known amounts of the cloned DNA restriction fragments. Since signal strengths can be accurately compared only between identically sized DNA fragments, the signal produced by hybridization to the 1-kb Bgl II DNA fragment shared by both Y3002 and the yeast genome is compared. A densitometer scan of the autoradiograph indicates that the amount of probe hybridized to the 1-kb band is equivalent in lanes 2 and 4, consistent with the presence of a single genomic copy of the probe sequences. Moreover, the densitometer scan of this band in lane 3, where one gene equivalent of Y3002 DNA has been added to the genomic DNA, confirms the expected result for a single-copy sequence; that is, twice as much label is hybridized to this position than in lanes 2 and 4. All other signals (the 1.5-kb and 8-kb Bgl II fragments of genomic DNA and the 17.8-kb Bgl II fragment containing λ DNA from Y3002) correspond to those expected with this particular probe. Finally, since Y3002 insert DNA contains no internal Eco RI cleavage sites, the presence of a single 8-kb Eco RI genomic fragment is consistent with the interpretation of a single-copy sequence.

of differences in the expression of particular genes. In contrast, the representation of coding sequences in cDNA libraries reflects differences in cellular mRNA levels. Thus, expression libraries containing genomic DNA rather than complementary DNA may be more suitable for a thorough examination of coding capacity of eukaryotes with small genome sizes.

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Directed Mutagenesis of Dihydrofolate Reductase

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The enzyme dihydrofolate reductase (DHFR; E.C. 1.5.1.3) (*1*), is found in every kind of organism, from bacteria to mammals, and in large amounts in rapidly dividing cell lines. It catalyzes the NADPH-dependent (NADPH, reduced form of nicotinamide adenine dinucleotide phosphate) reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, which in turn plays a central metabolic role as a carrier of one-carbon units in the biosynthesis of thymidylate, purines, and some amino acids (*2*). An unusual feature of thymidylate biosynthesis in particular is that tetrahydrofolate is oxidized to dihydrofolate in the course of a one-carbon transfer to uridylate. Thus blockade of the DHFR-catalyzed reduction of dihydrofolate back to tetrahydrofolate in a rapidly proliferating cell results in the depletion of its tetrahydrofolate pool, with consequent cessation of DNA synthesis and, ultimately, stasis and cell death.

Furthermore, DHFR is especially susceptible to inhibition by synthetic folate analogs (the antifolates), some of which show a high degree of species selectivity. These peculiarities of DHFR enzymology have assumed practical importance in the clinical treatment of a number of diseases, notably the leukemias and other cancers, and certain bacterial and protozoal infections, against which chemotherapy with antifolates such as methotrexate, trimethoprim, and pyrimethamine proves to be effective (*3*). Not surprisingly, therefore, DHFR has become an enzyme of considerable interest to pharmaceutical chemists and drug designers.

Being the smallest of the well-characterized nicotinamide-dependent oxidoreductases, DHFR is also of interest to the structural biochemist. For example, the *Escherichia coli* enzyme consists of just a single 159-residue polypeptide chain. Thus DHFR presents an opportunity to study the stereochemistry of nicotinamide activation by high-resolution x-ray diffraction methods.

Motivated by these considerations, Matthews and Kraut and their co-workers (*4, 5*) have during the past few years determined the x-ray crystal structures of DHFR's derived from three species—*E. coli*, *Lactobacillus casei*, and chicken (the last being representative of all vertebrate DHFR's, which are highly homologous—at the maximum practical resolution and degree of refinement. Examination of these structures reveals a characteristic, highly conserved backbone chain fold, even though their amino acid sequences are only about 25 percent homologous, and provides a catalog of enzyme-substrate interactions, about half of which are also conserved. One can guess how some of these interactions might contribute to the catalytic mechanism, but reasons for the evolutionary conservation of others remain obscure. In addition, a few residues that do not interact directly with the substrate or cofactor are also conserved in the known DHFR sequences. Thus, many new questions are raised by the x-ray structures; but what is of overriding importance is that these questions are specific and highly focused.

It has been somewhat easier to ask incisive questions about the function of some part of an enzyme, given its three-dimensional structure, than it has been to devise equally incisive experiments to

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