Molecular cloning of hormone-responsive genes from the yeast Saccharomyces cerevisiae

(transcriptional regulation/pheromone action/poly(A)+ RNA/4-thiouridine/recombinant DNA)

GARY L. STETLER* AND JEREMY THORNER†

Department of Microbiology and Immunology, University of California, Berkeley, CA 94720

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ABSTRACT A method for identifying yeast genes whose transcription is differentially regulated was developed. The technique is based on incorporation of the analog 4-thiouridine into nascent RNAs, which allows their purification. The purified RNAs are used to prepare cDNA copies for screening of genomic DNA libraries by hybridization. Using this procedure, several cloned yeast DNA segments were found whose transcription in MATa haploids in vivo is apparently modulated in a dramatic fashion within 10-15 min after exposure to the mating pheromone, α factor. Subsequent analysis indicated that these sequences fall into three major classes: (i) genes expressed in vegetatively growing cells that are no longer transcribed after α-factor administration ("turn-off" genes); (ii) genes whose expression is increased 10-20-fold after exposure of the MATa cells to α factor ("turn-on" genes); and (iii) genes that are expressed only after α-factor treatment ("turn-on" genes). The first class may encode products required for cell cycle progression; the third class may code for products uniquely involved in the mating process.

One manifestation of peptide hormone action is the regulation of gene expression (1, 2). The two haploid cell types (a and α cells) of baker’s yeast (Saccharomyces cerevisiae) can be induced to conjugate ("mate") with each other by the action of oligopeptide pheromones (a factor and α factor, respectively) that resemble the peptide hormones of multicellular eukaryotes (3). Among the responses evoked by pheromone administration are: induction of cell surface agglutinins, which promote adhesion between cells of the opposite mating type; inhibition of progression past the G1 phase of the cell cycle, which synchronizes the mating partners; activation of certain enzymes involved in cell wall biosynthesis; and, ultimately, conversion of the normally spherical cells to a characteristically elongated morphology (for review, see ref. 4). Although a growing catalogue of such pheromone-induced changes exists, the molecular mechanisms whereby the pheromones elicit these effects is not well known nor is it known whether any of these effects reflects changes in gene expression. Furthermore, yeast mating is a genetically complex process. The mating type locus (MAT) and at least 10 other unlinked (STE) genes appear to be involved in the ability of one or the other (or both) haploid cell types to mate (5, 6). Another dozen or so genes that also have some effect on various aspects of the mating process have been identified (4, 7). Aside from MAT itself, it is currently unknown which, if any, of these genes have a regulatory role and which are structural genes for products that are directly involved in conjugation.

As one approach to understanding the biochemical basis of pheromone action and as an independent means to identify genes involved in the mating process, we devised a method to directly determine if exposure to α factor rapidly alters the pattern of expression of specific genes in a haploid, in either a positive or negative sense. By using this procedure, it was indeed possible to identify and isolate certain a cell genes that are transcriptionally regulated in dramatic fashion shortly after α-factor treatment. The technique we developed may be generally applicable for the identification and isolation of differentially expressed genes in other organisms.

MATERIALS AND METHODS

Labeling and Purification of 4-Thiouridine-Substituted RNA. Two cultures (1 liter each) of S. cerevisiae strain XS3-6b (MATa ura1 met2 lys2 his4-580 cry1 SUP4-3) were grown at 30°C to an A600 of 1.5–1.65 (about 8 × 107 cells per ml) in SC medium (8) containing 20 μg of uridine per ml (in place of uracil). Cells were collected by centrifugation at 4°C for 5 min at 5000 × g and washed by resuspension and recentrifugation in 100 ml of SD medium (8) lacking both glucose and uridine. The washed cell pellets from the two cultures were each resuspended in 1 liter of SC containing 75 μM 4-thiouridine (Sigma) and 0.05 μCi (2 nM) of [6-3H]uridine (1 Ci = 3.7 GBq; New England Nuclear) per ml. After incubation for 10 min at 30°C with shaking, purified natural α factor (9) (gift of H. Liao) in 0.1 M NaOAc (pH 5) was added to one culture at a final concentration of 0.3-0.4 μM (=50 units/ml); an equal volume of 0.1 M NaOAc (pH 5) was added to the second (control) culture. After 30 min, the cells were chilled and collected by centrifugation. Total RNA fractions of the control and α-factor-treated cells were purified immediately by phenol extraction (10). Poly(A)+ RNA was selected by chromatography on oligo(dT)-cellulose (Collaborative Research, Waltham, MA) (11) and concentrated by ethanol precipitation. Poly(A)+ RNA (up to 150 μg) was redissolved in 10 ml of 0.1% NaDodSO4/0.15 M NaCl/50 mM NaOAc, pH 5.5, heated to 65°C for 5 min, cooled rapidly in ice, and applied to a bed (1 ml) of phenylmercury agarose (12) (Affi-Gel 501, Bio-Rad) in a column (0.5 × 2 cm) at a flow rate of 5 ml/hr. The column was washed with 10 ml of the same buffer to remove nonspecifically bound RNA. The 4-thiouridine-containing RNA was eluted with the same buffer containing 10 mM 2-mercaptoethanol, at a flow rate of 5 ml/hr. Fractions (0.5 ml) were collected and the radioactivity present in samples (10 μl) of each was determined by liquid scintillation counting. Fractions containing the bulk of the RNA were pooled and concentrated by ethanol precipitation.

Construction of Yeast Genomic Library. DNA purified (13) from S. cerevisiae strain 381G (MATa ade2-1 his4-580 lys2 trp1 tyr1 cry1 SUP4-3) (6) was partially digested with either BamHI or Sau3A restriction endonuclease (New England

Abbreviations: kb, kilobases(s); nt, nucleotide(s).


†To whom reprint requests should be addressed.
BioLabs or Bethesda Research Laboratories) as recommended by the suppliers. Fragments in the size range 12–18 kilobases (kb) were purified by sucrose gradient centrifugation (14). The fragments were inserted into the bacteriophage vector λ1059, using T4 DNA ligase prepared by minor modifications of the procedure of Tait et al. (15), and packaged in vitro into phage particles, all essentially as described by Karn et al. (16).

Preparation of Hybridization Probes. 32P-labeled cDNA was prepared from the 4-thiouridine-containing (A) 32P RNA by using avian myeloblastosis virus reverse transcriptase (gift of N. Maizels), deoxyribonucleoside (α-32P)-triphosphates (Amersham), and random hexanucleotide primers generated from calf thymus DNA (gift of S. Hughes) (17). These probes were used for differential plaque hybridizations conducted by the method of Woo (18).

Recombinant λ phage were propagated in Escherichia coli BNN45 (hsdR R’ hsdM’ supE44 supF thi’ mer’) (19) and phage DNAs were purified as described by others (20). Whole recombinant bacteriophage λ DNAs used as probes were labeled by nick-translation (21).

Electrophoretic Analysis of RNA. Samples of poly(A) 32P RNA (1–2 μg), purified as above, were denatured and subjected to electrophoresis in gels (11 × 12.5 cm) of 1.5% agarose, transferred to nitrocellulose paper (Sartorius), hybridized to probes, and examined by autoradiography, all by only minor modifications of the methods of others (22, 23).

RESULTS

Detection of α-Factor Regulation of MATa Cell Gene Expression. Any method to determine whether α factor has a direct effect on the pattern of gene transcription should: (i) permit detection of both the appearance (or increase) and disappearance (or decrease) of specific mRNA species; (ii) increase the likelihood that any observed changes represent effects on the rate of transcription of specific genes (rather than alterations in RNA levels due to post-transcriptional events); and (iii) monitor relatively immediate primary responses to the pheromone (rather than long-term indirect responses due to the ultimate imposition of cell cycle arrest).

By isolating the newly synthesized mRNAs made in a cells shortly after pheromone treatment and those made in untreated a cells, cDNA probes could be prepared for screening a library of yeast genomic DNA by differential hybridization. Enrichment for the nascent mRNAs made by pheromone-treated and control cells was accomplished by the incorporation of 4-thiouridine into these molecules during a brief pulse (30 min, 0.25 of a generation). The presence of 4-thiouridine in nascent RNA permits its purification by chromatography on phenylmercury agarose. To enhance the efficiency of labeling, a pyrimidine auxotroph was used. At the concentration of 4-thiouridine employed, the rate of total RNA synthesis was at least 90% of that observed in control cultures in which the analog was replaced by an equivalent concentration of uridine (results not shown).

Typically, about 1–2% of the total RNA (absorbance at 260 nm) in exponentially growing cells was recovered as poly(A) 32P RNA by chromatography on oligo(dT)-cellulose. As judged by absorbance, only 3–5% of these species were retained on phenylmercury agarose. However, 65% of the radioactivity in the total poly(A) 32P RNA was present in the 4-thiouridine-containing fraction. Hence, the incorporation of 4-thiouridine provided at least a 10-fold enrichment for a specific class of transcripts, the newly made mRNAs. The overall yield of 4-thiouridine-containing poly(A) 32P RNA from 1-liter cultures was 5–5 μg. cDNAs representing the nascent mRNA population from vegetatively growing a cells (cDNAa) and the nascent mRNAs species in α-factor-treated a cells (cDNAa) were used as probes to screen a library of yeast genomic DNA cloned in a bacteriophage vector, λ1059. Two types of differential hybridizations were performed. First, duplicate filters were hybridized either with cDNAa or with cDNAa, and the hybridizations patterns were compared. Plaques displaying clear-cut differences in their degree of hybridization to the two different probes were picked for further testing. Because the average insert size in the recombinant phages (16.5 kb) was large enough to encode several genes, an inherent problem with the direct screen was that the presence on a phage of a gene whose transcription is responsive to α factor might go undetected if it is surrounded by several genes that are transcriptionally active under all conditions. In an attempt to minimize this difficulty, a second set of differential plaque hybridizations was performed in which a 300 to 400-fold excess of unlabeled competitor poly(A) 32P RNA was added along with the probe. To identify genes expressed only after exposure to α factor, competitor mRNA isolated from vegetatively growing cells was added along with cDNAa. Conversely, to identify genes no longer transcribed after pheromone treatment, competitor mRNA isolated from pheromone-treated cells was added along with cDNAa. Again, autoradiograms of the pairs of filters were compared and plaques displaying obvious differences in their degree of hybridization under the two different conditions were picked for retesting.

From a total of >30,000 individual plaques screened by either of these two methods, we recovered only 6 recombinant phages that reproducibly behaved, upon three successive plaque purifications and rescreenings, as if they carried genes whose transcription responded to the presence of the pheromone. Five of the phages were unique; the sixth phage contained an insert very similar or identical to one of the other five and was not studied further. This result suggests that, within the limits of detection of our method, α factor does not cause global changes in a cell gene transcription, at least within 30 min, but does affect the transcription of a highly selected set of genes. Nonetheless, because we chose for further examination only those phages that showed the most pronounced signals and only obtained one duplicate isolate, we consider it likely that not all of the sequences whose transcription is affected by α-factor treatment were recovered.

α Factor Is Both a Positive and Negative Regulator of Gene Expression. The number and size of the transcripts encoded by the five phages, and the magnitude and kinetics of the α-factor-induced changes in their transcription, were determined. Poly(A) 32P RNA was extracted from samples of a culture of a cells at various intervals after their exposure to α factor (Fig. 1). The spectrum of complementary RNA molecules was revealed by using as probe the entire DNA of each recombinant phage. To ensure that the α-factor concentration would remain relatively constant throughout the course of such experiments, the a haploids used carried an sstl (also called bar1) mutation (24–26), which prevents a cells from proteolytically degrading α factor (9). This analysis confirmed that all five phages are unique and carry DNA sequences that are transcribed differentially in vegetatively growing a cells and α-factor-treated a cells. Three overall classes of responses were found (Fig. 1).

The insert (10.4 kb) in one phage, λScG2, encodes two transcripts that are no longer transcribed or are transcribed at very reduced rates, after a cells are exposed to α factor (Fig. 1A). As judged by densitometer tracings of autoradiograms, the level of the major transcript, about 1500 nucleotides (nt), decays with a half-life of 30 min. We have termed this class of response “turn-off.”

Two phages (λScG8 and λScG11) each encode three transcripts that are present in vegetative cells; however, one of the transcripts on each phage shows a dramatic elevation (10 to 20-fold) after pheromone treatment. The steady-state level
of the \( \alpha \)-factor-induced transcript coded for by \( \lambda \)ScG8 (12.2-kb insert) appeared to increase progressively in abundance over the course of 60 min after exposure to \( \alpha \) factor (Fig. 1B). In contrast, the pheromone-induced transcript encoded by \( \lambda \)ScG11 (11.7-kb insert) reproducibly showed a so-called "burst-attenuation" response—that is, the level of transcript found at 60 min was always less than that observed at either 15 or 30 min (Fig. 1C). We have called these types of responses "turn-up."

The fourth phage, \( \lambda \)ScG7 (14.0-kb insert), encodes seven transcripts; however, three of them are present at an extremely low level, virtually undetectable, in vegetatively growing cells. Within 15 min after exposure of the cells to \( \alpha \) factor, these three transcripts have become nearly as abundant as the other transcripts encoded by the insert and they continue to accumulate for at least 60 min. The last phage, \( \lambda \)ScG6 (15.0-kb insert), also apparently codes for seven transcripts, ranging in size from 450 to 3,600 nt, the smallest of which behaves very similarly to the \( \alpha \)-factor-induced transcripts carried by \( \lambda \)ScG7 (results not shown). We have designated this third type of response "turn-on."

None of these effects could be attributed to differential loading of RNA samples or to differential recoveries of poly(A)\(^+\) RNA in the preparations made from cells at the different time points. For example, the exact same nitrocellulose filter that was used to demonstrate that \( \lambda \)ScG8 encodes a turn-up transcript (Fig. 1B) was washed and reprobed with \( \lambda \)ScG2 and confirmed that it specifies two turn-off transcripts (Fig. 1A). Also, reprobing of all such blots with other cloned yeast genes, specifically \( \text{LEU2} \) (8) and \( \text{LY52} \) (unpublished data), indicated that the level of these control mRNAs did not vary between samples within any given experiment by >20–30%. Finally, all but one of the phage inserts contained sequences complementary to one or more transcripts that did not show any obvious response to pheromone addition and thus served as internal controls to normalize for the uniformity of sample application.

To be certain that these responses were not due to some minor contaminant in our preparation of natural \( \alpha \) factor, \( \text{MATa sst1} \) cells were exposed to various concentrations of chemically synthesized \( \alpha \) factor and the kinetics of appearance of the turn-on transcripts specified by \( \lambda \)ScG7 were examined by gel electrophoresis and blotting. It was clear, first, that the synthetic pheromone induced the appearance of all three of the \( \alpha \)-factor-regulated transcripts. Second, a pronounced response was observed even at concentrations of the pheromone as low as 0.3 \( \text{nM} \) (Fig. 2). This level of pheromone is in good agreement with the range of concentrations reported to be physiologically efficacious, under similar conditions, for evoking certain biological responses, such as agglutinin induction (28) and cell cycle arrest (24, 25). Third, better resolution of the higher molecular weight species revealed that the induction involves only one of the two large (about 3600 nt) transcripts encoded by \( \lambda \)ScG7. Another of the positively regulated pheromone-responsive transcripts, that encoded by \( \lambda \)ScG6, also was clearly induced at 0.3 \( \text{nM} \) synthetic \( \alpha \) factor (results not shown).

In contrast, the negatively regulated pheromone-responsive transcripts specified by \( \lambda \)ScG2 did not show a significant decrease in their abundance at 30 min unless the cells were exposed to a concentration of synthetic \( \alpha \) factor of 2 \( \text{nM} \) or above (results not shown). Similarly, induction of the positively regulated transcripts encoded by \( \lambda \)ScG8 and \( \lambda \)ScG11 was not apparent below 2 \( \text{nM} \) \( \alpha \) factor. At least one

![Fig. 1.](image)

**Fig. 1.** Time course for transcriptional responses of yeast genes to \( \alpha \)-factor mating pheromone. *S. cerevisiae* RC634 (\( \text{MATa sst1-3 ade2-1} \) his6 met1 ura1 can1 cyh2 ram1 GAL) (24) was grown to a cell density of \( 5 \times 10^9 \) per ml in SC medium buffered at pH 5 with 20 mM sodium succinate and exposed to a final concentration of \( \alpha \) factor of 0.24 \( \mu \text{M} \); samples of the culture were removed at the times (in min) indicated. Poly(A)\(^+\) RNA was isolated, subjected to electrophoresis, and transferred to nitrocellulose. The filter-bound RNA was then hybridized to \( \text{32P} \)-labeled DNA isolated from each of the indicated pheromone-responsive phage clones. Molecular weight markers were a mixture of fragments from three separate digests (EcoRI, *Tel 1*, and *Rsa I*) of pBR322 (19) denatured by formaldehyde treatment (22). Probes were: \( \lambda \)ScG2 (A), \( \lambda \)ScG8 (B), \( \lambda \)ScG11 (C), and, \( \lambda \)ScG7 (D).
absence of the pheromone that show a greatly elevated level of expression after pheromone treatment; and (iii) genes that are no longer expressed after pheromone administration. The products of the genes in the first category are likely to be functions specifically involved in the mating process—for example, agglutinins or products of the various STE genes.

The products of the genes in the second category may not encode mating-specific functions because they are expressed to a certain extent in the absence of pheromone and are expressed equivalently in both haploid cell types and in diploids (unpublished results). Therefore, these genes may code for functions that have some role in normal vegetative growth but are required at elevated levels in conjugating cells. An example of such functions might be cell wall biologically response elicited from a cells, morphological elongation, is known to require levels of the pheromone in this range (3).

There exist a number of temperature-conditional mutations that cause yeast cells to arrest their cell division cycle apparently at the same stage as that brought about by exposure to mating pheromone (29, 30). One such lesion is the cdc28 mutation (29, 31). None of the transcripts described here exhibited altered expression due to the imposition of cell cycle arrest per se because the abundance of these species was unchanged in poly(A)+ RNA isolated from MATa cdc28 mutants shifted to the restrictive temperature for 60 min (results not shown).

DISCUSSION

We have identified five genomic clones of S. cerevisiae DNA that appear to encode a variety of polyadenylated RNAs whose intracellular concentrations change during the response of a cells to α factor. The screening procedure used to isolate these clones was dependent on differences in the amounts of newly synthesized mRNA rather than on differences in the steady-state level of total mRNA. Although post-transcriptional mechanisms may play a role in controlling the levels of these pheromone-regulated transcripts, we are unaware of effects of this magnitude and rapidity that are attributable solely to changes in RNA stability. For these reasons, we consider it very likely that the rate of transcription of these genes is controlled by the pheromone.

Because the tactic of labeling nascent RNA with 4-thiouridine could be applied under a variety of conditions imposed by an investigator, the method we have described should be generally useful for identifying and isolating genes that are transcribed differentially under other circumstances—for example, shifts of temperature-conditional mutants from one temperature to another, transfer of cells from one carbon source to another, or after heat shock of normal cells.

One of the more remarkable aspects of our findings is the variety of apparent transcriptional responses to the pheromone. These effects appear to define pheromone-responsive genes of three types: (i) genes not expressed, or expressed at very low levels, in vegetatively growing cells that are rapidly induced after exposure to α factor; (ii) genes expressed in the

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