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Transcriptional Activation of Yeast Nucleotide Biosynthetic Gene *ADE4* by GCN4*

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The yeast transcriptional regulator protein GCN4 harbors the bZIP DNA binding motif, which is common to a family of DNA-binding proteins in eukaryotic organisms from yeast to man. GCN4 and the mammalian activator protein AP-1 (*jun/fos*) regulate transcription by binding the same consensus DNA sequence ATGA(C/G)TCAT. GCN4 positively regulates the production of precursors of protein synthesis in yeast cells in response to the environmental signal "amino acid starvation." We find three GCN4 responsive elements (GCREs) in the 5′-flanking region of the purine biosynthetic gene *ADE4* and demonstrate that GCN4 efficiently activates transcription of *ADE4*. Two GCREs are essential to synergistically activate *ADE4* transcription by binding GCN4. The distal GCRE1 is also required for basal transcription of *ADE4*. Therefore, transcription factor GCN4 affects, in addition to protein biosynthesis, also nucleotide biosynthesis and, comparable to its mammalian counterpart AP-1, has a more general function within the yeast cell than previously assumed.

The bZIP motif is a common feature of a family of DNA-binding proteins which includes the yeast GCN4 protein, the *jun* and *fos* oncoproteins as well as the C/EBP enhancer protein (Agre *et al.*, 1989). The bZIP DNA-binding domains of GCN4 and the oncogene *jun* have been shown to be functionally homologous (Struhl, 1987). GCN4 binds to the GCN4 responsive element (GCRE),[1] that has been well characterized as the palindromic sequence 5′ ATGA(C/G)TCAT 3′ (Hope and Struhl, 1985; Hill *et al.*, 1986). This sequence has also been shown to be an optimal binding site for the human trans-activator protein complex AP-1 (Bohmann *et al.*, 1987) and been referred to as ARE, AP-1 responsive element, or TRE, 12-*O*-tetradecanoylphorbol-13-acetate responsive element, respectively (Kouzarides and Ziff, 1989). GCRE sequences in yeast have been found upstream of 30–40 unlinked genes encoding enzymes in 11 different amino acid biosynthetic pathways and two tRNA synthetase genes (Hinnebusch, 1988; Mirande and Waller 1988). The GCN4 protein stimulates transcription in response to amino acid starvation, a system called the "general control" of amino acid biosynthesis of yeast (reviewed by Hinnebusch, 1988). Therefore, GCN4 is a positive regulator of protein synthesis by controlling the production of precursors as amino acids and tRNA synthetases. Here we present evidence that GCN4 also activates transcription of the purine biosynthetic gene *ADE4* via several GCRE elements. Therefore, GCN4 is a still more general transcription factor which affects in addition to protein biosynthesis also nucleotide biosynthesis.

EXPERIMENTAL PROCEDURES

Sequencing of the ADE4 Gene 5′-Flanking Region—A *Bgl*II-*Mlu*I fragment of 985 bp containing the regulatory region of the *ADE4* gene with a portion of its structural gene was isolated from plasmid pPM7 (Mäntsälä and Zalkin, 1984) and, after trimming its 5′ ends blunt using Klenow polymerase, inserted into the *Sma*I cleavage site of plasmid M13mp10 (Vieira and Messing, 1982). Nucleotide sequencing was then performed with the chain termination method (Sanger *et al.*, 1977).

Site-directed Point Mutagenesis—Oligonucleotide-directed point mutations on GCRE sequences were generated using the Muta-Gene *in vitro* mutagenesis kit from Bio-Rad, based on a method described by Kunkel (Kunkel, 1985). Mutations were: GCRE1 (5′-TTGACTCTT-3′) to gcre1 (5′-TTTACGCTT-3′); GCRE2 (5′-ATGAATAAT-3′) to gcre2 (5′-ACGAATAGT-3′); GCRE3 (5′-ATGACTGCT-3′) to gcre3 (5′-ACGAATGCT-3′). Promoter region of all *ADE4* mutant alleles obtained by this procedure were sequenced using the chain termination method (Sanger *et al.*, 1977) thereby ruling out possible second site mutations.

Construction of Yeast Strains Carrying ADE4 Mutant Alleles—All yeast strains carrying *ADE4* mutant alleles were constructed using the gene replacement technique (Rudolph *et al.*, 1985). The complete *ADE4* promoter was first removed (position −510 to +230) and then substituted with the *URA3* gene. *ADE4* promoter mutant alleles were then reintroduced replacing the *URA3* gene. Linear fragment yeast transformations were performed using the lithium-acetate treatment method (Ito *et al.*, 1983). The integration of the mutant alleles at the original *ADE4* locus on the chromosome was confirmed using the Southern blot technique (Southern, 1975). *ADE4* replacements were performed in the following two isogenic derivatives of the *Saccharomyces cerevisiae* laboratory strain S288C: RH1408 carrying the *gcn4-103* mutation containing a large deletion of the *GCN4* gene (Hinnebusch, 1985) and RH1378 harboring the *gcd2-1* mutation that causes constitutively high amounts of GCN4 protein in the cell (Niederberger *et al.*, 1986).

DNase I Footprint Analysis—DNase I protection analysis was performed with modifications as described (Galas and Schmitz, 1978). 2×10^4 cpm of 5′-radiolabeled *ADE4* wild-type or mutant promoter fragments were incubated with 2–8 μg of partially purified GCN4 protein expressed in *Escherichia coli* in 20 mM Hepes, pH 7.0, 8% glycerol, 40 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 20 ng/μl poly(dI-dC) in a 50-μl assay. After 20 min of incubation on ice, DNase I was added to a final concentration of 10 ng/μl, and the reaction was terminated after 90 s by adding 125 μl of 0.12% SDS, 12 mM EDTA, 0.36 M NaAc containing 5 μg of yeast

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M74073.

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[1] The abbreviations used are: GCRE, GCN4 responsive elements; bp, base pairs; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tRNA. Samples were separated on a standard sequencing gel and autoradiographed. A G/A sequencing ladder was used as size marker (Maxam and Gilbert, 1980). GCN4 protein was produced using an *E. coli* expression system as previously described (Arndt and Fink, 1986).

Northern Analysis—Poly(A)$^+$ RNA was isolated as described earlier (Furter *et al.*, 1986). For Northern hybridization poly(A)$^+$ RNA was separated on a formaldehyde-agarose gel, electroblotted onto a nylon membrane, and hybridized with DNA fragments labeled according to the "oligo-labeling" technique (Feinberg and Vogelstein, 1984).

Primer Extension Analysis—Primer extension analysis was performed according to Kassavetis and Geiduschek (1982) using 100 μg of Poly(A)$^+$ RNA and 5×10^6 cpm of a 5′-end-labeled 35-bp primer (from position +38 to +72 relative to the translation start site of the *ADE4* gene).

Media and Enzyme Assays—Yeast strains were cultivated in yeast extract peptone dextrose complete medium or minimal vitamin medium supplemented with uracil (40 mg/liter) and arginine (40 mg/liter) as described earlier (Miozzari *et al.*, 1978). For enzyme level determination, 100-ml cultures of identical yeast strains as used for transcript level measurements were grown to an OD_{546} of 2 and permeabilized using Triton X-100 as described (Miozzari *et al.*, 1978). Specific APRTase enzyme activities were then assayed by the glutamate dehydrogenase method (Messenger and Zalkin, 1979).

RESULTS

The ADE4 Gene Promoter Contains Three GCRE Sites That Bind GCN4 Protein in Vitro—The *ADE4* gene of *S. cerevisiae* encodes for the enzyme glutamine phosphoribosylpyrophosphate amidotransferase (APRTase; EC 2.4.2.14). An extension of the previously published 5′-flanking sequence of the *ADE4* gene (Mäntsälä and Zalkin, 1984) of a further 220 bp up to position −510 revealed three putative GCREs that were designated as GCRE1, GCRE2, and GCRE3, respectively (Fig. 1*A*). These sites each consist of sequences that deviate only by 2 bp from the consensus 5′ ATGA(C/G)TCAT 3′ GCRE sequence. Deviations of 1–2 bp have been found for most naturally occurring GCREs (reviewed by Struhl, 1989). GCN4 protein specifically bound to all of these GCRE sequences in the *ADE4* promoter *in vitro* when tested in a DNase footprint analysis (Fig. 1*B*). The binding affinities of GCRE1 and GCRE3 for GCN4 *in vitro* corresponded to the affinities of functional GCREs as found in the yeast *TRP4* or *ARO3* genes (Braus *et al.*, 1989; Mösch *et al.*, 1990; Paravicini *et al.*, 1989), whereas the GCN4 affinity for GCRE2 was estimated to be approximately 8–10-fold lower (Fig. 1*A* and Fig. 2*B*).

GCN4 Synergistically Activates Transcription of the ADE4 Gene via GCRE1 and GCRE3—The presence of GCN4 protein in the cell-stimulated transcription of the *ADE4* gene severalfold when assayed in a Northern RNA hybridization analysis (Fig. 2*C*). This stimulation of *ADE4* transcription by GCN4 correlated with a 3-fold increase in the specific enzyme activity of the *ADE4* gene product as measured by *in situ* APRTase enzyme level determinations (Fig. 2*C*). A 2–5-fold stimulation of transcription has been found for most genes under the control of GCN4 (Hinnebusch, 1988).

Since *in vitro* binding of GCN4 does not necessarily determine an *in vivo* function for a putative GCRE site (Schmidheini *et al.*, 1990), we created point mutations in the *ADE4* promoter region in GCRE1, GCRE2, and GCRE3, respectively (Fig. 2*A*). All base pair exchanges resulted in the GCN4 protein no longer being able to bind *in vitro* to any of the mutated GCRE sequences even at high concentrations of the protein (Fig. 2*B*). The different mutant promoter alleles were substituted for the wild-type *ADE4* promoter by gene replacement in the genomic *ADE4* locus. Expression of the *ADE4* gene under control of these mutant promoter alleles was measured in yeast cells containing either no GCN4 protein or constitutively high levels of GCN4. Fig. 2*C* summarizes the data of the *ADE4* transcript analysis as well as the

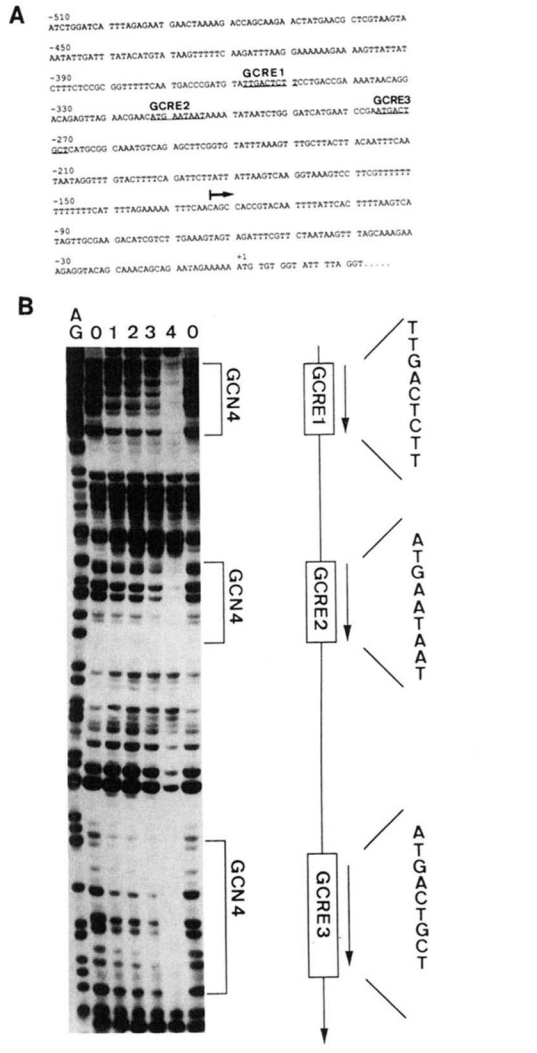

FIG. 1. **Analysis of GCN4 responsive elements (GCRE) in the *ADE4* promoter.** *A*, nucleotide sequence of the *ADE4* gene promoter of the 5′-untranslated region and the first six translated codons are shown. *Numbers* above the sequence show nucleotide positions relative to the translational start site indicated as *+1*. The major transcriptional initiation site at position −124 is marked by an *arrow* (|→), GCRE sequences are *underlined* and indicated as *GCRE1*, *GCRE2*, and *GCRE3* respectively. *B*, DNase I footprint analysis. DNA probe was a *Bgl*II/*Dra*I *ADE4* promoter fragment (positions −511 to −235) 5′-radiolabeled on the coding strand at the *Dra*I cleavage site using [γ-^{32}P]ATP and polynucleotide kinase. DNA probe was incubated with *E. coli* extracts containing GCN4 protein (1 μg in *lane 1*, 2 μg in *lane 2*, 4 μg in *lane 3*, 8 μg in *lane 4*) or without GCN4 protein (*lane 0*). After treatment with DNase I, the samples were separated on a standard sequencing gel. An *A/G* sequence ladder was used as size marker. Sequences protected by GCN4 protein are *bracketed*. GCRE sequences are represented as *boxes* and the corresponding DNA sequences are indicated on the *right*.

different specific enzyme activities of the *ADE4* gene product APRTase. A complete loss of any transcriptional activation of *ADE4* by GCN4 was found when the GCRE1 box was mutated, and mutations in GCRE3 allowed a transcriptional stimulation by GCN4 of only approximately 10–20% compared with wild-type activation. These findings imply a synergistic interaction between GCRE1 and GCRE3 in the *ADE4* promoter. A similar situation has been found in the amino acid biosynthetic *TRP4* gene of yeast (Mösch *et al.*, 1990). 60–70% transcriptional activation of *ADE4* by GCN4 was still possible when the GCRE2 sequence was mutated in the *ADE4* promoter. GCRE2, therefore, has only a minor role for the

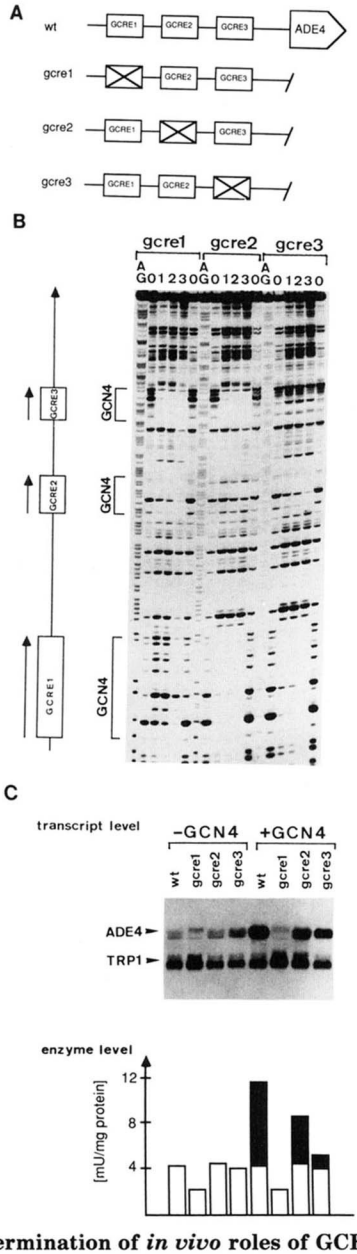

FIG. 2. **Determination of *in vivo* roles of GCRE sites in the *ADE4* promoter region.** *A*, schematic representation of *ADE4* promoter wild-type (*wt*) and mutant alleles (*gcre1*, *gcre2*, and *gcre3*). Mutated GCRE sequences are shown as *crossed boxes*: gcre1: 5′-TTGACTCTT-3′ to 5′-TTTACGCTT-3′; gcre2: 5′-ATGAATAAT-3′ to 5′-ACGAATAGT-3′; gcre3: 5′-ATGACTGCT-3′ to 5′-ACGAATGCT-3′. *B*, DNase I footprint analysis of *ADE4* promoter mutant alleles. Footprinting with GCN4 protein was carried out as described in Fig. 1*B* using mutated *ADE4* promoter fragments (gcre1, gcre2, and gcre3) that were radiolabeled at position −396 on the noncoding strand. DNA probes were incubated with *E. coli* extracts containing GCN4 protein (2 μg in *lane 1*, 4 μg in *lane 2*, 8 μg in *lane 3*) or without GCN4 protein (*lane 0*). *AG* is an A/G sequence ladder as size standard, GCN4-protected regions are *bracketed*, GCRE sequences are represented as *boxes*. *C*, expression of the *ADE4* gene under control of promoter mutants and GCN4. For *transcript level* poly(A)$^+$ RNA from yeast strains containing no GCN4 protein (*−GCN4*) or constitutively high levels of GCN4 protein (*+GCN4*) and carrying different *ADE4* promoter mutant alleles (*wt*, *gcre1*, *gcre2*, and *gcre3* according to *A*) at the chromosomal *ADE4* locus were cohybridized against radiolabeled *ADE4* and *TRP1* probes. The *TRP1* transcript was chosen as an internal standard for the amount of mRNA as it is not under the control of GCN4 (Braus *et al.*, 1988). Transcript sizes are: *ADE4*, 2.2 kb; *TRP1*, 1.0 kb. For *enzyme level* determinations specific APRTase enzyme activities were assayed by the glutamate dehydrogenase method (Messenger and Zalkin, 1979). The given values (in *mU/mg protein*) are the means of four independent cultivations each measured twice with a standard deviation that did not exceed 20%. GCN4-dependent expression is indicated by *black bars* determined by subtracting basal expression (*−GCN4*, *open bars*) from constitutively derepressed expression (*+GCN4*).

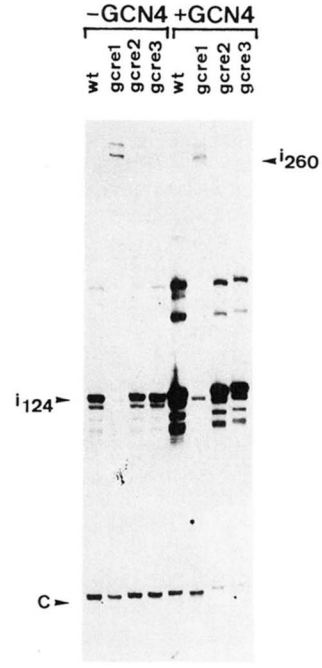

FIG. 3. **Transcription start site selection patterns.** 100 μg of poly(A)$^+$ RNA from identical preparations as used for quantitative Northern hybridizations in Fig. 2*C* were hybridized to completion with an excess of a γ-^{32}P-labeled *ADE4* primer (from position +38 to +88 relative to the translational start site at +1) and subsequently elongated using avian myeloblastosis virus reverse transcriptase. Initiation sites of *ADE4* transcription are designated as *i* (subscripts indicate their position relative to translational start at +1) and are marked by an *arrow*. *C* indicates an unspecific control transcript.

GCN4-mediated activation. This correlates with the low binding affinity of GCRE2 for GCN4 *in vitro*.

GCRE1 Forms Part of the Basal Promoter of the ADE4 Gene—Typical amino acid biosynthesis promoters are dual promoters and hence can be regulated by two control systems, namely general (GCN4-dependent) and basal (Struhl, 1986; Arndt *et al.*, 1987). Whereas the general control promoter is only active under conditions of amino acid starvation, the basal control promoter is not regulated by amino acid levels and is responsible for the basal level of transcription of the amino acid biosynthesis genes, even when amino acids are present in the growth medium. The basal transcription of the *ADE4* gene was affected when the GCRE1 element was mutated. Transcription efficiency dropped down to about half and the normal *ADE4* transcript was substituted for a transcript of larger size that was detected in the Northern hybridization analysis. Mutations in GCRE2 or GCRE3, however, did not affect basal transcription of *ADE4*. When we determined and quantitated the 5′ start sites of wild-type *ADE4* transcripts (Fig. 3), basal and GCN4-dependent transcription of *ADE4* mainly initiated at position −124 (*i124*) relative to the translational start site. However, when the GCRE1 element was mutated, transcription of *ADE4* initiated at position −260 (*i260*) independent of the presence or absence of GCN4 protein. GCRE1, therefore, is not only essential for the activation of *ADE4* by GCN4 but also seems to be a recognition site for a factor involved in the basal transcription of the gene.

DISCUSSION

The regulator protein GCN4 has been found to control protein synthesis by activating transcription of amino acid biosynthetic and aminoacyl-tRNA synthetase genes. GCN4 generally acts via the GCN4-dependent promoter of these genes (Struhl, 1989). In specific cases GCN4 can also regulate basal transcription as shown for only a few genes including the yeast *ARO3* and *LEU2* genes (Paravicini *et al.*, 1989; Brisco and Kolhaw, 1990). Evidence for a possible TATA-factor function of GCN4 has also been proposed (Chen and Struhl, 1989), and GCN4 has been suggested to fulfill a second role as the general transcription factor TFIID in the regulation of the GCN4-dependent promoter of the *TRP4* gene (Mösch *et al.*, 1990). The finding that GCN4 regulates transcription of the purine biosynthetic gene *ADE4* implies a regulatory role of GCN4 in an additional metabolic network, namely nucleotide biosynthesis. For basal promoters an interconnection between histidine and purine biosynthesis pathways has been suggested (Tice-Baldwin *et al.*, 1989), but no direct link on the molecular level has been found so far. It furthermore remains to be elucidated whether other purine biosynthetic genes are also under the control of GCN4.

Eukaryotic cells from yeast to human contain structurally similar and functionally analogous transcription factors that recognize essentially identical sequences, as for instance the yeast GCN4 protein and the vertebrate AP-1. Although these eukaryotic transcription factors are structurally related, the homologues often seem to perform different functions in their respective organisms. Whereas, for example, AP-1 was found to activate a variety of genes whose functions seem to be unrelated, its evolutionary counterpart GCN4 appeared to be specific only for genes involved in protein biosynthesis. The finding that GCN4 regulates transcription of the purine biosynthetic gene *ADE4* points to a yet more general function for this transcription factor in yeast.

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