Subunits of Yeast RNA Polymerase II Transcription Factor TFIIH Encoded by the CCL1 Gene*

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Both 45- and 47-kDa subunits of TFIIK, a subcomplex of RNA polymerase II general transcription factor TFIIH, are encoded by the yeast cyclin gene CCL1. In all likelihood, these two subunits individually form cyclin-dependent kinase/cyclin dimers with Kin28 protein, a key enzyme in phosphorylation of the C-terminal domain of RNA polymerase II concomitant with transcription.

The initiation of RNA polymerase II transcription requires the five general transcription factors TFIIA, -ID, -IIe, -IIIf, and -IIF. Yeast TFIIH has been described as a holoenzyme (holoTFIIH), comprising Sis2 protein, a five-subunit core complex (core TFIIH), and polypeptides of 33, 45, and 47 kDa, collectively referred to as TFIIK (Svejstrup et al., 1994, 1995; Feaver et al., 1993). Sis2 protein and core TFIIH are required for both transcription and DNA repair (Svejstrup et al., 1995; Wang et al., 1994). TFIIK is a protein kinase which may play dual roles as well (Feaver et al., 1994b; Svejstrup et al., 1995): in addition to phosphorylation of the C-terminal domain of RNA polymerase II, TFIIK has been shown in vertebrate systems (as the cyclin-dependent kinase-activating kinase) to phosphorylate cell cycle control protein kinases (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993; Fisher and Morgan, 1994; Makela et al., 1994; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). The 33-kDa subunit of yeast TFIIK is encoded by the KIN28 gene (Feaver et al., 1994b), originally studied on the basis of its homology to the cyclin-dependent kinase CDC28 (Simon et al., 1986). KIN28 was further shown to interact with a cyclin homolog, CCL1, in vivo (Valay et al., 1993). Here we demonstrate the association of Cd1 with TFIIK in vitro.

EXPERIMENTAL PROCEDURES

HoloTFIIH Purification and Microsequencing—HoloTFIIH was purified from yeast whole cell extracts essentially as described (Svejstrup et al., 1994) except that the Phenyl HR5/5 step was omitted. HoloTFIIH was at least 50% pure in the final (Mono Q) fractions. Purified holo-

TFIIH (100 μg) was separated in a 9% SDS-polyacrylamide gel, the polypeptides transferred to a polyvinylidene difluoride membrane (Bio-Rad), and tryptic peptides from the 45- and 47-kDa bands were microsequenced by the Harvard Microchemistry Facility.

Expression and Purification of Recombinant CCL1—The open reading frame encoding Cd1 was amplified by the polymerase chain reaction from yeast genomic DNA by the "touchdown" method as described (Feaver et al., 1994a). Primers were used which introduced HindIII and XhoI sites at the 5' and 3' ends of the reading frame, respectively. Primer sequences were: 5'-ATAATCCCGGGAAGCTTACGGATAT-3' and 5'-ATAGAATTCCTCGAGTTTTTTGCTTTTTCTCAA-3'. The HindIII-Xhol fragment was cloned into the corresponding sites of the bacterial expression plasmid pET-20b (Novagen) introducing a leader peptide and a six-histidine tag at the 5' and 3' ends of the open reading frame, respectively. Use of this vector enabled expression of the otherwise toxic Cd1 protein in bacteria, by virtue of leader peptide-mediated export to the periplasmic space. Recombinant Cd1 protein was expressed in BL21(DE3) cells (Novagen) by growth at room temperature to an A<sub>600</sub> of 0.6, and then addition of isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.1 mM. Cells were grown for 4 h after induction. Recombinant Ccl1 was only partially soluble and was purified from the insoluble fraction essentially as described previously for recombinant Kin28 protein (Feaver et al., 1994b). The yield was about 3 mg/liter starting culture.

Preparation of Cd1 Antiserum and Affinity Purification—Purified, recombinant Cd1 protein was fractionated by SDS-PAGE * and visualized by staining with 0.1% Coomassie Blue R-250. The protein band was excised and used to inoculate rabbits (Berkeley Antibody Co.). The antibody was affinity-purified on a recombinant Cd1-Sepharose column, essentially as described previously for anti-Kin28 antibody (Feaver et al., 1994b).

Other Methods—Immunoblots were performed as described (Chasman and Kornberg, 1990). The affinity-purified anti-Cd1 antibody and the affinity-purified anti-Kin28 antibody were both used at a final dilution of 1/250, whereas the secondary antibody/detection reagent was a goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad). Silver staining was done as described (Blum et al., 1987).

RESULTS AND DISCUSSION

Three approaches were taken to reveal CCL1 gene products in TFIIK. Surprisingly, both the 45- and the 47-kDa subunits of TFIIK were identified in this way. First, affinity-purified anti-Cd1 antibodies, raised against recombinant Cd1 protein produced in bacteria, reacted with the 45- and 47-kDa polypeptides in immunoblots (Fig. 1). Second, these polypeptides, revealed both by silver staining and by immunoreactivity (Fig. 2), precisely coeluted with Kin28, with the other subunits of TFIIH, and with C-terminal domain kinase activity (Svejstrup et al., 1994) in the final step of TFIIH purification. Finally, tryptic peptides derived from both 45- and 47-kDa subunits corresponded identically with the deduced amino acid sequence of the CCL1 gene (Table I).

How might both 45- and 47-kDa subunits of TFIIK derive from the single CCL1 gene? Two possibilities, phosphorylation and proteolytic degradation, were rendered unlikely by further analysis. Treatment with calf intestinal alkaline phosphatase had no effect on the mobility of either subunit in SDS-PAGE, although the same treatment did cause a mobility shift of Kin28, indicative of its phosphorylation, as described previously (Feaver et al., 1994b). Likewise, both forms of Cd1 protein were detected at similar levels in crude extracts from cells disrupted with hot SDS, arguing against proteolysis during the course of purification.

*The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
**Affinity-purified anti-Ccl1 antibodies specifically cross-react with the 45- and 47-kDa subunits of holoTFIIH.** Purified holoTFIIH (60 μl of Mono S fraction 46 (Svejstrup et al., 1994)) was analyzed in a 10% SDS-polyacrylamide gel and visualized by staining with silver (Silver) or transferred to nitrocellulose and detected with affinity-purified anti-Ccl1 antibody (CCL1ab) or preimmune serum (Preimmune). The subunits of holoTFIIH are indicated at the left. Protein bands migrating slower than Ssl2 are the only apparent contaminants of the fraction.

**Fig. 2.** Ccl1 protein copurifies with subunits p45 and p47 and with Kin28 in highly purified holoTFIIH. Consecutive fractions (60 μl) from Mono S (Svejstrup et al., 1994) were analyzed in a 10% SDS-polyacrylamide gel and visualized by staining with silver (top panel) or transferred to nitrocellulose and probed with affinity-purified anti-Ccl1 antibodies (panel labeled CCL1) or with affinity-purified anti-Kin28 antibodies (panel labeled KIN28).

An alternative explanation for the multiple forms of Ccl1 protein may lie in the occurrence of a second AUG, 19 codons downstream from the first AUG in the open reading frame of the CCL1 gene. Translation initiation from this second AUG would give rise to a protein product 2199 daltons smaller than that from the first AUG, consistent with the apparent difference in molecular mass of the two forms of Ccl1 revealed by SDS-PAGE. While the “first AUG-rule” of eukaryotic translation posits that the AUG codon closest to the 5’-end of the mRNA is a unique site of initiation (Kozak, 1987), one of two escape mechanisms that account for most exceptions to this rule (Kozak, 1991) may apply in the case of CCL1: the sequence context for 40 S ribosomal subunit binding appears far more favorable at the second AUG (GUCAAUGU), matches to the vertebrate consensus sequence, boldfaced) than at the first (GAUAGAUGA). Another feature of the region upstream from the CCL1 gene which might result in dual sites of translational initiation is the short distance (less than 80 bases) from the putative TATA-box of the promoter to the first ATG. As the site of transcriptional initiation in yeast is between 40 and 120 bases downstream from the TATA-box, the 5’-leader might be too short for translational initiation exclusively at the first AUG. Deletion analysis of the yeast HIS4 translational initiator region has shown that a short leader (20 bases from 5’-end to position +1) can result in bypass of the first AUG and usage (up to 20% depending on sequence context surrounding the translational start sites) of a downstream AUG (Cigan et al., 1988). Attempts to confirm translation initiation at both AUGs in CCL1 by N-terminal sequencing of the 45- and 47-kDa polypeptides have so far proved unsuccessful, probably due to blocked N termini of the proteins.

These findings alter our view of the oligomeric state of TFIIK. It is most likely a two-subunit protein, comprising Kin28 and either 45-or 47-kDa forms of Ccl1, rather than a complex of all three polypeptides as originally surmised. It would seem, then, to differ from its mammalian counterpart, the MO15/cyclin H pair, which are found in a complex, termed cyclin-dependent kinase-activating kinase, with a distinct, third polypeptide. Recently, however, we and others have found that the third subunit of cyclin-dependent kinase-activating kinase is homologous to a subunit of the yeast TFIIH core complex2,3 (Fisher et al., 1995), so the difference between the yeast and mammalian systems may only reflect relative affinities of subunits within holoTFIIH and be of no functional significance.

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