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Recessive Mutations in the Second Largest Subunit of TFIIIC Suggest a New Step in RNA Polymerase III Transcription

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An analysis of mutant *S. cerevisiae* strains selected for their ability to increase transcription by RNA polymerase (pol) III has identified 14 isolates in which this phenotype is recessive. Genetic linkage and complementation studies suggest that all 14 isolates contain recessive alleles of *PCF1*. *PCF1* encodes the 131-kDa subunit of transcription factor IIIC ($TFIIIC_{131}$) and was identified previously by dominant mutations that also increased transcription by pol III. The recessive mutation, *pcf1-3*, results in a conservative substitution (R728 → K) towards the carboxyl-terminus of the protein. This position is distinct from the site of the dominant mutation *PCF1-1* (H190 → Y), which maps to a tetratricopeptide repeat (TPR). Site-directed mutagenesis at amino acid 728 generated one allele, *pcf1-4*, with a stronger phenotype than *pcf1-3*. Extracts from *pcf1-3* and *pcf1-4* strains increase pol III transcription two- to threefold and ninefold, respectively, over wild-type under conditions that permit either single or multiple rounds of initiation. The entire effect of these mutations in vitro can be accounted for by an increase in the amount of transcriptionally active TFIIIB. In contrast, *PCF1-1* primarily affects the rate of preinitiation complex assembly. The genetic, molecular, and biochemical data suggest that amino acid 728 in $TFIIIC_{131}$ constitutes part of a structural domain in this protein that affects TFIIIB activity by influencing a previously undefined step in transcription. This step is suggested to occur after the recruitment of TFIIIB to DNA.

RNA polymerase III Transcriptional activation Nonsense suppression

IN all eukaryotic organisms the transcription of tRNA, 5S RNA, and many other small RNA genes by RNA polymerase (pol) III requires at least two multisubunit transcription factors, TFIIIB and TFIIIC. These factors can be subfractionated in some systems but the basic functional properties of the complete assemblies appear to be conserved across species (Geiduschek and Kassavetis, 1992; Willis, 1993; White, 1994). TFIIIC binds to the A and B block promoter elements of tRNA-type genes and to TFIIIA-5S gene complexes and then recruits TFIIIB to form a preinitiation complex capable of binding the polymerase. In addition to sequence-specific DNA binding, the TFIIIC complex, which comprises six subunits in yeast, has been attributed with numerous activities. For example, TFIIIC behaves as an enhancer binding factor in that its primary binding site, the B block, functions within certain limits in a distance- and orientation-independent manner (Burnol et al., 1993b). Additionally, through the A block, TFIIIC plays a role in start site selection, presumably by influencing the placement of TFIIIB (Eschenlauer et al., 1993; Burnol et al., 1993b). DNA bending and antirepression by TFIIIC have also been reported (Leveillard et al., 1991; Burnol et al., 1993a). TFIIIB is the initiation factor of the pol III system and in yeast is a complex of three polypeptides (TBP, $TFIIIB_{70}$, and $TFIIIB_{90}$; Kassavetis et al., 1992). This complex is analogous to the basal transcription complex in the pol II system in that once TFIIIB is bound upstream of the

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transcription start site, it alone is able to recruit the polymerase for multiple rounds of initiation (Kassavetis et al., 1990).

Pol III gene transcription is subject to positive and negative regulation under a variety of circumstances. Regulation has been documented to occur upon cell differentiation (White et al., 1989), during embryogenesis (Wolffe and Brown, 1988), in response to altered cell growth rate (Tower and Sollner-Webb, 1988), and during the cell cycle (Gottesfeld et al., 1994). Additionally, infection of mammalian cells by viruses (Gaynor et al., 1985; White et al., 1990; Fradkin et al., 1987; Kwee et al., 1992) and other external stimuli (Garber et al., 1994) also affect the level of pol III gene transcription. In several of these cases, altered transcription is known to be mediated by changes in the activity of TFIIIB or TFIIIC. However, information concerning the mechanism(s) of regulation of these factors is rather limited (reviewed in White, 1994, and see below). Moreover, in no case has it been determined which of the many subunits of TFIIIB and TFIIIC is responsible for eliciting a particular regulatory effect.

There are only a few accounts of negative regulation of pol III gene transcription. Cultured mouse cells, in response to treatment with cycloheximide or growth into stationary phase, decrease transcription of 5S RNA and VA RNA genes (Tower and Sollner-Webb, 1988). The biochemical basis of this decrease has been examined by transcription reconstitution experiments using partially purified factors and involves a reduction in TFIIIB activity. Downregulation of TFIIIB activity also underlies the loss of pol III transcription activity during mitosis (Gottesfeld et al., 1994) and the reduced transcription of rodent B2 genes following retinoic acid-induced differentiation of F9 carcinoma stem cells (White et al., 1989). In mitotic regulation, TFIIIB activity is inhibited by the action of one or more cyclin-dependent protein kinases. In contrast to the preceding examples, poliovirus infection of HeLa cells reduces pol III transcription primarily by inactivating TFIIIC; TFIIIB activity is also diminished but to a lesser extent (Fradkin et al., 1987). The loss of TFIIIC activity in poliovirus-infected cells has been correlated with the disappearance of an active phosphorylated TFIIIC complex and the appearance of two inactive forms of the factor (produced by dephosphorylation and proteolysis, respectively; Clark et al., 1991). One final but classic example of negative regulation in the pol III system concerns the inactivation of oocyte-specific tRNA, 5S RNA, and other genes during development in *Xenopus laevis* (Wolffe and Brown, 1988; Stutz et al., 1989; Andrews et al., 1991). Differential expression of oocyte-specific 5S RNA and OAX genes has been attributed to the reduced stability of TFIIIC/gene complexes (Wolffe, 1988; Reynolds and Johnson, 1992) and is thought to result in permanent gene inactivation by assembly into chromatin. In this context it is intriguing that a factor identified in fractionated *Xenopus laevis* oocyte extracts can mediate an ATP-dependent loss of TFIIIC DNA binding activity (Giardina and Wu, 1990).

Our laboratory has previously described a genetic strategy to isolate mutations in yeast that affect pol III gene transcription (Willis et al. 1989). The critical element in this strategy was a synthetic, dimeric nonsense suppressor tRNA gene, *sup9-e A19-supS1* (Fig. 1A) whose unique transcriptional properties allowed the selection of extragenic suppressors of an A block (A19) pro-

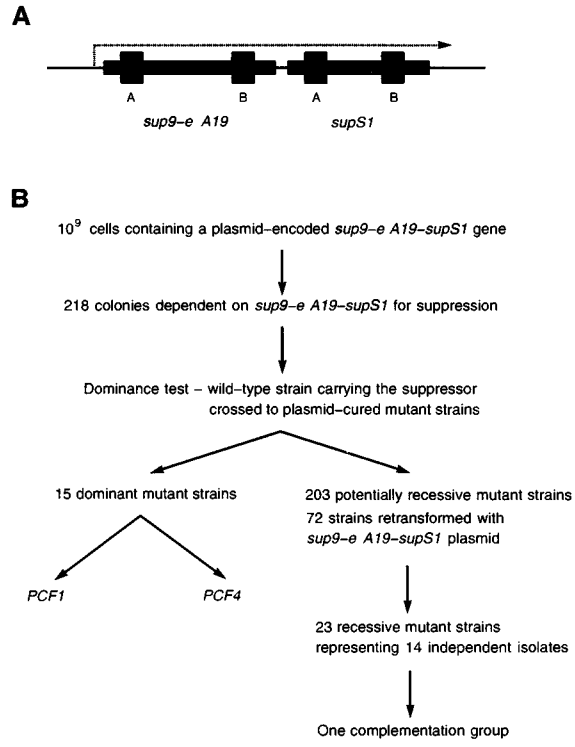

FIG. 1. The genetic strategy. (A) Schematic representation of the dimeric *sup9-e A19-supS1* suppressor. The A and B block promoter elements of the *sup9* and *supS1* genes are indicated. The *supS1* promoter elements are inactive in this construct. Transcription of the dimeric tRNA precursor is shown by a dashed line. (B) Genetic scheme used to identify dominant and recessive mutant strains expressing *supS1* suppressor activity. The 14 independent recessive isolates are represented by strains 1, 3, 5, 9, 22, 23, 27, 29, 46, 51, 59, 72, 89, and 113 from the mutagenesis. The 15 dominant strains represent 8 independent isolates (Willis et al., 1989).

moter mutation. Briefly, the transcriptional dependence of the *supS1* gene in this construct on the promoter of the *sup9-e* gene enabled the identification of suppressors of the *sup9-e A19* mutation by their ability to express *supS1* amber suppressor activity. Two genes, *PCF1* (*TFC4*) and *PCF4* (*TDS4/BRF1*), have been identified using this selection. They encode the 131-kDa subunit of TFIIIC ($TFIIIC_{131}$) and the 70-kDa subunit of TFIIIB ($TFIIIB_{70}$), respectively (Willis et al., 1989; López-de-León et al., 1992; Marck et al., 1993; Rameau et al., 1994). Dominant mutations in both genes activate pol III gene transcription in vitro. In *PCF4-1* cell extracts, increased transcription correlates with an increase in the amount of $TFIIIB_{70}$ that is stoichiometrically required and limiting in yeast cells (López-de-León et al., 1992). In contrast, the increased transcription exhibited by *PCF1-1* cell extracts appears to result primarily from the enhancement of a rate-limiting step in transcription complex assembly, namely recruitment of TFIIIB. Additionally, this mutation increases the amount of transcriptionally active TFIIIB in mutant versus wild-type cell extracts (Willis et al., 1992; Rameau et al., 1994). How these two effects are related is not yet clear.

This article describes the identification of recessive mutations in the *PCF1* gene that increase pol III gene transcription. The fact that pol III gene transcription can be increased by dominant and recessive *PCF1* alleles that map to structurally different regions of the protein suggests that this subunit of TFIIIC has multiple functions in transcription.

MATERIALS AND METHODS

Strains and Genetic Methods

Procedures for growth, mating, complementation analysis, and tetrad analysis of yeast are described in Sherman et al. (1986). Random spore analysis was performed according to Rockmill et al. (1991). Nonsense suppressor activity was monitored by growth on solid synthetic minimal medium lacking tryptophan and methionine. The phenotype was scored 1–2 days after replica plating.

The yeast strains used in this work are described in Table 1. Recessive mutant strains expressing *supS1* amber suppressor activity were isolated by ethylmethane sulfonate mutagenesis of strain IW1B6 (Willis et al., 1989). Dominance tests were carried out by crossing plasmid-cured, mutant strains to the wild-type strain, IWD1. Strain U72 was obtained following two backcrosses of mutant strain 72 to IWD1. Crosses of this strain to the original plasmid-cured recessive mutant strains were performed to test for complementation. Strain ACE6 was obtained by random spore analysis after crossing strains U72 and ES25. Strains IS1 and IS7 were obtained by random spore analysis after crossing strains U72 and IW1B6. The suppression phenotype of *PCF1* alleles created by site-directed mutagenesis was analyzed as follows. The mutant genes contained in the plasmid pRS313 were transformed into strain IS17D and subjected to the random spore procedure. Haploid colonies with a His+Leu+ phenotype were tested for *supS1* suppressor activity. From this group, strains that were also *Mata* and Arg− were chosen for mating to IW1B6 to make diploid strains heterozygous for the *pcf1* mutations. Haploid strains containing the $PCF1^+$ gene or the *pcf1-4* gene on pRS313 that were derived from IS17D as described above were used for extract preparation. Whole-cell extracts were also prepared from a derivative of strain U72 (*pcf1-3*) in which the nonsense suppressor had been eliminated by growth on medium containing 5-fluoroorotic acid (1 mg/ml) and a corresponding wild-type strain.

Plasmid Constructs and Site-Directed Mutagenesis of $PCF1^+$

The wild-type *PCF1* gene and the *pcf1-3* gene were cloned from partial genomic libraries of strains IW1B6 and U72, respectively, by colony hybridization in *E. coli* using the *PCF1-1* gene as a probe. Both genes were recovered on 5.5-kb *Pst*I fragments in the yeast/*E. coli* shuttle vector pRS315 (Sikorski and Hieter, 1989). The construction of chimeric clones to map the *pcf1-3* mutation site involved replacing three restriction fragments from the mutant clone (*Not*I-*Bln*I, *Bln*I-*Nde*I, *Nde*I-*Apa*I) with the corresponding wild-type fragments. The three fragments span the entire length of the *Pst*I insert in pRS315. The *pcf1-3* mutation site was identified by dideoxy sequencing from the *Nde*I site to end of the coding region. Site-directed mutagenesis at amino acid 728 was conducted on the wild-type *PCF1* gene in plasmid pRS313 by using the helper phage R408 to produce uridine-containing single-stranded DNA in *E. coli* strain CJ236 (Kunkel, 1985) and the oligonucleotide (5′-TCTGCCACAGAATTGNNNGG ATTGTCGTATGA-3′). Mutations were identified by DNA sequence analysis using an adjacent primer.

TABLE 1
YEAST STRAINS

Strain	Genotype	References
IW1B6	*Matα ura3-52, leu2-3,112, his3-11,15, trp1-1, met8-1*	Willis et al. (1989)
IWD1	*Mata ura3-52::URA3(sup9-e A19-supS1), leu2-3, 112, arg4-17, trp1-1, met8-1*	Willis et al. (1989)
ES25	*Matα leu2-1, his5-2, ade2-101, ade3-26, lys2-801, trp1Δ, met8-1, ilv1-1*	Provided by C. L. Greer
U72	*Mata ura3-52::URA3(sup9-e A19-supS1), leu2-3, 112, arg4-17, trp1-1, met8-1, pcf1-3*	This work
ACE6	*Matα ura3-52::URA3(sup9-e A19-supS1), leu2-1, his5-2, ade2-101, ade3-26, lys2-801, trp1-1, met8-1, pcf1-3*	This work
T2D	*Mata ura3-52::URA3(sup9-e A19-supS1), leu2-3, 112, arg4-17, trp1-1, met8-1, PCF1-1*	Willis et al. (1989)
IS1	*Mata ura3-52::URA3(sup9-e A19-supS1), leu2-3, 112, his3-11,15, trp1-1, met8-1, pcf1-3*	This work
IS7	*Matα ura3-52::URA3(sup9-e A19-supS1), leu2-3, 112, his3-11,15, trp1-1, met8-1, pcf1-3, arg4-17*	This work
IS17	IS1 × IS7	This work
IS17D	As IS17, except that one of the *pcf1* genes was deleted and replaced by *LEU2 (pcf1Δ::LEU2)*	This work

Extract Preparation and In Vitro Transcription Assays

The analysis of pol III in vitro transcription was carried out using wild-type, *pcf1-3*, and *pcf1-4* whole-cell extracts prepared by glass bead disruption of 10–15 g of cells according to Klekamp and Weil (1982) as modified by Willis et al. (1989). To ensure that extracts prepared from wild-type and mutant strains were qualitatively similar, cell growth and extract preparation was carried out in parallel. The *PCF1-1* extract used in this work (and the corresponding wild-type control extract) have been described previously (Willis et al., 1992).

Nucleoside triphosphates and radiolabeled nucleotides for in vitro transcription experiments were purchased from Pharmacia and Amersham, respectively. Multiple round transcription assays were performed as described previously (Willis et al., 1989; Willis et al., 1992) using the *sup9-e* gene as a template. Reactions were incubated for 1 h at 15°C. Assays involving complex preassembly were carried out by incubating reactions lacking one or more NTPs at 25°C for 30 min. This is sufficient time for complex assembly to reach equilibrium (Kassavetis et al., 1989; Willis et al., 1992). The missing NTPs were subsequently added. Stalled transcription complexes containing a seven-nucleotide nascent RNA were assembled on the *sup9-e* gene by omitting CTP from the preincubation (Willis et al., 1992). Heparin (300 μg/ml) was used to limit transcription to single rounds (Kassavetis et al., 1989). After gel electrophoresis, the amount of ^{32}P incorporated into RNA was quantitated by measuring the Cerenkov radiation of excised bands.

RESULTS

Isolation of Recessive Mutations That Activate sup9-e A19-supS1 *Gene Expression*

While characterizing mutant strains that were dependent on the *sup9-e A19-supS1* gene for nonsense suppressor activity, we observed that the majority of these strains (203 out of 218) failed to express the *supS1* suppressor as heterozygous diploids (Fig. 1B). This result suggested that we may have isolated recessive mutations capable of activating pol III gene transcription. To pursue this possibility, we first demonstrated that the suppression phenotype could be regained in plasmid-cured haploid mutant strains by retransformation with the *sup9-e A19-supS1* suppressor gene. A total of 72 plasmid-cured strains from the 203 potentially recessive candidates were transformed and tested for *supS1* activity. Twenty-three colonies from 14 independent groups were found to regain this phenotype (Fig. 1B). These strains therefore contain recessive mutations that allow the expression of *supS1*. One simple way that recessive mutant cells could have acquired a *supS1* suppression

phenotype is by elevating the plasmid copy number (i.e., increasing the suppressor gene dosage). To eliminate this possibility, we examined whether *supS1* could be expressed from a chromosomal *sup9-e A19-supS1* construct. Seven of the 23 plasmid-cured mutants were crossed to the wild-type strain, IWDI, which contains a single copy of the dimeric suppressor integrated at the URA3 locus. Random spore analysis was performed and the resulting colonies were examined for isolates that both contained (Ura+) and expressed (Trp+, Met+) the *supS1* suppressor gene. The frequency of Ura+ colonies that were active in amber suppression was about 50% in all seven crosses. This finding suggests that the expression of *supS1* in recessive mutant strains results from increased synthesis or decreased turnover of the *supS1* suppressor tRNA.

Recessive Mutations in the Second Largest Subunit of TFIIIC Increase supS1 *Expression*

The *supS1* suppression phenotype of a recessive mutant strain (ACE6) and a heterozygous diploid strain derived from a cross between the mutant strain and a wild-type strain (IWDI) is shown in Fig. 2A. Growth of the haploid mutant strain was observed after 2–3 days on minimal medium in contrast to the heterozygous diploid and the wild-type haploid strain, which showed little or no growth at this time. Tetrad analysis of the sporulated heterozygote is shown in Fig. 2B. Colonies arising from dissected spores segregated *supS1* suppressor activity in a 2:2 pattern, indicating that this phenotype is conferred by a single nuclear mutation.

The number of complementation groups represented among the recessive mutants was determined by crossing a derivative of one mutant (U72) to each of the 23 isolates (including itself) that initially expressed *supS1* after transformation with the *sup9-e A19-supS1* suppressor. Diploid strains were selected and tested for their ability to express *supS1*. All of these strains had phenotypes that were indistinguishable from their suppressor-containing haploid counterparts (data not shown). No complementation was observed. We conclude that all 23 strains are likely to contain mutations in the same gene. An examination of different strains from this group showed that the recessive mutations did not confer additional phenotypes such as cold or heat sensitivity and did not affect the doubling time in complete medium.

As noted earlier, the same strategy that produced the recessive mutations described above also yielded dominant mutations in two genes, *PCF1* and *PCF4*. Accordingly, it was important to show whether the recessive mutations defined a unique gene or a regulatory domain of one of the positively acting *PCF* gene products. To do this, a linkage analysis was conducted by a random spore method (Rockmill et al., 1991). An analysis of 113 haploid progeny obtained from a cross between a *PCF1-1* strain (T2D) and a recessive mutant strain (ACE6) yielded only three nonsuppressing colonies (data not shown). The low frequency of this phenotype in the cross indicates that the two mutations are genetically linked. (This contrasts with the cross between *PCF4-1* and the recessive mutant in which 20% of the progeny were nonsuppressors.) The recessive mutation was therefore either in the *PCF1* gene or resided in a gene that is closely linked to *PCF1*. The possibility of allelism to *PCF1* was tested by cloning of the wild-type *PCF1* gene. A clone containing the *PCF1*$^+$ gene in the centromeric plasmid vector pRS315 was recovered by colony hybridization to a sized gene bank in *E. coli* (Rameau et al., 1994) and transformed into a recessive mutant strain (U72). As a control, the plasmid was also transformed into a wild-type yeast strain to monitor possible *PCF1*$^+$ gene dosage effects on suppressor activity. As was found for experiments using a 2-μm plasmid (Rameau, 1994), wild-type cells show no *PCF1*$^+$ gene dosage effect (Fig. 3A). However, a single wild-type copy of *PCF1* was able to complement the *supS1* suppressor phenotype of strain U72. Thus, all of the recessive mutant strains examined are likely to harbor alleles of *PCF1*. The specific mutant allele in strain U72 was designated *pcf1-3*.

Identification of the pcf1-3 *Mutation Site*

To map the site of the *pcf1-3* mutation, the mutant gene was cloned in plasmid pRS315 in the same orientation as *PCF1*$^+$. Subsequently, three hybrid genes were constructed in which restriction fragments encompassing the entire wild-type *PCF1* clone were substituted into the homologous sites in the *pcf1-3* clone (Fig. 3B). These constructs were tested for their ability to complement the *supS1* suppressor phenotype of strain U72. The fragment containing the mutation site was readily identified because its replacement with the wild-type sequence led to complementation (no growth). This phenotype was observed in reclone C (Fig. 3C), which contains the carboxy-terminal half of $TFIIIC_{131}$. DNA sequencing of this fragment revealed a single nucleotide substitution (G → A) in the second position of the codon corre-

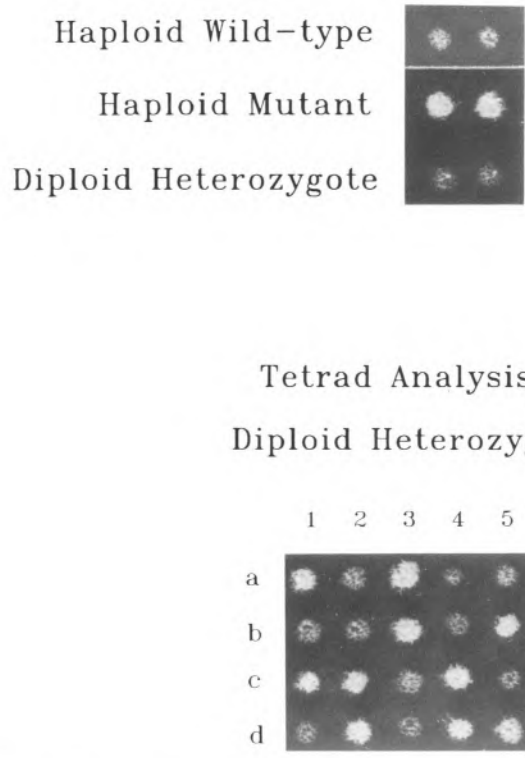

FIG. 2. The recessive mutant phenotype and tetrad analysis. The upper part of the figure shows the *supS1* suppressor phenotypes of a wild-type strain (IWD1), a recessive mutant strain (ACE6), and a diploid strain heterozygous for the mutation. The lower part of the figure shows a tetrad analysis of the heterozygous diploid strain. Colonies arising from dissected asci were picked onto YPD medium and replica plated to test for suppressor activity.

sponding to amino acid 728. The mutation results in a conservative arginine to lysine substitution at this position. Interestingly, the *pcf1-3* mutation resides in a region of the protein that is physically and structurally distinct from the dominant mutation isolated previously in our laboratory (Rameau et al., 1994) (Fig. 4).

Isolation of New Alleles at Amino Acid 728

Due to the conservative nature of the *pcf1-3* mutation and its relatively modest stimulation of transcription in vitro (two- to threefold, see below), we carried out a structure–function analysis at amino acid 728 in the hope of isolating a stronger allele. This seemed feasible because the dominant mutations *PCF1-1* and *PCF4-1* had stronger phenotypes than *pcf1-3* and increased transcription in vitro from 6- to 10-fold. Using site-directed mutagenesis six new alleles of *PCF1* were created (Table 2). They included substitutions with histidine (*pcf1-4*, R728H), methionine *pcf1-5*, R728M), valine (*pcf1-6*, R728V), glycine *pcf1-7*, R728G), and glutamic acid (*pcf1-8*, R728E). Additionally, one substitution produced a truncated protein (*pcf1-9*, R728STOP). Analysis of the suppression phenotypes of the mutations was conducted in a strain deleted for the chromosomal *PCF1* gene (see Materials and Methods). Two of the mutations showed substantial suppressor activity (Table 2, Fig. 5). One of these, *pcf1-4*, had a stronger phenotype than the original *pcf1-3* mutation whereas the other, *pcf1-5*, was weaker. Two other mutations (*pcf1-6* and *pcf1-7*) showed only marginal suppressor activity and the *pcf1-8* mutation was suppressor minus. Finally, the *pcf1-9* mutation, which produced a protein lacking 297 residues from the carboxyl-terminus, failed to rescue viability. A comparison of the suppressor phenotypes of haploid and heterozygous diploid strains containing *pcf1-3*, *pcf1-4*, and *PCF1-1* is shown in Fig. 5. In a haploid strain containing *pcf1-4*, the *supS1* suppressor is ex-

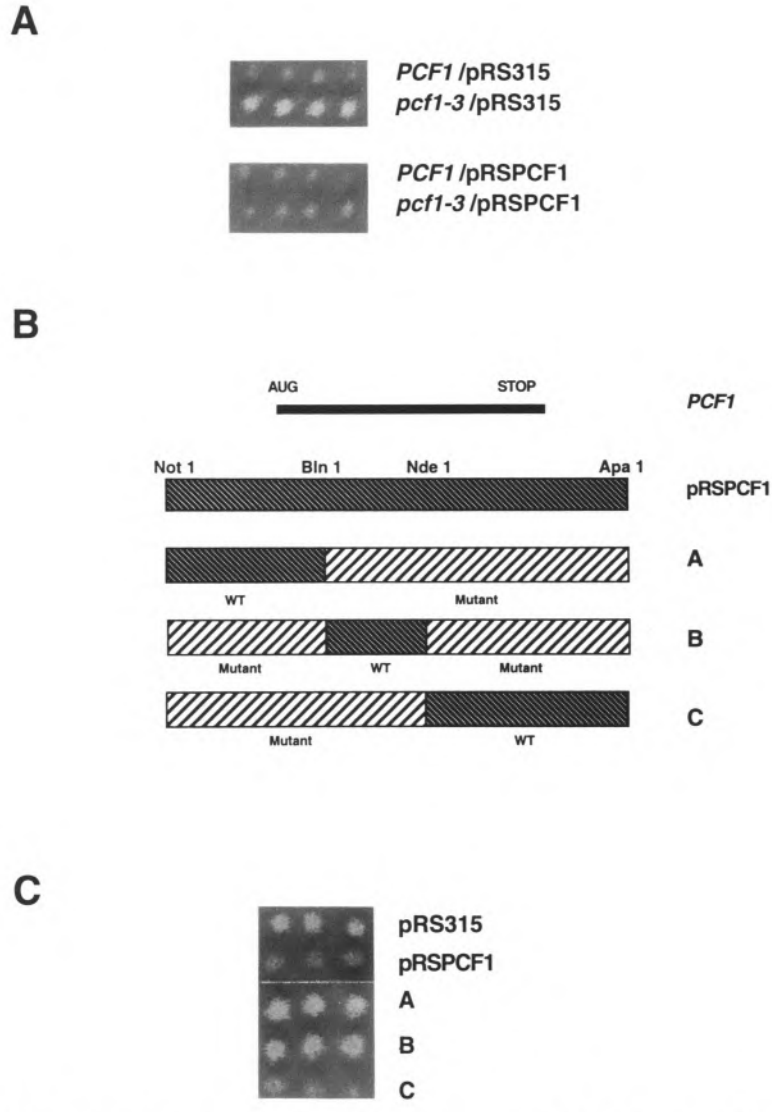

FIG. 3. Complementation by *PCF1*$^+$ and mapping the *pcf1-3* mutation site. (A) Complementation of the recessive mutation in strain U72 by *PCF1*$^+$. Strains IWD1 (*PCF1*$^+$) and U72 (*pcf1-3*) were transformed with a centromeric plasmid containing the wild-type *PCF1* gene (pRS*PCF1*) or empty vector (pRS315). The suppressor phenotype of four colonies from each transformation is shown. (B) Chimeric constructs used to define the location of the *pcf1-3* mutation site. The restriction sites used to construct the clones and their position relative to the *PCF1* coding sequence are indicated together with the origin (wild-type or mutant) of each fragment. (C) The constructs illustrated in (B) were transformed into a strain containing the *pcf1-3* mutation (U72) and tested for *supS1* suppressor activity.

pressed at a similar level to the dominant allele, *PCF1-1*, whereas in a diploid strain heterozygous for *pcf1-4*, suppressor activity is reduced, albeit not to the wild-type level. In contrast, the *supS1* suppressor activity of a haploid *PCF1-1* strain is unchanged in a heterozygous diploid. The relatively weak suppressor phenotype of *pcf1-3* is complemented in diploid and merodiploid cells (Figs. 2A, 3C, and 5). The other suppressor active alleles are also complemented as heterozygotes (Table 2).

Analysis of Transcription in pcf1-3 *and* pcf1-4 *Cell Extracts*

To examine whether the *pcf1-3* and *pcf1-4* mutations affect transcription in vitro as observed for the dominant *PCF* mutations, whole-cell extracts

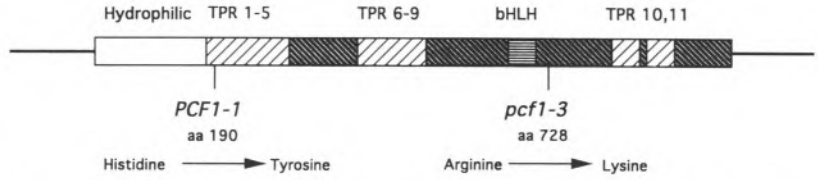

FIG. 4. Structural domains in $TFIIIC_{131}$. The $TFIIIC_{131}$ polypeptide (1025 amino acids) is represented schematically according to the domains identified by Marck et al. (1993) and Rameau et al. (1994). These domains include a hydrophilic amino-terminus (residues 1–120), 11 putative tetratricopeptide repeats (TPRs) arranged in four groups (residues 128–297, 432–569, 875–908, and 959–992), and a putative basic helix–loop–helix region (residues 611–698). The site and nature of the *PCF1-1* and *pcf1-3* mutations are also indicated.

were prepared in parallel from mutant and wild-type strains. Initially, extract titration experiments were performed at a constant excess of template to establish the linear range for transcription and to determine the relative activity of the extracts. In multiple experiments involving two independent extracts of a wild-type strain and a *pcf1-3* strain, the relative activity of the mutant was increased two- to threefold (Fig. 6, and data not shown). The small but reproducible difference seen in these experiments is consistent with the weaker suppression phenotype of *pcf1-3* compared to *PCF1-1* and *PCF4-1* and with the higher stimulation of transcription seen for the dominant mutations (Rameau et al., 1994; López-de-León et al., 1992). For *pcf1-4*, the strength of its suppressor phenotype correlates with an increase in transcription in vitro similar to that seen for the dominant mutations (Figs. 5 and 6). When normalized for protein concentration, the transcription activity of a *pcf1-4* cell extract was found to be nine times that of wild-type (Fig. 6).

A variety of yeast pol III genes, including tRNA genes, a 5S RNA gene, and the U6 snRNA gene, were tested for transcription in a *pcf1-3* extract to examine whether the recessive mutations display a template preference. For all these templates, a two- to threefold increase in transcription

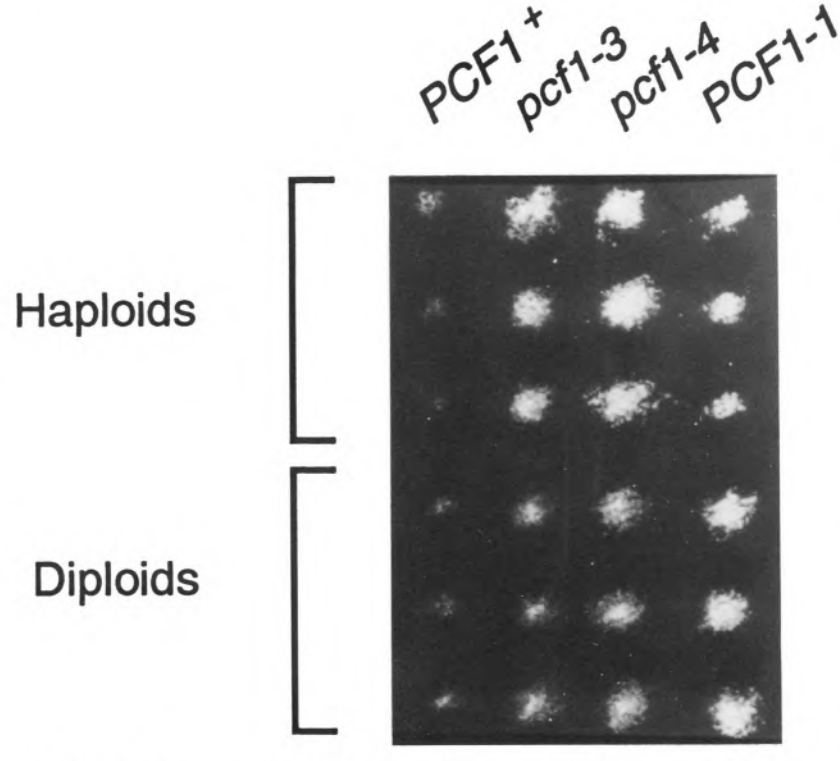

FIG. 5. *supS1* suppressor phenotypes of site-directed mutants at amino acid 728. Suppressor activity is compared for haploid strains containing the indicated mutations and for diploid strains heterozygous for these mutations.

TABLE 2
PHENOTYPE OF *PCF1* MUTATIONS

PCF1 Allele	Amino Acid*	Relative Suppressor Phenotype	Complementation
Wild-type	H190, R728	–	N.A.
PCF1-1	H190Y	+++	No
pcf1-4	R728H	+++	Partial
pcf1-3	R728K	++	Yes
pcf1-5	R728M	+	Yes
pcf1-6	R728V	±	Yes
pcf1-7	R728G	±	Yes
pcf1-8	R728E	–	N.A.

*Wild-type residues are shown together with the changes in various mutants.

was observed relative to wild-type; no template preference was observed. The *pcf1-3* mutation is therefore likely to affect the transcription of most, if not all, pol III genes. Additionally, the *pcf1-3* mutation does not exhibit allelic template preference because transcription of the *sup9-e A19* gene was increased to the same extent as *sup9-e* (data not shown).

Previous studies on *PCF1-1* have shown that the difference in transcription between mutant and wild-type cell extracts is minimized when preinitiation complexes are assembled in the absence of one or all four nucleoside triphosphates. This indicates that the *PCF1-1* mutation affects a rate-limiting step in preinitiation complex assembly (Willis et al., 1989; Willis et al., 1992; Rameau et al., 1994). To determine whether mutations at amino acid 728 also affect this step, transcription was analyzed in multiple-round reactions where preinitiation complexes had or had not been preassembled. In addition, single-round reactions were carried out by forming stalled ternary complexes under equilibrium conditions and then extending the nascent RNAs in the presence of heparin (Kassavetis et al., 1989). The results obtained using *pcf1-3* and *pcf1-4* cell extracts are compared to *PCF1-1* in Figs. 6B and C. In contrast to *PCF1-1*, the fold increase in transcription by extracts of the *pcf1-3* and *pcf1-4* strains is unaffected by complex preassembly. Reactions involving preformed complexes showed no reduction in the fold stimulation of transcription compared to their unassembled counterparts. For the single-round reactions this finding indicates that the entire effect of these mutations can be accounted for by an increase in the number of transcriptionally competent preinitiation complexes. The data show that the step in transcription affected by *pcf1-3* and *pcf1-4* is distinct from that affected by the dominant mutation *PCF1-1*.

DISCUSSION

In this work we have described the isolation of recessive mutations that suppress the negative effects of a tRNA gene A block promoter mutation in vivo and activate transcription of pol III genes in vitro. Genetic analysis suggests that the recessive mutations in 14 independently isolated strains all map to the *PCF1* gene. Interestingly, this gene, which encodes the second largest subunit of TFIIIC, was identified previously by dominant mutations isolated using the same selection strategy. Thus, both dominant and recessive mutations in the *PCF1* gene increase pol III gene transcription.

A New Function for TFIIIC

The transcription properties of *pcf1-3* and *pcf1-4* cell extracts indicate that these mutations do not influence the rates of preinitiation complex assembly, polymerase elongation, or recycling of the polymerase (Fig. 6B). Changes at these steps would affect the amount transcription in multiple-round reactions performed without complex preassembly but would not affect transcription in single-round reactions where complex assembly is at equilibrium, elongation proceeds to termination, and recycling is not permitted. A mutation affecting any one of these steps would show a difference in the fold stimulation of transcription (over wild-type) in the two assays. However, the fold stimulation of transcription by *pcf1-3* and *pcf1-4* cell extracts does not change in these assays (Fig. 6B). In contrast, differential stimulation of transcription is observed when these assays are performed with *PCF1-1* cell extracts (Fig. 6B) (Willis et al., 1989; Willis et al., 1992). These data suggest that the *pcf1-3* and *pcf1-4* mutations affect a different step in the transcription process than that affected by *PCF1-1*. This conclusion is supported by the dif-

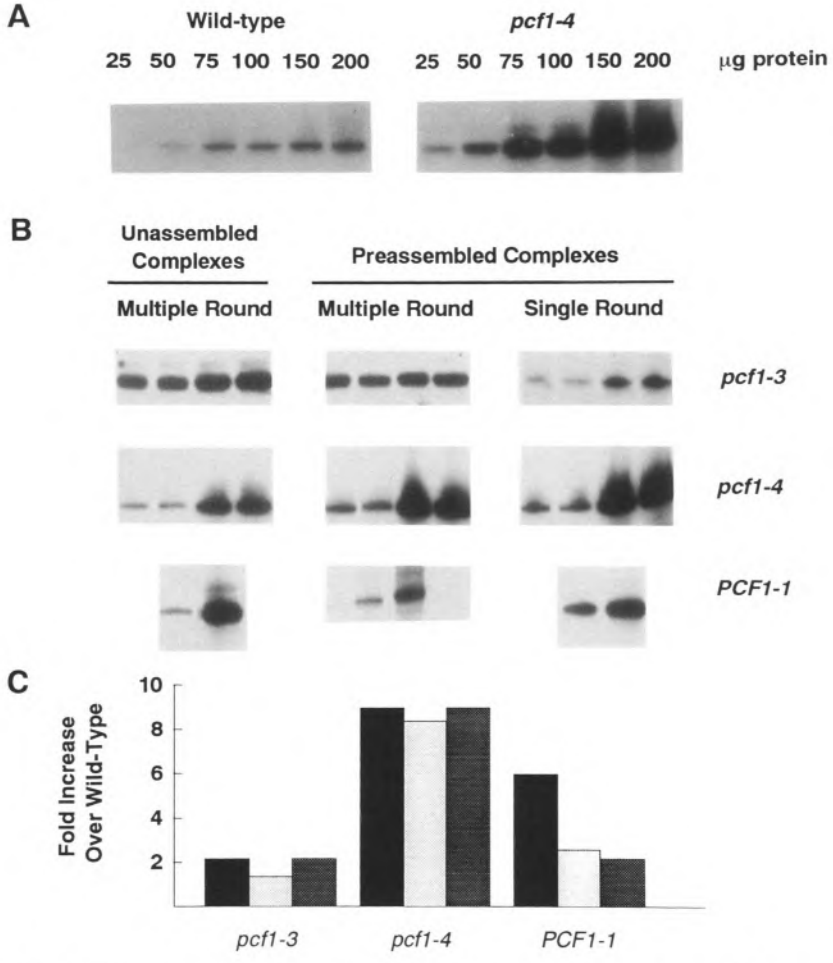

FIG. 6. Transcription in *pcf1-3* and *pcf1-4* cell extracts. (A) Wild-type and *pcf1-4* extract titration. The indicated amounts of extract were assayed under multiple-round conditions (no preincubation) with excess *sup9-e* template. A ninefold difference in transcription was determined by linear regression. (B) Comparison of transcription in *pcf1-3*, *pcf1-4*, and *PCF1-1* extracts. Three reaction conditions are compared: standard multiple-round reactions (shown as unassembled complexes), multiple-round reactions on preassembled complexes, and single-round reactions (see Materials and Methods). For *pcf1-3* and *pcf1-4*, transcription reactions are shown duplicate with the wild-type on the left and the mutant on the right. For *PCF1-1*, a single lane is shown for the wild-type and the mutant. Protein concentrations were normalized and were in the linear range for transcription in each set of reactions. All reactions were performed using the *sup9-e* gene as the template. (C) The fold increase in transcription was calculated for each set of reactions shown in (B). Solid bars compare multiple round reactions from unassembled complexes, light stippled bars compare multiple rounds of transcription from preassembled complexes, and the dark stippled bars compare transcription under single-round conditions. The data are representative of multiple experiments in which the standard deviation for the fold increase in transcription over wild-type is less than 20%. The data for *PCF1-1* reproduce previously published findings (Willis et al., 1989; Willis et al., 1992).

ferent phenotypes of the mutants (Fig. 5, Table 2) and by the fact that the mutations map to different structural domains in $TFIIIC_{131}$ (Fig. 4); *PCF1-1* mutates amino acid 190 within TPR2 whereas *pcf1-3* and *pcf1-4* mutate amino acid 728 in a region for which a structural motif has not yet been defined.

What can be deduced about the step affected by the recessive mutations? The biochemical properties of *PCF1-1* suggest that the primary effect of this mutation involves an increase in a rate-limiting step in preinitiation complex assembly (Rameau et al., 1994). Other studies have shown that this step corresponds to the recruitment of TFIIIB (Kassavetis et al., 1989). We conclude, therefore, that the *pcf1-3* and *pcf1-4* mutations affect an event that follows TFIIIB recruitment but precedes initiation (Fig. 7) (TFIIIC has no de-

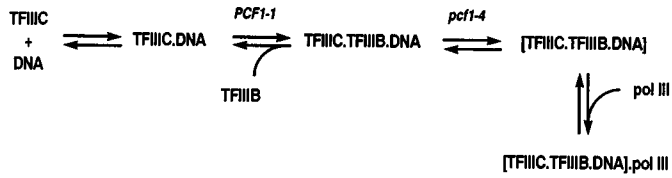

FIG. 7. Events in the assembly of an active pol III transcription complex. The first step represents the binding of TFIIIC to a tRNA gene or a 5S gene-TFIIIA complex. This is followed by the rate-limiting step in transcription complex assembly, recruitment of TFIIIB to the DNA (Kassavetis et al., 1989). The principal effect of the *PCF1-1* mutation is the enhancement of this rate-limiting step (Rameau et al., 1994). As indicated in the text, the *pcf1-4* mutation is proposed to affect a step that follows TFIIIB recruitment. This step is hypothesized to involve the activation of TFIIIB to transcriptional competence (the complex in square brackets). We infer that TFIIIB activation precedes polymerase binding but the order of these steps has not been determined.

fined postinitiation function; Kassavetis et al., 1990). This finding is significant insofar as it suggests a new TFIIIC-mediated step in transcription that is distinct from the role of this factor in recruiting TFIIIB to the DNA.

Several observations indicate that the *pcf1-3* and *pcf1-4* mutations ultimately increase the activity of TFIIIB. First, the dominant mutations, *PCF4-1* and *PCF1-1*, increase transcription in crude extracts by directly or indirectly increasing TFIIIB activity (López-de-León et al., 1992; Rameau et al., 1994). This was expected for *PCF4-1*, which encodes the 70-kDa subunit of TFIIIB, but it was not anticipated for *PCF1-1*. Nonetheless, TFIIIB fractions and subfractions (IIIB″), which have been purified from *PCF1-1* extracts and are devoid of detectable TFIIIC, have increased activity. Second, TFIIIB is the limiting activity in wild-type yeast whole-cell extracts (Kassavetis et al., 1989; I. Sethy and I. Willis, unpublished data). Consequently, changes in the amount of active TFIIIB affect the level of transcription (Kassavetis et al., 1989; Willis et al., 1992; López-de-León et al., 1992; Rameau et al., 1994). These changes are observable and can be quantified in single-round reactions. The fact that *pcf1-3* and *pcf1-4* cell extracts increase transcription under single-round conditions suggests that these mutations, like *PCF1-1*, increase the amount of active TFIIIB. This conclusion is supported by extract fractionation studies showing that the activity of a TFIIIB fraction from *pcf1-4* cell extracts is increased over wild-type (I. Sethy, unpublished data).

Implications of Dominant and Recessive Mutations in the Same Gene

Recessive mutations usually indicate that some function has been lost in the mutant cells. However, in certain genetic systems where a protein functions stoichiometrically rather than catalytically, some loss of function mutations may appear to be codominant. Because the implications arising from dominant and recessive phenotypes are diametrically opposed and the *PCF1* gene product is believed to function stoichiometrically as a subunit of TFIIIC, the issue of whether the *pcf1-3* or *pcf1-4* mutations are gain or loss of function mutations warrants some discussion. The dominant allele, *PCF1-1*, stimulates transcription in crude extracts about sixfold (Willis et al., 1989) and is not complemented to any detectable degree (Fig. 5). This indicates that the mutant TFIIIC factor outcompetes the wild-type factor in heterozygous diploid cells and implies that the mutation increases the affinity of the factor for DNA and/or increases the stability of the TFIIIC-DNA complex. In contrast, the *pcf1-4* allele, which has a similar *supS1* suppressor strength as *PCF1-1* and increases transcription ninefold, is partially complemented. This phenotype suggests that there is very little or no competitive advantage of the mutant factor over wild-type with regard to DNA binding or the ability to form a stable preinitiation complex. Furthermore, because DNA binding must occur in order to see the effects of TFIIIC mutations on later steps in the transcription process, such mutations may be expected to show a partial complementation phenotype irrespective of whether they cause a gain or a loss of function. In light of this, we cannot attribute the increased transcription exhibited in *pcf1-3* and *pcf1-4* strains to a positive or a negative effect of the mutations. We note, however, that the higher frequency at which the recessive mutations were isolated relative to the dominant mutations (14 out of 72 vs. 8 out of 218, Fig. 1B) favors a loss of function. Definitive proof of the positive or negative nature of these mu-

tations in $TFIIIC_{131}$ must await a more detailed understanding of the functions of the protein.

If *pcf1-3* and *pcf1-4* improve a function of $TFIIIC_{131}$, then the region around amino acid 728 could be considered a TFIIIB activation domain. If, however, they cause a loss of function, this region would serve to regulate TFIIIB activation negatively. This leads us to consider the circumstances in a yeast cell where negative regulation of pol III transcription might be manifested. One possibility is that $TFIIIC_{131}$ may be involved in coordinating the level of pol III transcription with the growth state of the cell. Growth control of pol III transcription has been reported previously in mouse cells (Tower and Sollner-Webb, 1988) and operates to regulate the supply of gene products in actively growing versus stationary phase cells. Alternatively, negative regulation of pol III transcription may occur during the cell cycle in yeast, as has been demonstrated in mitotic extracts of *Xenopus laevis* (Gottesfeld et al., 1994). Interestingly, in both cell cycle and growth control of pol III transcription, TFIIIB has been implicated as the factor whose activity is regulated. Whether TFIIIC is also involved in these cases has not been addressed. Studies are underway to address the possible role of TFIIIC in these events in yeast.

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