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A selection for mutants of the RNA polymerase III transcription apparatus: *PCF1* stimulates transcription of tRNA and 5S RNA genes

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A genetic approach has been developed to study transcription by RNA polymerase III. A pair of *Schizosaccharomyces pombe* nonsense suppressor tRNA genes were arranged in tandem such that expression of the downstream (*supS1*) tRNA suppressor was dependent upon transcription initiated by the internal promoter of the upstream (*sup9-e*) gene. Dominant mutant strains of *Saccharomyces cerevisiae* were isolated that suppress in *trans* the effect of an A block promoter mutation (A19) in the *sup9-e* gene and restore *supS1* suppressor activity. Fifteen mutant strains, eight of which were independently isolated, all have elevated steady-state levels of *sup9-e A19* RNA consistent with an increase in gene transcription. Extracts of a strain carrying the dominant mutant gene, *PCF1*, show a general 6-fold stimulation in transcription of mutant (A19) and wild-type tRNA genes and increase 5S gene transcription 4-fold compared with extracts from a wild-type strain. A transcription factor exclusion assay was used to show that the *PCF1* mutation affects two distinct stages in transcription: one prior to and one after stable complex formation; and that these effects are mediated by a component of the stable complex. Further evidence of an effect during complex assembly was obtained in a time-course experiment that showed a shortened lag phase in the *PCF1* extract. The results indicate that *PCF1* is either a component of the stable complex or a positive regulator of its activity.

Key words: nonsense suppressor mutants/RNA polymerase III/transcriptional activation

Introduction

RNA polymerase III (pol III) is responsible for the synthesis of RNAs belonging to three distinct classes of genes, each of which is distinguished by their promoters and by the complement of protein factors required for their transcription. Two of these classes are characterized by genes with different types of internal promoters. 5S RNA genes, which are the sole representatives of one group (type I) (Geiduschek and Tocchini-Valentini, 1988), contain at least three transcriptionally important intragenic regions (Pieler *et al.*, 1987; Tyler, 1987; Sharp and Garcia, 1988). The second group (type II genes) which includes tRNA and adenovirus VA RNA genes among its most studied members, contain only two internal promoter elements (Sharp *et al.*, 1985). Transcription of both type I and type II genes involves a common set of protein factors, TFIIIB and TFIIIC, with an additional factor, TFIIIA, required for 5S gene transcription.

For type II genes, the intragenic promoter regions are known as the A and B blocks. These regions are the sites of interaction of TFIIIC. DNase I footprinting and gel mobility shift experiments using TFIIIC fractionated from various sources have shown that the B block is a high affinity binding site for this factor (reviewed by Geiduschek and Tocchini-Valentini, 1988). In contrast, A block interactions with TFIIIC are much weaker and contribute very little to the overall binding constant (Baker *et al.*, 1986). The interactions in this region are sensitive to the spacing between the control regions (Baker *et al.*, 1987; Fabrizio *et al.*, 1987) and are influenced by ionic strength (being more stable at low salt concentrations (Baker and Hall, 1984). Mutations which reduce homology to the A block promoter consensus sequence do not dramatically affect stable complex formation (in contrast to B block mutations; Allison *et al.*, 1983) but can reduce promoter activity markedly (Folk and Hofstetter, 1983; Willis *et al.*, 1984). Thus, the importance of TFIIIC–A block interactions for efficient initiation of transcription is well documented. Exactly how these weak interactions promote transcription is unknown.

Native TFIIIC from yeast (also known as tau) has a mol. wt of ~320 kd (Ruet *et al.*, 1984; Stillman *et al.*, 1985). This factor, however, is not a single polypeptide. Rather, it appears to contain multiple, non-identical subunits; several protein bands of mol. wt 100–150 kd copurify with TFIIIC activity (Camier *et al.*, 1985). The heterogeneous composition of this factor is supported by results from other systems. The human and *Bombyx mori* TFIIIC fractions have been resolved into two components; both must be combined together with TFIIIB and pol III to reconstitute specific transcription (Ottonello *et al.*, 1987; Yoshinaga *et al.*, 1987). The human component designated TFIIIC2 footprints the B block region of the adenovirus VA1 RNA gene. The complementing factor, TFIIIC1, while not footprinting by itself, is able to extend the TFIIIC2 footprint over the A block. Recent studies on the activation of pol III gene transcription by the adenovirus E1a protein have provided evidence for post-translational regulation of TFIIIC activity (Hoeffler *et al.*, 1988). Two forms of human TFIIIC have been resolved by chromatography on phosphocellulose; both forms bind to the internal promoter of a type II gene producing complexes of different mobility on native gels. Notably, the slower migrating complex was significantly more active in transcription and could be converted into the faster migrating form by treatment with phosphatase. Activation of TFIIIC by E1a was suggested to involve the phosphorylation of pre-existing inactive factor. At present, it is not known which component of TFIIIC is modified or how this modification affects transcription. Indeed, *in vitro* phosphorylation of TFIIIC has yet to be demonstrated.

The other common factor, TFIIIB, has been purified extensively from yeast (Klekamp and Weil, 1986) and

HeLa cells (Waldschmidt *et al.*, 1988). This factor is a single polypeptide with a mol. wt of ~60 kd. The function of TFIIIB in transcription does not appear to involve sequence-specific binding to DNA (Geiduschek and Tocchini-Valentini, 1988; Kassavetis *et al.*, 1989). Rather, a large body of biochemical data is available which suggests that the factor may stabilize TFIIIC–A block interactions (e.g. see Baker and Hall, 1984; Bieker *et al.*, 1985; Klekamp and Weil, 1987). Interactions between TFIIIB and the C2 component of TFIIIC as well as pol III have also been hypothesized based on order of addition experiments (Bieker *et al.*, 1985; Dean and Berk, 1988).

In vitro pol III systems have been available for ~10 years. However, with the exception of the 5S gene-specific factor, TFIIIA, which is present in great abundance in *Xenopus* oocytes, the biochemical purification of pol III transcription factors has been a difficult task. We have taken a genetic approach to investigate pol III gene transcription thereby circumventing the biochemical difficulties with this system. In this report we describe a genetic selection that targets mutations in the pol III transcription apparatus. A collection of dominant mutant strains affecting tRNA gene transcription was isolated and one mutation was characterized in some detail. Extracts of strains carrying the dominant mutant gene *PCF1* exhibit a general stimulation of both tRNA and 5S RNA gene transcription compared with extracts from a wild-type strain. We show that this increase in transcription is mediated by a factor with properties similar to TFIIIC that is sequestered in stable transcription complexes. We propose that similar mechanisms may operate in yeast and human cells to control pol III transcriptional activity.

Results

Design of a genetic selection for mutants of the RNA polymerase III transcription apparatus

The approach we have taken in designing a selection for mutants of the RNA polymerase III transcription apparatus involves the isolation of extragenic suppressors of mutations affecting tRNA promoter function. In previous studies we have identified ~60 different second-site mutations in two homologous nonsense suppressors, *sup3-e* and *sup9-e*, from *Schizosaccharomyces pombe*. A subset of these mutations decrease gene transcription (Willis *et al.*, 1984; Pearson *et al.*, 1985; Nichols, 1988; Nichols *et al.*, 1989). The efficiency of transcription of these mutant genes and their ability to compete for binding of transcription factors has been determined using whole-cell extracts derived from *Saccharomyces cerevisiae* and in reactions containing partially purified factors and polymerase. Based on this information we chose the A block promoter mutant *sup9-e A19* to select mutations that would compensate for the poor transcription of this gene; within the A block, mutations at position 19 cause the greatest reduction in *sup9-e* (and *sup3-e*) gene transcription (Willis *et al.*, 1984; Pearson *et al.*, 1985).

The location of tRNA gene promoters within the tRNA coding sequence presents a major problem in selecting extragenic suppressors of a promoter mutant. It cannot be assured, indeed, it is most unlikely, that compensation of the transcriptional defect (regardless of the mutation) will lead to expression of nonsense suppressor activity since mutations in the dihydrouridine and TΨC stems and loops

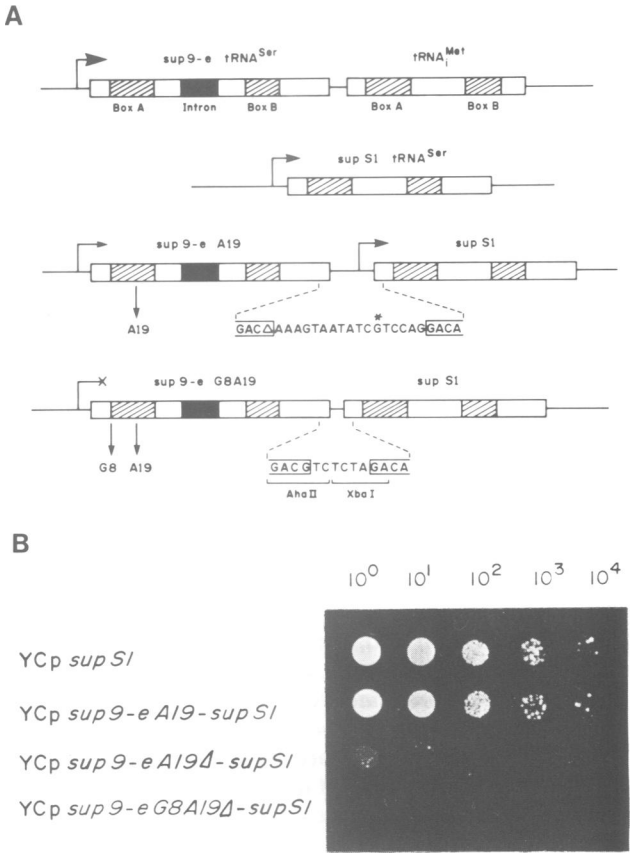

Fig. 1. Construction and expression of hybrid dimeric nonsense suppressor tRNA genes. (**A**) The different tRNA gene constructs used in this work are represented schematically. From top to bottom they include the parental dimeric *sup9-e* tRNA^{Ser}–tRNA^{Met} gene, *supS1*, *sup9-e A19-supS1* and *sup9-e G8A19Δ-supS1*. The construct used for mutant selection, *sup9-e A19Δ-supS1*, is identical to the *sup9-e G8A19* allele but contains the wild-type base (T) at position 8. The initiation site of *supS1* transcription is shown by an asterisk. The 3′ terminal base pair of *sup9-e* was lost during construction of *sup9-e A19-supS1* and is indicated by a Δ in the sequence. Transcription control regions are hatched and the intron of the *sup9-e* gene is shown as a solid block. (**B**) *supS1* suppressor activity in *S.cerevisiae* strain IW1B6 (Materials and methods). Ura$^+$ transformants bearing different *supS1*-containing plasmids were grown to saturation in liquid minimal medium lacking uracil. The cultures were washed in water and four, serial 1 in 10 dilutions were prepared. Aliquots (5 μl) were spotted on a minimal medium plate selecting for the plasmid marker (*URA3*) and for amber suppressor activity.

perturb tRNA structure so as to prevent or seriously impair RNA processing, aminoacylation or function in translation (Willis *et al.*, 1986b, and references therein). To overcome this problem we have exploited the unusual (for eukaryotes) tandem arrangement of the *sup9-e* gene with a tRNA$_i^{Met}$ gene and the co-transcription of these two genes into a dimeric tRNA^{Ser}–tRNA$_i^{Met}$ precursor. Studies on this dimeric gene pair (Willis *et al.*, 1984) and on different variants of a *S.cerevisiae* tRNA^{Arg}–tRNA^{Asp} gene pair (Reyes *et al.*, 1986; Straby, 1988) have shown that expression of the downstream gene relies on transcription directed by the internal promoter of the upstream gene. We therefore replaced the tRNA$_i^{Met}$ gene with a reporter gene whose activity could be monitored and selected for *in vivo*. The reporter gene chosen for this purpose was the *S.pombe* amber nonsense suppressor *supS1* since it shared similar *in vitro* transcriptional properties with the tRNA$_i^{Met}$ gene

that it was replacing; both genes are poor templates for transcription and weak competitors for transcription factors in a stable complex formation assay (Hottinger-Werlen *et al.*, 1985).

A series of hybrid, dimeric suppressor tRNA genes was prepared as shown in Figure 1(A). In the initial construct, *sup9-e A19-supS1*, the *supS1* gene was positioned 16 bp downstream from the 3′ end of the *sup9-e A19* gene. The spacer sequence was then reduced to 6 bp by primer-directed mutagenesis to give *sup9-e A19Δ-supS1*. In this construct, the spatial arrangement of the suppressors is more reminiscent of the parental $tRNA^{Ser}-tRNA^{Met}$ gene pair where the two genes are separated by 7 bp. Finally, an additional mutation (G8) was introduced into the A block of the *sup9-e* gene to give *sup9-e G8A19Δ-supS1*. Among the A block mutations that we have characterized, G8 is second only to A19 with regard to the severity of its effect on transcription (Nichols, 1988).

The monomeric and dimeric *supS1* constructs were cloned separately into the centromeric plasmid vector, YCp50, which carries the *URA3* gene as a selectable marker and transformed into *S.cerevisiae* strain IW1B6 (see Materials and methods). The ability of the different *supS1* constructs to suppress the UAG nonsense mutations, *trp1-1* and *met8-1*, is shown in Figure 1(B). The level of amber suppression observed for the dimeric *sup9-e A19-supS1* gene was indistinguishable from that obtained using the monomeric *supS1* gene indicating that a spacing of 16 bp between the two tRNA genes is sufficient to allow independent expression of *supS1*. By shortening the intragenic spacer sequence to 6 bp in *sup9-e A19Δ-supS1*, amber suppressor activity was reduced to $<0.1\%$ of the level obtained for *supS1* alone. This residual *supS1* activity was abolished by the addition of another mutation (G8) to the A block of the *sup9-e* gene. From this we conclude that the small amount of suppressor activity detected with *sup9-e A19Δ-supS1* must arise via transcription of a dimeric *sup9-e A19Δ-supS1* precursor and subsequent RNA processing (to yield mature *supS1* tRNA) rather than by a persistent low level of transcription directed by *supS1* itself. Thus, these results show that expression of *supS1* suppressor activity from the *sup9-e A19Δ-supS1* gene pair is dependent upon transcription directed by the defective internal promoter of the upstream *sup9-e* gene. This system provides a simple and sensitive *in vivo* assay of tRNA promoter strength over a dynamic range of $>10^4$. In addition, the effective lack of expression of *supS1* in this genetic context allows for the selection of mutations that suppress the transcriptional defect of the upstream gene.

Isolation of dominant extragenic suppressors of an A block promoter mutation

Transformants of the haploid strain IW1B6 containing YCp*sup9-e A19Δ-supS1* were mutagenized and the cells were plated with selection for the plasmid (Ura^+) and for amber suppressor activity (Trp^+, Met^+). Subsequent identification of strains in which amber suppressor activity was dependent on the presence of the YCp*sup9-e A19Δ-supS1* plasmid was achieved after growth on medium containing 5-fluoro-orotic acid. From 10^9 mutagenized cells, Ura^- segregants were recovered for 1765 suppressor active strains. Of these, 218 strains co-segregated the Ura^-, Trp^- and Met^- phenotypes indicating that each required the plasmid to express the nonsense suppression phenotype.

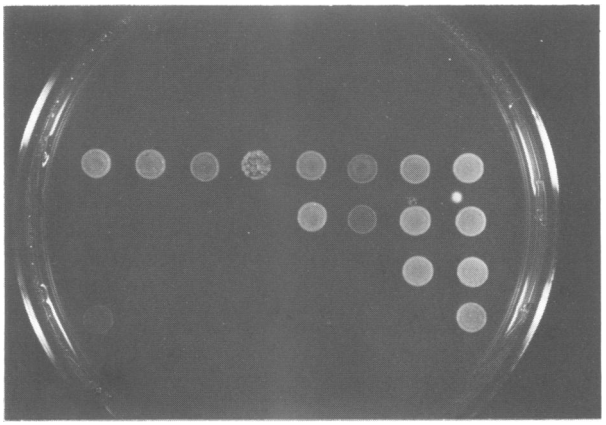

Fig. 2. *supS1* suppressor activity in heterozygous mutant diploid strains. Cultures of the 15 diploid strains that expressed *supS1* were grown to saturation in complete medium, washed in water and equal numbers of cells were spotted onto minimal medium plates selecting for suppressor activity. A diploid strain obtained by crossing the unmutagenized parent strain, IW1B6, with the tester strain, IWD1, was used as a negative control. Strain designations from left to right are: top row, 12-11, 18-10, 20-11, 45-4, 51-20, 52-3, 59-4, 60-4; second row, 51-23, 52-7, 59-24, 60-24; third row, 59-25, 60-25; bottom row, control, 60-36. Mutant strains in different columns represent independent isolates.

Further characterization of the mutant strains required reintroduction of the *sup9-e A19Δ-supS1* suppressor and identification of mutants in which *supS1* suppressor activity was restored. This was accomplished genetically following the construction of a tester strain (IWD1) containing a single chromosomal copy of the *sup9-e A19Δ-supS1* dimeric gene (see Materials and methods). Since the ability of the transcription apparatus to direct RNA synthesis from an inactive promoter requires, in effect, the creation of a new promoter specificity, we expected that the desired mutants would be phenotypically dominant. Accordingly, each of the 218 plasmid-cured mutant strains was crossed to the tester strain carrying *sup9-e A19Δ-supS1* and examined directly for amber suppressor activity. Diploid cells (His^+, Arg^+) were selected by complementation and then tested for *supS1* suppression of the homozygous suppressible markers, *trp1-1* and *met8-1*. Fifteen strains, eight of which represented independent isolates, were able to grow in the absence of tryptophan and methionine (Figure 2). Thus, these strains contain dominant mutations that permit expression of *supS1* from the dimeric *sup9-e A19Δ-supS1* gene.

Extragenic suppressors of the A19 transcriptional defect increase the steady-state levels of sup9-e A19 RNA

Expression of *supS1* in transcriptionally altered mutant strains could potentially occur in one of two ways: by compensation of the A19 defect in *sup9-e* and transcription of a dimeric tRNA precursor (from which mature *supS1* can be obtained by RNA processing); or by independent transcription of *supS1* from its own internal promoter (refer to Figure 1A). To examine these possibilities, the mutant strains were separately transformed with the plasmids YCp*sup9-e A19Δ-supS1* or YCp*sup9-e G8A19Δ-supS1*. A comparison of the level of *supS1* expression in several strains is shown in Figure 3. Similar results were seen for all the other strains. No mutant was found in which the expression

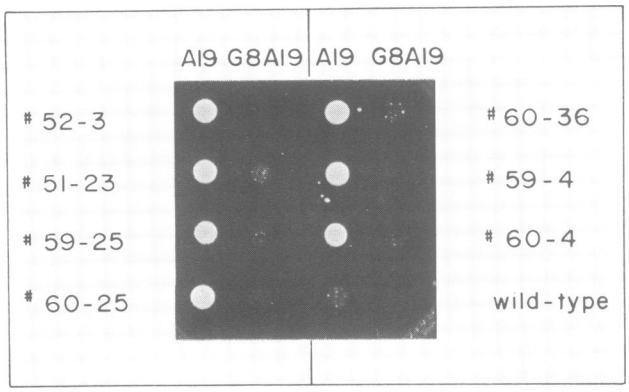

Fig. 3. *sup9-e A19Δ- and sup9-e G8A19Δ*-dependent expression of *supS1* in wild-type and mutant strains. Plasmid-transformed strains were grown as described in the legend to Figure 1. After washing in water, equal numbers of cells (2.5×10^5) were spotted onto a minimal medium plate lacking tryptophan and methonine and incubated for 2.5 days at 24°C.

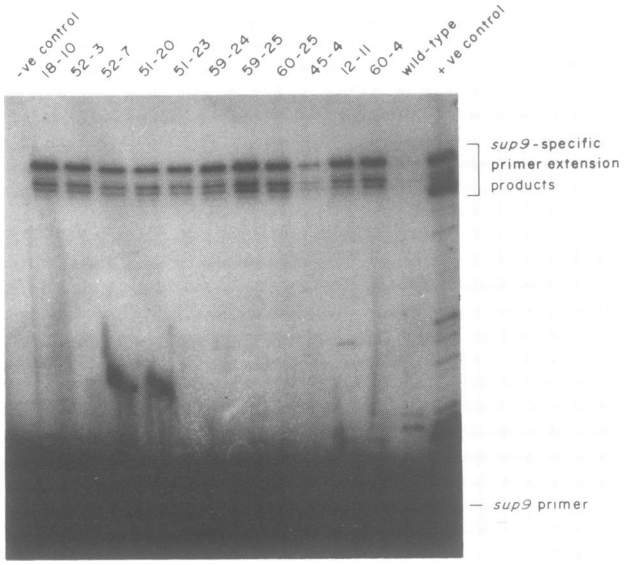

Fig. 4. Detection of *sup9-e A19Δ-supS1* transcripts in RNA from mutant strains by primer extension. Cultures of the diploid strains shown in Figure 2 were grown to mid-log phase for the preparation of RNA. Equal amounts of RNA were hybridized to a 5′ ^{32}P-labeled 26mer complementary to the *sup9* anticodon/intervening sequence region and extended using MLV reverse transcriptase. Extension products were resolved on a 10% denaturing polyacrylamide gel. RNA from *S.cerevisiae* strain IW1B6 which lacks *sup9* sequences was used as a negative control. The positive control lane shows the products obtained with RNA from a *S.cerevisiae* strain transformed with a multi-copy plasmid, YRp*sup9*$^+$, containing the wild-type *sup9* gene. The two major extension products in this lane correspond to *sup9* precursors with and without the 5 nucleotide 5′ leader sequence (Willis *et al.*, 1986a).

of *sup9-e A19Δ-supS1* and *sup9-e G8A19Δ-supS1* was equivalent; addition of the G8 mutation to *sup9-e A19* significantly reduced the level of *supS1* expression in all strains. In contrast to the wild-type strain which showed no expression of *sup9-e G8A19Δ-supS1*, several of the mutant strains were able to express this construct weakly. The level of expression of *sup9-e G8A19Δ-supS1* in those mutants (e.g. 51-23, 59-25 and 60-36) was roughly equivalent to that observed for *sup9-e A19Δ-supS1* in the wild-type strain. The effect of the G8 mutation in *sup9-e* on *supS1* suppressor activity argues that the mode of *supS1* expression in the mutant strains is via transcription of a dimeric tRNA precursor.

Since expression of the *supS1* suppressor in the mutants was most probably due to an increase in the transcription of the dimeric *sup9-e A19Δ-supS1* gene, we expected that the steady-state concentration of *sup9-e A19* transcripts would be elevated in these strains. The experiment shown in Figure 4 demonstrates that this is indeed the case. RNA was isolated from the wild-type and mutant diploid strains shown in Figure 2 and analyzed by primer-extension using an oligonucleotide complementary to the *sup9-e* anticodon/intervening sequence region. In the wild-type strain, the amount of *sup9-e A19Δ-supS1* RNA is very low and *sup9* primer-extension products are barely detectable. The amount of these products is significantly increased in RNA isolated from the mutant strains. Similar results were obtained with the remaining mutants (data not shown).

Genetic analysis of dominant transcription activators

The dominant nature of the mutants and the requirement that the *sup9-e A19Δ-supS1* gene be present for the mutant phenotype to be expressed complicates their analysis by genetic methods. To follow the segregation of the *sup9-e A19* compensatory mutations, diploid strains must be constructed which are homozygous for the integrated dimeric suppressor gene and heterozygous for the dominant mutation which expresses it. Then, after establishing that the mutant phenotype in each strain results from a mutation in a single nuclear gene (by tetrad dissection), the number of different genes in the collection can be determined by a linkage analysis. This work is currently in progress. However, because of the time consuming nature of these experiments, we decided to focus our attention initially on one particular mutant strain. Isolate 59-24 (Figure 2) was chosen among those mutants exhibiting the strongest nonsense suppression phenotype. The remainder of the work described here deals with the genetic and biochemical characterization of this strain. The original isolate of mutant 59-24 has been outcrossed twice to the wild-type tester strain (IWD1) to obtain a haploid strain which can express a chromosomally integrated copy of the *sup9-e A19Δ-supS1* suppressor. Tetrad analysis of the spores obtained after crossing this strain back to wild-type for a third time showed 2:2 segregation of the suppressible markers, *trp1-1* and *met8-1*. Thus, a single nuclear mutation is responsible for the *supS1* suppression phenotype of this mutant. Based on the previous *in vivo* characterization of this strain and *in vitro* data described below, this mutant has been designated *PCF1* for polymerase C factor 1.

PCF1 increases transcription of type I and type II pol III genes in vitro

To investigate whether the increase in steady-state *sup9-e A19Δ-supS1* RNA in the *PCF1* strain was due to an increase in gene transcription, whole-cell extracts were prepared in parallel from equal amounts of physiologically equivalent *PCF1* mutant and wild-type cells (see Materials and methods). Initially, transcription reactions contained equal, non-limiting amounts of template DNA and were normalized for the amount of extract protein. Under these conditions we found that the initial rate of *sup9-e A19* transcription was 5- to 7-fold higher in the *PCF1* cell extract than in the wild-

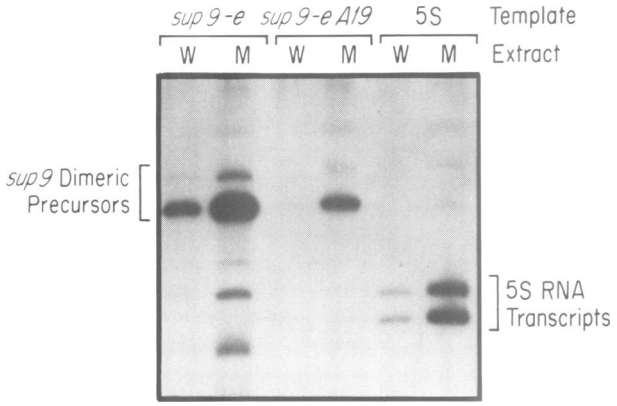

Fig. 5. Stimulation of tRNA and 5S RNA gene transcription in extracts of the *PCF1* mutant strain. Whole-cell extracts were prepared in parallel from equal amounts of wild-type (W) and *PCF1* mutant (M) cells (see Materials and methods). Reactions were normalized for extract protein and were limiting for transcription (i.e. template DNA was added in excess). DNA templates included *sup9-e*, *sup9-e A19* and 5S RNA genes.

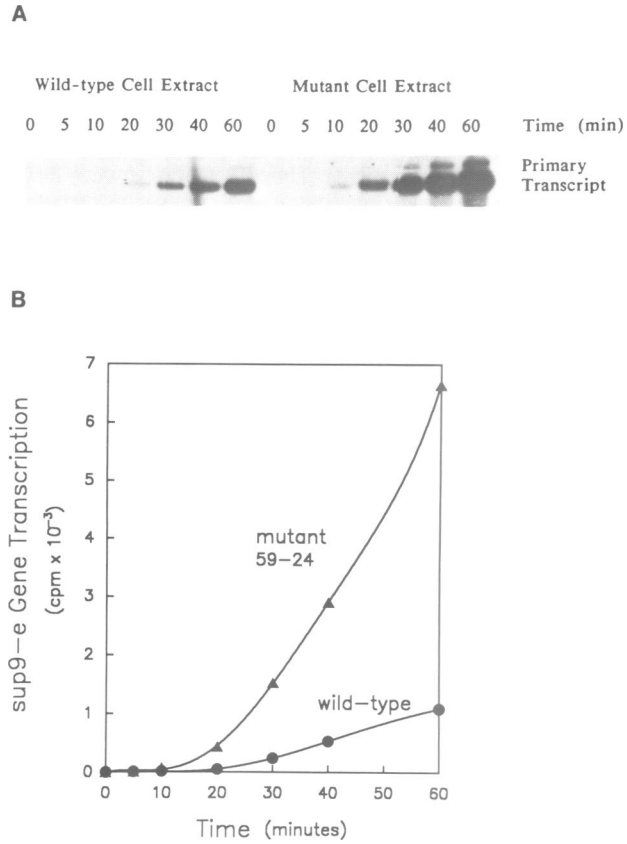

Fig. 6. Time-course of *sup9-e* transcription in wild-type and *PCF1* cell extracts. Reaction conditions for this experiment were the same as in Figure 5. (**A**) An autoradiogram of the transcripts synthesized after incubation at 15°C for various times. (**B**) A plot of transcription versus time for this experiment.

type cell extract (Figures 5–7). Interestingly, transcription of the parental *sup9-e* suppressor gene in mutant cell extracts was also increased by the same amount. Thus, the magnitude of the A19 transcriptional defect is preserved in extracts from *PCF1* cells but the overall level of transcription is increased relative to wild-type cells. As a result, transcription of the mutant gene in the mutant extract is now comparable to

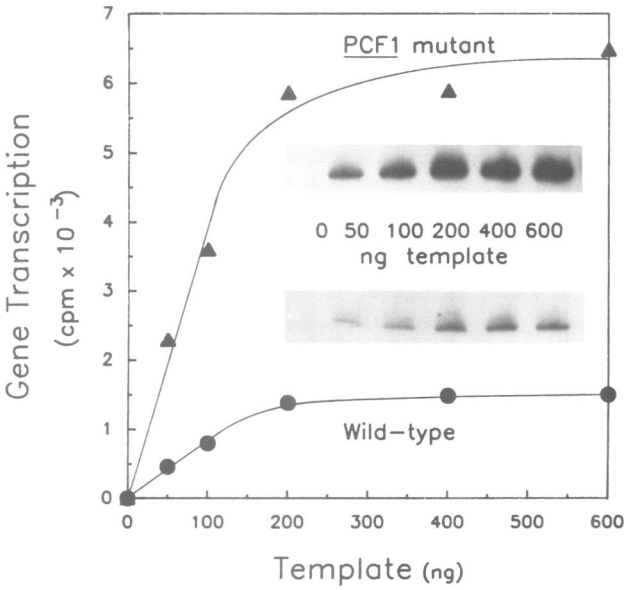

Fig. 7. Titration of *sup9-e* DNA in cell extracts. Transcription reactions contained equal amounts of extract protein and the total DNA concentration was kept constant by addition of carrier plasmid DNA. Specific conditions are given in Materials and methods.

transcription of the wild-type gene in the wild-type extract. These findings indicate that the *PCF1* mutation causes a general elevation of tRNA gene transcription. This effect is not restricted to tRNA genes, however. The transcription of a yeast 5S RNA gene is also increased in the mutant extract (Figure 5). In this case, a 4-fold difference in transcription between wild-type and mutant extracts was observed. Clearly, a gene product involved in the transcription of both of these types of pol III genes must be altered in the mutant strain.

A time-course of tRNA gene transcription in mutant and wild-type extracts was performed using the above conditions (i.e. excess template DNA and equal amounts of extract protein) and the results are shown in Figure 6. From this experiment we see that linear transcription continues for at least 60 min in both extracts and that, as above, the transcription rate in the mutant extract is six times that of the wild-type. Both extracts exhibit the typical lag phase (representing the formation of pre-initiation complexes) prior to attaining a linear rate of transcription. However, the length of the lag phase in the *PCF1* extract is only about half that of the wild-type. This finding suggests that at least part of the difference between the two extracts may be attributable to events which take place during the formation of stable transcription complexes.

To examine further the relationship between factors stably associated with the *sup9-e* gene and the *in vitro* behavior of the *PCF1* extract, we established conditions to perform extract mixing experiments. Initially, a template titration experiment was carried out with both extracts, again using equal amounts of extract protein. The result, shown in Figure 7, indicates that the template concentration corresponding to half maximal transcription appears to be the same for both extracts. Thus, the concentration of the limiting transcription component is apparently unchanged by the *PCF1* mutation. We next used a competition experiment to determine the preincubation conditions required for complete formation of

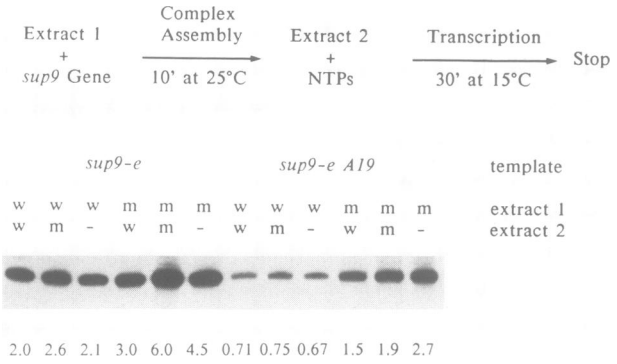

Fig. 8. Use of a transcription factor exclusion assay to examine the biochemical effect of the *PCF1* gene product. Experimental details are described in the text and are illustrated in the upper part of the figure. w, wild-type; m, *PCF1* mutant. The amount of transcript (fmol) synthesized in each reaction is shown below the gel and was determined by Cerenkov counting of the excised bands.

stable transcription complexes. We established that preincubation of the *sup9-e* gene with limiting amounts of wild-type extract under favorable ionic conditions (see Materials and methods) for 10 min at 25°C precluded transcription of a dimeric $tRNA^{Arg}-tRNA^{Asp}$ gene that was added subsequently to the reaction (data not shown). The same preincubation conditions also eliminate the lag phase in wild-type extracts. These conditions were then used to conduct a transcription factor exclusion assay (Figure 8). In this assay, a limiting amount of gene is preincubated with either wild-type or mutant cell extract in the absence of ribonucleoside triphosphates to allow complete assembly of stable transcription complexes. The other extract is then added together with ribonucleotides to initiate transcription. Using either *sup9-e* or *sup9-e A19* DNA as the template, the amount of transcription which ensues reflects the activity of the extract that was preincubated with the gene (Figure 8). Thus, the increased transcription in the *PCF1* cell extract must be mediated by a component of the stable complex. Interestingly, in contrast to the 6-fold stimulation of tRNA gene transcription seen in previous experiments (Figures 5–7), a lesser effect is observed if stable complexes are preformed. In several repetitions of this experiment we have measured increases in transcription of only 2- to 3-fold. Thus, to account for the overall *in vitro* effect, we propose that the *PCF1* mutation influences two distinct stages in the transcription process. Consistent with the reduced lag phase (Figure 6), one effect of *PCF1* is presumably exerted during stable complex formation; the other occurs after the formation of stable complexes, in the active transcription phase of the reaction (Figure 8).

Discussion

We have described a genetic selection that targets mutations in the pol III transcription apparatus. The selection is based on suppression of a transcriptional defect resulting from a point mutation in the internal promoter of one tRNA gene (*sup9-e*) and concomitant expression of another downstream, co-transcribed nonsense suppressor tRNA (*supS1*). A hunt for mutants that compensate in *trans* for an A block promoter mutation (A19) in the *sup9-e* gene and express *supS1* amber suppressor activity yielded 15 strains, eight of which were isolated independently. In each of these cases, expression of *supS1* suppressor activity is dominant in diploids heterozygous for the compensatory mutations indicating that a gain of function is responsible for the mutant phenotype. This fact, together with the demonstrated increase in the steady-state level of *sup9-e A19* transcripts is most readily explained by an increase in the transcription of this gene in the mutant strains. The long half-life of tRNA and the need to invoke a hypothetical positive regulator of RNA stability, makes it unlikely that reduced tRNA turnover is responsible for *supS1* expression.

From our current understanding of the functions of the pol III transcription factors and the mechanism of tRNA gene transcription, at least three different groups of mutants may be obtained using this strategy. The first and most obvious of these are mutants of the component of TFIIIC (presumably the yeast analog of the human C1 factor) (Yoshinaga *et al.*, 1987) that interacts with the A block region of tRNA genes. These mutants are expected to compensate for the A19 mutation by making new contacts in the A block region of the internal promoter. The second group may comprise mutants of TFIIIB. Work from several laboratories involving TFIIIB from different sources, including yeast, indicates that this factor participates in protein–protein interactions with TFIIIC and pol III (Bieker *et al.*, 1985; Klekamp and Weil, 1987; Dean and Berk, 1988). The interaction between factors IIIB and IIIC has been hypothesized to stabilize the relatively weak binding of the latter to the A block. Potentially, hyperstability mutants of TFIIIB could be generated that are capable of stabilizing the interaction of TFIIIC with a mutant A block. The final class of mutants that we anticipate includes genes which function to enhance the amount or the activity of the transcription factors. One intriguing scenario is that mutants of a protein kinase may be obtained that activate TFIIIC in a manner analogous to the putative human enzyme responsible for activating pol III gene transcription in adenovirus-infected cells (Hoeffler *et al.*, 1988).

Dominant mutants in any of these groups could potentially increase *in vitro* transcription of both type I and type II pol III genes as described here for *PCF1*. Currently, we cannot exclude any of these possibilities. However, several noteworthy similarities exist between our *in vitro* data and previously described properties of human TFIIIC. These similarities relate to the finding that the *PCF1* mutation influences transcription at two discrete stages, during and after stable complex formation. Firstly, activation of human TFIIIC by the adenovirus transforming protein E1a has been shown to increase the rate of association of this factor with the internal promoter of a type II gene (Hoeffler *et al.*, 1988). Potentially, the shortened lag phase in extracts of the *PCF1* mutant strain may reflect an increase in TFIIIC binding affinity. Although the binding of TFIIIC to the internal promoter of pol III genes is normally very rapid (compared with the subsequent entry of TFIIIB into the complex) (Bieker *et al.*, 1985), the suggestion that TFIIIC may mediate the lag phase effect gains appeal with the knowledge that this factor is limiting for transcription in the yeast *in vitro* system used in this work; addition of partially purified TFIIIC but not TFIIIB or pol III to whole-cell extracts results in increased gene transcription (I.Willis, unpublished results). Secondly, activation of human TFIIIC by E1a is known to

result from an increase in the activity, not the amount of the factor (Hoeffler *et al.*, 1988). Thus, if TFIIIC is primarily responsible for the increased transcription in *PCF1* extracts, this must also result from an increase in the activity of the factor since the template titration experiment showed that the concentration of the limiting factor (TFIIIC) was unchanged. Of course, the other similarity between the *in vitro* properties of *PCF1* and E1a-activated TFIIIC is the ability to stimulate transcription of both tRNA and 5S RNA genes (Gaynor *et al.*, 1985; Hoeffler and Roeder, 1985). Based on these comparisons, we suggest that *PCF1* is more likely to encode a positive regulatory or a component of TFIIIC than TFIIIB. Clearly, additional experiments will be required to resolve this issue.

The ability to generate mutants in the pol III transcription apparatus provides a new approach to study the structure and function of the transcription factors and the mechanisms for regulating their activity. The dominant phenotype of the mutants described here not only permits the cloning of the respective mutant genes but also signals the generation of novel biochemical activities. The analysis of *PCF1* (and any other mutant genes identified in this collection) will contribute important new information to our understanding of pol III gene transcription.

Materials and methods

Plasmid constructions

Separation of the *sup9-e A19* $tRNA^{Ser}$ gene from the downstream $tRNA_i^{Met}$ gene was achieved by creating an *Aha*II site in the spacer sequence between the two genes (Nichols *et al.*, 1989). A 17mer (GGTGACGTCATTTGTGC) and an M13mp8 clone of the mutant *sup9* gene (Willis *et al.*, 1984) were used for this purpose. The plasmid YCp*sup9-e A19-supS1* was constructed by the ligation of three restriction fragments including: a 700 bp *Hin*dIII–*Aha*II fragment of *sup9-e A19* obtained by digestion of the spacer mutant (above) with *Aha*II, filling in the 5′ overhang with Klenow fragment and finally, digestion with *Hin*dIII; a 140 bp *Dra*I–*Hin*dIII fragment of *supS1* (Krupp *et al.*, 1985); and *Hin*dIII-cleaved YCp50. Further alterations to the hybrid *sup9-e A19-supS1* dimeric gene were made by oligonucleotide-directed mutagenesis after recloning the 840 bp *Hin*dIII fragment from YCp*sup9-e A19-supS1* into M13mp18. Shortening of the spacer sequence was achieved using a 21mer (GGTGACGTCTCTAGACACTAT) and the G8 mutation was introduced using a 13mer (GTCACTAGGTCCG). Each of these constructs was then transferred back into the YCp50 vector on the *Hin*dIII fragment. The plasmid YCp*supS1* contains a 450 bp *Bam*HI–*Hin*dIII *supS1* fragment in YCp50. YIp*sup9-e A19Δ-supS1* was obtained by transferring the *Hin*dIII fragment of YCp*sup9-e A19Δ-supS1* into YIp5.

Yeast strains and methods

Genetic manipulations with yeast and growth media are described in Sherman *et al.* (1986). The strains used in this work are: YH-G4, *MATα trp1-1 met8-1 his4-38 arg4-17* (Hottinger *et al.*, 1984); S648 and S649, *MATα ura3-52 leu2-3,112 his3-11,15* (from Dr G.S. Roeder); IW1B6, *MATα ura3-52 trp1-1 met8-1 leu2-3,112 his3-11,15*; IW2A4, *MATα ura3-52 trp1-1 met8-1 leu2-3,112 arg4-17*; and IWD1, *MATα ura3-52::URA3(sup9-e A19 Δ-supS1) trp1-1 met8-1 leu2-3,112 arg4-17*. Strain IW1B6 was obtained by random spore analysis from a cross between strains YH-G4 and S648. Strain IW2A4 was obtained in a similar manner from a cross between strain YH-G4 and S649. Strain IWD1 was constructed by targeted integration of *Stu*I-cleaved YIp*sup9-e A19Δ-supS1* into the *ura3-52* locus of strain IW2A4. Confirmation that a single copy of the plasmid was integrated at this site was obtained by Southern analysis using a YIp5 probe.

Mutant isolation

The haploid strain IW1B6 (10^9 cells) containing YCp*sup9-e A19Δ-supS1* was mutagenized with ethylmethanesulfonate to 25% lethality (Sherman *et al.*, 1986). Cells were divided equally into 100 tubes, each containing 2 ml synthetic complete medium lacking uracil and grown at 24°C for 2 days. An aliquot ($\simeq 10^7$ cells) from each tube was washed in water and plated at 24°C on minimal medium with selection for the plasmid (Ura^+) and for amber suppressor activity (Trp^+, Met^+). Colonies that had grown after 4 days were replica-plated onto fresh minimal plates (as above at 24°C) and onto duplicate complete media plates (24 and 36°C) to identify suppressor active mutant strains that exhibited reduced growth at elevated temperatures. [This secondary phenotype was sought in an effort to enrich for the desired mutants by eliminating many of the strains containing chromosomal nonsense suppressors (most of which are non-conditional) that are generated by mutgenesis. Initially, we reasoned that mutant strains capable of transcribing a promoter mutant (e.g. A19) might not be able to transcribe wild-type genes at rates high enough to sustain rapid growth at 36°C and therefore might show reduced growth compared wtih a wild-type strain. However, the final group of 15 mutants failed to co-segregate this phenotype with suppression. We now suspect that in screening for reduced growth we selected cells that were more heavily mutagenized and therefore more likely to contain the mutants of interest.] Candidate strains were picked onto synthetic complete medium containing 5-fluoro-orotic acid and grown at 24°C to select for Ura^- cells that had failed to inherit the plasmid in the previous mitotic division. Colonies that formed from Ura^- segregants were replica-printed to identify mutants that co-segregated Ura^-, Trp^- and Met^- phenotypes. After reconfirming this phenotype, further characterization of candidate strains was performed by mating them to the test strain, IWD1, which contained a single chromosomal copy of the *sup9-e A19Δ-supS1* suppressor gene integrated at the *URA3* locus (see Yeast strains and methods). Replica-plating was performed to confirm that mating had occurred (by complementation of the *his3* and *arg4* mutations) and to identify diploid cells in which *supS1* suppressor activity was expressed.

Primer extension analysis

Diploid cells heterozygous for the extragenic A19 suppressor mutations and the *sup9-e A19Δ-supS1* gene (see Figure 2) were grown in complete medium (YPD, 100 ml) to a density of 2×10^7 cells/ml for the preparation of low mol. wt RNA (Willis *et al.*, 1984). The oligonucleotide TAGATGACTAGAATACAGGATTCAAG, complementary to the *sup9* anticodon/intervening sequence region (45 pmol) was phosphorylated at its 5′ end with [γ-^{32}P]ATP (240 μCi, 6000 Ci/mmol) and T4 polynucleotide kinase and isolated after electrophoresis on a denaturing 15% polyacrylamide gel. Reverse transcription primed with this oligonucleotide initiates at nucleotide 21 of the mature *sup9-e* serine tRNA and was performed as follows: RNA (20 μg) and the 5′ ^{32}P-labeled oligonucleotide (0.2 pmol, 1.5×10^6 c.p.m.) were boiled in 20 μl of 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 75 mM KCl for 2 min and then rapidly chilled in ice. The reaction volume was increased to 40 μl by addition of 4 μl 5 × M-MLV reaction buffer (BRL), dithiothreitol (DTT) to 10 mM, each of the four dNTPs to 0.25 mM and M-MLV reverse transcriptase (400 U, BRL). After incubation for 60 min at 37°C reactions were deproteinized by phenol extraction and precipitated with ethanol. The products were analyzed on a denaturing 10% polyacrylamide gel.

In vitro transcription

Wild-type (IW1B6) and mutant (*PCF1*) cells that had been cured of the *sup9-e A19Δ-supS1* gene were grown in YPD medium (3 l) to OD_{600} = 3.0. This yields ~10 g of cells. Whole-cell extracts were prepared in parallel by the method of Klekamp and Weil (1982) as modified by Nichols *et al.* (1989). After the final $(NH_4)_2SO_4$ precipitation, the pellet was resuspended in BC100 buffer (~5 ml) and desalted on a Sephacryl S200 column (12 cm high × 2.5 cm diameter) equilibrated with BC100 buffer containing 1 mM phenylmethylsulfonyl fluoride. An excluded fraction (no larger in volume than the sample itself) was collected, aliquoted and stored at −70°C. Apart from rapid desalting of the extract, this gel filtration step achieves some fractionation of extract components; the transcription machinery, which is excluded from this matrix, is separated from low mol. wt proteases, nucleases and nucleic acids.

Transcription reactions containing excess template DNA (200 ng) and normalized amounts of extract protein (45 μg, determined by Biorad assay) were performed in a volume of 50 μl with 10 mM HEPES–KOH (pH 7.9), 10 mM $MgCl_2$, 130 mM NaCl, 2 mM DTT, 10% (v/v) glycerol, 1.2 mM ATP, 0.6 mM each of CTP and UTP, 25 μM [α-^{32}P]GTP (8 Ci/mmol) and 200 ng carrier YRp17 or pGEM plasmid DNA. Reactions were incubated at 15°C (to minimize RNA processing of the transcripts) for 60 min. In the time-course experiment, reactions were stopped at the times indicated in Figure 6. For the template titration experiment, reactions were incubated for 45 min and contained 90 μg of extract protein. The amount of gene was varied from 0 to 600 ng and the total DNA concentration was maintained at 12 μ/ml by addition of carrier DNA. RNA was purified and analyzed on denaturing 8% polyacrylamide gels (Willis *et al.*, 1984). After autoradiography, bands were excised and quantified by Cerenkov counting.

DNA templates include the dimeric *sup9-e* $tRNA^{Ser}-tRNA_i^{Met}$ gene and the corresponding *sup9-e A19* mutant gene (Willis *et al.*, 1984). The plasmid pRIBs1 contains a single *S.cerevisiae* 5S RNA gene and was provided by Dr J.Warner.

Transcription competition experiments were performed by preincubating 200 ng of *sup9-e* gene with 45 μg protein extract (from either wild-type or mutant cells) in a 25 μl reaction for 10 min at 25°C, under ionic conditions known to favor complex formation [10 mM HEPES–KOH, (pH 7.9), 10 mM $MgCl_2$, 70 mM NaCl, 2 mM DTT, 10% (v/v) glycerol] (Baker and Hall, 1984). After preincubation, reactions were transferred to a 15°C bath for 2 min before addition of the second gene (a dimeric $tRNA^{Arg}-tRNA^{Asp}$ gene in pYM205; 200 μg; Hottinger-Werlen *et al.*, 1985), NTPs and extra salts. The final reaction conditions were the same as described above. Transcription was allowed to proceed for 60 min at 15°C. Under these conditions transcription of second gene was precluded. In the transcription factor exclusion factor assay, a limiting amount of gene (25 ng) and carrier plasmid (175 ng) was preincubated as above, in a volume of 25 μl with 45 μg of extract protein. After a further 2 min incubation at 15°C, an equal amount of extract protein was added together with additional carrier DNA (200 ng), NTPs and salts. Reactions in which no second extract was added contained, instead, an appropriate amount of BC100 buffer. Transcription was allowed to proceed for 30 min.

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