Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure

Vishwanath Iyer and Kevin Struhl[1]

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

[1]Corresponding author

Many yeast promoters contain homopolymeric dA:dT sequences that affect nucleosome formation *in vitro* and are required for wild-type levels of transcription *in vivo*. Here, we show that poly(dA:dT) is a novel promoter element whose function depends on its intrinsic structure, not its interaction with sequence-specific, DNA-binding proteins. First, poly(dA:dT) stimulates Gcn4-activated transcription in a manner that is length dependent and inversely related to intracellular Gcn4 levels. Second, Datin, the only known poly(dA:dT)-binding protein, behaves as a repressor through poly(dA:dT) sequences. Third, poly(dG:dC), a structurally dissimilar homopolymer that also affects nucleosomes, has transcriptional properties virtually identical to those of poly(dA:dT). Three probes of chromatin structure including HinfI endonuclease cleavage *in vivo* indicate that poly(dA:dT) increases accessibility of the Gcn4 binding site and adjacent sequences in physiological chromatin. These observations suggest that, by virtue of its intrinsic structure, poly(dA:dT) locally affects nucleosomes and increases the accessibility of transcription factors bound to nearby sequences.
*Key words*: chromatin/eukaryotic promoters/gene regulation/transcriptional activation

Introduction

Eukaryotic RNA polymerase II promoters typically consist of a TATA element and upstream regulatory sequences. The TATA element is a binding site for TFIID, the general transcription factor that nucleates the assembly of the basic RNA polymerase II machinery (Conaway and Conaway, 1993; Zawel and Reinberg, 1993; Buratowski, 1994). Upstream promoter elements are target sites for sequence-specific, DNA-binding proteins that stimulate transcription (Mitchell and Tjian, 1989; Struhl, 1989; Ptashne and Gann, 1990). Such activator proteins typically contain a DNA-binding domain that interacts with its cognate site and an activation domain that stimulates transcription. The paradigm regarding the function of upstream elements is that they serve merely to bring activator proteins to the promoter, thus permitting the activation domains to stimulate transcription by direct or indirect interactions with a component(s) of the basic machinery and/or chromatin. By dictating potential arrangements of specific regulatory proteins at a promoter, upstream regulatory elements play a major role in determining transcription patterns in response to environmental or developmental cues.

Many yeast promoters contain homopolymeric poly(dA:dT) sequences (Struhl, 1985; Karlin et al., 1993), and in several cases they have been shown to be necessary for wild-type levels of transcription *in vivo* (Struhl, 1985; Lowry et al., 1990; Schlapp and Rodel, 1990; Thiry-Blaise and Loppes, 1990; Schultes and Szostak, 1991). Poly(dA:dT) sequences have an unusually short helical repeat (10.0 bp instead of 10.6 typical of B-DNA) (Peck and Wang, 1981; Rhodes and Klug, 1981) and a distinctively narrow minor groove (Alexeev et al., 1987), and they are structurally rigid (Nelson et al., 1987). As a consequence of these unusual properties, poly(dA:dT) does not favor the assembly or stability of nucleosomes *in vitro*. Initial chromatin reconstitution experiments involving dialysis from high salt indicated that long dA:dT tracts could not be wrapped into nucleosomes (Kunkel and Martinson, 1981; Prunell, 1982). More recent reconstitution protocols involving histone exchange in lower salt indicate that nucleosomes can form on poly(dA:dT), but at lower efficiency on longer tracts (Puhl et al., 1991). These nucleosome destabilizing properties are likely to be related to the strong bias of short dA:dT tracts to be located on the outside surface and the difficulty of bending longer tracts around the histone core (Drew and Travers, 1985; Satchwell et al., 1986).

It has been suggested that poly(dA:dT) sequences might function by virtue of their intrinsic structure, a mechanism distinct from that of conventional upstream elements that interact with DNA-binding, transcriptional activator proteins (Chen et al., 1987). In yeast cells, transcription by bacteriophage T7 RNA polymerase can be stimulated by the *DED1* promoter, which contains an imperfect (28 out of 34 bp) poly(dA:dT) element, but not by the Gal4-dependent enhancer (Chen et al., 1987). However, important aspects of this hypothesis are untested or controversial. First, the imperfect poly(dA:dT) element in the *DED1* promoter can stimulate transcription from naked DNA templates *in vitro* (Lue et al., 1989), indicating that it interacts with a specific DNA-binding activator protein. Thus, the mechanism by which this *DED1* element stimulates transcription by T7 RNA polymerase is unclear. Second, even in cases involving perfect poly(dA:dT) sequences, transcriptional effects might be mediated by the action of an activator protein bound to the site rather than by the intrinsic structure of poly(dA:dT). Indeed, yeast cells contain a poly(dA:dT)-binding protein, Datin, that specifically recognizes homopolymeric tracts >10 bp in length (Winter and Varshavsky, 1989). Third, there is no evidence that poly(dA:dT) sequences affect chromatin structure and increase accessibility of transcription factors to the chromosomal template *in vivo*.
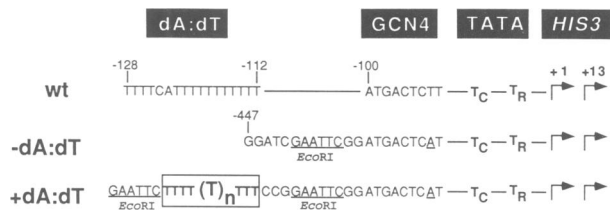
Fig. 1. Schematic representation of *his3* promoters. The wild-type *his3* promoter (wt) contains an imperfect poly(dA:dT) element, Gcn4 binding site, two TATA elements (T_C and T_R) and two mRNA initiation sites (+1 and +13). The −dA:dT promoter contains the Δ101 deletion, which removes sequences between −103 and −447, and an optimal Gcn4 binding site (underlined base indicates different from wild-type sequence). Promoters containing poly(dA:dT) sequences of various lengths and orientations (T residues on coding strand shown) were generated by inserting oligonucleotides at the indicated EcoRI site. The downstream end of the inserted poly(dA:dT) sequence occurs at the position identical to that of the natural *his3* element at −112.

In this paper, we investigate the mechanism by which poly(dA:dT) sequences promote transcription using a combination of functional analyses and probes of chromatin structure. Our results suggest that poly(dA:dT) stimulates Gcn4-activated transcription by increasing the accessibility of the Gcn4 binding site in chromatin. Further, they suggest that poly(dA:dT) is a novel promoter element whose function depends on its intrinsic structural properties, not its ability to bind transcription factors. The physiological importance of poly(dA:dT) sequences in yeast cells is discussed.

Results

### Poly(dA:dT) sequences stimulate Gcn4-activated transcription

The yeast *his3* promoter region contains two proximal TATA elements, T_C and T_R, that respectively are primarily responsible for transcription from the +1 and +13 mRNA initiation sites (Struhl, 1986). Located upstream of these TATA elements are a binding site for the activator Gcn4 (nucleotides −100 to −92) and an imperfect poly(dA:dT) sequence (15 of 17 residues between −112 and −128; see Figure 1). Deletion analysis indicates that this imperfect poly(dA:dT) sequence is important for wild-type levels of *his3* expression (Struhl, 1985).

To investigate the properties of poly(dA:dT) in more detail, we used a modified version of the *his3* promoter that contains an optimal Gcn4 binding site (Hill *et al.*, 1986), but lacks all upstream sequences between −103 and −447 (Figure 1). Perfectly homopolymeric (dA:dT) oligonucleotides were inserted upstream of the Gcn4 binding site at the equivalent position of the native *his3* poly(dA:dT)-like sequence, and the resulting promoter constructs were integrated at the *his3* locus by gene replacement. In rich medium (YPD), the poly(dA:dT) sequences stimulate *his3* transcription, with initiation occurring primarily from the +13 site (Figure 2). In accord with previous results (Russell *et al.*, 1983; Struhl, 1985), poly(dA:dT) tracts function in both orientations and longer tracts are more stimulatory than shorter tracts. The stimulation of *his3* transcription due to poly(dA:dT) ranges from a 3-fold increase for a 17 bp tract to a 6-fold increase for a 42 bp tract.

The observation that poly(dA:dT) stimulates *his3* tran-
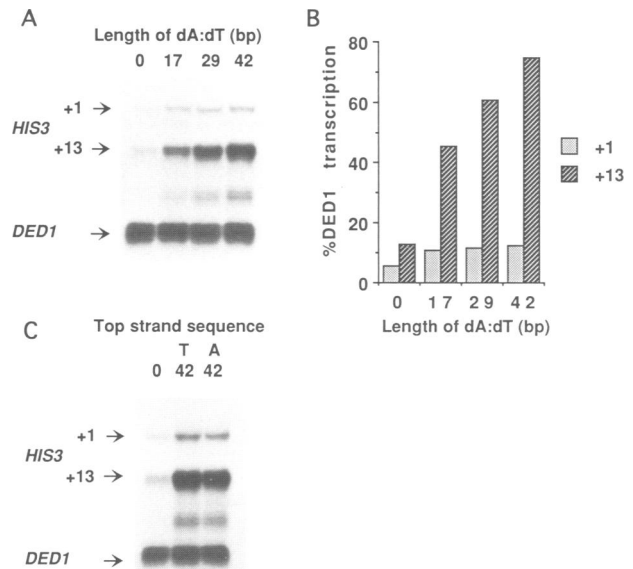
Fig. 2. Poly(dA:dT) stimulates transcription in a length-dependent, orientation-independent manner. RNAs from strains containing the indicated *his3* promoters that were grown in YPD were subjected to quantitative S1 analysis; positions of the *his3* (+1 and +13) and *ded1* transcripts are indicated. (**A**) *his3* promoters with different lengths of poly(dA:dT), all with T residues on the top (coding) strand. (**B**) Quantification of +1 and +13 *his3* RNAs (as % *ded1* RNA levels) indicating that the stimulatory effect of poly(dA:dT) depends on its length. (**C**) Comparison of transcription between promoters having (dA:dT)$_{42}$ with T or A residues on the top strand, showing that the stimulatory effect is independent of orientation.
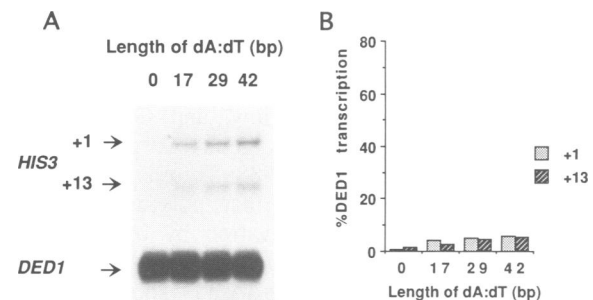
Fig. 3. Effect of poly(dA:dT) in the absence of Gcn4. RNAs from *gcn4-Δ1* strains containing *his3* promoters that were grown in YPD medium were subjected to quantitative S1 analysis; the positions of the *his3* (+1 and +13) and *ded1* transcripts are indicated. (**A**) *his3* promoters with different lengths of poly(dA:dT), all with T residues on the top (coding) strand. (**B**) Quantification of +1 and +13 *his3* RNAs (as % *ded1* RNA levels).

scription primarily from the +13 site is reminiscent of the situation that arises upon Gcn4 activation (Struhl, 1986). As the poly(dA:dT) sequences are located just upstream of the Gcn4 binding site, their stimulatory effect might reflect an increase in Gcn4-dependent activation. Indeed, the high level of *his3* transcription dependent on poly(dA:dT) is not observed in isogenic *gcn4* deletion strains (Figure 3). At this lower level of *his3* transcription, there is a modest stimulatory effect of poly(dA:dT) that increases somewhat with the length of the sequence. Thus, poly(dA:dT) strongly stimulates Gcn4-dependent activation in a length-dependent manner.
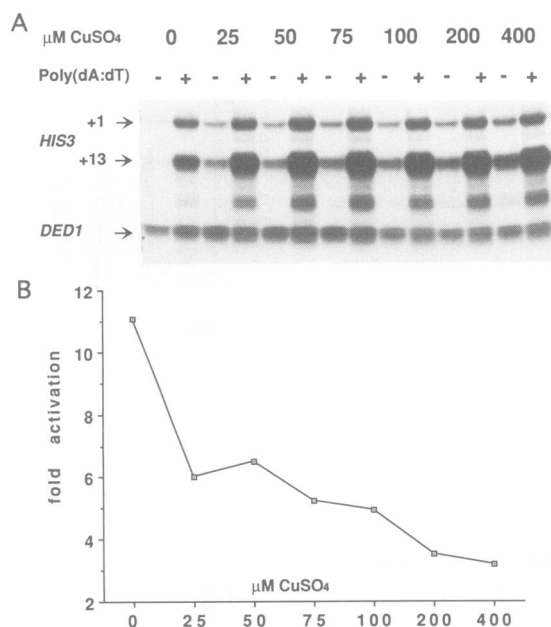
Fig. 4. Stimulation by (dA:dT) varies inversely with intracellular Gcn4 levels. *gcn4-Δ1* strains containing *his3* promoters with or without poly(dA:dT) and harboring a plasmid expressing Gcn4 under a copper-inducible promoter were grown in different concentrations of CuSO₄. (**A**) Quantitative S1 analysis; positions of the *his3* (+1 and +13) and *ded1* transcripts are indicated. (**B**) Fold-stimulation by (dA:dT)₄₂ (ratio of total transcription from the promoter containing the homopolymer to the promoter lacking it) as a function of CuSO₄ concentration.

Fig. 5. Datin acts as a transcriptional repressor through poly(dA:dT) sequences. (**A**) Quantitative S1 analysis of RNAs from *DAT1* or *dat1-Δ* strains containing *his3* promoters with or without (dA:dT)₄₂ that were grown in YPD; the positions of the *his3* (+1 and +13) and *ded1* transcripts are indicated. (**B**) Quantification of +1 and +13 *his3* RNAs (as % *ded1* RNA levels). (**C**) Quantitative S1 analysis of RNAs from *DAT1*, *dat1-Δ* or *DAT1* overexpressing (p*DAT1*) strains containing promoters with or without (dA:dT)₄₂ that were grown in synthetic complete medium lacking tryptophan.
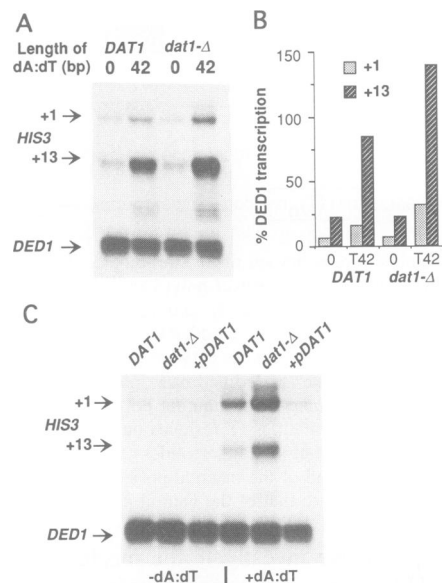
Stimulation by poly(dA:dT) varies inversely with the GCN4 concentration

It is noteworthy that the strong enhancement of Gcn4-dependent activation by poly(dA:dT) occurs when cells are grown in YPD medium. Under these conditions, Gcn4 protein levels are extremely low, and Gcn4-activated transcription of natural target genes is generally not observed (Hinnebusch, 1984, 1988; Thireos *et al.*, 1984). These considerations suggest that poly(dA:dT) might stimulate Gcn4-dependent activation by improving the accessibility of the adjacent Gcn4 binding site, and they predict that the effect of poly(dA:dT) should be more pronounced at low Gcn4 concentrations.

To test this prediction, we expressed Gcn4 from a copper-inducible promoter such that Gcn4 levels could be regulated by varying the concentration of CuSO₄ in the growth medium. With increasing CuSO₄ concentration, increased *his3* transcription was observed from promoters lacking or containing a 42 bp poly(dA:dT) sequence, a reflection of higher Gcn4 levels (Figure 4). However, the fold-stimulation rendered by the presence of the poly(dA:dT) element decreases at higher CuSO₄ concentrations. Thus, the magnitude of the poly(dA:dT) effect on *his3* transcription is inversely related to the amount of Gcn4.

Datin is not required for transcriptional stimulation by poly(dA:dT)

There are two basic hypotheses that could account for the transcriptional stimulatory properties of poly(dA:dT). First, as is the case for all previously characterized eukaryotic promoter elements, poly(dA:dT) might function by binding a specific activator protein. Second,

poly(dA:dT) might function by virtue of its unusual DNA structure. To decide between these hypotheses, we carried out three independent lines of experiments.

First, we considered the possibility that transcriptional activation by poly(dA:dT) might be mediated by Datin, the only known yeast poly(dA:dT)-binding protein (Winter and Varshavsky, 1989; Reardon *et al.*, 1993). Datin specifically recognizes perfectly homopolymeric dA:dT tracts that are >10 bases in length. To analyze the transcriptional role of Datin, promoters that contain or lack (dA:dT)₄₂ were analyzed in *gcn4* deletion strains in which the gene encoding Datin (*DAT1*) was either completely deleted or present in multiple copies (Figure 5). In the absence of datin, there is a reproducible 70% increase in transcription from the promoter containing (dA:dT)₄₂. Conversely, overexpression of Datin leads to a 3-fold decrease in transcription from this promoter. These effects depend on (dA:dT)₄₂ because the promoter lacking this sequence is not affected by the absence or overexpression of Datin. Thus, Datin behaves as a transcriptional repressor that functions specifically through poly(dA:dT) sequences.

Poly(dG:dC) functions in a similar manner to poly(dA:dT)

As a second test, we examined the transcriptional effects of poly(dG:dC), the other homopolymeric DNA sequence. Poly(dG:dC) also has an unusual DNA structure that is marked by inflexibility (McCall *et al.*, 1985), but in many respects its properties are opposite those of poly(dA:dT). Specifically, poly(dG:dC) has a wide minor groove (McCall *et al.*, 1985), and when wrapped into nucleosomes, short dG:dC tracts have the opposite rotational preferences from short dA:dT tracts (Drew and Travers, 1985;
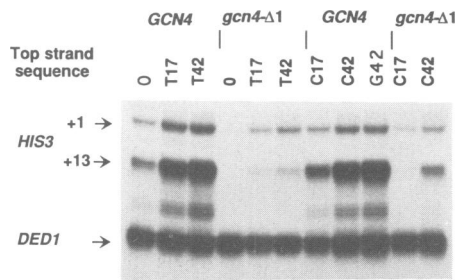
Fig. 6. Poly(dG:dC) acts similarly to poly(dA:dT). RNAs from *GCN4* or *gcn4-Δ1* strains containing *his3* promoters with the indicated poly(dA:dT) or poly(dG:dC) tracts (length of top strand sequence) that were grown in YPD medium were subjected to quantitative S1 analysis; the positions of the *his3* (+1 and +13) and *ded1* transcripts are indicated.
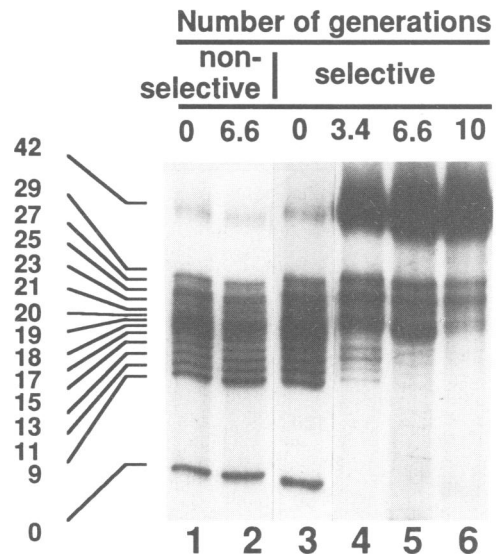

Fig. 7. Poly(dA:dT) function increases continuously with length. PCR analysis of promoter DNA from a mixed culture of strains with different lengths of poly(dA:dT) in the *HIS3* promoter, grown for the indicated number of generations in non-selective (lanes 1 and 2) or selective (lanes 3–6) conditions. Positions of bands corresponding to the different lengths of poly(dA:dT) are indicated on the left. Lanes 1 and 3 are zero time points, showing the relative amounts of the strains at the beginning.

Satchwell *et al.*, 1986). The unusual structures of poly(dA:dT) and poly(dG:dC), though radically different from each other, are both disfavorable to nucleosome formation *in vitro* (Rhodes, 1979; Simpson and Kunzler, 1979; Drew and Travers, 1985; Satchwell *et al.*, 1986; Jayasena and Behe, 1989).

When inserted upstream of the Gcn4 binding site in the *his3* promoter, poly(dG:dC) behaves in a remarkably similar manner to poly(dA:dT). Poly(dG:dC) stimulates Gcn4-activated transcription from the +13 site in both orientations and in a length-dependent manner (Figure 6). Moreover, dG:dC and dA:dT homopolymers of similar lengths stimulate transcription to comparable extents. In addition, poly(dG:dC) is similar to poly(dA:dT) in its ability to stimulate basal, Gcn4-independent transcription. Thus, two very different DNA sequences, which are unlikely to interact with a common activator protein and which affect nucleosome formation *in vitro* as a consequence of their unusual structures, mediate similar transcriptional effects *in vivo*.

Activity of poly(dA:dT) increases continuously with its length

The third experiment to discriminate between the two hypotheses for poly(dA:dT) function was suggested by Leonard Guarente. If the stimulatory function of poly(dA:dT) depends on its intrinsic structure, then the level of transcription should increase continuously as the dA:dT tract is gradually lengthened. Conversely, if transcriptional stimulation is due to a poly(dA:dT)-binding activator, the length-dependent increase should occur in discrete steps corresponding to the number of protein molecules that can simultaneously occupy a given poly(dA:dT) tract. Typically, a single DNA-binding protein occupies 10–20 bp.

We therefore measured *his3* transcription in a set of yeast strains in which poly(dA:dT) tracts in the *his3* promoter were varied by 1 or 2 bp increments. Standard assays involving S1 nuclease analysis or growth rates in a medium containing aminotriazole indicated a length-dependent increase in *his3* transcription, but they were insufficiently accurate to distinguish between the two hypotheses (data not shown). To circumvent this problem, we devised a competitive growth assay in which strains with different poly(dA:dT) lengths were directly compared with each other in the same selective environment. Equal

numbers of cells of the individual strains were inoculated together into a medium containing 10 mM aminotriazole, a competitive inhibitor of the *his3* gene product that preferentially affects the growth of cells with lower *his3* expression levels. At various times during growth of the mixed culture, genomic DNA was isolated and the *his3* promoter region was amplified by PCR. The relative intensity of the bands corresponding to the different lengths of poly(dA:dT) is a measure of the relative growth of the strains. Advantages of this competitive growth assay are that growth rates are internally controlled and that small differences among the various strains are magnified.

Upon continuous growth of the culture in medium containing 10 mM aminotriazole, there is a progressive increase in the relative intensity of bands corresponding to longer poly(dA:dT) elements (Figure 7, lanes 3–6). For example, after three or four generations of selection, bands representing derivatives containing dA:dT tracts of 9, 11, 13 and 15 bp show a relative increase in intensity; after 10 generations of selection, bands representing derivatives containing dA:dT tracts of 19, 20, 21, 25 and 27 show a relative increase. These increases occur only when cells are grown in selective medium; preferential growth of cells with longer poly(dA:dT) elements is not observed in non-selective conditions (lanes 1 and 2). This experiment indicates that poly(dA:dT) function increases continuously with its length rather than in discrete steps, consistent with the hypothesis that the transcriptional stimulatory effects of poly(dA:dT) are due to its intrinsic structure.

Poly(dA:dT) improves the accessibility of the Gcn4 binding site in vivo

To ascertain directly whether poly(dA:dT) increases the accessibility of the Gcn4 binding site in the *his3* promoter,
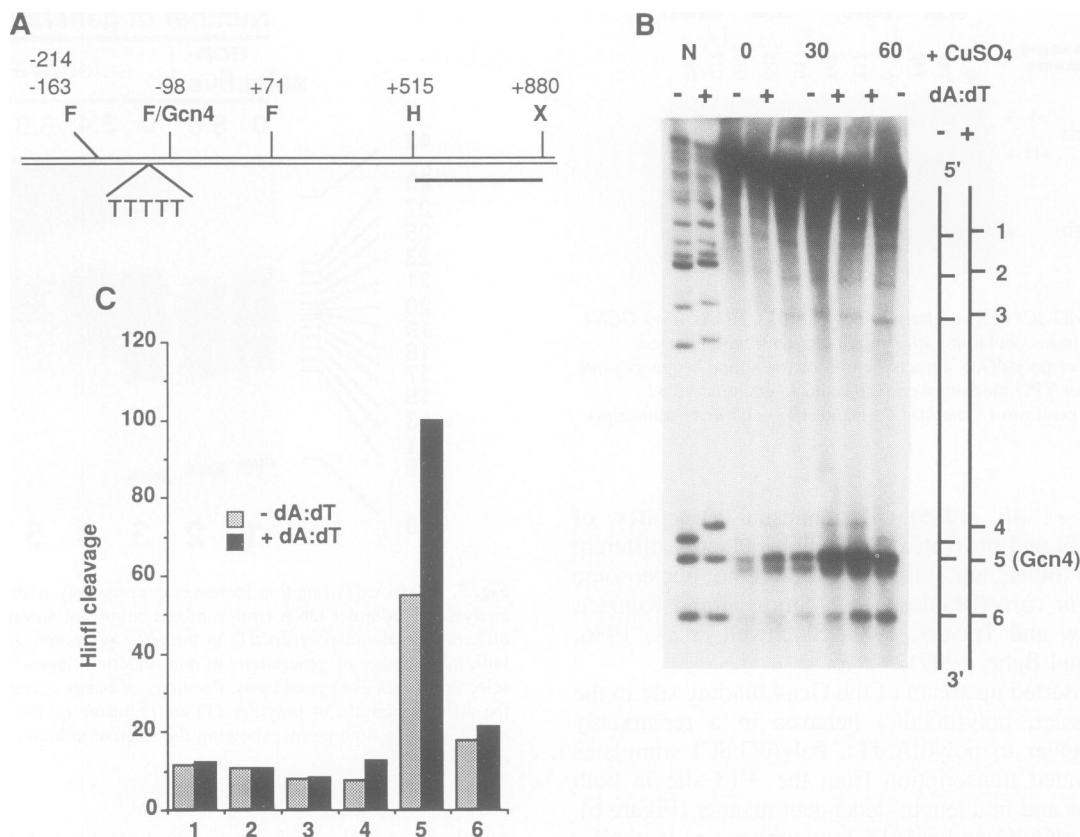
Fig. 8. Accessibility of the Gcn4 site determined by HinfI cleavage *in vivo*. (**A**) Structure of *his3* region from promoters with and without $(dA:dT)_{42}$ with cleavage sites for XhoI (X), HindIII (H), HinfI (F; optimal Gcn4 binding site shown) indicated. Coordinates are listed with respect to the +1 transcriptional initiation site (different coordinates reflect the inserted homopolymer); owing to the Δ101 allele, the relevant HinfI site corresponds to position −497 of the wild-type *his3* gene which actually maps in the *PET56* structural gene. Probe for genomic hybridization (XhoI–HindIII fragment; solid line) is indicated. (**B**) Hybridization analysis to compare Hinfl cleavage in promoters containing or lacking $(dA:dT)_{42}$. Genomic DNA from the indicated *gcn4* deletion strains after inducing HinfI for various times (in minutes) after addition of $CuSO_4$ was digested with XhoI, electrophoretically separated, and hybridized with the XhoI–HindIII fragment of *his3* DNA. Lanes marked N refer to the naked DNA controls. Bands marked 1–6 indicate HinfI cleavage sites; 6 corresponds to +71, 5 corresponds to the optimal Gcn4 binding site, and 4 corresponds to the sites at −163 (−dA:dT) or −214 (+dA:dT). (**C**) Quantification of HinfI cleavage of bands 1–6 in arbitrary units. Approximately 10% of the chromosomes are cleaved at the Gcn4 binding sites at the 60 min time point.

we utilized a novel assay of chromatin structure *in vivo* that will be described elsewhere. In this assay, which is analogous to standard assays performed on isolated nuclei, expression of a restriction endonuclease is induced in yeast cells for a short time, and cleavage sites are mapped in genomic DNA. We utilized HinfI endonuclease because its recognition sequence, GANTC, is present in the core of an optimal Gcn4 binding site (TGACTCA). Thus, the extent of HinfI cleavage is a direct measure of the accessibility of the Gcn4 binding site in the *his3* promoter under physiological conditions.

To induce HinfI endonuclease rapidly without significantly altering the cell physiology, we utilized a copper-inducible promoter based on the Ace1 transcription factor described previously (Klein and Struhl, 1994b). Copper induction involves a peripheral aspect of cellular metabolism, and it occurs by a simple and rapid mechanism, copper-dependent folding of the Ace1 DNA-binding domain (Furst *et al.*, 1988). Using an assay in which cleavage at the Gcn4 site and adjacent HinfI sites in the genome are determined simultaneously (Figure 8A), we compared strains that either contain or lack $(dA:dT)_{42}$ (Figure 8B). Quantitative analysis (Figure 8C) indicates that $(dA:dT)_{42}$ causes a 70% increase in HinfI cleavage

at the Gcn4 binding site (located 16 bp downstream) and a 60% increase at the HinfI site located 57 bp upstream; cleavage at all other HinfI sites is not affected and serves as an internal control. This experiment also reveals considerably more cleavage at the Gcn4 site than at adjacent HinfI sites, an effect that is not observed on purified DNA. This observation, while not yet understood, indicates that the Gcn4 site in the *his3* promoter is relatively more accessible to HinfI than adjacent genomic sites. More importantly, these results provide direct evidence that poly(dA:dT) increases accessibility of the Gcn4 binding site *in vivo*.

To confirm and extend these results, we analyzed chromatin structure in the vicinity of the Gcn4 binding site by two independent methods. First, we expressed *Escherichia coli dam* methylase *in vivo* (Singh and Klar, 1992) and determined the extent of methylation of a suitably located recognition site, GATC (Figure 9). This was accomplished by a primer extension assay on genomic DNA that was digested with restriction endonucleases specific for cleavage of methylated or unmethylated GATC sequences. Methylation was observed to a greater extent in the *his3* promoter containing $(dA:dT)_{42}$ (90% methylated, 10% unmethylated) than on the analogous promoter
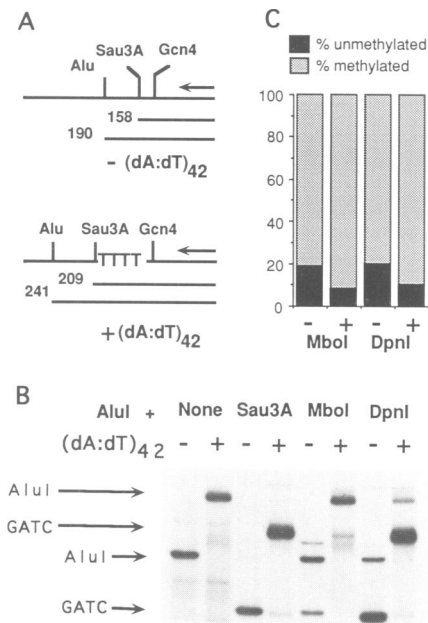
Fig. 9. Analysis of chromatin *in vivo* by *E.coli dam* methylase. Genomic DNA was prepared from *gcn4* deletion strains containing *his3* promoters with or without (dA:dT)$_{42}$ and harboring a plasmid that expresses *E.coli dam* methylase. (**A**) Structures of *his3* promoters with positions of the Gcn4 binding site, *dam* methylase recognition site (GATC, in Figure 1), and *Alu*I site relative to the end-labelled oligonucleotide probe (arrow). The length of primer extension products (in nucleotides) terminating at these sites is indicated. (**B**) Primer extension analysis of genomic DNA that was previously cleaved with *Alu*I and the indicated methylation-sensitive restriction enzymes. Positions of fragments from the promoter lacking (dA:dT)$_{42}$ (short arrows) or containing (dA:dT)$_{42}$ (long arrows) are indicated. (**C**) The level of methylation was determined by calculating the intensity of the *Dpn*I band as a fraction of the sum of the *Alu*I and *Dpn*I bands, and also as the intensity of the *Alu*I band as a fraction of the sum of *Alu*I and *Mbo*I bands in the relevant digests.

lacking this sequence (80% methylated, 20% unmethylated). Second, analysis of micrococcal nuclease sensitivity in permeabilized spheroplasts (Figure 10) indicates that poly(dA:dT) causes increased cleavage at three sites (bands a, b and c) in the immediate vicinity of the element. In contrast, cleavage of numerous sites further upstream or downstream of (dA:dT)$_{42}$ is not affected by the presence or absence of the homopolymeric sequence. Thus, these two independent lines of evidence confirm that the poly(dA:dT) locally alters chromatin structure such that adjacent DNA in the promoter is more accessible to proteins.

Discussion

Poly(dA:dT) function depends on its intrinsic structure, not its interaction with transcription factors

Promoter elements are specific DNA sequences that contribute to the overall transcriptional properties of a given gene. It is generally assumed that promoter elements are recognized by specific DNA-binding transcription factors. Here, we demonstrate that poly(dA:dT) is distinct from all previously characterized eukaryotic promoter elements in that it stimulates transcription *in vivo* by virtue of

its unusual DNA structure, not by binding a specific activator protein.

Three independent lines of evidence argue compellingly against the possibility of a specific DNA-binding protein that activates transcription through poly(dA:dT). First, the only known yeast poly(dA:dT)-binding protein, Datin, actually represses transcription through poly(dA:dT) sequences (Figure 5). While it is experimentally impossible to exclude absolutely the existence of other poly(dA:dT)-binding proteins, it is noteworthy that deletion of the *DAT1* gene eliminates poly(dA:dT)-binding activity in yeast cell-free extracts (Winter and Varshavsky, 1989). Given the extremely high number of poly(dA:dT) tracts in the yeast genome (Struhl, 1985; Karlin *et al.*, 1993; see below), a biologically significant poly(dA:dT)-binding protein is likely to be abundant and hence observed by conventional assays of cell-free extracts.

Second, poly(dA:dT) and poly(dG:dC) homopolymers of similar lengths stimulate transcription to comparable extents (Figure 6). This observation is remarkable given that these two homopolymers have DNA sequences that are completely dissimilar and DNA structures that are radically different from each other and from normal B-form DNA. It is extremely unlikely that poly(dA:dT) and poly(dG:dC) interact specifically with a common protein, or that yeast cells contain distinct poly(dA:dT)- and poly(dG:dC)-binding proteins with strikingly similar transcriptional activation properties. Nevertheless, despite their extensive differences, poly(dA:dT) and poly(dG:dC) share the property of affecting nucleosome formation *in vitro* as a consequence of their unusually inflexible structures. Thus, the striking functional similarity of poly(dG:dC) and poly(dA:dT) provides convincing evidence that the stimulatory effect of poly(dA:dT) arises as a direct consequence of its unusual DNA structure.

Third, poly(dA:dT) function improves continuously when its length is increased by small increments (Figure 7). This observation supports the hypothesis that poly(dA:dT) function is due to its intrinsic structure, because longer dA:dT tracts are progressively more unusual in the context of normal DNA. In contrast, the hypothesis of a poly(dA:dT)-binding protein predicts that the length-dependent increase in transcriptional activity should occur in discrete steps corresponding to the number of protein molecules that can bind simultaneously to a given poly(dA:dT) tract. DNA-binding proteins typically occupy 10–20 bp of DNA, a region that is much larger than the 1–2 bp increments in dA:dT tract length in the *his3* promoters tested here.

The conclusion that poly(dA:dT) functions by virtue of its inherent structure pertains only to perfectly homopolymeric sequences. For imperfect dA:dT tracts, which arise frequently in yeast promoter regions, it is possible that transcriptional effects might be mediated in part or completely by specific DNA-binding proteins. Indeed, the imperfect dA:dT tract in the *ded1* promoter (six imperfections in 34 bp) can stimulate transcription *in vitro* in a manner that is competed by specific oligonucleotides, indicating that it interacts with a specific activator protein (Lue *et al.*, 1989). Thus, the relative contributions of the unusual structure or the protein-binding activity of the *DED1* poly(dA:dT)-like element towards its transcriptional stimulatory properties on RNA polymerase II and T7
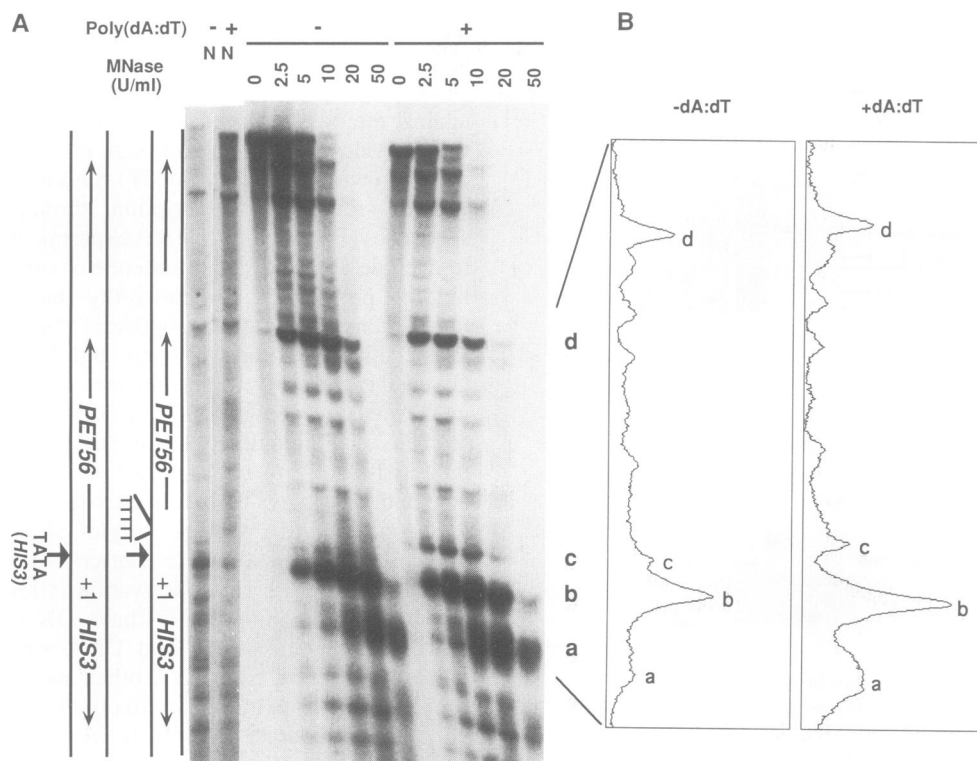
Fig. 10. Analysis of chromatin by micrococcal nuclease cleavage. (**A**) Permeabilized spheroplasts from *gcn4* deletion strains containing *his3* promoters with or without (dA:dT)$_{42}$ along with naked DNA controls (N) were treated with the indicated amounts of micrococcal nuclease and analyzed by hybridization to a *his3* fragment (positions 515–880). The location of the *HIS3*, *PET56* and adjacent 1.7 kb RNAs (Struhl and Davis, 1980) are indicated along with the *HIS3* TATA region and dA:dT. Bands a–d were used for quantitative analysis; band b maps at the *HIS3* TATA region (Struhl, 1982). (**B**) Quantification of autoradiogram with bands a–d indicated.

RNA polymerase *in vivo* (Struhl, 1985; Chen *et al.*, 1987) are not easily assessed. However, the uncertainty in the case of the *DED1* (or other) imperfect poly(dA:dT) tracts does not apply to perfect poly(dA:dT) sequences, and in the experiments of Lue *et al.* (1989), it was notable that a perfectly homopolymeric stretch of 39 residues failed to support transcriptional activation *in vitro* or to compete with the *DED1* dA:dT-like element.

Poly(dA:dT) functions by improving accessibility of the promoter

Our results from a variety of experimental approaches are all consistent with the hypothesis that poly(dA:dT) elements stimulate transcription *in vivo* by improving the accessibility of DNA in the local vicinity to specific transcription factors. Poly(dA:dT) stimulates Gcn4-dependent activation under conditions when Gcn4 levels are sufficiently low to preclude activation of normal target genes. This suggests that poly(dA:dT) increases accessibility of an adjacent Gcn4 binding site in comparison to other Gcn4-binding sites in the genome, thereby allowing the limited amount of Gcn4 to bind and stimulate transcription preferentially from this site. As Gcn4 levels are progressively raised, binding sites are more easily occupied, thus accounting for the decreased effectiveness of poly(dA:dT) in promoting Gcn4-dependent activation (Figure 4). More directly, by determining the extent of *Hin*fI cleavage in yeast cells under physiological conditions, we have demonstrated that poly(dA:dT) increases accessibility of the Gcn4 binding site *in vivo*.

Three independent probes of chromatin structure, *Hin*fI,

E.coli dam methylase and micrococcal nuclease, indicate that poly(dA:dT) increases accessibility of adjacent DNA to proteins (Figures 8–10). The patterns of *Hin*fI and micrococcal nuclease cleavage indicate that the perturbation of chromatin structure occurs in both directions from poly(dA:dT) but is confined to sequences ~100 bp from the element. These perturbations in chromatin structure do not arise indirectly as a consequence of increased transcription because they are highly localized and are generally found in non-transcribed regions. Consistent with this view, the Gcn4 site in the *his3* promoter lacking poly(dA:dT) is preferentially accessible to *Hin*fI even when *his3* transcription is very low (Figure 8), and micrococcal nuclease sensitivity of the *his3* TATA region is not correlated with the amount of transcription (Struhl, 1982; Oettinger and Struhl, 1985).

The observed effects on chromatin structure *in vivo* are directly due to poly(dA:dT), and they are likely to be related to the effects of poly(dA:dT) on nucleosomes *in vitro*. These effects are unlikely to be related to those of bent DNA sequences that contain short, helically phased dA:dT tracts and activate *E.coli* promoters *in vivo* (Bracco *et al.*, 1989) and *in vitro* (Gartenberg and Crothers, 1991). Long poly(dA:dT) tracts are straight and structurally rigid (Nelson *et al.*, 1987), and they do not stimulate transcription of yeast promoters *in vitro* (Lue *et al.*, 1989). The overall similarity of micrococcal nuclease cleavage patterns in the presence or absence of poly(dA:dT) suggests that altered nucleosome phasing or nucleosome-free DNA is not involved. The local perturbation of chromatin structure extends over a region (~200 bp) that is somewhat

Transcription factors
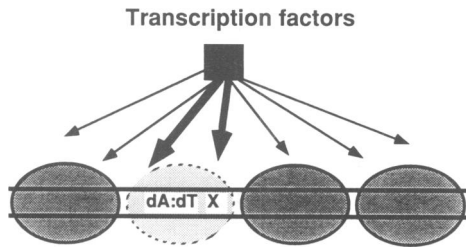
Fig. 11. Model for poly(dA:dT) function. A stretch of DNA (double solid line) containing a poly(dA:dT) sequence and a binding site for a transcription factor (X) is coated by nucleosomes (shaded ovals); the position of the nucleosomes with respect to the dA:dT tract is arbitrarily drawn to reflect the apparent lack of nucleosome phasing. The nucleosome covering the dA:dT tract is shown as being perturbed (lighter oval and dashed line) in comparison to adjacent nucleosomes; this perturbation could reflect decreased stability and/or altered conformation of the nucleosome. DNA sequences covered by this nucleosome (e.g. X) on either side of poly(dA:dT) are preferentially accessible (thicker arrows) to transcription factors (black box). As the position of the perturbed nucleosome can vary with respect to the dA:dT tract, the preferentially accessible region should be somewhat larger than a single nucleosome. See text.

larger than a single nucleosome. An attractive model, therefore, is that a nucleosome covering poly(dA:dT) will be destabilized relative to adjacent and otherwise normal nucleosomes such that it will be less effective in competing with transcription factors for DNA (Figure 11). In this view, longer dA:dT tracts would be more destabilizing to the relevant nucleosome, and the repressive effects of Datin might be rationalized by its occupancy of nucleosome-perturbing sequences.

Our results do not address whether increased accessibility reflects a local decrease in nucleosome occupancy and/or an altered nucleosomal conformation or organization. Whatever the precise mechanism, the local effect on nucleosome formation is quite subtle. However, as normal nucleosomal arrays very strongly reduce acccessibility of proteins in comparison with that observed on naked DNA, a perturbation in chromatin that even slightly reduces this inhibition would be expected to have a significant effect on transcription.

Physiological significance of poly(dA:dT) sequences

Aside from TATA elements, poly(dA:dT) is the most common sequence in yeast promoter regions. Analysis of yeast chromosome III reveals that 40 out of 184 open reading frames contain perfect dA:dT tracts ≥10 bp that are located in promoter regions (defined as ≤1 kb from the ATG initiation codon). Assuming that the frequency of such sequences on chromosome III is typical of the yeast genome in general, this corresponds to ~1500 yeast genes whose promoters contain dA:dT tracts that have been shown here to stimulate transcription. This estimate does not include the long but slightly imperfect dA:dT tracts that frequently occur in yeast promoter regions, and many of these are likely to stimulate transcription by the same mechanism, although perhaps to a lesser extent. For example, a 17 bp dA:dT tract with two imperfections is required for wild-type levels of the divergently transcribed HIS3 and PET56 mRNAs (Struhl, 1985).

For individual yeast promoters, the importance of a poly(dA:dT) sequence(s) will depend on its length, perfec-

tion and location with respect to the TATA and upstream elements. However, there are several examples of natural yeast dA:dT tracts that are required for normal levels of transcription (Struhl, 1985; Lowry et al., 1990; Schlapp and Rodel, 1990; Thiry-Blaise and Loppes, 1990; Schultes and Szostak, 1991). Given their remarkable frequency in yeast promoter regions, it is very likely that poly(dA:dT) sequences are relevant for the expression of a significant fraction of yeast genes and hence play a major role in cell physiology. In addition, poly(dA:dT)-containing promoters could be potentially regulated by changes in the level or activity of Datin. While the role of poly(dA:dT) in other organisms has yet to be established, there are many long dA:dT tracts in humans and other eukaryotic species which have the potential to play important roles in transcription.

Materials and methods

Genetic manipulations

The starting his3 promoter used in these studies (his3-Δ101, 189) contains the wild-type T_R and T_C TATA elements, an optimal binding site for Gcn4, an EcoRI site 3 bp upstream of the Gcn4 site, and a deletion of upstream sequences between -103 and -447 (Tzamarias et al., 1992). Homopolymeric dA:dT or dG:dC sequences of varying lengths were inserted into the EcoRI site as double-stranded oligonucleotides. Except where specifically mentioned, poly(dA:dT) refers to a promoter with T residues on his3 coding strand. All promoter derivatives were integrated at the HIS3 locus of strain KY320 (or its derivatives) by two-step gene replacement (Chen and Struhl, 1988). Strains containing gcn4-Δ1 were generated by two-step gene replacement using YIp56-Sc3674, which lacks a SacI–BamHI fragment encompassing most of the GCN4 structural gene (Engleberg et al., 1994). The URA3 centromeric plasmid containing the copper-inducible promoter used to express Gcn4 and HinfI endonuclease has been described previously (Klein and Struhl, 1994b); the promoter contains a single binding site for the Ace1 activator (Thiele, 1988) upstream of the his3 TATA region at position -85 (Struhl and Hill, 1987). The coding sequence for HinfI endonuclease from positions 1174 to 1989 (Chandrasegaran et al., 1988) was PCR-amplified from a plasmid kindly provided by Keith Lunnen, and cloned downstream of the copper-inducible promoter; this molecule also contains 3' untranslated and transcriptional termination sequences from the GCN4 gene. The Gcn4 derivative expressed from the copper-inducible promoter contains the SV40 nuclear localization signal (Nelson and Silver, 1989) and the HA1 epitope from influenza virus (Field et al., 1988) at its N-terminus (Tzamarias and Struhl, 1994). DAT1 (Winter and Varshavsky, 1989) was cloned by PCR from yeast genomic DNA. dat1Δ::TRP1, which was generated by replacing the 0.8 kb DraIII-NdeI fragment of DAT1 (encodes the entire structural gene) by the AatII-BglII TRP1 fragment of YCplac22 (Gietz and Sugino, 1988), was introduced into yeast by gene replacement. DAT1 was also cloned into YEplac112, a multicopy TRP1 vector (Gietz and Sugino, 1988), for overexpression.

Transcriptional analysis

For most experiments, cells were grown in YPD medium, conditions in which wild-type strains have extremely low Gcn4 levels owing to a translational control mechanism (Hinnebusch, 1984, 1988; Thireos et al., 1984). For the experiment in which Gcn4 levels are regulated by the copper-inducible promoter (Figure 4), cells were grown in glucose minimal medium containing all 20 amino acids in the presence of various concentrations of $CuSO_4$. For experiments concerning the role of Datin (Figure 5), cells were grown in glucose minimal medium containing casamino acids and uracil in order to maintain the TRP1 plasmid. Under these conditions, Gcn4 levels are also extremely low, but they may differ slightly from those occurring when cells are grown in YPD.

Total RNA was prepared from logarithmically growing cells and quantified by absorbance at 260 nM. An amount weighing 20 μg of each RNA sample was hybridized to completion with a 10- to 100-fold excess of ^{32}P-labelled HIS3 and DED1 oligonucleotides and treated with S1 nuclease as described previously (Chen et al., 1987; Klein and Struhl, 1994a). HIS3 RNA levels were quantified with respect to the DED1 internal control using PhosphorImager analysis (Molecular Dynamics).

Each determination represents the average of three experiments; the values are accurate to $\pm 15\%$.

Growth competition assay

gcn4-Δ1 strains containing his3 promoters with dA:dT tracts of varying lengths (0, 9, 11, 13, 15, 17, 18, 19, 20, 21, 23, 25, 27, 29 or 42) were individually transformed with the plasmid containing Gcn4 under the control of the copper-inducible promoter. When grown in medium containing only residual levels of copper, these strains have low levels of Gcn4 that are not affected by amino acid starvation and that maximize the poly(dA:dT) effect on his3 transcription. After separate growth overnight in non-selective medium, approximately equal number of cells of each strain (assayed by A_{600}) were inoculated into a flask containing selective medium (which lacks histidine and contains 10 mM aminotriazole); as a control, a parallel culture was grown in non-selective medium. For each time point, most of the culture was harvested, and genomic DNA was isolated; the remaining cells were allowed to grow further after addition of the same medium. Time points were taken after every 10-fold increase in optical density of the culture. To analyze the composition of the cultures at the various time points, 2 µg of genomic DNA was digested with HindIII and subjected to PCR using 10 ng of ^{32}P-end-labelled oligonucleotide (HIS3 positions $+16$ to $+46$) and 10 ng of unlabelled oligonucleotide (HIS3 positions -548 to -519) as primers. Twenty-five cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min were carried out, and the products resolved on a 5% denaturing polyacrylamide gel.

Analysis of chromatin in vivo by HinfI cleavage

gcn4 deletion strains with or without (dA:dT)$_{42}$ in the HIS3 promoter and containing the plasmid with HinfI endonuclease controlled by the copper-regulated promoter were grown in synthetic complete medium lacking uracil to $A_{600} = 0.7$. gcn4 deletions strains were used to avoid competition between Gcn4 and HinfI for common recognition sites. This HinfI expression plasmid only marginally affects growth in the absence of added CuSO$_4$, but it strongly inhibits growth at a CuSO$_4$ concentration of 250 µM, and cells are inviable at 500 µM. To induce HinfI, CuSO$_4$ was added to the growing culture to a final concentration of 1 mM, and cells harvested for preparation of genomic DNA at various times thereafter. No detectable loss of viability was observed 2 h following the addition of CuSO$_4$, indicating that there are few general deleterious effects during the time-frame (1 h) of the experiment.

To compare HinfI cleavage in strains with and without (dA:dT)$_{42}$, genomic DNA was digested to completion with XhoI which cleaves just downstream of the HIS3 structural gene (position $+880$) and fractionated by electrophoresis on a 2% agarose gel. After transfer to a Nytran filter, the DNA fragments were hybridized with a HindIII–XhoI fragment from the 3' half of the HIS3 gene (positions 515–880) that was labelled with ^{32}P by random priming. As controls, DNA was isolated from parallel cultures of strains with a plasmid lacking the HinfI coding sequence, digested to completion with XhoI, and subjected to partial digestion with HinfI in vitro. The extent of HinfI cleavage in vivo at various sites was quantified using PhosphorImager analysis. Each determination represents the average of two experiments; the values are accurate to $\pm 10\%$.

Analysis of chromatin in vivo with dam methylase

The plasmid expressing E.coli dam methylase, pJS1 (Singh and Klar, 1992), was kindly provided by Dr Jagmohan Singh. gcn4 deletion strains with or without (dA:dT)$_{42}$ in the HIS3 promoter and harboring pJS1 were grown to exponential phase in synthetic complete medium lacking leucine, and the genomic DNA isolated. The extent of methylation in vivo was determined by digesting the DNA with methylation-sensitive restriction enzymes with GATC recognition sites (Singh and Klar, 1992) and by detecting the cleavage products by primer extension (Huibregste and Engelke, 1991). DNA was first digested with AluI which cleaves upstream of the GATC sequence near the Gcn4 binding site, and then with either Sau3AI (which cleaves regardless of methylation state), MboI (which cleaves only unmethylated DNA) or DpnI (which cleaves only dimethylated DNA). Reactions were done overnight using 100-fold excess of enzyme to ensure complete digestion. Primer extension was performed using 2 ng of a ^{32}P-end-labelled oligonucleotide (HIS3 positions $+16$ to $+46$) and 500 ng of digested genomic DNA as the template. Thirteen cycles of denaturing at 94°C for 2 min and annealing and extension at 72°C for 6 min were carried out, and the products resolved on a 6% sequencing gel and quantified using PhosphorImager analysis. The percentage methylation was determined from three independent experiments, and the values are accurate to $\pm 1\%$.

Chromatin analysis with micrococcal nuclease

gcn4 deletion strains with or without (dA:dT)$_{42}$ were grown to exponential phase in YPD medium, washed in 1 M sorbitol, and spheroplasted with yeast lytic enzyme (ICN, 70 000 U/g at a final concentration of 1 mg/ml) in 1 M sorbitol, 28 mM β-mercaptoethanol at 30°C for 20 min. After washing twice in buffer (1 M sorbitol, 50 mM NaCl, 10 mM Tris pH 7.5, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM β-mercaptoethanol and 0.5 mM spermidine), spheroplasts were permeabilized with the non-ionic detergent NP-40 (0.075% final concentration) which was added along with various concentrations (0–200 U/ml) of micrococcal nuclease (Sigma) as described by Kent et al. (1993). For naked DNA controls, deproteinized DNA from parallel cultures were treated with appropriate concentrations (0.1–10 U/ml) of micrococcal nuclease in the same buffer and processed identically. Reactions were set up on ice, transferred to 37°C for 5 min, and then stopped by the addition to 0.5% SDS, 25 mM EDTA; the samples were treated with RNase A and proteinase K, extracted twice with phenol–chloroform, and precipitated. This procedure generates a 160 bp nucleosomal ladder as evidenced by ethidium staining of the DNA on agarose gels. Sites of micrococcal nuclease cleavage in the HIS3 promoter region were mapped by with respect to the XhoI site (position 880) as described above for HinfI endonuclease cleavage in vivo. Southern blots were visualized by autoradiography and also scanned for quantification on the PhosphorImager.

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