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Short Communication

A Nucleosome-Free dG-dC-Rich Sequence Element Promotes Constitutive Transcription of the Essential Yeast *RIO1* Gene

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***RIO1* is an essential gene that encodes a protein serine kinase and is transcribed constitutively at a very low level. Transcriptional activation of *RIO1* dispenses with a canonical TATA box as well as with classical transactivators or specific DNA-binding factors. Instead, a dG-dC-rich sequence element, that is located 40 to 48 bp upstream the single site of mRNA initiation, is essential and presumably constitutes the basal promoter. In addition, we demonstrate here that this promoter element comprises a nucleosome-free gap which is centered at the dG-dC tract and flanked by two positioned nucleosomes. This element is both, necessary and sufficient, for basal transcription initiation at the *RIO1* promoter and, thus, constitutes a novel type of core promoter element.**

Key words: Chromatin analysis (*in vivo* footprinting) / Constitutive promoter / Essential gene / Gene shuffling / Promoter analysis / Protein serine kinase / *Saccharomyces cerevisiae*.

RIO1 is an essential gene which codes for a protein serine kinase highly conserved from Archaea to man (Angermayr *et al.*, 2002a; Angermayr and Bandlow, 2002). The biological role of *RIO1* and members of the *RIO1*-like kinase family is largely unknown. In *Saccharomyces cerevisiae* Rio1p plays an essential role in cell cycle progression, *i.e.* in the transition from G1 to S phase and in metaphase, before onset of anaphase. However, there are indications that Rio1p influences 18S rRNA maturation and certain steps in the assembly of the small ribosomal subunit as well (Vanrobays *et al.*, 2002).

RIO1 is transcribed constitutively at an extremely low level (Angermayr and Bandlow, 1997a,b). In contrast to core promoters of regulated genes (*e.g.* Chen and Struhl, 1988; Harbury and Struhl, 1989), little is known about the molecular basis of constitutive gene expression, and the DNA sequence elements involved are poorly defined. The *RIO1* promoter shares an 869 bp comprising upstream region with the divergently transcribed *GCY1* gene. This intergenic region provides an interesting model to study gene-specific regulation. Expression of *GCY1* is induced 25-fold by growth on galactose due to the binding of the galactose-inducible transactivator Gal4p to a single upstream activating sequence (UAS_{GAL}). Although Gal4p acts bidirectionally and over long distances, expression of *RIO1* is not influenced by this specific transactivator. Previous work (Angermayr and Bandlow, 1997a, 2003) has shown that the core promoter of *RIO1* allows assembly of a basal transcription complex that differs from the one recruited to the TATA box of *GCY1* and that does not respond to Gal4p. Here, we studied the molecular basis of the constitutive activation of the *RIO1* promoter.

Apart from the remote UAS_{GAL} (in front of *GCY1*), we found several remarkable nucleotide sequences in the region 5'-upstream of the *RIO1* gene, *i.e.* two closely spaced poly(dA:dT) tracts, as well as each a GC- and an AT-rich sequence block; however, a canonic TATA motif was absent. In a first attempt to define the minimal promoter required for constitutive transcription of *RIO1*, we truncated the intergenic region of *GCY1* and *RIO1* stepwise from the *GCY1* side. Since *RIO1* is essential, we could not create mutations that yield a non-functional promoter into the genuine genomic context. So we decided to use plasmid-based *lacZ* reporter constructs. In previous studies on the *GCY1* promoter, initiating a transcript divergent to *RIO1* (Angermayr and Bandlow, 2003), as well as on the *PFY1*-promoter (Angermayr *et al.*, 2003), we had observed that *lacZ* expression data obtained with plasmid-borne wild-type and mutant constructs fully paralleled transcript levels of the same mutant promoters in the genuine genomic background (however, *GCY1* and *PFY1* are non-essential genes in contrast to *RIO1*). We ligated the respective shortened promoter elements to pYLZ2 as a *lacZ* expression plasmid (Hermann *et al.*, 1992) and determined β-galactosidase activities (Angermayr and Bandlow, 1997b; Figure 1A). The promoter of *RIO1* is extremely weak (Angermayr and Bandlow, 1997a). On the basis of the transcriptome analysis, it has been calculated that the concentration of *RIO1*-mRNA in the steady-state is about one molecule per cell or even less (Holstege *et al.*, 1998). In the wild type, *RIO1*-mRNA

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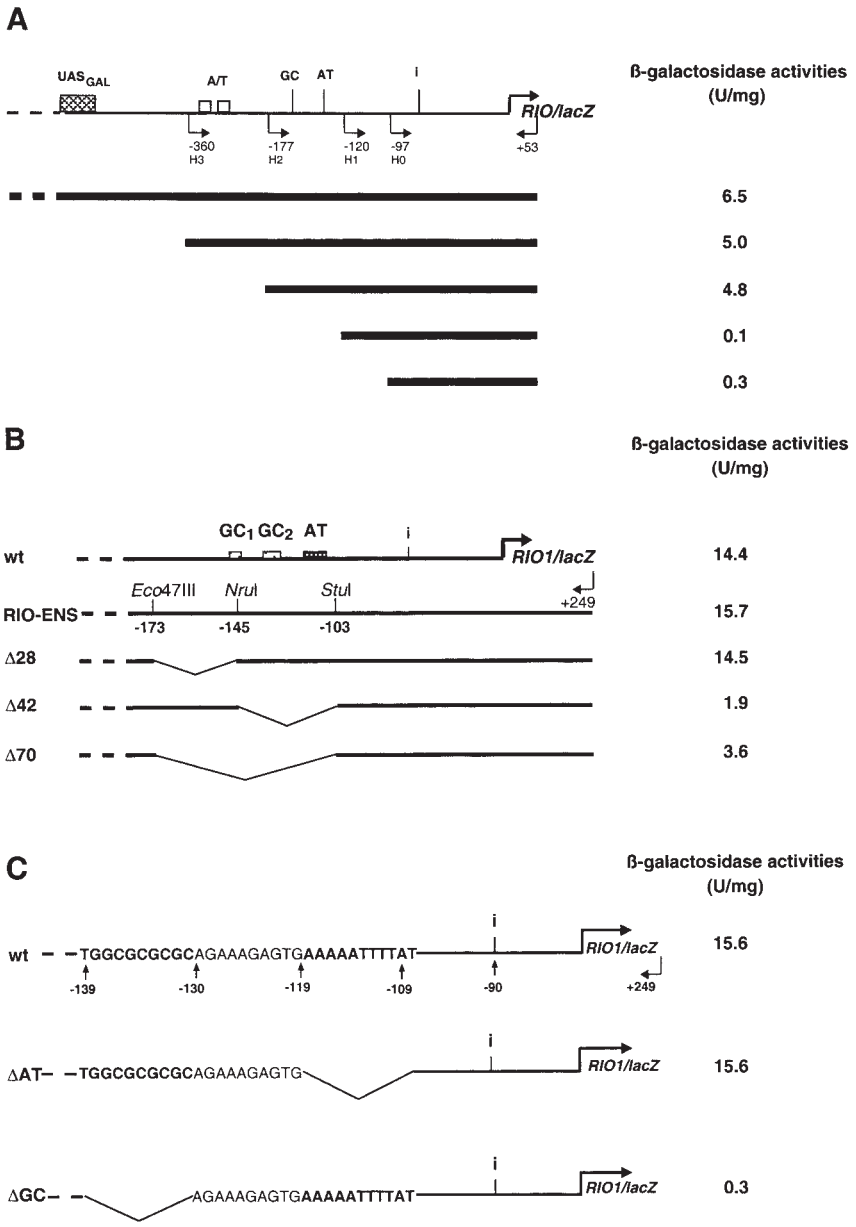

Fig. 1 β-Galactosidase Activities of Various Mutants of the *RIO1* Promoter.
(A) Schematic drawing of the promoter of the *RIO1* gene. Forward primers for the truncations and their positions are indicated. The right column gives β-galactosidase reporter activities (Angermayr and Bandlow, 1997a,b) of the yeast reporter plasmid pYLZ2 (Hermann *et al.*, 1992). (B) Introduction of restriction sites into the presumptive core promoter of *RIO1* and measurement of the respective reporter activities. GC- and AT-rich sequences are indicated. (C) Deletion of short sequence blocks and respective β-galactosidase reporter activities. Reporter activities differ between the experiments shown in (A) and (B, C) due to different constructs using different reverse primers (the construct in B and C contains a longer 5'-coding part of *RIO1* than in panel A).

escapes detection by Northern blotting, and expression of *RIO1* promoter-*lacZ* fusions from low copy plasmids hardly differs from background. For this reason, all reporter constructs were expressed from the multi-copy plasmid pYLZ2.

Truncation of the *RIO1* promoter by means of the forward primers H2, H3 or H4, reveals that reporter expression stays at a relatively constant level (Figure 1A) indicating that no important *cis* element lies within this region. This observation furthermore shows that the two (dA:dT) blocks, lying between primers H2 and H3, have no significant bearing on the expression of *RIO1*. Transcription of *RIO1* is eliminated when the region between positions –177 and –120 is deleted (primer H1). Thus, the *cis*-element essential for expression of *RIO1* lies within a 57 bp interval between positions –177 and –120. Transcriptional activation of unregulated genes frequently employs 'general regulatory factors'. These are highly abundant and frequently assist to recruit other factors and/or play an architectural role. They are easily detected both, by their strong retention signal in electrophoretic mobility shift assays (*e.g.* McLean *et al.*, 1995; Anger-

mayr and Bandlow, 1997b; Angermayr *et al.*, 2003) and/or *in silico* by their well-defined DNA-binding motifs. However, we did not find any indications for a specific transactivator protein binding to the promoter region of *RIO1*. The apparent absence of binding of a classical transactivator or general transcription factor argues that synthesis of *RIO1* mRNA exclusively is basal transcription.

To narrow down the promoter requirements for expression of *RIO1*, we introduced restriction sites into the above determined region by *in vitro* mutagenesis taking care of minimal sequence alterations. These constructs differed from those shown in Figure 1A in that a different reverse primer was used which annealed at nucleotide positions +249 to +231, whereas in the constructs displayed in Figure 1A the reverse primer was complementary to positions +53 to +35. The longer construct yielded higher expression as the respective fusion protein proved to be proteolytically more stable. We inserted an *Eco*47III site at the 5'-end (position –173), and a *Stu*I site at the 3'-end of the essential promoter of *RIO1* (position –103). In addition, we introduced an *Nru*I site at position –145. By these means, we were able to delete the whole essential promoter region on the one hand, and on the other hand eliminate either half of these sequences (Figure 1B). Neither the restriction sites introduced, nor the deletion of the most upstream 28 bp of the 70 bp comprising segment in question influenced promoter activities of *RIO1*. However, when we deleted the 42 bp *Nru*I/*Stu*I fragment, promoter activity was completely impaired. The same was the case for deletion of the 70 bp total promoter region.

The 42 bp segment essential for expression of *RIO1* excels by containing a block of nucleotides with extremely high GC content followed by an AT-rich stretch. It is located 13 to 55 bp 5' from the single transcriptional initiation site at position –90 (Angermayr and Bandlow, 1997b). In yeast, transcription usually starts about 40 to 120 bp downstream the core promoter. Therefore, it seems plausible that this region comprises the core promoter of *RIO1*. To test this possibility, we eliminated either the GC-rich or the AT-rich block by site specific *in vitro* mutagenesis (Figure 1C). The same reverse primer was used as described for Figure 1B. Deletion of the AT-rich sequences had no effect on *RIO1*/*lacZ* expression. However, deletion of the GC-block destroyed promoter activity completely. Thus, the eight bp-comprising GC block constitutes an essential promoter element, presumably the basal promoter specifically required for transcription of *RIO1*.

In order to obtain evidence that the GC-rich sequence block has functional importance for the expression of *RIO1 in vivo*, we performed a 'gene shuffling' experiment. A strain disrupted for *RIO1* was rescued by a wild-type copy of the gene on a *CEN*-based *URA3* plasmid (YMA51; Angermayr *et al.*, 2002a). In addition, it harboured a second (*LEU2*)-plasmid containing either a wild-type copy, too (control), or a deletion of the GC- or

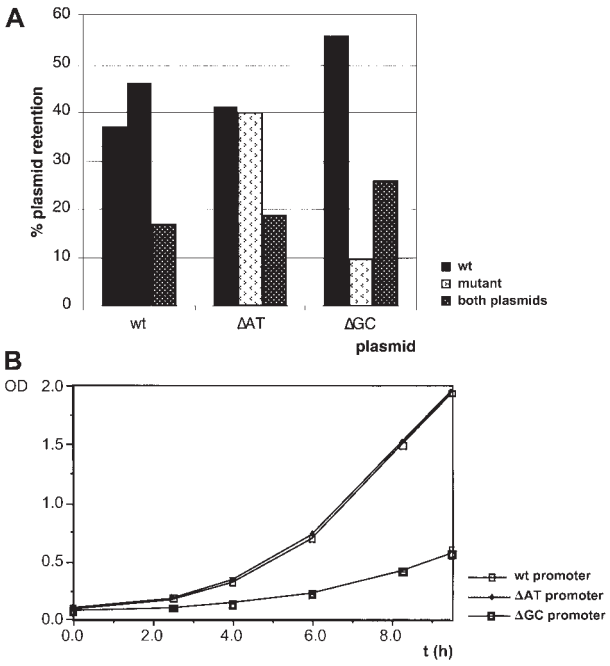

Fig. 2 Plasmid Segregation Test under Non-Selective Conditions.
A strain deleted for *RIO1* was rescued by a *RIO1* wild-type copy on a *CEN*-based *URA3* plasmid (YMA51) and simultaneously contained either a wild-type *RIO1* (control) or ΔGC or ΔAT promoter deletion mutant on a *LEU2 CEN* plasmid. The two plasmids were allowed to segregate for 30 generations in complete medium and then tested for the markers retained (panel A). Panel (B) displays growth curves of YMA52 (wild-type control) and one of the segregants containing either the ΔGC or the ΔAT mutation. Identical results were obtained with two other segregants each.

AT-rich blocks. Subsequent segregation was performed in the presence of 5-fluoro-orotate selecting against the presence of the *URA3* plasmid, under semi-selective or under non-selective conditions with similar results. Figure 2A shows the data obtained under non-selective conditions which are most meaningful as they reflect the importance of the respective plasmid for sustaining cell viability. In the control, either of the two wild-type *RIO1* copies are lost with comparable probability, although loss of the *URA3* plasmid generally is slightly more frequent (compare with Angermayr *et al.*, 2002a). The plasmid carrying the deletion of the AT block is retained at similar frequency as wild type, and its retention as the sole *RIO1* plasmid displays no phenotype (not shown). The plasmid harbouring the deletion of the GC block is retained in a few rare occasions as the sole *RIO1* copy, but the phenotype of these cells is very sick and closely resembles cells deprived of Rio1p (after shift from galactose to glucose medium of a *RIO1* deleted strain rescued by *RIO1* under guidance of the *GAL10* promoter): giant wrinkled cells, frequently arrested in G1 (not shown here, but compare with Angermayr *et al.*, 2002a). In addition, these cells display an extreme slow growth phenotype (Figure 2B) and enter stationary phase at a very low titre

(2×10^7). These observations demonstrate the importance of the GC-rich sequence block for the expression of Rio1p also *in vivo*, although its deletion allows survival at the border of viability. Similarly, residual transcription at a very low level has been observed with other genes after deletion of their TATA core promoter (*e.g.* Angermayr and Bandlow, 1997a). In addition, yeast cells frequently harbour more than one copy of CEN-plasmids (1 – 3 copies per cell). Therefore, more than one copy of *RIO1* under the mutant promoter likely sustains viability.

Next we examined the chromatin structure at the *RIO1* promoter in the genomic context. Generally, unregulated transcription reflects a static situation. It is assumed that constitutive promoters permanently are in an active state and kept free of nucleosomes and, thus, accessible to the basal transcription machinery. In chromatin, normal B-helical DNA is wrapped around histone octamers which usually are arranged in regular nucleosomal structures (Workman and Kingston, 1998; Becker and Hörz, 2002). To meet the specific requirements for transcriptional gene activation, core promoter sequences of constitutive promoters are presumed to lie in a nucleosome-free gap of the chromatin. Core promoters of actively transcribed genes have been found to be free of nucleosomes (*e.g.* Fascher *et al.*, 1990; Piña *et al.*, 1990). Revealingly, it has been observed that experimentally induced depletion of yeast cells of histone H4 leads to maximal activation of several genes even in the absence of activator (Han and Grunstein, 1988; Durrin *et al.*, 1992). This shows that in these instances the major task of the respective transcription factor(s) is to affect or mediate removal of repressing nucleosome(s) and that basal transcription can dispense with classical transactivators as long as the basal promoter provides a freely accessible platform for the assembly of the basal transcription complex.

The absence of binding sites for architectural proteins or classical transcription factors led us to presume that the DNA sequence or structure at the basal promoter could directly be responsible for providing the prerequisites for the assembly of the basal transcription complex. Therefore, we examined whether the promoter in fact is free of nucleosomes. We analysed the chromatin structure at the *RIO1* promoter by DNaseI or MNase digestion (Figure 3). In 'naked' DNA, the entire promoter region is readily sensitive to digestion by either nuclease indicating that it does not contain sequence-determined constraints hindering nucleolytic degradation. Also in native chromatin, the core promoter is freely accessible to nucleolytic attack by either nuclease and, consequently, is free of nucleosomes. Apparently, no additional DNA-binding proteins are necessary to remove nucleosomes in order to enable access of the basal transcription complex to the core promoter. The nucleosome-free gap spans about 130 bp of promoter DNA. It is centered at the GC-rich core promoter and comprises the transcriptional initiation site as well.

Upstream of the hypersensitive site, a region follows to the 5'-side of the promoter that is not well cleaved by either nuclease in 'naked' DNA. In chromatin, however, the

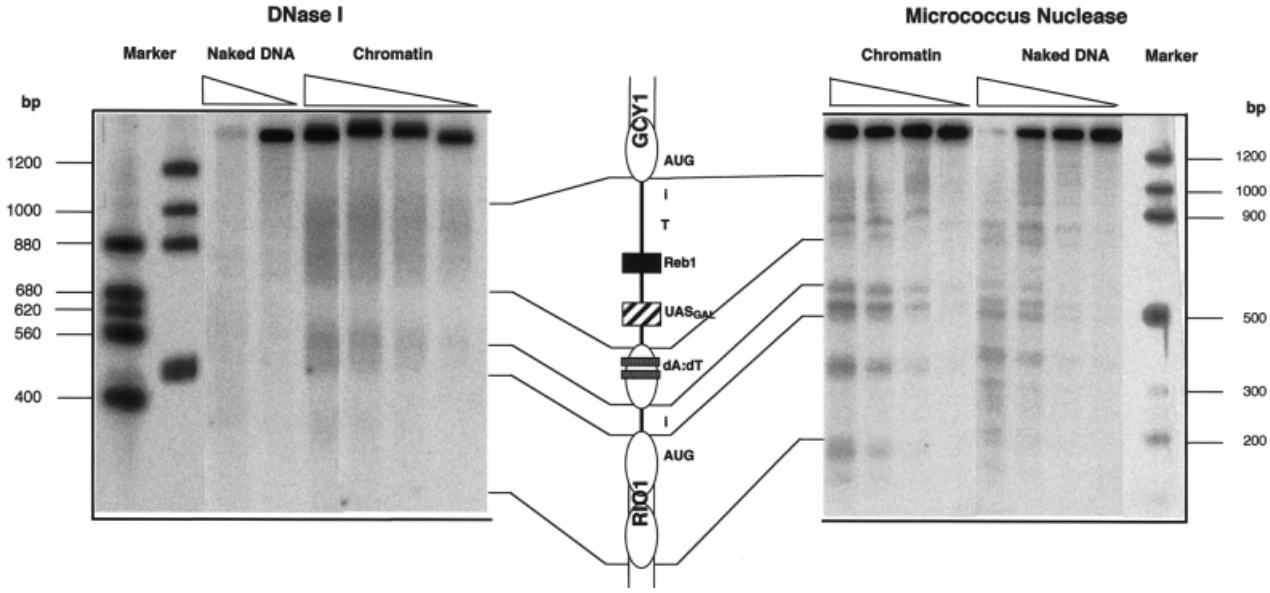

Fig. 3 Chromatin Structure Analysis of Promoter and Coding Region of *RIO1* in the Genomic Context.
Native chromatin of the wild type was digested with increasing concentrations of DNaseI or micrococcal nuclease (Thoma, 1996), and DNA then deproteinated, treated with restriction endonuclease to create defined ends, electrophoresed and detected by indirect end-labelling (see Angermayr *et al.*, 2002b and Angermayr and Bandlow, 2003 for details). DNA fragment length standards are given at the margins. The results are illustrated by the corresponding schematic drawing in the centre. Ellipses symbolise nucleosome-protected DNA, wide open bars indicate coding, narrow bars depict non-coding hypersensitive regions. The TATA box (T), the Reb1p-binding site and the Gal4p box of the adjacent *GCY1* promoter as well as the position of two dA:dT 13-meric blocks, the transcriptional initiation sites (i) and the AUG translational initiation sites are indicated.

nuclease-insensitive region is much wider and significantly extends in both 5' and 3' direction relative to 'naked' DNA, indicating that in chromatin it is protected by a nucleosome veiling about 150 bp of DNA. This nucleosome is centered at the 21 bp spacer between two 13-meric (dA:dT) blocks and, as the boundaries of the protected sequence are relatively sharp, appears to be positioned. Packaging of dA:dT sequences into nucleosomes is controversially discussed. It has been reported that DNA containing dA:dT blocks is incompatible with wrapping around nucleosomes due to their rigid structure (Struhl, 1985; Iyer and Struhl, 1995); however, arrangement of short tracts of homooligomeric dA:dT sequences in a nucleosomal structure is perfectly possible (Kunkel and Martinson, 1981). Thus, it appears that the 21 bp of spacer DNA between the two homooligomeric dA:dT blocks helps to position the single upstream nucleosome (see schematic drawing in Figure 3).

The two nucleosomes flanking the gap on the 3'-side protect 150 bp of DNA each. The boundaries are sharp, and the spacer is narrow so that they seem to be positioned as well. Thus, the core promoter lies in a gap between two positioned nucleosomes.

Several principles have been found to govern preferred positioning of nucleosomes and creation of nucleosome-free gaps. Sequence-dependent binding of two nucleosomes to two adjacent sites of prebent DNA may lead to translational positioning of nucleosomes and allow access of transcriptional activators to the extended linker between them. The linker DNA may, however, be too short to allow the binding of an additional nucleosome. Such an arrangement is the basis of accessibility of linker DNA to specific transactivators in several promoters (Fascher *et al.*, 1990; Piña *et al.*, 1990; Travers, 1990; Archer *et al.*, 1991; Straka and Hörz, 1991; Angermayr *et al.*, 2002b). As an alternative, poly(dA:dT) sequences have been observed to deviate from the B conformation of DNA and to be incompatible with packaging into nucleosomes due to their rigid DNA structure (Struhl, 1985; Chen *et al.*, 1987; Tanaka *et al.*, 1992; Iyer and Struhl, 1995). However, GC-rich sequences may display similar incompatibilities (Iyer and Struhl, 1995; Angermayr and Bandlow, 2003).

By DNA permutation analysis (Zinkel and Crothers, 1987), we failed to detect any indication of extensive structural DNA deformation or bending of the GC block (not shown) which could be the cause for nucleosome exclusion. However, it has been reported that in dG-dC-rich sequences the DNA structure is distorted in a way that the minor groove is wider than in random DNA (Yoon *et al.*, 1988), but presumably the deformation is too small to be detected by permutation analysis. Since on the one hand, the TATA-binding protein (TBP) binds to the minor groove (Lee *et al.*, 1991; Starr and Hawley, 1991) and bends it towards the major furrow (Horikoshi *et al.*, 1992; Starr *et al.*,1995) and since on the other hand, prebending of a core promoter sequence enhances the affinity of the basal transcription complex to DNA (Parvin *et al.*, 1995), an enlarged minor groove could facilitate recruitment of transcription factor IID (TFIID) to dG-dC sequences and allow transcription initiation even in the absence of transactivators. dG-dC-rich sequences have indeed been found to allow assembly of TFIID and to promote transcription *in vitro* (Singer *et al.*, 1990), and experimental evidence suggests that the TATA-binding protein-associated factor (TAF) composition of this TFIID differs from the one binding to the TATA box of the adjacent Gal4p-controlled *GCY1* promoter due to the difference in the structures of the TATA-containing and the GC-rich core promoters (Angermayr and Bandlow, 1997a). However, creation of a consensus TATA box in front of *RIO1* from the naturally occurring sequence TATAGA by a single base pair exchange increased expression about threefold and rendered it susceptible to induction by galactose (about one hundred-fold; Angermayr and Bandlow, 1997a). This finding demonstrates a principal difference of the two types of core promoters in their responsiveness to regulatory factors and indicates that the type of core promoter is responsible for the low constitutive transcription of *RIO1* and for the inability of the basal transcription machinery to interact with the adjacent Gal4p site.

The deviation of prebent dG-dC-rich DNA from the normal B-conformation could also be the cause for nucleosome exclusion. On the other hand, we have shown that the GC-rich promoter element is flanked by two positioned nucleosomes. However, the two 5'-upstream dA:dT blocks probably play only an accessory role in positioning the upstream nucleosome, as their deletion has no effect on Rio1/lacZ expression (Figure 1A). The intervening gap (130 bp) may be too narrow to accommodate an additional nucleosome (*cf.* Fascher *et al.*, 1990; Angermayr *et al.*, 2002b, as examples) providing an alternative or additional reason for the existence of the nucleosome-free gap.

Thus, two minimal parameters determine the strength of basal transcription in the absence of transcription factors: (i) accessibility of the core promoter to the basal transcription complex in a nucleosome-free gap of the chromatin and (ii) the structural deformation of core promoter DNA in order to promote binding of TFIID. In line with this conclusion, on the basis of extensive mutational analysis of the basal promoter for Gcn4p-independent transcription of the *HIS3* gene it has been postulated that structural rather than sequence peculiarities of the core promoter DNA are responsible for promoter recognition and basal transcription initiation *in vivo* and *in vitro* (Mahadevan and Struhl, 1990).

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