The interaction of $\omega_2$ with the RNA polymerase $\beta'$ subunit functions as an activation to repression switch

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Annex 1.
Partial $P_{\omega}$ occupancy by $\omega_2$ activates transcription

Protein $\omega_2$ has a high affinity for operators that contain at least two consecutive heptads. This affinity increases when four or more heptads are involved ($K_{\text{Dapp}} 5 \pm 1$) (1). However, with a single heptad or with heptads separated by one or more base pairs, binding is extremely poor ($K_{\text{Dapp}} > 800 \text{ nM}$) (1). The affinity also depends on heptad organization and is maximal for converging ($\rightarrow\leftarrow$, $K_{\text{Dapp}} \sim 20 \text{ nM}$), while lower for direct ($\rightarrow\rightarrow$, $K_{\text{Dapp}} \sim 40 \text{ nM}$) and diverging ($\leftarrow\rightarrow$, $K_{\text{Dapp}} 120 \text{ nM}$) heptad arrangements (1). The $\omega_2$ protein does not distort the DNA structure upon binding to $\rightarrow\rightarrow$ or $\leftarrow\leftarrow$ DNA (2). These structures show that a pair of positively charged, antiparallel $\beta$ strands of $\omega_2$ insert into the major groove of DNA and establish sequence-dependent contacts to symmetric or asymmetric repetitive sequences with a 0.3 Å deviation with respect to the central C-G pair of each repetition (2-5). The interaction between RNAP-$\sigma^\Lambda$ and $P_\omega$ DNA was enhanced with sub-stoichiometric concentrations of $\omega_2$ (Fig. 2). We hypothesised that, under these conditions, $\omega_2$ would preferentially bind to heptads with a $\rightarrow\leftarrow$ configuration (e.g, heptads 6 and 7) (Fig. S1 and 3A). These heptads overlapped the -35 element and the spacer region (positions -34 to -21). To test this hypothesis, the heptad 6 ($\leftarrow$, 5’-TGTGA-T-3’), 7 ($\leftarrow$, 5’-TaaTcT-3’), or heptads 6 plus 7 ($\leftarrow\leftarrow$, 5’-TGTGA-TTaaTcT-3’) were mutagenized. Run-off transcription experiments were then performed with each. As a control, equivalent mutations were introduced in heptad 1 ($\rightarrow$), 2 ($\rightarrow$) or heptads 1 plus 2 ($\rightarrow\rightarrow$) (Fig. S1 and 3A). With the exception of mutated sequences of heptads 6 plus 7 (which included mutations within the -35 element), RNAP-$\sigma^\Lambda$ transcribed the $P_\omega$ variants nearly as well as the wt in the absence of $\omega_2$ (data not shown). Similar levels of transcription were observed in the presence of limiting or stoichiometric amounts of $\omega_2$. This result suggests that the selective occupancy of heptads 6 and 7 play a minor role, if any, in $\omega_2$ regulation of $P_\omega$ utilization.

Annex 2.
Proteins $\omega_2$ or $\omega 2$ repress $P_\delta$ utilization

The $\omega_2$ protein has three functional regions: i) the unstructured NTD (residues 1-19) required for the $\omega_2\cdot\delta_2$ interaction (4); ii) the $\beta$-sheet domain (residues 28-32), which is necessary for DNA binding (2,3,5); and iii) the $\alpha$-helix $\alpha_1$ (residues 34-46) which in concert with the $\alpha$-helix $\alpha_2$ domain (residues 51-64) contribute to monomer-monomer and dimer-dimer interfaces (2,3). Whether or not residues 10-28 and the C-terminal residues 65-71 have anything more than a structural role to play is unknown (3,6).

Members of the superfamily of $\omega_2$ regulators can be divided into two sub-families, of which $\omega$ and $\omega_2$ are representative (7,8). Monomeric $\omega_2$ (79-residues long) shares 98% identity with $\omega$ (71-amino acids) within the first 55 residues, but the degree of identity drops to 18% in the remaining 24 residues (Fig. S7A). With the aim of mapping the $\omega_2$ domain involved in the interaction with RNAP-$\sigma^\delta$, and to test whether a similar mechanism of action applies to other $\omega_2$ regulated promoters, we compared $P_\delta$ promoter binding and consequent transcription repression activity, in vivo, of $\omega_2$, $\omega_2$ or $\omega_2$ΔN19, which lacks the 19 first amino acids (4). A single copy of $P_\delta$ was fused to the promoter-less $lacZ$ gene and integrated into the amyE locus of the $B. subtilis$ genome as a unique copy. This operation was performed in the BG508 strain (9). This construct was used to measure the effect of these $\omega$-like genes in trans. The results of $\beta$-galactosidase activity assays showed that $\omega_2$ repressed $P_\delta$ mediated transcription to levels comparable to those of $\omega_2$ or $\omega_2$ΔN19 (Fig. S7B) (2,4,9). The suggested that the dimer is the functional form of $\omega_2$ and that the different C-terminal domains of $\omega$ and $\omega_2$ are not involved in gene repression. In vivo experiments also revealed an increase in the repression of $P_\delta$ utilization in the presence of both $\omega_2$ and $\delta_2$ (Fig. 7B), suggesting that these proteins act in concert.

Annex 3.

The central and C-terminal regions of $\omega_2$ might not interact with RNAP-$\sigma^\delta$

The strain bearing the $P_\delta;lacZ$ fusion (see Annex 2) was transformed with a plasmid carrying a $\omega$ sequence that had a mutation either in its coiled ($\omega$K52A), $\alpha$-helix $\alpha_2$ ($\omega$E53A), or hypothetical $\alpha$-helix $\alpha_2^*$ ($\omega$R70A) regions. The effect of these mutants on $P_\delta$ expression was comparable to that of wt $\omega_2$ in trans. All of them repressed $P_\delta$ transcription by >50-fold (Fig. S7B). An intermediate effect was observed with the $\omega$R64A mutant, which repressed $P_\delta$ utilization by ~30-fold (Fig. S7B). However, the D56A mutant only reduced $P_\delta$ transcription
~6-fold. Since the intracellular concentrations of $\omega_2$ and its mutant variants were similar (data not shown), we tentatively proposed that $\omega_2$D56A was impaired in its interaction with either $P_\omega$ DNA, with itself or with RNAP-$\sigma^A$. To test these hypotheses, the $\omega$D56A gene was over-expressed and its product was purified and characterized in vitro.

In comparison with the wt, the $\omega$D56A protein binds $P_\omega$ DNA weakly (data not shown). Also, smaller proportion of this mutant protein assembles into dimers (Fig. S7C). In light of the fact that, in the dimeric form of $\omega$, the $\beta$-sheet adopts an antiparallel orientation before binding $P_\omega$ DNA, we propose that the main consequence of D56A mutation is poor $\omega$ dimerization. This is consistent with the observation that $\omega$D56A binds $P_\omega$ DNA with reduced affinity and suggests that D56 contributes to the $\omega$-$\omega$ interface (Fig. S7D). Therefore, $\omega$D56A was not analysed further. It would be very interesting to determine whether the $\omega_2$ NTD can by itself recruit RNAP-$\sigma^A$ to the $\omega_2$-$P_\omega$ DNA complex.

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**Figure legends**

**Fig. S1.** Protein $\omega_2$ or $\omega_2$ cognate sites. Illustration of the $P_\omega$, $P_{\omega 2}$, $P_\delta$, and $P_{\text{copS/R/F}}$ regions from plasmids pIP501$^a$, pSM19035$^b$, pAM$\beta$$^1$$^e$, pRE25$^d$, and pVEF23$^e$. The positions of nucleotides are numbered relative to the transcription start site. Contiguous 7-bp repeats and their relative orientations are represented with either $\rightarrow$ or $\leftarrow$. The -35 and -10 elements are indicated with rectangles and the transcription start sites indicated with solid arrows bent 90°. The dotted-line rectangles and the empty, bent arrows marked with a “?” denote uncharacterized promoters.

**Fig. S2.** The $\omega_2$ protein modulates RNAP-$\sigma^\Lambda$ binding to $P_\omega$ DNA. The 423-bp [$\alpha^{32}$P]-$P_\omega$ DNA (1 nM) was incubated with a range of concentrations of $\omega_2$ (7.5, 15 and 30 nM in [A] or 7.5, 15, 30 to 60 in [B]) or concentrations of RNAP-$\sigma^\Lambda$ (3.7, 7.5, 15 and 30 nM in A and B). Alternatively, the amount of $\omega_2$ was fixed either at 3.7 nM in (A, lanes 10-13), 7.5 nM (B, lanes 12-16) or 60 nM (B, lanes 17-21) while the concentration of RNAP-$\sigma^\Lambda$ was varied (3.7, 7.5, 15 and 30 nM). After DNase I treatment, the complexes were analysed by dPAGE. The +1 site is labelled in the diagram of the operator site-promoter $P_\omega$ region. The dotted-line rectangle in (A) highlight the extended footprint of RNAP-$\sigma^\Lambda$ in lanes 8-10.

**Fig. S3.** Cooperative binding of $\omega_2$, $\delta_2$ and RNAP-$\sigma^\Lambda$ to $P_\omega$ DNA. Linear 423-bp [$\alpha^{32}$P]-HindIII-KpnI $P_\omega$ DNA (0.2 nM) was incubated with either 0.75 or 6 nM of $\omega_2$, either 7.5 or 30 nM of RNAP-$\sigma^\Lambda$, or 37, 75, or 150 nM of $\delta_2$. Alternatively, the concentration of $\omega_2$ was fixed at 0.75 nM, while a range of concentrations of $\delta_2$ was varied (37, 75 and 150 nM). These reactions were performed for 15 min at 37 °C in buffer C in the presence (A) or absence (B) of 1 mM ATP. (C) $P_\omega$ DNA was incubated with increasing amounts of $\omega_2$ (0.75, 1.5, 3, 6, 12 and 24 nM, solid circles) or $\delta_2$-ATP (18.5, 37, 75, 150, 300 and 600 nM, solid squares). Alternatively, $\omega_2$ was fixed (0.75 nM) and increasing amounts of $\delta_2$-ATP (9.2, 18.5, 37, 7.5, 150 and 300 nM, empty squares) used, or apo-$\delta_2$ was fixed (37 nM) and increasing concentrations of $\omega_2$ (0.19, 0.37, 0.75, 1.5, 3 and 6 nM, empty circles) used. The signals present in the protein-DNA complex and in the free-DNA (FD) was determined by densitometry. The data presented here are averages and standard deviations of the results of at least three independent experiments. (D) Binding of $\delta_2$ and RNAP-$\sigma^\Lambda$ to $P_\omega$ DNA. A 423-bp [$\alpha^{32}$P] $P_\omega$ DNA was incubated with increasing amounts
of δ2 (37, 75 and 150 nM) or RNAP-σA (15, 30 and 60 nM), or a fixed concentration of RNAP-σA (15, 30 or 60 nM) and increasing concentrations of δ2 (37, 75, 150, 3 nM) in buffer A containing 1 mM ATP for 15 min at 37˚ C. The signals present in the protein-DNA complex and in the FD was determined by densitometry.

**Fig. S4.** Effect of ω2 on the formation of a RNAP-σA-promoter RP0 complex with Pω DNA. (A) 423-bp [α32P]-HindIII-KpnI Pω DNA (1 nM) was pre-incubated with increasing concentrations of ω2 (1.8, 3.5, 7.5, 15, 30 and 60 nM, solid circles). Then, a fixed amount of RNAP-σA (7.5 nM) in the absence (lanes 1-8) or presence (lanes 9-16) of the initiating nucleotides GTP and ATP was added. DNA melting was probed by KMnO4 footprinting. The positions hypersensitive to KMnO4 are marked (RP0 and RPINIT) and depicted at the bottom; the coordinates are indicated relative to the transcription start point. Purine (G +A) chemical sequencing reactions are shown. Also depicted are the relevant regions. (B) Plot of the relative intensity of the bands of the RP0 and RPINIT hypersensitive sites. Band intensities were determined by densitometry. The data presented here are averages and standard deviations of the results of at least three independent experiments.

**Fig. S5.** RNAP-σA-promoter RP0 at Pω is not affected by δ2. 423-bp HindIII-KpnI Pω DNA (1 nM) was incubated with a fixed amount of RNAP-σA (7.5 nM) in the absence (lane 5) or presence of increasing concentrations of δ2 (75, 150, 300 and 600 nM) in the presence of the initiating nucleotides (GTP and ATP) and DNA melting (open complex) was probed by KMnO4 footprinting. The positions hypersensitive to KMnO4 are marked (RP0 and RPINIT); the coordinates are indicated relative to the transcription start point. The data presented are representative of at least three independent experiments. Purine (G +A), chemical sequencing reactions are shown. The relevant regions of Pω are depicted.

**Fig. S6.** RNAP-σA retains ω2. A mixture containing 1.5 µg His-tagged RNAP-σA and of ω2 in buffer A was loaded onto a 50 µl Ni2+ micro-column at room temperature. After extensive washing, the retained proteins were eluted with 50 µl buffer B containing 1 M NaCl and 0.4 M imidazole. These were resolved by SDS-PAGE and stained with Coomassie blue. The data presented are representative of at least three independent experiments.
**Fig. S7.** Protein ω22 recognizes and represses $P_\delta$ utilization. (A) Clustal W2 sequence alignment of the transcriptional repressors ω and ω22 with secondary structure elements shown above. The identical and conserved amino acids denoted in red and in blue, respectively. (B) Activity specified by $P_\delta$ in the presence or absence of the indicated product. The β-galactosidase activity is expressed in Miller units (10). Values presented are the means of data from at least four separate experiments. (C) Either wt ω2 or the ωD56A variant was incubated with increasing concentrations of the DSS cross-linking agents. The proteins were resolved by 12.5% SDS-PAGE and the proportion of protein found in dimers was calculated. (D) Structural model of ω2-bound to $P_\omega$ DNA. This is derived from the 3D co-structure of the minimal operator site and ω2Δ19 (1IRQ PDB) (2). The position of D56 is highlight in red.

**Fig. S8.** A model of ω2 and RNAP-σA in a complex with $P_\omega$ DNA. Shown is the spatial occupancy model of three ω2 bound to $P_\omega$ in the presence or absence of β’ alone or RNAP-σA. The modelled structures were prepared with I-Tasser and visualized with PyMOL version 1.5.0.4. The structures of the RNAP subunits of *B. subtilis* are represented using the following colour scheme: the σ factor (green), α' and α'' (orange and light orange), β (blue) and the β’ subunit (red). The DNA is indicated in orange and the −35 and −10 sites in yellow. Protein ω2 is represented in pink and grey.
Fig. S4

A

- NTPs
- RNAP-αA
- 7.5 nM

+ ATP + GTP
- RNAP-αA
- 7.5 nM

B

Intensity, $I / I_0$ (%)

- $o$, protein (nM)

- $RP_0$
- $RP_{\text{INIT}}$
Fig. S5

RNAP-\(\sigma^A\)

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| + ATP + GTP | + | G | A |
| 7.5 nM | - | - | - | - | - |

+ATP + GTP

\(\delta_2\)

RP\(_{\text{INIT}}\)

RP\(_o\)

-35

-69

+18

+1

-10
Fig. S6

The diagram shows a gel electrophoresis analysis with three lanes: Control, Flow Through, and Elution. The molecular weight markers are labeled in kDa: 175, 80, 58, 46, 30, 25, 17, and 7. The proteins identified are:

- RpoB (β)
- RpoC (β')
- RpoD (σ)
- RpoA (α)
- RpoE (δ)
A

B

Gene in trans | β-Galactosidase activity | $P_6$
---|---|---
none | 2317 ± 22 |
ω | 32 ± 12 |
ω δ | 20 ± 8 |
ωΔN19 | 44 ± 15 |
ω2 | 55 ± 14 |
ωK52A | 46 ± 9 |
ωE53A | 41 ± 8 |
ωD56A | 411 ± 42 |
ωR64A | 80 ± 9 |
ωR70A | 46 ± 9 |
