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Warman, Emily A; Singh, Shivani S; Gubieda, Alicia G; Grainger, David C

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A non-canonical promoter element drives spurious transcription of horizontally acquired bacterial genes

Emily A. Warman, Shivani S. Singh, Alicia G. Gubieda and David C. Grainger

Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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ABSTRACT

RNA polymerases initiate transcription at DNA sequences called promoters. In bacteria, the best conserved promoter feature is the AT-rich -10 element; a sequence essential for DNA unwinding. Further elements, and gene regulatory proteins, are needed to recruit RNA polymerase to the -10 sequence. Hence, -10 elements cannot function in isolation. Many horizontally acquired genes also have a high AT-content. Consequently, sequences that resemble the -10 element occur frequently. As a result, foreign genes are predisposed to spurious transcription. However, it is not clear how RNA polymerase initially recognizes such sequences. Here, we identify a non-canonical promoter element that plays a key role. The sequence, itself a short AT-tract, resides 5 base pairs upstream of otherwise cryptic -10 elements. The AT-tract alters DNA conformation and enhances contacts between the DNA backbone and RNA polymerase.

INTRODUCTION

All living organisms transcribe their genomes using the enzyme RNA polymerase (1). The process initiates at defined DNA sequences called promoters (1). In Escherichia coli, a multisubunit core RNA polymerase (ωββ') binds one of seven dissociable σ factors to recognise promoter DNA (2). The housekeeping σ70 factor is best studied and targets two promoter regions; the -10 (5'-TATAAT-3') and -35 elements (5'-TTGACA-3') (2). The -10 sequence facilitates promoter DNA unwinding and is usually indispensable (3). Conversely, the -35 element aids initial RNA polymerase recruitment and can be replaced by transcription factors fulfilling the same role (3). In isolation, core promoter elements are ineffective (4). For instance, specific interactions between σ70 and the -10 element occur only in the context of single stranded DNA (5). Hence, -10 elements support DNA melting but not sequence specific RNA polymerase recruitment (4,5).

Sections of the E. coli genome acquired by horizontal gene transfer have an unusually high AT-content (6). Consequently, sequences resembling promoter -10 elements occur frequently (7). These can participate in spurious transcription initiation (8–10). The histone-like nucleoid structuring (H-NS) protein counteracts this by coating large AT-rich DNA islands (8,11,12). The resulting nucleoprotein complexes hinder transcription (13,14). Hence, H-NS acts as a xenogeneic silencer (15,16). Importantly, silencing prevents titration of RNA polymerase that otherwise perturbs housekeeping transcription (8). Given the lack of ancillary promoter elements, and binding sites for transcription factors, it is not clear how RNA polymerase initially recognizes promoters within horizontally acquired genes.

In this paper, we sought to understand recognition of spurious promoters by RNA polymerase. Our study reveals the importance of a short AT-tract, positioned upstream of the -10 element, at happenstance but not conventional promoters. This unusual sequence element permits transcription from otherwise cryptic -10 hexamers. Mechanistically, the AT-tract facilitates an interaction with σ70 residue R451, which recognizes the DNA backbone (17). We show that uncontrolled transcription of AT-rich genes is not restricted to σ70 dependence; the alternative σ38 factor also plays a role.

MATERIALS AND METHODS

Strains, plasmids and oligonucleotides

Strains, plasmids and oligonucleotides are described in Table 1. Standard procedures for cloning and DNA manipulation were used throughout. Promoter DNA fragments were made either using oligonucleotides or synthetic DNA fragments described in Table 1. All promoter DNA fragments were flanked by EcoRI and HindIII restriction sites to allow cloning in plasmid pRW50 or pSR. To construct DNA fragments with random sequence, but defined AT-content,
we used the oligonucleotide ‘Random R’ in combination with variants of the ‘Random F’ primer (Table 1). Each variant of the latter was synthesised using a different mixture of nucleotides to generate ‘any base’ (N). Whilst the A:T and G:C ratios were always the same the overall AT-content varied as indicated. Primers used to generate other synthetic promoter sequences, with or without AT-tracts, are also listed in Table 1. These promoters were made using pairs of oligonucleotides with short regions of complementarity at the 3’ end with the remainder of the sequence serving as a template for DNA polymerase. The ATR fragment introduced a random string of A or T bases (denoted W in Table 1). RPB104 Δhns was constructed by Gene Doctoring as described previously (8, 18).

β-Galactosidase assays

Assays were done following the Miller protocol using E. coli strain JCB387 (19). Cells were grown to mid-log phase in LB media, supplemented with 35 µg/ml tetracycline, at 37°C. All experiments, except for the ATR assays, were done in triplicate and mean values are shown. For the ATR assays we present the overall distribution of activities obtained from single experiments. Error bars represent standard deviation.

Proteins

RNA polymerase core enzyme was purchased from NEB. WT and R451A σ factors were purified as previously described (17).

In vitro transcription

In vitro transcription assays were done using the system of Kolb et al. (20) and the protocol of Savery et al. (21). Briefly, pSR carrying promoters of interest was isolated using a QIAGEN maxiprep kit. Plasmid DNA was mixed to a final concentration of 16 µg/ml with transcription buffer (20 mM Tris pH 7.9, 200 mM GTP/ATP/CTP, 10 mM UTP, 5 µM (o32P)UTP, 5 mM MgCl2 and 100 µg/ml BSA). RNA polymerase was mixed with either WT or R451A σ70 then added to reactions for 10 min at 37°C. RNA products were visualized on a 7% denaturing polyacrylamide gel. RNAI transcript was used as a loading control. Full gel images are shown in Supplementary Figure S1.

Promoter DNA bending assays

To compare differences in DNA bending, double stranded promoter fragments generated by PCR were separated on a 7.5% non-denaturing polyacrylamide gel. Electrophoresis was done in TBE buffer at 4°C. DNA was stained with ethidium bromide and viewed on a UV transilluminator. Full gel images are shown in Supplementary Figure S1.

ChiP-seq

Experiments were done according to the protocol of Haycocks et al. (22) using strain RPB104Δhns that encodes SPA-tagged rpoS. Duplicate cultures were grown to an OD600 of 3.0 in LB then crosslinked, lysed and sonicated. Next, α38-DNA complexes were immunoprecipitated with anti-FLAG antibody and Protein A sepharose beads. Fragments were blunted and poly(A) tailed with 5′-3′ exo-Klenow (NEB). NEXTflex barcoded adaptors (Bioo Scientific) were attached by ligation. Following elution, complexes were de-crosslinked by boiling. The resulting DNA libraries were amplified by PCR and quantified by Qubit analysis before pooling and sequencing using an Illumina MiSeq instrument. The raw data are available from Array-Express (E-MTAB-8778).

Bioinformatics

FastQ files were converted to Sanger format using Fastq-Groomer and aligned to the MG1655 genome using Bowtie for Illumina. The resulting SAM files were converted to BAM files and read depth per base was calculated using MultiBam summary. Data were normalised to the same average read count to allow comparison. Peaks for σ38 binding were called if the average read depth was 4 or above. The same analysis was applied to results of a ChiP-seq assay of σ38 binding in the parent strain RPB104 (23). To identify motifs in collections of putative promoter DNA sequences we used MEME (24).

RESULTS

Happenstance promoters share a conserved AT-rich sequence element

The starting point for this work was our previous analysis of promoters within horizontally acquired genes (8, 10). We speculated that such promoters were chance occurrences resulting from the high AT-content of foreign DNA (10). To quantify the relationship between DNA AT-content and promoter occurrence we generated 8 separate DNA fragment libraries. The fragments in each library were 43 bp in length and had random sequences. However, the overall AT-content of libraries was different and set between 40% and 75%. Fragments were fused to lacZ in plasmid pRW50 and used to transform E. coli strain JCB387. A total of 10,735 transformants were selected on MacConkey agar. This allowed 1,039 red lac+ colonies to be identified, corresponding to active promoters. Our experimental strategy is summarised in Figure 1A. For each fragment library, we calculated the percentage of all DNA fragments with promoter activity. These data are plotted against percentage AT-content in Figure 1B. There was a clear correlation between library AT-content and the number of promoters identified. Few promoters were generated in DNA fragments with an AT-content <50%. We also measured LacZ activity in lysates of cultures derived from each lac+ colony. This allowed us to determine the average activity of all promoters in each library (Figure 1C). Whilst a positive correlation was evident, there was no increase in average promoter activity when the AT-content exceeded 60% (Figure 1C). Each active promoter was sequenced and a DNA sequence logo was generated (Figure 1D, top). For comparison, we also made DNA sequence logos representing intragenic promoters subject to repression by H-NS (middle) and canonical intergenic promoters (bottom) (10, 25).
Synthetic promoter -10 elements are underlined and key base changes introduced by oligonucleotides are in bold.
Figure 1. Promoters are more prevalent and active in random DNA sequences with a higher AT-content. (A) Experimental strategy for generation and selection of promoters from random DNA sequences of defined AT-content. Briefly, 43 bp DNA fragments of random sequence, but a defined AT-content between 40% and 75%, were fused to the lacZ gene in plasmid pRW50. The resulting plasmid libraries were used to transform the E. coli Δlac strain JCB387. Transformants expressing LacZ were identified as red or pink colonies on MacConkey agar. Such lac+ colonies were selected and their random 43 bp DNA insertion was sequenced. (B) The number of lac+ colonies increases as AT-content increases. A total of 10,735 colonies were examined and the percentage of lac+ colonies is plotted against the % AT-content of the corresponding insertion library. (C) Average promoter activity increases in AT-rich DNA sequences. The LacZ activity, quantified for lac+ colonies by β-galactosidase assay, is plotted against library % AT-content. (D) DNA sequence motifs associated with different classes of promoter.
Figure 2. AT-tracts activate transcription from cryptic -10 elements and alter DNA bending. (A) AT-tracts increase promoter activity. Synthetic promoter sequences containing a consensus -10 element (yellow box), -35 element (blue box) and two different AT-tract sequences (AT\textsuperscript{i} and AT\textsuperscript{ii}, red box) were cloned upstream of the \textit{lacZ} gene. For each promoter, LacZ activity was measured in triplicate. Activity values are shown as a percentage relative to the ‘-10/-35’ promoter. The absolute activity of this promoter was 236 Miller units. Error bars show standard deviation. (B) An AT-tract is required for transcription initiation \textit{in vitro}. Bands on the gel are RNA transcripts produced \textit{in vitro} using the indicated promoter sequences. The RNAI transcript serves as an internal control. The control lane shows transcripts generated from empty pSR plasmid. (C) AT-tracts alter DNA bending. Bands on the gel correspond to DNA fragments with or without an AT-tract. All DNA fragments are the same length but have different electrophoretic mobility due to altered curvature.
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Figure 3. AT-tracts of any sequence can activate transcription from a cryptic -10 element. Promoter sequences containing a -10 element and a randomised AT-tract, with or without a partial -35 element, were fused to the lacZ gene in plasmid pRW50. The resulting library was used to transform the Δlac E. coli strain JC5387 and LacZ activity was determined for each transformant. Data are presented as a box plot with each point representing one colony. Measured LacZ activity is also shown for a promoter without an AT-tract (-10/-35) and the empty plasmid.

In all logos, the best conserved feature was the promoter -10 element, particularly bases one, two and six. Conversely, -35 elements were poorly conserved; only the 5′-TT-3′ dinucleotide at positions one and two was evident. Randomly generated and intragenic promoters had an AT-tract between promoter positions -17 and -23. This element was not enriched at canonical promoters. Note that AT-tract sequence differed in randomly generated and intragenic promoters (Figure 1D, compare top two panels). We subsequently refer to these variants as ATi and ATii.

AT-tracts can activate cryptic -10 elements

As noted above, the promoter -10 element alone is ineffective. Hence, transcription factors play a key role by activating canonical promoters. We reasoned that the AT-tract may circumvent the need for transcription factors, or a promoter -35 element, at happenstance promoters. To test this, we generated a set of synthetic promoters. The promoters all had a consensus -10 element. This was augmented with combinations of ATi, ATii and a partial -35 hexamer (5′-TT-3′). The different promoters are illustrated schematically alongside LacZ activity measurements in Figure 2A. As expected, the -10 element alone was unable to drive lacZ expression. Addition of the partial -35 element had no impact. Conversely, addition of either AT-tract variant resurrected promoter activity. This activation increased further when a partial -35 element was also present. To confirm our observations, selected promoters were cloned upstream of the λoop terminator in plasmid pSR. Transcripts terminating at λoop can be detected following electrophoresis. The RNAI transcript is derived from the pSR replication origin and serves as an internal control. No transcripts were produced from promoters lacking AT-tracts (Figure 2B, lanes 1–3). Addition of an AT-tract allowed transcription (lane 4) that increased further upon inclusion of the partial -35 element (lane 5).

AT-tracts alter promoter DNA bending

Changes to the DNA sequence, particularly the introduction of AT-tracts, can alter DNA bending (17). Altered bending affects electrophoretic mobility of DNA during native PAGE. We examined the mobility of different promoters with or without an AT-tract (Figure 2C). DNA fragments containing a -10 element, with or without a partial -35 hexamer, had the same electrophoretic mobility (Figure 2C, lanes 1 and 2). Addition of an AT-tract reduced mobility of DNA fragments during electrophoresis (lanes 3 and 4).

Most AT-tract sequences stimulate transcription

Interestingly, both ATi and ATii were able to activate transcription despite having different sequences (Figure 2A). We reasoned that many AT-tracts may be able to stimulate transcription. To test this we made a new library of promoter DNA fragments. The fragments had a consensus -10 hexamer in the presence or absence of a partial -35 element. Promoter positions -17 to -23 were a random combination of As and Ts (denoted ATR). We examined 103 promoters containing ATR; all were transcriptionally active (Figure 3). This was evident both in the presence (44 promoters) and absence (59 promoters) of the partial -35 element. We conclude that most, and potentially all, appropriately positioned AT-tracts stimulate transcription. In turn, this suggests that a broad range of DNA conformations can be beneficial.
Figure 4. Sidechain R451 of σ^70 is required for transcription at promoters dependent on an AT-tract. (A) The gel image shows transcripts generated by RNA polymerase associated with either WT σ^70 or the R451A mutant. The RNAI transcript is derived from the pSR replication origin and serves as an internal control. The -9A-10T promoter is constitutively active and does not have an AT-tract.

**Activation by AT-tracts requires σ^70 residue R451**

We previously showed that an A or T at promoter position -18 could stimulate transcription by enhancing a DNA backbone contact with σ^70 side chain R451 (17). We predicted that σ^70 R451 would also be important at promoters dependent on the AT-tract. To test this, we repeated our in vitro transcription analysis and compared wild type RNA polymerase with the σ^70 R451A derivative. The R451A mutation resulted in a total loss of transcription at all promoters dependent on an AT-tract (Figure 4, lanes evenly numbered up to 8). However, the mutant σ^70 was unimpaired at a control promoter (lanes 9 and 10). The control promoter is dependent on a near consensus -35 hexamer and has a G at position -18 (17).

**Many H-NS repressed intragenic promoters require AT-tracts**

We speculated that many naturally occurring promoters, within H-NS silenced genes, would be dependent on AT-tracts and σ^70 R451 for activity. To test this prediction, we used six intragenic promoters from our previous analysis of horizontally acquired genes (10,26). The promoters were within the coding sequences of ygaQ, yigG, wcaD, lpXD, yqiI or wzxB. We determined the size of transcripts generated from each promoter in vitro (Supplementary Figure S2). This allowed transcription start sites to be mapped. The annotated promoter sequences are shown in Figure 5A. All six promoters contain an appropriately positioned AT-rich sequence. However, only the promoters within yqiI and wzxB had -35 and -10 sequences near to the consensus. Transcription was measured in vitro using RNA polymerase or the σ^70 R451A derivative. Production of the ygaQ, yigG, wcaD and lpXD derived transcripts was greatly reduced by the R451A mutation (Figure 5B, lanes 1–8). Conversely, transcription from the yqiI and wzxB DNA fragments was unchanged (lanes 9–14). We next replaced the AT-tract upstream of each -10 element with a GC-rich sequence. Only promoters requiring σ^70 R451 were inactivated when the AT-tract was removed (Figure 5C, lanes 1 and 3, 5 and 7, 9 and 11, 13 and 15). Conversely, promoters not requiring R451 functioned independently of the AT-tract (lanes 17–24).

**Many H-NS repressed intragenic promoters function with σ^38 associated RNA polymerase**

Our search for promoter motifs provided evidence that AT-tracts play a key role at happenstance promoters (Figure 1D). Hence, we have focused on understanding this DNA
Figure 5. Effect of the $\sigma^{38}$ R451A mutation on spurious intragenic promoters. (A) Intragenic promoter DNA sequences. Transcription start sites and promoter elements are highlighted. The number of base matches to the consensus sequence for each element is also indicated. (B) The gel image shows transcripts generated by RNA polymerase associated with either WT $\sigma^{38}$ or the R451A mutant. Bands at 107/108 nt are RNAI transcripts derived from the pSR replication origin. The -9A-10T is constitutively active and functions independently of the AT-tract. (C) Effect of replacing AT-trtracts with GC-rich sequences (the -35 and -10 elements were unchanged). The sequence immediately upstream of the -10 element was replaced with 5′-CTTCGCAGTCAGCTGAC-3′ (or 5′-CTTCGCAG-3′ for extended -10 elements).

| Gene | Promoter sequence | -35 match | -10 match | R451 required |
|------|-------------------|-----------|-----------|---------------|
| -35 element | AT-tract | -10 element | +1 |
| TTGACA | WWWWWW | TATAAT | R |
| ygaQ | TTATGAGTTCATGACTGCTGAGATG | 3/6 | 3/6 | yes |
| yigG | CTTATGATTCTTACATG | 3/6 | 4/6 | yes |
| wcaD | TATGACTTGCTGACCTGAG | 3/6 | 5/6 | yes |
| lpxD | GCACATGCTGACAGGCGAGCGGTCG | 2/6 | 3/6 | yes |
| yqiL | TTTACCTGACATTACCATGACACAGCACTGATGATGCTGACT | 5/6 | 5/6 | no |
| wzxB | AATGAGACATTATACACATATTATGTAATGACATG | 5/6 | 5/6 | no |

Figure 6A, top. The logo depicts a 5′-TGN-3′ motif upstream of the sequence 5′-TATACT-3′. Previous work has shown that promoters used by the alternative $\sigma^{38}$ factor, encoded by $rpoS$, often have 5′-TGN-3′ motifs (27). Furthermore, ChIP-seq analysis identified 5′-TATACT-3′ as the consensus -10 element for $\sigma^{38}$ (Figure 6D, bottom) (23). Hence, $\sigma^{38}$ might also serve promoters within horizontally acquired genes. To test this, we used ChIP-seq and compared chromosome-wide $\sigma^{38}$ binding in E. coli RPB104 and the $\Delta hns$ derivative. We identified 890 $\sigma^{38}$ binding peaks in the starting strain and 905 peaks in the $\Delta hns$ derivative (Supplementary Table S1). The proportion of $\sigma^{38}$ binding peaks within genes increased in cells lacking H-NS (Fig-
Figure 6. The alternative $\sigma^{38}$ factor recognises many intragenic promoters. (A) Sequence logo showing the imperfect extended -10 element conserved in randomly generated promoters (top). The $\sigma^{38}$ promoter logo defined using ChIP-seq analysis is included for comparison (23). (B) The proportions of $\sigma^{38}$ ChIP-seq peaks found inside/outside of genes (blue charts) or regions highly H-NS bound by H-NS (purple charts) in a wild-type (WT) or $\Delta hns$ E. coli strain. (C) Examples of $\sigma^{38}$ ChIP-seq peaks inside genes in $\Delta hns$ (red) but not WT (blue) cells. H-NS binding signals are derived from the ChIP-seq data of Kahramanoglou et al. (32). (D) Scatter plot showing changes in $\sigma^{38}$ binding when hns is deleted. Each data point represents the log10 of $\sigma^{38}$ ChIP-seq signal in a 500 bp bin. Sequences usually bound by H-NS are shown in dark purple (high H-NS) and areas without H-NS are shown in pale purple (low H-NS).

We previously noted the widespread spurious transcription of AT-rich horizontally acquired genes (10). The phenomenon was attributed to increased occurrence of sequences resembling promoter -10 elements (8,10). However, promoter -10 elements alone are unable to drive transcription; the sequence cannot recruit RNA polymerase to the DNA (4). Structural analysis provides a rationale for this observation; base specific interactions between $\sigma^{40}$ and
the -10 hexamer only occur after DNA unwinding (5). In this work we show that spurious intragenic promoters frequently depend on an AT-tract located between -17 and -23 base pairs upstream of the transcription start site (Figures 2–5). Consistent with this, we and others have previously noted an A or T at positions -17 and -18 can be stimulatory (17,28–30). The AT-tract alters nucleic acid bending and facilitates a contact between σHNS side chain R451 and the double helix backbone. Hence, otherwise cryptic -10 elements are able to participate in the process of transcription initiation. Many intragenic promoters also function with σHNS bound RNA polymerase. This may explain why inactivation of rpoS is a pre-requisite for deletion of hns in Salmonella spp. (31).

The simplest explanation for the abundance of promoters within AT-rich genes is chance occurrence (8–10). However, this hypothesis is difficult to test. In an effort to address the issue, we compared DNA sequence properties of spurious and randomly generated promoters (Figure 1). Whilst not proof of accidental origin, both types of promoter frequently depend on the presence of an AT-tract (32). Interestingly, the majority of spurious promoters within AT-rich regions do not arise by chance but instead by horizontal gene transfer (33). Therefore, promoters within AT-rich genetic regions are more likely to occur by chance.

Intriguingly, the frequency of chance promoter occurrence increases only when the DNA AT-content exceeds that of the E. coli genome (i.e. ~50%, Figure 1B). This may be indicative of adaptations that reduce the initiation of transcription at unwanted locations. For instance, the E. coli RNA polymerase could be hardwired to initiate transcription inefficiently at DNA sequences with an AT-content resembling coding DNA. We speculate that the AT-content threshold, above which promoters spontaneously arise, will differ depending on the genome AT-content of a given organism. Similarly, we predict that RNA polymerases isolated from bacteria with AT-rich genomes could be adapted to such templates and generate fewer spurious transcripts compared to the E. coli enzyme. In summary, we provide an explanation for the widespread occurrence of spurious promoters within horizontally acquired sections of the E. coli genome. Our data also have implications for our understanding of RNA polymerase specificity and promoter evolution.

**DATA AVAILABILITY**

ArrayExpress accession E-MTAB-8778.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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