Overlapping promoters modulate Fnr- and ArcA-dependent anaerobic transcriptional activation of the *focApfl* operon in *Escherichia coli*

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The recently identified *P6A* promoter of the anaerobically inducible *focApfl* operon of *Escherichia coli* overlaps the Fnr (fumarate-nitrate reduction regulator)-dependent *P6* promoter. The Fnr-binding site of *P6* and the –35 hexamer sequence of *P6A* are shared between the promoters. Inactivation of *P6A*, through introduction of a –10 hexamer mutation, resulted in enhanced anaerobic induction of operon expression. The dependence on the ArcA (aerobic respiration control regulator) and Fnr transcription factors for anaerobic induction was tested for several *focA–lacZ* and *pfl–lacZ* gene fusions. Anaerobic induction became more dependent on Fnr in derivatives lacking a functional *P6A* promoter compared with wild-type constructs. Moreover, aerobic expression of the *focA* gene was reduced by the *p6A* mutation, as was the dependence on ArcA for anaerobic induction. Inactivation of *P6* severely reduced Fnr-dependent anaerobic induction, in accord with previous findings. Transcription analyses demonstrated that a mutation in the –10 hexamer sequence of either *P6A* or *P6* did not adversely affect transcription from the remaining promoter. Taken together, these results indicate that the *P6A* promoter moderates the Fnr-dependent activation of *P6* through competition for RNA polymerase binding.

Keywords: gene regulation, transcription factors, overlapping promoters, Fnr and ArcA, anaerobic metabolism

INTRODUCTION

The gene encoding the enzyme pyruvate formate-lyase (Pfl) forms an operon with a gene termed *focA* (Sawers & Böck, 1988, 1989; Suppmann & Sawers, 1994). Pfl is the central enzyme of anaerobic carbon metabolism in *Escherichia coli* and catalyses the non-oxidative, co-enzyme A-dependent cleavage of pyruvate to formate (Knappe & Sawers, 1990). The product of the *focA* gene is an integral membrane protein required to modulate the intracellular formate pool (Suppmann & Sawers, 1994). The *focApfl* operon is expressed at low levels in aerobic cells but upon oxygen depletion, expression is induced between 10- and 15-fold (Sawers & Böck, 1988, 1989). Anaerobic induction of expression is controlled by the two global transcription factors ArcA (aerobic

respiration control regulator) and Fnr (fumarate-nitrate reduction regulator) (Sawers & Suppmann, 1992; Sawers, 1993; Drapal & Sawers, 1995a, b; Kaiser & Sawers, 1995).

Transcription of the *focApfl* operon is directed by multiple promoters (Sawers & Böck, 1989; Sawers, 1993). Five promoters are located within the operon and lie directly upstream of the *pfl* gene (Fig. 1). A further three promoters are located in an approximately 500 bp regulatory region immediately upstream of the *focA* gene. One of these upstream promoters, *P6A*, was discovered in a recent study which examined *in vitro* and *in vivo* expression from the Fnr-dependent *P6* promoter (Kaiser & Sawers, 1995). *P6* and *P6A* overlap and their respective initiation sites are separated by 10 nt, which indicates that the promoters are separated by one helical turn. The *P6* promoter has a poorly conserved –10 hexamer sequence and lacks a –35 hexamer sequence. The *P6* promoter does, however, have a

Abbreviations: ArcA, aerobic respiration control regulator; Fnr, fumarate-nitrate reduction regulator; Pfl, pyruvate formate-lyase.

Fig. 1. Schematic representation of the *focApfl* operon promoter-regulatory region. The upper portion of the figure shows the *focApfl* operon promoter-regulatory region, the *focA* gene and the 5' part of the *pfl* gene. The DNA fragments used to construct the various *focApfl* promoter-*lacZ* gene fusions are designated λ RM20201, λ RM1031 and λ RM123. The lower portion of the figure shows the DNA sequence of the 'top' strand of the P6 and P6A promoters. The more 3' transcription initiation site of the P6 promoter is designated +1. The -10 hexamer sequences of the P6 and P6A promoters are overlined and the mutations introduced into the respective hexamer are shown by vertical, dashed arrows. The -35 hexamer sequence of the P6A promoter is boxed and the palindromic Fnr recognition sequences are highlighted by converging horizontal arrows. *fnr-1* indicates that the Fnr-binding site is mutated.

palindromic Fnr-binding site (Spiro & Guest, 1990) which is centred at -41.5 bp relative to the transcription initiation site and directs anaerobic, Fnr-dependent transcription (Kaiser & Sawers, 1995). The P6A promoter, in contrast, has a consensus -10 hexamer sequence and a -35 hexamer sequence that matches the consensus in three positions (Fig. 1). Consequently, transcription from the P6 promoter is regulated by Fnr, whilst transcription from P6A appears to be factor-independent (Kaiser & Sawers, 1995). The relationship between these promoters is complicated by the fact that the upstream 'half-site' of the Fnr recognition sequence and the -35 hexamer directly overlap. This has major implications for the functions of these promoters in controlling *focApfl* operon expression. The aim of this study therefore was to characterize the P6A promoter in an attempt to identify its physiological function. Through the use of specific mutagenesis of the -10 hexamer sequences we have inactivated each promoter individually. These studies have provided credence for the hypothesis that an important function of P6A is to modulate transcription initiation from P6 through competition between RNA polymerase and the Fnr transcription factor for a common binding site.

METHODS

Strains, plasmids, phages and growth conditions. Bacterial strains, plasmids and phages used in this study are listed in Table 1. Bacteria were grown in buffered rich medium as

described by Begg *et al.* (1977). Glucose was added to a final concentration of 20 mM for both aerobic and anaerobic growth and cells for both enzyme assay and RNA preparation were harvested in the mid-exponential phase of growth. Chloramphenicol was used at a concentration of 15 μ g ml⁻¹ while kanamycin was used at 50 μ g ml⁻¹. Media and buffers used for λ work were prepared as described by Sawers & Böck (1989).

Plasmid construction. The construction of pRS551 and pRS552 derivatives pRM23, pRM2307, pRM2317, pRM1031 and pRM20201, which harbour various portions of wild-type and mutant derivatives of the *pfl* regulatory region fused with the *lacZ* gene, has been described previously (Sawers & Böck, 1988; Sawers, 1993; Drapal & Sawers, 1995a; Kaiser & Sawers, 1995). These constructs were used directly or acted as templates for the construction of further mutant derivatives by PCR amplification with the appropriately designed oligonucleotides. Transfer of *pfl-lacZ* fusions to the specialized λ transducing phage λ RS45 was performed as described by Simons *et al.* (1987). The various promoter mutations had no influence on translation of either the *focA* or *pfl* genes, thus operon and protein fusions gave comparable results.

Primer extension analysis of total RNA. Total RNA was isolated from exponentially growing cells using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Primer extension analysis of *pfl* P6- and P6A-derived transcripts was performed with 10 μ g total RNA and 0.2 pmol ³²P-end-labelled primer according to Sawers & Böck (1989). Care was taken to ensure equal loading of RNA and the results of our primer extension analyses were highly reproducible. The oligonucleotides used in these studies included LACZ-1 (5' GGTCATAGCTGTTTCCTGTGTG 3'), which primes within the *lacZ* gene, and PFL16 (5' GTAAAAGATCAAAAGG-GTTGTCAG 3'), which hybridizes downstream of the P6 promoter. Products of the primer extension reactions were separated on 6% polyacrylamide sequencing gels containing 7M urea.

β -Galactosidase enzyme assays. β -Galactosidase enzyme activity was determined in cultures of exponentially growing cells, and the specific activity was calculated according to Miller (1972). Values reported are the mean of at least three independent experiments performed in triplicate. The SEM of the values reported was not more than 15%.

Other methods. Densitometric quantification of autoradiograms was performed using a Mirror 600 Plus Scanner and MacBas version 2.0 software. Several exposures of film were used to ensure that data were collected in the linear sensitivity range of the film.

RESULTS

Influence of -10 hexamer mutations on P6 and P6A transcription

The locations of the transcription initiation sites of the P6 and P6A promoters have been determined both *in vitro* and *in vivo* (Sawers & Böck, 1989; Sawers, 1993; Kaiser & Sawers, 1995). These initiation sites are separated by 10 bp (Fig. 1). In an attempt to characterize the relationship between these two promoters and their influence on *pfl* expression, -10 hexamer mutations, which inactivate the promoters, were introduced into the three *focA-lacZ* fusion constructs λ RM20201, λ RM1031 and λ RM123 (Fig. 1). The *p6-2* mutation was introduced into the P6 -10 hexamer and the *p6A*

Table 1. Strains, plasmids and phages used in this study

Strain, plasmid or phage	Genotype*	Reference or source
Strain		
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>ptsF25 deoC1 relA1 flbB530 rpsL150</i> λ^-	Casadaban & Cohen (1979)
RM102	Like MC4100 but Δ <i>fnr</i> Δ (<i>srl-recA</i>)306::Tn10	Birkmann <i>et al.</i> (1987)
RM313	Like MC4100 but <i>arcA1 zjj</i> ::Tn10	Sawers & Suppmann (1992)
RM315	Like MC4100 but Δ <i>fnr arcA1 zjj</i> ::Tn10	Sawers & Suppmann (1992)
RM2307	Like MC4100 but carrying the <i>p6A</i> mutation in the <i>pfl</i> regulatory region	Sawers (1993)
Plasmid		
pRS551	Km ^R Ap ^R <i>lacZ lacY lacA</i>	Simons <i>et al.</i> (1987)
pRM23	pRS552 Φ (<i>pfl'-lacZ</i>)1397(Hyb)	Sawers & Böck (1988)
pRM2307	Like pRM23 Φ (<i>pfl'-lacZ</i>)1397(Hyb) but carrying the <i>p6A</i> – 10 hexamer mutation	Sawers (1993)
pRM2317	Like pRM23 Φ (<i>pfl'-lacZ</i>)1397(Hyb) but carrying the <i>p6-2</i> – 10 hexamer mutation	Sawers (1993)
pRM1031	pRS551 Φ (<i>focA'-lacZ</i>) carrying the 493 bp insert from the <i>focApfl</i> regulatory region	Drapal & Sawers (1995a)
pRM1036	Like pRM1031 but carrying the <i>p6-2</i> mutation	This work
pRM1036A	Like pRM1031 but carrying the <i>p6A</i> mutation	This work
pRM20201	pRS551 Φ (<i>focA'-lacZ</i>) carrying the 119 bp DNA insert from the <i>focApfl</i> regulatory region	Kaiser & Sawers (1995)
pRM20203	Like pRM20201 but carrying the <i>p6-2</i> mutation	This work
pRM20203A	Like pRM20201 but carrying the <i>p6A</i> mutation	This work
Phage		
λ RS45	<i>lacZ' lacY imm²¹ ind</i>	Simons <i>et al.</i> (1987)

* Ap, ampicillin; Km, kanamycin.

mutation into the *P6A* – 10 hexamer. The wild-type and mutant *lacZ* derivatives were introduced in single copy in to the chromosome of various *E. coli* strains and used to study transcription and expression.

Initially we determined the effects of the promoter mutations on *P6* and *P6A* transcription *in vivo*. For this purpose total RNA from wild-type and *fnr* mutant strains bearing the λ derivatives λ RM20201, λ RM20203 (carrying the *p6-2* mutation) and λ RM20203A (carrying the *p6A* mutation) was isolated. These derivatives include only the *P6* and *P6A* promoters and the Fnr recognition sequence (Fig. 1; Kaiser & Sawers, 1995; Drapal & Sawers, 1995b). Using a primer which specifically hybridized within the *lacZ* coding sequence it was possible to determine directly the transcripts derived from the *lacZ* fusions (Fig. 2a). A single initiation site for *P6* could be identified, which is identical to that found for the wild-type chromosomal promoter (Sawers & Böck, 1989; Drapal & Sawers, 1995b). We have noted that transcription always initiates from a single site (the labelled G residue in Fig. 1) when short promoter region fragments are analysed and often initiates from two adjacent sites (the labelled C and G residues in Fig. 1) in constructs which include the complete promoter-regulatory region plus the downstream promoters, for example from λ RM123 (see Fig. 3; Sawers & Böck, 1989; Sawers, 1993).

Transcription from *P6A* initiated primarily at a site located at – 11 bp relative to the start site of *P6* (Fig. 2a). This start site also correlates with a site used by the promoter in its natural chromosomal location. A weaker signal initiating at a G residue located at – 14 bp was occasionally observed (Kaiser & Sawers, 1995; Drapal & Sawers, 1995b).

Primer extension analysis of λ RM20201 in a wild-type genetic background demonstrated that transcription from *P6* was increased anaerobically, while that from *P6A* was essentially unaffected by the cellular oxygen status (Fig. 2a). Quantification of the transcripts indicated that the combined level of *P6* plus *P6A* transcription increased approximately threefold under anaerobic conditions (Fig. 2b). Analysis of transcription in a *fnr* mutant revealed a drastic reduction in transcription from *P6* and abolition of anaerobic induction. This result is in accord with previous findings (Sawers, 1993; Kaiser & Sawers, 1995). The residual transcription from *P6* observed in the *fnr* mutant was due to low-level cAMP-receptor protein (Crp)-dependent initiation (G. Sawers, M. Kaiser, A. Sirko & M. Freundlich, unpublished results).

The *fnr* mutation resulted in a slight increase in *P6A* transcription anaerobically and a more frequent use of the secondary initiation site at – 14 bp both aerobically and anaerobically (Fig. 2a). The consequence of these

Fig. 2. Primer extension analysis of transcriptional *lacZ* fusions. (a) Total RNA was isolated from MC4100 and RM102 (*Δfnr*) carrying the *focA-lacZ* transcriptional fusions *λRM20201* (wild-type *P6* and *P6A* promoters), *λRM20203* (*P6* promoter mutant) or *λRM20203A* (*P6A* promoter mutant) after aerobic (+O₂) or anaerobic (-O₂) growth. Specific analysis of the transcripts generated by the fusions was achieved with an oligonucleotide which primed within the *lacZ* gene (see Methods). An autoradiogram is shown after the products were separated in a denaturing polyacrylamide gel. M designates the C reaction of a control DNA sequence. (b) Quantification of transcripts *P6+P6A* was performed after densitometric scanning of the autoradiogram shown in (a). The results presented show the total transcripts (*P6+P6A*) generated from the particular fusion. Open and filled bars represent analysis of RNA from aerobically and anaerobically grown cells, respectively. Strain MC4100 is designated as wt while strain RM102 is designated *Δfnr*.

slight alterations in *P6A* transcription to the overall transcription from *λRM20201* was an approximately twofold reduction in transcription compared with the wild-type both aerobically and anaerobically; however, a 2.5-fold anaerobic induction of transcription was still observed (Fig. 2b).

Fig. 2(a) shows that introduction of the *p6-2* mutation in derivative *λRM20203* abolished transcription from the *P6* promoter, as anticipated (Sawers, 1993). The *p6-2* mutation had two consequences for *P6A* transcription: firstly, transcription initiated from the G residue at position -12 in the mutant derivative, instead of the A residue at -11 bp; and secondly, the G residue at -14 bp was no longer used as a site of initiation.

Interestingly, transcription initiated primarily from the A residue at -11 bp in a *fnr* mutant both aerobically and anaerobically, indicating that Fnr can affect initiation from *P6A* even when transcription initiation at *P6* is prevented by the *P6* -10 hexamer. As was noted for *λRM20201*, the *fnr* mutation resulted in enhanced *P6A* transcription; however, in the *λRM20203* derivative the effect was observed both aerobically and anaerobically.

The *p6A* mutation in *λRM20203A* completely inactivated the *P6A* promoter while the regulation and levels of *P6* transcription remained unaffected (Fig. 2a). Anaerobic induction of *P6* transcription was 4.6-fold, while introduction of the *fnr* mutation resulted in a 14-fold reduction in anaerobic transcription (Fig. 2b). A similar reduction in *P6* transcription was observed for the *λRM20201* derivative but the overall effect on transcription was masked by transcription from *P6A* (Fig. 2a, b).

Effect of the *P6* and *P6A* promoter mutations on expression from the *λRM20201 focA-lacZ* fusion derivatives

The influence of chromosomal *arcA* and *fnr* mutations on the levels of *lacZ* expression from *λRM20201*, *λRM20203* and *λRM20203A* after aerobic and anaerobic growth was monitored (Table 2). Expression from *λRM20201* was induced 3.4-fold by anaerobiosis, which is in good agreement with the transcription data (compare Fig. 2). Anaerobic induction was strongly reduced in a *fnr* mutant and was reduced to twofold by an *arcA* mutation. Whilst the *fnr* mutation reduced the absolute level of anaerobic β -galactosidase enzyme activity but did not affect the aerobic activity level, the *arcA* mutation did not influence the anaerobic level of activity compared with the wild-type, but instead it resulted in a twofold increase in aerobic activity levels. This result is in agreement with the finding that ArcA has a negative influence on *focA-lacZ* expression in this short promoter fragment (Drupal & Sawers, 1995b). As expected from the transcription studies, the *p6-2* mutation in construct *λRM20203* abolished anaerobic induction of expression (Table 2). Expression was derived solely from the activity of the *P6A* promoter (see Fig. 2a). ArcA had no major effect on β -galactosidase activity, whereas Fnr repressed expression anaerobically. Fnr did not show this effect in the absence of a functional ArcA protein.

The *p6A* mutation on *λRM20203A* resulted in a threefold reduction in aerobic expression, indicating that the *P6A* promoter is responsible for the major proportion of the expression in aerobically grown cells (Table 2). Anaerobic growth resulted in a 13-fold induction of expression which was absolutely dependent on the Fnr protein. An *arcA* mutation had little effect on expression; the mutation resulted in a slight increase in aerobic expression and a small decrease in anaerobic expression. These results demonstrate that the *P6* promoter is responsible for the anaerobic induction of

Table 2. Expression of the *focA* gene driven by the *P6* and *P6A* promoters

<i>lacZ</i> derivative*	Host genotype	β -Galactosidase activity (Miller units)		
		Aerobic (+O ₂)	Anaerobic (-O ₂)	Induction ratio (-O ₂ /+O ₂)
λ RM20201 (wild-type)	wt	360	1215	3.4
	Δfnr	300	400	1.3
	<i>arcA</i>	710	1470	2.1
	<i>arcA</i> Δfnr	300	550	1.8
λ RM20203 (<i>P6</i> mutant)	wt	210	130	0.6
	Δfnr	220	245	1.1
	<i>arcA</i>	275	250	0.9
	<i>arcA</i> Δfnr	280	330	1.2
λ RM20203A (<i>P6A</i> mutant)	wt	100	1300	13.0
	Δfnr	74	60	0.85
	<i>arcA</i>	135	1040	7.8
	<i>arcA</i> Δfnr	100	82	0.82

*The λ derivatives of the correspondingly named plasmids were inserted in single copy into the chromosome of the strains to be tested.

focA expression, while the *P6A* promoter modulates the aerobic level of expression. It is also noteworthy that the absolute level of anaerobic expression in the wild-type promoter derivative and the *p6A* mutant is similar although there is a significant difference in anaerobic expression in the *fnr* mutant. These results suggest that the effect of both promoters on expression is not additive and when *P6* transcription is prevented by the *fnr* mutation, *P6A* expression becomes unimpeded. This agrees with the results of the transcription studies in Fig. 2(a).

The influence on *focA-lacZ* expression of the addition of promoter *P7* regulatory sequences

The λ RM20201 *focA-lacZ* fusion includes only a portion of the *focApfl* operon regulatory sequences and lacks promoter *P7* and sequences important for ArcA-dependent regulation of expression. To examine the effect of the *p6-2* and *p6A* mutations on expression directed by the complete upstream promoter-regulatory region of the operon, we constructed a series of *lacZ* operon fusions based on the λ RM1031 derivative (Fig. 1; Drapal & Sawers, 1995a, b). The results presented in Table 3 show that addition of the *P7* promoter sequences resulted in a 7.3-fold anaerobic induction which was strongly dependent on ArcA and only partially dependent on Fnr. Introduction of the *p6-2* mutation reduced anaerobic induction twofold; however, the absolute level of anaerobic expression was reduced threefold compared with the wild-type promoter fragment. Aerobic expression was also reduced partially by the *p6-2* mutation. The *p6-2* mutation had a comparable effect on expression as that observed when the wild-type promoter-regulatory region was analysed in a *fnr*

mutant. The residual anaerobic induction was absolutely dependent on a functional ArcA protein and was partially dependent on Fnr; in the *fnr* mutant an approximately threefold anaerobic induction was still apparent but the absolute level of β -galactosidase activity in both aerobically and anaerobically grown cells was reduced threefold. Taken together, these findings indicate that promoter *P7* activity accounts for the anaerobic induction of expression in derivative λ RM1036 and that ArcA mediates anaerobic induction when the Fnr-dependent *P6* promoter is inactivated.

The *p6A* mutation caused a threefold reduction in aerobic expression when compared with the wild-type grown under the same conditions (Table 3). This confirms the earlier observation that *P6A* activity is in part largely responsible for the expression of the *focA* gene in aerobically grown cells. A 15.5-fold anaerobic induction of *lacZ* expression from the λ RM1036A derivative in the wild-type strain was observed. This anaerobic induction was almost completely dependent on Fnr. An *arcA* mutation had a minimal effect, reducing enzyme activity by 20% and 30% in aerobically and anaerobically grown cells, respectively, relative to enzyme activity levels in the wild-type (Table 3). In an *arcA fnr* double null mutant, anaerobic β -galactosidase activity was reduced 60-fold compared with the wild-type. Inactivation of the *P6A* promoter, therefore, severely impairs ArcA-dependent anaerobic induction of *focA* gene expression. Since anaerobic induction becomes almost exclusively Fnr-dependent, these findings indicate that *P6A* controls the degree to which Fnr and ArcA control anaerobic induction of *focA* expression. These data also indicate that the *P6A* promoter is important for ensuring substantial levels of aerobic *focA* gene expression.

Table 3. Expression of the *focA* gene driven by the *P6*, *P6A* and *P7* promoters

<i>lacZ</i> derivative	Host genotype	β -Galactosidase activity (Miller units)		
		Aerobic (+ O ₂)	Anaerobic (- O ₂)	Induction ratio (- O ₂ /+ O ₂)
λ RM1031 (wild-type)	wt	500	3650	7.3
	Δfnr	440	1260	2.8
	<i>arcA</i>	265	380	1.4
	<i>arcA</i> Δfnr	245	260	1.1
λ RM1036 (<i>P6</i> mutant)	wt	330	1050	3.2
	Δfnr	125	360	2.9
	<i>arcA</i>	100	120	1.2
	<i>arcA</i> Δfnr	120	155	1.3
λ RM1036A (<i>P6A</i> mutant)	wt	160	2490	15.5
	Δfnr	115	135	1.2
	<i>arcA</i>	125	1670	13.4
	<i>arcA</i> Δfnr	100	43	0.43

Table 4. Influence of a *p6A* mutation on *pfl* gene expression

<i>lacZ</i> derivative	Host genotype	β -Galactosidase activity (Miller units)		
		Aerobic (+ O ₂)	Anaerobic (- O ₂)	Induction ratio (- O ₂ /+ O ₂)
λ RM23 (wild-type)	wt	455	5055	11.0
	Δfnr	230	1425	6.2
	<i>arcA</i>	390	3630	9.3
	<i>arcA</i> Δfnr	390	500	1.3
λ RM2307 (<i>P6A</i> mutant)	wt	470	7510	16.0
	Δfnr	250	730	2.9
	<i>arcA</i>	440	4360	9.9
	<i>arcA</i> Δfnr	250	190	0.8

The influence of the *p6A* promoter mutation on *pfl* expression directed by the complete promoter system

Table 4 shows that when promoters *P1*–*P7* are all placed before the *lacZ* gene in construct λ RM123, expression was induced 11-fold by anaerobic growth compared to the levels seen after growth in the presence of oxygen. These results are in agreement with previous findings (Sawers & Böck, 1988, 1989; Sawers & Suppmann, 1992; Sawers, 1993). The *fnr* mutation reduced the anaerobic induction ratio by approximately twofold, whilst the *arcA* mutation reduced this ratio by only 15%. Only in a double null mutant was anaerobic regulation abolished. These findings indicate that both Fnr and ArcA have important roles in mediating anaerobic induction of expression when the complete promoter-regulatory region plus the downstream promoters are present.

The influence of the *p6-2* mutation on anaerobic and ArcA/Fnr-dependent regulation of the *pfl* gene has been reported previously (Sawers, 1993). The *p6A* mutation resulted in an increase in the anaerobic induction ratio to 16-fold in λ RM2307 (Table 4). This is due to an increase in the level of anaerobic expression. The introduction of an *fnr* mutation reduced this level 10-fold, while concomitantly reducing the aerobic level of expression by almost twofold. This had the consequence that the anaerobic induction ratio was reduced to threefold. The *arcA* mutation resulted in a 40% reduction in the anaerobic level of expression, while in the *arcA fnr* double null mutant anaerobic induction was abolished. Thus, inactivation of the *P6A* promoter caused an increase in the anaerobic induction ratio and resulted in expression becoming more dependent on Fnr.

That the *p6A* mutation also abolished transcription

Fig. 3. Primer extension analysis of promoter *P6* and *P6A* transcription in MC4100 and RM2307. Lanes: 1, MC4100 grown anaerobically; 2, RM2307 grown anaerobically. The initiation sites of *P6* and *P6A* transcription are shown by arrows.

from the *P6A* promoter in the derivative including all the *focApfl* operon promoters is demonstrated in Fig. 3. Significantly, transcription from the *P6* promoter was unaffected by the *p6A* –10 hexamer mutation. Primer extension analysis also revealed that transcription initiated with almost equal efficiency from the adjacent C and G residues at –1 and +1, respectively. This is in contrast to the transcription observed with short promoter derivatives where a single initiation site from the G residue at +1 was observed (Fig. 2).

DISCUSSION

Overlapping *P6* and *P6A* promoters modulate Fnr-dependent anaerobic regulation

ArcA and Fnr control the extent of anaerobic induction of *pfl* operon expression. The results presented in this report demonstrate that the *P6A* promoter has an important function in determining the degree to which either transcription factor controls expression. The relative dependence on Fnr for anaerobic induction of expression was increased through inactivation of *P6A*. Although this effect was observed in three different *lacZ* fusion constructs analysed, it was most apparent in the two sets of *lacZ* fusions which lacked the five downstream promoters, *P1*–*P5*. Thus, the level of expression attained in anaerobically grown *p6A* mutant derivatives was reduced minimally by a factor of 18 through introduction of a *fnr* mutation compared with only a threefold reduction in derivatives with functional *P6A*

promoters (see Tables 2 and 3). In the construct including all of the *pfl* operon promoters, anaerobic expression from the *P6A*⁺ and *P6A*[–] derivatives was reduced 3.5- and 10-fold, respectively, by the *fnr* mutation. In each case, anaerobic induction was abolished in *arcA fnr* double null mutants in all the derivatives tested. Therefore, although transcription from the *P6A* promoter is weak relative to the other *pfl* promoters (Sawers & Böck, 1989; Sawers, 1993), it nevertheless has a significant role in the regulation of anaerobic *pfl* gene expression.

The major transcription initiation sites of the *P6* and *P6A* promoters are separated by 10 nt (Fig. 1). The *P6* promoter has a relatively poorly conserved –10 hexamer sequence and is lacking a –35 hexamer sequence, while the *P6A* promoter has a consensus –10 hexamer sequence and a poorly conserved –35 hexamer sequence. The *P6A* –35 hexamer sequence forms the left ‘half-site’ of the Fnr-binding site (Spiro & Guest, 1990) of the *P6* promoter. As a consequence, the relative affinities of RNA polymerase and Fnr for this sequence govern the frequency of transcription initiation from the *P6* and the *P6A* promoters. We noted previously that the upstream Fnr ‘half-site’ had one mismatch relative to the consensus Fnr-binding site (Kaiser & Sawers, 1995). Mutation of this mismatch to create a consensus Fnr-binding site resulted in increased *P6A* transcription rather than increased transcription from *P6*. This was because the mutation also brought the –35 hexamer sequence of the *P6A* promoter closer to the consensus, and hence increased the relative affinity of RNA polymerase for the promoter.

The requirement of *P6A* activity for ArcA-dependent anaerobic regulation

The primary effect of inactivation of the *P6A* promoter was to enhance the relative dependence on Fnr for anaerobic transcriptional activation and to reduce the effective requirement for ArcA. ArcA has several binding sites in the *pfl* promoter-regulatory region (Drapal & Sawers, 1995a), one of which abuts the *P6A* –10 hexamer. Mutation of this hexamer sequence had no effect on the ability of ArcA to bind to the promoter-regulatory region (data not shown). Rather, we believe that the marginally reduced dependence on ArcA for anaerobic regulation caused by the *p6A* mutation (see Table 3) is probably an indirect effect due to occlusion of its function through the unimpeded binding of Fnr to its binding site at the *P6* promoter. Therefore, together with integration host factor (Sirko *et al.*, 1993) and the *P7* promoter (Drapal & Sawers, 1995b), the *P6A* promoter has an important function in mediating ArcA-dependent regulation of the *focA* gene.

Oxygen-dependent promoter switching

The *pfl* operon *P6* and *P6A* promoters provide an example of overlapping promoters where an oxygen-dependent switch is involved. Fnr is inactive in aero-

bically growing cells and thus under these conditions only P6A is active. Anaerobiosis activates Fnr which results in strong transcription initiation from P6 and reduced initiation from P6A. One of the best characterized examples of a switch mechanism in overlapping promoters is presented by the P1 and P2 promoters of the *gal* operon (Adhya, 1987). Much in the same way that anaerobiosis controls the switch between the *pfl* P6 and P6A promoters, so cAMP concentrations modulate the switch between the overlapping Crp-dependent P1 and P2 promoters of the *gal* operon (Bingham *et al.*, 1986; Irani *et al.*, 1989). More recently described examples of overlapping promoters controlling anaerobic gene expression include the P₁ and P₂ promoters of the *fixRnifA* operon in *Bradyrhizobium japonicum* (Barrios *et al.*, 1995) and the P_a and P_b promoters of the *E. coli arcA* gene (Compan & Touati, 1994). The overlapping *arcA* gene promoters are of particular relevance, since, as with the *pfl* promoters, the downstream promoter is positively regulated by Fnr. During anaerobiosis this promoter augments transcription of three constitutive promoters to increase *arcA* expression fourfold (Compan & Touati, 1994).

The consequences of P6A promoter activity for cellular physiology

During aerobiosis the P6 and P7 promoters are inactive but the P1–P5 promoters exhibit low-level activity (Sawers & Böck, 1989; Sawers, 1993). Consequently, the *focA* gene can only be transcribed by the P6A promoter aerobically, whilst the *pfl* gene is transcribed from the P6A promoter and the low-level activity of the P1–P5 promoters. This dependence of *focA* expression on P6A promoter activity was reflected in the reduced aerobic expression observed in the operon fusion derivatives where the *lacZ* gene was fused with the promoter-regulatory region of the *focApfl* operon; in contrast, inactivation of the P6A promoter had little effect on aerobic *pfl* expression. A further difference between *focA* and *pfl* expression is the stronger dependence of *focA* expression on ArcA (compare Tables 3 and 4). This suggests that *focA* expression is induced, through ArcA, during the transition from aerobiosis through microaerobiosis, whereas *pfl* expression may be induced to high levels at a later stage when the cells become more anaerobic. ArcA has been shown to be activated at higher redox potentials compared with Fnr (Fu *et al.*, 1991; Tseng *et al.*, 1996). Therefore, these differences in ArcA and Fnr dependence could introduce a temporal aspect to enzyme synthesis whereby FocA is synthesized immediately prior to Pfl. Since the FocA protein functions in regulating the intracellular formate pool (Suppmann & Sawers, 1994), it would be of benefit to the cell to maintain low-level synthesis of FocA aerobically and to induce synthesis quickly in preparation for the shift to anaerobiosis with the concomitant rapid production of formate through the action of Pfl. Expression studies in chemostat cultures where oxygen concentration can be precisely controlled will provide experimental support for these proposals.

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