Transcriptional Regulation of Genes Encoding the Selenium-Free [NiFe]-Hydrogenases in the Archaeon *Methanococcus voltae* Involves Positive and Negative Control Elements

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ABSTRACT

Methanococcus voltae harbors genetic information for two pairs of homologous [NiFe]-hydrogenases. Two of the enzymes contain selenocysteine, while the other two gene groups encode apparent isoenzymes that carry cysteinyl residues in the homologous positions. The genes coding for the selenium-free enzymes, *frc* and *vhc*, are expressed only under selenium limitation. They are transcribed out of a common intergenic region. A series of deletions made in the intergenic region localized a common negative regulatory element for the *vhc* and *frc* promoters as well as two activator elements that are specific for each of the two transcription units. Repeated sequences, partially overlapping the *frc* promoter, were also detected. Mutations in these repeated heptanucleotide sequences led to a weak induction of a reporter gene under the control of the *frc* promoters in the presence of selenium. This result suggests that the heptamer repeats contribute to the negative regulation of the *frc* transcription unit.

IT was recognized early that RNA polymerases from archaea are much more complex than homologous enzymes from the other group of prokaryotes, bacteria (Zillig *et al.* 1989). This observation raised interest in the analysis of promoter structures in Archaea. Archaeal promoters resemble those of Eucarya and contain a TATA box and an initiator element (Thomm and Wich 1988; Reiter *et al.* 1990; Gohl *et al.* 1995). Indeed, two required transcription factors are homologues of the TATA-binding protein and TFIIB, constituents of the basic eucaryal transcription apparatus (for review see Thomm 1996). Given the particular mode of transcription initiation in archaeal cells, transcriptional regulation is also of interest. So far, only a few systems have been studied in some detail. In halophilic archaea, positive regulation by an activator protein governs the expression of gas vesicle genes (Röder and Pfeifer 1996; Krüger *et al.* 1998). Recently, a putative activator was described that is necessary for the transcription of the molybdenum formyl-methanofuran dehydrogenase in the methanogenic archaeon *Methanobacterium thermoautotrophicum* (Hochheimer *et al.* 1999). Negative regulation has been demonstrated for genes involved in nitrogen metabolism in *Methanococcus maripaludis* (Cohen-Kupiec *et al.* 1997, 1999). Classical repressors had earlier been described for lysogenic archaeal viruses (Ken and Hackett 1991; Stolt and Zillig 1992).

We have been studying the transcriptional regulation of genes encoding [NiFe]-hydrogenases in *Methanococcus voltae.* This archaeon harbors genetic information for four such enzymes (Halboth and Klein 1992), two of which contain selenocysteine residues as ligands of the Ni atom in their primary reaction sites. The other two have cysteinyl residues in the homologous positions. The two transcription units, *vhc* and *frc*, encoding the latter enzymes are transcribed only under selenium limitation (Berghöfer *et al.* 1994). They are linked by an intergenic region containing all the *cis*-elements for the transcriptional regulation (Beneke *et al.* 1995). We were interested in determining what type(s) of regulation govern the transcription of the *frc* and *vhc* genes and whether or not the apparent coordinate regulation was due to common regulatory elements in the intergenic region. Our mutational analysis presented here suggests that both negative and positive regulation are involved and that an apparent silencer region mediates the coordinate regulation of the transcription of both gene groups.

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MATERIALS AND METHODS

Strains and media: *M. voltae* PS, DSM 1537 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Escherichia coli* DH5α *supE44* Δ*lacU169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* was obtained from Stratagene (La Jolla, CA). BW313 HfrKL16 PO/45 (*lysA61-62*) *dut1 ung1 thi-1 relA1* (Kunkel *et al.* 1987) was a gift of H.-J. Fritz (Göttingen). The amino acid media used for *M. voltae* were described earlier (Berghöfer *et al.* 1994; Sniezko *et al.* 1998). Selective media for the isolation of transformants contained 5–10 μg/ml puromycin. *E. coli* was cultivated in LB medium, Terrific broth, 2YT medium

TABLE 1

Primers used for *in vitro* mutagenesis

Name	Sequence[a]	Application
IR Nde105(1)	CTATATAAAC***CATATG***ATTTCGATT	Introduction of *Nde*I site 1
IR Nde170(2)	TATTTAATAGA***CATATG***AAATATTATTAAC	Introduction of *Nde*I site 2
IR Nde233(3)	CAATTCA***CATATG***AGTAAACATTTAA	Introduction of *Nde*I site 3
IR Nde290(4)	CACAAATTAGTCA***CATATG***TGTTTATATAG	Introduction of *Nde*I site 4
IR Nde347(5)	TATTTATCGGTAG***CATATG***TTATTAAGAAT	Introduction of *Nde*I site 5
f55	GTTAGCATTATAGACTAATGAGAAATTAGAATACC	Removal of natural *Nde*I site in intergenic region
f295	ATTTAAAAGTCACAAA***GATATC***AATTATGTGTTTATATAG	Mutation of heptamer I
f395	ATTTAGTCATTAGTCATT***AAGCTT***TTTAGTTTATTA TATTTT	Mutation of heptamer II
f405	CCACAATTAGTATTTTATTT***AGATCT***AAATCATTAAGCTT TTTAG	Mutation of heptamers II, III, IV
PhmvA Nsi	CTAATAGGTGAA***ATGCAT***GTTACGTCCTG	Generation of an *Nsi*I site at the start of the *uidA* gene
PhmvA Cla	TAGGTGAA***ATCGAT***GTTACGTCC	Generation of a *Cla*I site at the start of the *uidA* gene
IR Nsi	GGTTTTCCC***ATGCAT***TCACCTATTTGTTAAGC	Generation of an *Nsi*I site at the *frc* end of the intergenic region
IR Cla	CTAATGAGGTGA***ATCGAT***GGCTGAAAACTAGTACC GAAAATTGTCG	Generation of a *Cla*I site at the *vhc* end of the intergenic region

[a] Relevant restriction sites in the primers are shown in bold italics.

(Sambrook *et al.* 1989), or Standard I (Merck, Darmstadt, Germany). Plates contained 1.5% agar. Selective media were supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin.

Plasmids and primers: The plasmids used (Figure 1) were derived from Mip integration vectors (Gernhardt *et al.* 1990; Beneke *et al.* 1995; Sniezko *et al.* 1998) into which the intergenic region (IR) between the *vhc* and *frc* gene groups was inserted and linked to the *treA* (Schöck *et al.* 1996) and/or *uidA* (Jefferson *et al.* 1986) reporter genes (Beneke *et al.* 1995; Sniezko *et al.* 1998) in between *Cla*I and *Nsi*I sites. Plasmid MipvhcΔ was derived from Mipvhc. In this plasmid the intergenic region of Mipvhc was shortened by deleting the *frc*-proximal part of the intergenic region between the newly introduced *Eco*RI site (compare also Figure 2) and the *tmcr* terminator. For mutagenesis phagemid vectors pBluescript KS(+) (Short *et al.* 1988) obtained from Stratagene and pSL1180 (Brosius 1989) purchased from Amersham Pharmacia Biotech (Braunschweig, Germany) were used. The primers used for *in vitro* mutagenesis are listed in Table 1.

DNA techniques: Standard techniques for plasmid preparation and cloning were those described in Sambrook *et al.* (1989) or Ausubel *et al.* (1996). *In vitro* mutagenesis followed the method of Kunkel *et al.* (1991). The method involves the introduction of uracil instead of thymine into DNA by an *E. coli* Dut^- Ung^- mutant. Upon transfection of this strain with a phagemid and helper phage, a uracil-containing single-stranded DNA of the phagemid template is produced. A primer carrying a mutation is then annealed to the template and the second strand is synthesized *in vitro*. The double-stranded, nicked phagemid is transformed into an *E. coli* Dut^+ Ung^+ strain. The repair system of the cell then recognizes uridyl residues and removes them. The strand with the mutation serves as template for the repair. Usually >95% of the analyzed clones carry the mutation. For the construction of mutants in the intergenic region the mutation was subcloned in phagemid vectors, mutagenized, and subsequently reinserted into the relevant plasmid after sequence verification.

Transformation: Transformation of *E. coli* was done by electroporation (Ausubel *et al.* 1996) using a Gene Pulser apparatus (Bio-Rad, Munich, Germany) at 2.5 kV, 25 μF, and 200 Ω. *M. voltae* was transformed employing liposomes (Metcalf *et al.* 1997; Sniezko *et al.* 1998). Two micrograms of DNA per 10^9 cells was used. Single colonies were picked and cultivated in liquid medium.

Extract preparation and enzyme assays: The cell extracts used were centrifugation supernatants from cell lysates. They were prepared as described earlier (Beneke *et al.* 1995). Protein concentrations were determined using the dye binding assay (Bradford 1976) with bovine serum albumin as a standard. The conditions of the β-glucuronidase and trehalase tests were previously described (Beneke *et al.* 1995; Sniezko *et al.* 1998). One unit of enzyme is defined as the activity leading to the hydrolysis of 1 μmol substrate per minute at 30°.

RESULTS

Coordinate regulation of the *vhc* and *frc* promoters: The transcription of the two gene groups *frc* and *vhc*, both encoding selenium-free [NiFe]-hydrogenases in *M. voltae*, is coordinately regulated. The gene expression increases after selenium deprivation. The gene groups are connected by an IR that contains the TATA-box-initiator-type promoters and Shine-Dalgarno sequences (see Figure 2). It was previously shown that the *cis*-elements for transcriptional regulation are contained in the intergenic region (Beneke *et al.* 1995). However, in these experiments the coordinate regulation of the *frc* and *vhc* promoters was not directly shown. In the meantime we have found that a second reporter gene, *treA* from *Bacillus subtilis* can also be expressed in *M. voltae* (Sniezko *et al.* 1998). Therefore, the coordinate

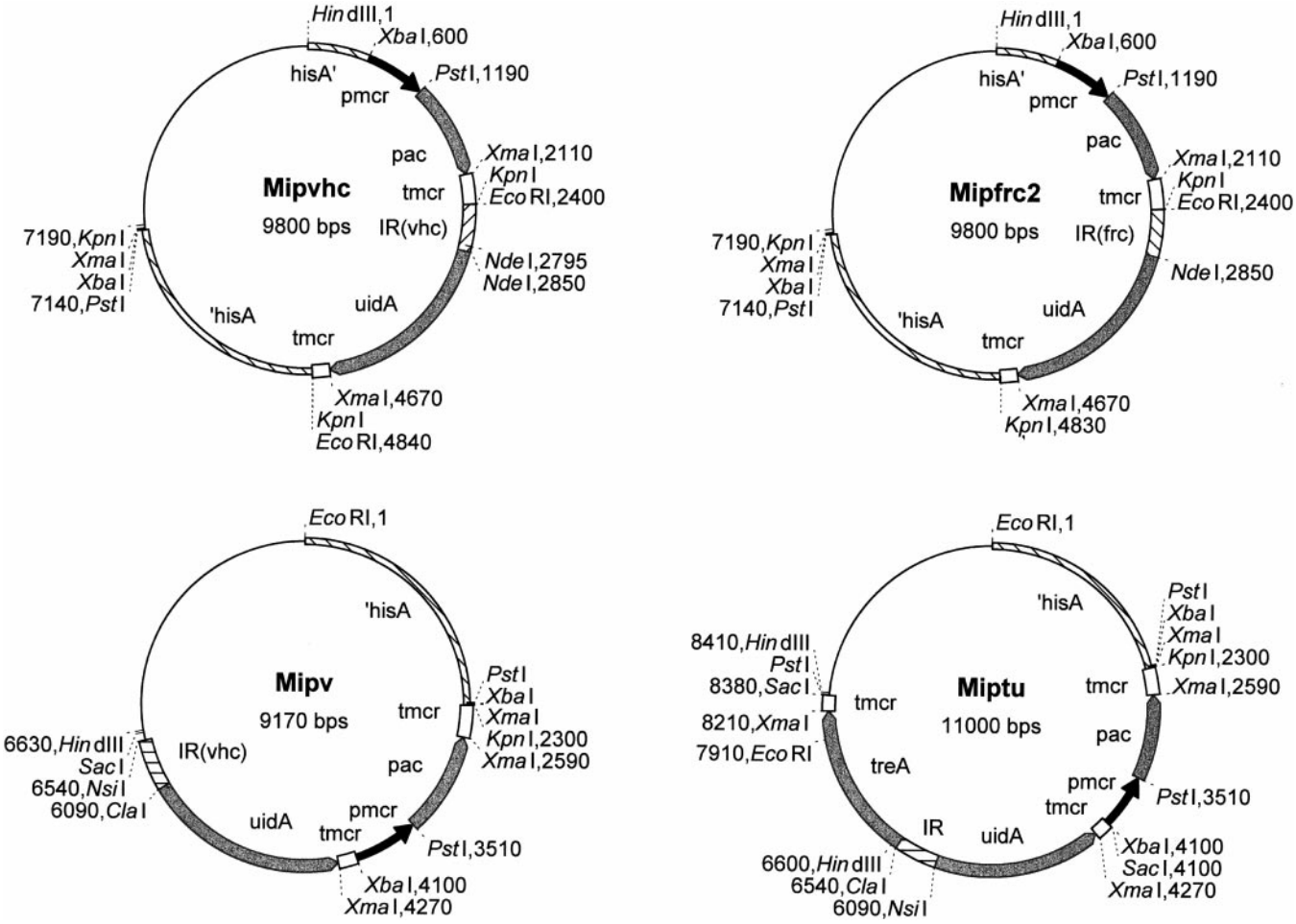

Figure 1.—All four plasmids are based on the Mip integration vectors (Gernhardt *et al.* 1990; Beneke *et al.* 1995; Sniezko *et al.* 1998). The reporter genes *uidA* from *E. coli* or *treA* from *B. subtilis* were put under the control of the *vhc-frc* IR or part thereof. The promoters thus linked to the reporter genes are indicated in parentheses. Mipvhc corresponds to Mipuid-vhc (Beneke *et al.* 1995) and served for the preparation of the deletion of the proximal part of the intergenic region yielding MipvhcΔ. Mipfrc2 is a derivative of Mipuid-frc (Beneke *et al.* 1995) in which two restriction sites were abolished: *Eco*RI at 4840 bp and a *Nde*I site within the intergenic region (compare Figure 2). Construction of the backbone for Mipv and Miptu was described by Sniezko *et al.* (1998). The IR-*uidA*-tmcr cassette for Mipv and Miptu was taken from Mipfrc2 and appropriately mutated. The source for the *treA* gene was Miptre (Sniezko *et al.* 1998). The direction of the genes is indicated by the pointed boxes. *pac*, puromycin transacetylase gene from *Streptomyces alboniger* (Lacalle *et al.* 1989) used as selection marker; *pmcr*, methyl CoM reductase promoter; *tmcr*, terminator of the methyl CoM reductase transcription unit from *M. voltae*. *hisA'* and *'hisA* are sections of the *hisA* gene (Cue *et al.* 1985) of *M. voltae* used as homologous integration sequences.

regulation of both promoters was demonstrated using cells transformed with a construct in which the *frc* promoter was linked to the *uidA* gene and the *treA* gene was connected to the *vhc* promoter (Miptu, Figure 1). Both activities were then determined in the same cell extracts. The results are shown in Table 2. Both reporter genes had only low activity in the presence of selenium. Both activities increased upon selenium deprivation. Thus the regulation of the *vhc* and *frc* promoters was coordinate.

Tandem heptamer repeats overlapping the initiator are involved in negative regulation of the *frc* promoter: As reported previously (Sorgenfrei *et al.* 1997), three heptameric sequences (II–IV, Figure 2b) repeated in tandem overlap the *frc* promoter initiator. The hep-

TABLE 2

Activity of the *vhc* and *frc* promoters dependent on selenium supply as simultaneously determined with the help of two reporter genes

	+ selenium	− selenium
β-Glucuronidase expression	1.8 ± 0.6	100.0 ± 2.7
Trehalase expression	4.7 ± 1.5	100.0 ± 4.5

The assays were performed with extracts from cells transformed with plasmid Miptu (Figure 1). All values are given in percent; 100% equals 1670 milliunits (mg of protein)$^{-1}$ in cell extracts for glucuronidase and 135 milliunits (mg of protein)$^{-1}$ for trehalase. Values are mean and standard error for 10 independent measurements.

(a)

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  1 CATCGATTCACCTCATTAGATAATCTAATATAGTCAATAGGTATTCTAATTTCTCATATG
    gtaGCTAAGTGGAGTAATCTATTAGATTATATCAGTTATCCATAAGATTAAAGAGTATAC
    vhc                                                  1     *
 61 TCTATAATGCTAACTAATATATATAATACAATTTTTAAATCGAAATCAAAAGGTTTATAT
    AGATATTACGATTGATTATATATATTATGTTAAAAATTTAGCTTTAGTTTTCCAAATATA
                                                            2
121 AGAAAATTTTAAAGACATTATATTGAATTATTAATATGGTTAATAATATTTCTAATATCT
    TCTTTTAAAATTTCTGTAATATAACTTAATAATTATACCAATTATTATAAAGATTATAGA
                                                          3
181 ATTAAATAAAGAAATACCATATTAGATTTTTTAATATATATTTAAATGTTTACTGATATG
    TAATTTATTTCTTTATGGTATAATCTAAAAAATTATATATAAATTTACAAATGACTATAC
                                                        4
241 TGAATTGTTTATTTTAAATTACAAAATAAAAACTAAACATCTATATAAACACATAATTGA
    ACTTAACAAATAAAATTTAATGTTTTATTTTTGATTTGTAGATATATTTGTGTATTAACT
    I                                                    5
301 CTAATTTGTGACTTTTAAATAAAAATATCTGTAAAAAATTCTTAATAACTTAAACTACCG
    GATTAAACACTGAAAATTTATTTTTATAGACATTTTTTAAGAATTATTGAATTTGATGGC
                                              II    *III    IV
361 ATAAATACTAGTTAAATTAAAATATAATAAACTAAAATGACTAATGACTAATGACTAAAT
    TATTTATGATCAATTTAATTTTATATTATTTGATTTTACTGATTACTGATTACTGATTTA
                                          frc
421 AAAATACTAATTGTGGCTTAACAAATAGGTGAATGCatg
    TTTTATGATTAACACCGAATTGTTTATCCACTTACGTAC
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(b)

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                  I          II     III    IV
vhc ------------TTGACTAA----ATGACTAATGACTAATGACTAA--------frc
                TGATATC     AGCTTAATGATTTAGATCTAA
                298-304          397-418
```

Figure 2.—(a) Intergenic region between the *vhc* and *frc* gene groups of *M. voltae*. The wild-type sequence is available in the GenBank file under the accession no. X 61203. At the indicated positions 1–5 *Nde*I sites were introduced that were used to create deletions as described in the text. The changed hexanucleotide sequences are shown in italics and underlined. The position of a newly created *Eco*RI site used for the construction of plasmid MipvhcΔ (Figure 1) is shown with the same signature. It needed a G to C mutation at position 247. The TATA box and initiator (ATGA) elements of the two promoters are in boldface. The transcription initiation points (the G nucleotides within the initiator sequences) are marked with asterisks. The roman numerals I–IV indicate the positions of the four TGACTAA heptamers. (b) Mutational changes introduced into the heptamers. In the top row the wild-type sequences are shown. Note that the three adjacent heptamers will also look identical to each other if read in a frame shifted by one nucleotide in the 5′ direction. The second row indicates the changes introduced by site-directed mutagenesis in the mutants described in Table 3. The mutational changes are indicated in bold italics. The positions of the nucleotides in the intergenic region are also given by numbers. Alternative mutants were also tested and gave the same results.

tamer can be defined either as TGACTAA or ATGACTA. Assuming the first sequence, a fourth copy of the sequence was detected further upstream in the section (I, nucleotides 298–304, Figure 2b). To assess the effect of these sequences on the *frc* promoter activity, we subjected the sequences to site-directed mutagenesis in a construct in which the *uidA* gene was linked to the *frc* promoter (Mipfrc2, Figure 1). Mutation of the three boxes overlapping the initiator resulted in a small increase of the promoter activity (Table 3). Mutation of the single TGACTAA sequence (heptamer I) did not influence the expression of *uidA*. Mutation of only heptamer II belonging to the triple heptamer repeat had a slight but significant effect. This might mean that the relevant heptameric sequence reads ATGACTA, because the three adjacent heptamers II, III, and IV can be read in this way if the frame is shifted by one position in the 5′ direction; the apparently irrelevant sequence of heptamer I would then change to *T*TGACTA (see Figure 2).

Deletion of the region upstream of the *frc* gene group changes the regulatory pattern of the *vhc* promoter: We have suggested earlier (Sorgenfrei *et al.* 1997) that the heptamer sequences might be negative regulatory elements that influence the expression of both the *frc* and *vhc* promoters, acting as operators and silencers at the same time. This model predicted that removal of

TABLE 3

Role of heptamer repeats on the regulation of the *frc* promoter

Construct	β-Glucuronidase activity	
	+ selenium	− selenium
Mipfrc2	2.1 ± 0.2	97.3 ± 5.0
Mipfrc2 Mut I, II–IV	10.0 ± 0.5	103 ± 6.1
Mipfrc2 Mut II–IV	9.0 ± 0.4	ND
Mipfrc2 Mut I, II	3.8 ± 0.5	ND
Mipfrc2 Mut I	1.2 ± 0.6	ND

All values are given in percent; 100% equals 2650 milliunits mg protein)$^{-1}$ in cell extracts. Values are mean and standard error for at least eight independent measurements. The measurements were done with extracts from cells transformed with mutant derivatives of plasmid Mipfrc2 (Figures 1 and 2). ND, not determined.

TABLE 4

***uidA* expression under control of the *vhc* promoter of the intact *vhc-frc*-intergenic region or a part lacking the *frc* promoter-operator region**

Construct	β-Glucuronidase activity	
	+ selenium	− selenium
Mipvhc	<1	98.6 ± 9.4
MipvhcΔ	22.7 ± 1.7	102.6 ± 2.6

All values are given in percent; 100% equals 2700 milliunits (mg of protein)$^{-1}$ in cell extracts. Values are mean and standard deviations for at least four independent measurements. Mipvhc contains the intact intergenic region. In MipvhcΔ the *frc*-proximal part of the IR was removed (compare Figures 1 and 2).

the heptamers would cause the induction of both the *frc* and *vhc* promoters in the presence of selenium. We therefore constructed an integration plasmid that carried the intergenic region linked to the *uidA* gene under the control of the *vhc* promoter and lacked the upstream region of the *frc* promoter including the heptamer repeats. While this construct exhibited *vhc* promoter activity (Table 4), the induction was incomplete. Thus, other regulatory *cis*-elements beside the heptamers probably influenced the *vhc* transcription. To identify those elements, we performed a deletion analysis of the intergenic region. Because the measurable trehalase activity in the cell extracts was comparatively low, the *uidA* reporter gene was used throughout in this approach. In an additional construct (Mipv, Figure 1) the intergenic region was therefore linked to the reporter gene so that the β-glucuronidase expression was governed by the *vhc* promoter.

Deletion analysis of the intergenic region leads to the identification of further positive and negative regulatory elements: To perform deletion analyses, we introduced pairs of evenly spaced *Nde*I sites into the IR sequence to allow the deletion of defined parts of the intergenic region (Figure 2a). With the intact intergenic region, the expression of the *uidA* reporter gene was turned off in the presence of selenium when attached to either the *frc* or the *vhc* promoter. Deletion of a *vhc*-proximal part of the intergenic region (Δ1-3) led to a loss of function of the *vhc* promoter but, surprisingly, also strongly affected the *frc* promoter. Deletion Δ2-3 partially relieved the negative effect of selenium on both the *frc* and *vhc* promoters. However, it also led to reduction of the *vhc* promoter activity in the absence of selenium without affecting the *frc* promoter activity under this condition (compare lines 3 of Tables 5 and 6). This indicated the existence of positive regulation of the *vhc* promoter by an element contained in the region between the *Nde*I sites 2 and 3. This conclusion was confirmed by the Δ2-4 construct. This deletion also affected the activity of the *frc* promoter, which was reduced

TABLE 5

Deletion analysis of the *vhc-frc*-intergenic region linked to the reporter gene *uidA* in *frc* direction

Construct	β-Glucuronidase expression	
	+ selenium	− selenium
Miptu	1.8 ± 0.6	99.0 ± 2.7
Miptu Δ (1-3)	<1	<1
Miptu Δ (2-3)	40.4 ± 1.3	100.9 ± 1.3
Miptu Δ (2-4)	50.6 ± 0.5	52.9 ± 1.0
Miptu Δ (3-4)	<1	48.9 ± 2.3
Miptu Δ (3-5)	<1	40.0 ± 2.2
Miptu Δ (1-5)	<1	1.0

All values are given in percent: 100% equals 1667 milliunits (mg of protein)$^{-1}$ in cell extracts. Values are mean and standard error for 10 independent measurements.

(Table 5, line 4). Similarly, deletions Δ3-4 and Δ3-5 both reduced *uidA* expression from the *frc* promoter. However, these deletions did not affect expression from the *vhc* promoter because the pattern of expression was similar to that observed with the complete intergenic region (compare Tables 5 and 6, lines 6). Upon deletion of region 1-5, both promoters were affected in the same way as with the Δ1-3 deletion.

DISCUSSION

Positive and negative regulatory *cis*-elements of transcriptional regulation are known to be involved in regulation of both bacterial and eucaryal genes. The classical elements of bacterial negative transcriptional regulation are the operators located close to or overlapping with the promoter sequence as first described for the *lac* operon of *E. coli* (for review see Beckwith and Zipser 1970). They are binding sites for repressor proteins that interfere with the binding of the RNA polymerase or its action and therefore with the initiation of transcription.

TABLE 6

Deletion analysis of the *vhc-frc* intergenic region linked to the *uidA* reporter gene attached to the *vhc* promoter

Construct	β-Glucuronidase expression	
	+ selenium	− selenium
Mipv	1 ± 0.1	101.1 ± 7.0
Mipv Δ (1-3)	9.6 ± 0.9	13.5 ± 1
Mipv Δ (2-3)	36.9 ± 0.5	56.2 ± 1
Mipv Δ (2-4)	38.0 ± 0.5	40.9 ± 1.5
Mipv Δ (3-4)	2.2 ± 0.2	103.8 ± 9.4
Mipv Δ (3-5)	2.0 ± 0.6	95.0 ± 9.4
Mipv Δ (1-5)	10.6 ± 1.0	11.2 ± 1

All values are given in percent: 100% equals 2440 milliunits (mg of protein)$^{-1}$ in cell extracts. Values are mean and standard error for 10 independent measurements.

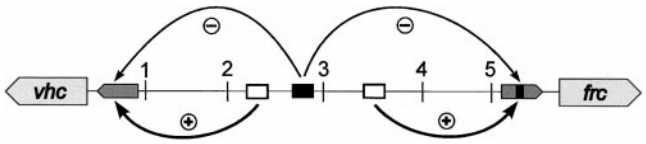

Figure 3.—Model describing the coordinate regulation of the gene groups encoding selenium-free hydrogenases in *M. voltae*. The gene groups are indicated as large pointed boxes. They point in the direction of their transcription. The promoters are shown as smaller pointed boxes proximal to the genes. Negative regulatory elements are shown in black; positive regulatory elements are shown as open boxes. The locations of the *Nde*I sites introduced for the deletion mutagenesis are numbered as in Figure 2. Note that the relative positions of the silencer element and the positive regulatory element shown within section 2-3 are arbitrary. They could be overlapping.

This type of regulation is also found in eukaryotes (for reviews see Renkawitz 1990; Hanna-Rose and Hansen 1996). Positive regulator elements can be either adjacent to the promoter as activator binding sites, like the classical Crp binding site (for review see Reznikoff 1992), or further upstream, like the NtrC binding site (Reitzer and Magasanik 1986) in *E. coli*. In the latter case they are functional counterparts of eucaryal enhancers. Silencers are regulatory elements that lead to a reduction of promoter activity at a distance. They are common regulatory elements in Eucarya (for review see Ogbourne and Antalis 1998). So far they have been found in only a few cases in Bacteria (Fletcher and Csonka 1995; Jubete *et al.* 1995; Schnetz 1995; Schnetz and Wang 1996; Murphree *et al.* 1997). These silencer regions can extend over more than 50 bp.

We were interested in understanding the coordinate regulation of the two transcription units encoding selenium-free [NiFe]-hydrogenases in the methanogenic archaeon *M. voltae*. Our experiments have revealed at least four regulatory regions in the intergenic region linking the two transcription units *frc* and *vhc*. They are depicted in Figure 3. The repeated heptamer overlapping with the initiator of the *frc* promoter resembles an operator. However, it plays only a minor role. It does not influence the *vhc* promoter, which was found to be inactive in the presence of selenium in a construct in which the tandem repeats were deleted (data not shown). The main negative regulatory region is located in region 2-3 (Figures 2 and 3), because its deletion can lead to roughly 40-fold induction of both promoters. It is therefore a common regulatory element. Still, further mutational analysis is needed to rule out that this interval contains two very closely neighboring independent promoter-specific elements. In any case, the element(s) would function at a distance of at least 100 bp, which is common for silencers and corresponds to the distances seen with bacterial enhancers and silencers or upstream regulatory sequences in eucaryal promoter regions.

As mentioned, negative regulation by silencers is rare in bacteria. In one of the described cases elements on both sides of the affected promoter are needed (Schnetz 1995). This is ruled out in our case because the affected promoters drive reporter genes that are followed by plasmid sequences. It is highly unlikely that a putative silencer protein would specifically interact with elements in these sequences. In contrast to the silencer region, the two positive regulation elements that we have detected in regions 2-3 and 3-4 are specific for the *vhc* or *frc* promoters, respectively. The coordinate regulation therefore appears to rely mainly on the silencer.

The results obtained with deletions Δ1-3 and Δ1-5 show that both lead to a strong reduction or complete loss of the *vhc* or *frc* promoter activity, respectively. We have found that a Δ1-2 deletion has the same effect on the *frc* promoter (data not shown). These results are not incorporated in the model shown in Figure 3. In principle, the 1-3 region could contain another activating element for the *vhc* promoter. However, the simultaneous negative effects of deletions Δ1-2, Δ1-3, and especially Δ1-5 on *both* promoters are difficult to understand, because in the latter case the silencing element located in section 2-3 is removed. Further investigations will therefore be needed to explain these findings that could be due to a more general effect such as a change in DNA or even chromatin structure, which could also influence the promoter activities as reported for the known bacterial silencers (Schnetz and Wang 1996).

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