Identification of the Promoter of the \textit{Bacillus subtilis} \textit{sdh} Operon

LARS MELIN, KERSTIN MAGNUSSON, AND LARS RUTBERG

Department of Bacteriology Karolinska Institutet, S-104 01 Stockholm, and Department of Microbiology, University of Lund, Sölvérgatan 21, S-223 62 Lund, Sweden

Received 30 December 1986/Accepted 16 April 1987

The \textit{Bacillus subtilis} \textit{sdhCAB} operon contains the structural genes for the three subunits of the membranebound succinate dehydrogenase complex. An \textit{sdh}-specific transcript of about 3,450 nucleotides was detected in vegetative bacteria. S1 nuclease mapping experiments showed that the \textit{sdh} operon is transcribed from a sigma-43 promoter; the transcript starts at a guanosine residue 90 base pairs upstream from the first gene of the operon, \textit{sdhC}. No \textit{sdh} transcript was found in \textit{B. subtilis} carrying the \textit{sdh-115} mutation, which decreases expression of the \textit{sdh} operon by more than 99%. The \textit{sdh-115} mutation is a G-to-A transition in the \textit{−35} region of the sigma-43 promoter. The \textit{sdh} operon is sensitive to glucose repression. When the \textit{sdh} promoter region was used to drive transcription of the \textit{cat-86} gene this gene also became glucose repressed.

The \textit{Bacillus subtilis} membranebound succinate dehydrogenase [SDH; EC 1.3.99.1, succinate:(acceptor) oxidoreductase] complex contains equimolar amounts of three subunits: a flavoprotein, an iron-sulfur protein, and cytochrome \textit{b}558 (13). The level of SDH activity in the bacteria varies up to 5- to 10-fold, depending on the growth conditions. SDH is subject to glucose repression. The SDH activity increases when growing bacteria enter stationary phase; this increase is repressed by glucose (11, 23). Both glucose repression and postexponential increase in enzyme activity are thought to reflect transcriptional control of the \textit{sdh} genes. The structural genes for the subunits of the SDH complex, \textit{sdhC} (cytochrome \textit{b}558), \textit{sdhA} (flavoprotein), and \textit{sdhB} (iron-sulfur protein), have been cloned and sequenced (12, 17, 18, 24). The designation of these genes has recently been altered to conform with the \textit{Escherichia coli} \textit{sdh} and \textit{frd} operons (24). All three genes are closely linked and are thought to constitute an operon (19, 24), which is transcribed in the order \textit{sdhC}, \textit{sdhA}, \textit{sdhB}. At least six sigma factors which recognize different promoter structures have been found in \textit{B. subtilis} (5). The use of alternate promoters has been described for several genes in \textit{B. subtilis}, and minor forms of RNA polymerase holoenzyme are known to be involved in temporal control of gene expression (5, 11, 15). In the \textit{sdh} operon, one sigma-43 and two possible sigma-32 promoter sequences have been found upstream from \textit{sdhC} (18). The aim of the present experiments was to identify the promoter(s) of the \textit{sdh} operon.

MATERIALS AND METHODS

**Bacteria and plasmids.** The bacterial strains used were \textit{B. subtilis} 3G18 (ade \textit{met} \textit{trp}C2) and 3G18 \textit{sdhA12} (ade \textit{met} \textit{trp}C2 \textit{sdhA12} [8a]) and \textit{E. coli} 5K (\textit{hsdR hsdM thi thr rpsT lacZ}). Plasmid pKIM4 (Fig. 1a) is a derivative of pHV32 (22), which carries the \textit{sdh} promoter region, \textit{sdhC}, and part of \textit{sdhA} (17). pKIM115 is pKIM4 carrying mutation \textit{sdh-115}. pLR1 is a derivative of the promoter-searching plasmid pPL603 (34); it carries a promoterless \textit{cat-86} gene and expresses resistance to kanamycin.

**Media.** The bacteria were maintained on tryptic blood agar base (Difco); antibiotics were used as needed. Liquid cultures were grown in LB broth (20) or in nutrient sporulation medium (8); details are given in the respective figure legends. When used, antibiotics were added at the following concentrations: chloramphenicol and kanamycin, 5 μg ml⁻¹; ampicillin, 35 μg ml⁻¹.

**Competent \textit{B. subtilis}** (1) and \textit{E. coli} (20) were prepared by published methods.

**Extraction of DNA and RNA.** Plasmid DNA was prepared by standard methods (20). RNA was purified from \textit{B. subtilis} and \textit{E. coli} by the hot-phenol method (32).

**Blotting techniques.** DNA was transferred to nitrocellulose filters after separation on agarose gels by the method of Southern (29). Hybridization with labeled probes was done with the same protocol. For RNA, total RNA was separated on agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized with a probe as described previously (2).

**S1 nuclease mapping.** S1 nuclease mapping was done essentially as described by Berk and Sharp (2).

**General methods.** Restriction enzymes, T4 polynucleotide ligase, DNA polymerase I (Klenow fragment), calf intestinal phosphatase, and polynucleotide kinase were from New England BioLabs, Inc., or Boehringer GmbH and were used as instructed by the manufacturers. Nick translation was done with a kit from Amersham Corp. DNA sequencing was done by the dideoxy chain-terminating method (27) by using the phage M13 system (21) and by using primers specific for the \textit{sdh} operon (18). SDH activity was measured as described previously (13), and chloramphenicol acetyltransferase (CAT) activity was measured by a colorimetric method (28).

RESULTS

**Identification of an \textit{sdh} transcript.** Inspection of the nucleotide sequence upstream from \textit{sdhC} has revealed a typical sigma-43 promoter sequence and two possible sigma-32 promoter sequences (18). All three sequences are located within 200 base pairs (bp) upstream from the ATG initiation codon of \textit{sdhC}. To show that this segment contains promoters which are active in vivo in \textit{B. subtilis} and also to facilitate later analysis of the \textit{sdh} promoter region, the following experiment was done. Plasmid pLR1 is a derivative of pPL603 (34). About 200 bp upstream, the promoterless \textit{cat-86} gene of pLR1 contains a cloning cassette from bacteria phase M13mp8 (21). A 520-bp Sau3A-PstI fragment from pKIM31 (Fig. 1a) was cloned into \textit{BamHi-PstI}-cleaved
The fragment harbors the promoter sequences described above and extends 290 bp into the sdh region. The resulting plasmid, pSDP4 (Fig. 1b), confers resistance to at least 50 μg of chloramphenicol ml⁻¹ in B. subtilis.

The cat-86 gene in pSDP4 is carried on a 3.2-kilobase (kb) BamHI-BgIII fragment which does not include the origin of replication of pLR1. This fragment was isolated, ligated, and used to transform B. subtilis 3G18, selecting for resistance to 5 μg of chloramphenicol ml⁻¹. Such transformants can arise only by integration of the fragment into the B. subtilis chromosome by homologous recombination (10, 22). Southern blotting (29) of DNA from one such transformant, 3G18::sdp8 cleaved with PstI, confirmed that the BamHI-BgIII fragment had integrated in tandem with the sdh operon (Fig. 2).

To demonstrate the presence of an sdh transcript in B. subtilis, RNA was extracted from several strains and fractionated by agarose gel electrophoresis under denaturing conditions. The RNA was then transferred to nitrocellulose filters and hybridized to a nick-translated 520-bp Sau3A-PstI fragment from pKIM4. In strain 3G18, a transcript of about 3,450 nucleotides (nt) was found (Fig. 3a). No transcript which hybridized with the probe was found in strain 3G18 sdhΔ12, a strain in which essentially all of the sdh operon, including the promoter region, is deleted (8a), which confirms the specificity of the probe. Also, no transcript was detected in bacteria which carried the sdh-115 mutation. This mutation decreases expression of all three genes of the sdh operon by more than 99% (19; L. Hederstedt, unpublished data); sdh-115 is a promoter down mutation (see below). A 3,450-nt transcript was detected also in strain 3G18(pSDP4) and in 3G18::sdp8 (Fig. 3b). In addition, a

FIG. 1. (a) General structure of the B. subtilis sdh operon, with some relevant restriction sites indicated. The sdh promoter is located within the 520-bp Sau3A-PstI fragment. Parts of the operon have been subcloned in plasmids pKIM4 (17) and pKIM31, which were used in the present work. Abbreviations: B, BamHI; E, EcoRI; P, PstI; and S, Sau3A. The bar represents 1 kb. (b) General structure of pSDP4. The 520-bp Sau3A-PstI fragment which contains the sdh promoter region is drawn with double lines. The single line indicates the vector, with the cat-86 gene marked as a hatched box. Abbreviations: Km', kanamycin resistance gene from pUB110; ori, origin of replication of pSDP4; T, TaqI. Other abbreviations are defined in the legend to Fig. 1a.

FIG. 2. Structure of part of the 3G18::sdp8 chromosome carrying the 3.2-kb BamHI-BgIII fragment from pSDP4. The arrows indicate the approximate start and direction of transcription from the duplicated sdh promoter. S(B/b) is the Sau3A site generated by self-ligation of the 3.2-kb BamHI-BgIII fragment. cytA denotes the truncated cytochrome b558 (sdhC) gene. b, BgIII; other abbreviations are defined in the legend to Fig. 1a.

FIG. 3. Filter hybridization of RNA (Northern blots) from different B. subtilis strains with a nick-translated 520-bp Sau3A-PstI fragment from pKIM4 which contains the sdh promoter region and the 5' half of the sdhC gene (see Fig. 1a). The bacteria were grown with shaking at 37°C in 100 ml of LB medium in 1-liter flasks having indentations. RNA was extracted from exponentially growing cells (A600 = 0.6) (32), fractionated by agarose gel electrophoresis under denaturing conditions, transferred to nitrocellulose filters, and then hybridized with the probe. RNA from the following strains was tested. (a) Lanes: 1, strain 3G18; 2, 3G18 sdhΔ12; 3, strain 3G18 sdh-115. (b) Lanes: 1, strain 3G18 sdh-115; 2, strain 3G18::sdh8; 3, strain 3G18(pSDP4).
smaller transcript of about 1,250 nt was found which represents cat-86 transcription driven by the sdh promoter. Both strains contain larger amounts of cat-86 transcript compared with sdh. For strain 3G18(pSDP4), this is an expected gene copy effect. In 3G18::sdp8, this could reflect, for example, duplication of the integrated BamHI-BglII fragment (36) or greater stability of the cat-86 transcript.

Mapping of the sdh promoter. The potential sigma-43 promoter starts 96 bp upstream from the ATG initiation codon of sdhC, and the two tentative sigma-32 promoters start 62 and 148 bp, respectively, upstream from the same ATG (18). The promoter sequences do not overlap. To identify the sdh promoter(s), the transcriptional start of the sdh transcript was mapped by S1 nuclease protection experiments. RNA was extracted from different B. subtilis strains and also from E. coli(pKIM4) and E. coli(pKIM115). The probe used was an SmaI-TaqI fragment from pSDP4. The TaqI site starts at nt 29 in the sdhC coding region; the SmaI site is located 5 bp upstream from the Sau3A site in pSDP4. In strain 3G18, a single protected fragment of 120 nt was found, whereas no protected fragment was found in RNA from bacteria carrying the sdh-11S mutation (Fig. 4a). A single 120-nt protected fragment was detected also in RNA from strain 3G18(pSDP4), 3G18::sdp8, and E. coli(pKIM4) (Fig. 4b). These results place the transcriptional start of the sdh operon at a guanosine residue about 10 bp downstream from the middle of the sigma-43 −10 sequence (Fig. 5). They also confirm a previous suggestion that, in pKIM4, sdhC is transcribed from its native promoter in E. coli (18). In addition to the 120-nt fragment, several minor fragments of about 100 nt each were found in RNA from E. coli(pKIM4). We do not know whether these latter fragments represent

FIG. 4. S1 nuclease mapping of the sdh promoter. The bacteria were grown in LB medium as described in the legend to Fig. 3 and harvested during exponential growth (A600 = 0.5 to 0.7) for RNA extraction. S1 nuclease mapping was done essentially as described in reference 2. The end-labeled probe used was a SmaI-TaqI fragment from pSDP4 (see Fig. 1b) which contains the whole sdh promoter region and which extends 29 nt into the sdhC gene. RNA from the following strains was tested. (a) Lane A, strain 3G18; lane B, strain 3G18 sdh-11S. (b) Lanes 1, E. coli 5K(pKIM4); 2, E. coli 5K(pKIM115); 3, strain 3G18; 4, 3G18(pSDP4); 5, 3G18::sdp8; 6, probe incubated without RNA. The rightmost lanes in panel a and the leftmost lanes in panel b are sequencing gels used for size determination of fragments.

FIG. 5. Nucleotide sequence of the sdh promoter region; the numbering of the nucleotides is based upon that in reference 18. The −35 and −10 sequences of the promoter are underlined, the marked G residue at +1 indicates the transcriptional start, the proposed ribosome-binding sequence for sdhC is underlined, and the start of sdhC is indicated. The G residue, marked with an asterisk in the −35 region, is mutated to an A residue in strain 3G18 sdh-11S. Within sets of parentheses are shown the new transcriptional start found in E. coli 5K(pKIM115) (+1X), the proposed ribosome-binding site (rbsX), and the start (orfX) and stop (stopX) of the region coding for a small peptide upstream of sdhC and which overlaps with this gene.
additional transcriptional starts or degradation at the 5' end of the 120-nt fragment.

In E. coli(pKIM115), the 120-nt protected fragment was not found; instead, a longer fragment of 167 nt appeared (Fig. 4b), suggesting that a new transcriptional start was used. This is surprising since we have not detected any B. subtilis cytochrome b558 in E. coli(pKIM115). Inspection of the sequence contained in the pKIM115 transcript revealed a strong ribosomal binding site (GGAGG) followed after seven bases by an ATG initiating an open reading frame which terminates with a TAA stop codon overlapping the start codon of sdhC (Fig. 5) (18). Translation of this short peptide may effectively block translation of sdhC from the same mRNA. Sequencing of the sdh-115 mutation has shown it to be a G-to-A transition in the sigma-43 −35 sequence (Fig. 5).

In the experiments described above, in which RNA was extracted from exponentially growing cells, some ambiguity is possible that a minor sdh promoter is activated, e.g., during sporulation. However, we have not been able to detect any increased SDH activity in 3G18::sdh-115 in stationary-phase cultures grown in several different media, including nutrient sporulation medium (8).

**Control of cat-86 expression from the sdh promoter.** The experiments described above have identified the (major) sdh promoter. In order to see whether control elements involved in glucose repression were located within the 520-bp Sau3A-PstI fragment in pSDP4, the effect of glucose on cat-86 expression was determined. In 3G18::sdp8, both SDH and CAT activities increased during stationary phase, and both activities were repressed by glucose (Fig. 6). Transcription of sdh starts at the same G nucleotide both in the presence and in the absence of glucose (data not shown). No glucose effect on cat-86 expression was found in 3G18(pSDP4); this finding most likely reflects a gene copy number effect.

**DISCUSSION**

The expected size of the B. subtilis sdh operon transcript is about 3,300 nt, as calculated from the transcriptional start determined here and the proposed stem-loop termination structure downstream from the sdhB gene (24). In Northern blots, an sdh transcript of about 3,450 nt was found; this 5% difference from the calculated size could well be within the error of the size determination. A new reading frame opens 65 bp downstream from sdhB (24). The present size estimate of the sdh transcript, as well as the location of the termination structure proposed above (24, 33), make it unlikely that this open reading frame is part of an sdh transcript.

S1 nuclease protection experiments using RNA from B. subtilis strains with the sdh promoter region in a chromosomal or plasmid location have shown that the sdh transcript starts at a G residue located 10 bp downstream from the middle of a typical sigma-43 −10 sequence. The sdh-115 mutation, which decreases expression of the sdh operon in E. coli(pKIM4) by more than 99%, is a G-to-A transition in the −35 sequence of the same sigma-43 promoter. The fact that we cannot detect any sdh transcript in bacteria carrying the sdh-115 mutation makes it very unlikely that an alternative promoter, other than the sigma-43, can be used to transcribe the sdh operon at a rate required for normal functioning of the Krebs cycle during growth. B. subtilis mutants which produce about 10% of wild-type SDH activity have an SDH-negative phenotype (L. Hederstedt, unpublished data); i.e., they accumulate and excrete succinate. Although we cannot exclude the possibility that some minor promoter can be used to transcribe the sdh operon, we conclude that the sigma-43 promoter described above is the (major) sdh promoter.

This promoter is also used to transcribe the sdhC gene in E. coli(pKIM4). However, when the promoter is weakened by the sdh-115 mutation, a new transcriptional start 47 nt upstream from the wild-type start appears. This start is not used in B. subtilis. Upstream from this start there is a perfect sigma-70gc −10 box. The plasmid pKIM115 carries the whole sdhC gene, yet the gene is not expressed in E. coli although it is clearly transcribed from the 'new' upstream promoter. A possible explanation is that translation of a small peptide that terminates within sdhC blocks translation of this gene.

Several enzymes in B. subtilis are glucose repressed (6, 7, 23). For aaconitase, there is fair evidence that glucose repression causes a decreased transcriptional rate of the respective gene, catB (26). The fact that cat-86 expression in 3G18::sdp8 is glucose repressed argues that, for sdh also, glucose repression affects translation.

In E. coli, glucose repression has been shown to operate via affecting the levels of cyclic AMP in the cells, and the mechanics of the regulation are known in considerable detail (4). Little is known about the corresponding mechanisms in B. subtilis. This latter bacterium is thought not to contain cyclic AMP (3, 14), although there is at least one report to the contrary (16). A number of glucose-resistant sporulation mutants of B. subtilis have been isolated (30, 31). One of these mutations has been located to the gene rpoD, which codes for the sigma-43 polypeptide (25, 33), and another mutation maps in the rpoBC operon (30).

If glucose affects transcription in B. subtilis catabolite-repressed genes, it might be possible to identify some conserved nucleotide sequences in the promoter region of this operon.
such genes. So far, very few glucose-repressed genes have been sequenced, and so very few of their promoters have been mapped carefully. A comparison between the promoter region for the gnt operon (9) and sdh aligned at the common TTG of the −35 sequence shows that 9 of 12 bases (AAAT\_TA\_\_AA\_TTA) from −49 to −60 are identical. However, since most B. subtilis sigma-43 promoters have very AT-rich −40 to −60 regions, the significance of this homology is uncertain. Promoter regions from more glucose-repressed genes have to be characterized before sequences specifically involved in repression may be identified.

ACKNOWLEDGMENTS

We thank Alexander von Gabain for much help.

This work was supported by a grant from the Swedish Medical Research Council.

LITERATURE CITED


