Transcription and transcript processing in the 
\textit{sdhCDAB–sucABCD} operon of \textit{Escherichia coli}

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The genes encoding succinate dehydrogenase (\textit{sdhCDAB}), the specific components of the 2-oxoglutarate dehydrogenase complex (ODH, \textit{E1o} and \textit{E2o}; \textit{sucAB}) and succinyl-CoA synthetase (\textit{sucCD}) form a cluster containing two promoters at 163 min in the chromosome of \textit{Escherichia coli}: \textit{Psdh sdhCDAB-P_\textit{suc sucAB–sucCD}}. The gene encoding the lipoamide dehydrogenase component of both the 2-oxoglutarate and pyruvate dehydrogenase complexes (\textit{E3; IpdA}) is the distal gene of another cluster containing two promoters located at 2.7 min: \textit{P_{pdh pdhR–ace€H,@ IpdA}}. The responses of the \textit{suc} and \textit{lpd} promoters to different environmental conditions and to regulator defects were investigated with appropriate \textit{lacZ} fusions, in order to understand how expression of the \textit{sucAB} genes is co-regulated with other genes in the \textit{sdhCDAB–sucABCD} cluster and with \textit{ipdA} expression. Expression from the \textit{su} promoter was repressed by IHF and partially activated by \sigma^{32} but it was not regulated by ArcA, FNR, CRP, FruR or Fis, and not repressed by glucose or anaerobiosis, indicating that the well-established catabolite and anaerobic repression of ODH synthesis is imposed elsewhere. In contrast, the \textit{lpd} promoter was repressed by both glucose (via a CRP-independent mechanism) and anaerobiosis (mediated by ArcA), and activated by Fis, but it was not regulated by FNR, FruR, IHF or \sigma^{32}. These observations support the view that transcription of the \textit{sucABCD} genes is primarily initiated and regulated at the upstream \textit{sdh} promoter, and that the \textit{lpd} promoter is independently co-regulated with \textit{P_{sdh}} (primarily by ArcA-mediated repression) rather than with \textit{P_{suc}}. Direct evidence for co-transcription of the entire \textit{sdhCDAB–sucABCD} region from \textit{P_{sdh}} was obtained by detecting a 10 kb transcript in \textit{mc} and \textit{me} mutants, but not in the parental strains. Three \text{RNasell}-specific processing sites, which contribute to the extreme instability of the readthrough transcript, were identified in the \textit{sdhCDAB–sucABCD} intergenic region. Other sites of endonuclease processing were located by interpreting the patterns of transcript subfragments observed in Northern blotting.

\textbf{Keywords:} 2-oxoglutarate dehydrogenase, succinate dehydrogenase, lipoamide dehydrogenase, mRNA processing, \text{RNaseIII}

\textbf{INTRODUCTION}

The pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (PDH and ODH) complexes are analogous multienzyme complexes that catalyse the oxidative decarboxylation of pyruvate to acetyl-CoA or of 2-oxoglutarate to succinyl-CoA (Guest \textit{et al.}, 1989; Perham, 1991). They perform important metabolic roles in funnelling glycolytic carbon into the citric acid cycle and its subsequent oxidation in the cycle. The synthesis of the complexes under different environmental conditions has been extensively studied at the enzyme and protein levels (Amarasingham \textit{&} Davis, 1965; Buck \textit{et al.}, 1986; Quail \textit{et al.}, 1994; Smith \& Niedhardt, 1983). Thus, the PDH complex is induced by pyruvate and partially repressed by growth on glucose or acetate and by anaerobiosis. In contrast, the ODH complex is most
highly induced during aerobic growth on acetate and citric acid cycle intermediates, and like succinate dehydrogenase and succinyl-CoA synthetase (SCS) it is severely repressed by excess glucose, anaerobiosis and cAMP deficiency. Indeed, the repression of these three co-ordinately regulated enzymes is a major feature of the conversion of the citric acid cycle into its branched or non-cyclic anaerobic form (Guest & Russell, 1992; Cronan & LaPorte, 1996).

The PDH and ODH complexes are encoded by two distinct gene clusters in the *Escherichia coli* chromosome (Fig. 1). The *pdh* operon (*pdhR-aceEF-lpdA*) at 2-7 min encodes a pyruvate-responsive repressor (*PdhR*), the specific pyruvate dehydrogenase (E1p) and lipoate acetyltransferase (E2p) components of the PDH complex, and lipoamide dehydrogenase (E3), which is a common component of both the PDH and ODH complexes (Quail et al., 1994). The genes are expressed from two major promoters: *P*$_{pdh}$ which generates a 7-4 kb *pdhR-lpdA* readthrough transcript; and *P*$_{lpdA}$, which generates an independent 1.7 kb *lpdA* transcript (Quail et al., 1994; Spencer & Guest, 1985). Quantitative S1 mapping studies indicated that transcription from *P*$_{lpdA}$ is co-regulated with synthesis of the ODH complex, so as to maintain a supply of E3 subunits when the synthesis of all components of the PDH complex (via *P*$_{pdh}$) is repressed, but synthesis of the ODH complex is induced (Spencer & Guest, 1985). The specific dehydrogenase and lipoate succinyltransferase components of the ODH complex are encoded by the *sucA* (E1o) and *sucB* (E2o) genes in the *gltA-sdhCDAB-sucABCD* gene cluster at 16.3 min (Fig. 1), which additionally encodes citrate synthase (CS, *gltA*), succinate dehydrogenase (SDH, *sdhCDAB*), and succinyl-CoA synthetase (SCS, *sucCD*). All of the genes, except *gltA*, have the same polarity, and S1 mapping studies established that the *gltA-sdhA* intergenic region contains two *gltA* promoters and a divergent but non-overlapping *sdhA* promoter, *P*$_{sdh}$ (Wilde & Guest, 1986). Similar studies by Spencer & Guest (1985) showed that there is a single *suc* promoter (*P*$_{suc}$) upstream of the *sucA* gene and that transcription continues between the *sucAB* and *sucCD* genes: no independent transcription of the *sucCD* genes was detected (Fig. 1). Transcript termination (or processing) sites were identified immediately downstream of the *sdhCDAB* and *sucAB* genes, but significantly, no transcript linking the *sdhB* and *sucA* genes was detected (Spencer & Guest, 1985). This suggested that the ODH complex and SCS are co-expressed independently of SDH. The *lpdA* gene is preceded by an IRU (ERIC) sequence and the *sucAB* genes are flanked by three and four REP sequences, but it is not known whether these have any effect on transcription.

Early studies with *galK* transcriptional fusions indicated that *P*$_{sdh}$ and *P*$_{lpdA}$ are more active than *P*$_{suc}$ and that *P*$_{sdh}$ is strongly repressed by anaerobiosis and repressed fivefold both by glucose and by crp cya double deletion,
whereas $P_{suc}$ and particularly $P_{lpd}$ are much less affected by such conditions; $P_{suc}$ and $P_{lpd}$ also differ in lacking potential CRP-binding sites (Spencer et al., 1986; Spencer & Guest, 1987; Guest et al., 1989). Indeed, to account for the discrepancies between the patterns of $P_{suc}$ activity and the corresponding ODH and SCS enzyme activities, it was suggested that the $sucABC$ genes may normally be transcribed and regulated from the $sdb$ promoter, despite the lack of evidence for $sdb$ readthrough transcription. More recently, studies with $lacZ$ fusions have shown that $sdb$ gene expression is anerabically repressed some 70-fold by the combined effects of ArcA and FNR, and repressed up to threefold by glucose in a CRP- (and FruR-) independent manner (Iuchi et al., 1994; Park et al., 1995). In contrast, the $pdh$ promoter is not strongly controlled by ArcA, FNR or CRP (Quail et al., 1994).

The present work was initiated to investigate the effects of global regulators on the $suc$ and $lpd$ promoters, in order to understand how expression of the $suc$ and $lpd$ genes might be co-regulated. The results support the view that the $suc$ gene is transcribed and regulated primarily from the $sdb$ promoter and that co-expression of the $sdb$, $suc$ and $lpd$ genes is mediated by ArcA. Direct evidence that the $sdb$ and $suc$ genes belong to a single operon transcribed primarily from the $sdb$ promoter was obtained by detecting an $sdbCDAB\text{--suc}ABC$ readthrough transcript in $rnc$ and $meC$ mutants.

**METHODS**

**Bacterial strains, plasmids and bacteriophages.** The strains of *Escherichia coli* K-12 are listed in Table 1: DH5$\alpha$ was the routine transformation host; MC4100 was the primary host for expression studies with $lacZ$ fusions; and several strains, including W3110, RS6521 ($rnc$) and N3431 ($rne^+$) were used as RNA sources. A $suc\text{--lacZ}$ transcriptional fusion was constructed by cloning the 1.65 kb BamHI fragment containing the $sdb\text{--suc}$ intergenic region and a small segment of vector DNA from pGS128 into the $lacZ$ fusion vector (pRS415) to produce pGS1048 and ultimately $\lambda$G266 (Fig. 1). The orientation of the subcloned BamHI fragment was established by defining the location of an asymmetric FisI site. An analogous $suc\text{--lacZ}$ translational fusion was constructed by cloning the same fragment 'in phase' into pMD1406, to generate pGS982 and ultimately $\lambda$G264 (Table 1). Transfer of fusions to $rZ5$ and the construction of monolysogens was described previously (Quail et al., 1994). The $lpd\text{--}lacZ$ translational fusion phase, $\lambda$G219 (Fig. 1), was constructed previously (Quail et al., 1994).

Monolysogens of MC4100 containing each of the fusion phases were constructed according to Cunningham et al. (1997) and further derivatives containing $arcA$, $frn$, $fruR$, $himA$, $rpoS$ and $fs1$ mutations were constructed by $P1$ transduction using the corresponding donor strains (Table 1). In the case of $crp$, the single copies of the fusion prophages were first established in JRG1999 (pGS279), $\Delta lac acr$ (crp$'$), from which the plasmid was subsequently cured. Appropriate multicopy plasmids were used to complement the $arcA$ (pRB38), $frn$ (pCH21) and $crp$ (pGS279) mutations in the corresponding monolysogenic derivatives.

**Microbiology and enzymology.** Bacteria were cultured in L broth with glucose (0-4% or 1%) when stated, and appropriate antibiotics (µg ml$^{-1}$): ampicillin, 100; kanamycin, 50; chloramphenicol, 25; tetracycline, 15. The minimal salts medium, M9 (Sambrook et al., 1989), was supplemented with glucose (11 mM) or with sodium acetate, sodium pyruvate or sodium 2-oxoglutarate (each at 40 mM) as carbon sources, and thiamin hydrochloride (5 µg ml$^{-1}$). For expression studies, monolysogens containing $\Delta lacZ$ fusion prophages were grown either aerobically in shaken flasks or under H$_2$ and CO$_2$ (95:5) in anaerobic jars. The $\beta$-galactosidase specific activities (nmol ONPG hydrolysed min$^{-1}$ (mg protein)$^{-1}$) were determined for permeabilized cells (Miller, 1972) using a Labsystems iEMS plate-reader and flat-bottomed microtitre plates to monitor changes in $A_{420}$ and to measure protein concentrations (Philips-Jones et al., 1993; Bradford, 1976). The quoted specific activities refer to exponential-phase cultures (OD$_{600}$ = 10 for L broth, 0-3 for acetate minimal medium, and 0-8 for all other minimal media); they were averaged from triplicate samples from three independent cultures, the overall variation being <20%.

**DNA manipulation, RNA extraction, Northern hybridization and primer extension analysis.** DNA was prepared and manipulated by standard procedures (Sambrook et al., 1989). RNA was extracted by the hot acid phenol procedure (Alib et al., 1981) from exponential-phase cultures (OD$_{600}$ = 0-6) that had been rapidly cooled to 4°C in liquid N$_2$. The cultures were grown aerobically in L broth at 37°C except for the rne$^+$ mutant and its parent, which were grown at 30°C to OD$_{600}$ 0-6 and then given a temperature shock (4 min at 43°C) to destroy RNaseE activity. Bacteria equivalent to 50 ml exponential culture (OD$_{600}$ = 0-6) were resuspended in 3 ml 20 mM sodium acetate (pH 5-5) containing 0-5% SDS and 1 mM EDTA, and then extracted at 60°C with phenol (equilibrated with 20 mM sodium acetate, pH 5-5). The aqueous phase was reextracted with phenol, and the RNA was recovered by ethanol precipitation and dissolved in diethylpyrocarbonate-treated water after two further ethanol precipitations.

Northern hybridization was performed after transferring denatured and electrophoretically fractionated RNA samples (20 µg in 1% agarose formaldehyde gels) to nylon membranes, using [$\alpha$-$\text{32P}$]dCTP-labelled probes according to the 'Ready to Go' labelling-kit instructions (Pharmacia). The $sucA$ probe was a 2-2 kb BamHI–BglII fragment of pGS128 (probe A; Fig. 1) and the $sucB$ probe was a 0-9 kb Avall–BglII fragment of pGS128 (probe B; Fig. 1). Specific probes for $sdb$, $sucC$ and $sucD$ regions (probes S, C and D, respectively; Fig. 1), were generated by PCR amplification using the following DNA templates and oligonucleotide primers (co-ordinates from GenBank accession number J01619): probe S, $\phi$G118 with S541 (3061–3078) and S542 (3378–3349); probe C, pGS128 with S543 (10921–10938) and S545 (11449–11432); and probe D, $\phi$G118 with S543 (12171–12125) and S546 (12693–12676). RNA stability was investigated by growing cultures to OD$_{600}$ 0-6, harvesting at OD$_{600}$ 0-5–0-6, and then transferring samples (10 ml) at 1 min intervals. These were chilled immediately to 4°C with liquid nitrogen and the RNA was isolated and used in Northern hybridization (as above) with $sdb$, $sucA$ and $sucB$ probes. The autoradiographs were analysed using a Bio-Rad 690 densitometer.

Primer extension analysis was performed by the method of Gerischer & Dürre (1992), modified to allow continuous incorporation of [$\alpha$-$\text{32P}$]dCTP, as described by Cunningham et al. (1997). Samples of total RNA (100 µg) from cultures of W3110, harvested at OD$_{600}$ 0-5–0-6, were used with 10 pmol primer. After processing, the samples were fractionated by electrophoresis in 6% acrylamide/7 M urea gels alongside a
sequence ladder derived from the corresponding DNA and primer. The oligonucleotide primers were (co-ordinates from GenBank accession number J01619): for sucA, S410 (6638–6609), S463 (9205–9183), S464 (8933–8910), S465 (8664–8641), S466 (8421–8401), S467 (8160–8141), S468 (7914–7893) and S469 (7646–7621); for sucB, S410 (6638–6609), S463 (9205–9183), S464 (8933–8910), S465 (8664–8641), S466 (8421–8401), S467 (8160–8141), S468 (7914–7893) and S469 (7646–7621); for sucC, S459 (9493–9467) and S462 (9752–9728); and for sucD, S407 (10944–10915).

Materials. Restriction enzymes were from Northumbria Biologicals. AMV reverse transcriptase was from Life Sciences and the RNA calibration ladder was from Life Technologies. Radiolabelled [α-32P]CTP (110 TBq mmol−1) and nylon membranes were from Amersham.

RESULTS

Studies with transcriptional and translational fusions

The existence of an independent sucABCD promoter (P\textsubscript{suc}) was originally inferred because the suc genes are expressed from subcloned fragments of the \textit{sdhCDAB–sucABCD} region, and this was confirmed in early studies with a sucA–galK transcriptional fusion. However, the activity of the suc promoter appeared not to be correlated with the synthesis of the ODH complex and SCS, particularly with respect to cAMP-dependent catabolite repression and anaerobic repression. The control of P\textsubscript{suc} was accordingly reinvestigated with a series of monolysogens containing λ\textit{sucA–lacZ} transcriptional and translational fusions (\textit{iG264} and \textit{iG266}, respectively; see Methods and Table 1). The activity of P\textsubscript{suc} was also compared with that of P\textsubscript{pyc} in an analogous series of λ\textit{ipdA–lacZ} (\textit{iG219}) monolysogens, in order to study the co-regulation of \textit{sucAB} (E10 and E20) and \textit{ipdA} (E3) expression. The relative activities of the \textit{sucA–lacZ} and \textit{ipdA–lacZ} fusions in cultures sampled when the enzyme activities are at their highest are summarized in Tables 2 and 3. The activities of the \textit{sucA–lacZ} translational fusion were uniformly about 10-fold lower than those of the corresponding transcriptional fusion, and were not included in the Tables.

Regulation of \textit{sucA–lacZ} expression. The activity of the suc promoter was relatively unaffected by the growth substrate, the presence of glucose, or the absence of oxygen (Tables 2 and 3). The activity varied within a threefold range compared to an overall 70-fold range for the corresponding enzymes (Buck et al., 1986). It is not known whether the 1.5–1.7-fold induction observed in 2-oxoglutarate minimal medium compared to other mini-
Table 2. Effects of growth substrate on sucA-lacZ and lpd-lacZ expression

The β-galactosidase specific activities [nmol ONPG hydrolysed min⁻¹ (mg protein⁻¹)] of exponential-phase cultures of monolysogenic derivatives of MC4100 containing isucA-lacZ transcriptional or ilpdA-lacZ translational fusion phages are shown (see Methods). The overall variation between triplicate samples from three independent cultures was <20%.

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>β-Galactosidase specific activity</th>
<th>suca-lacZ (jG266)</th>
<th>lpdA-lacZ (jG219)</th>
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<tr>
<td>Glucose</td>
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<td>923</td>
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<tr>
<td>Pyruvate</td>
<td>581</td>
<td>2282</td>
<td></td>
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<tr>
<td>Acetate</td>
<td>513</td>
<td>2051</td>
<td></td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>863</td>
<td>2111</td>
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<tr>
<td>L broth</td>
<td>385</td>
<td>1183</td>
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</table>

Table 3. Effects of mutations in global regulatory genes on sucA-lacZ and lpdA-lacZ expression

The β-galactosidase activities of exponential-phase L-broth cultures of isucA-lacZ and ilpdA-lacZ monolysogens containing specified global regulatory mutations of the corresponding regulatory plasmid (see Methods) are shown. The specific activities are expressed as percentages relative to the values for aerobic L-broth cultures of the parental strains (*), 385 or 1183 nmol ONPG hydrolysed min⁻¹ (mg protein⁻¹), respectively, as shown in Table 2. In each case the overall variation between triplicate samples from three independent cultures was <20%. The concentration of added glucose was 0.4% (w/v) except in studies with the crp and fruR mutants (†), where it was 1.0% (w/v). Increasing the glucose concentration had negligible effects on the parental strains. −, Not done.

<table>
<thead>
<tr>
<th>Strain or genotype</th>
<th>β-Galactosidase specific activity (% of parental control)</th>
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<tr>
<td></td>
<td>sucA-lacZ (jG266)</td>
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<td>L (aerobic)</td>
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<td>MC4100*</td>
<td>100*</td>
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<tr>
<td>arcA</td>
<td>71</td>
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<tr>
<td>arcA + pRB38</td>
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<tr>
<td>fnr</td>
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<tr>
<td>fnr + pCH21</td>
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<tr>
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<tr>
<td>crp + pGS279</td>
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<tr>
<td>fruR</td>
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<td>fis</td>
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<tr>
<td>rpoS</td>
<td>53</td>
</tr>
<tr>
<td>himA</td>
<td>299</td>
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</table>
activity was expected because enzyme synthesis is controlled by transcription from $P_{pgh}$ as well as from $P_{lpd}$ (Fig. 1). The parental activity of $P_{lpd}$ varied over a sixfold range depending on the substrate and growth conditions, being lowest during anaerobic growth with glucose and highest with pyruvate under aerobic conditions (Tables 2 and 3). This pattern of induction during growth on non-fermentable substrates in minimal media, and repression by glucose and anaerobiosis, is what would be expected for a citric acid cycle enzyme. The anaerobic repression was mediated by ArcA, as witnessed by the fivefold anaerobic derepression (and up to twofold aerobic derepression) in an arcA mutant (Table 3). The potential for further aerobic derepression indicates that $P_{lpd}$, like other citric acid cycle promoters, is poised in a partially repressed state in aerobic cultures, such that it can be further derepressed by arcA mutation, and repressed under all conditions in strains containing a multicopy $arcA^+$ plasmid. In contrast, inactivation of the fnr gene had no significant effect on expression of the $lpdA$–$lacZ$ fusion, but expression was repressed by the multicopy $fnr^+$ plasmid, suggesting that excess FNR can either invade the promoter and impair transcription or alternatively function indirectly by activating ArcA synthesis (Compan & Touati, 1994). The role of CRP is difficult to assess; it is certainly not serving as a typical cAMP-dependent activator. On the contrary, it seems to be responsible for a negative regulatory effect which is only apparent in the presence of glucose or in multicopy crp$^+$ situations (Table 3). These effects may be indirect, and it is concluded that neither CRP nor FruR is responsible for the observed repression by glucose. Inactivation of the other regulatory genes tested ($fis$, rpoS and himA) revealed that Fis is a potential activator of $lpdA$ gene expression (Table 3). There is a potential Fis-binding site, GTTTAAAAATTGtTa, centred at –29 in the $lpd$ promoter region (co-ordinates 5770–5784, Stephens et al., 1983), which is mismatched at two positions relative to the 15 bp consensus, GNT[c/t]A[AG]A[a/t]-[T/A][t/a]T[G/c]anc (Finkel & Johnson, 1992). However, Fis-binding at this position would be expected to repress, rather than activate, transcription.

**Transcript analysis of the sucABCD genes by Northern blot hybridization**

The lack of correlation between $sucA$–$lacZ$ expression and ODH enzyme synthesis revived an earlier suggestion that expression of the $suc$ genes may be regulated at the upstream $sdh$ promoter (Fig. 1). This view was strengthened because the regulatory profile of the $lpd$ promoter more closely resembled that of the $sdh$ promoter than the $suc$ promoter, and thus provided a plausible mechanism for co-expression of the $lpdA$ and sucAB genes based on shared features of the $lpd$ and $sdh$ promoters. Direct evidence was therefore sought for transcription across the $sdhB$–$sucA$ region containing the $suc$ promoter, even though no transcript had been detected by nuclease S1 mapping. Northern blot hybridization was used with a series of gene-specific probes containing parts of the $sdhC$, sucAB, C and D genes (probes S, A, B, C and D; Fig. 1) and RNA extracted from two RNase mutants and the corresponding parental strains (P90C and N3433).

**Hybridization with RNA from the parental strains.** Using samples of parental RNA, one major band of 3·5 kb, presumed to be the $sdhCDAB$ transcript extending from $P_{suc}$ to the $sdhB$–$sucA$ intergenic region, was detected with the $sdh$ probe (S; Fig. 1). Another major band of 6·6 kb, corresponding to a sucABCD transcript, was detected with each of the four suc gene probes (A–D; Fig. 2). All of the probes hybridized non-specifically to the rRNA components and several other specific bands were detected with parental RNA (Fig. 2). The 4·4 kb band best corresponds to a specific sucAB transcript, generated by transcript termination or processing within the $sucB$–$sucC$ intergenic region. Evidence for such termination, as well as readthrough transcription across this region, was obtained previously (Spencer & Guest, 1985). A potential 3·6 kb sucBCD transcript was also detected with the sucB, sucC and sucD probes (B–D; Fig. 2) and there was a very weakly hybridizing 2·1 kb component having 1–10% of the intensity of the 3·6 kb transcript, possibly representing a sucCD transcript or processed fragment (not visible in Fig. 2). However, no transcript corresponding to the entire $sdhCDAB$–sucABCD region was detected in parental RNA samples, even after prolonged exposure. This could mean that the two gene clusters are not co-transcribed or that the relevant transcript is very short-lived. It should also be stressed that it is not possible to discriminate between transcripts synthesized from an internal promoter, e.g. $P_{suc}$, and those derived from longer transcripts by processing or degradation.

**Studies with RNA from an rnc mutant.** Samples of RNA from a mutant lacking RNaseII (RS6521, rnc) were analysed with the same series of probes. This produced markedly different hybridization patterns, whose interpretation was to some extent complicated by the presence of similarly sized fragments of differing origin. Most significant was the 10 kb transcript detected with the $sdh$ probe after prolonged exposure of the autoradiographs (Fig. 2). It provided the first direct evidence for the existence of a full-length $sdhCDAB$–sucABCD transcript. As a result, most of the other RNA molecules can be regarded as processed or degradation products of the full-length transcript. The full-length transcript was accompanied by a strong 6·5 kb band in addition to the 3·5 kb $sdhCDAB$ transcript observed above with RNA from the parental strain. It was inferred that the 6·5 kb transcript extends from $P_{suc}$ to the 3' end of the sucA coding region ($sdhC$–$sucA$); this transcript would co-migrate with the sucABCD transcript when parental RNA is hybridized with probe A (Fig. 2), but it is not clear why the $sdhC$–$sucA$ transcript was not detected by probe A in the rnc mutant.

The 10 kb full-length $sdh$–suc transcript was detected with all of the suc probes after prolonged auto-
Fig. 2. Northern blot hybridization showing the transcripts detected with specific probes for citric acid cycle genes. RNA from me and mc mutants and the corresponding parental strains was used with probe S (sdh), probe A (sucA), probe B (sucB), probe C (sucC) and probe D (sucD). An extra track is included to show the 10 kb transcript detected after prolonged exposure of a hybridization of RNA from the mc mutant with probe S. The origins deduced for different RNA species (denoted by lower-case Roman numerals) are shown below the map of sdh-suc operon.

Radiography (data not shown). Using the same probes with RNA from the rnc mutant, the 6.6 kb sucABCD and 4.4 kb sucAB components that were prominent in parental RNA could not be detected (Fig. 2). This indicates that RNaseIII is responsible for their formation, by cleavage in the sdhB-sucA intergenic region. Retention of the 3.5 kb sdhCDAB transcript can be explained if a proportion of the transcripts initiating at the sdh promoter are terminated at a natural termination site(s) located immediately downstream of the sdhB gene, before traversing the sdhB-sucA intergenic region. The sucB, C and D probes showed that the 3.6 kb sucBCD transcript is retained as a major component in rnc mutant RNA as well as parental samples. The 3.6 kb sucBCD and 6.5 kb sdhC-sucA transcripts are presumably derived from the full-length transcript by specific endonucleolytic cleavage at or near the sucA-sucB gene boundary; the presence of both fragments in RNA from the rnc mutant clearly indicates that RNaseII is not responsible for their production (Fig. 2).

Studies with RNA from an rne mutant. There was a general increase in the relative amounts of the transcripts detected in analogous hybridization studies with RNA from the mutant lacking RNaseE (Fig. 2). This is because the overall rate of mRNA decay is lowered when RNaseE is inactivated. All of the transcripts observed with RNA from the parental strains were present in RNA from the rne mutant. In addition, the 10 kb full-length transcript could be detected with all of the probes (including the sdh probe after longer exposure than shown in Fig. 2), presumably due to general transcript stabilization. Some new components, which probably represent decay intermediates, were also observed, e.g. the 5-6 kb suc'ABCD fragment detected by all of the suc probes and a potential 3.4 kb suc'AB transcript detected by the sucA and sucB probes (Fig. 2).

Transcript stabilities. The stabilities of the four major transcription products were investigated by quantifying the temporal changes that occurred after adding rifampicin to block transcript initiation (Fig. 3). The relative amount of the sdhCDAB transcript declined at a rate corresponding to a chemical half-life of 3.3 min. However, in the case of the sucABCD transcript and its subfragments, the relative amounts increased for up to 4 min before declining with half-lives of 3-6 min for the sucABCD and sucAB transcripts, and 5-4 min for the sucBCD transcript. The reason for this behaviour is not clear. It would appear that a significant proportion of the suc transcripts is for some reason not accessible to hybridization, or not formed until after the rifampicin is
added. The turnover rates may be such that suc transcripts are generated after rifampicin addition by active RNA polymerase molecules that are stalled for example, in the sdhB–sucA intergenic region.

Primer extension analysis

Primer extension analysis was used to define the 5' extremities of the major transcription products encoding suc genes. Three distinct start-sites or processing sites (A, B and C) were detected in the sdhB–sucA intergenic region using S410 as primer (Fig. 1) with RNA from the two parental strains (Fig. 4a). The same products were detected with increased intensity in RNA from the rme mutant but, very significantly, none were detected with RNA from the rnc mutant (Fig. 4a). This indicates that all three ends are generated by RNaseIII cleavage in the sdh–suc intergenic region. The existence of three RNaseIII cleavage sites explains why transcripts spanning the sdh–suc intergenic region were so difficult to detect. Their precise locations are shown in Fig. 4(b) and it is interesting to note that sites A and B are within 3 bp and 1 bp of the putative sucA transcriptional start-sites identified previously by the less precise nuclease S1 mapping procedure (Spencer & Guest, 1985). It is not known whether one of the less intense products generated with primer S410 corresponds to the start-site of the independent sucA promoter. Such a site should have been more apparent with RNA from the rnc mutant, especially if it is located upstream of one or more of the RNaseIII processing sites. Unfortunately, potential sucA start-sites are masked by the minor artefactual bands which are generated by primer extension in regions of RNA secondary structure, and there are no recognizable promoter sequences in the sdh–suc intergenic region, which is consistent with the weak activity of the sucA promoter.

Analogous studies with RNA from both mutant and parental strains and primers spanning the sucA coding and sucA–sucB intergenic regions (S459, S462–469; Fig. 1) revealed two 5' extremities within the sucA coding region, but no start-sites or processing sites were detected immediately upstream of the sucB gene (data not shown). The internal sucA sites were 1.3 kb and 1.8 kb from the beginning of the sucA gene, and both were detected in parental RNA and more prominent in rme mutant RNA. Specific cleavage at the first site would generate the 5.6 kb suc'ABCD fragment (Fig. 2). No specific cleavage site was detected in or near the sucA–sucB junction, needed to explain the origin of the 6.5 kb sdhC–sucA and 3.6 kb sucBCD fragments.

Primer extension analysis with primer S407 (Fig. 1) revealed a series of putative 5' ends in the sucB–sucC intergenic region and in the distal segment of the sucB coding region (data not shown). These were probably due to pausing of the reverse transcriptase in the region containing four REP sequences rather than to specific initiation or processing events. The products were more intense in the rnc and rme mutants than in the parents, but the significance of this is not known. Transcription was previously shown to read across the sucB–sucC
Fig. 4. Primer extension mapping and location of RNaseIII processing sites in the sdhB-sucA intergenic region. (a) Primer extension analyses with RNA extracted from me and mc mutants and the corresponding parental strains, aligned to a sequence ladder generated with the same primer. The major products are denoted by the arrows, A, B and C. There is a 0–5 bp discrepancy between the primer extension products and the sequence ladder, but the validity of the interpretation is supported by other experiments. (b) Details of the sdhB-sucA intergenic region showing the positions of three RNaseIII processing sites (A–C) deduced from the primer extension products detected in (a). The positions of three REP sequences, another potential stem-loop, and the sdh transcript end points (↑) and sucA transcript start-sites (↓) defined previously by S1 mapping (Spencer & Guest, 1985), are shown.

intergenic region, and although a termination or processing site was identified 220 bp downstream of sucB, no evidence for the existence of an independent sucC promoter was found (Spencer & Guest, 1985). There is no potential promoter sequence in the intergenic region and the corresponding sucC-lacZ fusions are inactive (Park et al., 1997; data not shown).

DISCUSSION

The lacZ fusion studies showed that the regulatory profile of the suc promoter bears virtually no resemblance to those of the corresponding enzymes (ODH and SCS) or the lpd promoter. Compared to the lpd promoter, there was no ArcA- or FNR-dependent anaerobic repression, no glucose repression, nor any other common feature that could provide a mechanism for co-regulating sucABCD and lpdA expression. This contrasts with the features shared by the regulatory profiles of the sdh promoter (Iuchi & Lin, 1988; Iuchi et al., 1994; Park et al., 1995) and the lpd promoter, and synthesis of the corresponding enzymes (SDH, ODH and SCS). Indeed, the similarities provide strong support for the view that sucABCD transcription is initiated and regulated primarily from the sdh promoter, and this in turn provides a plausible mechanism co-ordinating expression of the sucAB and lpdA genes for synthesis of the ODH complex. Direct support for co-transcription of the sdh and suc genes has now been obtained by detecting a full-length sdhCDAB-sucABCD transcript and independently from parallel studies with a sdhCDAB–sucA-lacZ fusion (Park et al., 1997). This raises questions concerning which factors are primarily responsible for co-regulating the lpd and sdh promoters, and what is the normal role of the suc promoter.

In addition to being repressed by anaerobiosis and glucose the sdh promoter is more active in the absence of Fis and αB (Xu & Johnson, 1995) whereas the lpd promoter is activated by Fis and unaffected by αB. The regulatory coupling of the sdh and lpd promoters would thus appear to depend primarily on their ArcA-mediated anaerobic repression. The co-regulatory importance of glucose-mediated repression is uncertain because different mechanisms may be involved at each of the two promoters. Early work with an sdh–galK fusion had indicated that crp and cya are required for maximum expression, and S1 mapping studies not only confirmed that sdh transcription is severely repressed by glucose but indicated that there is a potential CRP-binding site.
at −80 in the sdh promoter region (Wilde & Guest, 1986). This promoter and its CRP-binding site can now be deemed responsible for the co-ordinate expression and the glucose-mediated repression of ODH and SCS synthesis observed by Buck et al. (1986). Indeed, the absence of a CRP-binding site in the suc promoter region was used to support the early suggestion that the promoter is not CRP-dependent nor is it associated with sdh by glucose (Table 3). Recent work has likewise shown the promoter probably supplies excess E3 subunits, due to synthesis observed by Buck (1997). The regulatory features of the suc promoter, lack of anaerobic repression and activation by σ88, suggest that it may provide E1o, E2o and SCS during anaerobic growth and in stationary phase, when the sdh promoter is severely repressed and some SDH functions are replaced by furamate reductase. Indeed, a slight anaerobic activation of the sucA promoter was observed by Park et al. (1997). The corresponding enzyme activities are presumably needed under anaerobic conditions to provide succinic semialdehyde-TPP (from 2-oxoglutarate) for menaquinone biosynthesis (Meganathan, 1996) and to provide succinyl-CoA (from either succinate or 2-oxoglutarate) for use in diaminopimelate and lysine biosynthesis (Patte, 1996) and methionine biosynthesis (Greene, 1996). The present location of the sucAB genes could mean they were inserted into an ancestral sdhCDAB–sucABCD (SDH–SCS) operon during the evolution of the citric acid cycle or that this organization facilitates switching between the cyclic and non-cyclic forms of the citric acid cycle associated with the aerobic and anaerobic metabolic modes adopted by E. coli. So, the suc promoter may be a vestigial remnant of an ancestral suc operon, that may retain specific anaerobic roles to allow some differential expression relative to the sdh promoter.

Detection of the full-length sdhCDAB–sucABCD transcript, albeit only in rnc and rne mutants, has now established that the eight genes encoding three citric acid cycle enzymes are co-transcribed from the sdh promoter. The operon contains one internal promoter (Pint) and termination can occur downstream of the sdhB and sucB genes in the intergenic regions that contain REP elements (Fig. 1). The extreme lability of the readthrough transcript is attributed to RNaseE-mediated cleavage at one or more of three sites in the sdhB–sucA intergenic region. This intergenic region is potentially rich in double-stranded RNA and hence targets for RNaseE (Fig. 4b). Indeed, the asymmetric organization of the cleavage sites A and B conforms to that of the RNaseE cleavage-site consensus (Krinke & Wulff, 1990). This does not apply to site C, which is seemingly located in a single-stranded region. It is important to note that even in the rnc mutant, the 3.5 kb sdhCDAB transcript is generated, presumably by natural termination at the previously identified sites (Spencer & Guest, 1985) located upstream of the RNaseE processing sites (Fig. 4b). The sucB–sucC intergenic region is similarly rich in potential secondary structure but the terminator or processing site is located downstream of the REP sequences (Spencer & Guest, 1985) and RNaseE is not involved here. The role of RNaseE in processing transcripts of the sdhCDAB–sucABCD operon is less defined. The general increase of stability of the major transcripts, and the presence of extra components in RNA from the rne mutant, show that RNaseE is involved in transcript processing and degradation, but the sequence specificity of this enzyme is still unknown (Cohen & McDowall, 1997). The degradation of mRNA by RNaseII and RNaseE is an important factor in defining the functional life of a transcript product (Kushner, 1996). In the case of the sdhCDAB–sucABCD operon it will in future be important to define the relative contributions of transcription initiation, transcription termination and transcript degradation to the overall patterns of enzyme synthesis under different conditions. It would be particularly interesting to understand the mechanism controlling natural termination relative to readthrough transcription at the internal sites and its physiological significance.

ACKNOWLEDGEMENTS

We are very grateful to Dr J. Green for helpful discussions. This work was supported by The Wellcome Trust.

REFERENCES


Received 2 February 1998; revised 24 March 1998; accepted 17 April 1998.