An acetate-sensitive mutant of *Neurospora crassa* deficient in acetyl-CoA hydrolase

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(Received 21 January 1992; revised 22 April 1992; accepted 18 June 1992)

The predicted amino acid sequence of the product of the acetate-inducible acu-8 gene of *Neurospora crassa*, previously of unknown function, has close homology to the recently published sequence of *Saccharomyces cerevisiae* acetyl-CoA hydrolase. An acu-8 mutant strain, previously characterized as acetate non-utilizing, shows strong growth-inhibition by acetate, but will use it as carbon source at low concentrations. The mutant was shown to be deficient in acetyl-CoA hydrolase and to accumulate acetyl-CoA when supplied with acetate. As in *Saccharomyces*, the *Neurospora* enzyme is acetate-inducible.

Introduction

The *Neurospora crassa* acu-8 gene is one of three cloned by Thomas *et al.* (1988) on the basis of their preferential transcription when sucrose was replaced by acetate as carbon source. The two others were found to code for acetyl-CoA synthetase and malate synthase respectively, but the function of acu-8 has remained obscure. Marathe *et al.* (1990) sequenced the gene, and by making use of the RIP (repeat induced point mutation) phenomenon (Selker *et al.*, 1987) obtained a mutant strain in which the acu-8 sequence was extensively disrupted. The effect of the RIP phenomenon in *Neurospora* is to cause premeiotic disruption of duplicated sequences in the same nucleus of specialized dikaryotic cells. Transformed and resident sequences therefore participating in this process become heavily cytosine-methylated and the subjects of extensive GC–AT base transitions. The resulting acu-8 mutant, as expected, failed to grow on 40 mM-acetate as carbon source (the concentration optimal for the wild-type), but, surprisingly, was able to use ethanol. The mutant was found to be normal in its ability to take up acetate from the growth medium.

The sequence of acu-8 includes an open reading frame of 521 codons interrupted by two introns. Initially, it was not found to resemble any other published sequence, but the recently published sequence of *Saccharomyces cerevisiae* glucose-repressible acetyl-CoA hydrolase (Lee *et al.*, 1990) shows close homology with acu-8. Lee *et al.* (1990) suggested that the function of acetyl-CoA hydrolase in yeast was to control the level of acetyl-CoA, which might otherwise become toxic under conditions of glucose derepression. Acetate toxicity seemed a likely explanation of the failure of the acu-8 mutant to grow on acetate. This communication provides supporting evidence for this hypothesis.

Methods

**Fungal culture.** *Neurospora crassa* was grown in liquid medium as previously described (Thomas *et al.*, 1988). Acetate was added from a stock solution of 1 M-acetic acid adjusted to pH 5.2 with NaOH.

**Acetyl-CoA hydrolase assay.** Extracts were made by grinding mycelium with sand in 0.4 M-sucrose, 10 mM-Tricine, pH 7.5, and centrifuging successively at 3000 g for 10 min to remove the cell debris and at 23500 g for 30 min to pellet the membrane fraction at 4 °C. Malate synthase, which would otherwise interfere with the assay, was predominantly in the pellet. Acetyl-CoA hydrolase, which remained in the supernatant, was assayed in a reaction mixture containing 50 mM-potassium phosphate, pH 7.2, and 0.2 mM-acetyl-CoA at 30 °C. Hydrolysis of acetyl-CoA was assayed by following the appearance of free -SH groups, measured by addition of dithiothreitol(2-nitrobenzoic acid) and determining the increase in absorbance at 412 nm. Acetyl-CoA synthetase was assayed as described previously (Thomas *et al.*, 1988).
Fig. 1. Comparison of the predicted amino acid sequences of the N. crassa acu-8 product (upper line) and S. cerevisiae acetyl-CoA hydrolase (lower line). Vertical lines indicate identical amino acid residues and horizontal lines indicate gaps inserted to maximize homology. These sequences appear in the PIR database under the accession numbers A35195 and A36331 for S. cerevisiae and N. crassa respectively.

\[
\begin{align*}
1 & \text{MASPIASAALRQ-GPSMLKKCLNPEMLQHFPNGAYIGWSGFPSATP} \\
50 & \text{R-SYLAHVEKASPAVQPLRCLRRRRDREEWALDMIAHRRRA} \\
48 & \text{KAVPEALIDHEKNNQKGLRNLVFGASAGPEENRWAIDMII---KRA} \\
99 & \text{PHQGVKNIAKIGRNEFDEKIDLSFPSVGYYTQDR-QKNLNDV} \\
148 & \text{VEATEIKEDGSIIVGATPELLIQMDKVIENVTAIPFSDDLHDITF} \\
145 & \text{IEATAIKEDGSIIVGATPELLIQMDKVIENVTAIPFS} \\
198 & \text{SDLPRKPYLIQCRCDRGIYTSVFVDEKVIGIIECTPDQTLNPSAD} \\
195 & \text{PNPFRKPPYLBKVDKCQVDVSIPVDFPKVAIVESTMRDQPPNTSD} \\
248 & \text{ETATAIGHLIEFEHEVAHRLPKNNLPLQSGIGNIANAVIGLETSNF} \\
245 & \text{DSMEAIAGHLVEFRENRKHLPENLPLQSGIGNIANAVIEGLAQGF} \\
298 & \text{KLNWTEVTQDFTLDLSGKLDATATSIRFPSTQFERFYKNWDNY} \\
295 & \text{KHTWETQVOLSDLDFENQLDAYSSTAVRLTEKFDFANWNKF} \\
348 & \text{KLLRSQSVNAPAEPYRLGVMNPVEVYAHANSTNVMSRLMG} \\
345 & \text{RLCLRSQVSNPFIRESPFVAMNPVEVYAHANSTNVGSRMLG} \\
398 & \text{GGASDFLRNSKYSIMHTPSRPSKTDAHGVSCIVFMCTHDLQTEHDLDV} \\
395 & \text{GGASDFLRNKLSIMHAPSRTPKVDPTGISTIVPMASHVDQTEHDL} \\
448 & \text{VTENGADLRGPVRARVIKDCAHVDKPILKAYEKEAEFLEKRM} \\
445 & \text{VTDQGLADRGPVREREI INKCAHPYQALLTDYLDRAEHYAKHN} \\
498 & \text{GHEPHLFINSPDMKALVEEGSMAKVF} \\
495 & \text{LHEPMLNKAFKFPHTNLAEKGM-KVDSWEPVD} \\
\end{align*}
\]

[13C]Acetate feeding experiments. The fate of [13C]acetate fed to the acu-8 mutant was investigated by NMR in perchloric acid extracts essentially as described by Thomas & Baxter (1987).

Database searches and sequence alignments. These were done using the UWCGG suite of programs of Devereux et al. (1984), including the FASTA program of Pearson & Lipman (1988).

Results and Discussion

Fig. 1 shows the comparison of the predicted amino acid sequences of yeast acetyl-CoA hydrolase and the N. crassa acu-8 product. The two sequences show 61% amino acid identity. There is significant similarity over virtually the entire sequences, with the greatest differences near to the N- and C-termini.

The acu-8 mutant is apparently totally deficient in acetyl-CoA hydrolase (Table 1), as would be predicted from the extensive modifications made in the gene by the RIP process (Marathe et al., 1990). The Neurospora enzyme resembles that from yeast in being repressed on succrose as carbon source and derepressed on acetate.
of the acu-8 type. At higher concentrations growth was delayed, and growth on ethanol (Marathe et al., 1990), which presum-
ably defective in isocitrate lyase and acetyl-CoA synthetase, were also inhibited by acetate when grown on glycerol as carbon source. As shown in Table 2, they differed from acu-8 in showing no growth on acetate alone at any concentration.

NMR analysis showed that acu-8 mycelium did not accumulate \(^{13}C\)acetate after transfer from sucrose to 40 mM-acetate as carbon source, but it did accumulate acetyl-CoA. Unlike the wild-type, acu-8 mycelium exhibited no significant flux through the glyoxylate cycle 10 h after transfer to 40 mM acetate.

These results are entirely consistent with the conjecture of Lee et al. (1990) that acetyl-CoA hydrolase performs the function of preventing a toxic build-up of acetyl-CoA under conditions of carbon catabolite de-
pression. It seems unlikely that it is involved in export of acetyl groups across the mitochondrial membrane, as discussed by Kohlhaw & Tan-Wilson (1977), because, as noted above, it appears to be cytosolic and not associated with the mitochondrial fraction.

One may ask how the cytosolic location of acetyl-CoA hydrolase is compatible with the maintenance of an adequate supply of acetyl-CoA to the mitochondria and glyoxysomes. It may be that the organelles can assimilate acetyl-CoA at high efficiency from a steady-state cytosolic concentration that is well below the Michaelis constant of the hydrolase, estimated by Kohlhaw & Tan-
Wilson (1977) as 2.2 \( \times 10^{-4} \) M for the yeast enzyme. Acetyl-CoA hydrolase may be a safety-valve which, even though necessary, is most of the time hardly used.

An additional possible reason why sufficient active acetate survives transit through the cytosol is that it is stored in some energetically equivalent derivative not subject to cytosolic enzymic degradation. Two alternative protected derivatives discussed by Kohlhaw & Tan-Wilson (1977) are citrate, through which acetate could cycle via citrate synthetase and ATP:citrate lyase, and acetylcarb
tine interchangeable with acetyl-CoA through carnitine-CoA acetyltransferase. These authors suggested that the latter enzyme, which they found to have a much higher affinity than acetyl-CoA hydrolase for acetyl-CoA, may be instrumental in the export of acetyl groups through the mitochondrial membrane. Both ATP:citrate lyase and carnitine-CoA acetyltransf
erase have been shown to be present in fungi (Ratledge & Gilbert, 1985; Jernejc et al., 1991), and the acetyltrans
erase has been demonstrated in Neurospora (I. F. Connerton, unpublished result). In the n-alkane-utilizing yeast Candida tropicalis, two forms of the carnitine acetyltransferase have been reported, one in the mito-
ochondria and one in the peroxisomes, which fulfil the function of glyoxysomes in this organism (Ueda et al., 1982). It will be interesting to see whether similar isoenzymes exist in Neurospora.

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Table 1. Acetyl-CoA synthetase and hydrolase activities in N. crassa wild-type and the acu-8 mutant

The activities recorded are means of duplicate assays and are representative of three independent determinations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Synthetase</th>
<th>Hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Sucrose</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>(74A)</td>
<td>Sucrose then 8 h glycerol</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Sucrose then 8 h acetate</td>
<td>92</td>
<td>36</td>
</tr>
<tr>
<td>acu-8</td>
<td>Sucrose</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Sucrose then 8 h glycerol</td>
<td>5</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Sucrose then 8 h acetate</td>
<td>85</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Table 2. Growth of N. crassa wild-type and acu mutants on different acetate concentrations

Standing liquid cultures (15 ml) were each inoculated with about 10⁶ conidia. Values for growth are means of duplicates. –, Already harvested; tr., trace (visible fuzz of germinated spores not recoverable as weighable mycelium); ND, not detectable.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incubation time (h)</th>
<th>Sucrose 13-3 mm</th>
<th>Acetate (mg dry wt) on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Wild-type (74A)</td>
<td>48</td>
<td>31.4</td>
<td>10</td>
</tr>
<tr>
<td>acu-8</td>
<td>83</td>
<td>33.3</td>
<td>tr.</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>acu-3</td>
<td>48</td>
<td>23.1</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>acu-5</td>
<td>48</td>
<td>36.1</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

The results of a representative experiment on growth of the acu-8 mutant on acetate as carbon source are shown in Table 2. On concentrations of acetate below 5 mM, acu-8 grew only slightly less well than the wild-
type. At higher concentrations growth was delayed, and at 40 mM, the concentration hitherto used for growth tests because it is near-optimal for the wild-type, the mutant showed only a trace of growth even after 5 d at 30 °C. These findings explain the ability of the mutant to grow on ethanol (Marathe et al., 1990), which presumably provides a sustained supply of acetate at low concentrations.

Other experiments (data not presented here) showed that growth on 0.5 mM-glycerol, a poor carbon source used by acu-8 as well as by the wild-type, was strongly inhibited by 5 mM-acetate in acu-8 but enhanced by the same acetate concentration in the wild-type. Two other acetate non-utilizing mutants, acu-3 and acu-5, respec-
We thank the SERC for support for I. F. C. and for making available NMR facilities. We also thank Dr John Archer for helpful discussion.

References


