MOLECULAR AND CELLULAR BIOLOGY, June 1990, p. 2638–2644 Vol. 10, No. 6
0270-7306/90/062638-07$02.00/0
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Duplication-Induced Mutation of a New *Neurospora* Gene Required for Acetate Utilization: Properties of the Mutant and Predicted Amino Acid Sequence of the Protein Product

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Received 30 November 1989/Accepted 21 February 1990

A cloned *Neurospora crassa* genomic sequence, selected as preferentially transcribed when acetate was the sole carbon source, was introduced in extra copies at ectopic loci by transformation. Sexual crossing of transformants yielded acetate nonutilizing mutants with methylation and restriction site changes within both the ectopic DNA and the normally located gene. Such changes are typical of the duplication-induced premeiotic disruption (the RIP effect) first described by Selker et al. (E. U. Selker, E. B. Cambareri, B. C. Jensen, and K. R. Haack, Cell 51:741–752, 1987). The mutants had the unusual phenotype of growth on ethanol but not on acetate as the carbon source. In a cross to the wild type of a mutant strain in which the original ectopic gene sequence had been removed by segregation, the acetate nonutilizing phenotype invariably segregated together with a RIP-induced *Eco*RI site at the normal locus. This mutant was transformed to the ability to use acetate by the cloned sequence. The locus of the mutation, designated *acu-8*, was mapped between *trp-3* and *un-15* on linkage group 2. The transcribed portion of the clone, identified by probing with cDNA, was sequenced, and a putative 525-codon open reading frame with two introns was identified. The codon usage was found to be strongly biased in a way typical of most *Neurospora* genes sequenced so far. The predicted amino acid sequence shows no significant resemblance to anything previously recorded. These results provide a first example of the use of the RIP effect to obtain a mutant phenotype for a gene previously known only as a transcribed wild-type DNA sequence.

In a previous paper, Thomas et al. (20) reported the cloning of four *Neurospora crassa* genomic sequences that were transcribed at a low or undetectable level during growth on a medium containing sucrose but were strongly transcribed when acetate was the sole carbon source. Two of them, clones 1 and 2, have been identified as coding for malate synthase and acetyl coenzyme A sythetase, respectively (20). Of the other two, clone 4 seemed likely to contain only the 3′ end of a coding sequence (20; S. Marathe, unpublished observations), but clone 3 appeared to contain a whole gene. The object of the present work was to use clone 3 to disrupt the corresponding genomic sequence, with a view to determining its function. The possibility of introducing unknown DNA sequences into the *Neurospora* genome at nonhomologous genomic loci by cotransformation (20), together with the finding (17) that artificially duplicated *Neurospora* genomic segments tend to be disrupted in the dicaryotic ascogenous hyphae prior to meiosis, offered the opportunity of inducing mutations specifically in the clone 3 gene.

MATERIALS AND METHODS

Neurospora strains. The standard *N. crassa* wild-type STA (74A) was used throughout; the wild type of the opposite mating type, ST*a*, and strains of both mating types carrying the mutation am^{132} (deletion of the structural gene for NADP-specific glutamate dehydrogenase [12]) were all inbred with ST*A* through at least five generations of backcrossing.

DNA clones. Clone 3, which has a 14.0-kilobase (kb) *Eco*RI *Neurospora* genomic fragment inserted into the Charon 4A lambda vector, was selected from a Charon 4A library of fragments from a partial *Eco*RI digest by G. H. Thomas et al. (20) on the basis of its strong hybridization with mRNA from acetate-grown mycelium and its failure to hybridize with mRNA from sucrose-grown mycelium. Plasmid pC3 was made by inserting a clone 3 5.7-kb *Eco*RI fragment into the polylinker of plasmid pEMBL9. The 2.6-kb *Neurospora* *Bam*HI fragment containing the entire am^+ (NADP-specific glutamate dehydrogenase) gene (11) was provided with terminal *Xba*I sites by successive insertion into the polylinker of pEMBL18, excision with *Hin*dIII plus *Sma*I, insertion into pEMBL19 between the *Hin*dIII and *Hin*cII polylinker sites, and final excision with *Xba*I. It then had an *Xba*I site at each end, one derived from the pEMBL18 and the other from the pEMBL19 polylinker, and could be inserted into pC3 at the *Xba*I site downstream of the clone 3-transcribed region to make plasmid pC3-*am* (Fig. 1). Subcloning was carried out by the method of Maniatis et al. (13). The pEMBL series of vectors is described by Dente et al. (2).

Transformation. Transformation of conidial protoplasts from am^{132} mutant strains was carried out as previously described (12, 21). Selection for the am^+ phenotype was made on minimal medium supplemented with 0.02 M glycine.

Testing for acetate utilization. Conidial inocula were tested for growth in standard minimal medium, except that 40 mM acetic acid, adjusted to pH 5.6 with sodium hydroxide, was the sole carbon source. For spot tests on agar plates, 0.1% L-sorbose was added to induce compact growth.

Southern blotting and hybridization. Transfers from agar-

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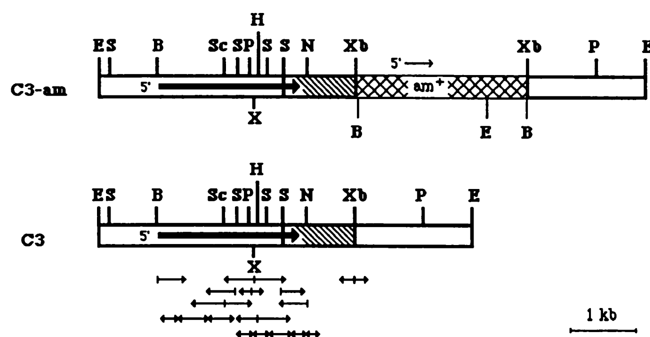

FIG. 1. Restriction site maps of the C3 genomic sequence and the C3-*am* construction inserted into pEMBL9 to make pC3 and pC3-*am*, respectively. The singly crosshatched segment represents the restriction fragment originally shown to hybridize to acetate-specific cDNA, and the doubly crosshatched bar represents the 2.64-kb *Bam*HI fragment, including the *am* gene, transcribed in the direction indicated by the thin arrow. The thin arrows below C3 indicate the sequencing strategy, and the thick arrow indicates the open reading frame encoding a 525-residue polypeptide (see Fig. 5). Restriction sites: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Xb, *Xba*I; X, *Xho*I; Sc, *Sac*I; and N, *Nsi*I.

ose electrophoretic gels were made to a nylon (Hybond) membrane, and the transfers were probed with [^{32}P]DNA according to the instructions of the manufacturers of the membrane (Amersham International). DNA probes were labeled by nick translation (13) or, generally more effectively, by the random-priming procedure (3).

DNA preparation. Mycelium was grown from heavy conidial inocula in 50-ml lots of liquid Vogel's minimal medium, with sucrose as the carbon source and a supplement of 0.5 mg of monosodium glutamate per ml, in 250-ml Erlenmeyer flasks without shaking. The flasks were incubated overnight at 30°C and for a further 24 h at 25°C. Mycelium was washed with 0.9% NaCl, freeze-dried, and used for DNA preparation as described previously (J. N. Stevens and R. L. Metzenberg, Neurospora Newsl. **29**:27–28, 1982). For preparation of DNA with minimal methylation, mycelium was grown with 50 μM 5-azacytidine (filter sterilized) added to the growth medium. In order to get yields of mycelium comparable to those obtained in the absence of azacytidine, growth had to be continued for an additional 24 h.

DNA sequence determination. Restriction fragments for sequencing were subcloned into the phage M13-based vectors mp18 and mp19 (22) and sequenced by the dideoxy-chain termination method by using the Sequenase system (19).

Acetate uptake measurements. Conidiospores at a concentration of ca. 5×10^6/ml were suspended in liquid minimal medium without a carbon source, except for 10 mM acetate added either at the start or at the end of a 5.5-h period of incubation with shaking at 35°C. At the end of this period, a tracer amount of [^{14}C]acetate was added and the incubation was continued. Samples of conidia were collected by membrane filtration (Millipore Corp.) at times from zero to 30 min after the ^{14}C addition, and the radioactivity retained after washing was measured in a scintillation counter.

RESULTS

Transformant T37 and its progeny. Strain am^{132} *a* was transformed to am$^+$ with DNA of plasmid pC3*am*, which contained both the transcribed region of clone 3 and the complete am^+ gene. The frequency of transformation was approximately 4×10^3/μg of DNA. Since most of the primary transformants were presumably heterocaryotic, a number were genetically purified by crossing to am^{132} *A* and selecting phenotypically am$^+$ ascospores germinated on plates of sucrose minimal medium. All of the transformants so purified were able to use acetate as a carbon source. Most subsequent experiments were carried out on transformant T37 and its derivatives. T37 was crossed again to am^{132}, random ascospores were selected for the am^+ phenotype, and 10 out of 64 gave acetate nonutilizing cultures; one of these, T37-3, was investigated further.

DNA analysis of T37 and an acetate nonutilizing derivative. As shown in Fig. 2A, *Bam*HI digestion of DNA from a T37-derived acu$^-$ mutant, T37-3, yielded fragment patterns that differed dramatically depending upon whether or not the mycelium had been grown on medium containing azacytidine. These differences are indicative of methylation of the clone 3 *Bam*HI sites. The fragments yielded by the azacytidine-grown culture (Fig. 2A and other data not presented here) are consistent with T37-3 containing, in addition to the normal clone 3 gene, an ectopic copy of pC3*am* that has undergone some internal deletion and duplication. A full restriction site map of the integrated sequence has not been made, but it evidently includes a complete copy of the 3.0-kb *Bam*HI fragment containing the whole of the clone 3 open reading frame. Both the normally located clone 3 gene and the ectopic pC3*am* sequences are clearly methylated, as shown by comparison of *Hpa*II and *Msp*I digests (Fig. 2A) and also of *Sau*3A and *Mbo*I digests. Other data, not presented here, show that the *Bam*HI restriction fragment pattern of T37-3 resembles very closely that of the original T37 after its first purification by crossing and also that the T37 sequences hybridizing to clone 3 were already methylated after the first purifying cross.

The fact that T37-3 was acetate nonutilizing suggested that its normally placed clone 3 gene had been effectively inactivated. To test this prediction, T37-3 was crossed to the wild type and phenotypically am$^-$ ascospores were selected after germination on unsupplemented minimal agar plates with sucrose as the carbon source. Several am$^-$ ascospores from this cross were all acu$^-$. The DNA analysis of one of them, T37-3-8, is shown in Fig. 2A (lanes 10 to 15). This segregant has lost that part of the T37-3 restriction fragment pattern due to ectopic pC3*am* sequences and retains only hybridizing fragments attributable to the normal locus. When T37-3-8 was grown with azacytidine to unblock methylation-protected restriction sites, it could be shown that although it retained the single *Bam*HI site present in clone 3 (Fig. 2A), it had suffered several changes in other restriction sites. There was an additional site for each of the enzymes *Eco*RI and *Hin*dIII and loss of the *Pst*I site, at least one *Sau*3A/*Mbo*I site, and, probably, a *Sal*I site (Fig. 2B).

The acu$^-$ phenotype is due to disruption of the clone 3 gene. To confirm that the changes in the genomic sequence probed by clone 3 and the appearance of the acu$^-$ phenotype were causally rather than coincidentally related, T37-3-8 was crossed to the wild type. Cultures grown from randomly isolated ascospores showed a close approximation to a 1:1 ratio of acetate-utilizing to -nonutilizing progeny. Twenty acu$^+$ and 20 acu$^-$ progeny cultures were used for DNA preparations, which were then tested for the presence or absence of the additional *Eco*RI site demonstrated in Fig. 2B. All of the 20 acu$^+$ progeny had the normal-sized *Eco*RI fragment, and all the acu$^-$ mutant progeny had it cut into two (Fig. 3). It thus seems safe to attribute the new *acu*

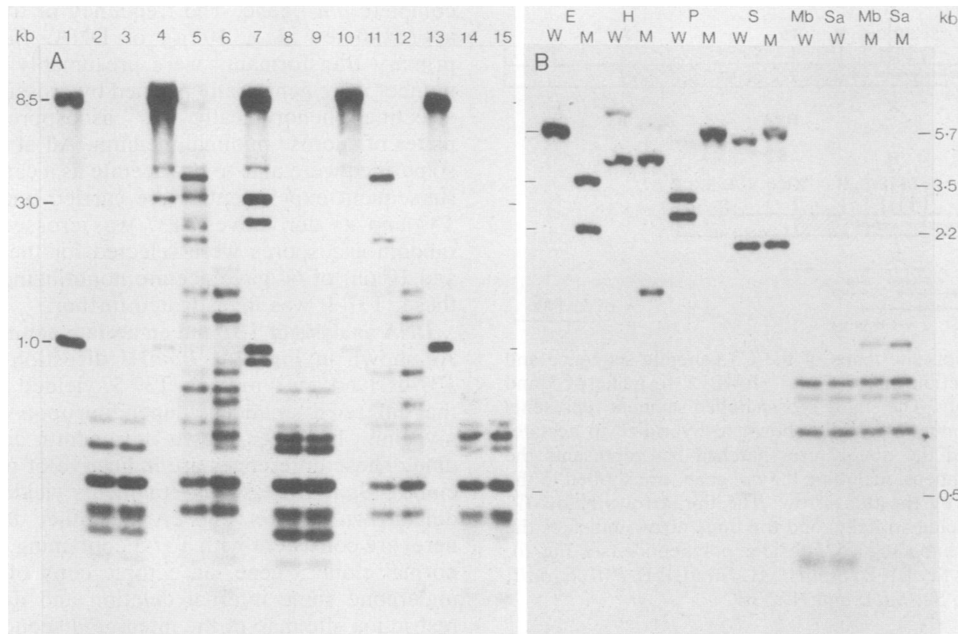

FIG. 2. Southern blot analysis of DNA from wild type and transformant T37 and derivatives. C3 DNA (see Fig. 1) was used as probe throughout. Fragment sizes in kilobases are indicated in the margins. (A) Lanes 1 through 3, Wild type; lanes 4 through 6, T37-3, an acetate nonutilizing mutant derived by outcrossing transformant T37; lanes 7 through 9, T37-3 grown on medium containing 5-azacytidine; lanes 10 through 12, T37-3-8, an acetate nonutilizing derivative of T37-3 with the ectopic sequences of T37 removed by meiotic segregation; lanes 13 through 15, T37-3-8 grown on medium containing azacytidine. Digestion was with *Bam*HI (lanes 1, 4, 7, 10, and 13), *Hpa*II (lanes 2, 5, 8, 11, and 14), or *Msp*I (lanes 3, 6, 9, 12, and 15). (B) Wild-type (W) and T37-3-8 (M) DNA digested with *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Sal*I (S), *Sau*3A (Sa), and *Mbo*I (Mb). T37-3-8 was grown on medium containing azacytidine to minimize methylation. By comparison with the wild type, the T37-3-8 hybridizing sequence has additional *Eco*RI and *Hin*dIII sites and has lost the *Pst*I site and one of the *Sau*3AI/*Mbo*I sites. The apparently real increase in size of the larger hybridizing *Sal*I fragment and the loss of the 0.5-kb *Sal*I fragment in T37-3-8 suggests the loss of one of the two closely placed (ca. 0.5 kb) *Sal*I sites (see Fig. 1).

mutant to a RIP-induced change in the clone 3 sequence. The new mutant has been designated *acu-8*.

To further test the conclusion that clone 3 carried the wild-type allele of *acu-8*, pC3*am* was used to transform T37-3-8 to ability to use acetate. Since direct selection for acetate utilization is inefficient (20), transformants were selected on the basis of am^+ function. A transformation frequency of approximately 6,000/μg of DNA was obtained. Fifty am^+ transformants were tested by spotting on to acetate plates, and 46 were acetate utilizing. Further tests of the 46 acu^+ transformants on liquid acetate medium showed a range of growth rates; although most were indistinguishable from the wild type, a few were clearly slow, perhaps because of integration of only a part of a normal *acu-8* upstream transcription-activating sequence.

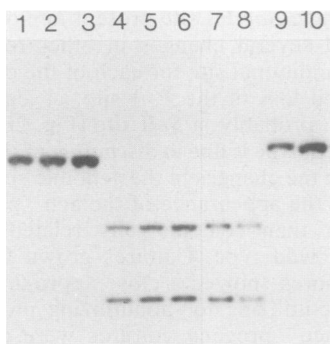

FIG. 3. DNA of 10 single ascospore isolates, representative of 20 analyzed (see text) from the cross T37-3-8 × wild type, digested with *Eco*RI and probed with pC3. The photograph is a composite of two electrophoretograms: lanes 1 through 3 and 9 and 10 acu^+; lanes 4 through 8 acu^- mutants.

Mapping of *acu-8*. As a first step to mapping *acu-8*, the 5.7-kb *Eco*RI fragment of pC3 was used to probe *Eco*RI-digested DNA from 18 progeny strains constituting the "cross 2" restriction-fragment-length-polymorphism mapping kit described by Metzenberg et al. (14; R. L. Metzenberg and J. Grotelueschen, Fungal Genet. Newsl. **35**:30–35, 1988). The Oak Ridge parental strain generated the expected 5.7-kb fragment, but the Mauriceville parent generated two fragments of 5.0 and 6.1 kb, respectively. The progeny strains showed segregation of the 5.7 from the 5.0-kb fragment, with the 6.1-kb fragment segregating independently. An investigation of the apparent additional copy of the clone 3 sequence in the Mauriceville strain will be reported elsewhere. The 18 progeny strains had already been characterized with respect to restriction-fragment-length polymorphisms marking different chromosomal arms. The 5.0/5.7-kb clone 3 polymorphism was clearly linked closely to polymorphisms in the right arm of linkage group 2 (IIR) detected by three different *Neurospora* DNA clones.

To define the position of *acu-8* more precisely, a double-mutant strain (*acu-8 trp-3*) was recovered from the cross T37-3-8 × *trp-3* and it was crossed in turn to *un-15*, a functionally uncharacterized temperature-sensitive mutant mapping about 15 centimorgans distal to *trp-3* on IIR. The results of the three-point cross are shown in Table 1; *acu-8*

TABLE 1. Mapping of *acu-8* in random ascospore cultures from *trp-3 acu-8 un-15*$^{+}$ × *trp-3*$^{+}$ *acu-8*$^{+}$ *un-15*[a]

Progeny genotype	No. observed	Progeny genotype	No. observed	Interpretation
trp-3 acu-8 +	77	+ + *un-15*	95	Parental
trp-3 + *un-15*	8	+ *acu-8* +	7	Crossover 1
trp-3 acu-8 un-15	1	+ + +	5	Crossover 2
trp-3 + +	0	+ *acu-8 un-15*	0	Double crossover

[a] Map order: *trp-3 acu-8 un-15*. Distances: 7.8 centimorgans for *trp-3* to *acu-8*; 3.1 centimorgans for *acu-8* to *un-15*.
[b] Plus denotes wild-type allele.

clearly maps between *trp-3* and *un-15*, perhaps somewhat closer to the latter. The seven classes of acetate nonutilizing mutants previously identified all clearly map at other loci (6).

Status of *acu-8* in the original T37 transformant. The initial transformant, T37, purified by crossing, which had the am^{132} deletion at the *am* locus and the am^{+}/clone 3 construct integrated ectopically, was crossed to T37-3-8 (*acu-8* am^{132}). Eight dissected asci each segregated 4 to 4 with respect to *am*, and six of them also segregated 4 to 4 with respect to *acu-8*. In the latter asci, *am* and *acu* segregated independently; there was evidently no functional *acu-8* gene associated with the ectopic am^{+} marker. The remaining two asci yielded only acetate nonutilizing ascospore progeny. These results indicate that the ectopic C3 sequence in the purified T37 was not functional, either because of its mode of integration or because of premeiotic disruption of duplicated sequences (called the RIP phenomenon and first described by Selker et al. [17]) in the purifying cross. The finding, already mentioned, that it was methylated supports the latter hypothesis.

Properties of *acu-8*. T37-3-8 and several other *acu* mutants isolated from crosses of other pC3*am*-transformed strains had a property not previously seen in acetate nonutilizing mutants: they were all found to utilize ethanol. Ethanol is a poor carbon source for *Neurospora* spp., but the *acu-8* mutants grow on it just as well as does the wild type (Fig. 4). In contrast, mutant *acu-5*, defective in acetyl coenzyme A synthetase (7), and *acp*, reported to be defective in acetate uptake (16), were severely deficient in ability to grow on ethanol. Other experiments showed that *acu-3*, *acu-6*, and *acu-7* were also unable to use ethanol. Unlike the mutants (*acu-3*, *acu-5*, and *acu-6*) with clear-cut enzyme deficiencies (7), the inability of *acu-8* to grow on acetate is not absolute. Over a long period, it does use acetate as carbon source, but markedly less well than it uses ethanol (Fig. 4). The *acu-8* lesion has no significant effect on inducible levels of isocitrate lyase, malate synthase, phosphoenolpyruvate carboxylase, or acetyl coenzyme synthetase (data not shown).

Since there is no known way that ethanol can be used as a carbon source other than conversion to acetate, the utilization of ethanol by *acu-8* mutants suggested that the lesion in these mutants was likely to affect acetate transport rather than acetate metabolism. However, an experiment on short-term uptake of [^{14}C]acetate by conidia, with and without prior incubation with acetate, showed that both T37-3 and a derived *acu-8* strain (used in the growth test shown in Fig. 4) had a strongly inducible acetate uptake system (16) of about the same activity as the wild type (data not shown).

Nucleotide sequence of the clone 3 open reading frame. The complete nucleotide sequence of a 2,248-base-pair portion of clone 3, including the region hybridizing to cDNA (Fig. 1), is shown in Fig. 5 (GenBank/EMBL accession number M31321). The sequence contains an open reading frame of 525 codons if one subtracts two segments, of 73 and 67 base pairs, respectively, that bear all the hallmarks of introns, including the conserved CTPuAC "lariat" branch sequence typically found about 15 base pairs from the 3′ end of *Neurospora* introns (11). The cDNA used to locate the transcription unit (Fig. 1) was presumably highly enriched with respect to the 3′ end of the transcribed sequence.

The codon usage (Table 2) is biased in ways typical of all

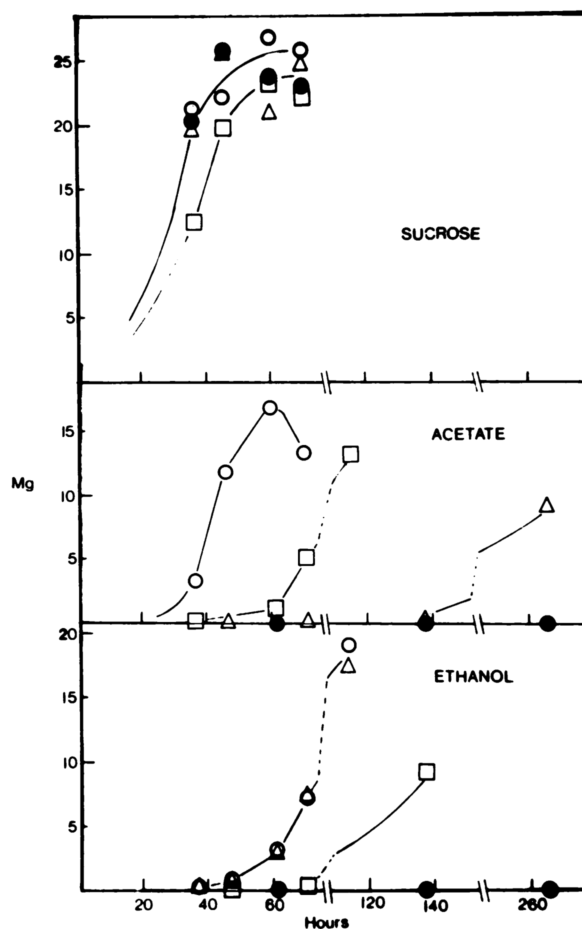

FIG. 4. Growth of wild type (○), *acu-5* (deficient in acetyl coenzyme A synthetase) (●), *acp* (reported deficient in acetate uptake) (□), and *acu-8* (△) on equivalent amounts of sucrose, acetate, and ethanol as sole carbon sources. The *acu-8* strain was an am^{+} derivative from the cross T37-3-8 × wild type. Approximately 10^5 conidia were inoculated into 20-ml lots of liquid Vogel's medium in 100-ml conical flasks with 40 mM acetate (at pH 5.6), 40 mM ethanol, or 6.67 mM sucrose and incubated at 30°C without shaking for the times indicated. Concentrations of acetate higher than 40 mM are inhibitory.

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GCAAAGCCATCCCGGACCTGTTGTAATTACCGTCATCATCTCCCCCTGTGTGACTGGCCTGGATTTGTTTTTTCTGCTTGTTCCGCTTCC
        10        20        30        40        50        60        70        80        90

GTCCCTTTTTTCGACTTCTTGCGGCATCTTTTTCCACTTCTTTTCTTCTTCTTCTTCTCTCCTCCAGCTCCTCCTGCTCCTCCTCCT
       100       110       120       130       140       150       160       170       180

TGACTTCCTTCGCGATCCTTTTATTTCTGTGTCGTCAAGGAATCCTCATCTTCCTCATCTTCCCTTCTTCCCTCTCTCCCTCTCCACTCT
       190       200       210       220       230       240       250       260       270

                                    MetAlaSerProIleAlaSerAlaAlaLeuArgAlaArgGlnGlyProSerMetLeu
ACACTCTCTACTAATCACACCACATCACCAACCATGGCGTCGCCCATTGCCTCGGCGGCCCTCAGGGCGCGTCAAGGCCCTTCCATGCTC
       280       290       300       310       320       330       340       350       360

LysLysLeuCysAsnProGluAspMetLeuGlnHisPheProAsnGlyAlaTyrIleGlyTrpSerGlyPheThrAlaSerAlaThrPro
AAGAAGCTCTGCAACCCCGAGGACATGCTCCAGCACTTCCCCAATGGCGCATACATCGGCTGGTCCGGTTTCACCGCGTCGGCTACCCCA
       370       380       390       400       410       420       430       440       450

A>-----------------------------------------------------------------------<rgArgSerTyrTyrLeu
AGTAAGTCGCCCATGTCGCTGGAGGAAATATAGCGCATGCCAAACCAAGGAAGTTGCTAACCCATGTGCTACAGGAAGGTCCTACTATCT
       460       470       480       490       500       510       520       530       540

 AlaAspHisValGluLysAsnAlaSerArgProAlaGlnValGlnProLeuArgArgCysLeuArgArgArgArgAspArgGluArgTrp
CGCTGACCACGTCGAGAAGAACGCCTCCAGGCCAGCTCAAGTACAGCCTCTTCGTCGGTGCCTCCGCCGGCGCCGAGACCGAGAACGCTG
       550       560       570       580       590       600       610       620       630

 AlaAlaLeuAspMetIleAlaHisAspArgArgArgAlaProHisGlnValGlyLysAsnIleAlaLysGlyIleAsnGluGlyArgIle
GGCTGCTCTCGACATGATCGCCCATGATCGCCGTCGCGCTCCTCACCAGGTCGGCAAGAACATTGCCAAGGGCATCAACGAGGGCCGCAT
       640       650       660       670       680       690       700       710       720

 AsnPhePheAspLysHisLeuSerMetPheProValAspLeuValTyr>-----------------------------------------
CAACTTCTTCGACAAGCACTTGTCCATGTTCCCCGTTGACCTTGTCTACGTAAGTGGTCGCGAATCTCGCTGGCGCATCGCAAACAGTTT
       730       740       750       760       770       780       790       800       810

-------------------------<GlyTyrTyrThrLysAspArgGlnAsnLysAsnLeuAspValValCysValGluAlaThrGluIle
GCTAACCCCTATCGCTCAATCAACAGGGCTACTACACCAAGGACCGCCAGAACAAGAACCTCGATGTCGTTTGCGTTGAAGCTACCGAGA
       820       830       840       850       860       870       880       890       900

 LysGluAspGlySerIleValLeuGlyAlaSerValGlyAlaThrProGluLeuIleGlnMetAlaAspLysValIleIleGluValAsn
TCAAGGAGGACGGTTCCATCGTCCTCGGTGCCTCGGTCGGTGCCACCCCCGAGCTCATCCAGATGGCCGACAAGGTCATCATTGAGGTCA
       910       920       930       940       950       960       970       980       990

 ThrAlaIleProSerPheAspGlyLeuHisAspIleThrPheSerAspLeuProProAsnArgLysProTyrLeuIleGlnGlnCysArg
ACACGGCCATCCCCAGCTTCGACGGTCTCCACGACATCACCTTCTCCGACCTCCCTCCCAACCGCAAGCCCTACCTCATCCAGCAGTGCA
      1000      1010      1020      1030      1040      1050      1060      1070      1080

 AspArgIleGlyThrThrSerValProValAspProGluLysValValGlyIleIleGluCysThrThrProAspGlnThrLeuProAsn
GGGACCGCATCGGTACTACTTCGGTCCCCGTCGACCCCGAGAAGGTTGTCGGTATCATCGAGTGCACCACCCCCGATCAGACCCTCCCCA
      1090      1100      1110      1120      1130      1140      1150      1160      1170

 SerProAlaAspGluThrAlaThrAlaIleAlaGlyHisLeuIleGluPhePheGluHisGluValAlaHisGlyArgLeuProLysAsn
ACTCCCCCGCCGACGAAACGGCTACGGCCATTGCCGGCCACCTGATTGAGTTCTTCGAGCACGAGGTCGCCCACGGCCGTCTGCCCAAGA
      1180      1190      1200      1210      1220      1230      1240      1250      1260

 LeuLeuProLeuGlnSerGlyIleGlyAsnIleAlaAsnAlaValIleGlyGlyLeuGluThrSerAsnPheLysAsnLeuAsnValTrp
ACCTTCTCCCTCTGCAGTCCGGTATCGGCAACATCGCCAACGCCGTCATTGGTGGTCTCGAGACCTCCAACTTCAAGAACCTCAACGTCT
      1270      1280      1290      1300      1310      1320      1330      1340      1350

 ThrGluValIleGlnAspThrPheLeuAspLeuPheAspSerGlyLysLeuAspPheAlaThrAlaThrSerIleArgPheSerProThr
GGACTGAGGTTATCCAGGATACCTTCCTCGACCTCTTCGACTCCGGCAAGCTTGACTTTGCTACCGCCACCTCGATCCGCTTCTCGCCCA
      1360      1370      1380      1390      1400      1410      1420      1430      1440

 GlyPheGluArgPheTyrLysAsnTrpAspAsnTyrTyrAspLysLeuLeuLeuArgSerGlnSerValSerAsnAlaProGluIleIle
CCGGCTTCGAGCGCTTCTACAAGAACTGGGACAACTACTATGACAAGCTCCTCCTCCGCTCTCAGTCCGTCTCCAACGCTCCCGAGATCA
      1450      1460      1470      1480      1490      1500      1510      1520      1530
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other highly expressed *Neurospora* genes so far sequenced; in particular, guanine is strongly preferred in third codon positions to adenine, pyrimidines are strongly preferred to purines, and cytosine is less strongly preferred to uracil. The predicted amino acid sequence does not show any clearly significant resemblance to any other published sequence available in the GenBank/EMBL database. It contains nothing suggestive of a transmembrane sequence.

DISCUSSION

RIP involves both methylation of many cytosine residues and a high frequency of GC-to-AT transition mutations (1). It was predictable that it would create null mutations with high frequency, and this prediction has been confirmed by studies of the effects of crossing on duplicated (5, 18) and triplicated (5) copies of the *N. crassa am* (NADP-specific glutamate dehydrogenase) gene. The RIP phenomenon clearly provides a general method for obtaining null mutations of any gene that has been cloned but which, for want of mutations, is of unknown function. It can provide for *Neurospora* spp., and very probably for other filamentous ascomycete fungi (9), the equivalent of the targeted gene disruption that is readily available in *Saccharomyces cerevisiae* through homologous integration of transforming DNA (4). The latter method can, in fact, be made to work in *Neurospora* spp. (15), but because of the relatively high frequency of nonhomologous integration of transforming DNA, it is far less efficient in *Neurospora* spp. than in yeast.

An interesting feature of the RIP-induced disruption at the normal *acu-8* locus in T37-3 is that it was instigated by the T37 ectopic gene copy that had evidently already undergone the RIP effect. It is clear that the normally located gene copy in T37-3 had further damage, manifested in restriction site changes, not present in T37. Evidently the ability of the duplicated sequence to induce RIP was not seriously impaired by its having undergone some previous RIP itself.

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  ArgArgLeuGlyValIleGlyMetAsnThrProValGluValAspIleTyrAlaHisAlaAsnSerThrAsnValMetGlySerArgMet
TCCGCCGTCTCGGTGTTATCGGCATGAACACCCCCGTCGAAGTCGACATCTACGCCCACGCCAACTCCACCAACGTCATGGGCTCCCGCA
     1540      1550      1560      1570      1580      1590      1600      1610      1620

  LeuAsnGlyLeuGlyGlySerAlaAspPheLeuArgAsnSerLysTyrSerIleMetHisThrProSerThrArgProSerLysThrAsp
TGCTCAACGGTCTCGGTGGTTCCGCCGATTTCCTCCGCAACTCCAAGTACTCCATCATGCACACCCCCTCCACCCGTCCCTCCAAGACCG
     1630      1640      1650      1660      1670      1680      1690      1700      1710

  AlaHisGlyValSerCysIleValProMetCysThrHisValAspGlnThrGluHisAspLeuAspValIleValThrGluAsnGlyLeu
ACGCCCACGGTGTTTCGTGCATTGTCCCCATGTGCACCCACGTCGACCAGACCGAGCACGATCTCGACGTCATCGTTACCGAGAACGGTC
     1720      1730      1740      1750      1760      1770      1780      1790      1800

  AlaAspValArgGlyLeuSerProArgGluArgAlaArgValIleIleAspLysCysAlaHisAspValTyrLysProIleLeuLysAla
TCGCCGATGTCCGCGGCCTCAGCCCCCGCGAGCGTGCCCGCGTCATCATTGACAAGTGCGCCCACGACGTCTACAAGCCCATCCTCAAGG
     1810      1820      1830      1840      1850      1860      1870      1880      1890

  TyrPheGluLysAlaGluPheGluCysLeuArgLysGlyMetGlyHisGluProHisLeuLeuPheAsnSerPheAspMetHisLysAla
CCTACTTTGAGAAGGCCGAGTTCGAGTGCCTTCGCAAGGGTATGGGCCACGAGCCCCACCTTCTCTTCAACTCTTTCGACATGCACAAGG
     1900      1910      1920      1930      1940      1950      1960      1970      1980

  LeuValGluGluGlySerMetAlaLysValLysPhe***
CTCTTGTCGAGGAGGGCTCCATGGCCAAGGTCAAGTTCTAAATGCTTGGCTAGAGTCAGAGAGTGTATATCTCGGTGGGGAAGAGATAAT
     1990      2000      2010      2020      2030      2040      2050      2060      2070

GAGAAAACCAGAGAGGATCAACAGTTGATCTTTGAATAAAACAAAGAGAACTGTCTAAACAAACAAACGTGGTATGGGGGAAATGCATTG
     2080      2090      2100      2110      2120      2130      2140      2150      2160

GTTAGGTGTTCATTCTTTCCGGTTCACGTATATATCTTTATTGGAAGGGGAAGAGAGAGAGAGAGAGATCGAAGAGAGGAGTGGATC
     2170      2180      2190      2200      2210      2220      2230      2240      2250
```

FIG. 5. Sequence of the part of clone 3 containing the 525-codon *acu-8* open reading frame interrupted by two introns. Underlining indicates intron internal consensus sequences and a possible polyadenylation signal. GenBank/EMBL accession number M31521.

Indeed, a third-round restriction site change (loss of the distinctive *Eco*RI site) has been seen in one derivative of the cross T37-3-8 × wild type. Presumably, repeated rounds of RIPing of an ectopic gene copy should lead to progressive decay of sequence similarity to the normal gene and eventual loss of ability to disrupt its function. This prediction is clearly testable.

The function of *acu-8* is still not clear. It is difficult to see how a mutation could block acetate but not ethanol utilization other than through some effect on acetate transport, either into the cell or, perhaps, into the glyoxysome. The results of short-term acetate uptake experiments do not support the hypothesis that *acu-8* controls an acetate permease operating at the cell membrane nor is the coding sequence of the gene suggestive of a membrane protein (Fig. 5). Investigation of the biochemical phenotype of *acu-8* mutants is continuing.

A feature of the *acu-8* sequence is the strong bias in codon usage: pyrimidines strongly preferred to purines, guanine to adenine, and, to a lesser extent, cytosine to uracil in third codon positions. This bias is also seen in two other *acu* genes in *Neurospora* spp., *acu-5* encoding acetyl coenzyme A synthetase and *acu-9*, a newly identified gene encoding malate synthase (R. Sandeman, I. F. Connerton, J. R. S. Fincham, and M. J. Hynes, unpublished data) and in virtually all the *Neurospora* genes that have been sequenced except for those involved in quinate utilization (8), in which there is a much closer approach to random codon usage.

TABLE 2. Codon usage in *acu-8*

Amino acid	Codon	Frequency	Amino acid	Codon	Frequency	Amino acid	Codon	Frequency	Amino acid	Codon	Frequency
F	TTT	2	S	TCT	2	Y	TAT	2	C	TGT	0
F	TTC	20	S	TCC	21	Y	TAC	12	C	TGC	9
L	TTA	0	S	TCA	0	*[a]	TAA	1	*	TGA	0
L	TTG	1	S	TCG	8	*	TAG	0	W	TGG	4
L	CTT	7	P	CCT	5	H	CAT	1	R	CGT	7
L	CTC	31	P	CCC	23	H	CAC	17	R	CGC	19
L	CTA	0	P	CCA	2	Q	CAA	2	R	CGA	2
L	CTG	3	P	CCG	0	Q	CAG	12	R	CGG	2
I	ATT	8	T	ACT	3	N	AAT	1	S	AGT	0
I	ATC	26	T	ACC	22	N	AAC	26	S	AGC	2
I	ATA	0	T	ACA	0	K	AAA	0	R	AGA	1
M	ATG	14	T	ACG	3	K	AAG	27	R	AGG	4
V	GTT	8	A	GCT	11	D	GAT	7	G	GGT	17
V	GTC	26	A	GCC	26	D	GAC	27	G	GGC	17
V	GTA	1	A	GCA	1	E	GAA	4	G	GGA	0
V	GTG	0	A	GCG	4	E	GAG	25	G	GGG	0

[a] *, Chain termination.

There are strong indications (10) that preferred usage of codons, corresponding to generally abundant tRNA species, is a characteristic of strongly and/or constitutively expressed genes. The strong codon bias of the *acu* genes, which are strongly regulated, seems likely to be connected with their high maximum levels of expression.

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