SEQUENCING STUDIES OF ICR-170 MUTAGENIC SPECIFICITY IN THE *AM* (NADP-SPECIFIC GLUTAMATE DEHYDROGENASE) GENE OF *NEUROSPORA CRASSA*

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ABSTRACT

The acridine half-mustard ICR-170-induced reversion of the mutant *am*15, which has a single base-pair deletion, at a frequency of between 9 and 28 × 10^{-6}. In each of three classes of revertants, the mutagen had induced the insertion of a $\begin{smallmatrix}\text{-G-}\\ \text{-C-}\end{smallmatrix}$ base pair at a $\begin{smallmatrix}\text{-G-G-}\\ \text{-C-C-}\end{smallmatrix}$ site. The mutant *am*6, which has a single base pair insertion, is known to be revertible, with UV light, by deletion of a $\begin{smallmatrix}\text{-G-}\\ \text{-C-}\end{smallmatrix}$ base pair at a $\begin{smallmatrix}\text{-G-G-G-}\\ \text{-C-C-C-}\end{smallmatrix}$ site. This mutant reverted with ICR-170 at a frequency of 0.1 × 10^{-6}. These results show that ICR-170 is able to induce addition frameshifts in *Neurospora crassa* within short, monotonous runs of G:C base pairs, but indicate a lack of deletion activity at such sequences.

THE *am* gene, coding for the NADP-specific glutamate dehydrogenase of *Neurospora crassa*, is the first in filamentous fungi to have been used to study frameshift mutagenesis at the level of DNA sequence. The mutant *am*6 was shown by SIDDIG *et al.* (1980) to have a single base-pair insertion in codon 5; various ultraviolet-induced revertants had compensating single base-pair deletions in nearby codons, including the deletion of a $\begin{smallmatrix}\text{-G-}\\ \text{-C-}\end{smallmatrix}$ from a $\begin{smallmatrix}\text{-G-G-G-}\\ \text{-C-C-C-}\end{smallmatrix}$ sequence. As a result of the analysis, a sequence of 17 bases was predicted in the mRNA, and this sequence, synthesized as DNA, was used as a probe in the cloning of the gene (KINNAIRD *et al.* 1982), which was subsequently sequenced (KINNAIRD and FINCHAM 1983).

The mutant *am*15, which was isolated following nitrous acid mutagenesis (FINCHAM and STADLER 1965), was provisionally characterized a number of years ago as a frameshift mutant on the basis of its high revertibility with the acridine half-mustard ICR-170 (L. E. KELLY and A. RADFORD, unpublished

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Abbreviations used in text: ICR-170, 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)-aminopropylamino]acridine.2HCl. CAS number (146-59-8); EMS, ethyl methanesulfonate.

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results). STREISINGER and OWEN (1985) have suggested that acridine compounds may induce frameshifts by stacking extrahelically with looped-out bases either in the template or the nascent strand, leading to a deletion in the former case and an insertion in the latter. Looping-out, or strand-slippage, is thought to occur preferentially within repetitive sequences, and the transient loops would be expected to have greater stability if the repeats involved G:C base pairs.

In prokaryotic systems, acridine half-mustards (ICR-191 has been mainly used) induce both deletions and insertion-type frameshifts at runs of G:C base pairs (AMES, LEE and DURSTON 1973; CALOS and MILLER 1981). In contrast, sequencing studies in yeast (MATHISON and CULBERTSON 1985; ERNST, HAMPSEY and SHERMAN 1985) show that ICR-170 primarily induces insertion-type frameshifts, also at runs of G:C base pairs. The evidence available until now from filamentous Ascomycetes has been indirect and somewhat conflicting. ICR-170 is a potent mutagen in *N. crassa* (BROCKMAN and GOBEN 1965). BRUSICK (1969) concluded that ICR-170-induced *ad-3* mutants of *N. crassa* were probably of the frameshift type, and he found that most were revertible with the same mutagen. However, in the *b2* (spore color) locus of *Ascobolus immersus*, ICR-170 appeared to act unidirectionally, in opposition to EMS (LEBLON 1972). LEBLON concluded that ICR-170 induced additions rather than deletions, with the interesting implication that the bias in meiotic gene conversion (characteristic of Ascobolus frameshift mutants) favors the longer strand. We present here the first direct evidence as to the mode of action of ICR-170 in *N. crassa*.

MATERIALS AND METHODS

Neurospora strains: The wild-type strain used was ST74*A*. The mutant strains *am*6-6-1*a* and *am*15-6-*a* are products of six generations of crossing and backcrossing to ST74*A*. Revertants R1-R16, isolated following ICR-170 treatment of *am*15-6-*a*, were genetically purified by crossing to *am* mutant strains of *A* mating type (also intensively inbred with ST74*A*), followed by isolation of ascospores of am^+ phenotype.

Mutagen treatments and selection of revertants: Conidia from approximately 1-week-old cultures were suspended in 0.1 M phosphate buffer, pH 7, at concentrations of between 1 and 5 × 10^7/ml and were mutagenized as described in Table 1.

Treated conidia were collected by membrane filtration, washed and plated on Vogel's minimal agar, with the usual sucrose replaced by 1% L-sorbose plus 0.2% sucrose (to induce colonial growth), and 0.02 M glycine to inhibit the "leaky" growth of *am* conidia. Revertant colonies began to appear after 3 days of incubation at 25° and were scored after 5 days.

GDH extraction and assay: 50-ml liquid cultures were grown for enzyme extraction, and crude extracts were prepared and assayed essentially as described by SIDDIG *et al.* (1980). GDH activity (rate of NADP reduction) was expressed as change in optical density at 340 nm per min × 100. Specific activity was expressed as activity per milligram of protein, with protein concentration being assayed by the micro-Biuret procedure.

Electrophoretic separation of GDH varieties: Samples of crude extracts were electrophoresed on nondenaturing 7% polyacrylamide slab gels and were stained for GDH activity essentially as described by CODDINGTON, FINCHAM and SUNDARAM (1966).

Cloning and Sequencing: DNA was prepared from *am*15 and revertant strains as previously described (KINNAIRD *et al.* 1982). A genomic library was made from each by

TABLE 1

Reversion of *am6* and *am15* with different mutagenic treatments

Mutant	Treatment	Percent survival	No. of revertants	Reversion frequency per 10^6 survivors[a]
am6	None	100	0	<0.1
am15	None	100	0	<0.1
	ICR-170[b]			
am6	4 μg/ml	65	0	<0.1
	6 μg/ml	43	1	0.1
am15	2.5 μg/ml	59	114	9.2
	5.0 μg/ml	11	74	26.4
	7.5 μg/ml	3	22	27.5
	UV[c]			
am6	30 secs	35	88	16.6
	45 secs	10	38	25.3
am15	40 secs	45	2	0.2
	65 secs	21	2	0.2
	EMS[d]			
am6	2% (v/v)	49	20	4.0
	5%	17	9	5.1
am15	2%	83	0	<0.1
	5%	18	0	<0.1

[a] Typical results from at least two experiments involving the screening of at least 10^7 surviving conidia.

[b] ICR-170 was dissolved in H_2O and added to conidial suspension to desired concentration. Treatment was at 30° for 2 hr with shaking.

[c] Conidia were treated in suspension while being stirred at room temperature.

[d] EMS was added directly to suspension, and treatment was for 40 min at 30°.

ligating 1 μg of *Hin*dIII-digested DNA into the *Hin*dIII site of the lambda-"spi" cloning vector L47, followed by *in vitro* packaging. Clones containing the *am* gene were identified by plaque hybridization, using a ^{32}P-labeled (nick-translated) 2.7-kb wild-type am^+-containing *Bam*HI fragment (KINNAIRD *et al.* 1982) as a probe. The 2.7-kb *Bam*HI fragment was purified from each clone by elution from an agarose gel, and subfragments generated by digestion with *Xho*I were then ligated at random into the *Sal*I site of M13mp8 (MESSING and VIEIRA 1982). Genetic mapping of the *am15* mutational site (J. R. S. FINCHAM, unpublished results) indicated that it was likely to fall near the center of the second exon and within the 0.7-kb *Xho*I fragment that spans this exon, probably within sequencing range of its "upstream" end.

The appropriate M13mp8 clones for sequencing across the *am15* site were identified by the ability of their DNA to anneal with the complementary single-stranded 0.7-kb wild-type *Xho*I fragment that had already been cloned by KINNAIRD and FINCHAM (1983). DNA sequencing was carried out by means of the "dideoxy" chain-termination method (SANGER *et al.* 1980).

RESULTS

Mutagen specificity: As shown in Table 1, *am15* reverted at a high frequency with ICR-170, but showed little or no response to UV or EMS, re-

TABLE 2

DNA and amino acid sequence alterations in *am*15 and representative revertants between codons 52 and 61

Strain	Sequences									
	52	53	54	55	56	57	58	59	60	61
Wild type	Trp	Glu	Asp	Asp	Asn	Gly	Asn	Val	Gln	Val
	TGG	GAG	GAC	GAC	AAC	GGC	AAC	GTC	CAG	GTC
*am*15	TGG	GAG	GAC	GAC	AA_	GGC	AAC	GTC	CAG	GTC
					−1					
					Lys					
*am*15 R12 (class I)	TGG	GAG	GAC	GAC	AAG	GGC	AAC	GTC	CAG	GTC
						+1				
					Lys	Ala	Thr	Ser		
*am*15 R15 (class II)	TGG	GAG	GAC	GAC	AAG	GCA	ACG	TCC	CAG	GTC
								+1		
			Gly	Arg	Gln					
*am*15 R11 (class III)	TGG	GAG	GGA	CGA	CAA	GGC	AAC	GTC	CAG	GTC
			+1							

spectively, under the conditions of the experiments. In contrast, *am*6 showed little response with ICR-170, but reverted at relatively high frequencies with UV and EMS under the conditions used.

Classification of ICR-170-induced *am*15 revertants: ICR-170-induced revertants from *am*15 were divided into three classes on the basis of two criteria: GDH thermal stability and electrophoretic mobility. Of the 16 revertants tested, four resembled wild type in producing GDH that, in the normal extraction buffer (0.05 M sodium phosphate, pH 7.4, with 1 mM EDTA), was almost stable for 10 min at 60°. Extracts of the remaining 12 lost most of their GDH activity during 5 min at 60°. All 16 revertants, whether they belonged to the heat-stable or the heat-labile class, produced GDH varieties with reduced net negative charge as compared with wild type. Within the heat-stable group (class I), including revertant R12, GDH migrated in polyacrylamide gel electrophoresis approximately 7% slower than wild type. The heat-labile group fell into two classes: one (class II), represented by R15, resembled the heat-stable group in electrophoretic mobility (*i.e.*, 7% slower), and the other (class III), represented by R11, showed a more extreme electrophoretic difference of approximately 18% slower than wild type. Class III revertants also were distinct in that they gave more faintly staining GDH bands from similar quantities of extracts.

DNA sequences of *am*15 and revertants: Table 2 shows the sequence differences from wild type found in *am*15 and in the revertants R12 (class I), R15 (class II) and R11 (class III). All share a single $\begin{matrix}-\mathrm{C}-\\-\mathrm{G}-\end{matrix}$ base-pair deletion in codon 56 (AAC for asparagine in wild type). Revertants R12 and R11 have a single $\begin{matrix}-\mathrm{G}-\\-\mathrm{C}-\end{matrix}$ insertion at a $\begin{matrix}-\mathrm{G}-\mathrm{G}-\\-\mathrm{C}-\mathrm{C}-\end{matrix}$ site in *am*15, and revertant R15 has a single $\begin{matrix}-\mathrm{C}-\\-\mathrm{G}-\end{matrix}$ insertion at a $\begin{matrix}-\mathrm{C}-\mathrm{C}-\\-\mathrm{G}-\mathrm{G}-\end{matrix}$ site. The insertion is in codon 57 (GGC) in R12, in codons 53–54 (GAGGAC) in R11 and in codons 59–60 (GTCCAG) in R15. The sequence changes are consistent with the observed electrophoretic differences. Class I and class II revertants have a charge change of +1, Asn → Lys$^+$, and class III revertants have a charge change of +3, Asp^-Asp^- → Gly Arg$^+$.

DISCUSSION

In each of the three analyzed *am*15 revertants, ICR-170 induced the addition of a $\begin{smallmatrix}-G-\\-C-\end{smallmatrix}$ base pair into a $\begin{smallmatrix}-G-G-\\-C-C-\end{smallmatrix}$ sequence, consistent with Streisinger *et al.*'s (1966) model of frameshift mutation by strand slippage and looping out. The apparent ineffectiveness of ICR-170 in inducing single base-pair deletions within a $\begin{smallmatrix}-C-C-C-\\-G-G-G-\end{smallmatrix}$ sequence to give *am*6 revertants suggests that loop-outs of the template strand occur less readily than do those of the nascent strand during DNA synthesis. Our finding that ICR-170 induces addition rather than deletion-type frameshifts in runs of G:C base pairs is in complete agreement with the results of two recent studies in yeast. Mathison and Culbertson (1985) sequenced 16 sites of ICR-170-induced mutation at the *his*4 locus of yeast and found that each one represented the addition of a G:C base pair into a monotonous run of two or more G:C base pairs. In another study (Ernst, Hampsey and Sherman 1985), ICR-170 was found to primarily induce additions of G:C base pairs at sites containing monotonous runs of G:C base pairs within the *CYC1* gene of yeast. Why ICR-170 should apparently specifically induce or stabilize slippage of the nascent strand during DNA synthesis at monotonous runs of G:C base pairs is not clear.

In prokaryotic studies there is no clear evidence of a preferential induction of additions by acridine compounds. In Salmonella, ICR-191 and ICR-346-OH treatment can cause the insertion of a G:C base pair into a run of three monotonous G:C base pairs, and the deletion of a $\begin{smallmatrix}-C-G-\\-G-C-\end{smallmatrix}$ sequence from a $\begin{smallmatrix}-C-G-C-G-C-G-C-G-\\-G-C-G-C-G-C-G-C-\end{smallmatrix}$ site (Isono and Yourno 1974). In the *lacI* gene of *E. coli*, 98% of ICR-191-induced mutations are single G:C base-pair deletions from, or additions to, either $\begin{smallmatrix}-G-G-G-\\-C-C-C-\end{smallmatrix}$ or $\begin{smallmatrix}-G-G-G-G-\\-C-C-C-C-\end{smallmatrix}$ sequences (Calos and Miller 1981).

Although the apparent difference in addition/deletion specificity between the eukaryotic and prokaryotic studies may be due to differences in the acridine compounds used, it may also reflect a constraint, perhaps mediated by proteins, on slippage events in the template strand during DNA synthesis in eucaryotes.

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Note added in proof: Since this paper was accepted, we have characterized another frameshift mutant and, from that, two ICR-170-induced revertants. The mutant, *am*129, isolated after nitrous acid treatment (Kinsey and Hung 1981), has a deletion of a $\begin{smallmatrix}-C-\\-G-\end{smallmatrix}$ base pair in codons 119–120.

The first revertant has the wild-type sequence restored, and the second revertant (R6) has a single $\begin{array}{c}-\mathrm{G}-\\-\mathrm{C}-\end{array}$ insertion at a $\begin{array}{c}-\mathrm{G}-\mathrm{G}-\mathrm{G}-\\-\mathrm{C}-\mathrm{C}-\mathrm{C}-\end{array}$ site in codons 121–122.

Strain	Sequences			
	119	120	121	122
Wild type	Asp	Pro	Lys	Gly
	GAC	CCC	AAG	GGC
*am*129	GAC	CCA	AGG	GC
*am*129R6	Asp	Pro	Arg	Gly
	GAC	CCA	AGG	GGC

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