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Genetics

Point mutations and DNA rearrangements 5′ to the inducible *qa-2* gene of *Neurospora* allow activator protein-independent transcription

(enhancers/upstream activator sequence/*qa* gene cluster/promoters/eukaryotic gene regulation)

ROBERT F. GEEVER, MARY E. CASE, BRETT M. TYLER, FRANK BUXTON*, AND NORMAN H. GILES

Department of Molecular and Population Genetics, University of Georgia, Athens, GA 30602

Contributed by Norman H. Giles, August 31, 1983

ABSTRACT Expression of the *qa-2* gene of *Neurospora crassa* normally requires a functional activator protein encoded by *qa-1F*. Twelve transcriptional mutants of the *qa-2* gene have been isolated in *qa-1F*$^-$ strains, and these allow partial expression of *qa-2* (1–45% of induced wild type) in the absence of functional activator protein. All 12 mutants have been characterized by genomic (Southern) blot hybridization and the DNAs of 5 have been cloned and sequenced. Eight mutations consist of large DNA rearrangements within a 500-base-pair region 5′ to the *qa-2* gene. One large rearrangement mutation, located 378 base pairs before the normal site of transcription initiation, causes exceptional levels of *qa-2* transcription (45% of induced wild type) from near the normal initiation site. Two of the other four mutations cloned involve tandem duplications (68 and 84 base pairs) of the same upstream region (centered at nucleotide −145), and two involve "point" mutations (at nucleotides −200 and −95) that closely flank the duplicated region. With one possible exception, none of the mutations appears to involve changes directly associated with RNA polymerase II binding and hence they differ from analogous mutations in comparable prokaryotic systems. The overall results suggest that at least some of the large DNA rearrangement mutations may be acting as upstream activator elements, possibly by juxtaposing enhancer-like sequences, whereas the duplications and point mutations may define a region of *qa-2* regulation, for instance at the level of RNA polymerase II access.

Genetic regulation in prokaryotic systems such as the *lac* and *ara* operons involves both negative and positive control mechanisms (1, 2). Positive control is mediated by the action of one or more activator proteins normally required for the initiation of transcription by RNA polymerase. However, in both the *lac* and *ara* systems, it has been possible to select promoter mutants in which RNA polymerase can initiate transcription in the absence of functional activator proteins (3, 4). In general, such activator-independent (initiator-constitutive) mutants have undergone single point mutations in the −35 or −10 regions that facilitate transcription by enhancement of RNA polymerase recognition and binding (5, 6). The possibility of selecting for comparable activator-independent mutants in a genetically analogous eukaryotic regulatory system provides an opportunity to examine the determinants of positive control for a eukaryotic gene. This paper describes the results obtained in studies with such a system.

The *qa-2* gene, which encodes the inducible enzyme catabolic dehydroquinase, is one of three *qa* structural genes in the *qa* gene cluster controlling quinic acid catabolism in *Neurospora crassa* (7). The *qa* gene cluster also includes two regulatory genes, *qa-1S*, which appears to encode a repressor, and *qa-1F*, which encodes an activator protein that positively controls the expression of *qa-2* and other genes in the cluster (ref. 8; L. Huiet, personal communication). Hence, *qa-1F*$^-$ (noninducible) mutants are pleiotropic negative, lacking activities for all *qa* enzymes. The entire *qa* cluster has been cloned (9), and coordinate regulation of this system by the inducer quinic acid and the activator protein (the product of the *qa-1F* gene) has been shown to occur at the level of transcription (10). Transcriptional initiation sites for *qa-2* have been located, and the DNA sequence for the *qa-2* gene and its flanking sequences has been determined (11).

To isolate activator protein-independent mutants of *qa-2*, the ability of catabolic dehydroquinase (encoded by *qa-2*) to substitute for the biosynthetic isozyme (encoded by *arom-9*) (7) was utilized. Since *qa-2* expression normally requires a functional *qa-1F* gene, a *qa-1F*$^-$ *arom-9*$^-$ double mutant lacks activity for both dehydroquinases and cannot grow on unsupplemented minimal medium. This situation provided the opportunity to select for *qa-1F*-independent transcriptional mutants of *qa-2* as "revertants" of a *qa-1F*$^-$ *arom-9*$^-$ strain producing sufficient catabolic dehydroquinase to replace the biosynthetic activity and permit growth on minimal medium. In these studies, such "activator-independent" mutants have been isolated and cloned and their DNA sequences have been determined. The mutations all occur in a region 5′ to the *qa-2* gene and exhibit a variety of DNA sequence alterations, including point mutations, small duplications, and large DNA rearrangements.

MATERIALS AND METHODS

Strains. The *N. crassa* wild-type strain used in these studies was 74A. Mutant strains contained a stable, pleiotropic *qa-1F*$^-$ allele (158 or 162) and an unlinked *arom-9*$^-$ allele (M6-11) (8). Double mutants, *qa-1F*$^-$ *arom-9*$^-$, lacking both biosynthetic (*arom-9*) and catabolic (*qa-2*) dehydroquinase activities are unable to grow on minimal medium. *qa-2* activator-independent mutants, initially obtained as heterokaryons on minimal medium, were crossed to the methionine-requiring strain 4894 (*me-7*$^-$) (this gene is closely linked to the *qa* cluster) to obtain homokaryotic isolates.

Mutant Induction and Enzyme Assays. Plating and crossing techniques, media composition, and procedures for enzyme assay have been described (8). Mutations were induced as follows: 4×10^8 macroconidia were suspended in an aqueous solution at 5×10^6 macroconidia per ml. Three 40-ml aliquots were gently mixed on a magnetic stirrer during exposure to UV, such that the resulting survival frequency was 15–20%. The conidial sus-

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Abbreviations: kb, kilobase(s); bp, base pair(s).

* Present address: Department of Genetics, University of Leeds, Leeds LS2 9JT, England.

pensions were diluted 1:10 with minimal plating medium and dispensed into Petri dishes. An unirradiated 40-ml control sample was plated in the same manner.

Analysis of DNA and RNA. Genomic DNA was prepared as described (12) and further purified by banding in cesium chloride with ethidium bromide. Transfer of DNA from agarose gels to nitrocellulose was as described (13). DNA sequence analysis was performed by using the chain-terminator method (14) with phage M13 vectors (15). Isolation of poly(A)$^+$ RNA (10), electrophoresis of RNA in agarose gels containing methylmercuric hydroxide, and transfer to diazobenzyloxymethyl paper (16) were as described. RNA and DNA filter hybridizations (17, 18) and quantitation of mRNA by S1 nuclease of RNA·DNA hybrids (17) were as described.

Cloning. One to 5 μg of genomic DNA digested with appropriate restriction enzyme(s) was ligated to 0.5–2.5 μg of appropriately digested pBR325 DNA at a concentration of 25 μg/ml. Transformation of *Escherichia coli* strain SK3430 (*aroD6 leuC hsdR4 endA1 sbcB15*, F$^-$) was performed as described (19), except that transformed cells were allowed to recover in L-broth at 37°C for 3–4 hr with shaking, centrifuged, and resuspended in 0.2 ml of 0.15 M NaCl, then spread on a minimal plate (M56/2) supplemented with 0.5% glucose, 0.01% leucine, and chloramphenicol at 100 μg/ml. Selection of the desired recombinant clone was possible because the expression of the *Neurospora qa-2* gene in *E. coli* is required to complement an *aroD6* mutation in the host strain (20).

RESULTS

Mutant Induction and Characterization by Enzyme Assays. Three UV mutagenesis experiments were performed using strain 158 and two using strain 162. All colonies capable of growth on minimal medium were assayed for the presence of heat-stable catabolic dehydroquinase. *qa-2* activator-independent mutants should produce at least low levels of heat-stable catabolic dehydroquinase, whereas *arom-9*$^+$ revertants would produce only heat-labile biosynthetic dehydroquinase (7). Between 100 and 150 colonies were obtained from each experiment. Enzyme assays revealed that >95% of the colonies were revertants of the *arom-9*$^-$ allele; 3–5% of the colonies from strain 158 showed heat-stable dehydroquinase activity, but only one such isolate was obtained from strain 162. This corresponds to a UV-induced mutation rate for presumptive *qa-2* activator-independent mutants of 10^{-7} per viable macroconidium for strain 158 and $\approx 10^{-8}$ for strain 162. None of the mutants was capable of growth on quinic acid as a sole carbon source.

N. crassa strains of genotype *qa-1F*$^+$ *arom-9*$^-$ express all *qa* enzymes due to an accumulation of the internal inducer, dehydroquinate. However, in the presence of the *arom-9*$^+$ allele, internal induction does not occur (7). To determine whether the activator-independent mutants express catabolic dehydroquinase constitutively or are subject to induction by the internal inducer, dehydroquinate, these mutants were crossed to an *arom-9*$^+$ *me-7*$^-$ strain and homokaryotic *me-7*$^+$ *arom-9*$^+$ segregants were recovered and assayed for catabolic dehydroquinase activity.

Relative activities are presented in Table 1. All activator-independent mutants show levels of catabolic dehydroquinase activity significantly higher than the progenitor strains (158 and 162) and the noninduced wild-type (74A). Most activities range between 3% and 10% of induced wild type, 158-33 representing an exceptional extreme, having about 45% of wild type. The data in Table 1 also show that individual activator-independent mutant strains carrying either an *arom-9*$^-$ or an *arom-9*$^+$ allele have similar levels of catabolic dehydroquinase activity, indicating that this activity is expressed constitutively and is not subject to further induction by the presence of dehydroquinate.

Table 1. Quantitation of *qa-2* enzyme and mRNA levels in the activator-independent mutants

Strain	cDHQase levels*		*qa-2* mRNA levels†	
	arom 9$^-$	*arom 9*$^+$	*arom 9*$^-$	*arom 9*$^+$
74A I	—	100	—	100
74A NI	—	0.1	—	0.3
158	0.2	ND	2.0	ND
162	0.2	ND	1.0	ND
162-4	3.0	ND	ND	5.0
158-6	3.2	9.1	ND	11.0
158-21	ND	4.4	ND	10.7
158-33	41.8	45.3	ND	46.9
158-35	4.0	6.3	ND	13.1
158-41	1.5	ND	ND	ND
158-48	5.0	ND	8.7	ND
158-99	8.4	10.0	ND	6.1
158-106	5.4	6.1	ND	10.3
158-108	5.8	4.9	ND	5.8
158-109	ND	3.4	ND	4.3
158-110	3.4	ND	5.4	ND

I, induced; NI, noninduced; ND, not determined.

* Catabolic dehydroquinase (cDHQase) activity was determined as μmol of dehydroshikimate produced per min at 37°C per mg of soluble protein and expressed relative to wild-type levels under inducing conditions (0.3% quinic acid for 6 hr in minimal medium).

† *qa-2* mRNA levels in the mutant and wild-type strains were determined from densitometry of a number of exposures of the autoradiogram shown in Fig. 1B, using a Beckman DU-8 spectrophotometer, and are expressed relative to induced wild type.

At present, we cannot account for the apparent disparity in mutation rates between the two progenitor strains. However, this may reflect the difference in the nature of the stable *qa-1F*$^-$ mutations themselves. Southern blots show that strain 162 contains a DNA rearrangement in the region of the *qa-1F* gene, whereas strain 158 shows no detectable variation in the restriction sites of 16 different enzymes (L. Huiet, personal communication).

***qa-2* Transcription Is Increased in the Activator Protein-Independent Mutants.** To determine whether the mutations were acting at the transcriptional level, we isolated poly(A)$^+$ RNA from several of the mutants and analyzed it by gel blot hybridization (16), using a *qa-2* DNA probe. Fig. 1A shows that the three mutants examined have significantly higher *qa-2* mRNA levels than their progenitor strain 158. Mutant 158-33, which had the highest *qa-2* enzyme levels, shows especially high levels of *qa-2* mRNA. It is also evident from Fig. 1A that the *qa-2* mRNAs in the mutants are similar in size to wild-type *qa-2* mRNAs and are presumably initiated and terminated at the normal sites. The two species of *qa-2* mRNAs observed (1.0 and 1.2 kb) differ at their 3′ ends (unpublished data).

To determine if the remainder of the mutants were transcriptional in nature, and to quantitate the effect of each of the mutations, poly(A)$^+$ RNAs from all of the mutants and from wild type were assayed for *qa-2* mRNA by S1 nuclease digestion of RNA·DNA hybrids (Fig. 1B). A DNA fragment spanning the 3′ end of the 1.0-kb *qa-2* mRNA was end-labeled with ^{32}P at the 3′ position at an *Eco*RI site within the *qa-2* gene (Fig. 1C), hybridized to poly(A)$^+$ RNA, and digested with S1 nuclease, and the protected DNA fragments were resolved by electrophoresis. In Fig. 1B, the 335- and 350-nucleotide bands correspond to the heterogeneous 3′ end of the 1.0-kb *qa-2* mRNA, while the 355-nucleotide band results from protection by the

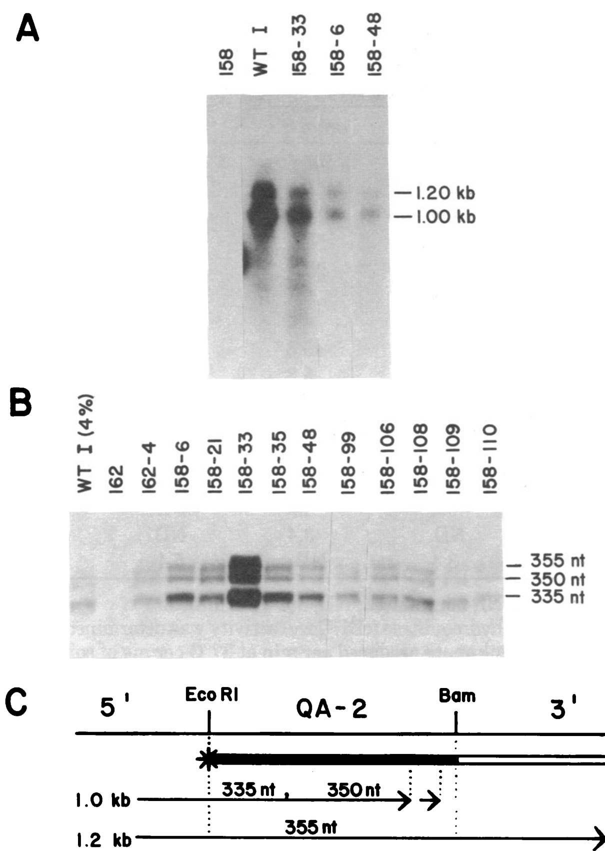

FIG. 1. *qa-2* mRNA in the activator-independent mutants. (*A*) Blot analysis of *qa-2* mRNA in three mutants. Fifteen micrograms of poly(A)$^+$ RNA from the strains indicated was loaded in each track. WT I, wild type induced. The probe DNA was a plasmid containing the *qa-2* gene. kb, Kilobases. (*B*) Quantitation of *qa-2* mRNA in the mutants by S1 nuclease digestion of RNA·DNA hybrids. The end-labeled probe fragment shown in *C* was hybridized to 25 μg of poly(A)$^+$ RNA from each mutant or 1 μg (= 4% of 25 μg) of induced wild-type poly(A)$^+$ RNA. The hybrids were digested with S1 nuclease and then resolved by electrophoresis in a denaturing 6% acrylamide gel. nt, Nucleotides. (*C*) Strategy for assay of *qa-2* mRNA. The asterisk indicates the site of 3' end-labeling of the probe fragment. The filled portion of the probe fragment represents *qa-2* DNA, and the open portion, plasmid DNA. The two *qa-2* mRNA species (see *A*) are indicated by 1.0 and 1.2 kb.

1.2-kb *qa-2* mRNA and corresponds to the junction between *Neurospora* and plasmid sequences. All of the mutants have significantly increased levels of *qa-2* mRNA.

The total amounts of DNA probe protected by each RNA preparation were quantitated by comparison to small known amounts of undigested probe DNA, using densitometry of the autoradiograms. The relative amounts of *qa-2* mRNA in each mutant are given in the last two columns of Table 1. There is a strong correlation between the relative levels of *qa-2* enzyme and mRNA in each mutant, indicating that the effect of each of the mutations is primarily at the transcriptional level.

Characterization of Activator-Independent Mutants on Southern Blots. Previous studies have described the cloning of the entire *qa* gene cluster and the construction of a detailed restriction enzyme map of this region (9). Presumptive *qa-2* activator-independent mutants showing heat-stable catabolic dehydroquinase activity were examined further for variation in restriction enzyme sites on Southern blots. Ten of 12 mutants showed detectable changes. In each case the altered region was associated with a restriction fragment containing the *qa-2* gene.

The results from an experiment in which the DNAs were digested with *Bgl* II are presented in Fig. 2*A*. Fig. 2*B* shows

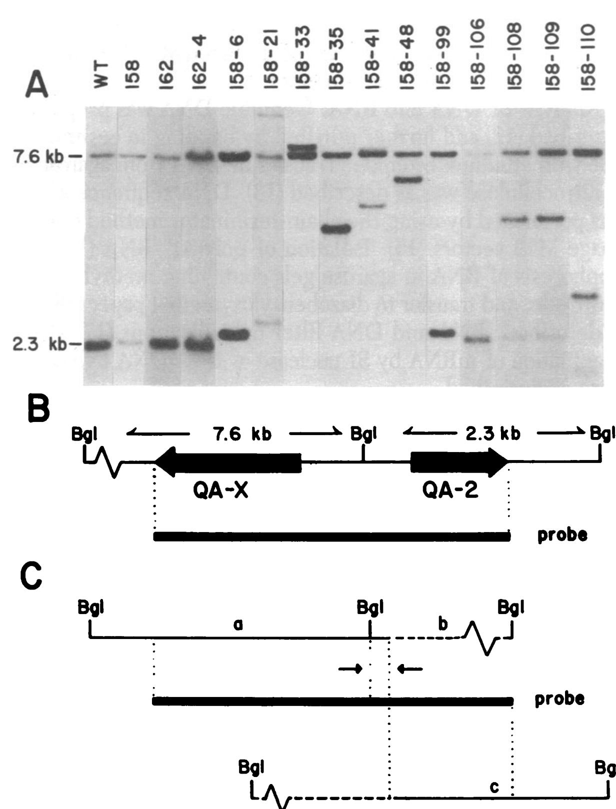

FIG. 2. Analysis of activator-independent mutations on Southern blots. (*A*) Genomic DNA isolated from the indicated mutants was digested with *Bgl* II, electrophoresed in 0.8% agarose gels, transferred to nitrocellulose, and hybridized with the ^{32}P-labeled DNA fragment diagrammed in *B*. WT, wild type. (*C*) An illustration of the types of *Bgl* II fragments (designated a, b, and c) generated by hybridization of the *qa-x*/*qa-2* probe in DNA rearrangement mutations. Continuous lines represent the *qa* region and broken lines represent the sequences introduced by the DNA rearrangement. Arrows indicate the region of variable homology with the probe, the extent of which depends on the location of the breakage point in each mutation.

the normal location of the *qa-x* and *qa-2* genes on adjacent 7.6- and 2.3-kb *Bgl* II fragments, respectively (10). A ^{32}P-labeled probe that spans the central *Bgl* II site was used to visualize the bands shown in Fig. 2*A*. Mutants 162-4 and 158-106 appear identical to strains 158, 162, and wild type, whereas the 2.3-kb *Bgl* II band has been slightly altered in mutants 158-6 and 158-99, suggesting the presence of an additional DNA sequence. The eight remaining mutants show considerable variation caused by a variety of DNA rearrangements that occur between the *Bgl* II cleavage site and probably before the *qa-2* coding region. The close proximity of the breakage points to the *Bgl* II site results in the appearance of an additional, less intense band (see Fig. 2*C* for diagram). The nature of the DNA rearrangements is not clear. Because the adjacent 7.6-kb band remains unaltered by these mutations, the DNA rearrangements cannot be due to deletions. Moreover, restriction maps constructed from other digests and Southern blots indicated that if any of the mutants resulted from a simple DNA insertion, the minimal size of such insertions would have to be between 10 and 20 kb, depending on the mutant. Further comparison of these restriction maps among the eight mutants shows that 158-108 and 158-109 appear to involve the same DNA rearrangement, while the other mutants all exhibit different rearrangements (Fig. 2*A*). Additional variations in Southern blots attributable to *qa-2* activator-independent mutations were not detected anywhere else in the *qa* gene cluster.

Cloning of the *qa-2* Gene from Mutant Strains. To investigate the nature of these mutations further, one rearrangement mutant (158-33) and the four mutants that showed little or no alteration (158-6, 158-99, 158-106, and 162-4) were cloned. The choice of restriction enzymes used for cloning the *qa-2* gene was determined by constructing restriction maps from Southern blots of mutant *N. crassa* DNA and knowing the location of these sites in wild-type (74A) *N. crassa* DNA. Generally, genomic DNA was digested with two restriction enzymes and ligated into compatible sites in pBR325. For example, DNA from strain 158-33 was digested with *Hin*dIII/*Xho* I and ligated into *Hin*dIII/*Sal* I sites of pBR325, which had also been digested with *Sph* I to minimize self-ligation. The selection for recombinant clones requires the expression of the *Neurospora qa-2* gene in *E. coli* (see *Materials and Methods*). Clones of 158-33 contained the *qa-2* gene and flanking sequences on a 2.2-kb insert as predicted from the mapping data.

Sequence Determination of Activator-Independent Mutants. The DNA sequence of the upstream region of the *qa-2* gene from the *Bgl* II site to the ATG initiation codon is presented in Fig. 3. The sequence is that of wild-type (74A) *N. crassa* and includes some differences from the previously reported sequence, which was determined from the *qa-1S* constitutive strain 105C (11). In addition, the DNA sequences of the two progenitor strains 158 and 162 have been determined. Both are identical to the wild-type sequence except for a single A·T to G·C transition at nucleotide residue −343. This transition creates a *Dde* I restriction site and has been traced to the original *arom-9*⁻ isolate M6-11 from which the progenitor strains 162 and 158 were derived. All descendants of M6-11 that have been examined contain this sequence.

DNA sequences 5′ to the *qa-2* gene were determined from the five *qa-2* activator-independent mutants cloned. The following mutations were observed (see Fig. 3). (*i*) Mutation 158-99 is a duplication of the sequence between residues −105 and −190 producing a direct repeat of 84 base pairs (bp). (*ii*) Mutation 158-6 has a similar duplication between residues −107 and −176, producing a direct repeat of 68 bp. (*iii*) Mutation 162-4 shows a substitution in sequence between residues −88 and −98 that results in a net 2-bp deletion. (*iv*) Mutation 158-33 is caused by a DNA rearrangement that begins at residue −378. Southern blot analysis has revealed that the first 400 bp of this rearranged sequence now adjacent to the *qa-2* gene are unique to the *Neurospora* genome (data not shown). (*v*) Mutation 158-106 has a C·G to A·T transversion at −200.

DISCUSSION

Expression of the *qa-2* gene of *N. crassa* normally requires the presence of a functional activator protein, encoded by *qa-1F*. Twelve mutations in the *qa-2* gene have been identified that allow partial expression of catabolic dehydroquinase (the *qa-2* gene product) in the absence of the functional activator protein. The relative increase in catabolic dehydroquinase enzyme activity correlates in each case with a proportional increase in *qa-2* mRNA transcription. All 12 mutations have been characterized by Southern blots and 5 have been cloned and sequenced. Eight of the 12 consist of large DNA rearrangements in the 5′ region of the *qa-2* gene. Except for mutations 158-108 and 158-109, which appear to be identical, each rearrangement represents a distinct mutational event involving a different DNA sequence. It is not known whether any of the rearrangements contain transposable elements (such elements have not yet been described in *Neurospora*).

One rearrangement mutation (158-33) shows very high levels of *qa-2* expression (45% of wild type) and has been investigated further by cloning and sequencing. This mutation is caused by the fusion of a unique sequence of *Neurospora* DNA at position −378 with respect to the normal transcription-initiation site. The 158-33 mutation does not appear to have joined a new pro-

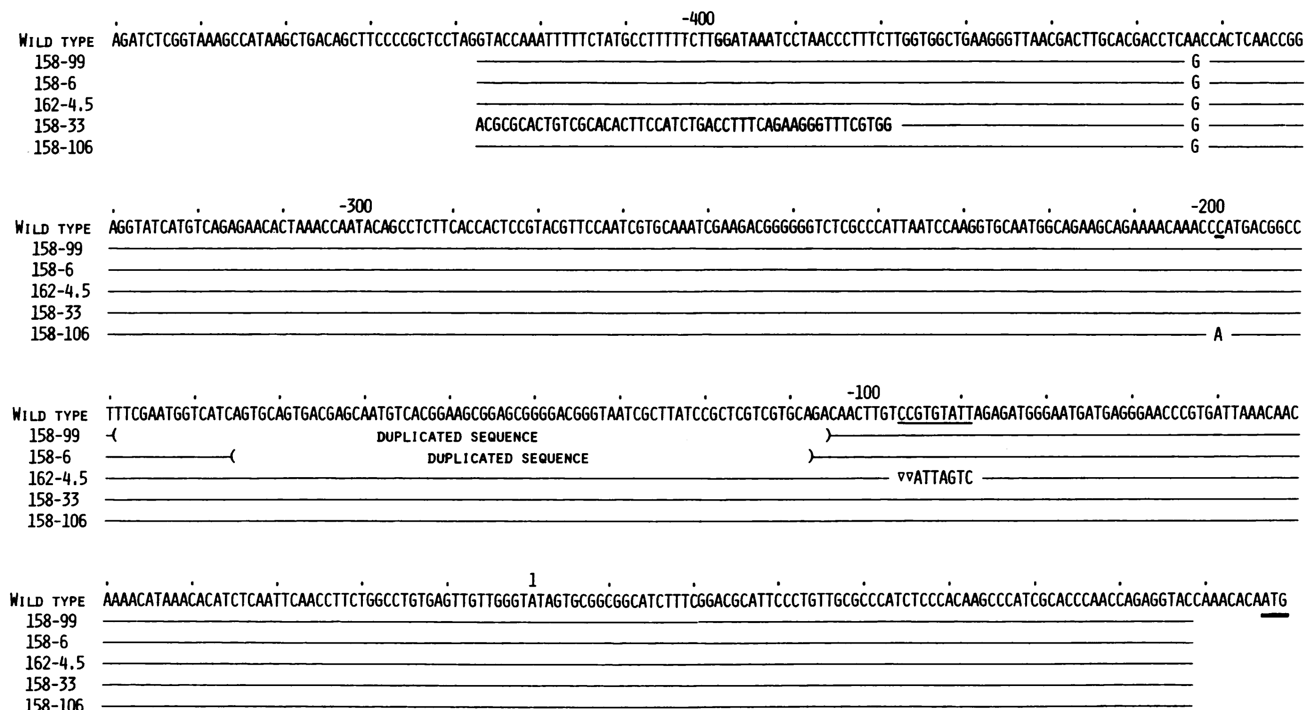

FIG. 3. DNA sequence 5′ to the *qa-2* gene and the location of the transcriptional mutations. The sequence represents that of wild type (74A). The ATG initiation codon for *qa-2* is heavily underlined. The regions sequenced in each mutant are indicated by the horizontal lines. Nucleotides in the wild-type sequence that have been altered in 158-106 and 162-4 are underlined. The A to G transition originates from strain M6-11 and is present in all mutant strains derived from it.

moter to *qa-2*, since in such a case transcription should start near the new promoter approximately 400 bp before the normal site of transcription initiation. However, *qa-2* mRNA in 158-33 is the same size as in wild type (Fig. 1A) and is not initiated within the new DNA sequence (unpublished data). This is comparable to the *CYP3-15* mutation described in yeast, in which a unique yeast DNA sequence has been fused 285 bp before the initiation codon of the *CYC7* gene, causing an overproduction of iso-2 cytochrome *c* while maintaining the wild-type transcription-initiation site (21). It is also unlikely that the 158-33 rearrangement alters a RNA polymerase II recognition sequence, since in other eukaryotic genes (22–24) such promoter sequences are confined to regions more proximal (within 100 bp) to transcription initiation. In this respect, regulation of the *qa-2* gene differs from analogous prokaryotic systems, such as *lac* and *ara*, where genetically comparable activator-protein-independent transcription mutations alter the promoter to enhance RNA polymerase recognition and binding (5, 6). The 158-33 mutation appears to increase *qa-2* transcription by an alternative mechanism. The simplest explanation is that the 158-33 mutation has introduced a so-called upstream activator sequence, which functions from a distance to increase basal levels of *qa-2* transcription. Similar upstream activator sequences appear to be present in the normal *his3* (23), *cycl* (25), and *gal10* (24) genes of yeast and the herpesvirus *tk* gene (22). Whether all of the large *qa-2* rearrangement mutations act by this mechanism is unclear. Additional evidence from Southern blots and nuclease S1 transcriptional mapping suggests that some of the other DNA rearrangements occur within close proximity to the transcription-initiation site and may involve promoter fusions (unpublished data).

In contrast to 158-33, 162-4 may alter RNA polymerase binding, since *qa-2* transcription in this mutant initiates only 45 nucleotides downstream from the site of the mutation (unpublished data).

Mutations 158-6 and 158-99 consist of tandem duplications of 68 bp and 84 bp, respectively, at the same location 5′ to the *qa-2* gene. In this respect they are reminiscent of the 72-bp repeat of the simian virus 40 enhancer (26). Although there is little homology within the duplicated regions to the conserved enhancer core sequences found in various animal viruses (including simian virus 40) and human cells (26), a characteristic property of enhancers is that they increase transcription from a distance, and the two duplication mutations as well as the point mutation 158-106 all alter the DNA sequence at positions upstream of nucleotide −176. If these mutations are acting as enhancers, then presumably they mediate *qa-2* transcription independently of *qa-1F* by increasing (enhancing) the normally very low level of *qa-2* transcription present in the *qa-1F*$^-$ progenitor strains. It is interesting to note that the "point mutations" (158–106 and 162-4) closely flank the region duplicated in 158-6 and 158-99. This suggests an alternative explanation, which is that these four transcriptional mutations define a region of *qa-2* regulation at the level of RNA polymerase II access. In the absence of the inducer, quinic acid, or of a functional activator protein, this upstream region would be inaccessible to RNA polymerase II entry. The transcriptional mutations would serve to destabilize the chromatin structure in this region, either creating a new entry site or rendering the existing site *qa-1F* independent. Whether or not these transcriptional mutants create enhancer-like sequences or cause a localized destabilization of chromatin (or both), it is clear that the mechanism of positive regulation in this eukaryotic system differs substantially from that in prokaryotes.

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