The *Drosophila melanogaster ade5* Gene Encodes a Bifunctional Enzyme for Two Steps in the *de novo* Purine Synthesis Pathway

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ABSTRACT

Steps 6 and 7 of *de novo* purine synthesis are performed by 5-aminoimidazole ribonucleotide carboxylase (AIRc) and 4-[(*N*-succinylamino)carbonyl]-5-aminoimidazole ribonucleotide synthetase (SAICARs), respectively. In vertebrates, a single gene encodes AIRc-SAICARs with domains homologous to *Escherichia coli* PurE and PurC. We have isolated an AIRc-SAICARs cDNA from *Drosophila melanogaster* via functional complementation with an *E. coli purC* purine auxotroph. This cDNA encodes AIRc yet is unable to complement an *E. coli purE* mutant, suggesting functional differences between Drosophila and *E. coli* AIRc. In vertebrates, the AIRc-SAICARs gene shares a promoter region with the gene encoding phosphoribosylamidotransferase, which performs the first step in *de novo* purine synthesis. In Drosophila, the AIRc-SAICARs gene maps to section 11B4-14 of the X chromosome, while the phosphoribosylamidotransferase gene (*Prat*) maps to chromosome 3; thus, the close linkage of these two genes is not conserved in flies. Three EMS-induced X-linked adenine auxotrophic mutations, *ade4*^{1}, *ade5*^{1}, and *ade5*^{2}, were isolated. Two gamma-radiation-induced (*ade5*^{3} and *ade5*^{4}) and three hybrid dysgenesis-induced (*ade5*^{5}, *ade5*^{6}, and *ade5*^{8}) alleles were also isolated. Characterization of the auxotrophy and the finding that the hybrid dysgenesis-induced mutations all harbor P transposon sequences within the AIRc-SAICARs gene show that *ade5* encodes AIRc-SAICARs.

THE *de novo* purine synthesis pathway involves 10 enzymatic steps for the synthesis of inosine monophosphate (IMP), followed by one of two paths to produce either adenosine monophosphate (AMP) or guanosine monophosphate (GMP). In bacteria, the *de novo* purine synthesis enzymes are encoded by separate genes, while their eukaryotic counterparts are often encoded by genes for multifunctional polypeptides (Henikoff 1987; Zalkin and Dixon 1992). One example of such variation in gene organization is found with the enzymes operating at steps 6 and 7 in the *de novo* synthesis of purines (Figure 1), 5-aminoimidazole ribonucleotide carboxylase (AIRc) and 4-[(*N*-succinylamino)-carbonyl]-5-aminoimidazole ribonucleotide synthetase (SAICARs). Three types of organization for these two enzymes can be found in bacteria, yeasts, and vertebrates. First, *Escherichia coli* AIR carboxylase and SAICAR synthetase are encoded in the operons *purEK* and *purC*, respectively (Tiedeman *et al.* 1989, 1990; Watanabe *et al.* 1989). Here, AIR carboxylase function requires both PurE and PurK, where PurK is required to assist PurE in the formation of the 5′-phosphoribosyl-5-aminoimidazole carboxylate (CAIR) product (see below) (Mueller *et al.* 1994). *Bacillus subtilis* has a single *pur* operon containing 12 cistrons, including *purE*, *purK*, and *purC*, which encode the three proteins as found in *E. coli* (Ebbole and Zalkin 1987). The second type of organization has the PurE and PurK AIR carboxylase functions fused. In the yeast *Saccharomyces cerevisiae*, the fused PurK-PurE-homologous sequences are encoded by the *ADE2* gene (Stotz and Linder 1990), with ~23 linker amino acids between the two domains (Zalkin and Dixon 1992). Yeast SAICAR synthetase is encoded by the *ADE1* gene (Myasnikov *et al.* 1991). The fusion of PurK and PurE domains is also found for the *ADE6* gene of fission yeast *Schizosaccharomyces pombe* (Szankasi *et al.* 1988). The third type of organization has a SAICAR synthetase domain fused with an AIR carboxylase domain to form a bifunctional enzyme encoded at a single locus (AIRc-SAICARs). This arrangement has been found for chickens (*Gallus gallus*; Gavalas *et al.* 1993), humans (Minet and Lacroute 1990), and rats (Iwahana *et al.* 1995). Interestingly, in these cases the AIR carboxylase domain lacks a PurK-homologous region (Zalkin and Dixon 1992). The nematode *Caenorhabditis elegans* also appears to have a locus encoding a bifunctional enzyme as found in the vertebrates (Genome Sequencing Center, personal communication; GenBank accession no. U39848).

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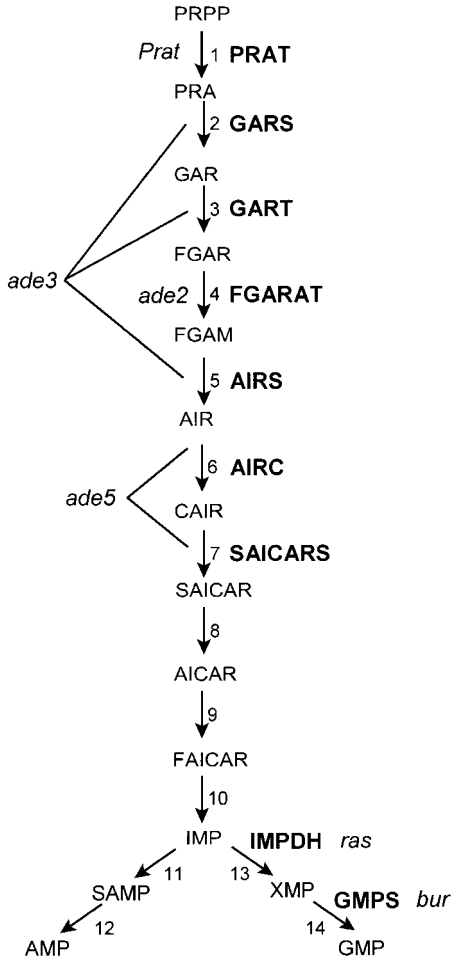

Figure 1.—Pathway for *de novo* purine synthesis. Names of Drosophila genes known to correspond to pathway enzymes are shown in italics. Pathway intermediate abbreviations are: PRPP, 5′-phosphoribosyl-1-pyrophosphate; PRA, 5′-phosphoribosyl-1-amine; GAR, 5-phosphoribosylglycinamide; FGAR, 5′-phosphoribosyl-*N*-formylglycinamide; FGAM, 5′-phosphoribosyl-*N*-formylglycinamidine; AIR, 5′-phosphoribosyl-5-aminoimidazole; CAIR, 5′-phosphoribosyl-5-aminoimidazole carboxylate; SAICAR, 5′-phosphoribosyl-4-(*N*-succinocarboxamide)-5-aminoimidazole; AICAR, 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole; FAICAR, 5′-phosphoribosyl-4-carboxamide-5-formamidoimidazole; IMP, inosine monophosphate; SAMP, adenylosuccinate; XMP, xanthosine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate. Pathway enzymes are: (1) PRAT, phosphoribosylamidotransferase; (2) GARS, GAR synthetase; (3) GART, GAR transformylase; (4) FGARAT, FGAR amidotransferase; (5) AIRS, AIR synthetase; (6) AIRC, AIR carboxylase; (7) SAICARS, SAICAR synthetase; (8) and (12) adenylosuccinate lyase; (9) AICAR transformylase; (10) inosinate cyclohydrolase; (11) SAMP synthetase; (13) IMPDH, IMP dehydrogenase; (14) GMPS, GMP synthetase. Figure after Henikoff (1987).

Along with these three alternate forms of gene organization, there is variation in the mechanism employed by AIR carboxylase (Figure 2). In chickens, the conversion from AIR to CAIR proceeds in one step directly from substrate to product with the use of the AIR carboxylase (PurE-like) domain (Firestine and Davisson 1994). In *E. coli*, the conversion from AIR to CAIR proceeds in two steps via an unstable intermediate called N^5-CAIR (N^5-carboxyaminoimidazole ribonucleotide). This intermediate is the result of the conversion of AIR by PurK in the presence of a bicarbonate ion fueled by the hydrolysis of ATP. The N^5-CAIR is then converted to CAIR by PurE (Firestine *et al.* 1994; Mueller *et al.* 1994). The PurK and PurE functions have fused into a single polypeptide in fungi, although it appears that they also have the bacterial two-step mechanism (Firestine *et al.* 1998). Thus, there are two AIR carboxylase mechanisms with different substrate, energy, and cofactor requirements, and the *E. coli* and fungal *de novo* purine biosynthesis pathways are one step longer than for vertebrates.

With this difference in catalytic mechanism, the question arises as to whether different AIR carboxylases are interchangeable *in vivo*. In some cases this was shown to be true; a chicken cDNA and a methanobacterial gene were cloned by functional complementation of an *E. coli purK* mutant, yet their sequences have PurE- but no PurK-homologous sequences (Hamilton and Reeve 1985; Chen *et al.* 1990). These same sequences can also complement *E. coli purE* mutants. A possible explanation for why the chicken and methanobacterial AIR carboxylases can complement a *purK* mutant is that, rather than providing a functional version of PurK, they provide a completely different route for the production of CAIR. On the other hand, a human AIR carboxylase cDNA, cloned by functional complementation of a *S. cerevisiae ade2* mutant defective in its PurE domain (Minet and Lacroute 1990; Schild *et al.* 1990), was unable to complement a *S. cerevisiae ade2* mutant defective in its PurK domain (Minet and Lacroute 1990). The divergence in AIR carboxylase function has been speculated to be a reflection of the level of intracellular CO_2 in the organism, where those organisms lacking the PurK function have higher CO_2 levels (Firestine *et al.* 1998).

The existence of multifunctional enzymes for *de novo* purine synthesis in eukaryotes may serve two related functions. First, the proximity of two enzyme domains in a single polypeptide may allow for substrate channeling and, second, enzymes can be expressed stoichiometrically (Henikoff 1987). An extension of this idea is the prediction that expression of different purine genes is somehow coordinated. Coordinated expression of purine genes has been found in microbial organisms. Extensive analysis of purine gene regulation in *E. coli*, *B. subtilis*, and *S. cerevisiae* has resulted in the identification of mechanisms that coordinate transcription in response to cellular concentrations of purines. In *E. coli*, transcriptional regulation is carried out by the PurR repressor, which represses expression of seven *pur* operons in response to increased purine concentrations (Zalkin and Dixon 1992). In *B. subtilis*, a PurR repressor controls expression of the single *pur* operon (Weng *et al.* 1995). In *S. cerevisiae*, purine starvation results in

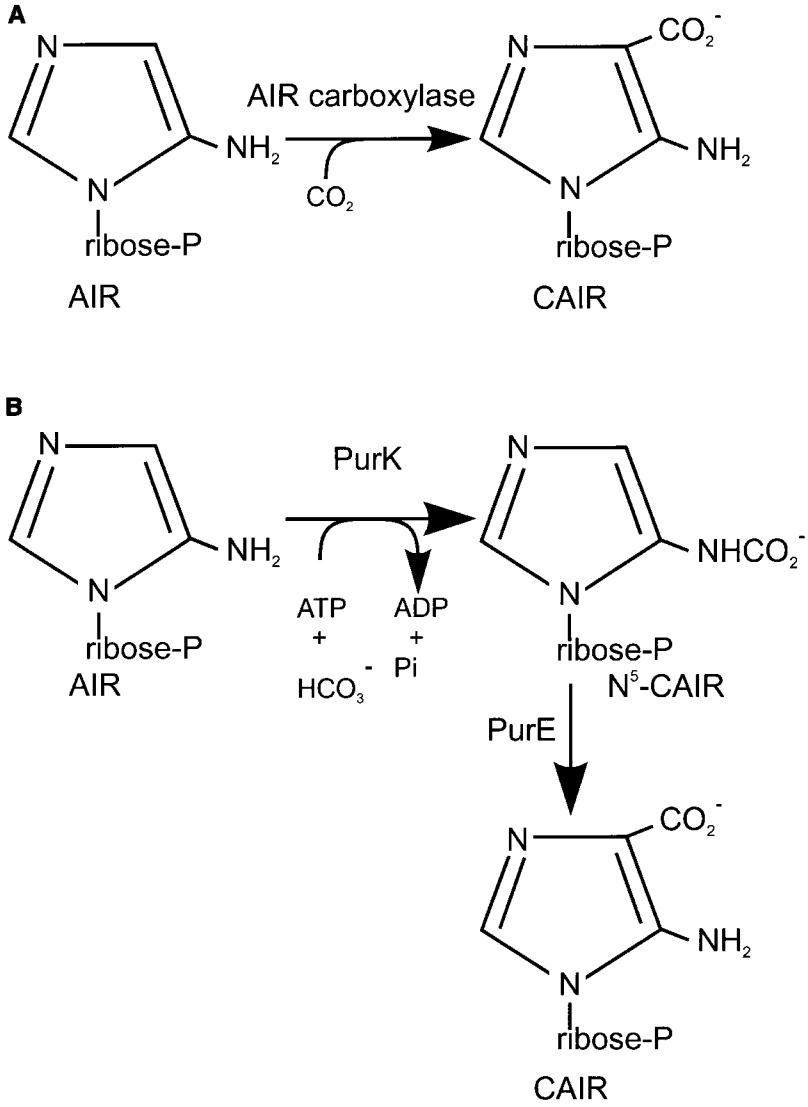

Figure 2.—Divergent catalytic mechanisms for AIR carboxylase. (A) Chicken mechanism. (B) *E. coli* mechanism (Firestine and Davisson 1994; Firestine *et al.* 1994, 1998). Abbreviations: AIR, 5′-phosphoribosylaminoimidazole; CAIR, 5′-phosphoribosyl-5-aminoimidazole carboxylate; SAICAR, 5′-phosphoribosyl 4-(*N*-succinocarboxamide)-5-aminoimidazole; N^5-CAIR, N^5-carboxyaminoimidazole ribonucleotide, ribose-P, 5′-phosphoribosyl moiety. Figure after Firestine and Davisson (1994).

the transcriptional activation of several *ADE* genes by the transcription factors GCN4 (Rolfes and Hinnebusch 1993), BAS1, and BAS2 (Daignan-Fornier and Fink 1992; Denis *et al.* 1998).

There is little evidence for coordinated expression of purine genes in multicellular organisms; however, one case has been found for coordinated expression of the two genes encoding phosphoribosylamidotransferase (PRAT; Figure 1) and AIRc-SAICARs in vertebrates. PRAT performs the first committed step in *de novo* purine synthesis and is considered a rate-limiting point in the pathway (Wyngaarden and Kelley 1983). These two genes are divergently transcribed from common promoter regions, ranging from 230 to 625 bases, in chickens, rats, and humans (Gavalas *et al.* 1993; Brayton *et al.* 1994; Iwahana *et al.* 1995). The use of bidirectional promoters occurs at other loci in eukaryotes, but this type of promoter is usually shared by evolutionarily conserved genes (Gavalas *et al.* 1993). This purine gene arrangement is the only known instance where such a bidirectional promoter has been found between two genes that function at different points in the same pathway. Very little is known about the mechanisms for transcriptional regulation of these two genes in eukaryotes. In a recent study of the human promoter region, binding sites for nuclear respiratory factor-1 (NRF-1) and Sp1 transcription factors were identified and shown to be required in part for transcription of both genes *in vitro* (Chen *et al.* 1997). However, these two transcription factors are not sufficient to explain the regulation of these genes and further studies are required to confirm their activities *in vivo.* While there are still many questions to be answered regarding the nature of the PRAT-AIRc-SAICARs bidirectional promoter, the unique nature of this structural feature and involvement of the rate-limiting enzyme for *de novo* synthesis of purines makes this a very intriguing point in the genetic regulation of the pathway.

Here we describe the isolation of a *D. melanogaster* AIRc-SAICARs cDNA through functional complementa-

tion of an *E. coli* purine auxotroph. This cDNA rescued an *E. coli purC* (SAICAR synthetase) mutant but was unable to rescue a *purE* mutant even though, as determined by sequence alignments, it contains both SAICAR synthetase and AIR carboxylase domains as found in vertebrates. This raises interesting questions about the mechanism of AIR carboxylase action in Drosophila in comparison to the chicken and bacterial mechanisms.

We have also determined the structure of the AIRc-SAICARs gene (*ade5*) from Drosophila and have identified potential sites for its transcriptional regulation. In addition, we show that the close linkage between the PRAT and AIRc-SAICARs genes found in vertebrates is not conserved in Drosophila. To learn more about the function of the *ade5* gene in Drosophila and to identify sequences important for its expression, we have generated several purine auxotrophic and lethal alleles of this gene using EMS, X rays, and *P*-element mutagenesis and have linked a subset of these mutations with structural defects in the gene.

MATERIALS AND METHODS

***E. coli* functional complementation screen:** NK6051 (Δ[*gpt-lac*]5 *purEK79*::Tn*10* λ-*relA1 spoT1 thi-1*) and NK6056 (Δ[*gpt-lac*]5 *purC80*::Tn*10* λ-*relA1 spoT1 thi-1*) were obtained from the *E. Coli* Genetic Stock Center. These purine auxotrophs were grown on minimal media, described by Vogel and Bonner (1956) as Medium E, supplemented with 0.5% glucose, 0.1% acid hydrolyzed casein, 0.2 μg/ml thiamin, 125 μg/ml tetracycline, and 100 μg/ml adenine. For functional complementation tests of Drosophila cDNAs, nonpermissive growth conditions were employed by omitting the adenine supplement.

Two *D. melanogaster* 0- to 24-hr embryonic cDNA libraries were generously supplied by Carl Thummel. One library was constructed using random-primed cDNA synthesis while the other was oligo(dT) primed. Both were housed in Stratagene's λZAPII expression vector, where cDNAs were directionally inserted, with the *Eco*RI restriction site at the 5′ end and the *Xho*I site at the 3′ end of the pBluescript SK plasmid (Carl Thummel, personal communication).

The λZAPII screening method was modified to facilitate the functional complementation screen being performed. The *in vivo* excision protocol (Short *et al.* 1988) was followed until the cDNAs had excised to yield Drosophila libraries in the pBluescript phagemid packaged in filamentous phage particles. The particles were then concentrated via polyethylene glycol (PEG) precipitation (Sambrook *et al.* 1989) from 300 CFU/ml to 2×10^5 CFU/ml. The titer of the phagemid library stocks was determined using a number of hosts: SOLR [*e14*$^-$ (*mcrA*), Δ(*mcrCB-hsdSMR-mrr*) 177, *sbcC, recB, recJ, umuC*::Tn*5* (*kan*^{R}), *uvrC, lac, gyrA96, relA1, thi-1, endA1*, λ^R, [*F′, proAB, lacI*^{q} *ZΔM15*] *Su*$^-$], XL1 Blue-MRF′ {*end A1, hsp R17, sup E44, thi-1, recA1, gyrA96, relA1, lac*, [*F′, proAB, lacI*^{q} *ZΔM15, Tn*10 (*tet*^{R})]}, NK6051, and NK6056; and plating on 100 μg/ml ampicillin containing media for phagemid selection.

The phagemid libraries were used to infect NK6051 and NK6056, in the absence of helper phage, directly followed by platings under nonpermissive conditions to test for complementation. An AIRc-SAICARs chicken cDNA (kindly supplied by Howard Zalkin; Chen *et al.* 1990) was used as a positive control for this procedure.

Genomic library screening, subcloning, and sequence analysis: Genomic λ phage library screening, λ DNA isolation, restriction digests, and subcloning followed standard protocols (Sambrook *et al.* 1989). The *D. melanogaster* genomic library (Maniatis *et al.* 1978) was obtained from the American Type Culture Collection. DNA fragment subcloning used the plasmid vector pVZ1 (Henikoff and Eghtedarzadeh 1987). Purified plasmid DNA was obtained using QIAGEN (Valencia, CA) plasmid preparation kits. DH5α (*F*$^-$, *endA1, hsdR17* (r_k^-, m_k^+), *supE44, thi-1, recA1, gyrA96, relA1*, Δ(*argF-laczya*)*U169*, ϕ*80dlacZΔM15*, λ$^-$) cells were made competent by standard calcium chloride method and used for transformations (Sambrook *et al.* 1989).

Hybridization probes were labeled using the random primer fluorescein-12-dUTP labeling kit (DuPont NEN) and detection followed the nucleic acid chemiluminescence reagent kit (DuPont NEN) protocol.

DNA sequencing was performed using the ABI 310 prism genetic analyzer (PE Applied Biosystems, Foster City, CA) with dideoxynucleotide triphosphate terminator sequencing chemistry (Sanger *et al.* 1977). In this particular method the dRhodamine terminator cycle sequencing ready reaction DNA sequencing kit (PE Applied Biosystems) was employed using the manufacturer's protocol, except that the suggested reaction volumes were halved. To facilitate the sequencing of the large genomic subclones, 5.5EL(d) and 5.5EL(r), Exonuclease III nested deletions were generated (Clark and Henikoff 1994). SeqEd software (PE Applied Biosystems) was used to edit, align, and produce consensus sequences from raw sequence data. Sequence alignments were performed using MULTALIN software with default parameters (Corpet 1988).

***In situ* hybridization to polytene chromosomes:** Canton-S third instar larval salivary gland polytene chromosomes were hybridized (Ashburner 1989) with a probe made using the 12.6-kb insert from the LibD genomic clone as a template. The probe was labeled with biotin-conjugated dUTP [Life Technologies (Rockville, MD) Bioprime kit], and biotin detection followed the procedure described by Ashburner (1989) using a Detek-1-hrp kit (Enzo Diagnostics, Farmingdale, NY).

RNA methods: Total RNA was extracted from ~1.5 g of frozen adult flies of various genotypes using TRIzol reagent (Life Technologies). RNA yields were assessed using ethidium bromide staining in agarose gels and optical density measurements from GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Piscataway, NJ). Polyadenylated RNA [poly(A)-RNA] was purified from 0.5 mg total RNA using the poly(AT) tract kit (Promega, Madison, WI).

For Northern blots, 2–5 μg of poly(A)-RNA was fractionated on a 1% agarose, 0.66 m formaldehyde gel (Sambrook *et al.* 1989) and transferred to GeneScreen Plus positively charged nylon membrane (Dupont NEN).

For primer extension, the AIRc-SAICARsPE primer was 5′-GGTTGTGGTGGTGGACATCTCGTTAATCGC-3′. This primer was radiolabeled by 5′ phosphorylation with [γ-^{32}P]ATP using the primer extension kit protocol (Promega). The primer extension reaction was performed using 10 μg of total RNA from adult Canton-S flies and 100 fmol of the ^{32}P-labeled AIRc-SAICARsPE primer. Size markers were a ^{32}P-labeled ϕX174 ladder standard (Promega) and a ^{32}P-labeled sequencing ladder made using the AIRc-SAICARsPE primer and p5.5EL(d) plasmid (see results) as template according to the Silver Sequencing protocol (Promega).

Drosophila strains and mutant screens: Unless otherwise indicated, flies were cultured at 25° on "standard medium," which was Instant Drosophila medium (Carolina Biological) supplemented with Fleischmann's yeast. Canton-S was used as the wild-type strain wherever needed. Genotypes of strains carrying EMS-induced *ade4* or *ade5* mutations were *y cv v ade4*^{1}

f/In(1)FM6, y^{31d} sc^{8} dmB, y cv v $ade5^{1}$ f/In(1)FM6, y^{31d} sc^{8} dmB, y cv v $ade5^{2(7)}$ f/In(1)FM6, y^{31d} sc^{8} dmB, y cv v $ade5^{2(25)}$ f/In(1)FM6, y^{31d} sc^{8} dmB, and *y cv v H23-14(44)f/In(1)FM6, y^{31d} sc^{8} dmB.* Strains carrying gamma-radiation-induced mutations were *$ade5^{3}$/In(1)FM6, y^{31d} sc^{8} dmB* and *$ade5^{4}$/In(1)FM6, y^{31d} sc^{8} dmB.* Strains carrying hybrid dysgenesis-induced mutations were *v $ade5^{5}$/In(1)FM6, y^{31d} sc^{8} dmB, v $ade5^{6}$/In(1)FM6, y^{31d} sc^{8} dmB,* and *v $ade5^{8}$/In(1)FM6, y^{31d} sc^{8}.*

For EMS-induced mutations, isolation of X chromosome-linked mutations was as described previously (Nash and Janca 1983; Janca *et al.* 1986). This screen was designed to locate lethal mutations on the X chromosome in the 9E1-10A11 region. At the same time, all strains carrying nonlethal mutagenized X chromosomes (*y cv v f/FM6*) were screened for a reduction in red eye pigmentation, and these were then tested for auxotrophy on Sang's defined medium (Sang 1956) under axenic culture conditions. From this screen, three mutants that responded to supplementation of the medium with adenosine were recovered (*$ade4^{1}$*, *$ade5^{1}$*, and *$ade5^{2}$*). For nutritional supplementation experiments on *$ade4^{1}$* and *$ade5^{1}$*, five *ade/FM6* females and three to five hemizygous *ade* mutant males were mated on a yeast-sugar medium (Nash and Bell 1968) for 3 days and then transferred to a defined medium for 5 days. Additives to Sang's medium (Sang 1956) were all at 3.2 mm except RNA, which was at 4 mg/ml or 8 mg/ml (Table 1).

Gamma-ray mutagenesis screens were performed to isolate noncomplementing alleles of *$ade5^{1}$* and, in doing so, *$ade5^{3}$* (C. Sherf and D. Nash, unpublished results) and *$ade5^{4}$* (S. Tiong, unpublished results) were produced.

A P-M hybrid dysgenesis screen was carried out to isolate *P*-element insertion-induced alleles of *$ade5^{1}$*. In this screen, P strain π2 males, carrying many *P* elements, were crossed with females of an isogenic *vermilion* (*v*) M strain. The *v* dysgenic male progeny, with germ-line *P*-element mobilization, were mated with *y cv v $ade5^{1}$ f/FM6* females. The females from this cross were screened for mutations that failed to complement *$ade5^{1}$*. Failure of complementation was scored on the basis of "purine syndrome" characteristics (Tiong and Nash 1990), such as reduced red eye pigmentation and wing defects. Thus, these females had the genotype *v*/y cv v $ade5^{1}$ f* (where * represents the *P*-element-induced mutation). For each line, the mutagenized X chromosome was balanced by crossing with *FM6*-bearing males. Females of genotype *v*/FM6* were then mated with *FM6* males again to obtain a stock isogenic for the X chromosome. For each mutant, two healthy and stable lines were retained and the *v** X chromosomes were then retested for failure to complement *$ade5^{1}$*. Three independently isolated alleles of *ade5* were isolated from this screen: *$ade5^{5}$*, *$ade5^{6}$*, and *$ade5^{8}$*.

Viability and complementation tests: To determine the viability of each *ade* mutant strain on Instant Drosophila Medium (Carolina) supplemented with yeast, one of the following crosses was performed: (1) 5 *adeX* males × 30 *adeX/FM6* virgin females or (2) 5 *FM6* males × 30 *adeX/FM6* virgin females, where *X* represents 1 of the 10 *ade* mutations listed above. In the first cross, female progeny were scored on the basis of the dominant Bar-eyed marker on *FM6*, where the ratio of *adeX/adeX* non-Bar-eyed females to *adeX/FM6* Bar-eyed females provided a measure of viability for X chromosome homozygotes. In the second cross, male progeny were scored on the basis of Bar-eyed phenotype, where the ratio of *adeX* males to *FM6* males provided a measure of viability for X chromosome hemizygotes.

For complementation tests between *$ade4^{1}$* and various alleles of *ade5*, 10 *$ade4^{1}$* males and 30 *$ade5^{X}$/FM6* virgin females were crossed and cultured on standard media, where *$ade5^{X}$* refers to the *ade5* alleles 1, 3, 4, 5, 6, and 8. Progeny were scored based on the Bar-eyed marker.

Amplification of AIRc-SAICARs segments in *ade5* strains: PCR using the eLONGase amplification system (Promega) was used to amplify regions of the AIRc-SAICARs gene from genomic DNA isolated from the *P*-element-containing *$ade5^{5}$*, *$ade5^{6}$*, and *$ade5^{8}$* strains and the wild-type Canton-S strain. The AIRc-SAICARs gene primers were: 5′-ATGAAGTTGCCCAGC CCCAGAATGG-3′ (primer 4), 5′-CACTTGCGGGCGATGA AGGCCTTGG-3′ (primer 6), 5′-AGGAACAGCGGCGGATAG GACGC-3′ (primer 11), and 5′-TCGGCCATTTGGGAACT GCAGGG-3′ (primer 12). The position and orientation of these four primer sequences in the gene are indicated in Figure 4. The *P*-element inverted repeat (PIR) primer was 5′-CGACGGGACCACCTTATGTTATTTCATCATG-3′.

RESULTS

Isolation of a Drosophila AIRc-SAICARs cDNA by functional complementation of an *E. coli purC* mutant: Previously, a chicken cDNA had been isolated by complementation of *E. coli purC* and *purEK* mutants (Chen *et al.* 1990). It therefore seemed reasonable that this would be a route for isolation of a corresponding Drosophila cDNA. Two 0- to 24-hr embryonic cDNA expression libraries were used in the functional complementation screens. These libraries were made with the λZAP vector (Short *et al.* 1988). Typically, with this vector, the library would be excised, infected into a standard host strain, and subsequently purified as a double-stranded plasmid for further use in a functional complementation screen. In this case, the double-stranded plasmid would have been transformed into the *E. coli* purine auxotrophs to assess complementation. This procedure requires a number of time-consuming manipulations that could reduce library complexity. To circumvent some of these steps, the filamentous phage generated by *in vivo* excision from the library were concentrated via PEG precipitation and used to directly infect the *E. coli* purine auxotrophs.

Following infections of *purC* and *purEK* mutants, cells were plated on restrictive media lacking a purine source. As a positive control for this novel procedure, the pZD1 phagemid containing the chicken AIRc-SAICARs cDNA (Chen *et al.* 1990) was used to infect the same strains in parallel. We screened 2.8×10^5 oligo(dT)-primed and 2.8×10^5 random-primed cDNAs for rescue of *purEK*, and 1.5×10^4 oligo(dT)-primed and 3.5×10^4 random-primed cDNAs for rescue of *purC*. Of these, a single colony was isolated from the *purC* strain infected with the oligo(dT)-primed cDNAs after 96 hr of growth at 37°. This phagemid, named pAlly1, was further tested by transforming its double-stranded DNA form into both the *purC* strain and the *purEK* strain. Growth under nonpermissive conditions was observed for *purC*, confirming that pAlly1 is capable of complementing the purine auxotrophy of the *purC* strain. However, pAlly1 failed to rescue the *purEK* defect. Therefore, pAlly1 supplied *E. coli* with SAICAR synthetase function but could not rescue the *purEK* AIR carboxylase function.

The 1.8-kb cDNA sequence from pAlly1 (GenBank

TABLE 1

Nutritional supplementation of *ade4*[1] and *ade5*[1] auxotrophs

Mutation	Supplement[a]	% Survival[b]	Females		Males	
			ade/FM6	*ade/ade*	*FM6/Y*	*ade/Y*
ade4[1]	Adenine	42	36	15	0	19
	Adenosine	147	277	408	1	328
	AICA	96	28	27	0	10
	AICAR	65	85	55	0	22
	Cytidine	0	1298	0	0	0
	Guanine	0	412	0	0	0
	Guanosine[c]	89	89	79	1	62
		2	188	3	0	1
	Hypoxanthine	0	162	0	0	0
	None	0	1240	0	0	1
	RNA	26	1595	417	4	559
	RNA × 2	10	370	36	0	54
	Uridine	0	1189	1	0	0
	Yeast	67	2678	1782	1510	1648
ade5[1]	Adenine	307	29	89	0	59
	Adenosine	155	322	499	0	324
	AICA	119	84	100	0	57
	AICAR	162	100	162	0	91
	Cytidine	3	1238	32	0	23
	Guanine	0	301	1	0	2
	Guanosine[c]	71	145	103	0	100
		26	244	63	0	55
	Hypoxanthine	3	199	5	0	5
	RNA	43	2027	867	3	775
	RNA × 2	33	387	126	0	123
	None	10	1788	170	0	165
	Uridine	1	1114	12	0	7
	Yeast	54	2882	1566	1156	1798

[a] AICA, aminoimidazole carboxamide; AICAR, aminoimidazole carboxamide ribonucleotide; RNA, 4 mg/ml; RNA × 2, 8 mg/ml; yeast, yeast-sugar medium (Nash and Bell 1968).

[b] Percentage survival of *ade/ade* relative to *ade/FM6* female siblings is indicated. This measure was not calculated for males due to the inviability of *FM6*-bearing males on all but yeast-sugar medium.

[c] Since they were equivocal, the results for two trials with guanosine are entered on separate rows.

accession no. AF102579) was conceptually translated and aligned to AIRc-SAICARs sequences from other organisms (Figure 3). Despite its inability to complement the *purEK* mutation, it appears to contain the entire coding region for both AIR carboxylase and SAICAR synthetase. Overall, the Drosophila cDNA encodes a protein more closely related to the vertebrate sequences (57 and 58% amino acids identical with the human and chicken proteins, respectively) than those for *E. coli* or *S. cerevisiae* (25 and 23% amino acids identical, respectively). On the basis of the alignment, the fly cDNA appears to contain the entire coding region for both AIR carboxylase and SAICAR synthetase. The first 262 amino acids correspond to the SAICAR synthetase domain, which has 58 and 28% amino acids identical with the chicken domain and *E. coli* PurC, respectively. Amino acids 263–429 correspond to the AIR carboxylase domain, which has 58 and 23% amino acids identical with the chicken domain and *E. coli* purE, respectively. The two domains are defined here based on mutational and sequence alignment analyses of a chicken AIRc-SAICARs cDNA (Chen *et al.* 1990).

Isolation and structural characterization of the AIRc-SAICARs gene from Drosophila: The pAlly1 cDNA fragment was used as a probe to screen a *D. melanogaster* genomic DNA library (Maniatis *et al.* 1978) housed in the λ vector, Charon 4A. Approximately 3.6×10^5 λ phage were screened and, of these, one clone called LibD showed intense and reproducible hybridization when hybridization was carried out at high stringency. DNA isolated from the LibD clone was mapped using several restriction enzymes and the location of the hybridization signal was narrowed down to 5.5- and 1.3-kb *Eco*RI fragments (Figure 4A). The 5.5-kb *Eco*RI fragment from LibD was subcloned in both orientations into the plasmid vector pVZ1 (Henikoff and Eghtedarzadeh 1987) to produce p5.5EL(d) and p5.5EL(r). A series of nested deletions on both p5.5EL(d) and p5.5EL(r) was generated and the complete sequence for 4.69 kb of both strands was produced (GenBank accession no.

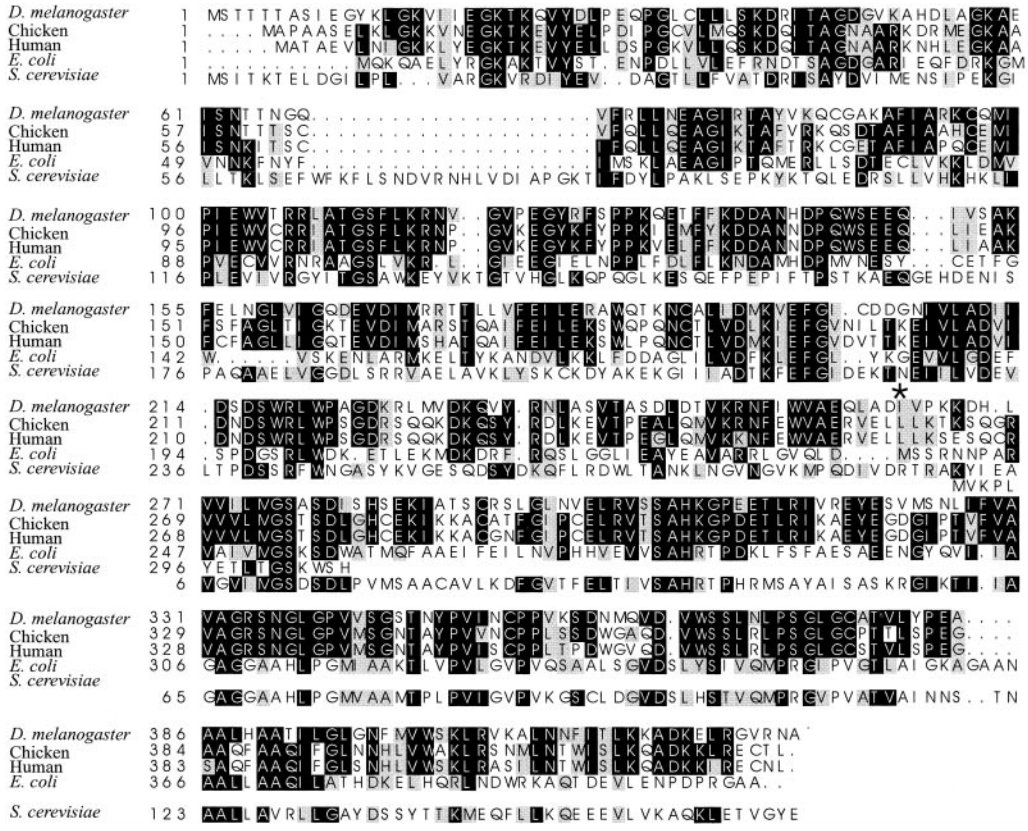

Figure 3.—Multiple sequence alignment of AIRc-SAICARs. Standard single-letter amino acid abbreviations were employed. Identical and conservative amino acids are boxed in black and gray, respectively. The approximate division between the SAICAR synthetase and the AIR carboxylase domains is indicated with an asterisk above the Drosophila sequence. The chicken and human sequences were derived from cDNAs (Chen *et al.* 1990; Minet and Lacroute 1990). It should be noted that the *E. coli* and *S. cerevisiae* sequences used in this alignment are not continuous, but were derived from two separate sequences. For *E. coli*, these were *purC* (Tiedeman *et al.* 1990) and *purE* (Tiedeman *et al.* 1989; Watanabe *et al.* 1989), respectively. For *S. cerevisiae*, these were *ADE1* (Myasnikov *et al.* 1991) and *ADE2* (Stotz and Linder 1990), respectively. The *S. cerevisiae* sequences are entered on two lines, as there is a small region of overlap between the end of the *ADE1* sequence (first) and the beginning of the *ADE2* sequence (second). For purposes of this alignment, the PurK-homologous domain [amino acids 1∼402 (Stotz and Linder 1990)] was removed from the *S. cerevisiae ADE2* sequence. The Drosophila sequence represented here has GenBank accession no. AF102579.

AF102579). In addition, the sequence of a single strand was determined for 0.71 kb at the 3′ end of the 5.5-kb *Eco*RI LibD subclone and 1.18 kb beyond the 5′ end of this subclone. Thus, in total, a region that spans 6.58 kb of the LibD clone was sequenced.

On the basis of sequence comparisons between the cDNA and the genomic sequence, several structural features of the AIRc-SAICARs gene have been identified (Figure 4B). There are four introns of varying length, 447, 100, 427, and 68 bases, respectively, all of which are contained in the SAICAR synthetase domain. In addition, a polyadenylation consensus sequence, AAUAAA (Sheets *et al.* 1990), is located 19 bases prior to the poly(A) addition site in the cDNA.

We detected a single AIRc-SAICARs mRNA of ∼1.9 kb in wild-type adult polyadenylated mRNA (Figure 5A). This corresponds to the length of the AIRc-SAICARs cDNA. To identify the transcription initiation site(s) for AIRc-SAICARs, primer extension mapping was performed using the AIRc-SAICARsPE primer and total RNA from adult Canton-S flies as the template for extension (Figure 5B). Two transcription initiation sites were detected; the major site occurs 243 bases upstream and a minor site occurs 220 bases upstream of the initiator methionine. In addition, the AIRc-SAICARs cDNA 5′ end lies in this vicinity as well, at 226 bases upstream of the initiator methionine. The cDNA 5′ end sequence is identical to the genomic DNA sequence in this region, indicating there are no introns in the 5′ UTR of this gene. To date, 14 AIRc-SAICARs cDNA 5′ ends have been sequenced as a part of the Berkeley Drosophila Genome Project Expressed Sequence Tag (EST) Database. The 5′ ends of these cDNAs fall within a 25-base interval from 201 to 225 bases upstream from the initiator methionine (not shown). These sites are all in the vicinity of the two transcription initiation sites determined by primer extension mapping for AIRc-SAICARs, but none extends back to the major initiation site.

A search for promoter consensus sequences was performed for the AIRc-SAICARs gene. The two most common promoter element sequences in Drosophila are the TATA-box, found in approximately half of Drosophila promoters, and the initiator element (Inr), found in about one-third of Drosophila promoters. The TATA-box consensus sequence in Drosophila is $^{A}/_{G}/_{C}$ TAT AAA $^{G}/_{A}$ $^{G}/_{C}$ and it is typically found at the −25 to −30 position with respect to the transcription initiation site (Arkhipova 1995). The AIRc-SAICARs gene has the sequence CCATAAAGT, beginning at position −23 with respect to its major transcription initiation site. This

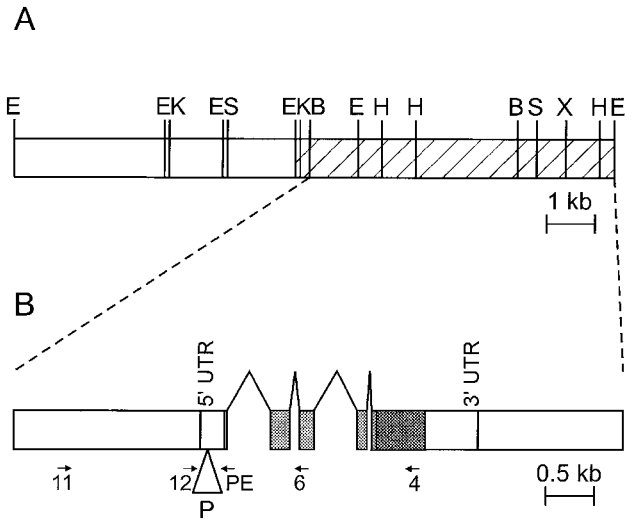

Figure 4.—Characterization of the AIRc-SAICARs gene. (A) Restriction map of the 12.6-kb LibD genomic DNA clone. *Eco*RI sites at either end are part of the Charon4A vector (Maniatis *et al.* 1978). Hatching indicates the two *Eco*RI fragments that hybridized with the cDNA. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; S, *Sst*I; X, *Xba*I. (B) Sequence organization of the AIRc-SAICARs gene. The transcribed region lies between the vertical lines marked as 5′ UTR (untranslated region) and 3′ UTR. Within the transcribed region, boxes represent exons and connecting lines represent introns. Arrows below the gene map indicate primers used in PCR analysis of the *P*-element insertions. Triangle below the gene map points to the site of *P*-element insertions within the 5′ UTR. The *P*-element inverted repeat (PIR) primer would anneal to sequences in either orientation within the region marked "P." Light gray represents the SAICAR synthetase domain; dark gray represents the AIR carboxylase domain. The sequence has GenBank accession no. AF102579.

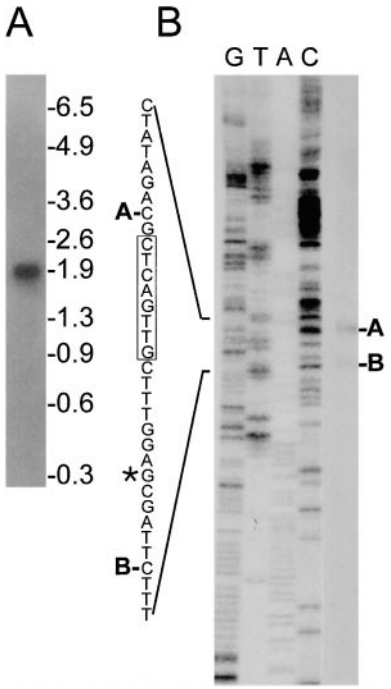

Figure 5.—Transcription of AIRc-SAICARs. (A) Northern blot of 1 μg of Canton-S adult polyadenylated RNA. (B) Primer extension identifies major and minor transcription initiation sites. A sequencing reaction primed with the same primer used for primer extension is shown as a marker. A and B indicate 5′ ends within a few nucleotides of resolution. Initiator sequence downstream from the major start site is boxed. Asterisk indicates the 5′ end of the cDNA isolated by functional complementation.

sequence matches seven out of nine bases in the TATA-box consensus sequence. The Inr consensus sequence in Drosophila is $^{T}/A/_{G}$ TCA $^{G}/_{T}$T $^{T}/_{C}$G and is typically found in the −10 to +10 interval with respect to transcription initiation. The AIRc-SAICARs gene has the sequence CTCAGTTG, beginning at position +3 with respect to the major transcription initiation site, which matches the Inr consensus sequence at seven of eight positions. Given the locations of these two sequences and their similarities to the consensus sequences, they are candidates for a TATA-box and initiator element in the AIRc-SAICARs gene.

Localization of the AIRc-SAICARs gene: The AIRc-SAICARs gene was localized within the *D. melanogaster* genome by *in situ* hybridization of a biotinylated DNA probe to Canton-S polytene chromosome squashes. The AIRc-SAICARs gene gave a single site of hybridization on the X chromosome at 11B4-14. The consistently diffuse nature of this region of the X chromosome (Lefevre 1976) did not allow us to localize the hybridization signal further.

The *ade4* and *ade5* genes colocalize with the AIRc-SAICARs gene: The *ade4* and *ade5* genes were originally identified in a screen for EMS-induced X chromosome-linked recessive mutations associated with reduced red eye pigmentation and adenine auxotrophy. Recombination mapping placed both *ade4*^{1} and *ade5*^{1} in the *v-f* interval (D. Nash, unpublished data), corresponding to sections 10 through 15 on the cytogenetic map (Lefevre 1976). Colocalization of *ade4*^{1} and *ade5*^{1} with the AIRc-SAICARs gene on polytene chromosomes made one or both of these mutations candidate alleles of the gene.

The *ade5*^{2} allele was isolated in the same screen, but the initial strain (*H23-14*) was only partially responsive to an RNA dietary supplement (data not shown). It appeared that there were two classes of flies in this strain, one carrying a mutation with reduced red eye pigmentation and adenine auxotrophy and the other carrying a mutation with normal red eye pigmentation and auxotrophy where only slowly developing survivors occur on an RNA supplement. Two *ade5*^{2} stocks, *ade5*$^{2(7)}$ and *ade5*$^{2(25)}$, were derived from the original H23-14 strain, and these both failed to complement *ade5*^{1} and have both reduced red eye pigmentation and adenine auxotrophy (data not shown). These two strains most likely carry the same mutation. The nature of the other mutation in the original *H23-14* stock, which was subsequently isolated in a stock called *H23-14(44)*, has not been resolved.

The two gamma-radiation-induced *ade5* alleles, *ade5*^{3}

and *ade5*^{4}, and the three hybrid dysgenesis-induced alleles, *ade5*^{5}, *ade5*^{6}, and *ade5*^{8}, were all isolated as mutations that failed to complement *ade5*^{1}, as indicated by a purineless phenotype that had been associated with mutations in other *de novo* purine synthesis genes (Tiong *et al.* 1989; Tiong and Nash 1990; Clark 1994). This phenotype is seen in the presence of standard Drosophila medium where there is an exogenous supply of adenine in the form of yeast or yeast extract and features adults with reduced pteridine (red) eye pigments and wing and leg defects. The null phenotype is pupal lethality.

Nutritional supplementation of *ade4*^{1} and *ade5*^{1} auxotrophic mutants: Supplementation of Sang's defined medium (Sang 1956) with various nutrients was done to further characterize the auxotrophic phenotype of *ade4*^{1} and *ade5*^{1} (Table 1). Only the data for females could be considered here since, for unknown reasons, the FM6-bearing males were only responsive to the yeast-sugar medium. Both *ade4*^{1} and *ade5*^{1} responded positively to supplementation with adenine, adenosine, and RNA, as has been found for auxotrophic mutations in *ade2* and *ade3* (Nash and Henderson 1982), which affect steps in the pathway prior to IMP. Likewise, *ade4*^{1} and *ade5*^{1} did not respond to supplementation with cytidine, guanine, uridine, or hypoxanthine. The data for supplementation with guanosine were equivocal. Since guanosine is not converted to adenine nucleotides in flies (Nash and Henderson 1982), guanosine must somehow act indirectly, perhaps through a mechanism for balancing nucleotide pools. Such indirect action may be unusually sensitive to experimental variation and this could explain these results.

The above results suggest that both *ade4*^{1} and *ade5*^{1} affect *de novo* purine synthesis at steps prior to IMP. The AICA and AICAR supplementation data help to localize the point in IMP synthesis that is affected in *ade4*^{1} and *ade5*^{1}. Both mutants responded to supplementation with AICAR. AICAR is synthesized from SAICAR by adenylosuccinate lyase, which performs the eighth step in the pathway (Figure 1). AICA also rescues the mutants, consistent with the observation that AICA can be converted to AICAR by adenine phosphoribosyltransferase (APRT; Thomas *et al.* 1973). These data place the function affected in these mutants at or before the eighth step in the IMP synthesis pathway.

Complementation test crosses were performed reciprocally between *ade4*^{1} and *ade5*^{1} on both Sang's medium (Sang 1956) and yeast-sugar medium (Nash and Bell 1968). The data indicate that the mutations complement with respect to the auxotrophic phenotype when grown on Sang's medium (Table 2). Thus, although *ade4*^{1} and *ade5*^{1} are adenine auxotrophic mutations mapping to the same region of the genome, it appears that they affect different functions in IMP synthesis. The *ade5*^{2} alleles fail to complement *ade5*^{1} with respect to the auxotrophic phenotype (D. Nash, unpublished data).

Complementation tests between *ade4* and *ade5* alleles: The two gamma-radiation-induced alleles, *ade5*^{3} and *ade5*^{4}, and the three hybrid dysgenesis-induced alleles, *ade5*^{5}, *ade5*^{6}, and *ade5*^{8}, were isolated as mutations that failed to complement *ade5*^{1}, giving a purine syndrome phenotype on standard medium. Although *ade4*^{1} and *ade5*^{1} complement, they are mutations with similar phenotypes and map positions and thus could be complementing alleles of the same gene. Therefore, the gamma-radiation- and hybrid dysgenesis-induced *ade5* alleles were also tested for complementation with *ade4*^{1}. In Table 3, the proportion of *ade4*^{1}/*ade4*^{1} females to total females represents the viability of the *ade4*^{1} chromosome on standard medium. If a cross produces a higher proportion than for *ade4*^{1}, then it shows improved viability and, therefore, the mutation must at least partially complement *ade4*^{1}. If a cross produces the same or a lower proportion than for *ade4*^{1}, then it shows a decrease in viability and, therefore, the mutation fails to complement *ade4*^{1}. Again, in comparing relative viabilities, the assumption is there is no secondary recessive mutation on the *ade4*^{1} chromosome that reduces viability.

From Table 3, it can be seen that all of the alleles tested except *ade5*^{3} complement *ade4*^{1}. One possible explanation for this result is that intragenic complementation is occurring, where *ade4*^{1} affects the enzymatic function of either the AIRc or SAICARs domain but not both, while the *ade5* alleles that complement it affect the other domain. *ade5*^{3} would then represent a mutation that affects both the AIRc and SAICARs domains and, therefore, fails to complement both *ade4*^{1} and *ade5*^{1}. An alternative explanation is that, since *ade5*^{3} is a gamma-radiation-induced mutation, it could be a double mutant or deficiency affecting both *ade5* and *ade4*. Thus, *ade4* and *ade5* may still represent two separate genes.

Viability of *ade4* and *ade5* mutants: To estimate viability of each *ade4* and *ade5* allele on standard medium, mutant homozygotes and/or hemizygotes were scored relative to siblings carrying the balancer *FM6*. The parental chromosomes for the *ade4* and *ade5* mutants were not available for this experiment; therefore, relative viabilities for the mutant alleles were examined rather than their viabilities with respect to the parental chromosome. We recognize that the reduced viability of any of the *ade4* and *ade5* homozygotes could be in part the result of a secondary recessive mutation on the X chromosome.

The chromosomes carrying the two gamma-radiation-induced alleles, *ade5*^{3} and *ade5*^{4}, are completely recessive lethal. On the basis of relative survival of *ade*/*ade* and *ade*/*FM6* siblings (data not shown), the following mutant chromosomes can be placed in order from least to most viable: *ade5*^{1}, *ade4*^{1}, *ade5*$^{2(7)}$, [*ade5*^{5}, *ade5*^{6}, *ade5*^{8}]. Thus, in general, the chromosomes carrying EMS-induced mutations are associated with lower viability than those carrying hybrid-dysgenesis-induced mutations.

Polymorphisms in the AIRc-SAICARs gene are associ-

TABLE 2

Complementation tests between *ade4*[1] and *ade5* alleles: Complementation of *ade4*[1] and *ade5*[1] with respect to auxotrophy

Cross			Females		Males		
♀	♂	Medium[b]	*ade/FM6*	*ade/ade*	*FM6/Y*	*ade/Y*	% Survival[c]
4	4	Sang's	87	0	0	3	0
4	5	Sang's	81	132	0	2	62.0 (±3.3)
5	4	Sang's	84	177	0	20	67.8 (±2.9)
5	5	Sang's	160	7	1	8	4.2 (±1.6)
4	4	Yeast	292	260	0	217	47.1 (±2.1)
4	5	Yeast	229	200	0	497	46.6 (±2.4)
5	4	Yeast	300	262	0	255	46.6 (±2.1)
5	5	Yeast	270	166	0	205	38.1 (±2.3)

[a] Crosses were *ade/FM6* × *ade/Y*, where the *ade* mutation is either *ade4*[1] or *ade5*[1].
[b] Flies were cultured on Sang's defined medium (Sang 1956) or yeast-sugar medium (Nash and Bell 1968). Complete medium is described in materials and methods.
[c] Percentage survival (± standard error of the mean) is the number of *ade/ade* out of total female progeny. This measure was not calculated for the males due to the inviability of *FM6*-bearing males with all supplements except yeast.

ated with the three hybrid dysgenesis-induced *ade5* alleles: To search for a link between *ade5* and the AIRc-SAICARs gene, we searched for restriction fragment polymorphisms in the hybrid dysgenesis-induced *ade5* strains using the 5.5-kb *Eco*RI fragment (Figure 4A) as a probe for Southern blots. All three of the hybrid dysgenesis-induced alleles (*ade5*[5], *ade5*[6], and *ade5*[8]) have larger AIRc-SAICARs *Eco*RI and *Bam*HI restriction fragments in comparison to wild type (only *Bam*HI data are shown in Figure 6A).

P elements vary in size, and the complete *P* element is typically 2.9 kb in length. Thus, one would expect a *P*-element insertion in the AIRc-SAICARs region to produce an increase of up to 2.9 kb in the size of a restriction fragment. This appears to be the case for the *ade5*[5], *ade5*[6], and *ade5*[8] alleles, as substantial increases in the size of AIRc-SAICARs restriction fragments with respect to Canton-S are observed for all three alleles. However, the increases in restriction fragment size are not always consistent for the two restriction enzymes, indicating that rearrangements or new restriction sites were introduced or deleted in the region for some of the alleles. The wild-type *Eco*RI fragment hybridizing to the probe is 7.4 kb while the major wild-type *Bam*HI fragment hybridizing to the probe is ~4.8 kb. The *ade5*[5] allele is associated with a 9.9-kb *Eco*RI fragment (2.5 kb longer) and a 8.7-kb *Bam*HI fragment (3.9 kb longer). This inconsistency is due either to a rearrangement, gain of an *Eco*RI site, or loss of a *Bam*HI site. The *ade5*[6] allele is associated with a 7.6-kb *Eco*RI fragment (0.2 kb longer) and a 6.4-kb *Bam*HI fragment (1.6 kb longer). A full-length 2.9-kb *P* element has an *Eco*RI site but no *Bam*HI site. Again, this is an inconsistency that could be explained in the same way as for *ade5*[5]. The *ade5*[8] allele is associated with an 8.6-kb *Eco*RI fragment (1.2 kb longer) and a 5.9-kb *Bam*HI fragment (1.1 kb longer). Within the resolution of this Southern blot, *ade5*[8] appears to have a partial *P*-element insert that did not create new restriction sites.

TABLE 3

Complementation tests between *ade4*[1] and *ade5* alleles: Complementation of *ade4*[1] and various *ade5* alleles on standard medium

adeX[a]	*ade4*[1]*/FM6*	*ade4*[1]*/adeX*	% Survival
ade4[1]	148	82	35.7 (±3.2)
ade5[1]	340	264	43.7 (±2.0)
ade5[3]	208	0	0
ade5[4]	406	395	49.3 (±1.8)
ade5[5]	312	300	49.0 (±2.0)
ade5[6]	227	193	46.0 (±2.4)
ade5[8]	248	278	52.9 (±2.2)

[a] Cross was *ade4*[1] males with *adeX/FM6* females.

The idea that the AIRc-SAICARs polymorphism observed for *ade5*[5], *ade5*[6], and *ade5*[8] is the result of *P*-element insertions is supported by the fact that, when the Southern blot was stripped and reprobed with a *P*-element sequence, the same polymorphic AIRc-SAICARs *Eco*RI and *Bam*HI fragments were detected (not shown). Taken together, these polymorphisms are a strong link between the *ade5* mutations and the AIRc-SAICARs gene.

Localization of *P*-element insertions in the 5′ untranslated region of the AIRc-SAICARs gene: A PCR-based approach was taken to further localize *P*-element insertions in the *ade5*[5], *ade5*[6], and *ade5*[8] alleles. Primers 11 and 4 (Figure 4B) were first used to amplify the entire AIRc-SAICARs region from the three *P*-element *ade5* mutants and wild-type flies. The wild-type 4-kb AIRc-SAICARs PCR product was increased to 5.8 and 5.4 kb

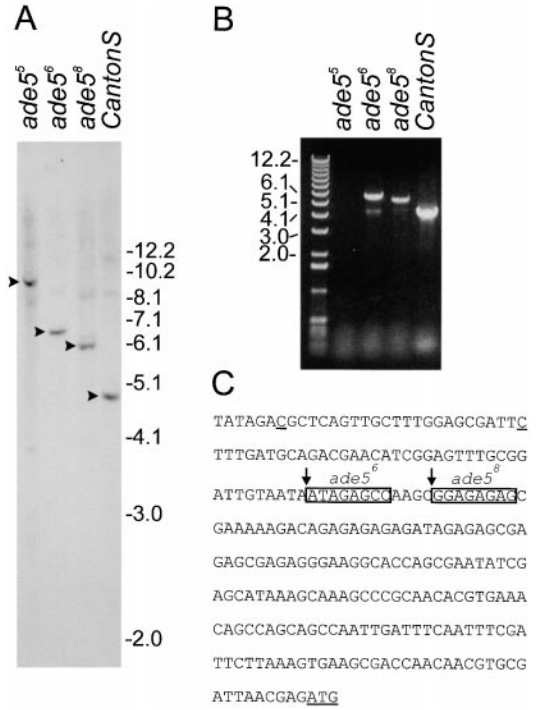

Figure 6.—Analysis of the AIRc-SAICARs gene in hybrid dysgenesis-induced *ade5* mutants. (A) Southern blot of genomic DNA isolated from females homozygous for *ade5* alleles, and Canton-S, and digested with *Bam*HI. Blot was probed with a 5.5-kb *Eco*RI LibD fragment that contains the AIRc-SAICARs gene (Figure 4A). AIRc-SAICARs-hybridizing bands that are altered between fly strains are indicated with an arrowhead. Fragment sizes are indicated in kilobases. (B) PCR amplification of the AIRc-SAICARs region of the three *ade5* hybrid dysgenesis-induced mutations. Genomic DNA of $ade5^5$, $ade5^6$, and $ade5^8$ homozygous and Canton-S females was amplified using primers 11 and 4 (see Figure 4B). DNA size standard is the Life Technologies 1-kb ladder. (C) Wild-type DNA sequence of the region around the *P*-element insertions for $ade5^6$ and $ade5^8$, showing the site of insertion (arrow) and the sequence duplicated in the mutants (boxed and to the right of the arrow). The site of insertion for $ade5^5$ is the same as for $ade5^6$ but the extent of the duplication is unknown (see text).

for $ade5^6$ and $ade5^8$, respectively (Figure 6B). Several attempts were made to generate this PCR product from $ade5^5$ genomic DNA without success. Thus, the $ade5^6$ and $ade5^8$ appeared to have *P*-element insertions of 1.8 and 1.4 kb, respectively. Since $ade5^5$ would not amplify with these primers, we suspect there is either a rearrangement or deletion of the region surrounding one of the primer binding sites. Alternatively, based on the Southern blot analysis, the predicted PCR product would be at least 6.5 kb, and so it may not have been generated due to inefficient amplification of a larger product.

Two other primer pairs were used in PCRs to localize the *P* element in the 4-kb region between primer sites 4 and 11. PCR with the PIR primer and primer 4 gave a 2.3-kb product for $ade5^5$, $ade5^6$, and $ade5^8$, indicating all three alleles had *P*-element sequences in the same region. PCR with primers 12 and 6 generated 3.0, 2.6, and 1.2 kb products for $ade5^6$, $ade5^8$, and Canton-S, respectively (not shown). For $ade5^6$ and $ade5^8$, these sizes are consistent with those generated using primers 11 and 4, indicating single *P*-element insertions of 1.8 and 1.4 kb in this 1.2-kb interval. As found with primers 11 and 4, the PCR results with primers 12 and 6 were inconclusive for $ade5^5$.

Sequencing of the PCR products across the insertion sites shows that all three alleles have *P*-element insertions that lie very close together in the 5′ untranslated region of the AIRc-SAICARs gene (Figure 6C). For $ade5^5$ and $ade5^6$, the insertions are 63 bp downstream from the major transcription start site. For $ade5^8$, the insertion is 75 bp downstream. *P*-element insertions typically generate an 8-bp duplication (O'Hare and Rubin 1983). Both $ade5^6$ and $ade5^8$ *P*-element insertion sequences have the typical 8-bp duplication. With only sequence data for the 3′ side of the $ade5^5$ insertion, the size of the duplication could not be determined.

Thus, for $ade5^6$, there appears to be a 1.8-kb *P* element in the 5′ untranslated region (UTR) of AIRc-SAICARs. The inconsistency between the *Eco*RI restriction fragment size (only 0.2 kb longer than wild type) might be explained by the addition of an *Eco*RI site in the *P* element; however, we did not determine the sequence far beyond the *P*-element inverted repeats. For $ade5^8$, the data are consistent with a 1.4-kb *P*-element insertion in the 5′ UTR of AIRc-SAICARs.

Altered AIRc-SAICARs mRNAs in *ade5* mutants: With the structure of these *P*-element insertions in mind, we examined polyadenylated mRNA isolated from $ade5^5$, $ade5^6$, $ade5^8$, and Canton-S females in a Northern blot using the 1.6-kb *Bam*HI AIRc-SAICARs cDNA fragment as a probe (Figure 7). The most notable result is that $ade5^8$ produces a major transcript that is ~1 kb longer than the wild-type transcript at 3 kb. The discrepancy between the transcript length and the length one would predict from the *P*-element insertion (3.3 kb) is likely due to limitations of gel resolution. Alternatively, there could be an additional or aberrant splicing event or a different transcription initiation site from within the *P* element. In any case, the reading frame in this mRNA may be altered. The $ade5^5$ transcript is wild type in length, but may not be present at wild-type levels, taking into account the RNA loading differences between $ade5^5$ and Canton-S RNA (Figure 7B). No transcript is detectable in the $ade5^6$ females. Thus, the analysis of the AIRc-SAICARs transcripts from $ade5^5$, $ade5^6$, and particularly $ade5^8$ mutants provides evidence for the link between the *ade5* gene first identified in a purine auxotrophy screen and the AIRc-SAICARs gene. The viability of flies carrying these alleles suggests that there must be some low level of AIRc-SAICARs produced from these alleles with *P*-element insertions in the 5′ UTR rather than in the open reading frame.

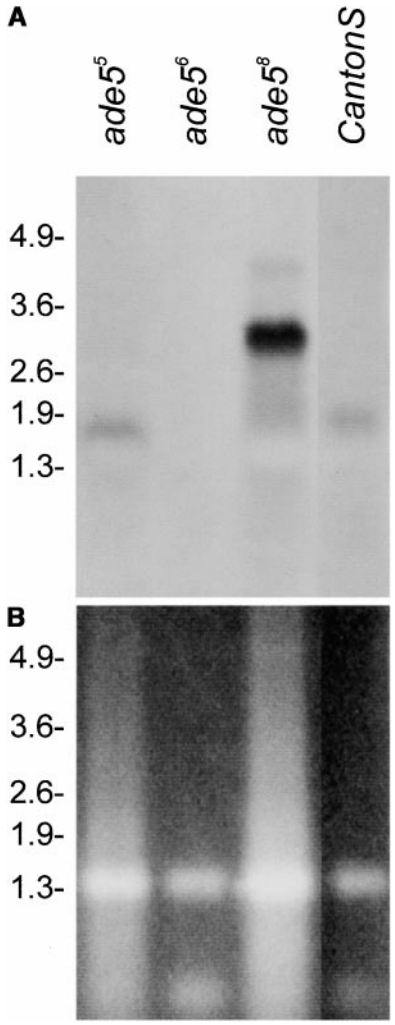

Figure 7.—AIRc-SAICARs mRNA in *ade5*^{5}, *ade5*^{6}, and *ade5*^{8} mutants. (A) A Northern blot of polyadenylated RNA isolated from a mixed population of adult males and females probed with the AIRc-SAICARs cDNA. One lane of the blot image was omitted between the *ade5*^{8} and Canton-S lanes. (B) Ethidium bromide staining of the gel prior to blotting.

DISCUSSION

A Drosophila cDNA encoding AIRc-SAICARs was cloned by functional complementation of an *E. coli purC* (SAICARs) adenine auxotroph. Using the same assay, this cDNA was unable to complement an *E. coli purE* mutant. Sequence alignments with AIRc-SAICARs from other organisms showed that this cDNA contains the entire coding region of both SAICARs and AIRc. Since the chicken cDNA can complement the *purE* mutant (Chen *et al.* 1990), there is a possibility that there are functional differences between the chicken and Drosophila AIR carboxylases.

The AIRc enzymes from *E. coli* and chickens operate by two distinct mechanisms (Firestine *et al.* 1994). The Drosophila AIRc domain has 58% amino acid identity with the chicken AIRc and only 24% amino acid identity with *E. coli* PurC. Therefore, based on amino acid identities, it is likely that the Drosophila AIRc enzyme functions in a similar fashion to the chicken AIRc. It has been proposed that the chicken AIRc is capable of substituting for both PurE and PurK because it offers a completely separate path to go from the AIR substrate to the CAIR product (Chen *et al.* 1990; Firestine and Davisson 1994; Mueller *et al.* 1994). If this is the case, then it is not clear why the Drosophila AIRc does not provide AIRc function in the *E. coli purEK* mutant. There are a number of possible explanations for this discrepancy. First, the Drosophila AIRc could function by the same mechanism as *E. coli* PurE and, as such, requires a functional version of PurK. However, our BLAST sequence searches with *E. coli* PurK have not identified any PurK-like homology in Drosophila or vertebrates, so there is no support for this explanation. Second, the Drosophila cDNA could contain a mutation in the AIRc domain. This possibility was ruled out when the exon sequences obtained for the AIRc-SAICARs gene were found to align exactly with the cDNA sequence. As there are several Drosophila AIRc-SAICARs cDNAs available from the Berkeley Drosophila Genome Project, we could test these cDNAs for consistency with the functional complementation behavior of our cDNA. A third explanation is that the AIRc mechanism is identical in Drosophila and chickens but, for some reason, the Drosophila enzyme may not be as efficient. Perhaps a post-translational modification unavailable in *E. coli* is required for Drosophila AIRc to function.

Transcription of the AIRc-SAICARs and the PRAT genes in vertebrates is divergent and controlled by common promoter elements in the intergenic region (Brayton *et al.* 1994; Gavalas and Zalkin 1995). We found that the AIRc-SAICARs gene maps to the X chromosome in section 11B 4-14. On the other hand, *Prat* maps to the right arm of chromosome 3 in section 84E1-2 (Clark 1994). A second *Prat* gene maps to the left arm of chromosome 3 (D. V. Clark, unpublished observations; D. Harvey, L. Hong, M. Evans-Holm, J. Pendleton, C. Su, P Brokstein, S. Lewis and G. M. Rubin, unpublished results). Thus, although the AIRc-SAICARs and *Prat* genes in Drosophila are not closely linked and divergently transcribed, this finding does not rule out the possibility that they have similar regulatory elements and their transcription is somehow coordinated.

The AIRc-SAICARs gene promoter region was identified by primer extension of adult RNA. A TATA-box and initiator (Inr) were found at typical positions relative to the major transcription start site, between −25 to −30 and −10 to +10, respectively (Arkhipova 1995). A second, minor transcription start site was also identified; however, no TATA-box or Inr consensus sequences were located in proximity to this initiation site. In contrast to the AIRc-SAICARs gene, *Prat* has a promoter lacking both TATA and Inr sequences with multiple transcription start sites (Clark *et al.* 1998). There are no obvious sequences shared between the *Prat* and AIRc-SAICARs

gene promoter regions. Further functional studies on their regulatory sequences and the expression patterns must be done to resolve this issue.

Two complementing EMS-induced adenine auxotrophic mutations, *ade4*^{1} and *ade5*^{1}, mapped to the same region of the Drosophila genome as AIRc-SAICARs. The EMS-induced alleles have the nutritional supplementation behavior typical of *de novo* purine synthesis genes. Furthermore, the mutants are rescued by compounds (AICA and AICAR) that place the defect at a point at or prior to step 8 in the pathway, which is consistent with a defect in AIRc-SAICARs at steps 6 and 7 in the pathway.

Two gamma-radiation-induced and three hybrid dysgenesis-induced alleles of *ade5* were isolated in subsequent screens. The *P*-element insertion alleles were isolated as independent noncomplementers of the EMS-induced *ade5*^{1} allele on the basis of the "purine syndrome" phenotype. In previous work, the first alleles of *ade2* and *ade3* were isolated in the same adenine auxotrophy screens (Nash and Janca 1983; Janca *et al.* 1986). More severe alleles were subsequently isolated as lethals or semilethals; these alleles show a purine-syndrome phenotype when flies are grown on standard medium (Tiong *et al.* 1989; Tiong and Nash 1990). The phenotypes of various *ade2* and *ade3* alleles served as the basis for the design of the gamma-radiation and hybrid dysgenesis screens for *ade5* alleles: new alleles would display a purine-syndrome phenotype in combination with *ade5*^{1} on standard medium.

Characterization of the lesions harbored in the independently isolated hybrid dysgenesis-induced *ade5* alleles indicates that the AIRc-SAICARs and *ade5* gene are synonymous. Evidence supporting this conclusion is threefold. First, Southern blot and PCR analyses of the *ade5*^{5}, *ade5*^{6}, and *ade5*^{8} hybrid dysgenesis-induced mutants showed polymorphisms associated with AIRc-SAICARs sequences in all three mutants. Second, sequencing of the regions flanking *P*-element inserts in *ade5*^{5}, *ade5*^{6}, and *ade5*^{8} localized *P* elements to the 5′ end of the transcribed but untranslated region of the AIRc-SAICARs gene. The *ade5*^{6} and *ade5*^{8} *P* elements are inserted at different sites in this region. Third, Northern blot analysis of the AIRc-SAICARs transcripts produced from the three mutants indicates a shift in the size of transcript produced by one of the alleles, *ade5*^{8}. Reductions in mRNA levels appeared to occur for the other two alleles, but these were not quantified. The position of the *P* element within the transcribed region, coupled with the increased transcript size for *ade5*^{8}, indicate that the *P* element in this strain is probably being transcribed.

Although all three hybrid dysgenesis-induced alleles have *P*-element sequences inserted in the 5′ untranslated region, the insertions are different sizes. The size variation could be due to insertion of different nonautonomous *P* elements already harboring deletions or due to insertion of a full-length *P* element followed by imprecise excision in subsequent generations before the mutation was backcrossed sufficiently to remove all sources of *P*-element transposase. This variation in insert size and sequence could explain the different behavior of the three alleles. For *ade5*^{8}, its longer 5′ end could interfere with the initiation of translation of AIRc-SAICARs or could result in initiation and termination of translation within the *P*-element sequence itself. Alternatively, initiation and translation through the *P*-element sequence into the AIRc-SAICARs sequence would result in additional amino acids or a reading frame shift. Any of these scenarios could impede the AIRc-SAICARs enzyme function, producing the mutant phenotype observed in *ade5*^{8}. The absence of detectable AIRc-SAICARs mRNA in *ade5*^{6} could be due to reduced transcription initiation as a result of the *P*-element insertion, or the presence of the *P* element may reduce mRNA stability.

On the basis of our nutritional studies, gene mapping, and molecular characterization of the hybrid dysgenesis-induced alleles, we conclude that the AIRc-SAICARs corresponds to the *ade5* gene. Further evidence could be provided by showing the *ade5* mutations could be rescued by transformation with a wild-type AIRc-SAICARs gene. The 5.5-kb *Eco*RI fragment from the LibD clone would be an ideal starting point for this type of experiment, since it contains the entire coding region of the gene and a considerable amount of flanking sequence.

As for the *ade4*^{1} mutation, further study is required to determine if it represents a separate gene or is a complementing allele of *ade5*. The latter scenario is reasonable, considering that AIRc-SAICARs is a bifunctional enzyme with independent domains (Firestine and Davisson 1994). The gamma-radiation-induced allele *ade5*^{3} fails to complement *ade4*^{1} completely; no *ade4*^{1}/*ade5*^{3} females were observed when cultured on standard medium. This result is unexpected, since males hemizygous for *ade4*^{1} are viable on standard medium. Perhaps this observation can be explained by a dosage compensation effect, where *ade5*^{3} is a null allele and females hemizygous for *ade4*^{1} have less transcription of this gene relative to hemizygous males. This finding is in contrast to the fact that *ade5*^{3} was isolated as a noncomplementing allele of *ade5*^{1}, where this heteroallelic combination is viable. Since *ade4* and *ade5* map in the same region of the X chromosome, and *ade5*^{3} is a gamma-radiation-induced mutation, it may be a deletion that encompasses both *ade4* and *ade5*. Further characterization of *ade5*^{3} may help to resolve the relationship between *ade4* and *ade5*.

If *ade4*^{1} is not an allele of *ade5*, the question becomes, what gene is it? Based on its behavior in the nutritional supplementation experiments, it is a good candidate for another *de novo* purine biosynthesis gene that operates at or prior to the eighth step in the pathway. Genes

encoding steps 1 (*Prat*); steps 2, 3, and 5 (*ade3*); step 4 (*ade2*); and now steps 6 and 7 (*ade5*) have been identified. Since *ade4*^{1} can be rescued with AICAR, it may be a mutation in the gene encoding the enzyme responsible for step 8 in the pathway, adenylosuccinate lyase, converting SAICAR to AICAR with the release of fumarate. One caveat to this interpretation is that adenylosuccinate lyase also catalyzes a very similar reaction in step 12 (Figure 1), converting SAMP to AMP with the release of fumarate (Henikoff 1987). If *ade4*^{1} corresponds to the gene encoding this enzyme, then the prediction is that it should not be supplementable with inosine or any intermediate prior to step 12, including AICAR (Nash and Henderson 1982). cDNAs encoding this enzyme as well as those for the other uncharacterized genes in the pathway are now available through the Berkeley Drosophila Genome Project (Harvey *et al.* 1997). Thus, it will be straightforward to clarify this issue using these cDNAs as probes for *in situ* hybridization to polytene chromosomes to localize the remaining purine pathway genes in the Drosophila genome.

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