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Mapping Mutations in Genes Encoding the Two Large Subunits of *Drosophila* RNA Polymerase II Defines Domains Essential for Basic Transcription Functions and for Proper Expression of Developmental Genes

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We have mapped a number of mutations at the DNA sequence level in genes encoding the largest (*RpII215*) and second-largest (*RpII140*) subunits of *Drosophila melanogaster* RNA polymerase II. Using polymerase chain reaction (PCR) amplification and single-strand conformation polymorphism (SSCP) analysis, we detected 12 mutations from 14 mutant alleles (86%) as mobility shifts in nondenaturing gel electrophoresis, thus localizing the mutations to the corresponding PCR fragments of about 350 bp. We then determined the mutations at the DNA sequence level by directly subcloning the PCR fragments and sequencing them. The five mapped *RpII140* mutations clustered in a C-terminal portion of the second-largest subunit, indicating the functional importance of this region of the subunit. The *RpII215* mutations were distributed more broadly, although six of eight clustered in a central region of the subunit. One notable mutation that we localized to this region was the α-amanitin-resistant mutation *RpII215*^{C4}, which also affects RNA chain elongation in vitro. *RpII215*^{C4} mapped to a position near the sites of corresponding mutations in mouse and in *Caenorhabditis elegans* genes, reinforcing the idea that this region is involved in amatoxin binding and transcript elongation. We also mapped mutations in both *RpII215* and *RpII140* that cause a developmental defect known as the Ubx effect. The clustering of these mutations in each gene suggests that they define functional domains in each subunit whose alteration induces the mutant phenotype.

Eukaryotic RNA polymerase II is the central component of the mRNA transcription machinery; during the course of synthesizing a pre-mRNA, it interacts with numerous other nuclear components, including the chromatin template, the RNA product, and an assortment of transcription factors (27, 46, 47). Perhaps reflecting the complexity of these interactions, RNA polymerase II is a complicated enzyme, consisting of 2 large subunits (220 to 240 kDa and 140 to 150 kDa) and 6 to 10 smaller subunits (10 to 40 kDa), depending on the organism (47, 50, 57). Despite the importance of RNA polymerase II in gene expression, however, many details of the functions of its individual subunits remain largely unknown.

Sequence analysis of the cloned genes encoding the two large eukaryotic subunits from different organisms has revealed that the largest subunit (2, 23) and the second-largest subunit (15, 53) each contain multiple conserved regions homologous to the β′ and β subunit of eubacterial RNA polymerase, respectively, suggesting a functional similarity of the corresponding subunits of prokaryotes and eukaryotes. Previous biochemical studies suggested that the two large subunits are probably involved in binding DNA templates and nascent RNA chains (4, 9, 18). Analysis of mutants of RNA polymerase II and suppressors thereof, from both *Saccharomyces cerevisiae* (3, 20, 44) and *Drosophila melanogaster* (11, 28), further suggests a role for the largest subunit in both transcription initiation and elongation and an influence of the second-largest subunit on the accuracy of mRNA start site selection. The second-largest subunit is also probably involved in binding the nucleotide substrates (41, 53) and, in bacteria, sigma factors. It may also contain the active site of RNA catalysis (40; see below) and an RNase-like domain (51), which might participate in RNA proofreading or in overcoming sequence-dependent blocks to transcription (8, 39).

A number of mutant alleles of the gene encoding the largest subunit of *Drosophila* RNA polymerase II, several of which cause phenotypes suggestive of selective alterations in specific functional domains, have been isolated (16, 17, 30, 31). Mapping of those mutations would thus help to define those domains. For example, the *C4* mutant RNA polymerase II is 250-fold less sensitive to inhibition by α-amanitin than is the wild-type enzyme (10, 16, 17) and shows a decreased elongation rate in an in vitro transcription assay (11). While the *C4* mutation has not been previously mapped, α-amanitin-resistant mutations in other organisms have been identified as single-amino-acid changes in or near a conserved region of the largest subunit (5, 25), thus potentially identifying a protein domain involved in elongation as well as drug binding.

The mutant alleles *C4*, *Ubl*, *C11*, *JH1*, and *WJK2*, when heterozygous with a normal *RpII215* allele, cause the capitellum of the haltere, an element of the third thoracic segment of flies, to be partially transformed into the wing blade, an analogous structure on the second thoracic segment (17, 30). This phenomenon is known as the Ubx effect, since a similar phenotype is seen in flies heterozygous for *Ultrabithorax* (*Ubx*) mutations. Furthermore, these *RpII215* mutant alleles enhance dramatically the otherwise modest defects seen in *Ubx*/+ individuals. None of these mutations has been mapped previously at the DNA level. Defects in

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differentiation and development have also been observed in amanitin-resistant mammalian cell lines (12) and in amanitin-resistant mutants of *Caenorhabditis elegans* (42). Apparently these mutations alter certain critical functions of the largest subunit in ways that are reflected as relatively specific defects in development. However, little is known about which functions are altered and how the transcription process is affected.

Another motivation for mapping mutations is to test for congruence between a locus defined genetically and a gene cloned molecularly. A test of this type was needed in the case of the second-largest subunit of *Drosophila* RNA polymerase II. Numerous recessive lethal mutant alleles define a complementation group (*A5*) on the third chromosome which from genetic data was concluded to be *RpII140*, the gene encoding the second-largest subunit (19, 29). We anticipated that mapping some of these mutations would verify this conclusion. In addition, the mapping results would contribute to the investigation of the Ubx effect, because a subset of these alleles enhance the Ubx phenotype in *Ubx*/+ flies. An interesting difference from the case of the *RpII215* alleles mentioned above is that these alleles do not display the Ubx phenotype in the absence of a mutant *Ubx* allele.

The availability of strains carrying these and other mutant alleles presents an opportunity to characterize functional roles and functional domains of the two large subunits of *Drosophila* RNA polymerase II and to investigate the molecular basis of the *Ubx*-like developmental defect. As the first step in these characterizations, we have used an approach combining the polymerase chain reaction (PCR) (45) with single-strand conformation polymorphism (SSCP) analysis (34, 35) to localize extant *RpII215* and *RpII140* mutations. In this approach, specific fragments of DNA are labeled and amplified simultaneously; then the DNA is melted, and the separated strands are subjected to nondenaturing gel electrophoresis. A mutation-containing fragment often displays a mobility shift due to SSCP. Once identified as containing a mutation, the DNA fragment is sequenced. Here we describe mapping of five mutant alleles of *RpII140* and eight mutant alleles of *RpII215* at the DNA sequence level, and we discuss the implications of our observations.

MATERIALS AND METHODS

Strains and crosses. Flies were maintained in vials on standard cornmeal-sugar-agar medium supplemented with live yeast cells or in 0.5-pt (ca. 0.24-liter) bottles on modified instant *Drosophila* medium (Carolina Biological) (16). Stocks containing *RpII215* mutant alleles were the following (see also Table 1): *v C4*, *v C20/FM7/Dp(1;Y) $B^{S-}Yy^{+}$*, *v E28/FM7/Dp(1;Y)$B^{S-}Yy^{+}$*, *y JH1 f; Ubx^{130}/Dl^{7}*, *ras v WJK1/FM7; Ubx^{130}/Dl^{7}*, *ras v WJK2/FM7; Ubx^{130}/Dl^{7}*, *y Ubl f/FM7*, *ras v JG1/FM7*, *ras v JG2*, and *ras v JG3/FM7*. Stocks containing *RpII140* mutant alleles were *A5 red e/TM6*, *M39 red e/TM3*, *Z19 red e/TM6B*, *Z36 red e/TM6B*, *Z43 red e/TM6B*, and *Z45 red e/TM6B*.

For homozygous viable mutant alleles, homozygous flies of *C4*, *C20*, *JH1*, *WJK1*, *WJK2*, *JG1*, *JG2*, and *JG3* were collected directly from expanded stocks grown at the permissive temperature. For recessive lethal mutant alleles, we generated heterozygous female progeny containing one copy of the mutant allele and one copy of the corresponding wild-type allele from which the mutant was derived. To do this for *RpII215*, males of *v E28/Dp(1;Y) $B^{S-}Yy^{+}$* were mated with virgin females of wild-type Oregon-R strain *P2*, and females of *y Ubl f/FM7* were mated with *y + f* males. For *RpII140*, balanced males containing different mutant alleles were mated individually with + *red e* virgin females, and appropriate female progeny were collected.

Suppressors of *Ubl*. Second-site suppressors of the Ubx effect-inducing, recessive lethal *Ubl* allele were sought as follows. Thirty separate stocks (*ras v Ubl/FM7c* females and *FM7c/Y* males) were maintained in 0.5-pt bottles on standard medium. Flies were mutagenized with ethyl methanesulfonate (EMS) as described previously (28) and subsequently examined for the presence of *ras v Ubl/Y* males. From an estimated 10^6 *ras v Ubl/Y* zygotes (based on the number of female progeny), we recovered three males. Each carried a new mutation, inseparable by recombination from the original *Ubl* mutation. These putative new *RpII215* alleles were named *JG1*, *JG2*, and *JG3*. These new alleles were examined for the ability to induce the Ubx effect. *JG1* still causes this effect, although the transformation of haltere to wing is one-fourth as strong as with the original *Ubl* mutation. In addition, we observed that *Ubl/JG1* flies do not survive to the adult stage. In contrast, neither *JG2* nor *JG3* causes the Ubx effect, and both survive in heterozygous combination with *Ubl*.

PCR-SSCP analysis. Genomic DNA was purified from adult flies. About 10 flies were ground with a 5-ml stripette (Costar) in 100 μl of buffer I (10 mM Tris [pH 7.5], 60 mM NaCl, 0.15 mM spermidine [Sigma], 0.15 mM spermine [Sigma], 10 mM EDTA, 5% sucrose), mixed with 100 μl of buffer II (0.1 M Tris [pH 9.0], 1.25% sodium dodecyl sulfate [SDS], 30 mM EDTA, freshly added 0.75% dimethyl pyrocarbonate), and incubated at 65°C for 15 min. After 30 μl of 8 M potassium acetate was added, the mixture was incubated on ice for 30 min and centrifuged for 2 min in a microcentrifuge (Vangard International). DNA was then precipitated from the supernatant with ethanol.

The PCR primers to amplify the coding region of *RpII140* (15) were as follows, listed 5′ to 3′, with the position of the first nucleotide corresponding to the published sequence indicated: 1a, A-631TGCTTCTGCACTTTAGAAAACTGA; 1b, T-1003GCCGTTCCGCCTGCAACTCAATGG; 2a, CT GAATTCA-945TGTCGGTGCAGCGCATTGTGG; 2b, T-13 36AGCCACCGGGATCCAACGGGCACTC; 3a, C-1281TT ACTGATCGTGATCTTACGGAGCTG; 3b, CTGAATTCC -1676ATCATCTCGGGATCGTCG; 4a, CTGAA-1632TTCT GGAGCATATCATCTATGA; 4b, C-1972AGCGAGATCA AGTCGCTTGTTTC; 5a, C-1916AGGAGAGAACTCGAT GATCGAGA; 5b, T-2294ATTGTGCAACTGACGAGGTTT GG; 6a, CT-2238GAATTCGCCAATTGGCAGAGACGG; 6b, C-2636ATCTGTATAGATTCGGATCTCACGA; 7a, G-2 584GTGTCTATGATTCGAGACATTCG; 7b, CTGCGGC-CGCA-2977CCCATAGCGCTTTGATAGGTG; 8a, C-29 32GATCACAATCAGAGTCCGCGTAA; 8b, T-3234GGT TGTAGCCAGTGTAGCAGAGA; 9a, A-3181CCAGCTGG CATCAATTCAATCGT; 9b, C-3494GTTTTCAGGAAGG GTAATGGTCT; 10a, CTG-3435AATTCGTGTATCTGGC GACGA; 10b, A-3737TGTGAAGGCCATGTCCTCCTG AC; 11a, G-3684ACAAAAGGGAACGTGCGGCATCC; 11b, C-4053AGGAAGACCTATAGTTAAGGGAC; 12a, CTGC GGCCGCA - 3988AATTGCTTAACTTTCAACTTGC; 12b, T-4310TGGCGATGGCAATCAGGCCACAG; 13a, C-426 1CGTACCGCGTACACATCTGCAAC; and 13b, CTGAA TTCA-4564GAAAATGCCACACAAAGCACC.

The PCR primers to amplify the coding region of *RpII215* (23), excluding the carboxy-terminal repeat domain were as follows: 0a, G-1020TCTAGTGGCCACCAGTATCGCTG; 0b, T-1260CGAATGAATCTGTGAGCCGCACT; 1a, G-18 09TGTTACTTCTTTCCTGCTTGCAG; 1b, G-2148CTGTC

CCCTAGACTTCATAACGA; 2a, C-2100CACACAATCCA AAGATCAAGGAG; 2b, T-2439GCCCAGAATAAAGCA CTCCTCAT; 3a, G-2382AGCGGGTTTGGGAAATCCTT AAG; 3b, C-2723GACTTTTGCATAGCCCTGGGCAT; 4a, T-2647CAAGATGCTGCAATTCCACGTCG; 4b, G-3012 ATCAATACGCTCGCCATTGTCGC; 5a, A-2962GTATCC GGGTGCCAAGTACATTG; 5b, T-3300CTGTCTGGGTG TGATGTGGATGT; 6a, T-3234TGCACGTTCCGCAGTCC ATGGAG; 6b, T-3572TCATCCTCCTCGTCCGGATGCGT; 7a, G-3520CAACGTGAACATGATACGCACAC; 7b, C-384 0CTGCTGGATCTCGTTGTAGGTCT; 8a, G-3789GTATT GGTGACACTATTGCCGAT; 8b, G-4100AAAATCGC TAGTCAGTAGCTTAC; 9a, T-4117GTGCTGGCATTTTT ATCTTCCAG; 9b, G-4453AATAAGCTGGCCCACCGAG TTAC; 10a, T-4406GGTTAACTACGACGGAACAGTGC; 10b, C-4740AGGTTGCACGGCAGTACCACCTT; 11a, G-4689AGACAAATCTTCCCTAACGGCGA; 11b, C-5052 TGTTGGAAACGCGTCTCGATTTC; 12a, C-4974ACAGA GGCGTTTGAGTGGTTGGT; 12b, A-5313GCCGTATTGG CCGTAACCTTGCG; 13a, T-5261ACTGTGCCGCCTGGA GCATACCA; 13b, G-5604TCCTGGAACTTGTTCTCTTC GTT; 14a, G-5556GTGCTGCGCATTAGGATCATGAA; 14b, T-5884CTCGCAAATATCGTTGGAGGATG; 15a, T-5 833CTGAGCGTGACGTGGACCCAATC; 15b, C-6159T CAGAGACGCCCCTCATGGGATC; 16a, T-6104GCTAA TGGATGCTGCCGCTCACG; 16b, G-6444CGTTGCTGA GTCGAGAGTAAAAT; 17a, T-6496CTCTTTCTTTCTTG TGCTTGCAG; 17b, C-6836GTCGACTGTGGATTGAAA TTTGG.

The 5′ end of primers (10 pmol) were labeled with [γ-^{32}P]ATP (11.4 pmol, 7,000 Ci/mmol; ICN) and polynucleotide kinase (4.5 U; Boehringer Mannheim) in 10 μl at 37°C for 30 min. Each 20-μl PCR reaction mixture contained 50 nM one labeled primer and 50 nM the other unlabeled primer, 75 μM each of the four deoxynucleotides, 0.1 μg of genomic DNA, and 1 U of *Taq* DNA polymerase (Boehringer Mannheim). Most PCRs were carried out in a Single Block System (Ericomp) as 30 cycles of 94, 66, and 72°C for 1 min each and one cycle of 94, 66, and 72°C for 1, 1, and 10 min, respectively. The annealing temperature was 64°C (when *RpII140* primers 1a-b and 5a-b were used) or 69°C (when *RpII140* primers 6a-b and 11a-b were used). After the reaction cycles, 1 μl of each PCR product was diluted by adding 11 μl of 0.1% SDS–10 mM EDTA and 12 μl of 95% formamide–20 mM EDTA–0.05% bromphenol blue–0.05% xylene cyanol. The mixture was heated at 95°C for 5 min and immediately placed on dry ice. Then 1 to 2 μl of the mixture was loaded immediately after thawing onto one or more of the following kinds of gels (30 cm by 40 cm by 0.35 mm): (i) 5% polyacrylamide (1 bisacrylamide:99 acrylamide)–5% glycerol–0.5× Tris-borate-EDTA (TBE); (ii) 5% polyacrylamide (1 bisacrylamide:19 acrylamide)–10% glycerol–1× TBE; or (iii) 0.5× MDE (mutation detection enhancement) gel (AT Biochem)–0.6× TBE. Electrophoresis was performed at 5 to 10 W for 12 to 16 h at room temperature in a sequencing apparatus with a metal cooling plate (model S2; Bethesda Research Laboratories). The temperature of the gel was maintained at about 26°C. The gel was dried on filter paper and exposed to X-ray film at −80°C for 1 to 12 h with an intensifying screen.

Subcloning and sequencing. PCR-amplified fragments were directly subcloned with the TA cloning kit (Invitrogen). Plasmid DNA purified by the alkaline lysis method (7) from a 3-ml overnight culture was mixed with one original PCR primer and denatured at 85°C for 5 min in 0.2 M NaOH–0.2 mM EDTA. Annealing and sequencing reactions were then carried out with the Sequenase kit (U.S. Biochemical). Sequences were analyzed on 5% Long Ranger gels (AT Biochem).

TABLE 1. *RpII140* and *RpII215* mutant alleles

Allele	Mutagenesis method used for isolation	Synonym[a]	Reference
RpII140			
A5	γ-Ray		29
M39	EMS		29
Z19	EMS		29
Z43	EMS		29
Z45	EMS		29
Z36	EMS		29
RpII215			
C4	EMS	*4*	16
C20	EMS of *C4*	*8*	17
E28	EMS of *C4*	*12*	17
WJK1	EMS	*K1*	30
WJK2	EMS	*K2*	30
JH1	EMS	*H1*	30
Ubl	EMS	*MGM179*	31
JG1	EMS of *Ubl*		This report[b]
JG2	EMS of *Ubl*		This report
JG3	EMS of *Ubl*		This report

[a] From reference 26.
[b] See Materials and Methods.

RESULTS

Detection of mutations. For recessive lethal mutant alleles, we first carried out genetic crosses to generate female progeny with one copy of the mutant allele and one copy of the corresponding wild-type allele from which the mutant was derived in order to eliminate nucleotide sequence polymorphisms that may occur in the *D. melanogaster* population.

We designed 18 and 13 pairs of PCR primers to amplify the coding regions of *RpII215* and *RpII140*, respectively; each pair of primers was to amplify a region of about 350 to 400 bp of genomic DNA. For each region, we performed separately two parallel PCR-SSCP analyses with only one of the two 5′-end-labeled primers. Thus, the single strands separated by heat denaturing the sample before electrophoresis could be visualized individually after nondenaturing gel electrophoresis and autoradiography. When homozygous mutant DNA (e.g., *C4/C4*) was subjected to the PCR-SSCP analysis, we expected to see one labeled band with a mobility different from that of wild type. When heterozygous DNA (e.g., *E28/+*) was subjected to the analysis, we expected to see two labeled bands, one with the same mobility as wild type and the other one with a different mobility.

We screened six mutant alleles of the *A5* locus, the presumptive *RpII140* gene, and eight *RpII215* mutant alleles (Table 1). We detected mobility shifts from four *RpII140* mutant alleles and all eight *RpII215* mutant alleles (Fig. 1 and 2). The alleles and the primer pairs of the PCR fragments affected by the mutations are listed in Table 2. Note that the *C20* and *E28* mutant alleles were derived from the *C4* chromosome, and we expected them to contain two alterations, one of which would be the original *C4* mutation. Indeed, one band with the same mobility shift appeared in the lanes for *C4* and *C20* homozygous DNA and for *E28/+* heterozygous DNA, strongly indicating that the *C4* mutation is located within the region amplified by primers 8a and 8b

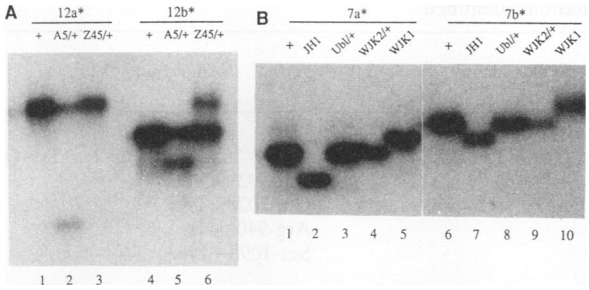

FIG. 1. PCR-SSCP analysis of *RpII140* and *RpII215*. (A) PCR-SSCP analysis of fragment 12 in *RpII140*. Genomic DNA of wild-type flies + (lanes 1 and 4) and mutation-containing flies *A5*/+ (lanes 2 and 5) and *Z45*/+ (lanes 3 and 6) was subjected to PCR-SSCP analysis as described in Materials and Methods. In lanes 1 to 3, only primer 12a was labeled; in lanes 4 to 6, only primer 12b was labeled. (B) PCR-SSCP analysis of fragment 7 in *RpII215*. Genomic DNA of wild-type flies +/+; Ubx^{130}/Dl^{7} (lanes 1 and 6) and mutation-containing flies *JH1* (lane 2 and 7), *Ubl*/+ (lanes 3 and 8), *WJK2*/+ (lanes 4 and 9), and *WJK1* (lanes 5 and 10) was analyzed by PCR-SSCP. In lanes 1 to 5, only primer 7a was labeled; in lanes 1 to 10, only primer 7b was labeled. PCR products were denatured by heating at 95°C for 5 min and electrophoresed at room temperature in a 5% polyacrylamide (1% bisacrylamide) gel containing 5% glycerol for 12 h at 5 W.

(Fig. 2). The *C20* and *E28* mutations themselves were also detected as mobility shifts in regions amplified by primers 10a-b and 4a-b, respectively (data not shown). Surprisingly, two different alterations were also detected for the *JH1* allele of *RpII215* in regions amplified by primers 7a-b and 8a-b (Fig. 1 and 2; see below).

In some cases, a mobility shift of the mutation-containing DNA fragment was shown by only one labeled single strand, not the other strand (e.g., *Z45* [Fig. 1]). In addition, we did not detect mobility shifts for two *RpII140* mutant alleles, *Z43* and *Z36*. Finally, two presumptive new mutations in *RpII215* that suppressed the lethality of the *Ubl* allele (*JG2* and *JG3*) did not show expected mobility shifts; however, we discovered that they were actually reversions to wild-type sequence (see Materials and Methods and below).

We also tested three alleles of the *Z6* locus, which maps very close to *RpII140* (19, 29), and found no differences from wild type (not shown).

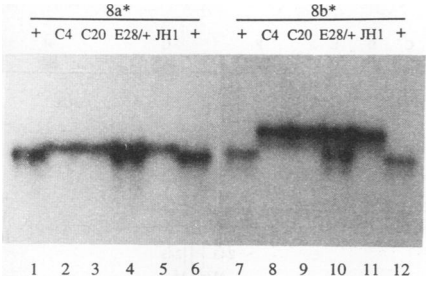

FIG. 2. PCR-SSCP analysis of fragment 8 in *RpII215*. Genomic DNA from wild-type flies *P2* (lanes 1 and 7) and +/+; Ubx^{130}/Dl^{7} (lanes 6 and 12) and mutation-containing flies *C4* (lanes 2 and 8), *C20* (lanes 3 and 9), *E28*/+ (lanes 4 and 10), and *JH1* (lanes 5 and 11) was PCR amplified and analyzed as described in Materials and Methods. In lanes 1 to 6, only primer 8a was labeled; in lanes 7 to 12, only primer 8b was labeled. Electrophoresis was performed under the conditions described in the legend to Fig. 1.

Determining mutations. With the PCR-SSCP analysis, we were able to localize the mutations to regions of 350 to 400 bp. To identify the mutations at the nucleotide level, we sequenced the affected region for each mutant allele (Fig. 3). For each region of interest, we sequenced several individual subclones to distinguish real mutations from random mutations potentially introduced during PCR. In addition, for each heterozygote, we sequenced about 12 subclones to distinguish mutant from wild type; in all cases, approximately 50% of the subclones were mutant. The sequencing analysis revealed one 15-bp deletion, three single-base substitutions for four *RpII140* mutant alleles, and eight single-base substitutions for eight *RpII215* mutant alleles. The mutations identified are listed in Table 2, and the distribution of the mutations is shown in Fig. 4.

Among the *RpII215* mutant alleles, we confirmed that *C20* and *E28* were double mutants, containing in common the *C4* mutation as expected and a second unique mutation, all of which were detected by PCR-SSCP. *JG1* was isolated as a suppressor of *Ubl* (see Materials and Methods); consistent with this isolation history, it was also a double mutant, containing the original *Ubl* mutation and the newly induced suppressor mutation.

One mutation that we mapped, *JH1*, was originally isolated as a suppressor in *trans* of the *C4*-caused Ubx effect (30). Interestingly, although *JH1* was induced on a wild-type chromosome, we find that it is actually a double mutant, containing the *C4* alteration in addition to a change at Ala-657→Thr. This arrangement of alterations may have arisen as a consequence of a gene conversion or double recombination event in either the original *v C4/JH1* non-Ubx effect female or a test progeny thereof (a less likely origin would be simultaneous induction of both mutations on the same chromosome). Because *JH1* homozygotes are viable, it will be possible to purify homogeneously mutant enzyme for biochemical characterization; for example, one question that immediately comes to mind is whether this enzyme is amanitin resistant or sensitive.

Since $RpII140^{Z43}$ caused the Ubx effect but was not detected by the mobility shift analysis, we attempted to locate this mutation by sequencing. We noticed that the four localized *RpII140* mutations were clustered in the C-terminal part of the second-largest subunit; therefore, we sequenced that region; we succeeded in identifying a single-base change from this mutant allele (Table 2).

DISCUSSION

Efficient mapping of point mutations in genes with large coding regions requires an approach that is sensitive and relatively easy to apply, especially if numerous alleles are to be mapped. Approaches that combine PCR with denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, chemical or enzymatic cleavage, and SSCP have been developed (see reference 43 for a review). We selected PCR-SSCP because this method is sensitive to a majority of single-base changes and is technically relatively simple. With this approach, we succeeded in detecting 12 mutations from 14 mutant alleles (86%), demonstrating that PCR-SSCP is a convenient and powerful tool. With one exception, all lesions were point mutations, predominantly G→A or C→T transitions, which is consistent with their having been induced by EMS mutagenesis. We found mutation $RpII140^{A5}$ to be a small deletion, which again, however, is consistent with its induction by γ-ray mutagenesis.

We note that our results, demonstrating that mutant alleles

TABLE 2. Summary of mutations identified

Allele	Recessive lethal	α-Amanitin resistant[a]	Ubx effect	Location by PCR-SSCP (primer pair)	Nucleotide change	Amino acid change
RpII140						
A5	Yes	No	No	12a-b	Δ4058–4072 (CCACCTACTATCAGC)	Δ1047–1051 (Pro-Thr-Tyr-Tyr-Gln)
M39	Yes	No	Yes	11a-b	G-3834→A	Gly-992→Glu
Z19	Yes	No	No	11a-b	G-3941→A	Glu-1028→Lys
Z43	ts[b]	No	Yes	ND[c]	G-3678→A	Arg-940→His
Z45	Yes	No	Yes	12a-b	C-4211→T	Ser-1098→Phe
RpII215						
C4	No	Yes	Yes	8a-b	G-3973→A	Arg-741→His
C20(C4)[d]	ts	Yes	Yes	10a-b	C-4447→T	Leu-878→Phe
E28(C4)[d]	ts	Yes	Yes	4a-b	G-2880→A	Ala-377→Thr
JH1(C4)[d]	No	ND	Yes	7a-b	G-3720→A	Ala-657→Thr
WJK1	ts	ND	No	7a-b	G-3784→A	Ser-678→Asn
WJK2	No	ND	Yes	9a-b	C-4273→T	Leu-820→Phe
Ubl	Yes	No	Yes	10a-b[e]	G-4471→A	Asp-886→Asn
JG1(Ubl)[d]	No	ND	No	15a-b	T-5963→A	Leu-1383→Gln

[a] Capable of growth on medium containing 10 μg of α-amanitin per ml.
[b] ts, temperature sensitive (not capable of growth at 28°C or above).
[c] ND, not determined.
[d] Double mutant also containing the mutation indicated within parentheses.
[e] Detected only with gel type iii (Materials and Methods).

of the *A5* locus contain lesions in the coding region for the 140-kDa RNA polymerase II subunit, have verified the conclusion drawn previously from genetic data (19, 29) that the *A5* locus, not the neighboring *Z6* locus, is indeed *RpII140*, the gene for the second-largest polymerase subunit.

Along with knowledge of the mutant phenotype and comparative information from work with related proteins, identification of the lesion causing a mutation provides information about possible functional roles played by the affected region of the protein. For example, in this work we have identified the alteration that generated the amanitin-resistant mutation *C4*. This mutation changes Arg-741 to His (Fig. 5D), altering a residue that is highly conserved among RNA polymerase II largest subunits and that lies near the location of amanitin resistance-causing mutations in mouse (Asn-793→Asp, corresponding to Asn-784 in the *Drosophila* sequence) (5) and *C. elegans* (Cys-777→Tyr and Gly-785→Glu, corresponding to Cys-779 and Gly-787, respectively, in the *Drosophila* sequence) (25). Considering this observation and another two facts; (i) RNA polymerase II from higher eukaryotes is normally sensitive to low levels of α-amanitin, which binds to the enzyme and inhibits the elongation stage of transcription, and (ii) the *C4* mutant enzyme binds the toxin with a 200-fold-lower binding affinity and has a 2-fold-slower elongation rate in vitro, we think that this conserved protein domain participates in the formation of the amanitin binding site and is importantly involved in the elongation process. One possible role is in the translocation of the enzyme along the DNA template (11). The sequence information in Fig. 5D, on the other hand, places some constraints on our interpretations. For example, the Arg residue altered in *C4* is found in the homologous subunit for yeast polymerase III and for the archaebacterial enzyme, which are not sensitive to amanitin. It seems likely that this Arg residue contributes to an essential function in all of these polymerases (related to elongation?) but that its contribution to the amanitin binding site is determined by context; in amanitin-insensitive enzymes, other differences from polymerase II have already reduced or eliminated amanitin binding. Because the distance from *JH1* to *Ubl*, the interval containing *C4* and other Ubx effect-causing alleles, is actually fairly large (229 amino acid residues), it is possible that

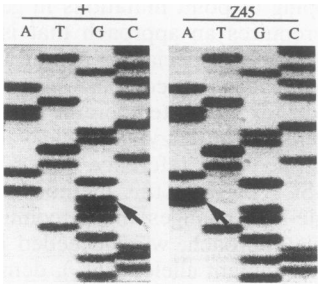

FIG. 3. Identification of the *Z45* mutation by DNA sequencing. PCR products amplified with *RpII140* primers 12a and 12b from wild-type and *Z45*/+ genomic DNA were directly subcloned with the TA cloning system. Several subclones were sequenced with primer 12b. The arrows indicate that a single G (position 4211) in the antisense strand of the wild-type allele is changed into A in the *Z45* mutant allele, corresponding to C-4211→T in the sense strand.

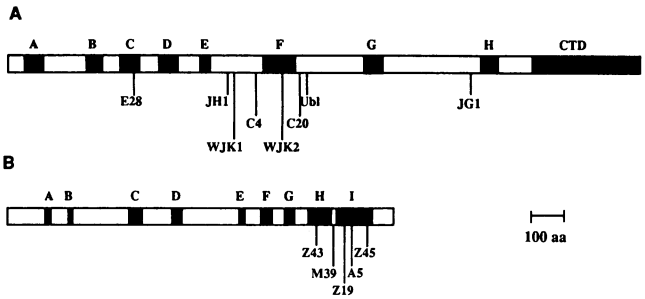

FIG. 4. Distribution of identified mutations. (A) The largest subunit of *Drosophila* RNA polymerase II; (B) the second-largest subunit of *Drosophila* RNA polymerase II. The black boxes represent the regions conserved between the two large eukaryotic subunits and the β′ (23) and β (53) subunits, respectively, of *E. coli* RNA polymerase. CTD, carboxy-terminal repeat domain; aa, amino acids.

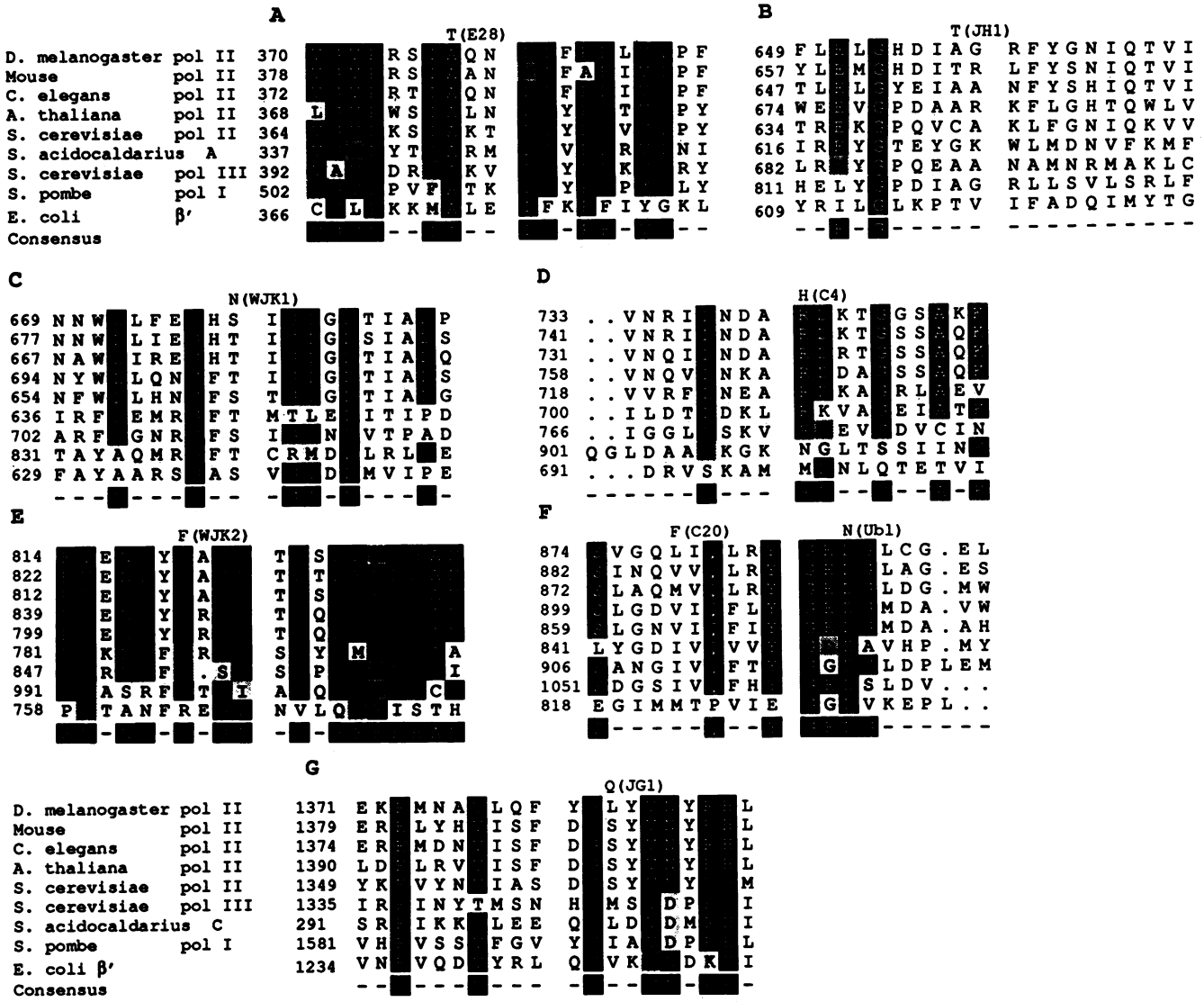

FIG. 5. Comparisons of the sequences around the mutations identified in *RpII215*. For each segment shown, the amino acid sequences of the largest subunit of mouse RNA polymerase II (1), *C. elegans* RNA polymerase II (6), *Arabidopsis thaliana* RNA polymerase II (33), *Schizosaccharomyces pombe* RNA polymerase I (21), *S. cerevisiae* RNA polymerases II and III (2), *Sulfolobus acidocaldarius* RNA polymerase (38), and *E. coli* RNA polymerase (36) are aligned under the sequence of *D. melanogaster* RNA polymerase II (23). Mutationally altered amino acids are indicated above the corresponding wild-type residue, with the mutation name in parentheses. The numbers of the first amino acids of each segment are also indicated. Black boxes indicate identical residues, and grey boxes indicate chemically similar residues. A consensus sequence is displayed along the bottom. The alignment was performed by using PILEUP and then displayed by using PRETTYBOX (Genetics Computer Group programs [14], available through the Duke Comprehensive Cancer Center sequence analysis facility).

different functional properties of the enzyme will be altered by different alleles. It is already known, for example, that an in vitro elongation rate change is not observed for all Ubx effect alleles (see below). Thus, it will be instructive to determine whether amanitin binding, elongation rate, or other functional properties are changed by mutant alleles in this region (e.g., *JH1*, *WJK1*, and *WJK2*).

A temperature-sensitive mutation, *E28*, changes Ala-377 to Thr in the largest subunit (Fig. 5A). The Ala-377 is completely conserved from *Escherichia coli* to humans within homology region C, one of the most conserved regions of the largest subunit, suggesting that the *E28* mutation might alter some basic function carried out by this domain. It has been pointed out that conserved region C of the largest subunits might be responsible for binding DNA or a DNA-RNA hybrid, since this region contains a potential two-helix motif resembling one found in *E. coli* DNA polymerase I and bacteriophage T7 DNA polymerase, which is thought to contact the major groove of the duplex DNA product (2). Future biochemical studies with the *E28* mutant enzyme, such as investigating its ability to bind DNA at higher temperatures, should help to identify basic functions associated with this essential domain.

Other temperature-sensitive lethal *RpII215* alleles (*WJK1* and *C20*) also affect either a very conserved residue or a residue very close to a completely conserved one (Fig. 5C

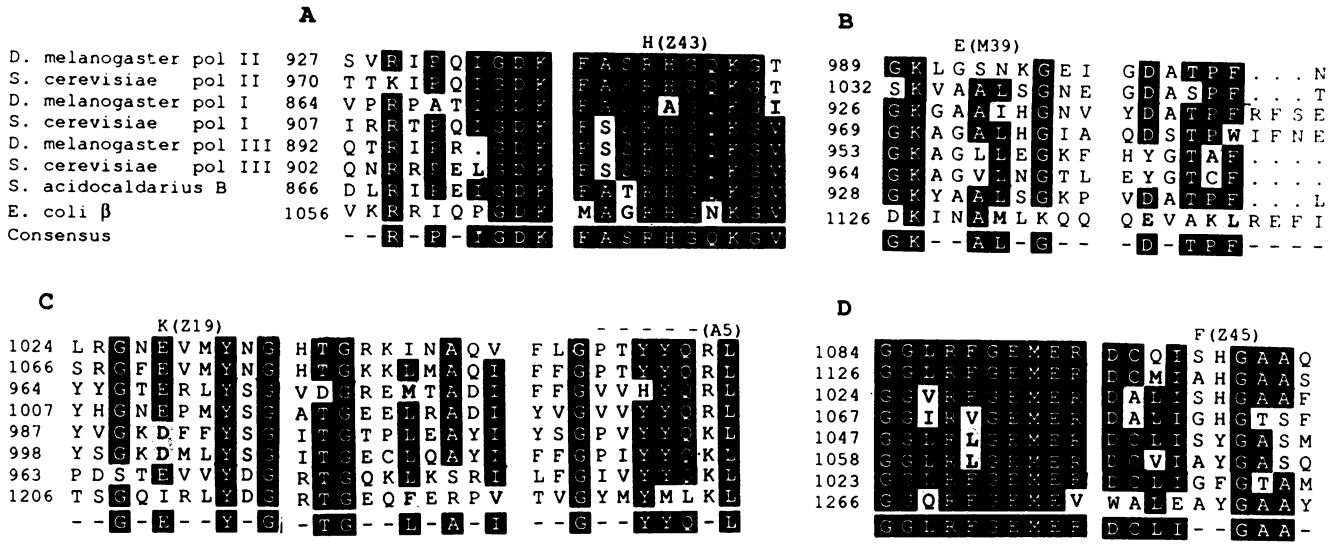

FIG. 6. Comparisons of the sequences around the mutations identified in *RpII140*. Sequence segments from the following second-largest subunits are aligned under the sequence of *D. melanogaster* RNA polymerase II (15, 52) as in Fig. 5: *D. melanogaster* RNA polymerase I (24), *D. melanogaster* RNA polymerase III (49), *S. cerevisiae* RNA polymerase I (56), *S. cerevisiae* RNA polymerase II (53), *S. cerevisiae* RNA polymerase III (22), *S. acidocaldarius* RNA polymerase (38), and *E. coli* RNA polymerase (13).

and F). Again, these alterations probably affect basic functions associated with the domain in which they reside. Alternatively, they might affect folding of the subunit or assembly or stability of the multisubunit polymerase molecule.

Interestingly, all of the *RpII140* mutations, temperature sensitive or recessive lethal, are clustered at the C-terminal part of the second largest subunit, emphasizing that this region is functionally critical; this nonrandom distribution of mapped lesions is reminiscent of a remarkably similar set of observations in yeast cells (48). Figure 6 shows the amino acid changes in context. The original γ-ray-induced lethal mutation *A5* is a deletion of five amino acids in highly conserved region I. Since *A5* behaves like a null, we presume that the deletion has inactivated an essential function of this subunit. This function is probably intimately involved with the catalytic activity of the enzyme, since a highly conserved histidine corresponding to position 1055 in the *D. melanogaster* subunit appears to form part of the catalytic pocket (His-1237 in *E. coli* β [32]). Another recessive lethal mutation, *Z19*, maps very close to *A5* in the same conserved region, changing a highly conserved Glu-1028 to Lys. This position is only two amino acids removed from a Gly-1026 that is altered by a conditional mutation in yeast cells, *rpb2-5* (48); interestingly, *Z19* is not a null but behaves like an antimorph (29a). An invariant residue in the highly conserved sequence block H is altered in the conditional lethal *Z43* allele, namely, Arg-940 to His. Again, this alteration occurs only four positions away from the invariant Lys-936 which corresponds to another residue (Lys-1065 in *E. coli* β) shown to be in the catalytic pocket (55) and critical for the transition from initiation to elongation (reference 32 and references therein). The remaining two mapped *RpII140* alleles (*M39* and *Z45*) affect somewhat less conserved residues but lie within or adjacent to the highly conserved blocks H and I and thus probably also affect directly or indirectly the catalytic center of the polymerase.

Certain *RpII215* mutant alleles (*C4*, *C11*, *Ubl*, *JH1*, and *WJK2*), when heterozygous with a normal *RpII215* allele (e.g., *C4*/+), produce a *Ubx*-like developmental defect (see the introduction). In contrast, homozygous females (e.g., *C4/C4*) or hemizygous males (e.g., *C4/Y*) do not exhibit this Ubx effect. In addition, when most of these mutant alleles are heterozygous with each other (e.g., *C4/WJK2*), the Ubx effect is suppressed. While the dependence of the Ubx effect on heterozygosity at the polymerase locus is poorly understood, the similarity in behavior of these mutant alleles has led to the suggestion that they produce the Ubx effect through similar mechanisms (30). We now provide a structural basis to support this suggestion by showing that the *C4*, *Ubl*, *JH1*, and *WJK2* mutations are clustered in or near homology region F (Fig. 5).

The Ubx effect is functionally equivalent to a reduced expression of *Ubx*. Two ideas have been proposed to explain this phenomenon. One idea is that the *RpII* mutations reduce the transcription of the *Ubx* gene itself. A second idea is that the effect is due to incorrect transcription of genes regulated by the Ubx protein (29). By this explanation, the *RpII* mutations would perturb regulatory interactions between the basic transcription machinery (possibly RNA polymerase II itself) and Ubx protein, a transcriptional regulator.

Additional hypotheses have been proposed to explain the role of heterozygosity in the Ubx effect. One posits that the interaction between the two different kinds of RNA polymerase II occurs at the level of autoregulation of the amount of enzyme; heterozygous cells might misread the amount of functional polymerase, while mutant cells could gauge correctly their reduced levels of activity and compensate by synthesizing more polymerase. An alternative class of hypotheses postulates that the interaction occurs between mutant and wild-type RNA polymerase II enzymes at some step in transcription. For example, one possibility is that during elongation, there is interference between enzymes with intrinsically different elongation rates. Another possibility is that the interaction occurs during initiation-related events. One scenario might be the following: if a mixed population of enzyme, competing for a limiting transcription factor, was less effective in initiation at some promoters than

a homogeneous population was, the level of transcription of a critical gene would fall below a certain threshold and the mutant phenotype would result. The mechanistic details of this competition remain obscure.

Do the mapping data provide support for any of these ideas? The *C4* mutation both causes the Ubx effect and alters the elongation rate under certain conditions in vitro (11). Another mutation, *WJK1*, which maps nearby appears to alter elongation in vivo (28), strengthening the idea that this part of the subunit plays a role in RNA chain elongation. These facts might suggest that the elongation interference model is correct. On the other hand, the in vivo elongation rate for *C4* enzyme, measured on the ecdysone-inducible locus *E74A*, does not differ from that of wild type (54). Apparently the difference observed in vitro is suppressed by other components or conditions in the nucleus. In addition, the in vitro elongation rate of *Ubl* polymerase is the same as that of wild type (11). On balance, it seems unlikely that a simple elongation interference model is correct. An indication that effects on initiation need to be kept in mind stems from the observation that in an in vitro system supporting accurate initiation by RNA polymerase II, *C4* enzyme appears to initiate less efficiently than wild-type enzyme (37).

As mentioned earlier, three of the mutant *RpII140* alleles (*M39*, *Z43*, and *Z45*) also enhance the Ubx phenotype in *Ubx*/+ flies, but unlike the *RpII215* alleles, none of the *RpII140* alleles causes the Ubx effect in the absence of a mutant *Ubx* allele. Because the mutations in the second-largest subunit cause the Ubx effect only in the presence of a mutant *Ubx* allele and because in genetic terms their interactions with the mutations in the largest subunit are additive rather than synergistic (29), it seems likely that they induce the Ubx effect by a mechanism different from that involving mutations in the largest subunit. These alleles contain alterations that probably affect the catalytic center of the enzyme (see above), and their apparent independence from the Ubx effect-causing alleles in *RpII215* might suggest that the altered region in the largest subunit, centered on conserved homology block F, acts in some sense independently from the altered region of *RpII140*, containing conserved blocks H and I.

Finally, the mutant allele *JG1* was also doubly mutant, as expected since it was isolated as a suppressor of *Ubl*-caused lethality. Consistent with its isolation history, *JG1* contains both the *Ubl* alteration (Asp-886→Asn) and a second change some 500 amino acids away in the primary sequence, a Leu-1383→Gln alteration near the C terminus. The *JG1* mutation suppresses both the Ubx effect and the recessive lethality of *Ubl*, indicating that the alteration near the C-terminal-most homology block affects aspects of enzyme function carried out by a more central domain and suggesting that these two parts of the subunit are actually close together in the native enzyme. Interestingly, two other *Ubl* suppressors isolated in the same screen (*JG2* and *JG3*) have in fact lost the *Ubl* mutation, reverting to wild-type PCR-SSCP mobility for all fragments and wild-type DNA sequence in fragment 10 (not shown). The mechanistic origin of these two independent revertants is not currently known.

The mapping data reported here represent a starting point for future investigations that may provide more detailed information about the actual functions of the domains altered by the mapped mutations. For mutant alleles that grow well as homozygotes, it should be possible to isolate enough RNA polymerase II for in vitro functional analyses. In vivo tests of functional alterations might make use of transgene constructs in which promoters of interest are linked to easily assayable reporter genes; possible effects on transcription of the reporter genes by lethally mutant RNA polymerases could then be assessed in heterozygotes or in early embryos, for example. It might also be valuable, for certain mutational alterations, to take advantage of the evolutionary conservation of polymerase subunits and introduce the same change by site-directed mutagenesis into the corresponding gene of *S. cerevisiae*. Subsequently, exploiting the manipulations possible in that organism to investigate the resulting functional consequences might effectively complement studies in *D. melanogaster* and add to our understanding of the roles played by specific domains of RNA polymerase II subunits. Finally, additional application of the mapping strategy described here should facilitate characterization of mutations in *RpII140* already isolated as second-site suppressors of mutations in *RpII215* (28); the results will identify domains likely to be involved in subunit-subunit interactions.

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