TIG — September 1988, Vol. 4, no. 9

Controlling P element insertional mutagenesis

Lynn Cooley, Celeste Berg and Allan Spradling

Two new methods for insertional mutagenesis in Drosophila *are now available. They are based on the ability to control experimentally the number of stable P element insertions per mutagenized strain. One method produces about 10 insertions per genome and has the potential to replace hybrid dysgenesis as a means for cloning genes by transposon tagging. The other mobilizes elements less frequently, producing strains containing a single P element that can be correlated with any resulting mutant phenotype. A library of single-element strains is currently being assembled and will be a valuable resource for* Drosophila *molecular genetics.*

P transposable elements have become powerful molecular and genetic tools for *Drosophila* research. In addition to their role as transformation vectors, P elements mobilized during hybrid dysgenesis have frequently been used to clone genes defined previously only by presumed point mutations[1,2]. Transposon tagging has also localized genetic functions to particular transcripts within cloned regions. Recently, methods for insertional mutagenesis with P elements have been improved[3,4]. It is no longer necessary to mobilize a heterogeneous collection of autonomous and defective elements, such as found in typical P strains, with sometimes unpredictable results. Instead, the structure and activity of the mutagenic elements can be placed under experimental control. These methods have the potential to advance many areas of *Drosophila* research including mutagenesis, gene cloning, recombination mapping and the production of chromosome rearrangements. In this article we discuss controlled P element mutagenesis and point out areas likely to undergo still further improvements.

All variations of controlled mutagenesis use strains containing two types of P element: 'mutator' elements are defective elements that may contain useful genetic markers, and that can be efficiently mobilized from their chromosomal insertion sites only when provided with a source of transposase; 'jumpstarter' elements produce transposase and can therefore catalyse transposition of mutator elements. During a controlled mutagenesis screen, a single jumpstarter element is crossed into a mutator-containing strain, initiating mutator transpositions. In the next generation, segregation of the chromosome bearing the jumpstarter element from the target chromosome stabilizes new mutator insertions. Mutagenesis schemes using controlled mobilization of mutator elements by a jumpstarter element can be grouped conveniently into two categories based on the average number of new insertions generated in each new strain.

Cloning known genes by constructing stable multiple-insert lines

Multiple-insert mutagenesis is an alternative to hybrid dysgenesis for producing insertion alleles of previously identified genes. High levels of mutator transposition are desirable to minimize the effort required to mutate a particular locus. Consequently, each mutagenized strain contains multiple insertions. Although mutator redundancy complicates gene cloning, insert stability guarantees that outcrossing, recombination and reversion tests can be used to identify the relevant mutator insertion and to simplify its molecular isolation.

Figure 1A illustrates the multiple-insert strategy described recently by Robertson *et al.*[4]. To initiate mutagenesis, a strain containing the Δ2-3(99B) jumpstarter element[5] is crossed to a mutator strain containing special 'ammunition' second chromosomes isolated from the Muller-5 Birmingham strain. Both the presence of 17 defective P elements on this chromosome as well as the high transposase levels produced by the Δ2-3(99B) jumpstarter element were chosen to generate the maximum possible insertion rate per F_2 genome. Despite its ability to stimulate high levels of new mutator insertions, the Δ2-3(99B) element rarely catalyses its own transposition. Chromosomes with new insertions that segregate away from the Δ2-3(99B)-bearing chromosome should therefore remain stable. Robertson *et al.* obtained a mutation rate in the *singed (sn)* locus of 2×10^{-3}, and also observed an average of two new X chromosome insertions in each F_2 progeny. Both rates are similar to those that occur during strong P/M hybrid dysgenesis. Thus, about 10 mutator elements are likely to be present in each new mutant strain. The actual design of multiple insert mutagenesis screens will differ somewhat from that shown in Fig. 1A depending on the chromosomal location and phenotype of the target genes.

Practical experience with controlled multiple-insert mutagenesis is still limited. The advantages of insert stability and high mutation rates must be weighed against some remaining drawbacks[4]. In particular, the somatic activity of the Δ2-3(99B) element causes temperature-dependent larval and pupal lethality in the presence of the Muller-5 Birmingham chromosome, presumably due to chromosome breakage. Progeny survive well only at 16°C. Several attempts to improve multiple-insert mutagenesis are currently under investigation. The Δ2-3(99B) jumpstarter is being combined with fewer mutator elements to determine if viability levels are increased. Another approach is to use the 17 element chromosome with a jumpstarter element that cannot function in somatic cells. The value of both modifications will depend on how much they reduce the total mutation rate. Premeiotic transposition events represent a second problem with multiple-insert schemes, since they lower the effective mutation rate. However, eliminating clusters of identical mutations caused by premeiotic transposition would require more effort than simply producing a compensating number of additional lines.

Identification of new mutations using single-insert lines

The generation of strains containing only a single marked mutator element has many advantages as a method of mutagenesis. Phenotypic and molecular analyses of new mutations are greatly simplified. The mutant gene can be mapped by identifying the transposon insertion site using *in situ* hybridization to polytene chromosomes. DNA flanking the insertion site can be cloned simply by screening a small library for P element homology. Revertants, including new alleles generated

by imperfect excision, can be recovered by reintroducing a jumpstarter element and scoring for loss of the phenotype specified by the mutator element's marker gene. Finally, as discussed below, single-insert lines have intrinsic long-term value for manipulating the *Drosophila* genome.

Single-insert lines are generated using mutator and jumpstarter elements that result in much lower transposition rates than those necessary for the multiple-element strategy. Methods for efficiently generating autosomal single-insert lines were described by Cooley *et al.*[3] and have subsequently been refined by C. Berg, D. McKearin and A. Spradling (unpublished). In the example shown in Fig. 1B, mutagenesis is initiated (F_0 generation) by crossing a strain bearing an X-linked ry11 mutator element[6] containing the *Drosophila rosy* gene to a strain with the third chromosome element jumpstarter-1 (Js-1)[3]. Individual males containing both elements are mated to *rosy*$^-$ females (F_1 generation). All daughters from this cross receive the X chromosome bearing ry11 from their fathers and therefore are *rosy*$^+$. Sons inherit their X chromosome from their mothers and are *rosy*$^-$ unless they develop from one of the rare germ cells in which the ry11 element transposed to an autosomal site. Since transposition often occurs premeiotically, saving only one *rosy*$^+$ son per cross ensures that each line contains an independent insertion. As in the multiple-element method, only progeny in which the jumpstarter element has segregated away are saved, thereby ensuring the stability of the new ry11 inserts. The insert-bearing chromosomes are balanced, and homozygotes scored for interesting phenotypes (Table 1). Cooley *et al.*[3] showed that nearly all the recovered mutations (with the exception of background mutations contaminating the starting strains) were caused by new mutator insertions. The X chromosome can be mutated by slightly modifying details of this scheme. For example, ry11 mutator insertions on a y^+Y chromosome were obtained recently (C. Berg and A. Spradling, unpublished), and should allow simultaneous mutagenesis of the X chromosome and autosomes.

Single-element mutagenesis methods can be improved further. The prototypical single-element mutagenesis screen used a mutator strain containing the pUChsneo transposon[7] and produced an average mobilization frequency of 8%; that is, 8% of all F_1 matings produced at least one fertile son inheriting a new autosomal insertion but lacking the jumpstarter element. This frequency improved dramatically to 44% (Table 2) when the ry11 mutator was used as described in Fig. 1B. Recently, even higher frequencies (80–90%) have been obtained using the Δ2-3(99B) jumpstarter element and several different mutators (Y. Hiromi and C. Goodman, pers. commun.). Another disadvantage of using the Js-1 element is a detectable frequency of self mobilization, estimated at 1–2%[3]. However, substituting the stable Δ2-3(99B) element for Js-1 appears to eliminate this problem.

Comparison with chemical mutagenesis

Under what conditions is single-element mutagenesis preferable to chemical mutagenesis? Most genes are likely to be susceptible to ethyl methanesulfonate (EMS) mutagenesis. In contrast, experience with hybrid dysgenesis suggests that P-induced alleles can be obtained with a reasonable effort for only about half the genes tested[2]. However, if molecular characterization is desired, chemical mutagenesis must often be followed by insertional mutagenesis. In many cases it will be more efficient simply to undertake single-element mutagenesis initially. This is especially true when a complex assay limits the total number of chromosomes that can be screened, or when a sizable class of genes with a similar mutant phenotype is to be studied. As discussed below, retention of single-insert lines in a stock center will further increase the usefulness of single-element mutagenesis relative to chemical mutagenesis.

Improving controlled mutagenesis methods

Mutator elements

Greater knowledge of P element transposition mechanisms would facilitate design of improved mutator elements. Element structure, position effects, transposase level and host factors may all influence mobilization rates. Information from transformation experiments provides some guidance to the effect of these variables on mutator transposition rates[8]. Transformation frequency is generally lower for large than for small transposons. However at least some cosmid-sized P elements successfully transpose. Certain sequences such as ribosomal DNA are associated with greatly reduced transformation frequencies, and other sequence-specific variations are probably common. Such information suggests that preference should be given to small mutator elements containing genes that have a history of successful use in transformation experiments.

Position effects on mobilization frequency represent a variable that is not easily assessed from transformation data. If the transposition frequency of a mutator element was highly sensitive to its site of insertion, the choice of a 'good' site might be as important as element structure or copy number. We compared the mobilization rates of two different *rosy*$^+$ transposons[6] from several X chromosome insertion sites (Table 2). All the elements were present in a similar genetic background and were mobilized following crosses to the same strain containing Js-1. The results of these experiments, and of our previous analysis of two sites of pUChsneo insertion, revealed no evidence of strong position effects on mobilization frequency. Thus, additional copies of ry11 may proportionately increase the frequency of useful transposition events. Element structure, however, significantly influenced the rate of jumping. Ry11, which contains a 7.2 kb *rosy*$^+$*Hin*dIII fragment, transposed 4–10 times more frequently than a similar mutator element, ry1, which contains a slightly larger *rosy*$^+$ segment (8.1 kb). It is not known whether an inhibitory sequence present

Table 1. Recessive phenotypes in lines homozygous for a single mutator element insertion

	Mutator	
	pUChsneo[a]	ry11[b]
Lethal	139 (10%)	105 (10%)
Semi-lethal	62 (5%)	39 (4%)
Female sterile[c]	27 (2%)	32 (3%)
Male sterile[c]	14 (1%)	13 (1%)
Visible	5 (0.4%)	5 (0.5%)
Viable, other[d]	1070 (81%)	825 (81%)
Total	1317	1019

[a] From Ref. 3. [b] C. Berg, D. McKearin and A. Spradling, unpublished. [c] The sterile categories include a few leaky lines with fertility ≤ 10% of wild type. [d] Includes lines with background mutations.

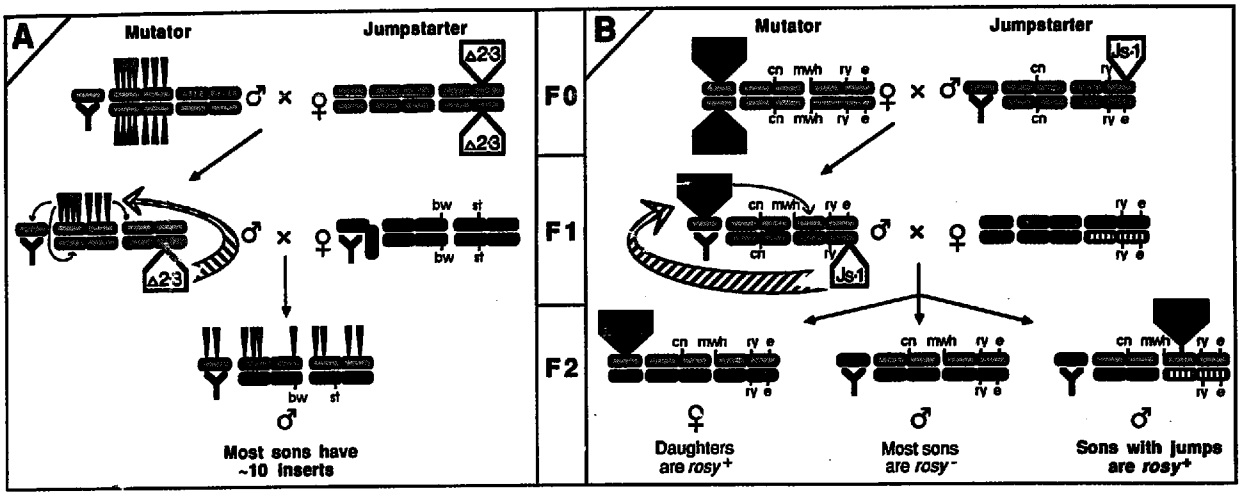

Fig. 1. Multiple- and single-element controlled mutagenesis. (A) Multiple-element mutagenesis as decribed by Robertson *et al.*[4]. The mutator strain contains 17 (seven are shown) defective P elements on a second chromosome isolated from the Muller-5 Birmingham strain. The jumpstarter element is Δ2-3(99B)[5]. Crosses are performed in bottles, en masse. The females used in the F_1 cross contain attached X chromosomes. (B) Single-element mutagenesis of the autosomes. The scheme shown is similar in design to the procedure described by Cooley *et al.*[3] but incorporates an improved mutator element marked with the *rosy* gene that transposes at a higher frequency. The F_0 cross is performed en masse and the F_1 cross in small vials (two males and three virgin females). One *rosy*$^+$ son (lacking jumpstarter) is retained per cross. Chromosomes are drawn in the order X, Y; 2nd; 3rd. Hatched symbols represent balancer chromosomes. Pentagons and triangles are P elements; red symbols indicate mutator elements and yellow symbols indicate jumpstarter elements. The large hatched arrow represents transposase.

near one of the ends of the 8.1 kb fragment or the different site of insertion of the *rosy* fragments within the P element[8] is responsible for these consistent differences. Table 2 also shows that the smaller pUChsneo element is mobilized less efficiently than the ry11 mutator, although the recovery of pUChsneo transpositions may also have been reduced somewhat due to the deleterious effects of G418 selection. Clearly, large changes in mobilization rate may result from even small changes in mutator structure.

Several new mutators exhibit potential advantages that should be tested further. The level of eye pigmentation varies between strains containing insertions of the *white* gene at different chromosomal locations. Unlike the *rosy* gene, the *white* gene is particularly susceptible to such 'position effects', sometimes allowing different insertion sites to be distinguished phenotypically[9,10]. Mutator elements containing a promoterless *lacZ* gene act as sensitive 'enhancer sniffers', allowing the presence of *cis*-regulatory sequences to be identified near the site of insertion by virtue of distinctive patterns of β-galactosidase expression[11]. Mutator elements with strong promoters or regulatory elements near their termini might more readily inactivate nearby genes.

Mutagenesis studies using hybrid dysgenesis suggested that the mutation frequency of certain genes might depend on the wild P strain employed (see Ref. 12). Strain-specific mutagenesis rates are expected on P-derived chromosomes since the sites of pre-existing elements become hotspots for imprecise excisions and chromosome rearrangements that generate local lesions. However site specificity of mutagenesis on chromosomes originally lacking P elements (M chromosomes) would imply that target sites for element insertion could be influenced by the structures or original locations of the elements within the P chromosomes. Because of the many uncontrolled variables in hybrid dysgenesis experiments it is difficult to assess the magnitude of such insertional preferences. However, any substantial preferences should become apparent from a comparison

Table 2. Mobilization of mutator elements from various X chromosome sites

Mutator	Site	Crosses[a]	Jumps[b]	%[c]
pUChsneo pUC8 *hsp neo*	5A	56	5	9
	9C	17 568[d]	1317[e]	8
ry11 Hind3 Hind3 Hind3 7.2 kb *rosy*$^+$	1F	3267[f]	1019[e]	31
	1F	5329[g]	2364	44
	9E	284	82	29
	18A	251	87	32
ry1 Hind3 Hind3 Hind3 1 kb 8.1 kb *rosy*$^+$	4D	99	3	3
	7D[h]	99	2	2
	9A–D[h]	103	8	8
	12D[h]	107	5	5

[a] F_1 crosses as indicated in Fig. 1. [b] Jumps are fertile F_2 sons that do not contain jumpstarter. Only one jump for any F_1 cross is counted. [c] Frequency is defined as percentage of F_1 crosses that yield at least one autosomal jump. [d] Mutagenesis screen reported in Ref. 3. [e] Phenotype distribution of these insertion lines is summarized in Table 1. [f] C. Berg, D. McKearin and A. Spradling, unpublished. [g] L. Yue, C. Berg and A. Spradling, unpublished. [h] The orientation of the 8.1 kb *rosy*$^+$ insert was not determined[6].

of the mutation rates during controlled mutagenesis involving different mutator elements or elements at different locations.

Jumpstarter elements

Mutation rates in controlled mutagenesis schemes may also be improved by changing the jumpstarter element to affect the level of active transposase it produces. The mobilization rate of a given mutator element is expected to be proportional to transposase concentration, at least at low levels. It is not clear whether high transposase levels continue to increase successful mobilization. Alternative pathways such as imprecise excisions or chromosome rearrangements may predominate, possibly even reducing the frequency of transposition of an intact element. High levels of the injected helper P element do not increase transformation rates[8]. Furthermore, the optimum level of transposase may depend on the structure of the particular mutator element under consideration. Finally, even if a high transposase level can increase transposition rates, it may also increase the rate of secondary mutations. Further experiments are required to determine if there is a maximum useful level of transposase in controlled jumping schemes.

Neither of the currently available jumpstarter elements is wholly satisfactory, regardless of the amount of transposase that proves to be optimal. The Δ2-3(99B) element produces high transposase levels and, since it rarely transposes, can be maintained as an inbred stock. While it is useful for single-insert mutagenesis, reduced viability in combination with the large number of elements required for multiple-insert mutagenesis presents practical problems. Autonomous elements, including Js-1, move at sufficient rates to require outcrossing at each generation, as well as occasional Southern blot analysis to ensure that a single element remains at the original site. An ideal jumpstarter element would lack *cis*-acting sequences necessary for mobilization, such as the 'wings-clipped'[13] element used in transformation experiments. Finally, it would be desirable to place transposase production within the jumpstarter strain under experimental control. Regulating the jumpstarter's transposase gene during strain propagation would minimize the possibility of its self-destruction. Control might be imposed by fusing the transposase gene to a tightly regulated promoter or by incorporating unlinked elements that induce the repressed state within the strain. At present neither approach appears very promising. The molecular basis of P element repression remains unknown and inducible promoters that can completely shut off transcription in germ-line cells are not available.

Insertion library

Controlled mutagenesis will generate large numbers of strains containing stable P element insertions. The retention, characterization and dissemination of such lines would facilitate the molecular and genetic definition of a large fraction of *Drosophila* genes. A collection of insertions at known chromosomal sites would allow genomic DNA clones to be linked into a contiguous map. Several factors influence which types of lines should be retained. Multiple-insert lines would initially be more efficient, since fewer strains per gene would have to be maintained; furthermore less work would be required to localize inserts by *in situ* hybridization. However, in the long run single-insert lines containing marked elements will prove to be a better resource. As the collection increased in size, single-insert strains with allelic insertions would be discarded, ultimately reducing the total number of strains that would have to be maintained. In addition, single-insert strains offer unique advantages that extend beyond gene identification and cloning. Any mutation could be genetically mapped between the closest two such insertions in the library and thus localized within specific physical limits. Chromosome deletions encompassing an insertion site can be produced by selecting for loss of the marker carried by the mutator element. This selection will also yield many new alleles of the gene identified by the original insertion. Furthermore, it appears likely that a library of single-insert strains can be used to construct specific chromosome rearrangements whose endpoints are defined by the position of integrated mutator elements (L. Cooley and A. Spradling, unpublished).

An insertion library of single-insert lines will be established at the *Drosophila* Stock Center at Bloomington, Indiana to facilitate these applications. One part of the library will contain insertions that have any associated phenotype that is easily scored in complementation tests. All new lines will have their insertions localized by *in situ* hybridization. They will be complemented with nearby genes, and with existing single-insert lines in the library having inserts in the same chromosomal region. About 2500 such lines could eventually be accumulated (providing an insertion on average every 50 kb throughout the euchromatin) that would identify most of the genes mutable by P element insertion. At present, about 500 single-insert lines have been generated in our laboratory with visible, sterile or lethal phenotypes. While most of these will be added to the insertion library, as the collection grows a smaller fraction of insertions will represent new loci. The approximately 1000 single-insert lines generated in many laboratories by P-element-mediated transformation should also contribute to the initiation of the insertion library. It is expected that only about 15% of such insertions will be responsible for a detectable phenotype (Table 1) thus providing about 150 additional candidate lines for *in situ* hybridization and complementation analysis. However, a potential problem with transformants is the existence of background mutations in some host strains used for transformations.

Insertion stains lacking an obvious detectable phenotype would also be useful. These lines will include strains with insertions causing subtle larval or adult defects whose detection requires behavioral tests, detailed anatomical characterization or biochemical screening. It would not be possible to eliminate allelic insertions from this collection as long as phenotypic effects remained undetected. Initially, only lines whose insertions have been localized by *in situ* hybridization will be accepted in this portion of the library.

As the library increases in size, it will offer alternatives to investigators undertaking mutagenesis. Investigators could receive copies of strains with relevant phenotypes that also bear insertions in genomic regions known to contain a gene of interest. An extensive library would greatly reduce the need for independent screens to isolate insertion mutations in such genes. Screening the library for subtle phenotypes would present greater logistical problems, since it would be impractical to copy and mail all the strains to numerous investigators. Summer courses sponsored by the Stock Center would offer an attractive solution. Each week a manageable

number of lines would be expanded and distributed to individual investigators who had set up equipment for their own assays on site. Providing a fraction of the strains per week to each investigator would be relatively easy. The entire library could be screened by the end of the course. The simultaneous study of the same lines by different investigators would almost certainly generate unexpected and informative surprises.

Acknowledgements

This work was supported in part by USPHS grant GM27875. L. Cooley is a postdoctoral fellow of the Carnegie Institution; C. Berg received postdoctoral support from the American Cancer Society.

References

1 Engels, W. R. in *Mobile DNA* (Berg, D. and Howe, M., eds), ASM Publications (in press)
2 Kidwell, M. (1986) in *Drosophila: A Practical Approach* (Roberts, D. B.,ed.), p. 59, IRL Press
3 Cooley, L. Kelley, R. and Spradling, A. (1988) *Science* 239, 1121–1128
4 Robertson, H. M. *et al.* (1988) *Genetics* 118, 461–470
5 Laski, F. A., Rio, D. C. and Rubin, G. M. (1986) *Cell* 44, 7–19
6 Spradling, A. and Rubin, G. (1983) *Cell* 34, 47–57
7 Steller, H. and Pirrotta, V. (1985) *EMBO J.* 4, 167–171
8 Spradling, A. C. (1986) in *Drosophila: A Practical Approach* (Roberts, D. B., eds), p. 175, IRL Press
9 Levis, R., Hazelrigg, T. and Rubin, G. M. (1985) *Science* 229, 558–560
10 Klemenz, R., Weber, U. and Gehring, W. J. (1987) *Nucleic Acids Res.* 15, 3947–3959
11 O'Kane, C. J. and Gehring, W. J. (1987) *Proc. Natl Acad. Sci. USA* 84, 9123–9127
12 Engels, W. (1985) *Drosophila Inf. Serv.* 61, 70–71
13 Karess, R. and Rubin, G. M. (1984) *Cell* 38, 135–146

L. Cooley, C. Berg and A. Spradling are at the Department of Embryology, Carnegie Institution of Washington, 115 W. University Pkwy, Baltimore, MD 21210, USA.

Transcriptional and post-transcriptional control of plastid mRNA levels in higher plants

Wilhelm Gruissem, Alice Barkan, Xing-wang Deng and David Stern

Accumulation of many plastid mRNAs, but not the relative transcription of their genes, changes considerably during chloroplast development and plastid differentiation. The independence of transcription activity and mRNA accumulation suggests the existence of mechanisms that can modulate the stability of individual plastid mRNAs during plant development. Inverted repeat sequences in the 3' nontranslated regions of most genes form the 3' end of processed mRNAs and may be structural components involved in RNA–protein interactions that could dictate mRNA stability.

The development of photosynthetically active chloroplasts is one of the most dramatic morphogenetic transitions during the formation of cotyledons and leaves in higher plants. Chloroplasts develop from undifferentiated progenitor organelles called proplastids, which lack pigmentation and internal membranes. Thus, the most visible changes are the rapid accumulation of chlorophyll and the development of the complex thylakoid membrane system. Many previously undetectable photosynthetic proteins and their mRNAs appear during the greening process. In contrast, in nonphotosynthetic organs, proplastids differentiate into other specialized plastid types such as amyloplasts, starch-containing plastids found in roots[1]. The mRNAs for photosynthetic proteins are undetectable or present at much lower levels in these organs. Photosynthetically active chloroplasts can also differentiate into plastid types of other distinct functions, such as the carotenoid-synthesizing chromoplasts in orange peel, carrots and tomato fruit. During chromoplast differentiation, the thylakoid membrane system disintegrates and photosynthetic proteins and their mRNAs disappear[2]. However, the differentiation of specialized plastids is not a terminal process, since they maintain their DNA and the competence to revert to chloroplasts[3].

The chloroplast and the nuclear genome together encode proteins of the photosynthetic electron transport chain and of the plastid transcriptional and translational apparatus. In addition, plastid genes code for a complete set of tRNAs and ribosomal RNAs[4,5] (Table 1). Because plastid protein complexes are partially specified in these distinct genomes, chloroplast development and plastid differentiation require communication between the nucleus and organelle to achieve the coordinated expression of interactive gene products. The regulatory role of the nuclear genome during chloroplast development and plastid differentiation in higher plants is obviously complex and less well understood at the present time. However, the complete DNA sequence analysis of the chloroplast genome[4,5] and the development of *in vitro* transcription systems has allowed us to examine the mechanisms that control transcription and mRNA levels in the organelle.

Chloroplast promoter regions: the first control step

The first step at which plastid gene expression might be regulated is transcription initiation. Chloroplast RNA polymerase recognizes specific upstream promoter regions, and for a number of genes, such as that of the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L), the sequences of these promoter regions are highly conserved in different higher plants[6]. Two sequence elements (ctp1 and ctp2) within these conserved regions are critical for promoter function *in vitro* as demonstrated by extensive deletion and mutational analyses, and their organization is similar to that of the *E. coli* consensus promoter (Fig. 1)[7–10]. Other sequence elements between ctp1 and ctp2 may be required for the