Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 400-407

FURTHER ANALYSIS OF A TRANSCRIPT NESTED WITHIN THE ACTIN 5C GENE OF *DROSOPHILA MELANOGASTER*

J. Papa Rao[1] and Ann Sodja[2]

Department of Biological Sciences
Wayne State University, Detroit, Michigan 48208

Received January 27, 1992

Previously we uncovered a 0.45-kb transcript within the 3' end transcribed untranslated region (3'UTR) of actin gene at 5C3-4 (act5C) of *Drosophila melanogaster*. We report here that its sequence bears no similarity to the known DNA or protein sequences. This and act5C transcripts are loaded on different polyribosomal classes. Gel retardation experiments performed with this fragment and several others from act5C reveal no DNA binding activity. The 0.45-kb transcript, initially isolated from different developmental stages of *D. melanogaster* embryogenesis, is also expressed in *Drosophila* Kc tissue culture cells, which will be used in transformation experiments designed to identify regulatory features of the nested gene and its possible interaction at some level with its "host" act5C gene.

In *D. melanogaster* actin encoding genes belong to an oligogene family of six members, each of which displays considerable spatial and temporal specificity of expression (1-5). Actin gene at 5C3-4 (act5C) is the first actin gene transcribed by early embryos and codes for cytoplasmic forms of actin involved in cytoskeletal functions (6,7). The act5C gene consists of at least three exons, two small leader (exons 1 and 2) and one large actin coding (exon 3) (8). Transcription initiation at each of the two leader exons is controlled by separate promoters (8,9). More recently, a third transcription initiation site has been found in exon 2; selection of transcription initiation from these three alternative promoters is developmentally regulated (10,11). Utilization of the three promoters and of three different polyadenylation signals as well as the observed differential intron splicing results in at least nine different transcripts giving rise to multiple actin isoforms, several of which display specific localization pattern during embryogenesis (8,12).

[1] Present address: Department of Crop Protection, Waite Agricultural Research Institute, Glen Osmond, South Australia 5064.

[2] To whom correspondence should be addressed.

0006-291X/92 $1.50

Previously we uncovered a novel 0.45-kb transcriptional unit within the 3'UTR of act5C gene (13). S1 nuclease protection and primer extension analyses revealed that its transcription initiation site was about 21 nucleotides upstream from act5C translation termination signal. The transcript co-terminates with and uses the first of the three polyadenylation signals of act5C gene. The two transcripts share coexpression over most stages of embryogenesis. In an *in vitro* translation system, the transcript translated into a protein of 7.4 kDa, as expected from the size of the open reading frame (ORF). Furthermore, the codon usage of the ORF is qualitatively consistent with that of several *Drosophila* structural genes (13). Overlapping gene arrangement within eukaryotic genomes is not uncommon. Partially overlapping genes on opposite DNA strands were observed in *Drosophila* and rodents (14-16). At the *Gart* locus of *D. melanogaster* and *D. pseudoobscura*, two genes encoding unrelated proteins exist on opposite DNA strands (17-19). In trout *in vitro* expression of two distinct proteins was observed from overlapping ORFs (20). However, none of these overlapping arrangements are identical to the nested organization observed between the act5C and the 3'UTR transcript. The only documented example to date, which approaches the act5C/3'UTR gene organization, is the partially overlapping arrangement of the duplicated sex-regulated genes at the *janus* locus of *D. melanogaster*. As in our case, these two have the same transcriptional orientation (21). Recently it was reported that partial overlap may exist between two duplicated esterase genes in *D. melanogaster* (22).

The temporal overlap in expression and nested arrangement between the act5C and the 0.45-kb genes suggest, but do not *a priori* prove a functional relationship between the two at some level. That one may exist is based on several observations regarding the actin associated 3'UTRs. In vertebrates these are gene specific, are conserved in an orthologeous fashion and diverge at rates lower than expected for a noncoding region (23,24). In some instances a functional role for these sequences *vis a vis* actin gene expression has been demonstrated (25). Extensive information on sequences and/or function of actin-associated 3'UTRs in invertebrates does not exist. We do observe some sequence similarities between 3'UTRs of several dipterans (Hadden and Sodja, manuscript in preparation). Furthermore, a regulatory role for a 3'UTR of an actin gene in *Caenorhabditis elegans* has been demonstrated (26). Hence, we wished to further characterize the act5C 3'UTR nested gene with the ultimate goal of determining its function and its regulation. Here we report that its DNA and protein sequence have no counterpart in the sequence data bases. Specific protein binding to the 3'UTR and to several other regions of act5C was not detected. The act5C and 0.45-kb transcripts associate with different polyribosomal classes. Both are expressed in a tissue culture cell line of *D. melanogaster*.

MATERIALS AND METHODS

Isolation of nuclei. Nuclei from staged 4-h embryos of *D. melanogaster* (Canton S) were prepared according to Hennighausen and Lubon (27) except that antipain, leupeptin and pepstatin A were excluded from the isolation buffer. Embryos were suspended in 5 pellet volumes of 0.3 M sucrose in buffer A containing 10mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM ethylene glycol-bis-(β-aminoethyl ether)-N'-tetraacetic acid (EGTA), 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Lysis was by 10 strokes in glass homogenizer and 2 strokes in the presence of 0.4% Nonidet P-40. The homogenate was spun at 12,000 g for 10 min

in Sorvall SS-34 rotor. The nuclear pellet was washed twice in 5 ml of 0.3 M buffer A without Nonidet P-40.

Nuclear extract preparation. Nuclear extract was prepared (28), as modified by Hennighausen and Lubon (27). Using 10 strokes in glass homogenizer, the nuclear pellet was resuspended in 2.5 ml of buffer containing 400 mM NaCl, 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol and 0.5 mM PMSF. The resuspended nuclei were stirred slowly for 30 min at 4°C and then centrifuged for 30 min at 25,000 g in Sorvall SS-34 rotor. The supernatant was dialyzed for 4 h against 50 volumes of buffer containing 20 mM HEPES-KOH, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol and 0.5 mM PMSF. After dialysis the extract was centrifuged for 20 min at 25,000 g in Sorvall SS-34 rotor to remove the precipitated material. The protein concentration was measured (29), and the extract stored in small aliquots at -70°C.

Isolation of DNA probe fragments and 3'end labeling. The fragments for gel retardation studies were generated as follows. An 8.7-kb actin containing EcoR1 genomic fragment was subcloned into EcoRI site of blue-scribe plasmid. The recombinant plasmid DNA was isolated and digested with EcoRI. The 8.7-kb DNA fragment was electroeluted and digested with SalI to obtain 0.7, 0.8 and 1.1-kb SalI fragments. The 0.45-kb HaeIII/ HindIII fragment was obtained from a pBR322 plasmid containing this fragment (13). A SalI/ EcoRI 2.7-kb fragment was further digested with HindIII to obtain 1.1-kb SalI/ HindIII and 1.6-kb HindIII/EcoRI fragments. All of these DNA probe fragments (Fig. 1) were eluted by freeze squeeze method (30) of appropriate agarose gel slices. The probes were 3'end labeled with [a-^{32}P] dCTP using the Klenow fragment (31); the specific activities were about 1-2 x 10^6 cpm/μg.

Electrophoretic mobility shift analyses. Protein-DNA binding assay was essentially according to Hennighausen and Lubon (27). One μg of nuclear extract was incubated with 0.5 - 1 μg of poly (dI-dC) in 25 ml of buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 12.5% glycerol, and 0.1% Triton X-100 for 30 min at 25°C or 30°C. One ng of labeled DNA probe was added and samples further incubated for 30 min and then analyzed on either 4% polyacrylamide gels (acrylamide:bis acrylamide, 30:1) or 0.5% agarose gels in a low ionic strength buffer of 6.7 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 3.3 mM sodium acetate. The gels were prerun at 50 V for 1 h before loading samples and the buffer was recirculated during electrophoresis. After electrophoresis the gels were placed in 5% glycerol, transferred to whatman 3 MM filter paper, dried in gel dryer at 80°C and autoradiographed.

Isolation and fractionation of polyribosomes. Polyribosomes from *D. melanogaster* (CantonS) 4-h staged embryos and *Drosophila* Kc cells were extracted as described (32). Embryos or Kc cells were homogenized in ice cold extraction buffer of 200 mM Tris-HC1, pH 9.0, 400 mM KC1, 200 mM sucrose, 35 mM $MgC1_2$ and 25 mM EGTA. The homogenates were centrifuged in Sorval SS-34 rotor at 15,000 rpm for 10 min at 4°C. The supernatant was layered on 3.5 ml sucrose cushion buffer composed of 40 mM Tris-HC1, pH 9.0, 200 mM KC1, 30 mM $MgC1_2$ and 5 mM EGTA, and centrifuged at 36,000 rpm in 40 Ti fixed angle rotor for 5 h at 4°C in L5 Beckman Ultracentrifuge. The polysomal pellet was drained and resuspended in 40 mM Tris, pH 8.5, 200 mM KC1, 30 mM $MgC1_2$, 5 mM EGTA, and used immediately or stored at -70°C.

Polyribosomes were layered on 12.5 - 50% sucrose gradient prepared in 40 mM Tris, pH 8.5, 200 mM KC1, 30 mM $MgC1_2$, and 5 mM EGTA (33). The gradients were centrifuged at 39,000 rpm for 75 min in SW 41 Beckman rotor andfractionated using an autodensiflow fractionator attached to UA 5 UV monitor. Fractions of 0.5 ml were collected and 2 volumes of ethanol were added to precipitate polyribosomes at -20°C overnight. Polyribosomes were collected by centrifugation at 4°C and used for RNA isolation.

RNA isolations and Northern hybridization analyses. Polyribosomes from Kc cells or from individual sucrose gradient fractions were diluted with 0.5 volume of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and RNA extracted with an equal volume of phenol-chloroform (1:1) containing 0.1% SDS. RNA was precipitated by addition of 0.12 volumes of 3 M sodium acetate, pH 5.2, and 2 volumes of cold ethanol at -20°C. Poly A+ RNA was obtained by affinity chromatography on oligo-(dT) cellulose (34).

The RNA so obtained was electrophoresed on 10 mM methylmercury hydroxide gels and transferred to Biodyne nylon transfer membranes (ICN) according to the ICN technical manual. The 0.8-kb SalI actin coding and 0.45-kb HaeIII/HindIII 3'UTR fragments (Fig. 1) were labeled

by nick translation to specific activities of 10^7 - 10^8 cpm/μg (31). Hybridizations were in 50% Formamide, (recrystallized and/or deionized), 5 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS, 2 x Denhardts (1 x Denhardts = 0.02% bovine serum albumin, 0.02% polyvinylpyrolidone, 0.02% Ficoll), 100 mg/ml sheared denatured salmon sperm DNA at 42°C for 48 h with gentle shaking. Filters were washed in 2 x SSC, 0.1% SDS buffer twice for 10 min at room temperature and in 0.1 SSC, 0.1% SDS at 50°C twice for 15 min. Autoradiography was performed using prefogged x-ray film and intensifying screens for 1-4 days at -70°C.

RESULTS AND DISCUSSION

Comparison of a sequence from a novel DNA fragment with other known sequences is useful in deducing the function of such newly isolated DNA. From our previous studies (13), the DNA and the deduced amino acid sequences of the 0.45-kb gene were known. A search of published sequences revealed no sequences similarities of the 0.45-kb gene to the known DNA or amino acid sequences. Thus, we could not speculate on a possible function of the 0.45-kb gene.

In attempts to gain some understanding about the origin of the 0.45-kb transcript (i.e. is it a product of a co- or postranscriptional event), gel retardation experiments were performed using nuclear extracts, which contain transcriptional and other regulatory factors with specific DNA binding properties. Previous band shift experiments identified several DNA sequences upstream from the act5C promoter regions to which some factor(s) from the nuclear extract binds (9,10,35). Hence we assayed the nuclear extracts for DNA binding ability to a number of restriction enzyme fragments from the 8.7-kb EcoR1 fragment (Fig. 1). The samples in lanes 1,3,5,7 (panel A) and 9 and 11 (panel B) of Fig. 2 are control DNA fragment probes not incubated with nuclear extracts. Lanes 2,4,6,8 (panel A) and 12 (panel B) of Fig. 2 are experimental, revealing no detectable complexes with probes A, B, C, D and F, respectively (Fig 1). However, in lane 10 of Fig. 2B,

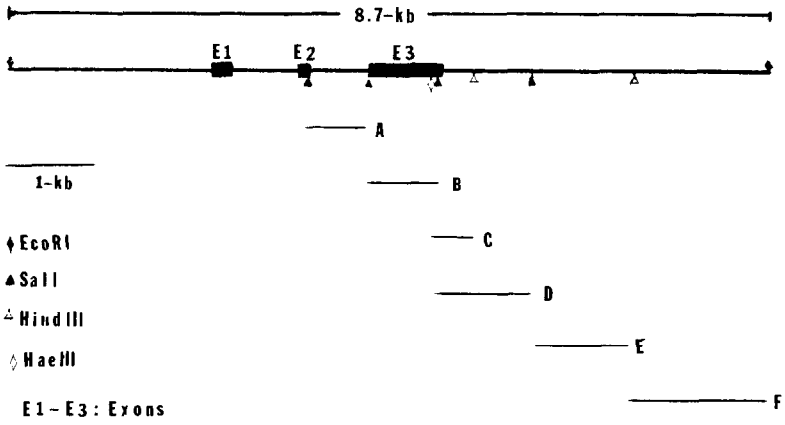

Fig. 1. Partial restriction enzyme map of act5C 8.7-kb EcoRI fragment and probes generated from it. The probes A-F are: (A) 0.7-kb SalI; (B) 0.8-kb SalI; (C) 0.45-kb HaeIII/HindIII; (D) 1.1-kb SalI; (E) 1.1-kb SalI/HindIII; and (F) 1.6-kb HindIII/EcoRI fragments. These fragments were 3'end labeled with [α-^{32}P] dCTP and Klenow fragment for use in gel mobility shift assays.

probe made to fragment E (Fig. 1), there is observed a shift in electrophoretic mobility, suggesting presence of a protein-DNA complex. This is an interesting observation the significance of which needs further clarification. Perhaps this defines a regulatory region for a transcriptional unit known to exist downstream from the 8.7-kb fragment (2). The results do not exclude the possibility that this downstream region may exert regulatory effects on the 0.45-kb gene expression. It is possible, but unlikely, that any one of the act5C promoters controls the expression of the 0.45-kb gene, since even the most proximal would have to act over an almost 3-kb distance, an unprecedented observation if true. It is unlikely that the nested transcript is the result of a splicing event since no canonical splice junction sequences were detected. Furthermore,

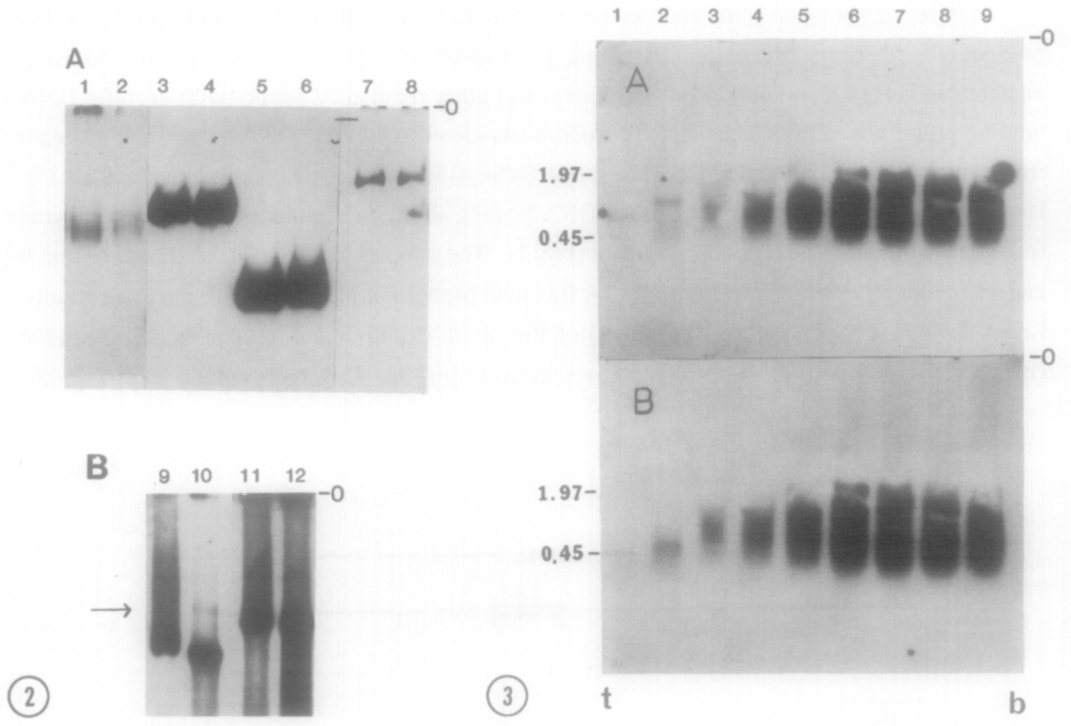

Fig. 2. Electrophoretic gel mobility shift assays. The binding reactions contained 1 μg of nuclear extract from 4-h embryos and 0.1 ng of ^{32}P-labeled DNA fragments A-F (Fig. 1). The samples in panel A were subjected to electrophoresis in polyacrylamide gels and those in panel B were subjected to electrophoresis in 0.5% agarose gels. Control in lanes 1,3,5,7,9 and 11 show naked probes of A-F, respectively; the binding reactions with probe fragments A-F are shown in lanes 2,4,6,8,10 and 12. A DNA-protein complex that shifted the mobility of probe fragment E in lane 10 is indicated by an arrow.

Fig. 3. Northern blot hybridization analysis of RNAs isolated from polysome fractions. RNAs were electrophoresed on 1% agarose gel containing 10 mM methylmercury hydroxide, transferred to Biodyne nylon membrane and hybridized with ^{32}P-labeled nick translated act5C 0.8-kb SalI actin coding sequence (panel A). The blot was dehybridized and reprobed with 0.45 kb HaeIII/HindIII act5C 3'UTR sequence (panel B). The size standard used was the RNA ladder (.5 - 0.24-kb) obtained from BRL. The origin of migration is indicated by the letter O; letters t and b denote the top and the bottom of the gradient, respectively.

if splicing were responsible for generation of the nested transcript, one would expect its appearance during embryogenesis to completely coincide with that of act5C. We observed such overlap in most but not in all developmental stages (13). In case of the duplicated genes at the *janus* locus each is under its own transcriptional regulation. In addition to the topological problems that the RNA polymerases encounter, the expression of the duplicated gene is hampered probably because its 5' end regulatory region resides within the 3' end region of the original gene. Presumably this region contains termination signals for its expression (36). It is possible that the regulatory signals for the 3'UTR sequence are within the act5C coding region and our binding assays did not detect these for some reason. Appropriate 5' end deletion constructs of the 8.7-kb fragment and a functional assay will enable us to identify regulatory regions.

In previous studies we demonstrated that the 0.45kb transcript was associated with polyribsomes (13). We wished to determine whether it associated with same polysomal class as the act5C transcript of about 1.97-kb or not. The former case might suggest the shorter transcript and act5C interact to somehow modulate translational efficiency. To this end polyribosomomes from 4-h embryos (earliest time at which the 0.45-kb transcript was detected) were fractionated on sucrose gradients and poly A+ RNA, isolated from individual polysome fractions, was analyzed by Northern blot hybridization. The Northern blots were probed with 0.8-kb SalI actin coding sequence and 0.45-kb HaeIII/ HindIII 3'UTR sequence. The results are shown in Fig. 3. The 0.8-kb probe visualized the 1.97-kb actin transcript in lane 6 of Fig. 3, panel A; and the 0.45-kb probe hybridized to the 0.45-kb 3'UTR transcript in lanes 2, and also to 1.97-kb act5C transcript in lane 6, panel B. In an *in vitro* translation system the 3'UTR transcript appears to translate into a protein whose size agrees with the size of the open reading frame (68 amino acids). The polysomal fractionation results further support that the 3'UTR transcript is most likely translated *in vivo*.

To determine whether *Drosophila* Kc cells express the 3'UTR transcript, polysomal poly A+ RNA was isolated and analyzed by Northern hybridization blots. The probes used were nick translated 0.8-kb SalI actin coding sequence and 0.45-kb HaeIII/ HindIII 3'UTR sequence of act5C. Fig. 4. shows that the 0.8-kb probe hybridized only to the 1.97-kb act5C transcript (lane a), whereas the 3'UTR probe hybridized to both the act5C as well as the 0.45-kb transcript (lane b). This is expected since the HaeIII/ HindIII 3'UTR fragment is a gene specific probe for act5C. The results obtained indicate that the Kc tissue cultures cells express the nested transcript, suggesting that it is not involved in development and differentiation. Because the actin isoforms in *D. melanogaster* share over 95% amino acid sequence similarity and all of them resemble vertebrate cytoplasmic actins, we postulated that the function of the 3'UTR gene product may be in specifying the function of the different actin isoforms (13).

The expression of the 0.45-kb gene in Kc cells was of practical importance to us, since we can utilize them in transient transformations with recombinant vectors containing the act5C promoters, followed by reporter gene sequence that will be flanked with act5C 3'UTR instead of the SV40 3' end sequence. The construction of these vectors is now underway. These will allow us to determine whether the 3'UTR transcript modulates in any way the expression of the act5C gene. Further studies should enable us to determine regulatory elements dictating 3'UTR gene expression. Results from these investigations should yield some insight into its function.

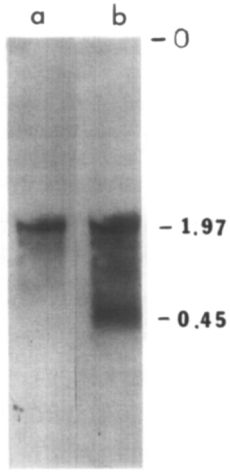

Fig. 4. Northern blot hybridizations of polysomal poly A$^+$ RNA from Kc cells. Ten μg of polysomal poly A+ RNA was electrophorsed on 1% agarose gel containing 10 mM methylmercury hydroxide, transferred to Biodyne nylon membrane and hybridized with ^{32}P-labeled nick translated act5C 0.8-kb SalI actin coding sequence (lane a). The blot was dehybridized and reprobed with 0.45 kb HaeIII/HindIII act5C 3'UTR sequence (lane b). The size standards used were as in Fig. 3. The origin of migration is indicated by the letter O.

ACKNOWLEDGMENTS

We are grateful to Drs. Stephen Krawetz (Wayne State University) for performing the search of sequence data bases and to Lucy Cherbas (University of Indiana, Bloomington) for her generous gift of Kc cells. We thank Mr. Timothy Hadden for critical evaluation of the manuscript. This research was supported by American Heart Association/MI, the NIH-GM08167, and the Center for Molecular Biology (CMB, Wayne State University) grants to A.S. J.P.R. was supported by a postdoctoral fellowship from the CMB.

REFERENCES

1. Tobin, S.L., Zulauf, E., Sanchez, F., Craig, E.A., and McCarthy, B.J. (1980) Cell 19, 121-131.
2. Fyrberg, E.A., Kindle, K.L., Davidson, N., and Sodja, A. (1980) Cell 19, 365-378.
3. Fyrberg, E.A., Bond, B.J., Hershey, N.D., Mixter, K.S., and Davidson, N. (1982) Cell 24, 107-116.
4. Fyrberg, E.A., Mahaffey, J.W., Bond, B.J., and Davidson, N. (1983) Cell 33, 115-123.
5. Sanchez, F., Tobin, S.L., Rdest, U., Zulauf, E., and McCarthy, B.J. (1983) J. Mol. Biol. 163, 533-551.
6. Sodja, A., Arking, R., and Zafar, R.S. (1982) Dev.Biol. 90, 363-368.
7. Sodja, A., Rizki, R.M., Rizki, T.M., and Zafar, R.S. (1982) Chromosoma 86, 293-298.
8. Bond, B.J., and Davidson, N. (1986) Mol Cell. Biol. 6, 2080-2088.
9. Bond-Mattews, B., and Davidson, N. (1988) Gene 62, 289-300.
10. Chung, Y., and Keller, E.B. (1990) Mol. Cell. Biol. 10, 206-216.
11. Vigoreaux, J.Q., and Tobin, S.L. (1987) Genes Dev. 1, 1161-1171.
12. Burn, T.C., Vigoreaux, J.O., and Tobin, S.L. (1989) Dev. Biol. 131, 345-355.
13. Rao, J.P., Zafar, R.S., and Sodja, A. (1988) Biochim. Biophys. Acta 950, 30-44.
14. Spencer, C.A., Gietz, R.D., and Hodgetts, R.B. (1986) Nature 322, 279-281.
15. Williams, T., and Fried, M. (1986) Nature 322, 275-279.

16. Adelman, J.P., Bond, C.T., Douglass, J., and Herbert, E. (1987) Science 235, 1514-1517.
17. Henikoff, S., Keene, M.A., Fechtel, K., and Fristrom, J.W. (1986) Cell 44, 33-42.
18. O'Hare, K. (1986) Trends Genet. 2, 33.
19. Henikoff, S., and Eghtedarzadeh, M.K. (1987) Genetics 117, 711-725.
20. Janowski, J.M., Krawetz, S.A., Walozyk, E., and Dixon, G.H. (1986) J. Mol. Evol. 24, 61-71.
21. Yanicostas, C., Vincent, A., and Lepesant, J-A. (1989) Mol. Cell. Biol. 9, 2526-2539.
22. Collet, C., Nielsen, K.M., Russell, R.J., Karl, M., Oakeshott, J.G., and Richmond, R.C. (1990) Mol. Biol. Evol. 7, 9-28.
23. Guning, P., Ponte, P., Blau, H., and Kedes,L. (1984) J.Mol. Evol. 20, 202-214.
24. Lee, J.J., Shott, R.J., Rose III, S.J., Thomas, T.L., Britten, R.J., and Davidson, E.H. (1984) J. Mol. Evol. 172, 149-176.
25. DePonti-Zilli, L., Seiler-Tuyns, A., and Paterson, B.M. (1988) Proc. Natl. Acad. Sci. USA 85, 1389-1393.
26. Krause, M., Wild, M., Rosenzweig, B., and Hirsch, D. (1989) J.Mol. Biol. 208, 381-392.
27. Hennighausen, L., and Lubon, H. (1987) In Methods in Enzymology (S.L. Berger and A.R. Limmel, Eds.), Vol. 152, pp. 721-735, Academic Press, New York.
28. Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475-1489.
29. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
30. Tautz, D., and Renz, M. (1983) Anal Biochem. 132, 14-19.
31. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 207-209.
32. Jackson, A.O., and Larkins, B.A. (1976) Plant Physiol. 57, 5-10.
33. Hari, V. (1980) Planta 148, 491-497.
34. Bantle, J.A., Maxwell, I.H., and Hahn, W.E. (1976) Anal. Biochem. 72, 413-427.
35. Parker, C.S., and Topol, J. (1984) Cell 36, 357-369.
36. Yanicostas, C., and Lepesant, J-A. (1990) Mol. Gen. Genet. 224, 450-458.