BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 217–221 (1998)
ARTICLE NO. RC988601

Generation of a Tropoelastin mRNA Variant by Alternative Polyadenylation Site Selection in Sun-Damaged Human Skin and Ultraviolet B-Irradiated Fibroblasts

Elaine Schwartz,[1] Joel M. Gelfand,* James C. Mauch, and Lorraine H. Kligman†
*Department of Dermatology, Mount Sinai School of Medicine, New York, New York 10029; *Harvard Medical School, Boston, Massachusetts 02138; and †Department of Dermatology, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Received April 1, 1998

The goal of this research was to delineate the post-transcriptional mechanisms responsible for the increased elastin synthesis characteristic of sundamaged skin. In this study, a unique molecular variant of the tropoelastin mRNA transcript was identified in human sundamaged skin that was derived from the usage of an alternate polyadenylation site. Nonsolar exposed human skin expressed one tropoelastin mRNA species whereas sundamaged human skin expressed the primary tropoelastin mRNA and a larger, alternate tropoelastin mRNA formed from the utilization of a second polyadenylation site. Cultured human skin fibroblasts expressed both tropoelastin transcripts and in vitro UV treatment increased the amount of the unique tropoelastin mRNA. Hairless mouse skin (normal and UV treated) expressed the primary tropoelastin transcript although UV irradiation increased the length of its poly (A) tail two-fold. Therefore, UV radiation may stimulate elastin production by affecting polyadenylation site selection and the poly (A) tail length of tropoelastin mRNA.

Elastin, a connective tissue protein, is responsible for maintaining the elasticity of the skin (1). It is synthesized in a soluble form (tropoelastin), becomes cross-linked and, in combination with microfibrillar proteins, forms elastic fibers in the extracellular matrix. Under normal circumstances, elastin production is significant in the fetal and neonatal periods and then decreases after birth (2). The amount of elastin remains relatively constant in normal adult skin due to a slow metabolic turnover (3). However, several disorders exist in adult skin that are characterized by increased elastin accumulation (4-7) including photoaging. Evidence suggests that posttranscriptional mechanisms control the cessation of elastin synthesis in mature tissues (2) and these mechanisms may be involved in the stimulation of elastin expression in these disorders.

Previous work demonstrated that posttranscriptional mechanisms are involved in the induction of tropoelastin synthesis by ultraviolet radiation. Ultraviolet B (UVB) irradiation of cultured human fibroblasts stimulates tropoelastin production by increasing the efficiency of translation of the tropoelastin mRNA (8). Furthermore, steady-state tropoelastin mRNA levels are similar in human skin biopsies taken from sundamaged and sunprotected sites (9). The hairless albino mouse is a commonly used animal model for the study of photoaging and photocarcinogenesis (10-12). In this model, steady-state tropoelastin mRNA levels in skins from 12 week UVB irradiated mice remain unaffected by UV exposure in spite of increased elastin accumulation (13). Regulatory mechanisms therefore are present in adult skin that can stimulate elastin synthesis in response to certain conditions including exposure to ultraviolet radiation.

Cis-acting sequences in the 3′ untranslated region (3′-UTR), polyadenylation site selection, and poly (A) tail length of several genes participate in the regulation of mRNA translation and stability (14-17). Many genes have multiple polyadenylation signals and the site selected has been correlated with development and tissue specificity (18, 19). The polyadenylation of the transcript influences the amount of protein produced due to changes in its mRNA stability and translatability.

In this study we describe the effect of ultraviolet radiation on polyadenylation site selection and poly (A) tail length in the tropoelastin transcript. The 3′ UTR from tropoelastin mRNA contains two polyadenylation signals located 230 bp apart (20). Our results demon-

[1] Corresponding author: Department of Dermatology, Bx 1047, Mt. Sinai School of Medicine, One Gustave L. Levy Pl, New York, NY 10029. Fax: (212) 348-7434.

0006-291X/98 $25.00

strated that exposure to ultraviolet radiation can affect polyadenylation site selection in sundamaged human skin and UVB irradiated fibroblasts and poly (A) tail length in UVB irradiated mouse skin.

MATERIALS AND METHODS

Preparation of RNA from skin samples. Two postsurgical cheek skin specimens obtained from 70 year old white males and one posterior neck skin specimen obtained from a 78 year old white male represented the sundamaged samples. One breast skin specimen (71 year old white female), one leg skin specimen (77 year old black female), and one foreskin specimen represented the nonsolar exposed samples. The skin specimens were immediately frozen in liquid nitrogen and total RNA was extracted from the skins using TRIzol reagent (Molecular Research Center, Inc., Cincinnati, OH). The yield of RNA was quantified from the optical density.

Cell culture of human skin fibroblasts. Cells were derived from skin biopsy explants of healthy individuals. Fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GibcoBRL, Grand Island, NY), 1X antibiotic-antimycotic (penicillin- streptomycin - fungizone; 100X GibcoBRL) and 1X nonessential amino acids (100X, GibcoBRL). Cells were passaged with a 1:3 split and fed twice weekly. Confluent cultures (early passages, 4-8) were irradiated in 100 mm^2 dishes using Westinghouse FS20 sunlamps (4.5 mJ/cm^2 of UVB). Cell layers were treated after removal of the media and gentle rinsing with Hanks' balanced salt solution. The dish lids were removed during the irradiation procedure. Immediately after irradiation, the cultures were incubated with fresh media. Sham-treated fibroblasts from the same strain served as the controls. Five days following irradiation, total RNA were extracted using RNA Stat 60 (Tel-test B, Inc., Friendswood, TX).

Animals. Female albino hairless mice (Skh - hairless - 1; Charles River Laboratories, MA) were irradiated with UVB thrice weekly for 10 weeks with a bank of 8 cellulose triacetate filtered Westinghouse FS-40 tubes. Each exposure was a previously determined 1 minimal erythema dose (MED: $0.04J/cm^2$) and required approximately 3 minutes. Appropriate age-matched, unirradiated mice served as the control group. Total RNA was extracted from the dorsal skin of the mice using TRIzol reagent.

Assessment of 3′ UTR of tropoelastin transcripts by 3′ RACE. The 3′ RACE system for rapid amplification of cDNA ends (RACE) was used according to manufacturer's instructions (GibcoBRL, Grand Island, NY). In the 3′ RACE reaction, the oligo (dT) primer contains a short sequence of bases at the 5′ end forming an anchor primer (AP). This anchor sequence is copied at the end of the coding strand during amplification (AUAP primer). Essentially, first strand cDNA synthesis was initiated at the poly (A) tail of mRNA using the adapter primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′; AP) and SuperScript II reverse transcriptase. Samples were incubated for 50 minutes at 42°C. The RNA template was removed from the cDNA:RNA hybrid molecule by digestion with RNase H.

Amplification of regions of the 3′ UTR of tropoelastin cDNA was performed using a sense primer specific for the 3′ UTR of tropoelastin and an antisense primer provided with the system (5′- GGCCAC-GCGTCGACTAG-TAC -3′; AUAP). An aliquot (2.5 μl) of the cDNA preparation was added to the PCR (polymerase chain reaction) mixture: PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM concentration of each deoxynucleotide, 0.2 mM of each primer, 5 μCi $dATP^{32}$, and 1 U of AmpliTaq DNA Polymerase (Perkin-Elmer, Foster City, CA) in a final volume of 25 μl. To prevent evaporation, 25 μl of mineral oil (SIGMA, St. Louis, MO) was added and the tubes were placed in a thermocycler (ERICOMP, San Diego, CA). PCR amplification proceeded for 30 cycles: 94°C for 1 min; 55°C for 2 min; 72°C for 2 min. The final elongation step was 72°C for 8 min. Table I summarizes the primers used in the amplification reactions.

TABLE 1

Nucleotide Sequence of Synthetic Primers Used for Amplification

Primers	Position[1]	Nucleotide sequence
EL3	595–617	5′-CCACACTGGGAATAGCCACCTTG-3′
EL4	1238–1260	5′-CCAGGCCAACAGTTGAAGGCAGA-3′
rEL1	150–170	5′-CACAGGACAAGGAAATCAGAC-3′
rEL2	877–899	5′-CTATTACAGAGCATGTCTCACCA-3′

[1] Numbered as described in (20) for human tropoelastin gene and as described in (22) for rat tropoelastin gene.

PCR products were separated on 4% NuSieve 3:1 Plus agarose gels (FMC BioProducts, Rockland ME) and visualized with ethidium bromide staining. However, only faint bands representing the alternative transcript was seen with ethidium bromide staining; the major product was the primary transcript. Therefore, aliquots of the amplification reaction using EL3 and AUAP primers were reamplified using primers EL3 and EL4 in order to purify sufficient quantities of the alternate transcript for sequencing. The products corresponding to the two tropoelastin transcripts were extracted from the agarose gel (QIAquick gel extraction kit, QIAGEN, Chatsworth, CA), and sequenced (Sequetech Corporation, Mountain View, CA). The specificity of the PCR product was determined by comparison of expected PCR product size with the actual size of the amplified DNA, by restriction enzyme digestion using ScaI and by direct sequencing. Radiolabeled PCR products were separated on a 10% polyacrylamide gel (Zaxis, Inc., Hudson, OH). Following electrophoresis, the gel was dried and exposed to film.

RESULTS

Induction of multiple tropoelastin transcripts by UV in human skin. An initial assessment of polyadenylation sites and poly (A) tail length of tropoelastin mRNA was performed by applying the 3′ RACE technique using EL3 and AUAP primers and subsequent digestion of the products with the restriction enzyme ScaI. PCR products were resolved on agarose gels and ethidium bromide staining revealed only faint bands corresponding to the alternative transcript (not shown). Therefore the PCR products were radiolabeled with $dATP^{32}$ in order to visualize the alternative tropoelastin mRNA species by autoradiography. Radiolabeled 3′ RACE products were separated on 10% polyacrylamide gels and visualized by autoradiography (Figure 1). Results shown in Figure 1 were obtained with total RNA prepared from two nonsolar exposed and two sundamaged skins. Similar results (not shown) were obtained with the remaining nonsolar exposed and sundamaged skins. The size of the major PCR product seen in lanes 1-4 was approximately 400 bp (primary tropoelastin mRNA species); an additional product of 700 bp (alternative tropoelastin mRNA) was produced using total RNA from sundamaged skin (lanes 3 and 4). Sequencing data identified these PCR products as tropoelastin

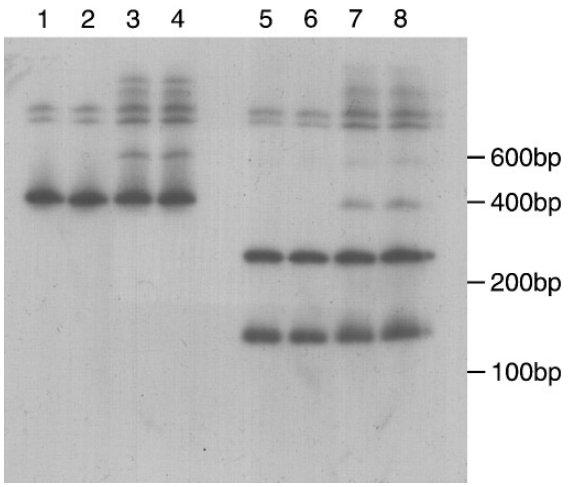

FIG. 1. 3′ RACE products and restriction endonuclease digestion patterns of radiolabeled 3′ RACE products obtained from the reverse transcription and amplification of total RNA prepared from sundamaged and nonsolar exposed human skins. Aliquots of the amplification reactions and ScaI digestions were run on 10% polyacrylamide gels and dried gels were autoradiographed. Lanes 1-4 show undigested PCR products from total RNA prepared from nonsolar exposed (lanes 1 and 2) and sundamaged (lanes 3 and 4) human skins. Lanes 5-8 show ScaI digestion patterns of PCR products from total RNA prepared from nonsolar exposed (lanes 5 and 6) and sundamaged (lanes 7 and 8) human skins.

cDNA and these products represent multiple tropoelastin transcripts produced from differential polyadenylation site usage. Nonsolar exposed human skin therefore expressed only the primary tropoelastin transcript that utilized the 5′ polyadenylation site. In contrast, human sundamaged skin expressed two variants of the tropoelastin transcript formed by the utilization of two polyadenylation sites.

The tropoelastin gene contains a ScaI site at 855 nt and therefore digestion of the 3′ RACE products generates two fragments. The 400 bp and 700 bp 3′ RACE products were susceptible to ScaI digestion. Fragments generated by ScaI digestion of 3′ RACE products are shown in lanes 5-8 (Figure 1). PCR products greater than 700 bp were not digested by ScaI and therefore were nonspecific products of the amplification reaction. The major fragments were about 260 bp (corresponding to EL3 to ScaI fragment) and 135 bp (corresponding to ScaI to 5′ polyadenylation site). The size of the fragment derived from ScaI to the 3′ polyadenylation site (lanes 7 and 8) was approximately 410 bp.

The location of the cleavage/polyadenylation site downstream of the poly (A) signal utilized in the primary tropoelastin transcript was determined by direct sequencing of the 400 bp 3′ RACE product (Table II). This site previously had not been identified in the human tropoelastin transcript and resembled the site found in the bovine tropoelastin transcript (21). The cleavage/ polyadenylation site downstream of the alternate polyadenylation signal was not identified because primer set EL3/EL4 was used to isolate this transcript for sequencing.

TABLE II

Partial Sequence of the 400-bp PCR Product at Cleavage/Polyadenylation Site

AATAAATATTTTATTTTTTGTCCTGG-$(A)_n$		
951		976

Note. 3′ RACE PCR products were generated from total RNA purified from human skin and were sequenced. The canonical polyadenylation signal is underlined and numbering is as in (20).

UV induction of multiple tropoelastin transcripts in cultured dermal fibroblasts. An initial assessment of polyadenylation site usage was performed by applying 3′ RACE with EL3 and AUAP primers (Table I) and subsequent digestion of the products with the restriction enzyme ScaI. Ethidium bromide staining revealed that normal fibroblasts expressed both tropoelastin mRNA species. The primary transcript was the major PCR product and only a faint band was seen for the alternate tropoelastin transcript. Radiolabeled 3′ RACE products were separated on 10% polyacrylamide gels and visualized by autoradiography (Figure 2). Cultured normal and UV treated fibroblasts expressed two forms of the tropoelastin transcript (lanes 1 and 2). The major ScaI fragments (lanes 3 and 4) were about 260 bp (EL3 to ScaI segment), 135 bp (ScaI to 5′ polyadenyl-

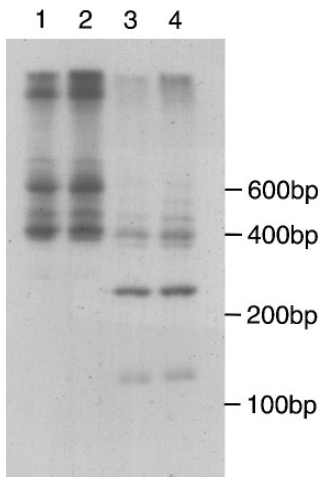

FIG. 2. 3′ RACE products and restriction endonuclease digestion patterns of radiolabeled 3′ RACE products obtained from the reverse transcription and amplification of total RNA prepared from control and UV treated fibroblasts. Aliquots of the amplification reactions and ScaI digestions were run on 10% polyacrylamide gels and the dried gels were autoradiographed. Lanes 1 and 2 show undigested PCR products from total RNA prepared from control (lane 1) and UV treated (lane 2) fibroblasts. Lanes 3 and 4 show ScaI digestion patterns of PCR products from total RNA prepared from control (lane 3) and UV treated (lane 4) fibroblasts.

ation site) and about 410 bp (ScaI to 3′ polyadenylation site). The relative intensities of the 410 bp and 135 bp bands were proportional to the amounts of the two transcripts. The autoradiograph was scanned and the ratio of the intensities were determined. Results demonstrated that total RNA prepared from UV treated fibroblasts contained almost twice the amount of the alternate tropoelastin transcript than did total RNA prepared from control fibroblasts (ratio of UV treated/ control fibroblasts = 1.8). In contrast, similar levels of the primary tropoelastin transcript was found in total RNA derived from control and UV treated fibroblasts.

Poly(A) tail length in tropoelastin mRNA from UV irradiated and control mouse skins. The results of the 3′ RACE analysis using primers rEL1 and AUAP are shown in Figure 3A. The major 3′ RACE product was about 800 bp and represented the primary tropoelastin transcript. Interestingly, the migration of the 3′ RACE product from total RNA from UVB treated skin was retarded (lane 1 vs lane 2). In order to accurately determine size differences, the 3′ RACE reaction was performed using rEL2 and AUAP to generate a smaller product and the results are shown in Figure 3B. The 3′ RACE product obtained with RNA from normal mouse skin migrated at about 120 bp whereas the PCR product obtained with RNA from UV treated mouse skin migrated at about 135 bp. The cleavage/polyadenylation site for rat tropoelastin mRNA has been identified (22). Therefore, the poly(A) tail length of the tropoelastin transcript in normal mouse was calculated to be about 18As whereas the poly(A) tail length of the transcript in UV treated mouse skin was about 33As.

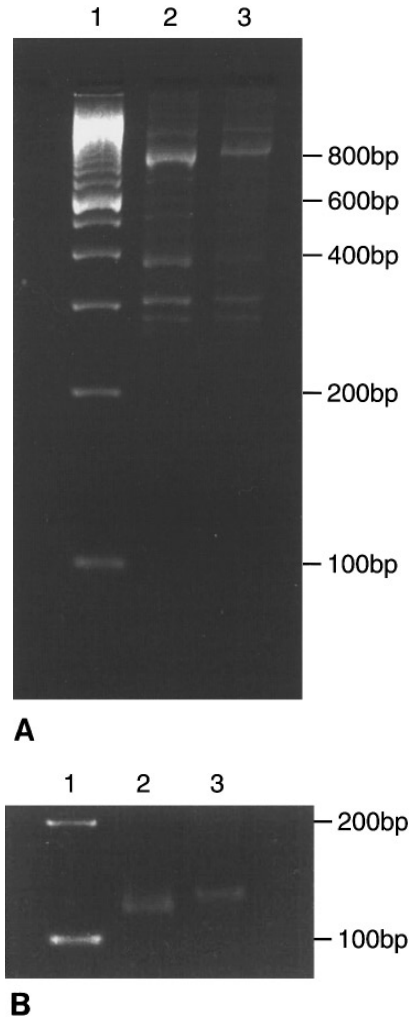

FIG. 3. 3′ RACE products obtained from the reverse transcription and amplification of total RNA prepared from normal and UV treated mouse skins using either rEL1 and AUAP primers (A) or rEL2 and AUAP primers (B). Aliquots of the amplification reactions were run on agarose gels and the products were visualized with ethidium bromide. Lane 1, 100 bp mol wt ladder; lane 2, PCR products from normal mouse skin RNA; lane 3, PCR products from UV treated mouse skin RNA.

DISCUSSION

The goal of this research was to delineate the posttranscriptional mechanism responsible for the increased elastin synthesis in sundamaged skin. Results from this investigation identify the modulation of the polyadenylation machinery as the posttranscriptional mechanism. In this study, a unique molecular variant of the tropoelastin mRNA transcript was identified in human sundamaged skin that was derived from the usage of an alternate polyadenylation site. The polyadenylation site chosen determines whether cis-acting sequences will be present in the 3′ UTR that can regulate mRNA stability and translatability. Interestingly, the alternative tropoelastin cDNA was amplified using the antisense primer EL4 which is located 70 bases downstream from the second canonical AATAAA signal. Since the cleavage/polyadenylation site typically is found 10-30 bases downstream from the poly(A) signal (19, 23, 24), these data suggest that an unusual polyadenylation signal exists downstream from the EL4 sequence.

Polyadenylation site selection is regulated by nuclear factors (19, 23-25) and can be affected by changes in the concentration of these factors. For example, when the concentration of a factor is limiting, the stronger polyadenylation site may be selected. In contrast, when the concentration of the factor is abundant, additional weaker polyadenylation sites may be utilized. Two factors known to be involved in polyadenylation site selection are cleavage/polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF) and modulation of the expression of CstF is associated with alternative polyadenylation. Under this scenario, ultraviolet radiation may stimulate the expression of a basal polyadenylation factor and thereby increase the use of an alternate polyadenylation site.

Cultured human skin fibroblasts expressed both forms of tropoelastin mRNA whereas normal human skin fibroblasts in vivo expressed only the primary transcript. Therefore, although cultured fibroblasts have been used to study the regulation of elastin synthesis, these in vitro experiments may not reflect the complex in vivo environment. The regulation of polyadenylation site selection may be tightly controlled in vivo and regulatory factors may be expressed in vitro that are not found in vivo. In contrast, both forms of the tropoelastin mRNA were found in sunexposed skin suggesting that expression of the alternative transcript is a response to UV radiation. This conclusion is supported by in vitro experiments in which exposure of cultured skin fibroblasts to UVB increased the expression of the alternative form of tropoelastin mRNA.

The augmented expression of an alternative tropoelastin mRNA provides the rationale for our previous results (8) which showed that UV treatment of human skin fibroblasts increases tropoelastin production without affecting steady-state levels of tropoelastin mRNA. Since the translation of tropoelastin mRNA from UV treated cells is increased, we concluded that altered translational efficiency results in increased tropoelastin accumulation. The alternative tropoelastin mRNA may display increased translatability which is consistent with these results. Further work is needed to isolate the full-length alternative tropoelastin mRNA and to compare its properties with those of the primary tropoelastin transcript.

Only the primary tropoelastin transcript was found in normal and UV treated mouse skins. However, the poly(A) tail length of the tropoelastin transcript in UV treated mouse skins was almost twice the length of the poly(A) tail of the tropoelastin transcript in normal mouse skin. The lack of effect of ultraviolet radiation on polyadenylation site selection may be due to the period of irradiation (10 weeks) or may be species specific. The increase in poly(A) tail length of the tropoelastin mRNA can stimulate its translation and thereby increase tropoelastin production.

Differential polyadenylation of tropoelastin transcripts may provide the molecular basis for the observed increase in tropoelastin production in sundamaged skin and UV irradiated fibroblasts. The yield of polypeptide product is directly related to mRNA decay rates and translatability and the larger, alternate tropoelastin mRNA species may display increased translatability and/or stability due to the presence of additional cis-acting elements. The relationship between UV and polyadenylation mechanisms has not been examined and represents an important area for future investigation.

REFERENCES

1. Parks, W. C., Pierce, R. A., Lee, K. A., and Mecham, R. P. (1993) *Adv. Mol. Cell. Biol.* **6,** 133–181.
2. Swee, M. H., Parks, W. C., and Pierce, R. A. (1995) *J. Biol. Chem.* **270,** 14899–14906.
3. Shapiro, S. D., Endicott, S., Province, M. A., Pierce, J. A., and Campbell, E. J. (1991) *J. Clin. Invest.* **87,** 1828–1834.
4. Uitto, J., Matsuoka, L. Y., and Kornberg, R. L. (1986) *in* Problems in Aesthetic Surgery (Rudolph, R., Ed.), pp. 307–338, Mosby, St. Louis.
5. Smith, J. G., Davidson, E. A., and Clark, R. D. (1962) *Nature* **195,** 716–717.
6. Smith, J. G., Davidson, E. A., Sams, W. M. Jr, and Clark, R. D. (1962) *J. Invest. Dermatol.* **39,** 347–350.
7. Schwartz, E., Cruickshank, F. A., and Lebwohl, M. (1990) *Exp. Mol. Path.* **52,** 63–68.
8. Schwartz, E., Feinberg, E., Lebwohl, M., Mariani, T. J., and Boyd, C. D. (1995) *J. Invest. Dermatol.* **105,** 65–69.
9. Werth, V. P., Williams, K. J., Fisher, E. A., Bashir, M., Rosenbloom, J., and Shi, X. (1997) *J. Invest. Dermatol.* **108,** 290–294.
10. Kligman, L. H. (1991) *Photochem. Photobiol.* **53,** 1–10.
11. Kligman, L. H., Akin, F. J., and Kligman, A. M. (1982) *J. Invest. Dermatol.* **78,** 181–189.
12. Kligman, L. H., Akin, F. J., and Kligman, A. M. (1985) *J. Invest. Dermatol.* **84,** 272–276.
13. Neocleous, V., Young, A. R., and Brownson, C. (1997) *Photochem. Photobiol.* **66,** 676–682.
14. Keller, W. (1995) *Cell* **81,** 829–832.
15. Jackson, R. J. (1993) *Cell* **74,** 9–14.
16. Jacobson, A., and Peltz, S. W. (1996) *Annu. Rev. Biochem.* **65,** 693–739.
17. Sachs, A. B. (1990) *Curr. Opin. Cell Biol.* **2,** 1092–1098.
18. Edwalds-Gilbert, G., Veraldi, K. L., and Milcarek, C. (1997) *Nucleic Acids Res.* **25,** 2547–2561.
19. Colgan, D. F., and Manley, J. L. (1997) *Genes Dev.* **11,** 2755–2766.
20. Indik, Z., Yoon, K., Morrow, S. D., Cicila, G., Rosenbloom, J., Rosenbloom, J., and Ornstein-Goldstein, N. (1987) *Conn. Tiss. Res.* **16,** 197–211.
21. Raju, K., and Anwar, R. A. (1987) *J. Biol. Chem.* **262,** 5755–5762.
22. Deak, S. B., Pierce, R. A., Belsky, S. A., Riley, D. J., and Boyd, C. D. (1988) *J Biol. Chem.* **263,** 13504–13507.
23. Sachs, A. B., and Wahle, E. (1993) *J. Biol. Chem.* **268,** 22955–22958.
24. Wahle, E., and Keller, W. (1992) *Annu. Rev. Biochem.* **61,** 419–440.
25. Bilger, A., Fox, C. A., Wahle, E., and Wickens, M. (1994) *Genes Dev.* **8,** 1106–1116.