Serine/threonine phosphorylation regulates binding of C hnRNP proteins to pre-mRNA

(casein kinase II/okadaic acid/protein phosphatase/heterogeneous nuclear ribonucleoprotein/mRNA splicing)

SANDRA H. MAYRAND, PAULETTE DWEN, AND THORU PEDERSON*

Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545

Communicated by Paul C. Zamecnik, May 27, 1993

ABSTRACT The C hnRNP proteins bind to nascent pre-mRNA and are thought to participate in an early step of the pre-mRNA splicing pathway. We report here that C hnRNP proteins are phosphorylated by a casein kinase II activity in a HeLa cell nuclear extract and that dephosphorylation of C hnRNP proteins is inhibited by the specific protein-serine/threonine-phosphatase 1/2A inhibitor okadaic acid. We further find that dephosphorylation of C hnRNP proteins is required for their binding to adenovirus and human β -globin pre-mRNAs. These results indicate that the participation of C hnRNP proteins in pre-spliceosome assembly is coupled to a dynamic cycle of their phosphorylation and dephosphorylation.

Pre-mRNA splicing takes place in complex ribonucleoprotein particles termed spliceosomes (1–5). The first described spliceosome proteins were called hnRNP proteins, based on their association with large heterogeneous nuclear RNA transcripts now known to include unspliced pre-mRNAs (6–12). The C-group hnRNP proteins are abundant nuclear proteins of M_r 42,000–44,000 that have been implicated in splicing (13, 14) and are known to be phosphorylated *in vivo* (8, 15–17) by a casein kinase II-type activity (18, 19). Because phosphorylation plays an important role in the regulation of protein binding to nucleic acids (20–25), we have investigated C hnRNP protein phosphorylation/dephosphorylation in a HeLa cell nuclear extract system used for *in vitro* splicing of pre-mRNA and have also examined C hnRNP protein binding to pre-mRNA as a function of phosphorylation.

MATERIALS AND METHODS

Phosphorylation, Immunoselection, and Electrophoresis of C hnRNP Proteins. Thirty percent (vol/vol) HeLa cell nuclear extract (26) was made 3.2 mM $MgCl_2$, 400 μM ATP, and 20 mM creatine phosphate and incubated for the indicated times with [γ - ^{32}P]ATP or [γ - ^{32}P]GTP at 0.2 mCi/ml (1 mCi = 37 MBq) or with a mixture of [γ - ^{32}P]ATP and [γ - ^{32}P]GTP each at 0.1 mCi/ml. After incubation with heparin (2 mg/ml) at room temperature for 10 min, immunoselection was carried out with protein A-Sepharose-bound 4F4 monoclonal antibody, specific for C hnRNP protein (27). The selected proteins were released by boiling the washed beads in 2 $\times$ gel sample buffer [0.125 M Tris-HCl, pH 6.8, 20% (vol/vol) glycerol/2% (wt/vol) SDS/5% (vol/vol) 2-mercaptoethanol] and analyzed by SDS/polyacrylamide gel electrophoresis and autoradiography. The 10% polyacrylamide gels were prepared from a 40% (wt/vol) monomer stock having a 29.6:1.0 acrylamide/*N,N'*-methylenebisacrylamide weight ratio.

[^{35}S]Methionine-labeled C hnRNP proteins were prepared from HeLa cells metabolically labeled with L-[^{35}S]methionine

(3.0 $\mu Ci/ml$) for 18 hr in medium containing half the normal concentration of methionine, and nuclear extracts were immediately prepared (28). In some experiments ^{35}S -labeled extracts were additionally labeled *in vitro* with [γ - ^{32}P]ATP and [γ - ^{32}P]GTP, as detailed in the figure legends.

Inhibition of Phosphorylation and Dephosphorylation. The inhibitors used were okadaic acid (LC Services, Woburn, MA), quercetin (Sigma), H-7 (Seikagaku America, Rockville, MD), and 2,3-diphosphoglycerate (Sigma). Inhibitors were preincubated in the nuclear extract for 10 min at 30°C prior to the addition of [γ - ^{32}P]ATP or [γ - ^{32}P]GTP.

Transcription of Biotinylated Pre-mRNAs. Biotinylated adenovirus and human β -globin pre-mRNAs were transcribed, respectively, from *Sca* I-cleaved pSP62 Δ i1 (3) and *Bam*HI-cleaved SP64-H β Δ 6 (29) by using SP6 RNA polymerase and a 1:1 molar ratio of biotin-11-UTP to UTP. The transcription reagents, including [5- 3H]UTP at a final concentration of 2.5–5.0 $\mu Ci/ml$ to facilitate subsequent transcript quantitation, were incubated together for 5 min at 35°C before the nonderivatized UTP was added. The RNAs were purified by Sephadex G-50 gel filtration.

Selection of Spliceosomes on Streptavidin-Agarose Beads. Biotinylated pre-mRNAs were bound overnight to streptavidin-agarose beads in binding buffer [50 mM Tris-HCl, pH 7.9/1 mM EDTA/15% (vol/vol) glycerol/0.05% Nonidet P-40] (30). Seventy to 85% of the RNA, monitored by incorporated [3H]UTP, routinely became bound to the streptavidin-agarose beads. The beads were washed five times in 1 ml of binding buffer followed by five 1-ml washes of splicing buffer which consisted of 30% buffer D (26) and 3.2 mM $MgCl_2$. Bovine serum albumin (200 $\mu g/ml$) and glycogen (200 $\mu g/ml$) were added to the last wash and allowed to incubate with the streptavidin-agarose-RNA complex for 30 min at 4°C. Separately, HeLa nuclear extracts were preincubated for 10 min at 30°C in the presence or absence of 1 μM okadaic acid and then for an additional 10 min at 30°C with [γ - ^{32}P]ATP and [γ - ^{32}P]GTP, 3.2 mM $MgCl_2$, 400 μM ATP, and 20 mM creatine phosphate. Streptavidin-bound pre-mRNA was then added to the nuclear extract and incubation was continued for an additional 30 min (30°C). The streptavidin-bound RNA and associated proteins were collected by centrifugation and the supernatant unbound fraction was also saved.

Selection of C hnRNP Proteins from Streptavidin-Bound RNA and Associated Proteins. The assembled RNA-protein complexes on streptavidin beads were washed extensively with binding buffer, and the RNA was released by nuclease digestion [micrococcal nuclease (400 units/ml) and RNase A (200 $\mu g/ml$) in the presence of 3 mM $CaCl_2$ for 45 min at 30°C]. The unbound fraction was treated similarly. Heparin (2 mg/ml) was then added to both the unbound and bound fractions and they were incubated for 10 min at room temperature before immunoselection with 4F4. The selected

proteins were subjected to electrophoresis and autoradiography.

RESULTS

Phosphorylation and Dephosphorylation of C hnRNP Proteins. A distinguishing feature of casein kinase II is its ability to use both ATP and GTP as the phosphate donor (31, 32). As shown in Fig. 1A, C hnRNP proteins became labeled by both [γ - 32 P]ATP and [γ - 32 P]GTP in HeLa nuclear extracts. As shown in Fig. 1B, phosphorylation of the C hnRNP proteins was inhibited by 6 and 12 mM 2,3-diphosphoglycerate (lanes 2 and 3) and by 50 and 100 μ M quercetin (lanes 4 and 5), both of which are specific inhibitors of casein kinase II (19, 33–35). Although 2,3-diphosphoglycerate and quercetin inhibited phosphorylation of C hnRNP proteins, the overall phosphorylation pattern of total nuclear extract proteins was not appreciably altered (data not shown). Phosphorylation of C hnRNP proteins also required Mg^{2+} (Fig. 1B, lane 6), as does casein kinase II activity (36). However, phosphorylation of C hnRNP proteins was not inhibited by 10 μ M H-7, a selective inhibitor of cAMP/cGMP-dependent protein kinases (data not shown).

To further investigate the phosphorylation and dephosphorylation of C hnRNP proteins, we used okadaic acid, a selective inhibitor of protein phosphatases 1 and 2A (37). C hnRNP proteins immunoselected from nuclear extracts of [35 S]methionine-labeled HeLa cells are shown in Fig. 2A, lane 1. There are three major bands, the two most rapidly migrating of which are labeled C1 and C2, based on previous

FIG. 1. Phosphorylation of C hnRNP proteins. (A) Both ATP and GTP are phosphate donors in C hnRNP protein phosphorylation. Phosphorylated hnRNP proteins were immunoselected with 4F4 monoclonal antibody. Lane 1, 10-min incubation with [γ - 32 P]GTP; lane 2, 30-min incubation with [γ - 32 P]GTP; lane 3, 10-min incubation with [γ - 32 P]ATP; lane 4, 30-min incubation with [γ - 32 P]ATP. (B) Inhibition of C hnRNP phosphorylation. The nuclear extract was preincubated with the indicated inhibitor for 10 min at 30°C and then incubated as in A for 10 min with both [γ - 32 P]ATP and [γ - 32 P]GTP (each at 0.1 mCi/ml) followed by immunoselection with 4F4 antibody. Lane 1, no inhibitors; lane 2, 6 mM 2,3-diphosphoglycerate; lane 3, 12 mM 2,3-diphosphoglycerate; lane 4, 50 μ M quercetin; lane 5, 100 μ M quercetin; lane 6, $MgCl_2$ was omitted from the reaction. The position of C1 hnRNP protein is indicated on the left. The dot denotes the hyperphosphorylated form of C1 protein (see Fig. 2).

FIG. 2. Hyperphosphorylated C hnRNP proteins. (A) 35 S-labeled C hnRNP proteins. C hnRNP proteins from HeLa cells metabolically labeled with L-[35 S]methionine were immunoselected from nuclear extract with 4F4 monoclonal antibody and subjected to electrophoresis and autoradiography. Lane 1, C hnRNP proteins selected from 35 S-labeled nuclear extract; lane 2, C hnRNP proteins selected from 35 S-labeled nuclear extract that had been preincubated with 1 μ M okadaic acid for 10 min at 30°C and then made 400 μ M ATP, 20 mM creatine phosphate, and 3.2 mM $MgCl_2$ and incubated for 30 min at 30°C; lane 3, C hnRNP proteins selected from 35 S-labeled nuclear extract made 400 μ M ATP, 20 mM creatine phosphate, and 3.2 mM $MgCl_2$ and incubated for 30 min at 30°C (no okadaic acid present). The hyperphosphorylated form of C1 hnRNP protein is denoted by a dot to the right of lane 3. (B) 32 P-labeled C hnRNP proteins. Experiments were carried out with the same 35 S-labeled nuclear extract as in A except that [γ - 32 P]ATP and [γ - 32 P]GTP (each at 0.1 μ Ci/ml) were added for the 30-min incubation at 30°C. Lane 1, 35 S-labeled C1 and C2 hnRNP proteins as markers; lane 2, 32 P-labeled C hnRNP proteins immunoselected after incubation with nuclear extract in the presence of 1 μ M okadaic acid; lane 3, 32 P-labeled C hnRNP proteins immunoselected after incubation of nuclear extract in the absence of okadaic acid. The dot at the right of lane 3 denotes the hyperphosphorylated C1 hnRNP protein.

studies (7, 38). The nuclear extract shown in lane 1 of Fig. 2A had not been incubated prior to immunoselection. When the 35 S-labeled nuclear extract was incubated for 30 min in the presence of ATP, creatine phosphate, and $MgCl_2$ (as in standard *in vitro* mRNA splicing reactions), several mobility changes occurred in the immunoselected proteins, as shown in lane 3 of Fig. 2A. The band migrating behind C2 in lane 1 became a doublet and a new band migrating behind this doublet appeared. In addition, another band migrating just behind C1 appeared, indicated by the dot at the right of lane 3. When the incubation of the nuclear extract with ATP, creatine phosphate, and $MgCl_2$ was also in the presence of 1 μ M okadaic acid, the band migrating behind C1 increased in intensity and the intensity of the C1 band itself decreased (Fig. 2A, lane 2). This suggests that the band indicated by the dot represents hyperphosphorylated C1 protein and that the inhibition of its dephosphorylation by okadaic acid increases the amount of the hyperphosphorylated form.

To confirm this, the same experiment was performed with [γ - 32 P]ATP and [γ - 32 P]GTP present during the incubation of nuclear extract prior to immunoselection. In lane 3 of Fig. 2B it can be seen that the new band migrating behind C1 (indicated by the dot) is 32 P-labeled. (Note also that the

FIG. 3. Binding of phosphorylated C hnRNP proteins to pre-mRNA. Biotinylated pre-mRNAs were bound to streptavidin and incubated in HeLa nuclear extracts which had been preincubated with [γ - 32 P]ATP and [γ - 32 P]GTP. Proteins in all lanes were immunoselected with 4F4 monoclonal antibody. Lane 1, proteins from adenovirus pre-mRNA unbound fraction (no okadaic acid); lane 2, proteins from adenovirus pre-mRNA bound fraction (no okadaic acid); lane 3, adenovirus pre-mRNA bound proteins from okadaic acid-treated nuclear extract; lane 4, proteins from adenovirus pre-mRNA unbound fraction, okadaic acid-treated nuclear extract; lane 5, β -globin pre-mRNA bound proteins, okadaic acid-treated nuclear extract; lane 6, proteins from the β -globin pre-mRNA unbound fraction, okadaic acid-treated nuclear extract. The dots at the right of lanes 4 and 6 denote the positions of the hyperphosphorylated species of C1 protein.

primary C1 band itself becomes 32 P-labeled.) When the incubation was carried out in the presence of 1 μ M okadaic acid, the 32 P labeling of the band migrating behind C1 was greatly increased, while much less 32 P-labeled C1 was detected (compare lanes 2 and 3). This confirms that this protein (denoted by the dot) is hyperphosphorylated C1 that undergoes active dephosphorylation by protein phosphatases 1 and/or 2A in the HeLa nuclear extract. In additional experiments it was found that okadaic acid inhibited dephosphorylation of C hnRNP proteins as completely at 100 nM as at 1 μ M (data not shown), suggesting that protein phosphatase 2A, rather than 1, is the enzyme involved (37).

Phosphorylation of C hnRNP Proteins and Pre-mRNA Binding. The incubation conditions leading to hyperphosphorylation of C1 hnRNP protein—namely, the presence of ATP, creatine phosphate, and $MgCl_2$ (Fig. 2)—are permissive for splicing of exogenous pre-mRNA. We and others have found that pre-mRNA splicing *in vitro* is inhibited both by okadaic acid and by 2,3-diphosphoglycerate, indicating that ongoing nuclear protein serine/threonine phosphorylation and dephosphorylation reactions are required for splicing (refs. 39 and 40; data not shown). Since the available evidence suggests that C hnRNP proteins bind to pre-mRNA at an early stage of the splicing pathway (41), we sought to determine whether binding of C hnRNP protein to pre-mRNA is related to phosphorylation. This was done by comparing the phosphorylation of C hnRNP protein bound to pre-mRNA in nuclear extract splicing reactions with that of C protein not bound to pre-mRNA. Biotin-labeled adenovirus pre-mRNA was first bound to streptavidin-agarose beads and then incubated in a nuclear extract (under splicing-permissive conditions) with [γ - 32 P]ATP and [γ - 32 P]GTP in the presence or absence of okadaic acid. The pre-mRNA bound factors were recovered by centrifugation and, after the beads were washed, the proteins were released by nuclease digestion (see

Materials and Methods). C hnRNP proteins were then immunoselected and analyzed by electrophoresis and autoradiography. As shown in Fig. 3, lane 2, phosphorylated C hnRNP proteins were bound to pre-mRNA. (Under these conditions of labeling and spliceosome assembly, we find that the major phosphorylated C hnRNP protein bound to the adenovirus pre-mRNA is C1.) However, when okadaic acid was included, a condition under which virtually all C1 protein is arrested in the hyperphosphorylated state (Fig. 2B, lane 2), no phosphorylated C1 protein became bound to the pre-mRNA (Fig. 3, lane 3), and all of the phosphorylated C hnRNP protein in the extract was found in the unbound fraction (lane 4). A similar result was obtained when a human β -globin pre-mRNA was used (Fig. 3, lane 5 vs. lane 6). These results indicate that there is a connection between the binding of C hnRNP proteins to pre-mRNA in a HeLa nuclear splicing extract and the phosphorylation state of these proteins.

DISCUSSION

Our observations are consistent with a dynamic phosphorylation/dephosphorylation cycle of C hnRNP protein modulating its binding to pre-mRNA, as depicted in Fig. 4. In step I, phosphorylated C hnRNP protein binds to pre-mRNA (Fig. 3, lane 2). In step II, the bound C hnRNP protein becomes hyperphosphorylated, which we envision as being either mechanistically coupled with or immediately leading to its release from the pre-mRNA. After its release, the C protein becomes dephosphorylated by protein phosphatase 2A in step III. However, when this phosphorylation/dephosphorylation cycle is interrupted at step III by a dephosphorylation inhibitor (okadaic acid), the hyperphosphorylated form of C hnRNP accumulates (Fig. 2B, lane 2) and the recycling of C hnRNP proteins onto new pre-mRNAs in step I cannot occur (Fig. 3, lanes 3 and 5).

Our data do not distinguish between complete (Fig. 4, dashed arrow) versus partial (solid arrow) dephosphorylation of the hyperphosphorylated C hnRNP protein prior to the next cycle of pre-mRNA binding. *In vivo* studies have indicated that initial sites phosphorylated by casein kinase II tend to turn over slowly and have pointed to the idea of partial dephosphorylation (32). Furthermore, consistent with our model is the observation that casein kinase II is often involved with multisite phosphorylation in which the first phosphorylation event triggers subsequent ones that then produce the protein's activity change (42). It is conceivable that if nonphosphorylated C protein is indeed part of this cycle, its initial phosphorylation (step I) is directly coupled to its pre-mRNA binding in the same way that the subsequent hyperphosphorylation (step II) may be directly coupled to release of C protein from the pre-mRNA.

Since the kinase responsible for C hnRNP protein phosphorylation is found in purified hnRNP particles (8, 15, 17, 18), it is clear that it associates at least transiently with pre-spliceosomes, which is consistent with the model we are proposing (Fig. 4). It is possible that the kinase responsible for hyperphosphorylating C hnRNP protein prior to or concurrent with its dissociation from pre-mRNA is actually an RNA-dependent protein kinase. Although to our knowledge such a kinase has not been reported, a double-stranded RNA-dependent protein kinase is a key component of the extensively studied interferon response system (43, 44), and RNA-dependent ATPases and helicases have been demonstrated to play essential roles in pre-mRNA splicing (45, 46). A DNA-dependent protein kinase has been reported which phosphorylates the transcription factor Sp1 only when it is bound to DNA (47).

Although we have focused on the phosphorylation/dephosphorylation of C hnRNP proteins, it is likely that the

FIG. 4. Schematic of C hnRNP protein phosphorylation and dephosphorylation in relation to pre-mRNA binding. In step I, phosphorylated C protein binds to pre-mRNA as part of the early pre-spliceosome assembly pathway. In step II, hyperphosphorylation causes bound C protein to be released from the RNA. Subsequently in step III, the released C protein is dephosphorylated by protein phosphatase 2A (PP2A). For clarity, only two sites of phosphorylation on the C hnRNP protein are shown.

phosphorylation or dephosphorylation of other nuclear proteins participates indirectly or cooperatively in spliceosome formation. For example, it has been shown that phosphorylation of another hnRNP protein, A1, eliminates its RNA-RNA annealing activity (48). In addition, it has recently been reported that dephosphorylation of the M_r 70,000 protein of the U1 snRNP particle is required for an early, precatalytic step in pre-mRNA splicing (49). Furthermore, many of the proteins that bind to the intron polypyrimidine tract and the 3' splice site of pre-mRNA are, like the C hnRNP proteins, phosphoproteins: for example, U2AF (50), the M_r 52,000 protein of the trimeric U4/U6/U5 complex (51), and SF2 (52). Since it is unlikely that all of these proteins bind to such a small region of the pre-mRNA simultaneously, it is attractive to suppose that a cascade of phosphorylations and dephosphorylations directs their sequential binding and release. Further studies of the phosphorylation states of these various pre-mRNA-binding proteins may provide important insights into their functions in the splicing pathway. Finally, we note that in addition to modulating their binding to pre-mRNA, the phosphorylation or dephosphorylation of hnRNP proteins may also influence their homotypic associations (53) or their binding to other spliceosome or nuclear proteins, by analogy with the recently described roles of phosphorylation in the assembly/disassembly of the nuclear lamina (54, 55) or the import of nuclear proteins (56).

We are grateful to Marty Jacobson and Jamal Tamsamani for advice and to Serafin Pinol-Roma and Gideon Dreyfuss, Howard Hughes Medical Institute/University of Pennsylvania School of Medicine, for generously providing the anti-C hnRNP protein monoclonal antibody. This work was supported by National Institutes of Health Grant GM21595-18 and by the G. Harold and Leila Y. Mathers Foundation.

- Brody, E. & Abelson, J. (1985) *Science* **228**, 963–967.
- Grabowski, P. J., Seiler, S. R. & Sharp, P. A. (1985) *Cell* **42**, 345–353.
- Frederick, D. & Keller, W. (1985) *Cell* **42**, 355–367.
- Bindereif, A. & Green, M. R. (1986) *Mol. Cell. Biol.* **6**, 2582–2592.
- Perkins, K. K., Furneaux, H. M. & Hurwitz, J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 887–891.
- Pederson, T. (1974) *J. Mol. Biol.* **83**, 163–183.
- Beyer, A. L., Christensen, M. E., Walker, B. W. & LeSturgeon, W. M. (1977) *Cell* **11**, 127–138.
- Karn, J., Vidali, G., Boffa, L. C. & Allfrey, V. G. (1977) *J. Biol. Chem.* **252**, 7307–7322.
- Pederson, T. & Davis, N. G. (1980) *J. Cell Biol.* **87**, 47–54.
- Mayrand, S., Setyono, B., Greenberg, J. R. & Pederson, T. (1981) *J. Cell Biol.* **90**, 380–384.
- Economidis, I. V. & Pederson, T. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1599–1602.
- Pederson, T. (1983) *J. Cell Biol.* **97**, 1321–1326.
- Choi, Y. D., Grabowski, P. J., Sharp, P. A. & Dreyfuss, G. (1986) *Science* **231**, 1534–1539.
- Sierakowska, H., Szer, W., Furdon, P. J. & Kole, R. (1986) *Nucleic Acids Res.* **14**, 5241–5254.
- Kish, V. M. & Pederson, T. (1978) *Methods Cell Biol.* **17**, 377–399.
- Dreyfuss, G., Choi, Y. D. & Adam, S. A. (1984) *Mol. Cell. Biol.* **4**, 1104–1114.
- Wilk, H. E., Werr, H., Friedrich, D., Kiltz, H. H. & Schäfer, K. P. (1985) *Eur. J. Biochem.* **146**, 71–81.
- Holcomb, E. R. & Friedman, D. L. (1984) *J. Biol. Chem.* **259**, 31–40.
- Friedman, D. L., Kleiman, N. J. & Campbell, F. E., Jr. (1985) *Biochim. Biophys. Acta* **847**, 165–176.
- Hershey, J. W. B. (1989) *J. Biol. Chem.* **264**, 20823–20826.
- Lu, H., Flores, O., Weinmann, R. & Reinberg, D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10004–10008.
- Lin, A., Frost, J., Deng, T., Smeal, T., Al-Alawi, N., Kikkawa, U., Hunter, T., Brenner, D. & Karin, M. (1992) *Cell* **70**, 777–789.
- Chestnut, J., Stephens, J. H. & Dahmus, M. E. (1992) *J. Biol. Chem.* **267**, 10500–10506.
- Takacs, A. M., Barik, S., Das, T. & Banerjee, A. K. (1992) *J. Virol.* **66**, 5842–5848.
- Jackson, S. P. (1992) *Trends Cell Biol.* **2**, 104–108.
- Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
- Choi, Y. D. & Dreyfuss, G. (1984) *J. Cell Biol.* **99**, 1997–2004.
- Lee, K. A. W. & Green, M. R. (1990) *Methods Enzymol.* **181**, 20–30.
- Kraimer, A. R., Maniatis, T., Ruskin, B. & Green, M. R. (1984) *Cell* **36**, 993–1005.
- Ruby, S. W., Goelz, S. E., Hostomsky, Z. & Abelson, J. N. (1990) *Methods Enzymol.* **181**, 97–121.

31. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56**, 567–613.
32. Tuazon, P. T. & Traugh, J. A. (1991) in *Advances in Second Messenger and Phosphoprotein Research*, eds. Greengard, P. & Robinson, G. A. (Raven, New York), Vol. 23, pp. 124–154.
33. Hosey, M. M. & Tao, M. (1977) *Biochim. Biophys. Acta* **482**, 348–357.
34. Cochet, C., Job, D., Pirollet, F. & Chambaz, E. M. (1981) *Biochim. Biophys. Acta* **658**, 191–201.
35. Hathaway, G. M. & Traugh, J. A. (1984) *J. Biol. Chem.* **259**, 2850–2855.
36. Kumar, R. & Tao, M. (1975) *Biochim. Biophys. Acta* **410**, 87–98.
37. Cohen, P., Holmes, C. F. C. & Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15**, 98–102.
38. Choi, Y. D. & Dreyfuss, G. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7471–7475.
39. Tazi, J., Daugeron, M.-C., Cathala, G., Brunel, C. & Jeanteur, P. (1992) *J. Biol. Chem.* **267**, 4322–4326.
40. Mermoud, J. E., Cohen, P. & Lamond, A. I. (1992) *Nucleic Acids Res.* **20**, 5263–5269.
41. Mayrand, S. H. & Pederson, T. (1990) *Nucleic Acids Res.* **18**, 3307–3318.
42. Roach, P. J. (1991) *J. Biol. Chem.* **266**, 14139–14142.
43. Galabru, J. & Hovanessian, A. G. (1985) *Cell* **43**, 685–694.
44. Hershey, J. W. B. (1991) *Annu. Rev. Biochem.* **60**, 717–775.
45. Ruby, S. W. & Abelson, J. (1991) *Trends Genet.* **7**, 79–85.
46. Guthrie, C. (1991) *Science* **253**, 157–163.
47. Jackson, S. P., MacDonald, J. J., Lees-Miller, S. & Tjian, R. (1990) *Cell* **63**, 155–165.
48. Cebianchi, F., Calvio, C., Stoppini, M., Buvoli, M. & Riva, S. (1993) *Nucleic Acids Res.* **21**, 949–955.
49. Tazi, J., Kornstadt, U., Rossi, F., Jeanteur, P., Cathala, G., Brunel, C. & Luhrmann, R. (1993) *Nature (London)* **363**, 283–286.
50. Zamore, P. D. & Green, M. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9243–9247.
51. Behrens, S. E. & Luhrmann, R. (1991) *Genes Dev.* **5**, 1439–1452.
52. Mayeda, A., Zahler, A. M., Krainer, A. R. & Roth, M. B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1301–1304.
53. Barnett, S. A., Friedman, D. L. & LeStourgeon, W. M. (1989) *Mol. Cell. Biol.* **9**, 492–498.
54. Georgatos, S. D., Stournaras, C. & Blobel, G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4325–4329.
55. Dessev, G., Iovcheva-Dessev, C., Bischoff, J. R., Beach, D. & Goldman, R. D. (1991) *J. Cell Biol.* **112**, 523–533.
56. Meir, U. T. & Blobel, G. (1992) *Cell* **70**, 127–138.