BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 523–527 (1997)
ARTICLE NO. RC977165

N-Myristoylation of the Catalytic Subunit of cAMP-Dependent Protein Kinase in the Free-Living Nematode *Caenorhabditis elegans*

Robert A. Aspbury,* Michael J. Fisher,* Huw H. Rees,* and Roger A. Clegg†,[1]
**Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, United Kingdom; and †Hannah Research Institute, Ayr KA6 5HL, Scotland, United Kingdom*

Received July 22, 1997

N-Myristoylation of the catalytic subunit (C-subunit) of cAMP-dependent protein kinase is widespread in animal cells. Some invertebrates express non-myristoylated isoforms of C-subunit but these co-exist with at least one myristoylated isoform. The generality of this observation implies an indispensable function for myristoylated C-subunit, but notwithstanding this, neither of the C-subunit isoforms hitherto described in *C. elegans* is apparently N-myristoylated. In light of this anomaly, the myristoylation status of the C-subunit has been examined in adult *C. elegans.* Evidence is presented for the presence of an N-myristoylated isoform. © 1997 Academic Press

Key Words: **protein kinase A; *Caenorhabditis elegans;* myristoylation.**

Enzymes belonging to the cyclic AMP-dependent protein kinase (PK-A) sub-group of the AAC family of protein kinases occur widely throughout eukaryotic organisms [1]. A considerable level of sequence homology is found between the catalytic (C-) subunits of these kinases. Furthermore, in multicellular organisms, N-myristoylation is an almost universal covalent modification among these molecules.

Although the C-subunit of mammalian PK-A was the first protein to be shown to be N-myristoylated [2], this modification is now known to be shared by a diverse group of proteins [3]. A feature common to many of these myristoylated proteins is that they are involved in intracellular signalling pathways and networks [4,5]. There is now quite general agreement that the function of the N-myristoyl modification is to co-operate in the generation of a targeting signal, pre-disposing the myristoylated protein to association either with a cellular membrane or perhaps with another soluble protein. The solvation energy for the fatty acyl functionality of a myristoylated protein inserting into a phospholipid bilayer is barely sufficient to stabilise the membrane-association of the protein [6]. This stabilisation is ensured by secondary interactions between protein and membrane - most commonly these are mediated by either further fatty acyl (usually palmitoyl) groups [7] or by electrostatic attraction between basic domains in the protein and acidic phospholipids in the membrane [3]. These secondary interactions may be subject to modulation (by depalmitoylation or phosphorylation of basic domains in the examples above) lending a conditional dimension to the membrane association of the myristoylated protein; other conditional signals or "switches" based on ligand-binding have also been described [8,9].

Although anchoring proteins have been described which restrict the location of PK-A holoenzyme heterotetramers via interaction with regulatory (R-) subunits [10], there is no evidence for a general association of free C-subunit (dissociated from R-subunits) with membranous intracellular structures. C-subunit is usually characterised as a soluble, cytosolic protein after liberation from R-subunit by the binding of cAMP or related analogues, although it is known to undergo migration into the nucleus which becomes discernible if its inability to recombine with cytosolic R-subunit persists longer than a few minutes [11]. The myristoylated N-terminus of C-subunit is apparently not obligatory in the mechanism of this nuclear migration [12] or in many other cellular functions [13] mediated by PK-A. However, there is limited evidence for selective utilization of phosphorylatable protein substrates by myristoylated C-subunit when compared *in vitro* with its non-myristoylated analogue [14,15]. This could imply that myristoylated C-subunit molecules are indispensable for a small sub-set of the many cellular func-

[1] To whom correspondence should be addressed. Fax: +44 (0)1292-678797. E-mail: cleggr@main.hri.sari.ac.uk.

Abbreviations used: IBMX, isobutylmethyxanthine; TBS, tris-buffered saline; n.i.s., non-immune serum.

0006-291X/97 $25.00
Copyright © 1997 by Academic Press
All rights of reproduction in any form reserved.

tions regulated by this enzyme. If this were the case, those multicellular organisms that express non-myristoylated C-subunit would be predicted also to express a myristoylated variant or isoform. This has been shown to be the case in *Aplysia* [16], although published information on the C-subunit analogue of *Caenorhabditis elegans* suggests that this nematode (and also *Ascaris suum* [17]) does not conform to this generalisation [18]. Although, on the basis of sequence analysis, both of the described isoforms in *C. elegans* are substantially homologous in overall size and sequence to the paradigm mammalian C-subunit, neither has a conventionally myristoylatable N-terminal glycine.

The work described here was undertaken to examine the N-myristoylation status of C-subunit in *C. elegans* with the objective of confirming or refuting the proposition that all C-subunit molecules in this organism lack this modification. The results clearly demonstrate the presence of a myristoylated protein having properties consistent with its identification as C-subunit.

MATERIALS AND METHODS

Organisms and biochemical preparations. Wild-type *C. elegans* (Bristol N2 strain) was cultured and harvested as described by Brenner [19]. Uniform labelling of myristoylated proteins was achieved by incubation in the presence of [9,10(n)-^{3}H]myristic acid (250μCi/ml) for 6h. The incubation medium was supplemented with amino acids, sodium pyruvate (5mM) and serotonin (2mg/ml). Following incubation, nematodes were harvested by centrifugation (300 $\times$ *g*, 0°C, 5min), washed three times in 20mM Tris-HCl, pH 8.0 and then stored as centrifugal pellets at −70°C until used.

Extracts of *C. elegans* were prepared by firstly resuspending nematode pellets (approx. 7 $\times$ 10^6 organisms) in 4ml 10mM MES, 0.2mM EDTA, 0.1mM DTT, 0.5mM IBMX, 0.5% Triton X-100, pH 6.8, containing the following protease inhibitors added (to the final concentrations given) immediately before use: PMSF, 2mM; benzamidine-HCl, 1mM; leupeptin, 10μg/ml; soya bean trypsin inhibitor, 1μg/ml. Nematode suspensions were then crushed and ground in a pestle and mortar at the temperature of liquid nitrogen. The resulting homogenates were allowed to thaw and incubated on ice for 30min. After centrifugation at 5000 $\times$ *g* for 10min at 4°C, the supernatants were either used immediately, or snap-frozen and stored at −70°C.

For use in control experiments, extracts of mammary tissue from rats in the 10th day of lactation were prepared as described previously [20].

C-subunit was prepared from various mammalian tissues as described by Flockhart & Corbin [21], or as a recombinant protein from *E. coli* [22]. The expression plasmid (pLWS-3) carrying the entire coding sequence of murine Cα was a generous gift from Prof. Susan S. Taylor. C-subunit was labelled with ^{125}I using the Iodogen method, giving specific radioactivities of approximately 25μCi/μg protein. Unlabelled C-subunit was added to ^{125}I-labelled material when experimental design required a lower specific radioactivity (see eg. Fig. 2).

Polyclonal anti-peptide antibodies were raised in sheep against the sequence H_2N-**GDTSNFDDYEEC**-COOH representing residues 322-332 (+ C-terminal cysteine) of murine Cα. The peptide was synthesised by conventional F-moc chemistry using a commercial automated synthesiser; it was resolved from other products by reversed-phase hplc and its purity was analysed by this technique and confirmed by mass spectrometry using a Finnegan Lasermat model MALDI mass spectrometer. It was then covalently coupled to keyhole limpet haemocyanin before use as an immunogen. Epitope(s) within this sequence, which is highly conserved between isoforms and across species, are located at the surface of the murine Cα-subunit molecule [23]. Complement-inactivated serum from a single animal (coded 365) was used either without further purification or after affinity purification of immunospecific IgG using C-subunit immobilized onto Sepharose 4B. Identical procedures were used for the preparation of antibodies against the peptide: H_2N-GNAAAKKGSEQY-COOH, representing the sequence 1-12 (+ C-terminal tyrosine) of murine Cα - this was coded 201.

Assay of PK-A activity. PK-A activity was measured in the presence of 1μM cAMP using Kemptide as a phosphate acceptor as described previously [20]. C-subunit activity was defined as that which was resistant to inhibition by PK-I; 1 unit of activity catalyses the transfer of 1μmol phosphate.min^{-1} under the specified conditions of assay.

Immunoprecipitations. For experiments in which immunodepletion of kinase activity was measured, incubations with antibody were at 0°C for 15min followed by 2min with Protein G-Sepharose. In those illustrated in Fig. 1, extracts of *C. elegans* or rat mammary tissue extract, containing initially 30μunits PK-A activity and 1μM cAMP, were incubated with antiserum 201 or antiserum 365 (sufficient to immunoneutralize 300μunits of activity) or with non-immune sheep serum. Incubations were supplemented with non-immune serum as necessary such that the total input of sheep serum per incubation was 5μl. Immune complexes were collected on Protein G-Sepharose beads and removed by centrifugation; the supernatant was sampled for the measurement of Kemptide kinase activity as above.

For investigation of quantitative aspects of C-subunit immunoprecipitation (see Fig. 2), ^{125}I-labelled C-subunit (0.4μg; 240000cpm) was added to aliquots of *C. elegans* extract (100μg protein) or buffer alone, both containing 1μM cAMP. Non-immune sheep IgG or affinity-purified antibodies (in large excess over antigen) were added and samples were incubated on a rotating mixer at room temperature for 1.5h; 100μl of a suspension consisting of 1 part settled volume Protein G-Sepharose beads: 1 part TBS buffer was then added and incubation was continued for a further 1.5h. Beads were sedimented and washed twice in 1ml of TBS. Bound proteins were solubilized by boiling the washed beads in Laemmli sample buffer, and analysed by SDS-PAGE [24] together with a standardizing sample of C-subunit (0.2μg; 120000cpm). Gels were dried for phosphorimaging. Quantitation of ^{125}I-radioactivity associated with C-subunit was done by applying appropriate computational routines to the phosphorimage data and by cutting-out and counting the C-subunit band at around 41kDa on the dried SDS-PAGE gel: both methods gave the same results. Similar immunoprecipitation methods were applied to extracts of [^{3}H]-myristate-labelled *C. elegans,* except that 1.4mg extract protein was treated per incubation.

RESULTS

PK-A activity in unfractionated extract of *C. elegans* was found to average around 12 munits/mg of extract protein. This compares with values of 1-2 munits/mg protein in the extracts of lactating rat mammary tissue. The titres of antibodies 365 and 201 were determined using mammalian C-subunit purified from tissue sources or from recombinant expression constructs of *E. coli.* Both antibodies were capable of immunoprecipitating C-subunit, whether or not the antigen was N-myristoylated (data not shown). In tissue extracts, where C-subunit is mostly associated with R-subunit, ab365 was ineffective in the immunodepletion of kinase activity unless cAMP was present. Similarly for ab201, its recognition of C-subunit was suppressed when C

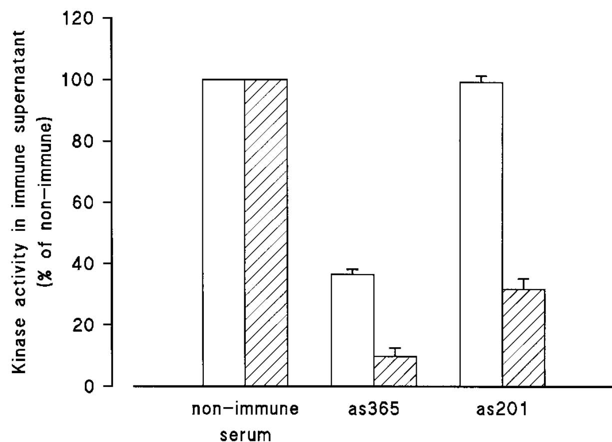


FIG. 1. Immunoprecipitation of kinase activity from extracts of *C. elegans* and mammary tissue: antiserum 201 is ineffective in *C. elegans.* Extracts of *C. elegans* (unhatched bars) and rat mammary tissue (diagonally hatched bars) were processed as described in Materials and Methods. The procedure itself was accompanied by some non-immune loss of activity such that the 100% values (for controls treated with non-immune serum) fell within the range 12-18μunits in the 4 experiments done. Results shown are means ± SEM.

was bound to R-subunit in holoenzyme heterotetramers (data not shown).

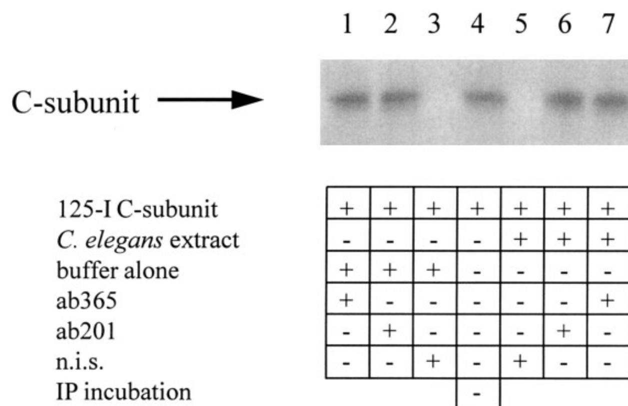
The Kemptide kinase activity in extracts of *C. elegans* (in the presence of cAMP) was used as an indicator of the concentration of C-subunit, assuming a specific activity of around 30units/mg protein which is typical of the mammalian enzyme in our hands [20]. Immunodepletion experiments were done in which varying amounts of antibody were added up to a maximum that was sufficient to precipitate a 10-fold excess of C-subunit over the quantity calculated to be present. As shown in Fig. 1, ab365 was able, at saturation, to immunodeplete 67% of the Kemptide kinase activity from *C. elegans* extracts, and around 90% from rat mammary tissue extracts. Whereas ab201 immunodepleted 72% of activity from rat mammary tissue, no immunodepletion with this antibody was observed in *C. elegans* extracts.

In the light of these results, experiments were done to assess whether the abilities of ab201 and ab365 to recognize authentic mammalian C-subunit were impaired when immunoprecipitation was attempted in extracts of *C. elegans.* Results of such an experiment are illustrated in Fig. 2, showing that immunorecognition of a defined quantity of mammalian ^{125}I-labelled C-subunit by an excess of ab201 or ab365 was equally efficient in the presence or absence of *C. elegans* extract. Thus, the incomplete immunodepletion by ab365 of Kemptide kinase activity intrinsic to *C. elegans* and the total absence of any immunodepletion by ab201 most probably reflect the lack of recognizable epitopes for these antibodies on some (ab365) or all (ab201) of the *C. elegans* C-subunit molecules. Confirming this, no signals were obtained from Western blots of *C. elegans* extracts with antiserum 201, whereas antiserum 365 detected a principal component at approx. 41kDa (data not shown) - the expected molecular size of the CeCATα isoform of C-subunit in this organism [18].

C. elegans C-subunit was immunoprecipitated by ab365 from an extract of nematodes that had been uniformly labelled with [^{3}H]myristic acid, and the immunoprecipiatates analysed by SDS-PAGE and phosphorimaging. As shown in Fig. 3, a clear labelling of the immunoprecipitated C-subunit was evident. This confirms that at least some of the C-subunit molecules recognised by ab365 had incorporated label from [^{3}H]-myristic acid. As anticipated from the inability of ab201 to immunodeplete kinase activity from *C. elegans* extracts (Fig. 1), this antibody immunoprecipitated no biosynthetically labelled C-subunit (Fig. 3). Further control experiments with non-immune sheep IgG demonstrated that precipitation by ab365 was immune-specific, and that it could be antagonised by an excess of the antigen peptide (not shown). Although insufficient radioactivity was present to enable chemical identification of the labelled compound in immunoprecipitates, analysis of total labelled protein in *C. elegans* extracts following pronase digestion (data not shown) confirmed myristoyl-glycine as the fatty acylated amino acid.

DISCUSSION

Analysis of the gene encoding C-subunit of *C. elegans,* and of the expressed C-subunit proteins, has



	1	2	3	4	5	6	7
125-I C-subunit	+	+	+	+	+	+	+
C. elegans extract	-	-	-	-	+	+	+
buffer alone	+	+	+	-	-	-	-
ab365	+	-	-	-	-	-	+
ab201	-	+	-	-	-	+	-
n.i.s.	-	-	+	-	+	-	-
IP incubation				-			

FIG. 2. Immunoprecipitation efficiency of anti-C-subunit antibody against mammalian C-subunit is unimpaired in extracts of *C. elegans.* The experiment shown is representative of 3 that have been done, in which the average recovery, as labelled intact C-subunit, of the total ^{125}I-radioactivity added to each incubation was, in *C. elegans* extracts, for 365, 47%; for 201, 44%; for NIS, 2% (tracks 7,6 & 5 respectively). In buffer alone, corresponding values were 43%, 43% and 5% (tracks 1,2 &3). For [^{125}I]C-subunit loaded directly onto the gel (track 4), 68% of added counts were recovered at the position of intact C-subunit protomer. The phosphorimage of this region of the gel is illustrated.

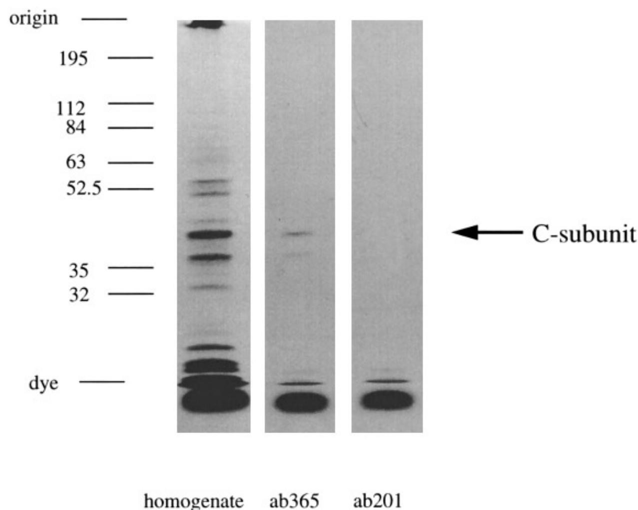


FIG. 3. Immunoprecipitation of a myristoylated C-subunit in extracts of *C. elegans* biosynthetically labelled in the presence of [^{3}H]-myristic acid. Phosphorimages of complete tracks from a representative 10% gel are illustrated; positions of M_r calibration markers and authentic mammalian C-subunit are indicated. The LHS track is of total extract protein (110μg); samples (1400μg extract protein) in the other 2 tracks, were treated with affinity-purified antibody 365 or antibody 201 as indicated and processed as described in Materials and Methods for immune precipitation and analysis by SDS-PAGE.

shown that two alternative splice variants are translated [18]. Both share an N-terminus that does not end in an N-myristoylation sequence. They differ in their C-terminal regions, one isozyme (CeCATα) having a sequence identical (except for 1 amino acid) to that of the antigen peptide for ab365, the other (CeCATα') having an alternative sequence [17] not containing this epitope. This is fully compatible with the outcome of our immunodepletion experiments (Fig. 1): the findings of Rubin's group [18] predict no epitope for ab201 on *C. elegans* C-subunit and a partial immunodepletion by ab365, depending in magnitude on the expression ratio of the C-terminal variant isoforms, CeCATα and CeCATα'. Our results indicate that around 30% of the C-subunit catalytic activity in adult *C. elegans* - that not depleted by ab365 - is attributable to the isoform CeCATα', and confirm that no molecules of *C. elegans* C-subunit are immunorecognizable by ab201.

In addition to this, our experiments on immunoprecipitation with ab365 from extracts of *C. elegans* radiolabelled with [^{3}H]myristic acid clearly demonstrate that an N-myristoylated isoform (approx. 41kDa) of C-subunit is expressed in this organism. In our experiments, only about 5% of the [^{3}H]myristoylated 41kDa protein from nematode extracts was immunoprecipitated by ab365. We interpret this fractional immunoprecipitation of the labelled 41kDa material as indicating simply that much of the labelled protein at this position of the gel is not C-subunit. Prominent among alternative candidate myristoyl-proteins of approx. 41kDa are α-subunits of selected heterotrimeric G-proteins - members of this family have been shown to be N-myristoylated in *C. elegans* (R.A. Aspbury, M.J. Fisher & H.H. Rees; unpublished observations). On this basis, it is clearly inappropriate to attempt to relate the extent of immunoprecipitation of [^{3}H]-labelled material, observed in the experiment illustrated in Fig. 3, to the immunodepletion of ~67% of C-subunit enzymic activity (be it myristoylated or not) by ab365 in the experiment illustrated in Fig. 1. Thus, although limitations on both the feasible availability of affinity-purified antibody and on the attainable specific radioactivity of biosynthetically labelled myristoyl-proteins in *C. elegans* made it impossible for us to confirm that we had achieved maximal immunoprecipitation of labelled C-subunit, it was clear that a significant subpopulation of the available C-subunit in *C. elegans* was myristoylated.

The sea-slug, *Aplysia,* expresses multiple isoforms of C-subunit, some of which arise from an alternative splicing event in the coding region of the N-terminus [16]. One N-terminal variant resembles the paradigm sequence of murine Cα and the other resembles that of both CeCATα and CeCATα' in *C. elegans.* Our results with ab201 show that the N-terminal sequence of the N-myristoylated C-subunit variant in *C. elegans* is not homologous with the murine Cα N-terminus, and that the additional polymorphism which we have demonstrated does not arise in this nematode by an analogous mechanism to that which operates in *Aplysia.*

The molecular mechanism by which N-myristoylation of C-subunit arises in *C. elegans* is unclear. In the light of the apparently exhaustive studies by Rubin's group of the genetics of C-subunit in this organism [18], it seems most likely that it must arise, contrary to accepted dogma [5, but see also 25], as a consequence of post-translational processing of a small proportion of the previously described CeCATα isozyme molecules.

ACKNOWLEDGMENTS

This work was supported in part by SOAEFD and by a BBSRC grant to R.A.C. R.A.A. was the recipient of a BBSRC postgraduate studentship.

REFERENCES

1. Hardie, G., and Hanks, S. (1995) The Protein Kinase FactsBook: Protein-Serine Kinases, Academic Press, London.
2. Carr, S. A., Biemann, K., Shoji, S., Parmelee, D. C., and Titani, I. (1982) *PNAS* **79,** 6128–6131.
3. McLaughlin, S., and Aderem, A. (1995) *TIBS* **20,** 272–276.
4. Towler, D. A., Gordon, J. I., Adams, S. P., and Glaser, L. (1988) *Ann. Rev. Biochem.* **57,** 69–99.
5. Resh, M. (1994) *Cell* **76,** 411–413.
6. Peitzsch, R. M., and McLaughlin, S. (1993) *Biochemistry* **32,** 10436–10443.

7. Milligan, G., Parenti, M., and Magee, A. I. (1995) *TIBS* **20,** 181–1862.
8. Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R. A., and Rothman, J. E. (1991) *Cell* **67,** 239–253.
9. Dizhoor, A. M., Chen, C.-K., Olshevskaya, E., Sinelnikova, V. V., Phillipov, P., and Hurley, J. B. (1993) *Science* **259,** 829–832.
10. Faux, M. C., and Scott, J. D. (1996) *TIBS* **21,** 312–315.
11. Nigg, E. A., Hilz, H., Eppenberger, H. M., and Dutly, F. (1985) *EMBO J.* **11,** 2801–2806.
12. Harootunian, A. T., Adams, S. R., Wen, W., Meinkoth, J. L., Taylor, S. S., and Tsien, R. Y. (1993) *Mol. Biol. of the Cell* **4,** 993–1002.
13. Clegg, C. H., Ran, W., Uhler, M. D., and McKnight, G. S. (1989) *J. Biol. Chem.* **264,** 20140–20146.
14. Panchal, R. G., Cheley, S., and Bayley, H. (1994) *J. Biol. Chem.* **269,** 23722–23730.
15. Adie, E. J., Thomas, P. H., Munday, M. R., and Clegg, R. A. (1995) *Biochem. Soc. Trans.* **23,** 451S.
16. Beushausen, S., Lee, E., Walker, B., and Bayley (1992) *PNAS* **89,** 1641–1645.
17. Jung, S., Hoffmann, R., Rodriguez, P. H., Mutzel, R., and Hofer, H. W. (1995) *Eur. J. Biochem.* **232,** 111–117.
18. Gross, R. E., Bagchi, S., Lu, X., and Rubin, C. S. (1990) *J. Biol. Chem.* **265,** 6896–6907.
19. Brenner, S. (1974) *Genetics* **77,** 71–94.
20. Gardner, R. A., Travers, M. M., Barber, M. C., Miller, W. R., and Clegg, R. A. (1994) *Biochem. J.* **301,** 807–812.
21. Flockhart, H., and Corbin, J. D. (1984) *in* Brain Receptor Methodologies, Part A (Marangos, P. J., Campbell, I. C., and Cohen, R. M., Eds.), pp. 209–215, Academic Press, New York.
22. Slice, L. W., and Taylor, S. S. (1989) *J. Biol. Chem.* **264,** 20940–20946.
23. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) *Science* **253,** 407–414.
24. Laemmli, U. K., and Favre, M. (1973) *J. Mol. Biol.* **80,** 575–599.
25. McIlhinney, R. A. J., and McGlone, K. (1990) *Biochem. J.* **271,** 681–685.