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IDENTIFICATION AND PARTIAL PURIFICATION OF A CHROMATIN BOUND CALMODULIN ACTIVATED HISTONE 3 KINASE FROM CALF THYMUS

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A calcium-calmodulin (Ca^{2+}-CaM) stimulated histone H3 phosphorylating activity was identified as a component of a nuclear protein complex purified from a 150 mM NaCl extract of calf thymus chromatin. This activity bound to a CaM-Sepharose affinity column in a Ca^{2+} dependent manner and was eluted off the column in the presence of EGTA. Equilibrium centrifugation of the EGTA eluate on a sucrose density gradient revealed that the activity is a component of a larger complex identified at 25% sucrose. This complex consisted of two major proteins, having M_r of 65 and 75 kDa. Using [^{125}I] CaM and the gel overlay technique it was shown that the 75 kDa protein is the major CaM binding protein in the complex.

In proliferating eukaryotic cells, a cascade of protein kinases, at least in part, regulates major transitions of the cell cycle, including the induction of mitosis (1). Among the specific proteins whose phosphorylation is induced at the G2-M transition and which remain phosphorylated throughout mitosis are several chromosomal proteins, including histones H1 and H3 (2-4) and a number of nonhistone proteins (5). The role that the phosphorylation of these proteins plays in chromatin condensation and/or control of mitotic events is currently unknown.

Probably the best characterized kinase of the cascade is the M phase-specific H1 kinase which is transiently activated at the G2-M transition in a wide variety of dividing cells (2, 6-10). Histone H1 is phosphorylated at sites specific to mitosis (11), an event concurrent with chromatin condensation during early metaphase (12). It has been proposed that H1 kinase is directly involved in chromatin condensation (13) and possibly the initiation of mitosis (14). Although the full range of natural substrates of this kinase is unknown, H1 kinase does not appear to phosphorylate H3.

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The abbreviations used are: CaM, calmodulin; H3, histone 3; EGTA, [ethylenebis (oxyethylenenitrilo)]-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris (hydroxymethyl) aminomethane; BSA, bovine serum albumin.

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H3 phosphorylation is also a strictly mitotic event which occurs at the G2-M transition and is rapidly reversed as cells leave the M phase (3,15,16). It has been further shown that H3 phosphorylation is regulated by Ca^{2+} at physiological concentrations (17). The fact that calmodulin (CaM) is known to be the primary mediator of Ca^{2+} dependent signaling in eukaryotic non-muscle cells (18,19), suggests that CaM might be involved in the regulation of H3 phosphorylation.

It has been reported that CaM can occupy intranuclear domains (20). Also, the nuclear matrix has been shown to contain several CaM binding proteins, including myosin light chain kinase (MLCK) (21) and a kinase characterized from neuronal nuclei (22). In addition nuclear CaM concentration was increased in proliferating rat liver cells activated by partial hepatectomy (23). These observations suggest that CaM may be important in regulating nuclear events during mitosis, including H3 phosphorylation.

EXPERIMENTAL PROCEDURES

Calf thymus histones were prepared according to Bonner et al. (24). CaM was purified by the method of Gopalackrishna and Anderson (25). CaM-Sepharose was prepared from purified CaM and cyanogen bromide-activated Sepharose 4B (Pharmacia Piscataway, N.J.) according to the manufacturer's instructions (26).

Calf thymus (200 gr) was homogenized in buffer 1 (10 mM potassium phosphate, 320 mM sucrose, 3mM $MgCl_2$, 10 mM $NaHSO_3$, pH 6.8) for 2 min at 6000 rpm in a Sorval Omni Mixer. This and all subsequent steps were performed at 4°C. The extract was filtered through 4 layers of cheese cloth and centrifuged at 1000 x g for 10 min. The pellet was suspended in buffer 1 and layered onto 20 ml of buffer 1 containing 2.4 M sucrose in polycarbonate tubes. The tubes were centrifuged at 70,000 x g for 120 min in a Beckman type 45 Ti rotor. The resulting pellet of chromatin was then washed twice in buffer 1. The purity of the chromatin preparation was confirmed by electron microscopy (data not shown).

The purified chromatin was suspended in 200 ml of buffer 2 (40 mM Tris-HCl, 150 mM NaCl, 4mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM DTT, pH 7.5), extracted by stirring for 30 min at 4°C and the extract centrifuged at 10,000 x g for 30 min. The resulting pellet was then re-extracted as described above. The pooled supernatants were applied to a CaM affinity column (15 x 1 cm), which was then washed with buffer 2 containing 500 mM NaCl, instead of 150 mM until the absorbance at 280 nm became less than 0.05. The column was then eluted with buffer 2 containing 500 mM NaCl and 2 mM EGTA instead of 0.5 mM $CaCl_2$. Fractions containing the protein peak were pooled and are referred to as the EGTA eluate.

A linear sucrose gradient (0-60%) was poured in 16 x 76 mm ultraclear tubes. The sucrose solutions used in preparing the gradient contained 40 mM Tris-HCl(pH 7.5), 500 mM NaCl, 4 mM $MgCl_2$, 2 mM EGTA, 0.1 mM DTT, 0.1 mM PMSF. The gradient had a total volume of 11 ml. Two-milliliters of EGTA eluate were layered on top of the gradients which were centrifuged for 36 hrs at 32,000 rpm at 4°C in a Beckman W 41 Ti rotor in a Beckman L7-65 centrifuge. The gradients were fractionated and the refractive index and H3 kinase activity determined.

Calf thymus histones were dissolved in 50 mM Tris-HCl, pH 6.8, to a final concentration of 5 $\mu g/\mu l$. The assay was performed in a final volume of 100 μl of 50 mM Tris (pH 6.8), 1 mM magnesium acetate, 0.5 mM 2-mercaptoethanol, 2 mM $CaCl_2$ (or 2 mM EGTA), 0.1 mM ATP, 10 μCi [^{32}P] ATP, 100 μg histones, variable amounts of CaM and an appropriate amount of enzyme (2-3 μg protein). Assays were carried out for 30 min at 30°C. The reaction was stopped by adding 20 μl SDS-buffer (5mM Tris-HCl, 20% SDS, 50% sucrose, bromophenol blue, pH6.7) and 10 μl 2-mercaptoethanol. Polyacrylamide gel electrophoresis was performed as described by Laemmli (27). Gels were stained with Coomassie Brilliant Blue and destained in 40% Methanol/10% Acetic Acid. Protein bands were cut out and counted in 5 ml of 3a70B scintillation fluid (Research Products International Corporation) using a Beckman liquid scintillation counter. Autoradiography was performed by exposing the dried gel to an X-ray film (Kodak, XAR-5). This assay was linear for a period of 60 minutes, and was used in screening column and gradient fractions as well as to quantitate recovery and the extent of purification.

Calmodulin was iodinated with Na^{125}I using Enzymobeads from Bio-Rad (28). Specific activity of the [^{125}I] CaM solution was 10^6 cpm/μg. Histone 3 kinase containing fractions were first separated by SDS-PAGE, then overlayed with [^{125}I] CaM as described by Alexander et al. (29).

RESULTS

Pooled extracts of chromatin in 150 mM NaCl were further purified by affinity column chromatography as described in Experimental Procedures. Washing of the CaM-affinity column with buffer containing EGTA instead of $CaCl_2$ resulted in the elution of a protein fraction which represented less than 5% of the total protein applied to the column (Table 1). This EGTA eluate could be shown to contain proteins which exhibited calcium dependent binding to calmodulin and to include a Ca^{2+}-CaM activated H3 kinase as described below.

Both the crude chromatin extract and the EGTA eluate were assayed for histone phosphorylating activity in vitro using calf thymus histones as substrate. It was observed that the crude extract contained protein kinase activity that phosphorylated all five histone fractions with H2B as the predominant substrate (Fig. 1). For the crude chromatin extract addition of Ca^{2+} and CaM to the assay did not appear to activate the phosphorylation of any of the histone fractions. This was also true when purified histone fractions were used as substrates. The EGTA eluate possessed a kinase activity that also phosphorylated all five histone fractions. However, phosphorylation of H3 was enhanced 5-6 fold in the presence of Ca^{2+}-CaM (Fig. 1). That H3 phosphorylation by the crude chromatin extract did not appear to be activated by Ca^{2+}-CaM could be due to the presence of other more abundant kinases in the extract, including a H2B kinase, that could phosphorylate H3 in a CaM independent manner and mask the appearance of the Ca^{2+}-CaM dependent phosphorylation mediated by H3 kinase.

In order to determine the concentration of CaM required to give maximum activation of H3 phosphorylation, assays were performed with calmodulin concentrations ranging from 0 to 50 μM. The histone concentration in the assay was 50 μM. Reactions were carried out in the presence of 200 μM Ca^{2+} or 500 μM EGTA and 2-3 μg enzyme. A minimum assay concentration of 100 μg CaM/ml (5.9 μM) was required to give maximum activation of H3 phosphorylation (Fig. 2). The phosphorylation of the other four histones was not affected by CaM in the concentration range used

TABLE I. Histone 3 phosphorylating activities of the different fractions in the presence and absence of calmodulin

	Total Protein (mg)	Specific activity cpm x 10^{-6}		Total activity cpm x 10^{-6}		Recovery (%)	Purification factor (fold)
		-CaM	+CaM	-CaM	+CaM	+CaM	+CaM
Crude Extract	61.6	2.4	2.3	146.4	141.1	100	1
EGTA Eluate	2.1	5.2	26	10.9	54.6	38.5	13.3
Peak I	0.35	1.3	1.6	0.49	0.56	4.0	
Peak II	0.36	10.2	50.2	3.7	18.1	12.8	21.8

Assays were performed as described in "Experimental Procedures". The assay mixtures contained 100 μg of histone and either 2mM EGTA or 2mM $CaCl_2$ and 10μg CaM in a final volume of 100 μl. The reaction was quenched by adding 30μl of a solution containing 5% SDS, 50% sucrose, 0.01% bromophenol blue, 30% 2-merceptoethanol and 25 mM Tris-Hcl, pH 6.7. Samples were boiled for 5 minutes and run on 16% acrylamide SDS-gel (20μl per lane). After staining the gel with coomassie blue and destaining it, the H3 region was cut out, dried and counted for ^{32}P. Peaks I and II are from sucrose density gradient centrifugation.

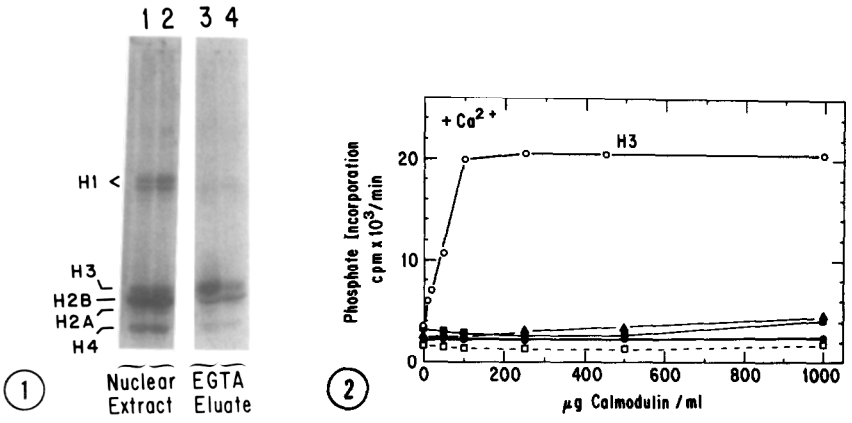

FIG. 1. **Autoradiogram of histones phosphorylated in the presence or absence of calmodulin.** Histones were phosphorylated in the presence of the 150 mM NaCl chromatin extract (Lanes 1 and 2) or the EGTA eluate (Lanes 3 and 4) in the presence (Lanes 1 and 3) or absence (Lanes 2 and 4) of calmodulin and calcium. Histone 3 is the predominant protein phosphorylated by the EGTA eluate in the presence of Ca^{2+} and calmodulin (Lane 3).

FIG. 2. **The histone phosphorylating activity of the EGTA eluate as a function calmodulin concentration.** Histones were phosphorylated in the presence of 0.5 mM $CaCl_2$ and equal volumes of EGTA eluate containing 2-3µg protein. Variable amounts of calmodulin were added to the assay mixtures. The reactions were quenched with SDS-buffer and samples were run on a 16% acrylamide SDS-gel. Histone regions were cut out and counted for ^{32}P incorporation. Histone fractions are: H1 (▲-▲), H3 (○-○), H2B (●-●), H2A (□-□) and H4 (■-■).

(Fig. 2). The inclusion of 0.1 mM trifluoperazine (CaM-antagonist) in the assay together with 200 μM Ca^{2+} and 10 μM CaM, suppressed phosphorylation of H3 to basal levels. This indicated that both Ca^{2+} and CaM are required for full activation of H3 phosphorylation under the assay conditions used.

Sucrose density equilibrium centrifugation of the EGTA eluate lead to the separation of two activity peaks (Fig. 3). Peak I contained a very small fraction of the activity and was unresponsive to CaM stimulation. Peak II contained the bulk of the kinase activity and this could be shown to be activated 5-6 fold by Ca^{2+}-CaM (Table 1). The highest activity in Peak II was identified at 25% sucrose, strongly indicating that the CaM activated H3 kinase is part of a larger complex obtained under the conditions of preparation.

Figure 4 shows the SDS-polyacrylamide gel electrophoresis profile of the isolated activity fractions. As can be seen from the gel the crude extract, EGTA eluate and peak II from the equilibrium centrifugation contained two prominent bands at about 65 kDa and 75 kDa. Both of these bands are most prominent in peak II, which is the fraction with highest specific activity (Table 1).

The binding of [^{125}I] CaM to specific proteins from the crude extract, EGTA eluate and peak II in the presence of Ca^{2+} or EGTA was studied as described in Experimental Procedures. Figure 5 shows that 2 proteins bound [^{125}I]CaM with high affinity in a Ca^{2+} dependent manner. Histones also bound CaM but in a Ca^{2+} independent manner. It is important to notice that the 75 kDa band showed the highest affinity. This is a further indication that the 75 kDa protein is either the kinase of interest or a component of it.

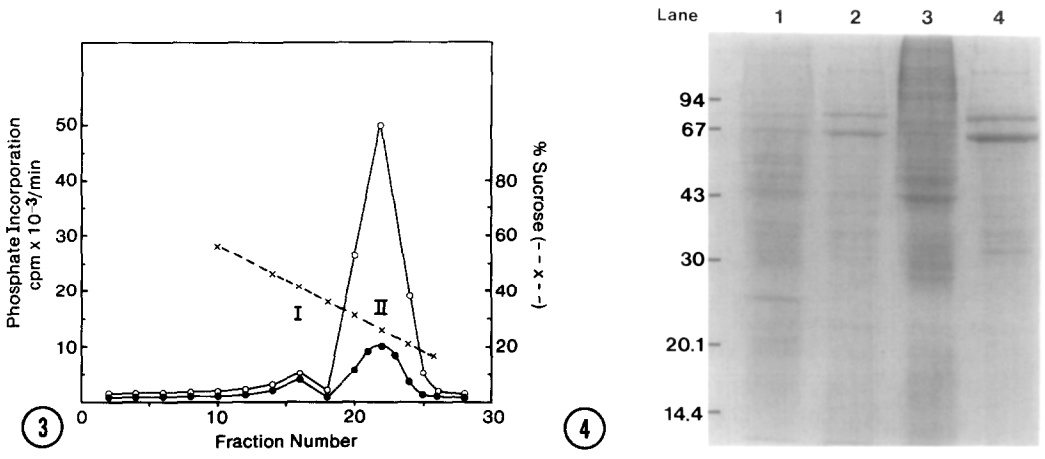

FIG. 3. **Equilibrium centrifugation of the EGTA eluate on a sucrose density gradient.** Fractions of 0.4 ml were collected from bottom to top of the tubes. The different fractions were assayed for their ability to phosphorylate H3. The reactions were quenched with SDS-buffer and run on a 16% acrylamide gel. Histone 3 regions were cut out and counted for ^{32}P incorporation which was about 5 times higher when assays were performed in the presence of calmodulin (O-O) than in its absence (●-●). Percentage sucrose (X-X).

FIG. 4. **SDS-polyacrylamide gel electrophoresis pattern of the different H3 kinase.** The polyacrylamide concentration in this gel was 12%. 1, 150 mM NaCl extract; 2, EGTA eluate; 3 and 4, activity peaks I and II respectively from the sucrose density gradient centrifugation. Arrow points to the 75 kDa protein.

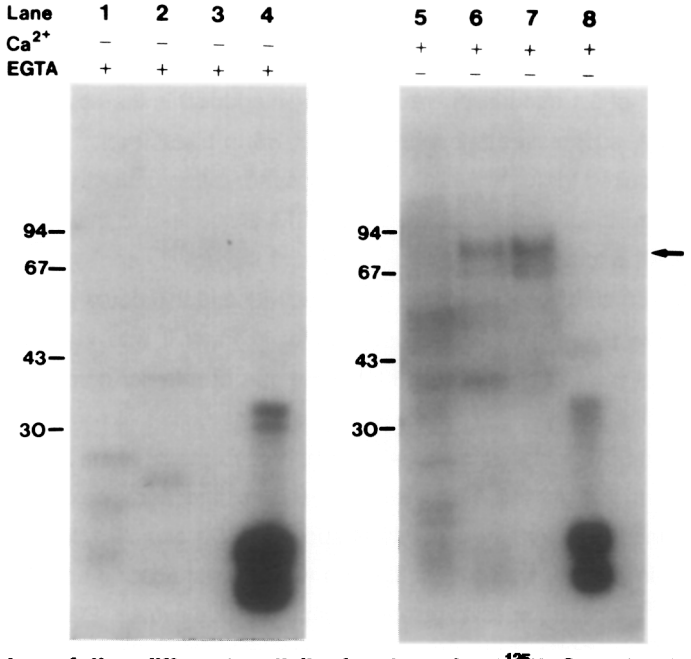

FIG. 5. **Overlay of the different activity fractions by [^{125}I] CaM in the presence and absence of Ca^{2+}.** The different activity samples were first separated on a 12% acrylamide gel. The gel was then overlayed with [^{125}I] CaM as in "Experimental Procedures." Lanes 1-4 were overlayed with [^{125}I] CaM in the presence of EGTA. Lanes 5-8 were overlayed with [^{125}I] CaM in the presence of Ca^{2+}. 1 and 5, 150 mM chromatin extract; 2 and 6, EGTA eluate; 3 and 7, peak II from the sucrose density gradient; 4 and 8, whole histones.

DISCUSSION

The foregoing results clearly demonstrate that calf thymus contains a chromatin bound protein kinase(s) which binds CaM in a Ca^{2+} dependent manner. Of particular interest to us is the fact that the phosphorylation of histone H3 by this kinase is enhanced about five fold in the presence of Ca^{2+} and CaM. Since it is well recognized that H3 phosphorylation is a strictly mitotic event (16) which is stimulated by Ca^{2+} at physiological concentrations (17), and since CaM is believed to regulate different stages of the cell cycle including mitosis (30,31), it may be postulated that H3 phosphorylation is one of a number of specific events that are controlled by the CaM mediated induction of mitosis.

The CaM activated H3 kinase (s) was initially identified by its binding to a CaM-Sepharose affinity column in the presence of Ca^{2+} and its elution from the column when Ca^{2+} was replaced with EGTA. Attempts to further purify the kinase using ion exchange and gel filtration chromatography as well as chromatofocusing and hydrophobic interaction chromatography proved unsuccessful. This lead us to believe that the kinase might be one component of a major complex. To test this hypothesis, a fraction of the EGTA eluate was subjected to sucrose density equilibrium centrifugation. As the results clearly show, the main CaM activated H3 kinase activity was identified at 25% sucrose. This could indicate that the kinase is in fact one component of a larger complex whose sedimentation coefficient is about equal to that of a ribosome. SDS-PAGE of different activity fractions revealed that two proteins with apparent molecular weights of 65 kDa and 75 kDa were the major proteins in the two fractions that displayed Ca^{2+}-CaM enhanced kinase activity, namely the EGTA eluate and peak II. In fact, these two proteins represented at least 80% of Peak II which in turn has twice the specific activity of the EGTA eluate. Peak I does not phosphorylate H3 in a Ca^{2+}-CaM dependent manner and contains very low amounts of the 65 kDa and 75 kDa proteins. We interpret these findings to mean that these two proteins are associated with the kinase of interest, possibly as constituent subunits of a multimeric kinase containing complex.

If either of the two proteins is the CaM activated H3 kinase that we are trying to identify it should bind CaM in a Ca^{2+} dependent manner. To answer this question the different activity fractions were run on an SDS-PAGE and overlayed with [^{125}I]CaM in the presence of EGTA or Ca^{2+}. Our results strongly indicate that while both the 65 kDa and the 75 kDa proteins bound CaM in a Ca^{2+} dependent manner, the 75 kDa protein seems to have a higher affinity for CaM. It is suggested that the 75 kDa chromatin bound protein is a CaM activated kinase or a regulatory subunit for which H3 is the best substrate. Phosphorylation of H3 leads to the formation of an acid labile histone phosphate (data not shown). This indicates that this kinase is most likely not related to CaM-kinase II nor to any of the CaM dependent kinases described thus far. This could also explain the very high CaM concentration needed for stimulation.

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REFERENCES

1. Russell, P., and Nurse, P. (1987) Cell 49, 559-567.
2. Bradbury, E.M., Inglis, R.J., and Mathews, H.R. (1974) Nature 247, 257-261.
3. Gurley, L.R., D'Anna, J.A., Barham, S.S., Deaven, L.L., and Tobey, R.A. (1978) Eur. J. Biochem. 84, 1-15.
4. Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988) Cell 55, 371-378.
5. Sahasrabuddha, C.G., Adlakha, R.C., and Rao, P.N. (1984) Ex. Cell Res. 153, 439-450.
6. Hardie, D.G., Mathews, H.R., and Bradbury, E.M. (1976) Eur. J. Biochem. 66, 37-42.
7. Lohka, M.J. (1989) J. Cell. Sci. 92, 131-135.
8. Meijer, L., Pelech, S.L., and Krebs, E.G. (1987) Biochemistry 26, 7968-7974.
9. Pelech, S.L., Meijer, L., and Krebs, E.G. (1987) Biochemistry 26, 7960-7968.
10. Woodford, T.A., and Pardee, A.B. (1986) J. Bio. Chem. 261, 4669-4676.
11. Langan, T.A. (1978) Meth. Cell. Biol. 19, 127-142.
12. Wu, R.S., Panusz, H.T., Hatch, C.L., and Bonner, W.H. (1986) CRC Crit. Rev. Biochem 20, 201-263.
13. Mueller, R.D., Yasuda, H., and Bradbury, E. M. (1985) J. Biol. Chem. 260, 5081-5086.
14. Inglis, R.J., Langan, T.A., Mathews, H.R., Hardie, D.G., and Bradbury, E.M. (1976) Exp. Cell. Res. 97, 418-42522.
15. Marks, D.B., Park, W.K., and Borun, T.W. (1973) J. Biol. Chem. 248, 5660-5667.
16. Gurley, L.R., Walters, R.A., and Tobey, R.A. (1974) J. Cell Biol. 60, 356-364.
17. Whitlock, J.P., Augustin, R., and Schulman, H. (1980) Nature 287, 74-76.
18. Means, A.R., Tash, J.S. and Chafouleas (1982) Physiol. Rev. 68, 1-38.
19. Means, A.R., (1988) Recent Prog. Hormone Res. 44, 223-262.
20. Harper, J.F., Cheung, W.Y., Wallace, R.W., Huang, H-L., Levine, S.N., and Steiner, A.L. (1980) Proc. natl. Acad. Sci. USA 77, 366-370.
21. Guerriero, V., Rowley, D.R., and Means, A.R. (1981) Cell 27, 449-458.
22. Sahyoun, N., LeVine, H. III, Bronson, D., and Cuatrecasas, P. (1984) J. Biol. Chem. 259, 9341-9344.
23. Serratosa, J., Pujol, M.J., Bachs, O., and Carafoli, E. (1988) Biochem. Biophys. Res. Comm. 150, 1162-1169.
24. Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura., F., Huang, R.C.C., Humberman, J., Jensen, R., Manishige, K., Ohlenbusch, H., Olvera, B., and Widholm, J. (1968) In "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 12, P. 3, academic Press, New York.
25. Gopalakrishna, R., and Anderson, W.B. (1982) Biochem. Biophys. Res. Comm. 104, 830-836.
26. Methods for coupling ligands to CNBr. activated sepharose 4B (1979). In "Affinity Chromatography, Principles and Methods", Pharmacia Fine Chemicals.
27. Laemmli, U.K. (1970) Nature 277, 680-685.
28. Neff, N.T., Boureet, L., Miao, P., and Dice, F. (1981) J. Cell Biol. 91, 184-194.
29. Alexander, K.A., Wakim, B.T., Doyle, G.S., Walsh, K.A. and Storm, D.R. (1988) J. Biol. Chem. 263, 7544-7549.
30. Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd, A.E. III, and Means, A.R. (1982) Cell 28, 41-50.
31. Sasaki, Y., and Hidaka, H. (1982) Biochem. Biophys. Res. Comm. 104, 451-456.
32. Eilam, Y., and Chernichovsky, D. (1988) J. Gen. Microbiology 134, 1063-1069.