Vol. 141, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 26, 1986 Pages 60-66

MONOCLONAL ANTIBODY THAT BLOCKS PHOSPHOINOSITIDE-DEPENDENT ACTIVATION OF MOUSE TUMOR DNA POLYMERASE ALPHA

V.L. Sylvia[1,2], J.O. Norman[3], G.M. Curtin[2], and D.L. Busbee[1,2]

[1]Department of Anatomy ,[2]Department of Physiology and Pharmacology ,
College of Veterinary Medicine, Texas A&M University, College Station, TX 77843
[3]Veterinary Toxicology and Entomology Research Laboratory
USDA, ARS, College Station, TX 77841

Received October 14, 1986

A monoclonal antibody (MaB) against mouse sarcoma DNA polymerase alpha was isolated from the culture medium of an IgG-secreting hybridoma. The MaB demonstrated reactivity against two murine DNA polymerase alpha preparations and a calf thymus DNA polymerase alpha. Murine sarcoma polymerase was activated in vitro by phosphatidylinositol-4-monophosphate (PIP) showing increased deoxynucleotidyltransferase activity and enhanced binding affinity to activated DNA template. The MaB did not neutralize polymerase activity, but blocked further activation of the enzyme by PIP. Treatment of polymerase with MaB prior to treatment with PIP inhibited both increased enzyme activation and increased binding of the enzyme to DNA template. Treatment of polymerase with MaB subsequent to treatment with PIP did not block enzyme activation or increased DNA template binding. The data suggest that this anti-DNA polymerase alpha IgG is directed against a regulatory subunit of the polymerase rather than the catalytic subunit. The antibody may serve to distinguish between DNA polymerase alpha preparations with distinctly different regulatory subunits.

DNA polymerase alpha, (deoxynucleotidyltransferase, E.C. 2.7.7.7, DNA pol-a), is the major enzyme of eukaryotic DNA replication during either mitosis or excision repair (1-3). It is a multimeric enzyme composed of a larger catalytic subunit (140-190 kD) and two or more smaller, presumably regulatory, subunits (40-70 kD). Monoclonal antibodies against the catalytic subunit have been developed for DNA pol-a preparations from a variety of cell types, including human KB cells (4,5), calf thymus (6,7), monkey kidney (8), and chicken embryo (9,10). These MaB have been used as specific probes to study the function of DNA pol-a in dividing cells (11-13) and as ligands for immunoaffinity chromatographic purification of DNA pol-a (10, 14).

We have investigated the structure and function of murine DNA pol-a isolated from Norman Murine Sarcoma (NMS) tissue. This enzyme is typical of alpha polymerases in that it is inhibited by aphidicolin (15) but is resistant to dideoxythymidine triphosphate inhibition (16). DNA pol-a specific activity and binding affinity to activated DNA are increased following treatment with phosphoinositides (17) or with phosphatidylinositol kinase (PIK) +

Abbreviations: DNA nucleotidyltransferase (E.C. 2.7.7.7), DNA pol-a; phosphatidylinositol, PI; phosphatidylinositol-4-monophosphate, PIP; phosphatidylinositol kinase, PIK; Norman murine sarcoma, NMS; immunoglobulin G, I.G; monoclonal antibody, MaB; anti-DNA polymerase alpha IgG-secreting hybridoma, BD101; enzyme-linked immunosorbent assay, ELISA; hybridoma-conditioned medium, HCM; Iscove's minimal essential medium, IMEM.

0006-291X/86 $1.50

phosphatidylinositol (PI) + ATP (18). Murine DNA pol-a exists as a tetramer composed of a catalytic subunit (150 kD) and three accessory subunits (50-60 kD) with the smallest subunit being a putative phosphoprotein. Phosphoinositide treatment enhances DNA pol-a binding to activated DNA cellulose and apparently increases the processivity of DNA pol-a on activated DNA template-primer.

We have developed a hybridoma secreting IgG with specificity for murine sarcoma DNA pol-a. We have examined the specificity of purified anti-DNA pol-a IgG against NMS, Ehrlich ascites, and calf thymus polymerases, and have tested the effects of this anti-DNA pol-a IgG on specific activity and DNA binding characteristics of the NMS polymerase.

MATERIALS AND METHODS

Chemicals: Deoxynucleoside triphosphates, activated DNA (Type XV), phosphatidylinositol-4-monophosphate (PIP), 50X hypoxanthine-aminopterin-thymidine (HAT) medium supplement, and anti-mouse IgG peroxidase conjugate were obtained from Sigma. Methyl-(^{3}H)-thymidine-5'-triphosphate (20 Ci/mmole) was obtained from ICN. Iscove's modified Minimal Essential Medium (IMEM) was obtained from Boehringer-Mannheim. Heat-inactivated fetal bovine serum (FBS) was obtained from Gibco.

Purification of DNA polymerase alpha: DNA pol-a preparations (6000 units/mg and 20,000 units/mg) were purified from NMS tissue and Ehrlich ascites cells, respectively. The purification protocols followed were Mechali's method (19) as modified by Busbee et al (20) coupled with increased purification using immunoaffinity chromatography (14). Size exclusion HPLC of DNA pol-a was performed using a Model 100 Perkin-Elmer HPLC system and Beckman TSK-4000 analytical columns. Polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE were performed to determine enzyme purity and subunit composition. Protein determinations performed throughout the purification process utilized the Bradford technique (21).

DNA polymerase alpha assay: DNA pol-a assay procedures were modified from Delfini et al (22). The reaction mixture contained activated DNA (100 g/ml); unlabeled dATP, dCTP, and dGTP (50 M each); dTTP (45 M); methyl-(^{3}H)-thymidine-5'-triphosphate (2.5 Ci/ml); bovine serum albumin (100 g/ml); dithiothreitol (0.5 mM); 50 mM Tris-HCl (pH 8); 3 mM $MgCl_2$; and 0.5 mM KCl. Aliquots of DNA pol-a (100 l) were incubated with equal volumes of the reaction mixture and buffer containing 50 mM Tris-HCl, 6 mM $MgCl_2$ and 1 mM KCl (pH 8). PIP and/or anti-DNA pol-a IgG were added to determine their effect on polymerase activity. Assay mixtures were incubated at 37 C for 90 minutes. The incubation was terminated by addition of 5 ml of cold 10% trichloroacetic acid (4C), and ^{3}H-dTMP incorporation into template DNA was radiometrically assessed (23). One unit of DNA pol-a activity is defined as the amount catalyzing the incorporation of 1 nmol of dTMP into template DNA in 60 min at 37 C.

Production and Selection of Hybridomas: Nine male BALB/c x C57BL/6 F_1 mice were intraperitoneally injected weekly with 200 units of DNA pol-a for 7 weeks. Choice of mouse strain and immunization schedule followed that of Tanaka et al (4). Splenocytes isolated from mice demonstrating the highest serum level of DNA pol-a specific antibody were fused to non-IgG secreting myeloma cells (P3/X63/Ag8.653) obtained from the American Type Culture Collection. Myeloma cells (5 X 10^7 cells/ml) were added to splenocytes (1 x 10^5 cells/ml) in 12 ml of IMEM total volume with stirring. To this mixture 0.5 ml of 50% polyethylene glycol (Kodak 1415, mw 8000) was added dropwise over 5 min and stirred for 10 min. The cell mixture was immediately diluted with 72 ml of IMEM containing 20% FBS and 1% 50X HAT solution. Cells were plated into 96-well Costar tissue culture plates and given fresh IMEM-HAT medium every three days for 4 weeks. Clones were expanded and maintained in IMEM. Enzyme-linked immunosorbent assays (ELISA) were used to test mouse sera and hybridoma-conditioned medium (HCM) for reactivity against 10 ng of purified murine sarcoma DNA pol-a in the initial screening phases.

Antibody purification and subclass determination: HCM from the hybridoma clone designated BD101 exhibited the greatest reactivity toward purified mouse sarcoma DNA pol-a. HCM was pooled, concentrated with 27% sodium sulfate and Protein A-Sepharose purified using the method of Ey et al (24) or using the System II IgG isolation kit (Isolab, Inc.). The sub-isotype of IgG secreted by BD101 hybridoma cells was determined using the Mouse Typer kit (Bio-Rad Laboratories).

Enzyme-linked immunosorbent assay (ELISA): Purified DNA pol-a preparations between 5 ng/ml and 10 g/ml were placed into 96-well microtiter plates and incubated 16 h at 4C to coat the well surface. Residual protein-binding sites were blocked by incubation for 30 min at room temperature with two changes of phosphate buffered saline containing 0.05% Tween-20 (PBST) and 5% FBS. Each well of the plates was treated sequentially with (1) 5 ng of BD101 IgG (100 l), (2) 1:1000 dilution of anti-mouse IgG peroxidase conjugate, and (3) substrate solution containing 1 mg/ml o-phenylenediamine and 40 l/ml 3% hydrogen peroxide in 0.1 M sodium citrate buffer, pH 4.5. The plates were incubated with each reagent for 30 min at room temperature with a PBST wash between steps. Following the last incubation, the absorbance (490 nm) was spectrally determined using the MR 600 microplate reader (Dynatech Laboratories, Inc.) at r = 490 nm and t = 410 nm.

DNA affinity assay: The binding affinity of DNA pol-a for activated DNA cellulose was determined using the method of Lawton et al (25). Purified murine DNA pol-a (150 units) was treated with control (50 mM Tris-HCl) buffer, PIP (10 g/ml), or BD101 IgG (10 g) for 30 min at 4C before being applied to an activated DNA-cellulose column (1.5 x 3.0 cm). In one series of experiments, the polymerase was incubated first with BD101 IgG for 15 min followed by the PIP treatment for 15 min, or vice-versa. After DNA pol-a was applied to the column the DNA cellulose was eluted for 20 min with 70 mM KCl and 20 min with 370 mM KCl at 0.5 ml/min, and 0.5 ml fractions were collected. The ratio of DNA pol-a eluting in the 70 mM (low affinity) and 370 mM (high affinity) washes was assessed for polymerase activity and for total protein.

RESULTS AND DISCUSSION

BD101 hybridoma secretes IgG recoverable at 40-50 g of anti-DNA pol-a IgG per ml of medium. Initial screenings of polyclonal HCM preparations suggested the primary immunoglobulin subisotype to be IgG_1 with trace amounts of IgG_{2a} and IgG_{2b}. However, the MaB was determined to be IgG_1 using the Mouse Typer test. Reactivity toward IgA, IgE or IgM was not observed for any HCM sample tested. Maximal production of BD101 IgG was observed in second passage cultures of the hybridoma with decreasing titers thereafter. Maximal cell viability was achieved for cells frozen in 96 well plates by rapidly thawing cells in the plates in a 37C $C0_2$ incubator and immediately transferring the well contents to culture flasks containing fresh prewarmed IMEM. Increased antibody production was observed in older hybridoma cultures following freezing and rethawing.

DNA pol-a, 0.5 ng to 1.0 g, purified from NMS tissue, Ehrlich ascites cells, or calf thymus was detected with 5 ng of IgG using ELISA techniques (Fig. 1). When incubated with concentrations of IgG up to 10 g, NMS DNA pol-a (6000 units/mg) did not exhibit a significant decrease in deoxynucleotidyltransferase activity compared to enzyme samples treated with control buffer. Treatment of DNA pol-a with PIP elicited an increase in enzyme specific activity, resulting in greater incorporation of dTMP into activated DNA template. The dTMP incorporation increase was dose responsive and was observed for DNA pol-a samples incubated with greater than 0.1 g/ml PIP or with a mixture of PIK (50 g/ml), PI (1 g/ml) and 50 mM ATP (18). In DNA pol-a assays (total vol. 0.3 ml), 5 ng of IgG was sufficient to block activation of the enzyme (150 units) by PIP (10 g/ml). As seen in Table

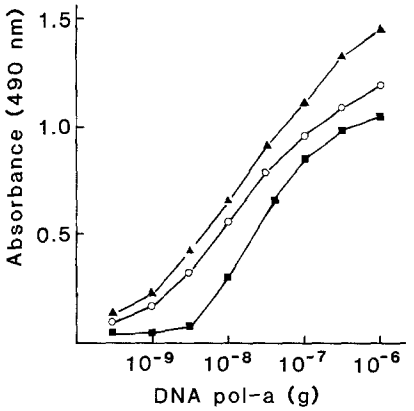

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) of 0.5 ng-1.0 g DNA polymerase alpha from murine sarcoma (O), Ehrlich ascites (▲) or calf thymus (■) incubated with 5 ng of BD101 antibody as described in Materials and Methods.

1, incubation of pol-a with PIP prior to treatment with IgG resulted in increased pol-a specific activity, but incubation of pol-a with IgG prior to treatment with PIP prevented the activation of pol-a.

Phosphorylation of murine sarcoma DNA pol-a is reported to increase its binding affinity for activated DNA-cellulose (18). Treatment of polymerase preparations (150 units) with PIK+PI+ATP prior to DNA-cellulose chromatography resulted in the loss of enzyme eluting in the low binding affinity fraction and significantly increased the polymerase fraction eluting in the higher ionic strength wash (Fig. 2). Incubation of pol-a with PIP (10 g/ml) prior to treatment with IgG (10 g) also increased the binding affinity of polymerase to the DNA-cellulose column (Fig. 3), whereas incubation of the enzyme with IgG prior to treatment with PIP decreased pol-a binding to the column (Fig. 4), with 95% of the polymerase eluting in the column void volume.

Table 1. Effects of Phosphatidylinositol-4-monophosphate and Anti-DNA Polymerase Alpha Antibody on DNA Polymerase Activity In vitro

Treatment	DNA Pol-a Activity
Control buffer	253 ± 27
BD101 IgG	264 ± 46
PIP	2425 ± 63
PIP 1st (15 min), BD101 2nd (15 min)	1943 ± 175
BD101 1st (15 min), PIP 2nd (15 min)	226 ± 25

The reaction mixture contained mouse sarcoma DNA pol-a (6000 units/mg) plus 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$ (control buffer), 5 ng BD101 IgG and/or 100 ng PIP. DNA pol-a activity is expressed as cpm of ^{3}H-dTMP incorporated into activated DNA/0.3 ml/90 min.

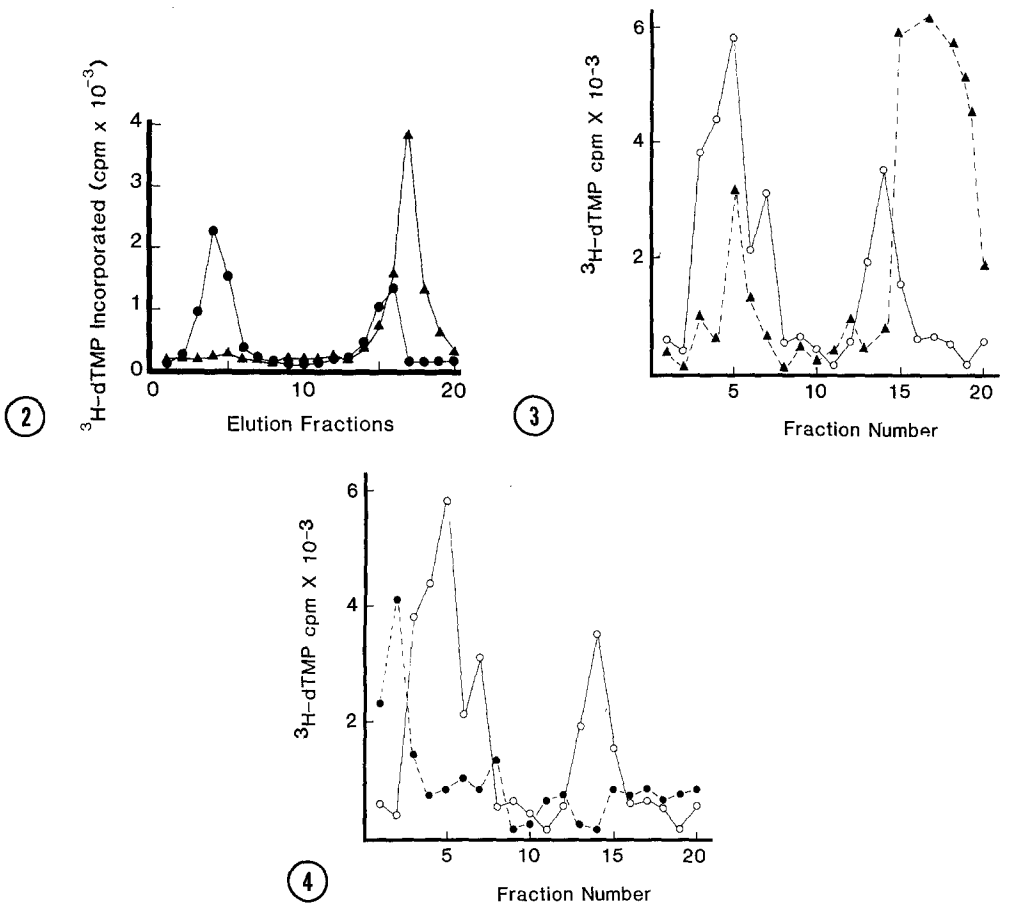

Fig. 2. An examination of mouse sarcoma DNA pol-a binding to DNA. An activated DNA cellulose column (0.5 x 3 cm) was prepared and equilibrated in 70 mM KCl. DNA pol-a (6000 units/mg, 150 units) treated with 50 mM Tris-HCl (pH 7.8) control buffer (●) or DNA pol-a treated with 10 g/ml PIP (▲) were incubated for 30 min at 4C and applied to the column. The column was washed with low ionic strength KCL buffer (70mM) and high ionic strength KCl buffer (370 mM) as described in Materials and Methods. Enzyme activity is expressed as cpm of ^{3}H-dTMP incorporated into template DNA/0.2 ml/ 90 min.

Fig. 3. Effect of phosphatidylinositol 4-monophosphate on DNA binding affinity. The affinity of mouse sarcoma DNA pol-a (150 units) for DNA was measured by activated DNA-cellulose chromatography. Untreated polymerase (O) or polymerase incubated with 1 g/ml PIP for 30 min followed by 10 g of BD101 IgG for 30 min at 4C (▲) before application to the DNA cellulose. The column was washed as stated for Fig. 3. Enzyme activity is expressed as cpm of ^{3}H-dTMP incorporated into acid insoluble product/ 0.2ml/ 90 min.

Fig. 4. Effect of BD101 antibody on DNA binding affinity. The affinity of mouse sarcoma DNA pol-a (150 units) for DNA was measured by activated DNA-cellulose chromatography. Untreated DNA pol-a (O) or polymerase incubated with 10 g of BD101 IgG for 30 min followed by 1 g/ml PIP for 30 min at 4C (●) before application to the DNA cellulose. The column was washed as stated for Fig. 3. Enzyme activity is expressed as cpm of ^{3}H-dTMP incorporated into acid insoluble product/0.2 ml/90 min.

A considerable degree of molecular heterogeneity has been reported for DNA pol-a (26), but a subunit approximating a molecular weight of 50 kD appears common to most murine and other mammalian pol-a preparations. DNA pol-a purified from Ehrlich ascites cells by immunoaffinity chromatography (14) displayed four subunits when treated with 50% ethylene

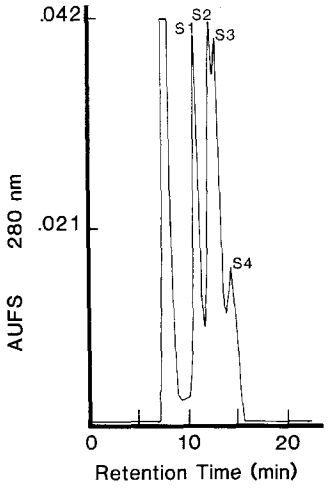

Fig. 5. Examination of DNA polymerase alpha subunits separated by HPLC. Ehrlich ascites cell DNA pol-a purified by immunoaffinity and DNA affinity techniques was treated with 50 % ethylene glycol for 45 min and subjected to size exclusion HPLC separation in phosphate buffered saline with 1 mM EDTA and 0.5 mM dithiothreitol at 1 ml/min. The system employed a TSK-125 guard column and a TSK-4000 analytical column (7.5 x 300 mm). The eluent was monitored at 280 nm with 0.042 absorption units full scale (AUFS). The initial peak at 7 min was ethylene glycol at the column void volume. ELISA of Ehrlich ascites DNA pol-a subunits in the peak samples were assessed using a Dynatech MR600 microplate reader at r=490 and t=410 nm, and are as follows: S1 (10.47 min) = 0.004, S2 (12.11 min) = 0.028, S3 (12.72 min) = 0.007, and S4 (14.26 min) = 0.473

glycol and separated by size exclusion HPLC (Fig. 5). Both the intact enzyme and the smallest subunit (about 50 kD) exhibited ELISA reactivity toward BD101 IgG, suggesting the presence of similar antigenic sites on both NMS and Ehrlich ascites DNA pol-a. Monoclonal IgG developed against human DNA pol-a catalytic subunit is reported to lack reactivity against rat or mouse DNA pol-a (11), whereas BD101 IgG demonstrated reactivity against both bovine and murine polymerases.

PIK-directed DNA pol-a activation has been proposed to occur by phosphorylation of a 50 kD enzyme subunit (18). Our data showing BD101 IgG to react with the DNA pol-a 50 kD subunit blocking PIP activation of the enzyme support the proposal (18) that DNA pol-a is activated by PIP interaction with the 50 kD subunit, and suggest that BD101 IgG may be directed against an epitope at or near a critical site on the 50 kD subunit. Reactivity of BD101 IgG to various DNA pol-a preparations (Fig. 1) suggests similarity of an IgG-binding region among polymerases. Phosphorylation of a DNA pol-a 50 kD subunit increases both specific activity of the enzyme and specific binding of the enzyme to a DNA-cellulose column. Since BD101 IgG is specific for the 50 kD subunit and DNA pol-a treatment with BD101 IgG blocks both pol-a activation and PIP-increased pol-a binding to a DNA-cellulose column, the 50 kD subunit apparently plays a critical role in the binding of DNA pol-a to DNA prior to the initiation of DNA replication. These data suggest the possibility that conservation of a DNA-binding subunit with structural similarities may occur for DNA pol-a from different species. Monoclonal IgG reactivity against specific DNA pol-a subunits may be useful in screening for such subunit similarities. These MaB may also be useful to distinguish

DNA pol-a preparations possessing or lacking specific subunits, and may provide a mechanism for studying the subunit function in eukaryotic DNA replication.

REFERENCES

1. Fisher, P. and Korn, D. (1977) J. Biol. Chem. 252, 6528-6535.
2. Weissbach, A. (1979) Arch. Biochem. Biophys. 198, 386-396.
3. Mosbaugh, D. and Linn, S. (1984) J. Biol. Chem. 259,N 1027-10251.
4. Tanaka, S., Hu, S., Wang, T. and Korn, D. (1982) J. Biol. Chem. 257, 8386-8390.
5. Wang, T., Hu, S. and Korn, D. (1984) J. Biol. Chem. 259, 1854-1865.
6. Masaki, S., Hiroshi, S., Tsuguhiro, K., Koiwai, O. and Yoshida, S. (1982) Nucleic Acids Res. 10, 4703-4713.
7. Masaki, S., Tanabe, K. and Yoshida, S. (1984) Nucleic Acids Res. 12, 4455-4467.
8. Karawya, E., Swack, J., Albert, W., Fedorko, J., Minna, J. and Wilson, S. (1984) Proc. Natl. Acad. Sci. USA 81, 7777-7781.
9. Matsukage, A., Yamaguchi, M., Tanabe, K., Nishizawa, M., Takahashi, T., Seto, M. and Takahashi, T. (1982) Gann 73, 850-853.
10. Hirose, F., Kedar, P., Takahashi, T. and Matsukage, A. (1985) Biochem. Biophys. Res. Commun. 132, 210-216.
11. Nakamura, H., Morita, T., Masaki, S. and Yoshida, T. (1984) Exp. Cell Res. 151, 123-133.
12. Miller, M., Ulrich, R., Wang, T. and Korn, D. (1985) J. Biol. Chem. 260, 134-138.
13. Miller, M., Seighman, C. and Ulrich, R. (1985) Biochemistry 24, 7440-7445.
14. Chang, L., Rafter, E., Augl, C. and Bollum, F. (1984) J. Biol. Chem. 259,14679-14687.
15. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) Nature 275, 458-460.
16. Waqar, M., Evans, M. and Huberman, J. (1978) Nucleic Acids Res. 5, 1933-1946.
17. Sylvia, V., Joe, C., Norman, J., Curtin, G. and Busbee D. (1986) Biochem. Biophys. Res. Commun. 135, 880-885.
18. Busbee, D., Joe, C., Sylvia, V., Norman, J. and Ragsdale-Robinson, S. (1986) Proc. Natl. Acad. Sci. USA, In Press.
19. Mechali, M., Abadiedebat, J. and deRecondo, A. (1980) J. Biol. Chem. 255, 2114-2122.
20. Busbee, D., Joe, C., Norman, J. and Rankin, P. (1984) Proc. Natl. Acad. Sci. USA 81, 5300-5304.
21. Bradford, M. (1976) Anal. Biochem. 72, 248-256.
22. Delfini, C., Alfani, E., DeVenezia, V., Oberholtzer, G., Tomasello, C., Eremenko, T. and Volpe, P. (1985) Proc. Natl. Acad. Sci. USA 82, 2220-2224.
23. Joe, C., Rankin, P. and Busbee, D. (1984) Mutation Res. 131, 37-43.
24. Ey, P., Prowse, S. and Jenkin, C. (1978) Immunochemistry 15, 429-436.
25. Lawton, K., Wiersocki, J., Schechter, S., Hilf, R. and Bambara, R. (1984) Biochemistry 23, 4294-4300.
26. Chen, Y., Bohn, E., Planck, S. and Wilson, S. (1979) J. Biol. Chem. 254, 11678-11687.