Metal Ion Content of *Dolichos biflorus* Lectin and Effect of Divalent Cations on Lectin Activity†

Carl A. K. Borrebaeck, Bo Lönnerdal,‡ and Marilynn E. Etzler*

ABSTRACT: The metal ion content of the *Dolichos biflorus* seed lectin has been determined by using atomic absorption spectrophotometry. Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} were all found in the native lectin and could be removed to different degrees by using a variety of techniques such as high ionic strength, low pH, chelating agent, and combinations of these procedures. By use of affinity electrophoresis, the remaining binding capacity of the lectin could easily be determined, and the *D. biflorus* lectin showed an absolute requirement for divalent cations to be able to bind *N*-acetyl-D-galactosamine. The association constant (K_a) of the lectin for *N*-acetyl-D-galactosamine was determined after remetallization of the lectin with each individual metal ion and a combination of them. The K_a of the lectin for the hapten differed depending on which divalent cation had been used for remetallization. Ca^{2+} ion alone was equal to a combination of all metal ions in its ability to confer the highest binding activity of the lectin for *N*-acetyl-D-galactosamine. The other cations, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}, could not restore the binding activity of the lectin to the same degree as Ca^{2+} or a combination of all ions. The *D. biflorus* lectin is, therefore, one of the first lectins that has been shown to require only a single cation to fully retain its binding activity in contrast to most lectins that require a combination of Ca^{2+}, Mn^{2+}, Mg^{2+}, or Zn^{2+}. Furthermore, we have shown that there is a selective process of ion uptake into the lectin since the metal ion ratios in the native lectin compared to the whole seed are quite different.

The requirements of some proteins for metal ions to retain their biological activity are well established. A relatively new class of metalloproteins are the lectins, the carbohydrate-binding proteins of nonimmune origin most widely found in leguminous plants. The lectins from jack bean (*Concanavalia ensiformis*; Agrawal & Goldstein, 1968), lima bean (*Phaseolus lunatus*; Galbraith & Goldstein, 1970, 1972), red kidney bean (*Phaseolus vulgaris*; Galbraith & Goldstein, 1970), and soybean (*Glycine max*; Jaffe et al., 1977), to mention a few of the most thoroughly examined, all have an absolute requirement for divalent cations to be able to bind carbohydrates.

The presence of metal ions has also been shown to confer other important features to the lectin molecules such as increased resistance against proteolytic enzymes (Blumberg & Tal, 1976; Thomasson & Doyle, 1975) and high thermal stability (Borrebaeck & Mattiasson, 1980; Doyle et al., 1976). Whether these effects reflect any in vivo roles of the metal ions remains, however, to be seen.

In this paper we report the metal ion content as well as the metal requirements of the lectin from horse gram (*Dolichos biflorus*) seeds. This lectin has a specificity for terminal nonreducing *N*-acetyl-α-D-galactosamine residues (Etzler & Kabat, 1970; Hammarström et al., 1977), and its structure has been extensively characterized (Carter & Etzler, 1975a,b,c; Etzler et al., 1977). Using an affinity electrophoretic system (Borrebaeck & Etzler, 1980), we have determined the effects of removal and subsequent addition of different metal ions on the association constant of this lectin for *N*-acetyl-D-galactosamine.

Materials and Methods

Materials. *D. biflorus* seeds were obtained from F. W. Schumacher Co., Sandwich, MA. Blood group A+H substance was isolated by ethanol precipitation (Kabat, 1956) from hog gastric mucin (Wilson Laboratories, Chicago, IL).

Purification of the Lectin. The *D. biflorus* lectin was isolated as previously described (Etzler & Kabat, 1970; Carter & Etzler, 1975a) by adsorption of the lectin onto insoluble polyleucyl hog blood group A+H substance and specific elution with 0.01 M *N*-acetyl-D-galactosamine. The ligand was removed by molecular exclusion chromatography, and the lectin was concentrated by ultrafiltration in a Diaflo ultrafiltration apparatus equipped with a PM-10 filter.

Demetallization of Glassware and Reagents. All glassware used in this investigation was allowed to soak 15 min in 5 M HNO_3 after the regular laboratory wash. It was then rinsed 5 times in distilled water and 5 times in water filtered through a Milli-Q Water Purification System apparatus (Millipore Corporation, Bedford, MA) and allowed to air-dry.

The blood group substance was dialyzed for 48 h against solution A of the affinity electrophoresis system (Borrebaeck & Etzler, 1980) before use. *N*-Acetyl-D-galactosamine (Pfanstiehl Laboratories, Inc., Waukegan, IL) was demetallized by using a strong cation exchanger (Dowex 50W) equilibrated with distilled water. The carbohydrate-containing fractions were collected after the chromatography, and the solution was lyophilized before use as the free ligand in the affinity system.

All reagents in the affinity electrophoresis system were tested for their metal ion content by atomic absorption spectrophotometry; Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} were analyzed, and only traces of Ca^{2+} were detectable, the levels always below 0.025 ppm.

Atomic Absorption Spectrophotometry. Seed samples were digested in concentrated nitric acid (Ultrex grade, J. T. Baker Co., San Francisco, CA) on a hot plate covered with a glass tray in order to avoid contamination. Lectin samples as well as digested seed samples were then aspirated into a flame (air–acetylene) atomic absorption spectrophotometer (Perkin-Elmer 370, Norwalk, CT). Standards were made by diluting commercial stock solutions of the respective metals (Fischer Scientific Co., Pittsburgh, PA).

Affinity Electrophoresis. A discontinuous nondenaturing polyacrylamide tube affinity electrophoretic system for *N*-

† From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received November 19, 1980.* This work was supported by U.S. Public Health Service Grant GM21882 from the National Institutes of Health and U.S. Department of Agriculture Grant SEA 5901-0-0242.

‡ Present address: Department of Nutrition, University of California, Davis, CA 95616.

0006-2960/81/0420-4119$01.25/0

Table I: Metal Ion Content of Native *D. biflorus* Lectin

preparation	mol of metal ion/mol of lectin						
	Ca^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}	Cu^{2+}	Fe^{2+}	Σ
Pl 42	3.55	1.55	0.70	0.51	0.64	0.00	6.95
Pl 43	3.28	1.77	0.64	0.25	0.27	0.00	6.21
Pl 44	3.18	1.46	0.60	0.43	0.38	0.00	6.05
seeds from *D. biflorus* (μmol/g of seed)	30.4	59.5	4.5	0.40	0.14	0.79	

acetyl-D-galactosamine-binding lectins, as previously described (Borrebaeck & Etzler, 1980), was used. Briefly, hog blood group A+H substance, containing terminal nonreducing *N*-acetyl-α-D-galactosamine residues, was entrapped in the polyacrylamide (5%) network of the running gel (pH 7.5). By use of different amounts of the free ligand in the running gel, the migration distance of the lectin can be used to deduct the association constants between the free ligand and the protein. It was possible to easily assess the binding properties of the de- and remetallized lectin by using small quantities (20–30 μg) of the protein. The total time for the electrophoresis was 3.5 h.

De- and Remetallization of the Lectin. The lectin was demetallized by using a variety of techniques such as high ionic strength, low pH, chelating agent, and combination of these procedures. The protein was dialyzed for a least 24 h against 4 × 1 L of each different buffer tested and against an additional 4 × 1 L of 0.1 M ammonium acetate buffer, pH 7.0, overnight before testing by atomic absorption and affinity electrophoresis. All pH changes of the protein sample were made rapidly by using 0.20 M HCl or KOH; otherwise, most of the protein precipitated at its p*I* of 5.05 (Carter & Etzler, 1975a).

The remetallization was performed by using 1 mM solutions, pH 6.0, of the chloride salt of each metal. The demetallized lectin was dialyzed against 4 × 1 L of each metal ion solution, respectively, for 24 h and against 0.1 M ammonium acetate buffer, pH 7.0, overnight before testing by atomic absorption spectrophotometry or affinity electrophoresis.

Results and Discussion

The metal ion content of the native *D. biflorus* lectin, a tetramer consisting of two different subunits (Carter & Etzler, 1975b), was first determined. Three different preparations of the lectin were tested for Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, and Fe^{2+}. As can be seen from Table I, Ca^{2+} was the most abundant ion with 3.35 mol/mol of lectin. It is apparent from the total metal ion content of the seed that a selective process of uptake into the lectin is involved since the metal ion ratios are quite different. The total content of metal ions was as high as 6.95 mol/mol of lectin, and it seemed likely that several ions could be loosely attached to the surface of the lectin.

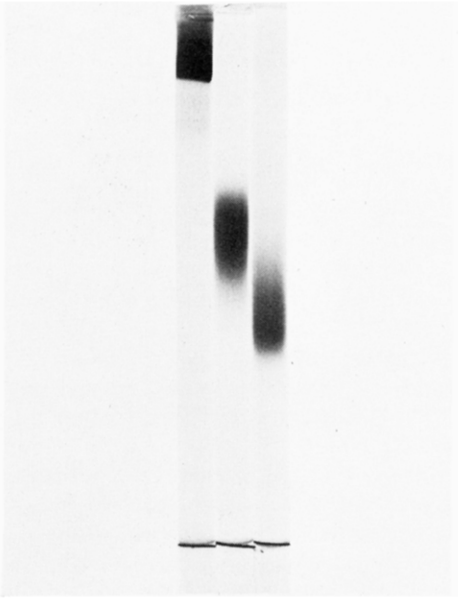

FIGURE 1: Polyacrylamide gels from an affinity electrophoresis run showing binding of (1, left) native lectin, (2, center) partially demetallized lectin, and (3, right) completely demetallized lectin. A 25-μg sample of protein was used in each case, and the concentration of blood group substance in the running gels was 0.5 mg/mL.

If the role of metal ions in lectins is to help maintain such a favorable tertiary structure that a binding site is generated, the lectin should lose the ability to bind to its ligand when the ions are removed. Several different techniques were tried for dissociating the metal ions from the lectin. High ionic strength, low pH, chelating agent, and combinations of these techniques were used (Table II). The most successful technique for removal of the ions from the *D. biflorus* lectin was the use of different concentrations of EDTA (Table II). Optimal demetallization conditions were achieved with dialysis against 10 mM EDTA for 86 h. In Figure 1 it can be seen that demetallized *D. biflorus* lectin completely lost its capacity to bind to the affinity gels as compared to native and partially demetallized lectin.

One metal ion at a time was then used to remetallize the lectin, and the association constant with *N*-acetyl-D-galacosamine was determined. All different metal ions found in the lectin were able to restore the binding abilities to different degrees as measured by affinity electrophoresis. Table III shows that Ca^{2+}-restored *D. biflorus* lectin seems to be the most active as judged by its K_a in comparison to the rest of the ions which give lower and indistinguishable values for the association constants. De- and remetallization of the isolated lectin obviously result in a different metal ion ratio than in the native lectin, metallized in vivo. A strong preference for calcium is, however, also observed in vitro. The reason for

Table II: Effect of Various Treatments on Metal Ion Content of *D. biflorus* Lectin (Pl 42)

dialysis buffer	dialysis time (h)	mol of metal ion/mol of lectin						ability to bind in affinity electrophoresis
		Ca^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}	Cu^{2+}	Σ	
(1) 1 M ammonium acetate, pH 7.0	35	3.70	0.22	0.63	0.81	0.56	5.92	++++
(2) 0.1 M glycine/HCl, pH 2.0	24	2.19	1.27	0.00	0.61	0.00	4.07	++++
(3) 0.1 M glycine/HCl + 1 mM EDTA,[a] pH 7.0	24	1.60	1.02	0.09	0.14	0.00	2.85	±
(4) 0.1 M glycine/HCl + 1 mM EDTA, pH 2.0	24	0.92	0.31	0.00	0.05	0.02	1.30	±
(5) 1 mM EDTA, pH 7.0, 35 h	35	0.44	0.40	0.00	0.00	0.00	0.84	–
(6) 1 mM EDTA, pH 7.0	86	0.48	0.09	0.00	0.00	0.00	0.57	–
(7) 10 mM EDTA, pH 7.0	35	0.60	0.00	0.00	0.00	0.00	0.60	–
(8) 10 mM EDTA, pH 7.0	86	0.37	0.00	0.00	0.00	0.00	0.37	–

[a] Part of sample 2 was dialyzed against 1 mM EDTA, pH 7.0. Use of this slow pH increase resulted in a 50% loss of protein due to precipitation.

Table III: Metal Ion Content and Association Constants of *D. biflorus* Lectin Remetallized with Divalent Cations

metal solution[a]	mol of metal ion/mol of lectin						
	Ca^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}	Cu^{2+}	Σ	K_a (M)
Ca^{2+}	8.50					8.50	4.8×10^2
Mg^{2+}		5.64				5.64	2.9×10^2
Mn^{2+}			7.18			7.18	2.9×10^2
Zn^{2+}				4.87		4.87	2.5×10^2
Cu^{2+}					6.25	6.25	2.5×10^2
Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}	6.83	0.69	0.56	1.77	2.88	12.73	4.5×10^2

[a] *D. biflorus* lectin was dialyzed against indicated 1 mM metal solution.

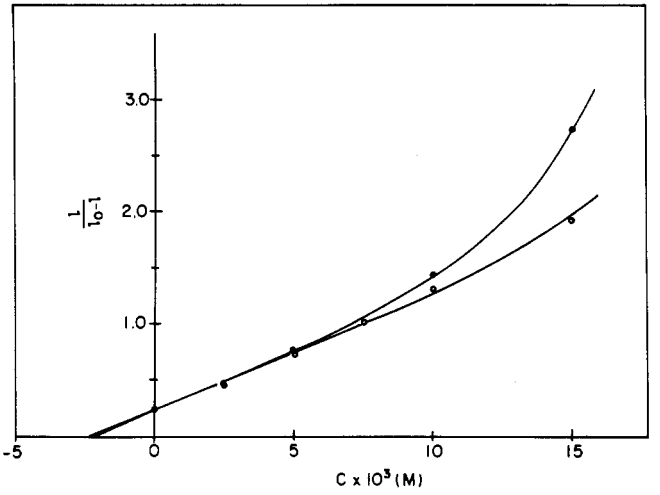

FIGURE 2: Affinity electrophoretic plots of the *D. biflorus* lectin remetallized with (1) Ca^{2+} ions (●) and (2) Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, and Cu^{2+} ions (○). l = electrophoretic mobility of the lectin at a given concentration of free ligand; l_0 = electrophoretic mobility of the lectin when no entrapped blood group substance A+H is present in the running gel (Borrebaeck & Etzler, 1980). The association constant of native lectin for *N*-acetyl-D-galactosamine is 2.2×10^2 M^{-1}. The higher values for the association constants between the remetallized lectin and *N*-acetyl-D-galactosamine may be due to a more favorable environment for metallization in vitro.

the remetallized lectin having a higher metal ion content than the native lectin is not known. However, the conditions for the remetallization in vitro are probably quite different from those when metallization of the lectin takes place in the plant.

D. biflorus lectin that was remetallized by using a mixture of all metal ions gave results similar to that of the lectin remetallized only with Ca^{2+} (Figure 2). Figure 2 also shows that remetallization of the lectin most likely caused some microaggregation of the protein as judged by the slightly curvilinear plot. This type of curve is thought to be due to multipoint interactions between lectin and ligand, accentuated because of protein aggregation (Borrebaeck & Etzler, 1980).

Although metal ions are an absolute requirement for the *D. biflorus* lectin to be able to bind to *N*-acetyl-D-galactosamine residues, it seems there is a marked difference among the metal ions in their ability to restore the binding activity when used to remetallize the lectin. *D. biflorus* lectin remetallized with Ca^{2+} alone has the same ability to bind its ligand as lectin remetallized with a mixture of all ions, i.e., Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}. This indicates that the *D. biflorus* lectin requires only Ca^{2+} ions for full carbohydrate binding. The other metal ions were not equally efficient in restoring the binding activity of the lectin.

The *Bandeiraea simplicifolia* I lectin (Hayes & Goldstein, 1974) and the *D. biflorus* lectin are the only lectins that have been found to require only one type of metal ion to restore full binding capacity. All other plant lectins studied to this date, including those from soybean (Galbraith & Goldstein, 1970; Jaffe et al., 1977), lima bean (Galbraith & Goldstein, 1970), kidney bean (Galbraith & Goldstein, 1970), lentil, and garden pea (Paulova et al., 1971a,b), require both Ca^{2+} and Mn^{2+} to restore the binding activity.

It seems, therefore, that lectins have different specificities for metal ions as cofactors. Furthermore, Ca^{2+} and Mn^{2+} along with Mg^{2+} are the most abundant ions in the lectin family, even if several other divalent cations can substitute for these ions and restore the binding activity to some extent. However, at least one other lectin, wheat germ agglutinin (Nagata & Burger, 1974), does not need any divalent cations at all in order to bind to carbohydrates, and concanavalin A has metal binding sites for lanthanides that do not control the binding activity of the lectin (Barber et al., 1975).

Acknowledgments

We thank John Corlett for his skillful technical assistance.

References

Agrawal, B. B. L., & Goldstein, I. J. (1968) *Arch. Biochem. Biophys. 124*, 218–229.

Barber, B. H., Fuhr, B., & Carver, J. P. (1975) *Biochemistry 14*, 4075–4082.

Blumberg, S., & Tal, N. (1976) *Biochim. Biophys. Acta 453*, 357–364.

Borrebaeck, C., & Etzler, M. E. (1980) *FEBS Lett. 117*, 237–240.

Borrebaeck, C., & Mattiasson, B. (1980) *Eur. J. Biochem. 107*, 67–72.

Carter, W. G., & Etzler, M. E. (1975a) *J. Biol. Chem. 250*, 2756–2762.

Carter, W. G., & Etzler, M. E. (1975b) *Biochemistry 14*, 2685–2689.

Carter, W. G., & Etzler, M. E. (1975c) *Biochemistry 14*, 5118–5122.

Doyle, R. J., Thomasson, D. L., & Nicholson, S. K. (1976) *Carbohydr. Res. 46*, 111–118.

Etzler, M. E., & Kabat, E. A. (1970) *Biochemistry 9*, 869–877.

Etzler, M. E., Talbot, C. F., & Ziaya, P. (1977) *FEBS Lett. 82*, 39–41.

Galbraith, W., & Goldstein, I. J. (1970) *FEBS Lett. 9*, 197–201

Galbraith, W., & Goldstein, I. J. (1972) *Biochemistry 11*, 3976–3984.

Hammarström, S., Murphy, L. A., Goldstein, I. J., & Etzler, M. E. (1977) *Biochemistry 16*, 2750–2755.

Hayes, C. E., & Goldstein, I. J. (1974) *J. Biol. Chem. 249*, 1904–1914.

Jaffe, C. L., Ehrlich-Rogozinski, S., Lis, H., & Sharon, N. (1977) *FEBS Lett. 82*, 191–196.

Kabat, E. A. (1956) in *Blood Group Substances: Their Chemistry and Immunochemistry*, Academic Press, New York.

Nagata, Y., & Burger, M. M. (1974) *J. Biol. Chem. 249*, 3116–3122.
Paulova, M., Entlicher, G., Ticha, M., Kostir, J. V., & Kocourek, J. (1971a) *Biochim. Biophys. Acta 237*, 513–518.
Paulova, M., Ticha, M., Entlicher, G., Kostir, J. V., & Kocourek, J. (1971b) *Biochim. Biophys. Acta 252*, 388–395.
Thomasson, D. L., & Doyle, R. J. (1975) *Biochem. Biophys. Res. Commun. 67*, 1545–1552.

Gene Switching in Myogenesis: Differential Expression of the Chicken Actin Multigene Family†

Robert J. Schwartz* and Katrina N. Rothblum

ABSTRACT: We described the construction of an α-actin complementary deoxyribonucleic acid (cDNA) clone, pAC269 [Schwartz, R. J., Haron, J. A., Rothblum, K. N., & Dugaiczyk, A. (1980) *Biochemistry 19*, 5883], that was used as a hybridization probe in the current investigation to examine the induction of actin messenger ribonucleic acid (mRNA) during myogenesis. A T_m difference of 10–13 °C between skeletal muscle α-actin and nonmuscle β- and γ-actin mRNAs and pAC269 allowed us to establish the highly stringent hybridization conditions necessary to measure separately the content of α-actin mRNA and β- and γ-actin mRNA during muscle development in culture. We observed low levels of α-actin mRNA (~130 molecules/cell) in replicating prefusion myoblasts. The vast majority of actin mRNA (2000 molecules/cell) present at this stage was accounted for by β- and γ-actin mRNA. Beginning at myoblast fusion, α-actin mRNA accumulated and within 30 h reached a level 270-fold greater than that observed in the undifferentiated state. At 95 h in culture when myotube formation was completed, α-actin content was at its peak (36 000 molecules/nucleus). Conversely, β- and γ-actin mRNA content began to decline at the beginning of fusion, and by the end of myotube formation β- and γ-actin mRNAs were undetectable by our techniques. A rapid depression of α-actin mRNA levels was observed after 95 h in the absence of cell death. At 6 days after the initiation of myotube formation, the content of α-actin mRNA was reduced by 80% in comparison to peak values and remained at that level. The switching of actin mRNA species was inhibited in myoblasts treated with bdU. The accumulation of α-actin mRNA and the disappearance of β- and γ-actin mRNA were observed following the reversal of the bdU block and coincident with the onset of myoblast fusion. We found that the expression of actin genes within the actin multigene family is switched in myogenesis through a strict developmental pattern.

Muscle development in culture has provided a cell differentiation system to study the regulation of contractile protein synthesis. Myogenesis follows a succession of morphological stages which includes the proliferation of myoblasts, the fusion of mononucleated cells, and the appearance of functional myofibrils (Dienstman & Holtzer, 1975). The fusion of myoblasts into multinucleated myotubes results in the coordinate appearance of a number of new muscle-specific contractile proteins (Buckingham, 1978; Devlin & Emerson, 1979; Strohman et al., 1977). The biochemical properties of one of these proteins, actin, has been studied extensively. Actin was once thought to be a single highly conserved protein but has recently been shown to represent at least six different polypeptides (Vandekerckhove & Weber, 1978) which are coded by a middle repetitive gene family in eukaryotic cells (Fryberg et al., 1980; Schwartz & Rothblum, 1980; Tobin et al., 1980). Of all the actins, α-actin appears to be selectively induced during myogenesis and is retained as a major constituent of the contractile apparatus in skeletal muscle (Gordon et al., 1977). Other types of actins including β and γ isoforms appear to be ubiquitous cytoskeletal proteins found in nonmuscle tissues including prefusion replicating myoblasts (Whalen et al., 1976; Garrels & Gibson, 1976).

Several studies on the appearance of mRNAs[1] in cultured muscle cells have suggested that myogenesis is regulated by transcriptional control (Strohman et al., 1977; Paterson & Bishop, 1977; Benoff & Nadal-Ginard, 1980). However, the mechanism(s) for the selective induction of α-actin mRNA within the actin multigene family during muscle development has not been previously elucidated. Overall actin mRNA content was detected in muscle cells by in vitro translation assays and shown to increase following myoblast fusion (Paterson et al., 1974). Other studies showed the qualitative appearance of α-actin during myogenesis but did not directly quantitate the induction of α-actin mRNA or the contribution of nonmuscle actin mRNA species to total actin mRNA content (Whalen et al., 1976; Hunter & Garrels, 1977). We have recently described the construction of a nearly full length α-actin cDNA recombinant DNA clone (95% full length), which can be used as a hybridization probe (Schwartz et al., 1980). However, due to a preexisting population of nonmuscle β- and γ-actin mRNA in prefusion myoblasts and their homology to the α-actin cDNA, it has been difficult to separately analyze the regulation of the α-actin gene during myogenesis. Therefore, we developed highly stringent hybridization conditions to specifically quantitate the content of α-actin mRNA.

† From the Department of Cell Biology and the Program in Neuroscience, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030. *Received November 20, 1980.* This work was supported by U.S. Public Health Service Grant NS-15050 and by a grant from the Muscular Dystrophy Association of America. R.J.S. is a recipient of a U.S. Public Health Service Research Career Development Award.

[1] Abbreviations used: mRNA, messenger ribonucleic acid; DNA, deoxyribonucleic acid; cDNA, complementary DNA; hnDNA, heterogeneous nuclear DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; poly(A), poly(adenylic acid); Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/81/0420-4122$01.25/0