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# Short Communication

## Conspicuous Accumulation of a Single-Stranded DNA Binding Protein in Skeletal Muscle Fibers in Inclusion Body Myositis

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***In muscle biopsies from patients with inclusion body myositis (IBM), multiple sites were found in many muscle fibers that bound single-stranded but not double-stranded DNA without sequence specificity, as exemplified by several different cDNA probes. This activity was attributable to a protein, because it was abolished by proteases but not by RNAse. Most of the sites of binding were myonuclei, whereas some were rimmed vacuoles, which probably result from nuclear breakdown. No comparable binding was seen in 27 control biopsies. A number of human and viral single-stranded DNA binding proteins exist but our data does not identify the protein responsible for DNA binding in IBM. Our findings reinforce the supposition that nuclear damage plays a basic role in the pathogenesis of IBM. (Am J Pathol 1994, 144:874–882)***

Inclusion body myositis (IBM) was recognized as a disease entity in the 1970s[1–3] but its etiology and pathogenesis remain obscure. Recent interest has arisen because of the finding of ubiquitinated areas, Congo red-positive deposits, and positive staining with antibodies to β-amyloid and other proteins in muscle fibers.[4–6] We attempted to assess whether there was an increase in the mRNA for the precursor protein of β-amyloid in IBM by using a cDNA probe.[7] We discovered to our surprise that in all IBM biopsies there was conspicuous binding of all the single-stranded DNA that we tested by an as yet unidentified protein. This finding appears to be specific for the disease and it has implications in its pathogenesis.

### Materials and Methods

The basic material consisted of muscle biopsies from 17 patients with sporadic IBM, 8 patients with familial IBM, and 27 control patients. One control had a histologically normal biopsy and the other 26 were disease controls, with diagnoses on biopsy of denervation atrophy (7), denervation atrophy with myopathy of uncertain type (2), nonspecific abnormality of muscle (2), limb girdle dystrophy (3), dystrophinopathy (1), dermatomyositis (3), polymyositis (1), necrotizing myopathy (2), myotonic dystrophy (1), myopathy of undetermined type (1), nemaline myopathy (1), lipid storage myopathy (1), and mitochondrial organelle depletion (1). Twelve of these control biopsies had cytoplasmic bodies, a feature often present in IBM. The clinical presentation of the sporadic IBM cases was typical,[3,8] and in all IBM cases the diagnosis was confirmed by the electron microscopic finding of characteristic abnormal tubular filaments in muscle fibers. Clinical details of the familial IBM cases have been published.[9–11]

Histological stains, antibodies, and DNA and RNA probes were applied to cryostat sections of muscle. They are listed in Tables 1 and 2 along with the number and types of biopsies to which they were applied.

Antibodies to the following proteins were used: transthyretin (TTR), Dako, Carpinteria, CA A002;

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Table 1. *The Number of Cases of Each Type on Which Stains and Antibody Reactions Were Performed*

| | Sporadic IBM Cases | Familial IBM Cases | Control Cases |
|---|---|---|---|
| Stains | | | |
| Hematoxylin and eosin | 17 | 8 | 27 |
| Congo red, Eastman's | 17 | 8 | 12 |
| Crystal violet | 17 | 8 | 12 |
| Thioflavin S | 5 | 3 | 24 |
| Acridine orange | 3 | 0 | 0 |
| Pyronin | 3 | 0 | 0 |
| Antibodies to | | | |
| TTR | 14 | 7 | 0 |
| PP | 5 | 2 | 0 |
| Ub | 18 | 8 | 24 |
| preA4 (BPP) | 14 | 6 | 16 |
| E1 (BPP) | 3 | 0 | 0 |
| β-amyloid (monoclonal) | 14 | 7 | 24 |
| β-amyloid (polyclonal) | 10 | 2 | 0 |
| Histone 1 | 3 | 0 | 0 |
| N-CAM | 3 | 0 | 0 |

Table 2. *The Number of Cases of Each Type on Which cDNA and RNA Probes Were Performed*

| | Sporadic IBM Cases | Familial IBM Cases | Control Cases |
|---|---|---|---|
| $^{35}$S probes | | | |
| Amy-1 | 1 | 3 | 12 |
| Amy-2 | 12* | 2 | 26 |
| Amy-2 after RNase | 4† | 0 | 0 |
| Amy-2 after SDS | 3† | 0 | 0 |
| Amy-2 after pronase | 3 | 0 | 0 |
| Amy-2 after trypsin | 3 | 0 | 0 |
| GPDH | 4 | 0 | 0 |
| M13 | 4† | 0 | 0 |
| M13 after RNAse | 3 | 0 | 0 |
| pUC 18 | 3 | 0 | 0 |
| pUC 18 after RNAse | 3 | 0 | 0 |
| α-actin | 4† | 0 | 0 |
| α-actin after RNAse | 3 | 0 | 0 |
| α-actin after trypsin | 3 | 0 | 0 |
| $^{33}$P probes | | | |
| Amy-2 | 3‡ | 0 | 0 |
| Amy-2 after RNase | 3‡ | 0 | 0 |
| M13 | 3‡ | 0 | 0 |
| GPDH | 3‡ | 0 | 0 |
| α-actin | 3‡ | 0 | 0 |
| α-actin after RNase | 3 | 0 | 0 |
| α-actin non denatured | 3 | 0 | 0 |
| α-actin after trypsin | 3 | 0 | 0 |
| α-actin after SDS | 3 | 0 | 0 |
| α-actin after dispase | 3 | 0 | 0 |
| RNA probes | | | |
| Amy-2 sense | 3 | 0 | 2 |
| Amy-2 antisense | 3 | 0 | 2 |

* Repeated two times in three biopsies, three times in one, four times in one.
† Repeated two times in three biopsies.
‡ Repeated two times in one biopsy.

monoclonal antibody to prion protein (PP), generously supplied by Dr. N. Cashman; β-amyloid precursor protein (BPP), Boehringer Mannheim, Laval, Quebec 1285-262; ubiquitin (Ub), Chemicon, Temecula, CA M481510; β-amyloid, monoclonal, Boehringer Mannheim 381–431; β-amyloid, polyclonal, Dako M872; histone, Chemicon M4B051; neuronal cell adhesion molecule (N-CAM), Becton Dickinson, San José, CA Leu 19. E1 is a monoclonal antibody against a synthetic polypeptide derived from BPP (peptide 313–324, numbered according to Kang et al[12]). Primary antibodies were used in dilutions of 1:5 to 1:25. Incubations were for 45 minutes. The bound antibodies were displayed by the streptavidin-biotin system, the biotin being labeled either with horseradish peroxidase or Texas red.

The DNA probes used were as follows: α-actin;[13] Amy-1, a cDNA probe for nucleotides 1795–2415 of BPP (numbering according to Kang et al);[12] Amy-2, a cDNA probe for nucleotides 875–1795; GPDH, glyceraldehyde-3 phosphate dehydrogenase, an essential enzyme in glycolysis;[14] M13 (GIBCO-BRL, Gaithersberg, MD), bacteriophage DNA; pUC-18 (GIBCO-BRL), a probe for a plasmid. Labeling of DNA probes with either $^{35}$S-dATP or $^{33}$P-dATP was by random priming (GIBCO-BRL). *In situ* hybridization was performed essentially as previously described.[15] The final posthybridization wash was conducted in 0.1× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 42 C. Sections were counterstained with hematoxylin and eosin (H & E). Some hybridizations using Amy-1 and Amy-2 were conducted without heat denaturation of the probe, leaving the probe as double-stranded DNA. In two sporadic IBM biopsies, after the distribution of grains with the Amy-2 probe had been observed and photographed, the emulsion was removed by warm water and the sections were compared with the photographs to see what lay beneath the locations where the grains had been.

The cRNA probes were synthesized by *in vitro* transcription (Ambion, Austin, TX) from a template of BPP cDNA (spanning nucleotides 875 to 1795) cloned into the plasmid vector T3/T7 18U (Pharmacia, Piscataway, NJ). After hybridization, the sections were treated with RNAse A (20 μg/ml in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for 30 minutes at 37 C before posthybridization washing. Antisense and sense cRNA probes were validated by parallel hybridization to mouse neuroblastoma cells that had been stably transfected with BPP751. The antisense probe produced significant grain accumulation over the cells, whereas with the sense probe the grains over cells were similar to the background.

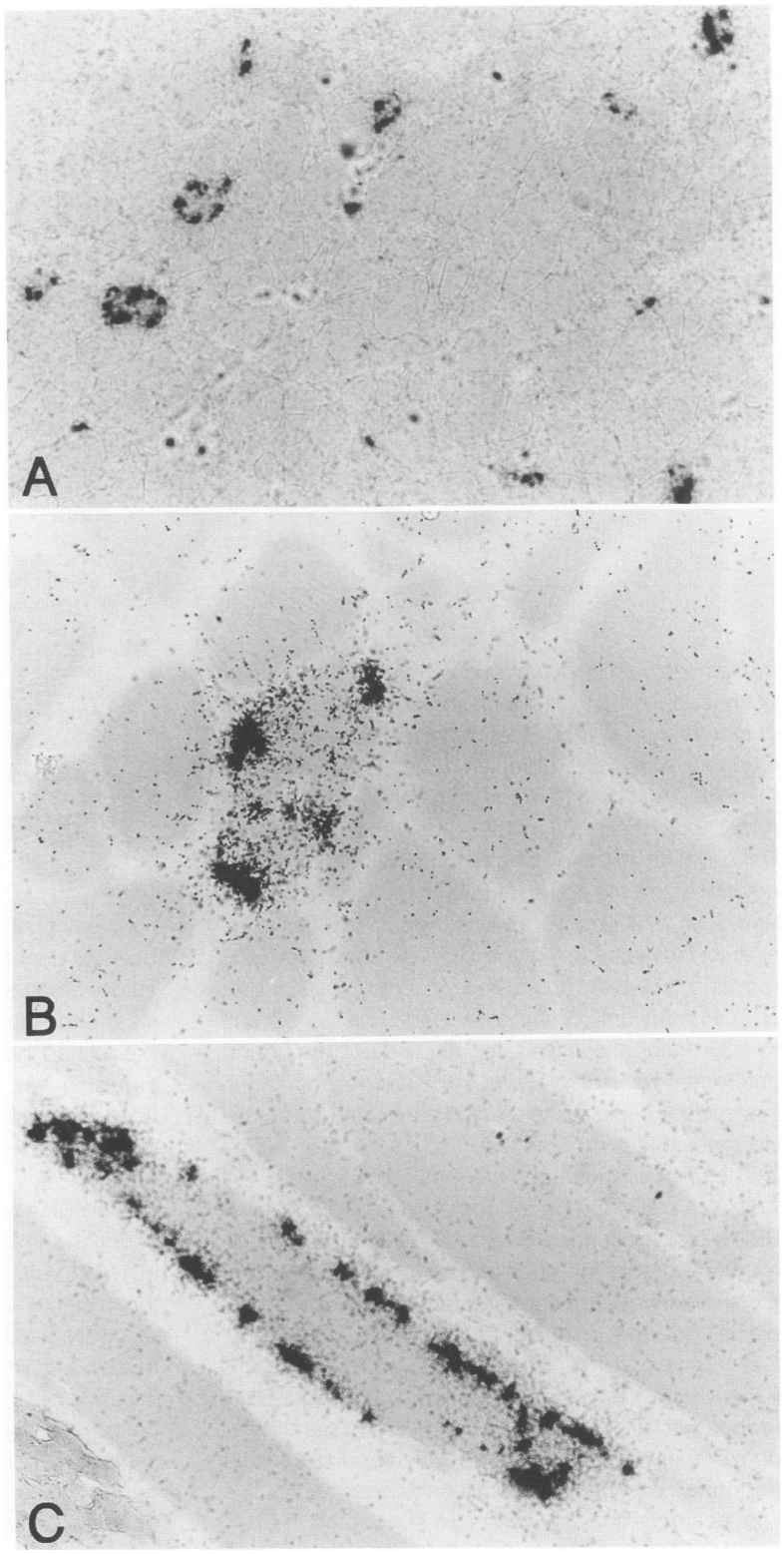


**Figure 1.** *Radiographic preparations of biceps muscle biopsy of sporadic IBM patients BG* (**A**) *and RC* (**B** *and* **C**) *using the Amy-2 cDNA probe for* in situ *hybridization.* **A:** *Transverse cryostat sections show several muscle fibers in which single or multiple dense pockets of silver grains are present;* ×*140.* **B:** *A fiber contains several collections of silver grains, mainly at its periphery. One collection in the interior of the fiber is near a vacuole. The appearance of the fiber is otherwise normal without significant atrophy;* ×*350.* **C:** *Longitudinal view of a positive fiber shows that the dense collections of silver grains, mainly at the fiber's periphery, extend over a segment of several hundred micrometers;* ×*350.*

## *Results*

Eosinophilic inclusions in muscle fibers were seen in muscle fibers in 15 biopsies from IBM patients. They are presumed to correspond to the ultrastructural finding of tubular filaments. Congo red-induced fluorescence was seen in 12 IBM biopsies (one of them familial). In four patients it involved five to eight fibers and in eight patients one to five fibers. Cystal violet-positive fibers were seen in 12 biopsies. Thioflavin S positivity was seen in eight IBM biopsies and one control. Antibodies to Ub stained a few fibers (especially fibers with vacuoles) in all sporadic IBM biopsies. Antibodies to β-amyloid stained one to five fibers in five sporadic IBM patients and none in controls. The EI antibody to BPP gave faint staining of the nuclear envelope in patients and controls. Antibodies to TTR and PP gave no reaction. Antihistone antibodies strongly stained all nuclei in all biopsies, whereas a slight amount of staining was seen in the cytoplasm of a few fibers in IBM cases. Antibodies to N-CAM outlined satellite cells in all biopsies and stained necrotic and regenerating fibers whenever they were present.

cDNA probes to Amy-1 and Amy-2 produced a striking picture of multiple positive sites (involving 5 to 50 fibers) in all IBM biopsies, both familial and sporadic (Figure 1), whereas normal controls and disease controls were uniformly negative except for a single positive fiber in one case. The silver grains were deposited in areas somewhat larger than a nucleus, many such concentrations usually being found in fiber segments up to at least 1 mM long. They were largely at the periphery of fibers. In the sections from which the emulsion was removed after photographing, the majority of the concentrations of grains were found to be centered on nuclei, whereas some were over rimmed vacuoles (Figures 2 and 3). A few vacuoles without basophilic granules in these fibers were not associated with grains. Pockets of grains were seen both in normal-sized muscle fibers and extremely atrophic ones that could be mistaken for interstitial cells or capillaries. RNAse pretreatment did not significantly affect the nuclear or cytoplasmic pockets of grains (Figure 4). Pronase, trypsin, and

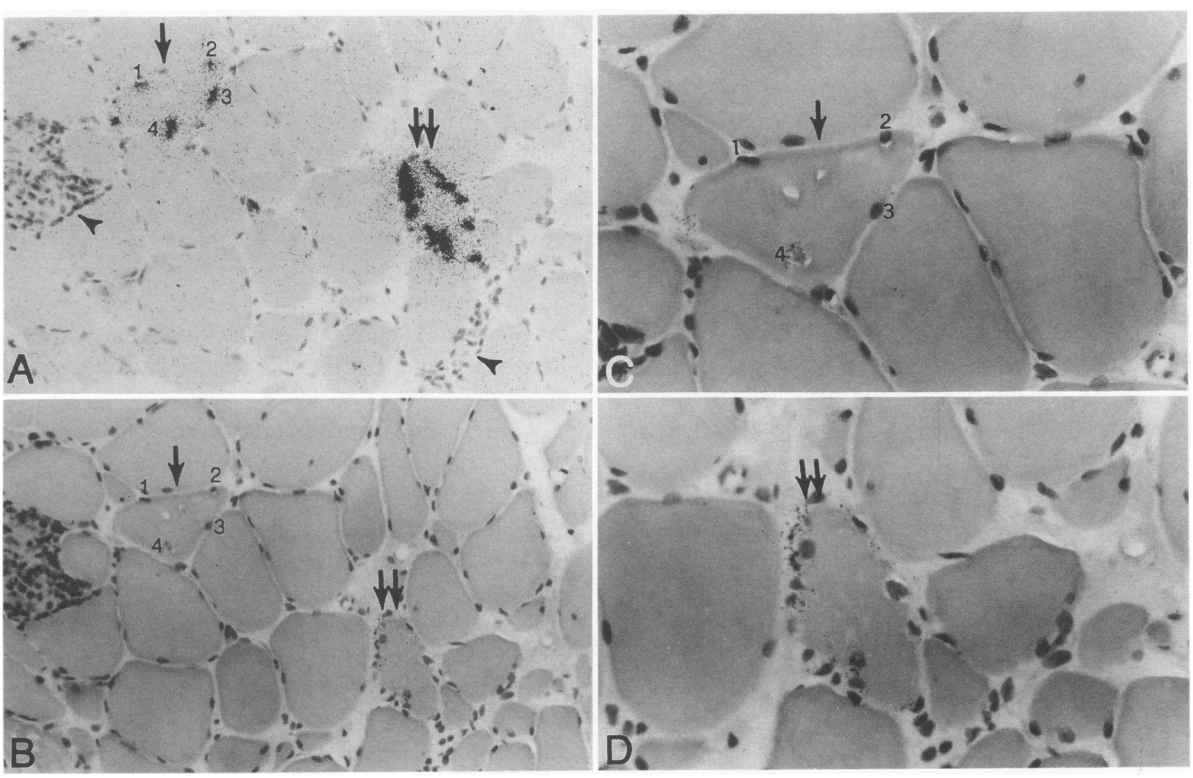


Figure 2. In situ *hybridization with the $^{35}S$ Amy-2 cDNA probe with slight hematoxylin counterstain in transverse cryostat sections of biceps from sporadic IBM patient RC. In* (A) *two positive fibers are present marked with single and double arrows. The positive fibers are not directly adjacent to endomysial mononuclear inflammatory infiltrates (arrowheads). In the top fiber (single arrow) four discrete pockets of silver grains are discernable and they are labeled 1–4; ×350. In* B, C, *and* D *the photographic emulsion has been removed, permitting the identification of the structures that underlay the silver grains shown in two fibers in* A. *In* B *and* C *it is clear that normal appearing myonuclei underlay grain pockets 1 to 3, whereas a vacuole containing an eosinophilic inclusion underlay pocket 4. In the second positive fiber (seen in* A, B, *and* D *marked with double arrow in* A) *the extensive pockets of grains tend to overlap but they are underlain by several peripheral myonuclei.* A, B: *×350,* C, D: *×520.*

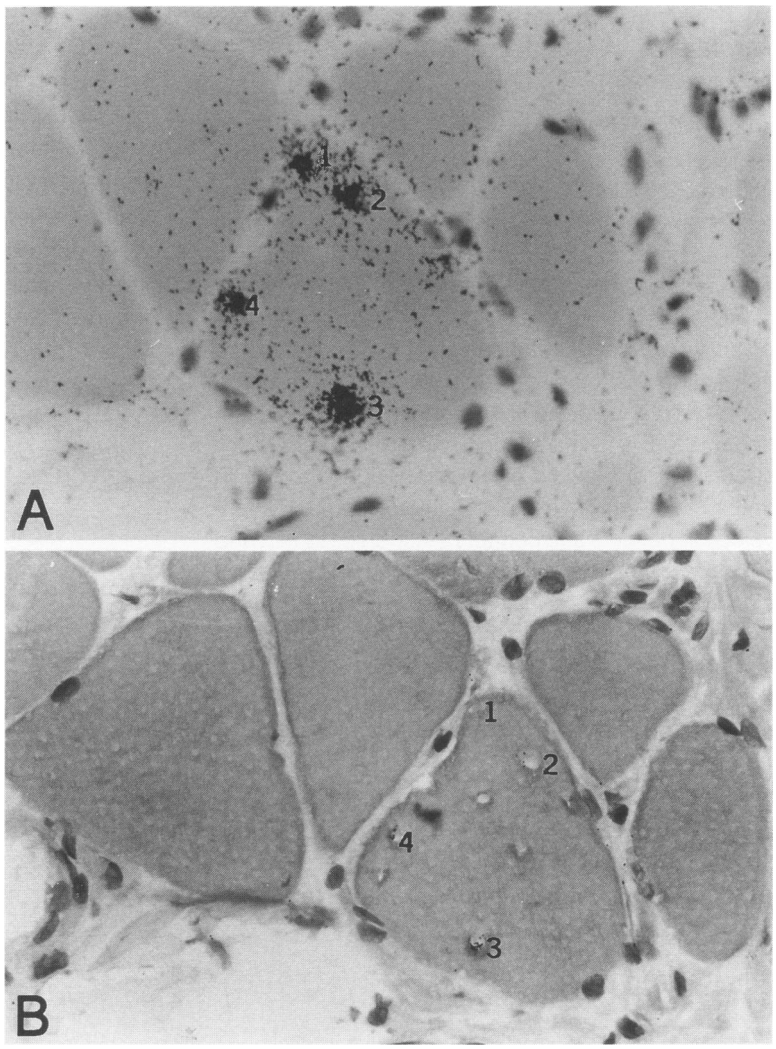


**Figure 3.** **A:** In situ *hybridization with Amy-2 cDNA probe and slight hematoxylin staining of transverse cryostat section of sporadic IBM patient RC. One fiber shows four major discrete pockets (marked 1 to 4) of silver grains.* **B:** *In the same section after removal of the emulsion, all four pockets correspond to vacuolar spaces, which are probably sites of nuclear disruption;* ×520.

SDS prevented DNA binding (data not shown). A similar picture of multiple pockets of grains in the fibers of all IBM patients tested was also seen with the probes to GPDH, α-actin, pUC 18, and M13 (Figure 5).

Neither the RNA sense nor antisense probe for β-amyloid produced any focal accumulation of grains in the muscle in excess of background. In particular no concentration was seen over vacuolated fibers.

## Discussion

In all IBM biopsies (both sporadic and familial cases) there were sites in multiple muscle fibers that bound all cDNA probes tested. The phenomenon was specific for IBM, because it was almost totally absent in the 26 control biopsies where it was tested. The binding was generally multiple in certain fibers. The grains were localized primarily over nuclei and less often over rimmed vacuoles. This provides additional evidence for the supposition that rimmed vacuoles result from breakdown of nuclei.[16]

What molecule could be responsible? It must be a protein, because binding was prevented by proteases and SDS but not by RNAse. It showed no sequence specificity. It bound only single-stranded DNA, because heat denaturation of the probe was essential for binding. It did not bind RNA probes.

A great variety of proteins exist normally in nuclei that will bind DNA, including histones, DNA and RNA polymerases, topoisomerases, gene activators, and repressors.[17] These may be excluded, most because they bind preferentially to specific sequences and others because they bind preferentially to double-stranded DNA, although they may have low affinity for single-stranded DNA. The major single-stranded binding protein in mammalian nuclei appears to be replication protein A (RP-A), a multisubunit chromosomal protein with polypeptides of 70, 34, and 13

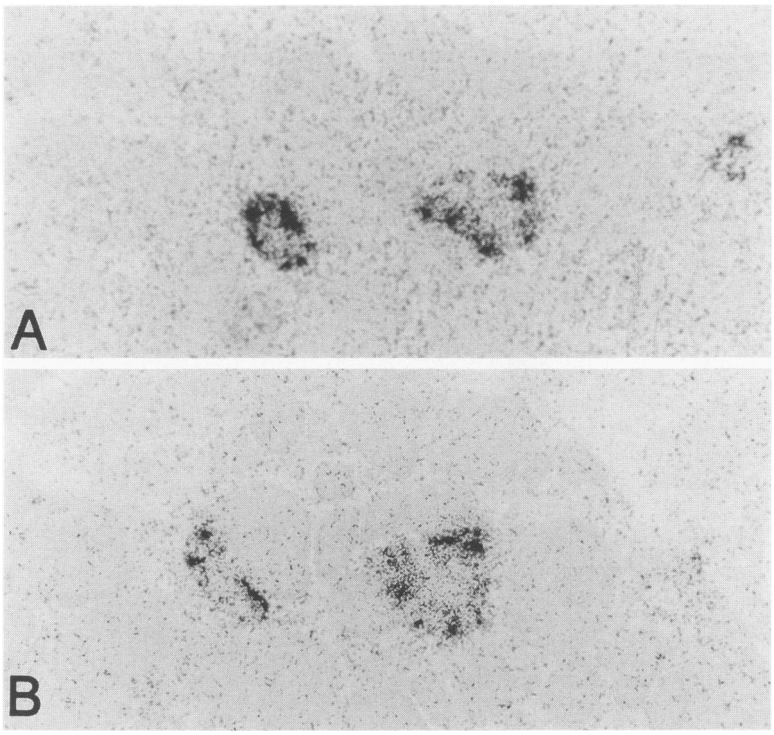


**Figure 4.** *Radioautographic preparations of serial transverse sections of biceps muscle from sporadic IBM patient BG; ×240. In both panels the probe was Amy-2 cDNA but in* (**B**) *the hybridization was preceded by a thorough RNAse digestion that failed to prevent the accumulation of silver grains.*

kd.[18] It has a role in chromosomal replication and probably in chromosomal excision repair. Other relatively abundant DNA binding proteins with a preference for single strands are the HMG-1 and HMG-2 proteins, whose role is elusive, and that at some stages in the cell cycle are most abundant in the cytoplasm.[19,20] Another largely cytoplasmic protein that can show considerable single-stranded DNA binding is lactic dehydrogenase.[21] Single-stranded-DNA binding proteins are an essential part of some viruses, such as adenoviruses,[22] herpes simplex,[23] and cytomegalovirus,[24] whereas others, such as SV-40, depend on the intrinsic proteins of infected cells.[25]

Our data at present are not sufficient to tell whether the DNA binding protein is a normal cell protein or a foreign, presumably viral protein. If it is a normal protein, it is present in great excess in IBM; controls showed no binding. Our technique does not pick up physiological levels of normal single-stranded DNA binding proteins. The amount of binding seen in some IBM nuclei implies a large amount of protein. Cytoplasmic proteins below a certain size may randomly enter the nucleus; if they become modified inside the nucleus, eg, polymerized, they could become trapped there. This appears to happen to actin in certain circumstances.[26] Nevertheless, it seems more likely that the DNA binding protein in IBM would be a nuclear protein, the most common nuclear protein that binds single-stranded DNA being RP-A. If there is widespread chromosomal damage in IBM, activation of DNA repair might up-regulate RP-A.

On the other hand, the possibility that this is a viral protein cannot be ruled out. There has been one report of isolation of an adenovirus from two biopsies of one IBM patient.[27] Presence of a viral protein would implicate a viral etiology of the disease, though making the familial cases more difficult to explain. We cannot totally exclude a coincident noncausal viral infection.

Until the DNA binding protein is identified, it is difficult to tell what role it may play in the pathogenesis of IBM and how it relates to other described abnormalities. The protein was largely localized to nuclei in specific segments of muscle fibers. Many, though not all, of these nuclei appeared structurally abnormal and one contained a prominent inclusion. The protein was also present in rimmed vacuoles, thus indicating that they are the result of nuclear breakdown, possibly as the result of liberation of phosphates. The number of nuclei seen in some of the affected nonatrophic fibers seemed excessive, suggesting that satellite cells had been stimulated to contribute excess nuclei to these fibers. It seems likely that eventually compensation for the destruction of myonuclei fails with the result that fibers atrophy.

Abnormal tubular filaments are a hallmark of the disease[3] but the masses of filaments are far less numerous, particularly in nuclei, than the DNA binding

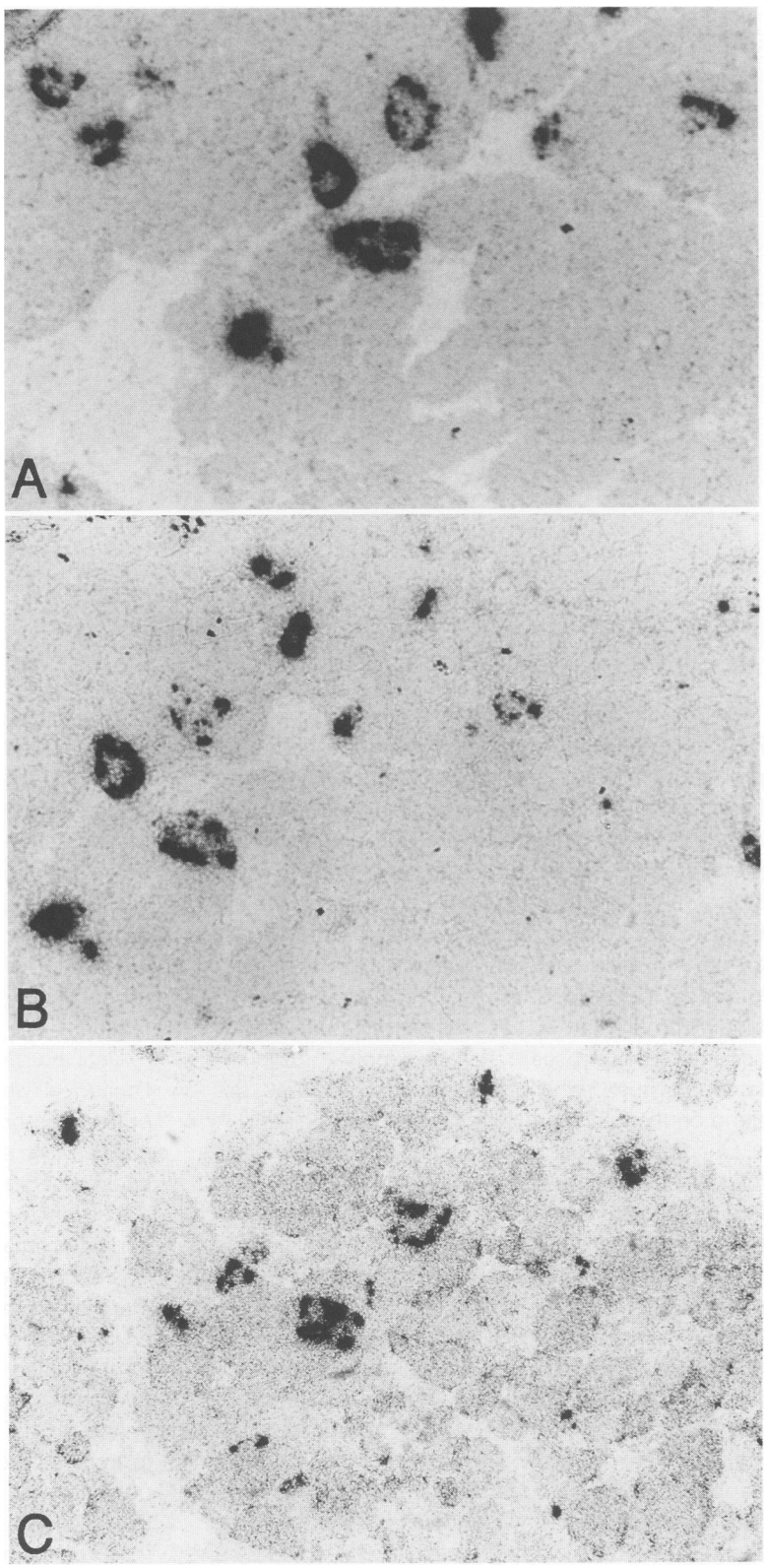


Figure 5. In situ *hybridization with three different cDNA probes shows numerous muscle fibers that contain the same type of dense collections of silver grains as shown with the Amy-2 probe in Figure 1.* A: *GPDH cDNA.* B: *M13 bacteriophage DNA.* C: α*-actin cDNA. Here the normally abundant actin mRNA produces an evenly distributed moderately high grain count over all fibers on which the abnormal dense collections are superimposed, whereas the background grain count remains low;* ×*350.*

activity. Likewise, accumulations of amyloid, as shown by Congo red staining or by antibodies to β-amyloid, appear much less widely distributed than the DNA binding protein. We found considerably less β-amyloid than has been reported by others.[6,28,29] Nuclear breakdown might liberate genomic DNA into the cytoplasm where it would eventually be degraded, but before that happened it is possible that unregulated transcription of many genes might occur, leading to accumulation of unexpected proteins, such as β-amyloid,[6] hyperphosphorylated tau,[29] and α-antichymotrypsin.[28] On the other hand, β-amyloid deposition might be a nonspecific component of autophagic vacuoles that may accompany the rimmed vacuoles. Some BPP is reported to be normally present at the nuclear envelope[30] and at neuromuscular junctions in skeletal muscle cells.[31] In contrast to the findings of Sarkozi et al[32] we were unable to show an increase in mRNA for BPP in IBM fibers by using antisense RNA probes. The focal accumulation of Ub is probably a nonspecific indication of abnormal proteins marked for proteolysis.

The inflammation that is inconstantly present in IBM may be related to extrusion and phagocytosis[33] of membranous whorls from muscle fibers in which they frequently are localized only a few score of nanometers from the sarcolemma.[3]

Evidence of nuclear damage has been shown in several accounts of IBM. Nuclei breaking down and liberating masses of abnormal filaments into the cytoplasm have been described by electron microscopists[1,34–36] and abnormalities in nuclear shape and proportion of heterochromatin have been described.[3] Tomé et al[37] found abnormal antibody staining of the nuclear envelope in many fibers. It is possible that the abnormal DNA binding protein has deleterious effects on nuclear structure and function. The identification of this protein or proteins should point the way toward a deeper understanding of the disease process.

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