The Sequence of the Dictyostelium myo J Heavy Chain Gene Predicts a Novel, Dimeric, Unconventional Myosin with a Heavy Chain Molecular Mass of 258 kDa*

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The complete sequence of the Dictyostelium myo J heavy chain gene has been determined from overlapping genomic clones. The gene spans ~7400 base pairs, is split by two small introns, and encodes a 2241-residue, 258-kDa heavy chain polypeptide that is composed of an N-terminal 944-residue myosin head domain, a central 863-residue domain that is predicted to form an α-helical coiled-coil containing six hinges, and a C-terminal 434-residue globular domain. The head domain is notable in that it contains a ~30 residue insert near the nucleotide binding pocket, and five potential calmodulin/myosin light chain binding sites at the head/tail junction. The existence within the Myo J tail domain of both an extensive coiled-coil structure and a large globular domain suggests that this myosin is dimeric and incapable of self-assembly into filaments. While these properties, as well as the overall predicted structure of the Myo J protein, are reminiscent of class V myosins, the sequence of the 434-residue globular tail piece of Myo J shows no similarity to that of either yeast or vertebrate myosins V. Consistent with this, phylogenetic analyses based on myosin head sequence comparisons do not classify Myo J as a type V myosin. These and other sequence comparisons indicate that Myo J and two as-yet-unclassified unconventional myosins from Arabidopsis represent members of the newest class within the myosin superfamily (class XI). Northern blots analyses suggest that Myo J may function predominantly in vegetative Dictyostelium cells. Finally, Southern blot analyses suggest that Dictyostelium possesses another myosin that is very closely related to Myo J.

To date, 10 classes of myosin have been identified, nine of which are considered to be unconventional (1-5). Like the myosins I, which were the first unconventional myosins identified (6), all of these unconventional myosins contain within their heavy chains the ~80-kDa mechanochemical domain that corresponds in sequence to the globular head of conventional, filament-forming myosins (myosin II). The sequences that comprise the remainder of their heavy chains are, on the other hand, widely divergent. While these sequences, which always extend C-terminal of the motor domain and in some cases N-terminal as well, are similar between members of the same class of unconventional myosins, they are unique relative to all other classes. These class-specific sequences are thought to confer functional specificity on a more or less generic motor domain by mediating specific interactions with different cellular proteins, structures, or membranes (for review, see Refs. 7-11).

The cellular slime mold Dictyostelium discoideum represents a reasonable system for the analysis of unconventional myosin structure and function because this ameboid cell demonstrates many different types of cellular and intracellular motility (12), and because it exhibits reasonable frequencies of homologous recombination, allowing null mutations to be created by targeted gene disruptions (13). This approach, when coupled with the characterization of the behavior of the mutant cells, should allow conclusions to be drawn regarding myosin function in vivo. To date, a single myosin II heavy chain gene (mhc a) and five myosin I heavy chain genes (myo A-E) have been cloned from Dictyostelium and completely sequenced (14-19). While analyses of myosin II-deficient cells have yielded a wealth of information about the function of this class of myosin in vivo (for reviews, see Refs. 20 and 21), the identification of myosin I-dependent functions has been complicated by the fact that at least some of the many myosin I isoforms expressed by this organism probably have partially overlapping functions (18, 22-24). The analysis of cell lines in which multiple, closely related myosin I isoforms have been eliminated from the cell is yielding more useful results, however (25, 26).

Southern blot analyses (18), together with the recent analysis of a Dictyostelium YAC library (27), indicate that this organism contains a total of 10-13 actin-based motor proteins (mhc a and myo A-L). While these additional four to seven uncharacterized myosin genes may simply represent additional myosin I heavy chain isoforms, it seems reasonable to suspect that at least some of them represent members of other classes of unconventional myosin, especially since several of these classes (e.g. myosin V) have been identified in both lower and higher eukaryotes (28-31). We set out, therefore, to identify within this group of four to seven putative myosin genes any that encode heavy chains other than for a type I myosin. Here we present the complete sequence of the Dictyostelium myo J heavy chain gene. The deduced amino acid sequence of the Myo J heavy chain polypeptide predicts a high molecular weight, dimeric myosin that is probably incapable of self-association into filaments and that may be regulated by calcium ions through multiple, heavy chain-associated calmodulin “light chains.” Myo J represents, therefore, the first unconventional myosin identified in this model system that is not a type I myosin. While the overall predicted structure of the Myo J protein is similar to that of class V myosins (32), sequence comparisons indicate that Myo J is not a type V myosin. More-

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over, Myo J does not fall neatly into any of the other nine classes of myosin identified to date. Therefore, by the criteria used to group previously identified myosins into 10 classes, Myo J represents the newest class within the myosin superfamily (class XI). Other sequence comparisons suggest that MYA1 and MYA2, two recently identified unconventional myosins from Arabidopsis (33, 34), may also be class XI myosins. Finally, Southern blot analyses indicate that Dictyostelium probably possesses a second type XI myosin gene. The analysis of cells in which both genes have been disrupted will hopefully provide definitive information regarding the function of class XI myosins in vivo.

MATERIALS AND METHODS

Common Procedures—Dictyostelium genomic DNA was purified using the maxi prep procedure described previously (12). Total Dictyostelium RNA was prepared by extraction in guanidine isothiocyanate and pelleting through a CsCl cushion, exactly as described by Sambrook et al. (35). For the analysis of RNA from cells undergoing chemotactic aggregation and early development, cells were developed on black filter supports equilibrated with “starvation buffer” as described previously (22). Southern blots were hybridized and washed under the “moderate stringency” conditions described previously (22), except that the hybridization temperature was 38 °C and the wash temperature was 50 °C. Northern blots were hybridized and washed as described elsewhere (22). Relative RNA expression levels were determined by scanning autoradiograms with an LKB Ultrascan XL laser densitometer. All probes were labeled with [α-32P]dATP using the random primer method (Amersham Corp.). Double stranded sequencing was performed on plasmid DNAs prepared using Qiagen columns (Qiagen Inc.). Plasmids were sequenced using the dideoxy cycle sequencing method, the dideoxy chain termination method of Sanger et al. (36), and either universal dye primers (Applied Biosystems kit 401112) or synthetic, gene-specific primers coupled with dye terminators (Applied Biosystems kit 401113). Synthetic primers were made on an Applied Biosystems model 380B DNA synthesizer, and purified using Qiaquick nucleotide purification cartridges (Applied Biosystems). Sequences were determined on an Applied Biosystems model 380B automated sequencer. All other common methods were performed as described by Sambrook et al. (35).

Library Construction and Screening—To create the various genomic sublibraries used here, restricted and size-fractionated Dictyostelium genomic DNA was electroeluted from agarose gels, ligated with the appropriate ZAP II vector (Stratagene), packaged using Gigapack Gold packaging extracts (Stratagene) according to the manufacturer’s instructions, and screened using Escherichia coli strain XL1 Blue (Stratagene). Bluescript plasmids were excised from the plaque-purified phage using helper phage R408.

Computer Analyses—General DNA sequence analysis methods (sequence entry, fragment overlap, translation, etc.) were performed using the appropriate menus within the program GCG (University of Wisconsin Genetics Computer Group Sequence Analysis Software). Data base searches were performed using the programs BLAST (37) and FAST A (38). Dot matrix comparisons were performed using the DOT PLOT menu of GCG (the exact parameters used are listed in the legend to Fig. 2). The program of Lupas (39) was used to identify regions of the Myo J heavy chain that are likely to from a helical coiled-coil. A probability of >90% was used to define regions of coiled-coil structure. The rooted phylogenetic tree was created using myosin head sequences (not including those portions of the neck domain that contain IQ motifs) and the program Clustal V (40). Branch lengths were corrected using the method of Kimura (41).

RESULTS

Identification and Cloning of the myoJ Heavy Chain Gene—DNA probes that span the highly conserved ATP-binding site region within the myosin head domain have been used successfully as heterologous probes to identify other myosin genes both within and between organisms (15, 42, 43). For example, a probe that corresponds to the ATP binding site of Dictyostelium Myo B recognizes eight other EcoRI fragments in Southern blots of Dictyostelium genomic DNA (18), and four myosin I heavy chain genes (myo A, C, D, and E) that correspond to four of these EcoRI bands were isolated using this Myo B fragment as a probe (22). In an effort to bias our search away from the identification of additional myosin I genes, in this study a 560-bp EcoRI fragment that spans the ATP binding site of the Dictyostelium myosin II heavy chain gene (mhc a) was used as a probe (14). This probe cross-hybridized with six EcoRI bands, of which 13.5, 3.4, and 3.2 kb were not derived from known Dictyostelium myosin genes (Fig. 1, lane 1). Interestingly, of the six cross-hybridizing bands, the 3.2- and 13.5-kb bands were recognized most strongly, and neither of these bands was recognized by probes that span the ATP binding site region of Myo A–E (Fig. 1, lane 2; Ref. 18).

In order to clone the gene (or portion of the gene) that corresponded to the 3.2-kb EcoRI band, an EcoRI genomic sublibrary was created. Specifically, genomic DNA was cut preparatively with EcoRI, fractionated by agarose gel electrophoresis, and DNA fragments ranging in size from 3.0 to 3.4 kb were obtained by electroelution. These fragments were then cloned into EcoRI-cut ZAP, and the subsequent sublibrary was probed with the 560-bp myosin II probe. As expected, this approach yielded a large number of genomic clones that contained the 3.2-kb EcoRI insert of interest (Fig. 2).

After partial DNA sequence analysis indicated that this 3.2-kb EcoRI fragment contained the coding sequence for the N-terminal two-thirds of a myosin head domain (see below), efforts were directed at obtaining the remainder of the gene. Because of the extreme AT bias of Dictyostelium DNA (12), large fragments of cloned Dictyostelium genomic DNA are very unstable. This fact precludes the use of cosmid libraries or standard phage libraries (containing ~20-kb inserts) and usu-

FIG. 1. Southern blot analyses. Lanes 1 and 2, whole Dictyostelium genomic DNA was digested with EcoRI and probed at moderate stringency with restriction enzyme fragments containing portions of the coding sequence surrounding the ATP binding site of Dictyostelium myosin II (lane 2) and Myo E (lane 2). The EcoRI fragments that correspond to myo A through myo E are indicated. The fragment assignments for myo C, Myo E, and the putative myosin gene corresponding to the ~13.5-kb band were confirmed by pulse-field gel electrophoresis (data not shown). The strong band at ~0.56 kb in lane 1 is from the myosin II gene. Lanes 3–6, whole Dictyostelium genomic DNA was digested with EcoRI, SstI, ClaI, and XbaI, respectively, and probed at moderate stringency with DNA fragments that together span most of the coding sequence for the coiled-coil tail domain of Myo J (Probe A in Fig. 2). Lanes 7–10, whole genomic DNA was digested with EcoRI, BclI, SstI, ClaI, and XbaI, respectively, and probed at moderate stringency with a DNA fragment that spans most of the coding sequence for the globular tailpiece of Myo J (Probe B in Fig. 2). The size standards for lanes 1 and 2, and for lanes 3–10, are included.

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MyoJ transcript (see below) suggested that at least several kilobase pairs each. In the case of MyoJ, the size of the MyoJ heavy chain gene (A) and the predicted domain structure of the Myo J heavy chain polypeptide (B). In A, 3.2 RI, 2.7 RI, 1.9 XBA, and 4.5 RI are the four overlapping genomic clones that span the myo J heavy chain gene. PCR 1.1 was the PCR-generated fragment used to identify the 2.7 RI genomic clone. Probe A and Probe B were the probes used in the Southern blots in Fig. 1 (lanes 3–10; see “Materials and Methods” for details). Probe A was also used for the Northern blots in Fig. 8.

ally forces one to engineer chromosome walks in small steps of several kilobase pairs each. In the case of MyoJ, the size of the MyoJ head sequence contains several interesting features, however. First, the flexible "25 kDa/50 kDa" loop that occurs just C-terminal of the conserved GESGAKT sequence contains considerably more residues in Myo J than in most other myosins (Fig. 3). For example, Myo J contains 64 residues between the T of GESGAKT and the beginning of the next highly conserved sequence below the "25 kDa/50 kDa" loop (LEAFGNAK), while Dictyostelium Myo A, B, C, D, and E and myosin II contain 33, 33, 39, 33, 36, and 34 residues, respectively, over this same distance. While different myosins do vary considerably in both the number of residues that comprise the "25 kDa/50 kDa" loop, and in the actual sequences of the loop, Myo J appears to be near the extreme end of the spectrum in terms of the size of this loop. The functional significance, if any, of differences in the size and sequence of this loop is not known, however (7, 44). Second, the sequence of the Myo J head domain begins at a position analogous to that of conventional myosins (7). In this regard, therefore, Myo J is more similar to the head domain of type II myosins than type I myosins, which begin at a position corresponding approximately to residue 75 in conventional myosin head sequences (7). Third, Myo J has cysteine residues in both of the positions (residues 725 and 735) that correspond to the reactive thiols in muscle myosins (Fig. 3). This fact contrasts with many unconventional myosins that lack one or both cysteines residues in these positions (7, 8).

With regard to the neck domain, between residues 821 and 939 there are five imperfect ~25-residue repeats that occur in tandem and that correspond to the "IQ motif" described by Cheney and Moosiker (9) (Fig. 4A). This motif, which is characterized by a conserved IQ (or LQ) pair and basic residues at two conserved positions C-terminal of the IQ pair, has been implicated as the heavy chain binding site for authentic calmodulin and for myosin light chains (for review, see Refs. 9 and 45). In addition to these five IQ repeats, there is a potential sixth repeat (residues 940–962) that immediately follows the fifth repeat. Whether or not this sixth repeat is a bona fide calmodulin/myosin light chain binding site is in question, however, since coiled-coil prediction programs (see below) indicate that the coiled-coil begins at residue 945. In summary, therefore, Myo J may bind per heavy chain a total of five to six light chains, which could be either authentic calmodulin, myosin...
light chains (which are members of the calmodulin superfamily), or a combination of both.

With regard to the central coiled-coil domain, the program of Lupas et al. (39), which predicts the probability of sequences forming a helical coiled-coils, indicates that most (but not all) of the MyoJ sequence between residues 945 and 1807 would form this supersecondary structure (a probability of 90% was used as a cutoff for defining segments of coiled-coil structure) (Fig. 5). Those areas that should not form coiled-coil exist as six short stretches of sequence that break up the coiled-coil into seven discrete regions (CCz1 through CCz7; see Figs. 2B and 3). These six hinges range in size from 16 to 25 residues. If all seven coiled-coil regions were actually to dimerize, and if one uses a rise of 0.1485 nm per residue in the coiled-coil structure (46), then the central domain of MyoJ should fold into a rod that is 110 nm in length and contains six discrete bendsites. Furthermore, if the central domain of MyoJ does form an extensive coiled-coil, which is likely, then the MyoJ motor protein should be dimeric, i.e. two-headed.

With regard to the remaining 434 residues of the MyoJ heavy chain (1808 through 2241), the program of Lupas et al. (39) reveals that this region exhibits no tendency to form coiled-coil. This 48-kDa C-terminal domain may, therefore, form a globular structure, as do the last 410 residues of type V myosins (32). The apparent existence of this large globular tailpiece, which in the folded MyoJ molecule would exist as a pair of globular domains C-terminal of the central rod, would appear to preclude the ability of MyoJ to form filaments. We predict, therefore, that MyoJ is not only two-headed, but is also incapable of self-assembly into filaments. Both of these are demonstrated properties of type V myosins (29, 32).

Globular Tail Domain Sequence Comparisons—Because the overall deduced structure of the MyoJ heavy chain resembles that shown for class V myosins, the sequence of the C-terminal globular tail domain of MyoJ was compared in detail to the...
globular tail domain sequences of the type V myosins from vertebrates (mouse dilute (28) and chicken myosin V (29)) and yeast (Myo2 (30) and Myo4 (31)) using the program BESTFIT and using dot matrix comparisons. As reported previously (29), the globular tail domains of the two vertebrate myosins V are extremely similar (98% identical by BESTFIT), which is reflected in the nearly uninterrupted identity line in the dot matrix comparison between them (Fig. 6A). Furthermore, while the degree of similarity between the globular tailpieces of the vertebrate and yeast myosins V is much lower (for example, the mouse dilute and yeast Myo2 tailpiece sequences are 28% identical), their relationship is significant (Refs. 1, 2, and 29; see below) and an identity line with some interruptions is clearly evident in the dot matrix (Fig. 6B). By contrast, dot matrix comparisons of the Myo J tail domain with that of vertebrate and yeast myosins V showed no clear evidence of an identity line in either case (Fig. 6C and D). BESTFIT comparisons indicate, for example, that the Myo J tail domain is only 19% and 16% identical to that of mouse dilute and yeast Myo2, respectively. Furthermore, neither the vertebrate nor the yeast myosin V globular tail domain sequences were identified in a BLAST search of the data base using the Myo J tailpiece sequence as a query (see below). Finally, comparisons using the program FAST A, in which percent similarities between two sequences are compared to similarity values obtained when the two sequences are randomly shuffled (38), indicated that the globular tail domain sequence of Myo J is not evolutionarily related to the tail domain sequences of class V myosins (data not shown).

To search for other sequences in the data base (both myosin and non myosin) that might exhibit significant similarity to the globular tailpiece sequence of Myo J, the program BLAST (37), which looks for local regions of homology, was used. This program did not identify any regions of significant sequence similarity between Myo J and any myosin that has previously been placed within the 10 current classes. The Myo J globular tailpiece also showed no regions of significant local sequence similarity with any non myosin sequence in the data base. The BLAST program did yield two sequences with reasonable scores, however, both of which are recently sequenced (and as-yet-unclassified) unconventional myosins from the plant Arabidopsis thaliana. MYA1 and MYA2 are closely related Arabidopsis myosin heavy chains whose overall deduced structure is again reminiscent of type V myosins, but whose globular tail domain sequences show only very limited similarity to that of vertebrate and yeast type V myosins (33, 34). Furthermore, while Kinekema et al. (34) did not formally classify MYA1 and MYA2, phylogenetic analyses based on myosin head sequence comparisons do not group the Arabidopsis myosins with the myosins V (Ref. 34; see below). The local regions of sequence similarity between Myo J and MYA1/MYA2 that were identified by the BLAST program are concentrated in the C-terminal half of the tailpiece and are evident in the dot matrices (Fig. 6, E and F), where an identity line containing many interruptions is present in the second half of both matrices. Fig. 4B shows the pairwise alignment of Myo J with MYA1 and MYA2 across the largest region of sequence similarity within the globular tail-

![Fig. 5. Presence of α helical coiled-coil domains within the Myo J heavy chain polypeptide. The program of Lupas et al. (39) was used to estimate the probability that the Myo J heavy chain sequence forms α helical coiled-coil. The plots indicate the probability from 0% to 100% (see scale). For comparisons sake, the complete heavy chain amino acid sequences of Dictyostelium myosin II (Ref. 14; MYOSIN II), Dictyostelium Myo B (Ref. 16; MYOSIN I), and chicken myosin V (Ref. 29, MYOSIN V) were also analyzed. The tail of myosin II forms an almost uninterrupted α helical coiled-coil, the tail of myosin I is devoid of coiled-coil, and the tail of myosin V contains a central coiled-coil domain that is interrupted in numerous places.

![Fig. 6. Dot matrix comparisons between the globular tail domain sequences of the vertebrate and yeast myosins V, Myo J, and Arabidopsis MYA1 and MYA2. Only the globular tail domain portions of these myosins are compared here. The window size was 11 residues, and the minimum acceptable score was 7 matches out of 11. Conservative substitutions, as defined by the PAM matrix, were counted. Chicken p190 refers to the myosin V homologue from chicken (29).]
myosins (labeled sentanewclass(classXI)withinthemyosinsuperfamily. Fig. myosin already identified. Myo J appears, therefore, to repre-
indicates that Myo J is not a type V myosin. Fig. 7 also shows
globular tail domain sequence comparisons described above,
cluster with the type V myosins. This result, together with the
950 are generally considered to represent a significant relation-
numerical values at the branch points (nodes) are the boot-
the tree were generated using the program Clustal V (40). The
multiple sequence alignments that were used to createthe
comparisons (1, 2). Fig. 7 shows a phylogenetic tree in which
groupingsthatonewouldmakebasedontaildomainsequence
based on head sequences only, they are in large part the exact
that the myosin heavy chain molecules sequenced to date
sequences performed using myosin head sequence comparisons indi-
ted that Myo J does not fall into any of the other nine classes of
Myo J cluster in 810 out of 1000 random resamplings that the sequences below a given node clustered together.
bootstrapping values that are greater than 950 indicate a significant
represents a new class (class XI) within the myosin superfamily. Fig.
with the coiled-coil domain of Myo J (see Fig. 2). The migration of the RNA standards is indicated. Lanes 2–6, 5 μg of total RNAs from cells starved for 0, 3, 6, 9, and 12 h were hybridized with probe A.
7 also shows that Myo J does cluster in 810 out of 1000 random reshufflings with a branch containing the Arabidopsis myosins MYA1, MYA2, and MYA3 (note that while MYA3 groups with MYA1 and MYA2 in head sequence comparisons, its tail sequence is very different from that of MYA1 and MYA2; see Ref. 34). While the pairing of Myo J with MYA1 and MYA2 in the tree is below the standard level for significance, it is consistent with the globular tailpiece sequence comparisons between Myo J and both MYA1 and MYA2 (Fig. 4) and suggests that Dictyo-
stelium Myo J and Arabidopsis MYA1 and MYA2 are all type XI myosins.
Southern Blots—In an attempt to determine whether Dictyo-
stelium contains another gene(s) that is closely related to Myo J, Dictyostelium genomic DNA was digested with several dif-
ferent restriction enzymes, resolved by gel electrophoresis,
transferred to nitrocellulose, and probed at moderate strin-
gency with (i) DNA fragments that encode most of the coiled-
col fragment that encodes most of the globular tailpiece (Probe A in Fig. 2) or (ii) a fragment that encodes most of the globular tailpiece (Probe B in Fig. 2). While probe A detected only the myo J gene (Fig. 1, lanes 3–6), probe B clearly detected not only myo J but a second gene as well (Fig. 1, lanes 7–10). This second gene falls within a 9-kb EcoRI band, an 5.2-kb BclI band, an 11-kb SstI band, and an 3.9-kb Xbal band. The fact that this second gene cross-hybridizes with the Myo J globular tailpiece probe to almost the same extent as does Myo J itself suggests that this second putative myosin gene is very closely related to Myo J. The absence of cross-hybridization between this second gene and the Myo J coiled-coil probe is consistent with the fact that coiled-coil sequences, while always conserved in their chemical nature (i.e. periodicities of hydrophobic and charged residues), are often not well conserved in terms of the actual amino acid sequence (14, 43).
Northern Blots—Probe A (see Fig. 1), which in Southern blots is specific for the myo J heavy chain gene (see above), was found to hybridize to an 7400-nucleotide mRNA in a Northern blot of total RNA isolated from mid-log vegetative cells (Fig. 8, lane 1). The existence of this mRNA, which is of the size expected for the fully processed Myo J heavy chain transcript and which is slightly larger than the myosin II heavy chain transcript (data not shown), suggests that the Myo J protein is present in growing cells. To determine if the steady state level of the Myo J transcript changes during early development, cells were seeded onto black filter supports at high density in a

![Fig. 7. Rooted phylogenetic tree of myosin head domain sequences.](image-url)

The tree was generated using the neighbor joining program Clustal V (40), with branch lengths corrected by the method of Kimura (41). The sequence divergence between any pair of sequences is equal to the sum of the horizontal branch lengths connecting the two sequences. The tree was rooted using the longest branch (Nina C). The bootstrapping values at each node indicate the number of times out of 1000 data resamplings that the sequences below a given node clustered together. Bootstrapping values that are greater than 950 indicate a significant relationship. The different classes of myosins (I–XI) are indicated to the right. The extra branch that encompasses both class V and class XI myosins (labeled Class V?) is meant to indicate the possibility that, as more sequences are added to the tree and/or other factors are consid-
ered in classification (e.g. function), class XI myosins may be reassigned at a later date as class V myosins (albeit divergent ones). To obtain the accession numbers for these sequences, see Ref. 47.

piece. These alignments suggest that Myo J and MYA1/MYA2 are related.

Phylogenetic Analyses—The most recent phylogenetic analyses performed using myosin head sequence comparisons indi-
cate that the myosin heavy chain molecules sequenced to date fall into 10 distinct classes (1–4). While these groupings are based on head sequences only, they are in large part the exact groupings that one would make based on tail domain sequence comparisons (1, 2). Fig. 7 shows a phylogenetic tree in which the Myo J head sequence has been included in such an analysis. The multiple sequence alignments that were used to create the tree were generated using the program Clustal V (40). The numerical values at the branch points (nodes) are the boot-
strapping values. These values, which provide an estimate of the degree of confidence in the trees branching pattern, indi-
cate the number of times that the sequences which were grouped together below a given node in the original tree were found to group together again in trees generated by random resampling 1000 times (1). Bootstrapping values greater than 950 are generally considered to represent a significant relation-
ship (1, 2, 40).

With regard to type V myosins, a bootstrapping value of 996 at the node that joins the vertebrate myosins V (mouse dilute and chicken myosin V) and the yeast myosins V (Myo 2 and Myo 4) strongly supports the idea that these four myosins are all members of the same myosin class (Fig. 7) (for details, see Refs. 1, 2, and 29). Myo J, on the other hand, clearly does not cluster with the type V myosins. This result, together with the globular tail domain sequence comparisons described above, indicates that Myo J is not a type V myosin. Fig. 7 also shows that Myo J does not fall into any of the other nine classes of

![Fig. 8. Northern blot analyses.](image-url)
non-nutrient buffered salt solution and allowed to undergo starvation-induced aggregation and early development. Parallel cultures were harvested for RNA extraction at 0, 3, 6, 9, and 12 h of development. At 3 h the cells showed no apparent change, at 6 h they were at “ripple stage” (active aggregation phase), at 9 h they were in tight aggregates, and at 12 h they had formed tipped aggregates. The results in Fig. 8 (lanes 2–6) show that the steady state level of the Myo J transcript falls gradually during the period of chemotactic aggregation and early development to a level at 12 h that is ~10% of the level in vegetative cells. This expression pattern suggests that while Myo J is probably present in vegetative cells, its function(s) may be required primarily in actively growing cells.

**DISCUSSION**

Myo J represents the first myosin heavy chain gene identified in Dictyostelium that is neither a class I or class II myosin. While the domain structure of the Myo J heavy chain and the predicted properties of the folded protein resemble those of type V myosins (32), the phylogenetic program that is currently being used to classify myosins according to their head sequences (Clustal V; Ref. 40) indicates that Myo J is not a type V myosin. Consistent with this, the sequence of the Myo J globular tailpiece shows no significant similarity to the globular tailpiece sequences of the bona fide vertebrate and yeast myosins V. Myo J also does not cluster with any of the other nine classes of myosin identified to date. Therefore, by the criteria used to define the previous 10 classes of myosin, Myo J represents a member of a new myosin class, class XI. We also found that some regions of the Myo J tailpiece showed limited similarity to the globular tailpiece sequences of MYA1 and MYA2, two unconventional myosins from Arabidopsis that have not yet been formally classified, but clearly do not group with type V myosins in phylogenetic analyses (33, 34). Furthermore, head sequence comparisons performed using Clustal V place Myo J closest to MYA1 and MYA2. For the moment, therefore, we are classifying MYA1 and MYA2 along with Myo J as type XI myosins (see also Ref. 47).

While it is true that these class XI myosins probably have the physical properties of class V myosins (i.e. two-headed and non-filamentous), these are also the predicted properties of class VI (48, 49) and class VIII (3) unconventional myosins, and possibly class VII myosins (50) as well. These properties appear, therefore, to be a common theme among distinct classes within the myosin superfamily. Having said this, however, we cannot completely exclude the possibility that Myo J, MYA1, and MYA2 are actually class V myosins (albeit with very divergent tail domains). The area of the phylogenetic tree that contains the four bona fide myosins V, Arabidopsis MYA1 and MYA2, and Dictyostelium Myo J is one that at the moment is difficult to interpret unequivocally. The addition of more sequences to the tree should allow a more confident assignment of Myo J in the future. In addition, future studies of the physiological roles of myosin in vivo may force a partial redefinition of the myosin superfamily that takes into account similarities in intracellular function. For example, two-headed myosins that do not self-assemble may be required features of actin-based motors that serve as intracellular vesicle motors.

When Dictyostelium amoebae are starved, they undergo a ~24-h-long process that involves chemotactic aggregation and differentiation of the aggregate into a fruiting body composed of a stalk supporting a spore-filled sac (12). Many cytoskeletal protein genes are strongly up-regulated during the early stages of development (5–10 h) when cells become highly motile and are chemotaxing toward cAMP (12). For example, the steady state level of the Myo B heavy chain transcript at 10 h of development is ~7-fold higher than in vegetative cells (16).

This enhanced gene expression is mirrored in elevated levels of Myo B heavy chain protein (25) and is consistent with the demonstrated role of Myo B in the locomotion of aggregation-stage cells (22, 23, 25). In contrast to Myo B, Myo J mRNA levels fall fairly dramatically from vegetative cell levels as cells progress through chemotactic aggregation and early development. This result suggests that Myo J’s primary function may be in actively growing cells.

A combination of approaches will probably be required to elucidate the function(s) of Myo J in vivo. For example, the localization of the protein at the light and electron microscope levels, and the biochemical identification of cellular proteins/structures that bind to its globular tail domain, should provide important clues as to the function of Myo J. Finally, the generation of Dictyostelium cell lines that lack Myo J, using either gene targeting techniques or antisense RNA approaches, and the identification of the behavioral abnormalities exhibited by these mutant cells, will hopefully allow well-conclusions to be drawn regarding Myo J function in vivo. Along these lines, however, we found strong evidence that Dictyostelium contains another myosin heavy chain that is closely related to Myo J and that may overlap functionally with it. In this vein, while single mutants of some myosin I isoforms have given reasonable phenotypes (22–24), single mutants of other isoforms have not (17, 18), presumably because of functional redundancy. Furthermore, while some of the defects exhibited by myosin I single mutants are clearly scorable (22–24), they are often not extremely striking. This is again probably due to functional overlap between closely related myosin I isoforms. To clearly define the functions of class XI myosins in Dictyostelium it may be necessary, therefore, to create single cells that lack both myosin XI isoforms. DNA fragments that encode the globular tailpiece of Myo J will be useful in cloning the putative second myosin XI isoform.

The presence of the five to six IQ repeats within the sequence of Myo J, together with recent studies of vertebrate myosins I and the myosin V homologue from chicken (see Ref. 45 for review), suggest that Myo J may bind numerous calmodulin molecules (up to 12 folded dimeric molecule) within the “neck” domain of the molecule. These calmodulins could play a critical role in regulating the ATPase and/or mechanochemical properties of Myo J. Additionally, Myo J may influence to a considerable extent the intracellular localization of calmodulin, much as do yeast myosin V homologue (Myo2) (51) and the 174-kDa Nina C myosin isofrom in Drosophila photoreceptor cells (52). In this regard, it will be interesting to see if Myo J plays a role in contractile vacuole function, since calmodulin is highly concentrated on the contractile vacuole membrane in Dictyostelium (53, 54) and since myosins have been implicated in contractile vacuole function (35).

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The Sequence of the Dictyostelium myo J Heavy Chain Gene Predicts a Novel, Dimeric, Unconventional Myosin with a Heavy Chain Molecular Mass of 258 kDa

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