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Tubulin and tektin in sea urchin embryonic cilia: pathways of protein incorporation during turnover and regeneration

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SUMMARY

Axonemal precursor tubulin is the major protein component of the detergent-soluble membrane/matrix fraction of sea urchin embryonic cilia. Its unusual abundance may reflect the rapid turnover of these cilia, a process that is further documented here. However, whether during induced regeneration or normal turnover and growth, most other newly synthesized axonemal proteins are not detectable in the membrane/matrix fraction, raising the question of how non-tubulin precursors transit the growing cilium to the distal tip where assembly is generally thought to occur. Three potential explanations were considered: (1) the assembly of these components is proximal; (2) their relative concentration is too low to detect; or (3) tubulin alone is conveyed via a membrane/matrix pathway while most other axonemal proteins are transported in association with the axoneme. Light microscope autoradiography of axonemes pulse-chase labeled with [^{3}H]leucine showed relatively uniform labeling, with no evidence for proximal incorporation. Fully grown cilia and cilia at early stages of regeneration were isolated from labeled embryos, fractionated into membrane/matrix, axonemal tubulin and architectural remnant components, and their labeled protein compositions were compared. Heavily labeled axonemal proteins, most notably the integral microtubule doublet component tektin-A, were not detected in the membrane/matrix fraction of emerging cilia, even though nearly half of the total ciliary tubulin appeared in that fraction, arguing against membrane-associated or soluble matrix transit for the architectural proteins at low concentrations. However, after thermal fractionation of axonemes from growing cilia, labeled proteins characteristic of the architectural remnant dominated the solubilized microtubule fraction, supporting axoneme-associated transport of the non-tubulin proteins during growth, in contrast to a membrane/matrix pathway for tubulin.

Key words: cilium, tubulin, tektin-A, membrane, turnover, regeneration, protein synthesis, protein transport, sea urchin embryo

INTRODUCTION

Ciliogenesis in the sea urchin blastula takes place prior to hatching by the coordinated, de novo synthesis of a number of 9+2 structural proteins. The resynthesis of most of these same proteins recurs after experimentally induced deciliation. At least one of these proteins, tektin-A, is synthesized in a limited, or 'quantal' amount, sufficient to construct a specific length of cilium (Stephens, 1989). The embryo can be deciliated many times without slowing the normal program of development (Stephens, 1977). A large pool of ciliary tubulin pre-exists in the embryo (Bibring and Baxandall, 1981) and this pool is approximately doubled when the embryo is 'animalized' and constructs cilia of twice-normal length (Harlow and Nemer, 1987; Stephens, 1991a). Ciliary dynein also exists in the unfertilized egg (Foltz and Asai, 1990, presumably awaiting incorporation into cilia at the blastula stage.

These various observations have led to a theoretical description of ciliogenesis and regeneration as a subroutine of development wherein the sea urchin embryo needs only to synthesize the architectural proteins (the nuts and bolts) required to assemble a cilium from a large pool of tubulin and dynein (a warehouse of building blocks), limiting ciliary length by limiting the synthesis of a few key components (Stephens, 1977, 1989). This frugal process would allow an embryo to expend minimal protein synthetic energy to maintain its ciliated epithelium while its developmental program could proceed unhindered (Stephens, 1994).

Tubulin, unequivocally identified by a variety of antibodies, is the major protein component of the detergent-solubilized membrane/matrix fraction of isolated sea urchin embryonic cilia, accounting for about one-fifth of the total tubulin content of the cilium. This tubulin is lipid-associated and can be recycled into monodisperse membrane vesicles. On the basis of preferential labeling during initial assembly, nearly identical pool labeling after further regeneration from both normal and up-regulated embryos, and parallel post-translational modifications coincident with ciliary elongation, this 'membrane' tubulin behaves as an axonemal tubulin precursor (Stephens, 1991a, 1992).

Embryonic cilia appear to be turning over at a rate approaching that of regrowth (Stephens, 1991b, 1992) and thus the strik-

ingly high tubulin content of the membrane/matrix fraction may simply reflect ciliary dynamics. But, whether during steady-state maintenance or active regrowth, the architectural (non-tubulin) proteins, especially the extraordinarily heavily labeled tektin-A, are absent from the tubulin-rich membrane/matrix fraction, posing the dilemma of how these other proteins transit the distance from the cell body to the growing tip of the cilium, undetected. Three possibilities can be considered: (1) the incorporation of architectural proteins takes place at the base of the cilium, a physically unlikely possibility; (2) these proteins have sufficiently high association constants such that their relative concentration in the membrane/matrix compartment during transit is very low; or (3) the architectural proteins are tightly associated with the axoneme proper during transport to the growing tip, whereas tubulin is transported in association with lipid via a membrane pathway.

In this report, I further evaluate the degree of ciliary protein turnover and test these alternative hypothetical routes for axonemal protein incorporation during growth. I find that turnover is indeed relatively high, consistent with the high tubulin content of the membrane/matrix fraction, and that most of the newly synthesized, non-tubulin proteins are axoneme-associated prior to assembly. Preliminary accounts of this work have been presented in abstract form (Stephens, 1991b, 1993).

MATERIALS AND METHODS

Sea urchin embryos

Eggs and sperm were obtained from the sea urchins *Strongylocentrotus droebachiensis* (Maine) and *Tripneustes gratilla* (Hawaii) by intracoelomic injection of 0.5 M KCl. The eggs were washed by decanting and then fertilized, washed again with Millipore-filtered sea water buffered at pH 8.0 with 10 mM Tris-HCl, and grown as a 1-2% suspension at 7.5-8˚C (*S. droebachiensis*) or at 22-24˚C (*T. gratilla*) in magnetically stirred 250 ml tissue culture flasks (Corning no. 26502-250 'Slow Speed Stirring Vessel System') at 30 rpm. Detailed methods for handling the delicate gametes and embryos of *S. droebachiensis* are given elsewhere (Stephens, 1972). Some embryos were animalized by addition of $ZnCl_2$ to a final concentration of 0.5 mM immediately after fertilization (Nemer et al., 1985). The developing embryos were washed thoroughly with Millipore-filtered sea water immediately after hatching to rid the cultures of debris or, in the case of zinc-animalized embryos, to hasten the action of the hatching enzyme. The embryos were returned to the stirred culture flasks at a concentration of 1-2%.

Cilia isolation

At the appropriate stage, the embryos were recovered by gentle manual centrifugation and treated for 2 minutes with double-tonicity sea water (32 g/l NaCl in sea water) at the growth temperature (Auclair and Siegel, 1966), resulting in complete deciliation through hypertonically induced breakage of the axoneme at the basal plate and release of the cilia with their resealed membranes intact. The deciliated embryos were quickly spun down by manual centrifugation and immediately resuspended in sea water. The cilia were recovered from the chilled hypertonic supernatant by centrifugation for 10 minutes at 10,000 ***g*** and washed once with cold normal sea water. This basic method has been described in detail elsewhere (Stephens, 1986). Resuspension of the deciliated embryos in normal tonicity sea water permitted full regeneration of cilia in 8 hours at 8˚C for *S. droebachiensis* (Stephens, 1977) and 2-2.5 hours at 24˚C for *T. gratilla*, after which the regenerated cilia were again isolated as above.

Protein labeling

For pulse-chase labeling, parallel cultures of 1 ml of developing or regenerating embryos in 25 ml of sea water were grown at 8˚C (*S. droebachiensis*) or 24-25˚C (*T. gratilla*) in siliconized 250 ml beakers (or 50 ml culture flasks) and pulse-labeled for 1 hour with the latter species or 4 hours with the former species using 62.5 μCi (2.5 μCi/ml) of tritiated leucine (New England Nuclear, NET-460, 147 Ci/mmol). The labeled amino acid was chased for 1 hour with *T. gratilla* or 4 hours with *S. droebachiensis* with a 0.5% solution of the unlabeled amino acid in sea water, reducing leucine incorporation into protein by >95%. The cultures were stirred continuously at 30 rpm by suspended magnetic paddles. The initially labeled virgin or regenerated cilia were isolated and the embryos were then typically allowed to develop an additional crop of cilia utilizing protein pools labeled (or depleted) during the first generation. Time variations on this general protocol are described in Results.

Cilia fractionation

The pelleted cilia were demembranated by extraction for 2-5 minutes at 0˚C with >10 volumes of 0.25% peroxide-free Triton X-100 (Pierce no. 28314) in 3 mM $MgCl_2$ buffered with 30 mM Tris-HCl, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride. The suspension was centrifuged for 5 minutes at 35,000 ***g***. The resulting pellet of 9+2 ciliary axonemes was either resuspended to the original extraction volume in fresh extraction solution for direct SDS-PAGE comparison with the membrane/matrix fraction or else further fractionated.

To thermally fractionate the axonemes into 9-fold ciliary remnants and solubilized axonemal tubulin (Stephens et al., 1989), the preparation was suspended in a stoichiometric volume of 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.1% 2-mercaptoethanol extraction, heated rapidly to 40˚C (*S. droebachiensis*) or 45˚C (*T. gratilla*) for 10 or 15 minutes, respectively, and then chilled on ice. The sample was centrifuged at 45,000 ***g*** for 15 minutes, the tubulin-containing supernatant was withdrawn, and the pellet of ciliary remnants was resuspended to the initial extraction volume with this same buffer.

In a limited number of cases, the remnants were further fractionated into soluble remnant components and insoluble tektin filaments by extraction with 0.5% Sarkosyl/2.5 M urea for 30 minutes on ice, followed by ultracentrifugation at 100,000 ***g*** for 30 minutes (Linck and Stephens, 1987). The pellet was resuspended stoichiometrically in the Sarkosyl/urea extraction buffer for SDS-PAGE analysis.

SDS-PAGE and fluorographic analysis

The membrane/matrix and axonemal fractions were mixed with concentrated SDS-sample buffer, boiled >2 minutes, and analyzed on uniform 8%T/2.5%C polyacrylamide gels (1.5 mm thick × 10 cm long × 15 cm wide), using 0.1% Sigma L-5750 SDS with the Laemmli (1970) discontinuous ionic system. Gels were stained by the equilibrium method of Fairbanks et al. (1971) using Coomassie Blue (Serva).

For fluorography, the gels were treated with En^{3}Hance (New England Nuclear), dried, and fluorographed at –80˚C against preflashed Kodak X-Omat AR film, typically for 1-4 days. By running the relevant comparisons on the same gel, specific activities were determined by video densitometry of the stained gel and its equivalent fluorograph, using multiple sample loadings and film exposures to remain within the linear limits of these methods. Images were analyzed with the Jandel Video Analysis (JAVA™) system (Jandel Scientific, San Rafael, CA), calibrated with neutral density standards to read absorbance directly, using white light transillumination. For absolute radioactivity measurements, tubulin bands were excised from dried gels, solubilized in 30% H_2O_2, and counted in a scintillation counter. The corresponding protein amounts were determined densitometrically from the gel, using known amounts of bovine serum albumin as an internal standard.

Light microscope autoradiography

Ciliary axonemes, suspended in 30 mM Tris-HCl, pH 8, and 3 mM $MgCl_2$, were spread on glass slides and allowed to air-dry. In some cases, they were fixed and stained for 5 minutes with 0.25% Fast Green FCF in 25% 2-propanol, 10% acetic acid, rinsed with 1% acetic acid, and air-dried; in other cases, they were fixed with 1% glutaraldehyde in suspension buffer. The slides were coated with LM-1 emulsion (Amersham) and processed according to the manufacturer's instructions. The emulsion protocol is basically that of Caro and van Tubergen (1962). After 1-2 weeks of exposure at 4˚C in the presence of silica gel, the slides were developed for 4 minutes in Kodak Microdol-X developer, stopped with 1% acetic acid, fixed for 4 minutes with Kodak rapid fixer, washed, dried and observed under video-enhanced phase-contrast or DIC-optics, using water as a temporary mounting medium.

RESULTS

The question of relative turnover

Leucine incorporation into virgin and regenerating cilia in a tropical sea urchin confirms rapid turnover

The initial evidence for a high degree of ciliary turnover in the cold water urchin *S. droebachiensis* was deduced indirectly from the ratio of specific activities between membrane/matrix and axonemal tubulin in steady-state cilia, yielding turnover estimates of >60% with respect to fully regenerated cilia, regardless of developmental stage (Stephens, 1991a). Similar rates can be obtained more directly from these data simply by using the ratio of specific activity of axonemal tubulin in steady-state versus regenerating cilia at both the hatched and mesenchyme blastula stage, and also for the late gastrula stage if one corrects for the transient stimulation of protein synthesis caused by deciliation. However, it would seem useful to confirm these observations in another species, particularly one in which neither cell division nor up-regulation of protein synthesis by deciliation would complicate interpretation.

To accomplish this, late gastrula and contemporaneous zinc-arrested mesenchyme blastula embryos of the tropical sea urchin *T. gratilla* were pulse-chase labeled in parallel for comparison of ciliary protein incorporation during both steady-state maintenance and ciliary regeneration for two distinct developmental stages. The cilia were isolated and all cultures were allowed to regenerate a second crop of cilia from the resulting uniformly labeled tubulin pools. These were fractionated into membrane/matrix and axonemal fractions, then analyzed by SDS-PAGE and fluorography. The basic results are illustrated in Fig. 1, while α-tubulin-specific activities are compiled Table 1 (α-tubulin is used, since β-tubulin is obscured by comigrating tektin-B). These embryos were chosen because: (1) deciliation does not stimulate ciliary protein synthesis at the late gastrula stage where ectodermal

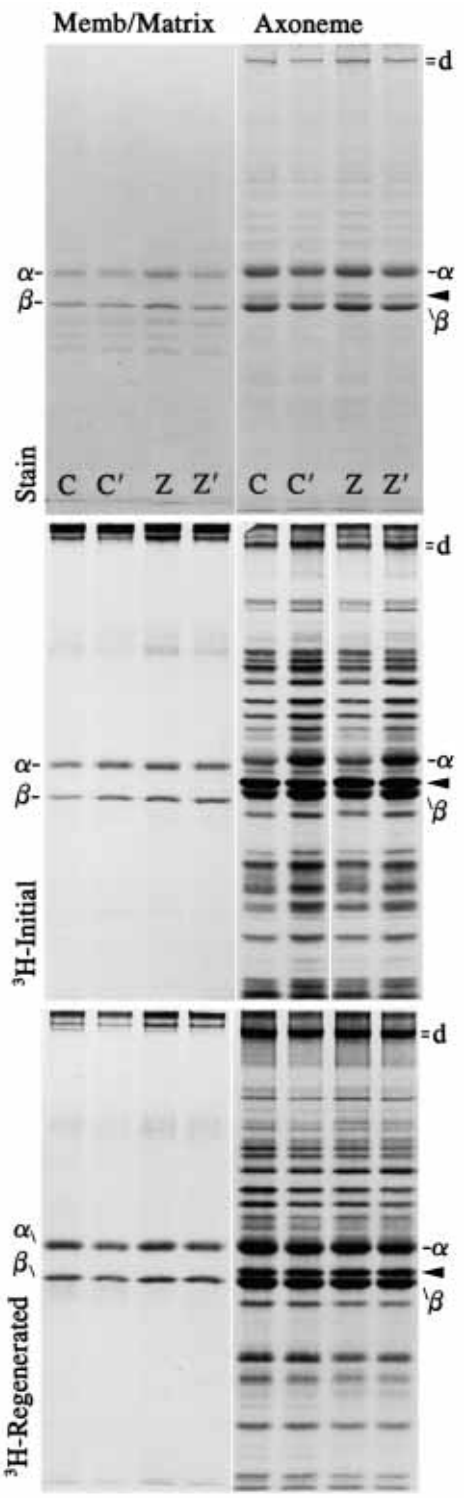

Fig. 1. Analysis of newly synthesized proteins incorporated into membrane/matrix and axoneme fractions of cilia from late gastrula and contemporaneous zinc-arrested *T. gratilla* embryos pulse-chase labeled at steady-state and during regeneration, followed by regeneration of cilia from labeled pools. Top panel, SDS-PAGE gel stained with Coomassie Blue; the membrane/matrix and axoneme fractions have been loaded stoichiometrically. Lanes C, fractions from control gastrula cilia labeled at steady-state; lanes C′, the same for control gastrula labeled during regeneration; lanes Z, the same for zinc-arrested blastula labeled at steady-state; lanes Z′, the same for zinc-arrested blastula labeled during regeneration. Center panel, ^{3}H-fluorogram of the initial labeling. Bottom panel, ^{3}H-fluorogram of the same fractions from cilia regenerated from the deciliated embryos analyzed above. d, Dynein heavy chain region; α and β, α- and β-tubulin; arrowhead, tektin-A. The similarity in overall labeling in each axoneme lane pair (center panel, C vs C′ or Z vs Z′) indicates that steady-state cilia incorporate protein to a level approaching that of fully regenerated cilia.

Table 1. Differential incorporation of labeled α-tubulin into cilia of late gastrula and contemporaneous zinc-arrested *T. gratilla* embryos undergoing steady-state turnover (0th) or total regeneration after deciliation (1st), followed, respectively, by regeneration or an additional regeneration (1st or 2nd) to evaluate pool labeling

	Steady-state				Deciliated		
	Membrane	Axoneme	Mem/Axo		Membrane	Axoneme	Mem/Axo
Gastrula:							
0th	1961	1160	1.69	1st	3274	2751	1.19
1st	2430	2878	0.84	2nd	2286	2458	0.93
Zinc-arrested:							
0th	1919	1145	1.68	1st	2916	2371	1.23
1st	2533	3067	0.83	2nd	2127	2193	0.97

Data are averages from scintillation counting of bands excised from duplicate gels, expressed in cts min^{-1} μg^{-1}. For the first entries in each data set, 0th specific activities were derived from lanes C and Z in Fig. 1, ^{3}H-Initial labeling (at steady-state), while 1st was derived from lanes C′ and Z′ in Fig. 1, ^{3}H-Initial labeling (while regenerating). The second entries in each data set, 1st or 2nd, were derived from lanes C and Z, or C′ and Z′, respectively, in Fig. 1, ^{3}H-Regenerated. The mass ratio of tubulin in the membrane/matrix fraction to that of the axoneme is 1:3.7 in all cases (Stephens, 1991a).

cell division has ceased; and (2) zinc-arrest likewise does not stimulate protein synthesis until the embryos are induced to make hyperlong cilia (Stephens, 1994). Both of these facts should be clear from the similarity of axonemal tubulin labeling in all of the full regenerations, within and between cultures (Table 1, 1st vs 2nd generations; gastrula vs zinc-arrest). The '0th' steady-state cases are critical exceptions since, by design, they began with cilia already assembled from unlabeled tubulin.

Four important confirmatory facts come from these data. First, the ratio of total label incorporated into steady-state versus regenerated ciliary axonemes, by direct scintillation counting, is 0.58±0.03 (n=6) for the late gastrula stage and 0.65±0.04 (n=6) for the zinc-arrested mesenchyme blastula, while the respective ratios obtained by using axonemal α-tubulin specific activities from Table 1 give values of 0.42 (1160/2751) and 0.48 (1145/2371). This provides additional independent evidence for protein turnover, with a magnitude quite comparable to that seen in *S. droebachiensis* at these same stages, but these data also indicate that the non-tubulin proteins must turn over somewhat more than tubulin itself, since the ratios derived from the total counts are higher. Second, while the specific activity of membrane/matrix tubulin in virgin cilia (i.e. the 0th point) exceeds that of axonemal tubulin by a factor of almost 2, as was seen before, the tubulin-specific activities nearly equalize in regenerated cilia (i.e. 1st generation in steady-state or 2nd generation in deciliated), supporting the axonemal precursor argument. Third, the fluorograms of the membrane/matrix fractions show the absence of most labeled axonemal proteins after either the first or the second regeneration, providing further reason to question the mode of incorporation. In addition, this species provides a clearer example of the absence of labeled dynein α/β heavy chain in the membrane/matrix fraction, uncomplicated by the prominent dynein-sized protein that is found in this fraction from *S. droebachiensis* (see below). Although heavily labeled very high molecular mass material does appear at the top of the gel, this is likely to be extracellular matrix proteins removed during the hypertonic deciliation. Finally, the amount of labeled tektin-A (here heavily overexposed to bring out minor bands) is reduced >70% in second generation cilia, as is the label of several other minor proteins, reinforcing the model for ciliary length regulation by 'quantal' or limited protein synthesis, heretofore demonstrated only in two *Strongylocentrotus* species (Stephens, 1989).

Ciliary length distributions indicate little ciliary regrowth

It could be argued that vigorous stirring in culture causes the loss of cilia by shearing and that the observed high degree of apparent turnover is simply the result of subsequent regrowth, but cilia from static and stirred cultures show virtually the same degree of incorporation (data not shown). Even though removal by mechanical shear can be eliminated as a major cause for ciliary turnover, spontaneous shedding or continual resorption and regrowth could still explain the unusually high labeling. Either of these events should be detectable from the length distribution profile of cilia isolated from steady-state embryos. Since the regeneration kinetics are near-linear for most of the regrowth, asymptotically approaching a final length in the last quarter of the growth phase (Stephens, 1992), one would expect a fairly uniform distribution of lengths, slightly weighted toward the final length, if all of the cilia were randomly regenerating. If none were regenerating, one would expect a narrow distribution centered about the mean (final) length, which is what is consistently seen after an embryo has finished regenerating cilia. If both situations were present, a mixed distribution should be seen. In fact, Burns (1973) has published length histograms of cilia from steady-state and regenerated control and zinc-animalized *T. gratilla* embryos, as used above, and these do show the narrow distribution predicted for populations not undergoing any appreciable random regeneration.

However, to address this point rigorously, one must be sure that all of the cilia were recovered by centrifugation and then properly scored. It has been customary to classify very short cilia as fragments and not count them, but we now know that even ciliary remnants, devoid of their microtubules, are very resistant to breakage (Stephens et al., 1989). Therefore, the length distribution of cilia recovered by high-speed centrifugation from control and animalized mesenchyme blastula embryos of *S. droebachiensis* was determined by measuring and scoring all recognizable axonemes. The results are illustrated in Fig. 2. In addition, the specific activities of α-tubulin and tektin-A were determined from parallel cultures of these embryos, undergoing both steady-state maintenance and induced regeneration. These data are given in Table 2.

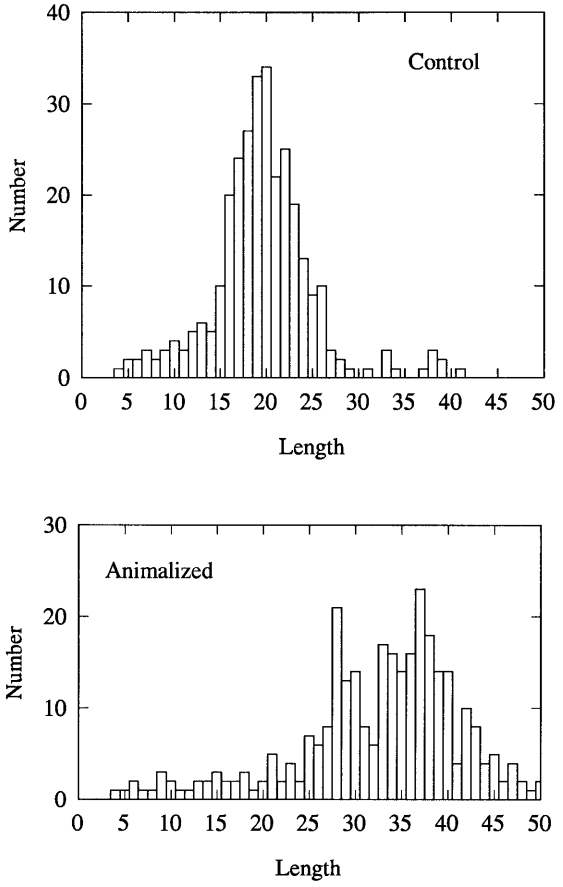

Fig. 2. Length histograms of steady-state cilia isolated from control early gastrula and contemporaneous zinc-animalized blastula of *S. droebachiensis*. Three-hundred cilia were scored in each case. These distributions indicate that <20% of either population could be regenerating.

The length histograms for cilia from control and zinc-animalized blastulae are consistent with the regeneration of only a relatively small fraction of cilia in each population. If each data set is treated as two populations, one growing randomly and the other being maintained at full length (and assuming that lengths above 20 μm in the animalized embryos represent the observed gradient of increasing length toward the animal pole while the bimodality reflects the abundant, shorter, motile cilia characteristic of the vegetal pole), one can arbitrarily assume (by inspection) that cilia below 16 μm in the control population and below 26 μm in the animalized embryos are growing. This assumption yields 15% by number or 8.8% by mass for the controls and 17% by number or 8.7% by mass for the animalized population. This is roughly what one would expect from the normal level of cell division that takes place during this period of development.

The leucine incorporation data from these cultures, obtained from a 4 hour pulse/4 hour chase during steady-state and regeneration (Table 2) gives further insight into the turnover process. Zinc-animalization doubles the degree of α-tubulin pool labeling, but, in spite of this, the ratio between steady-state and regenerated cilia is the same in both cases, ~65%. Tektin-A is up-regulated approximately 1.4-fold, roughly in proportion to the length of cilia that these embryos deploy (1.6-fold from data in Fig. 2). In both cultures, the labeling ratio between virgin and regenerated cilia averages 70% when tektin-A is used to evaluate relative turnover.

Table 2. Relative incorporation of [^{3}H]leucine into axonemal α-tubulin and tektin-A in ciliary axonemes of control and zinc-animalized mesenchyme blastula embryos of *S. droebachiensis* during steady-state turnover versus regeneration

	Virgin	Regenerated	Vir/Reg
α-Tubulin:			
Control	203±17	319±26	0.64
Animalized	450±47	683±43	0.66
Animalized/control	2.22	2.14	
Tektin-A:			
Control	11,161±892	16,414±984	0.68
Animalized	16,044±1203	22,606±1138	0.71
Animalized/control	1.44	1.38	

Data are averages from scintillation counting of duplicate bands excised from two different gels, expressed as cts min^{-1} μg^{-1} ± s.d. (n=4).

The question of transport route

Autoradiographic analysis of steady-state ciliary axonemes indicates uniform labeled protein distribution

It should be obvious from the foregoing that even virgin, steady-state cilia are in a dynamic equilibrium with newly synthesized proteins, yet these proteins do not appear in detectable amount in the membrane/matrix fraction in fully grown cilia. To test the hypothesis that these proteins incorporate at the base or proximal end of the cilium, late gastrula embryos were pulse-chase labeled for 2 hours and their virgin cilia were then isolated after various periods of steady-state turnover. The cilia were demembranated and the axonemes were subjected to light microscope autoradiography, after which the distribution of radioactivity along the length of the axoneme was evaluated by phase-contrast microscopy. The proximal end of the axoneme, marked by the basal plate, appears as a bead, while the distal tip tapers in the final micrometer of length, allowing the orientation to be determined. The cilia were scored in terms of the distribution of silver grains within the distal, central and proximal third of the 20-μm-long shaft. The results are sum-

Table 3. Distribution of silver grains in the proximal, central and distal third of ciliary axonemes determined by light-microscope autoradiography

	Turnover (hours)				End pulse
	2	4	6	8	6-8
Proximal	1.4±0.7	2.0±0.7	2.1±1.1	1.8±1.0	1.1±0.9
Central	1.3±0.7	2.0±0.7	2.6±1.2	2.1±1.0	1.0±0.7
Distal	1.5±0.6	2.2±0.7	2.5±1.0	2.4±0.9	1.2±1.0
Total	4.2±1.0	6.2±1.2	7.2±1.8	6.3±1.2	3.3±1.3
Sp. act.	5,425	9,053	16,765	14,176	4,112

Embryos were pulse-labeled for 2 hours, then isolated after 2, 4, 6 and 8 hours; unlabeled leucine was added after the 2 hour isolation. A parallel culture was deciliated and pulsed 2 hours before isolation at 8 hours of growth. After demembranation, 50 axonemes in each class were evaluated. The 6 and 8 hour time points were developed for 1 week; all others were for 2 weeks.

marized in Table 3. Although the total number of grains increased proportionately with labeling time, there were no marked differences in distribution of radioactivity along the lengths of the axonemes at the various time points tested. At any given time point, most cilia showed relatively uniform population labeling, arguing independently that few cilia were constructed de novo during the experiment. However, in the 2 hour time point there were some short cilia (<10 μm) whose labeling fell into the range of 4-6 grains, uniformly distributed, with these most likely corresponding to regenerating cilia. Such heavily labeled short cilia were far less obvious at later, post-chase time points, presumably because of equilibration and turnover. Direct attempts to determine the polarity of addition by pulse labeling at the final stage of growth yielded cilia with very low but still apparently random grain counts.

Long-term labeling of ciliary fractions reflects their protein composition

Analysis of the detergent-solubilized membrane/matrix and detergent-insoluble axonemal components limits our analyses to two defined compartments only. Previous work has shown that the ninefold structure of cilia can be retained after removal of 80% of the tubulin by 'melting' at low ionic strength (Stephens et al., 1989). This procedure yields a soluble fraction of axonemal tubulin and various doublet microtubule-associated proteins plus an insoluble ciliary remnant fraction retaining the tektins and other associated proteins characteristic of the intradoublet junction, radial spokes and interdoublet linkages. Such fractions can be analyzed in terms of their characteristic proteins, both early in regeneration or after full equilibration with labeled pools.

To establish the characteristic protein labeling patterns attained by fully equilibrated cilia, late gastrula embryos were pre-labeled for 2 hours with [^{3}H]leucine and then deciliated (these cilia being discarded). The embryos were allowed to regrow cilia from the now-labeled pools without further deciliation until the experiment was terminated after the equivalent of two full regeneration periods (15-16 hours), as spicules began to form. The incorporation of label into the various ciliary proteins should become uniform as a consequence of the initial regrowth and any subsequent turnover. Since leucine is a relatively constant component of most proteins, the radioactivity in each ciliary protein should approximate its amount in the various ciliary fractions. This is illustrated in Fig. 3 for duplicate embryo cultures, one of which was deciliated prior to the addition of label to assess possible long-term stimulation of protein synthesis by deciliation.

The patterns of label incorporation into the solubilized microtubule and remnant fractions are quite distinctive, basically paralleling their protein composition. For illustrative purposes, although the embryos were washed free of excess labeled leucine at the initial deciliation, an unlabeled leucine 'chase' was not used in this case so that some radioactivity would continue to appear in tektin-A and the other 'quantal' protein components. Even with the pools now uniformly labeled, such axonemal proteins are not obvious in the membrane/matrix compartment. This fraction does show heavy and complex labeling in the dynein region, although its characteristic dynein-sized polypeptide does not correspond in mobility to the major α/β axonemal heavy chains. Disproportionately labeled relative to its apparent (stained) amount is an acylated membrane protein (p, Fig. 3) described previously (Stephens, 1991a). The total lack of label in a prominent membrane/matrix protein (Δ, Fig. 3), whose mobility is sometimes coincident with tektin-A but is strongly calcium-dependent, confirms its origin as a maternal protein that is not further synthesized (Stephens, 1991a), even during this skeleton-forming stage.

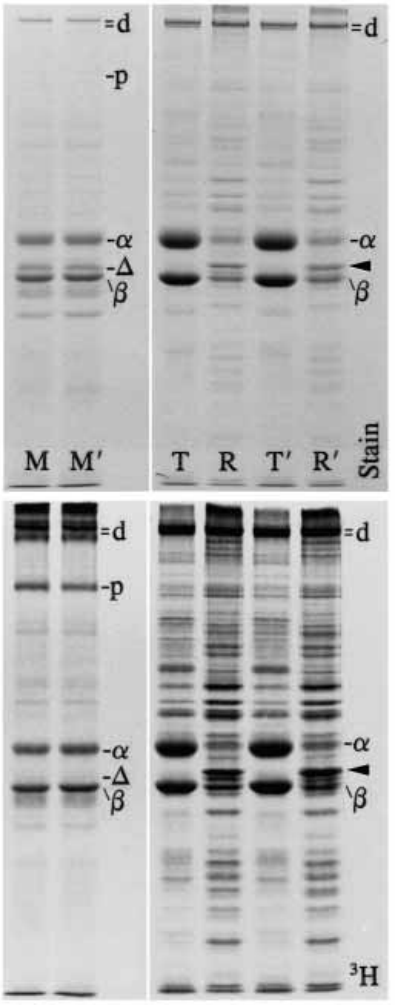

Fig. 3. Long-term incorporation of labeled proteins into membrane/matrix (M), solubilized microtubule (T), and ninefold remnant (R) fractions of cilia from *S. droebachiensis* embryos. Top panel, SDS-PAGE gel stained with Coomassie Blue; bottom panel, ^{3}H-fluorogram of the same. The primes designate fractions of cilia derived from embryos that were deciliated before the addition of isotope to demonstrate the lack of stimulation of protein synthesis by deciliation. d, Dynein heavy chain region; p, palmitoylated membrane protein; α and β, α- and β-tubulin; Δ, calcium-shift protein; arrowhead, tektin-A. The labeling pattern of each fraction is distinctive and closely reflects the protein composition of that fraction.

Labeling of fractions from early growth cilia reveals the axonemal association of newly synthesized proteins

An earlier effort to detect axonemal precursors in the detergent-soluble membrane/matrix fraction of cilia that were partially regenerated from pre-labeled embryos revealed that none of the heavily labeled 'quantal' components could be found (Fig. 3B in Stephens, 1992), a critical observation that gave rise to the present quandary concerning mode of incorporation. This previous analysis was done after 4 hours of regeneration, during which the cilia grew to better than half their final length. In our conventional 4 hour pulse/4 hour chase experiments, the consistent difference in specific activity between membrane/matrix and axonemal tubulin after a full regeneration was interpreted in terms of the necessary formation of much of the initial 1/5 of the cilium from unlabeled protein pools, the half-time for label uptake being about 45 minutes (Stephens, 1991a). In fact, direct measure-

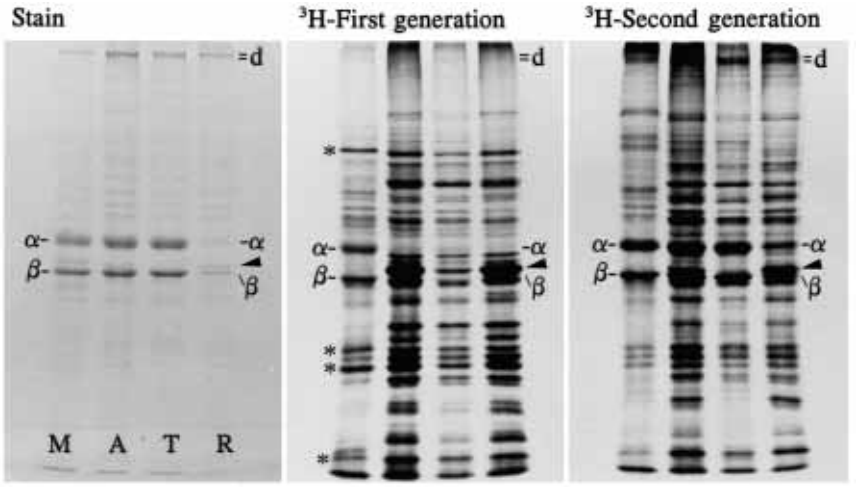

Fig. 4. Short-term incorporation of newly synthesized proteins into membrane/matrix (M), axoneme (A), solubilized microtubule (T), and ninefold remnant (R) fractions of partially regrown cilia from *S. droebachiensis* embryos. Left panel, SDS-PAGE gel stained with Coomassie Blue; center panel, ^{3}H-fluorogram of same; right panel, ^{3}H-fluorogram of the same fractions derived from a second crop of partially regrown cilia. d, Dynein heavy chain region; α and β, α- and β-tubulin; arrowhead, tektin-A; asterisks, major labeled proteins found in all fractions that are not normally seen in the membrane/matrix fraction after full growth. Heavily labeled proteins characteristic of the remnant fraction dominate the solubilized microtubule fraction in the first regeneration and also in the second, but to a lesser degree.

ment of labeled protein incorporation into *S. droebachiensis* gastrula cilia regrowing for 2 hours (to ~5 μm) indicates that the amount of label incorporated into α-tubulin is <10% of that seen after the full 8 hour regrowth period (to >20 μm), experimentally confirming this interpretation (data not shown).

Together, these observations suggest that the isolation of an earlier stage of regrowth, immediately following the uptake of [^{3}H]leucine, should provide axonemal structures formed mainly from proteins derived from initially unlabeled pools but containing - somewhere in the cilium - the most recently labeled axonemal proteins. Under these circumstances, the already assembled components of the axoneme would not dominate the pattern of radioactivity. To accomplish this, *S. droebachiensis* late gastrulae were pre-labeled for 1 hour, deciliated, and allowed to regrow cilia for 3 hours, after which the embryos were again deciliated. No leucine chase was used. A second crop of cilia was then regrown for another 3 hours and removed by deciliation. These first and second generation short cilia were fractionated into membrane/matrix, axoneme, solubilized microtubule and remnant fractions, separated by SDS-PAGE, and analyzed fluorographically by intentional over-exposure. The results are illustrated in Fig. 4.

Unlike the long-term labeling results illustrated above for fully grown steady-state cilia, the labeling profile of the solubilized microtubule fraction from the initial short regenerating cilia is almost identical, qualitatively, to that of the insoluble remnant fraction (Fig. 4, ^{3}H-First generation, 3rd vs 4th lane), thus reflecting heavily labeled architectural remnant proteins in the microtubule fraction. The axonemal α-tubulin itself (in contrast to that of the membrane/matrix) has little label, consistent with the initial assembly of most of the axoneme from unlabeled protein pools. This basic observation of remnant-destined proteins in the thermally solubilized microtubule fraction indicates that newly synthesized proteins are mainly associated with the 9+2 axoneme prior to incorporation. Some co-migrating axonemal proteins may also be found in the membrane/matrix fraction, although tektin-A and most other heavily labeled 'quantal' proteins are clearly not among them. This may reflect a certain degree of instability of such early regenerating cilia, although previous electron microscopy of this same time point indicated intact 9+2 structures after detergent extraction (Fig. 3D in Stephens, 1992). Furthermore, inclusion of 25 μM taxol during extraction does not change the relative amount of membrane/matrix tubulin (data not shown). Alternatively, these particular axonemal proteins may be membrane/matrix-associated during only the early stages of regeneration.

Note that the labeling patterns of the solubilized microtubule and insoluble remnant fractions in second generation short cilia (Fig. 4, ^{3}H-Second generation, 3rd vs 4th lane) are somewhat less alike and resemble more a composite of the first generation pattern plus the characteristic profiles of cilia at equilibrium with the labeled protein pools, as in Fig. 3. The membrane/matrix and axonemal tubulins, present in nearly equal amount at 3 hours of regeneration, are now equally labeled, as would be expected from the postulated precursor relationship. Labeled dynein heavy chains appear only in the second generation, presumably because of the time needed for transcription and translation of such large polypeptides. The presence of labeled dynein-like polypeptides in all of the fractions prevents any clear conclusion about dynein's incorporation route.

Incorporation dynamics in steady-state cilia confirm axoneme-associated precursors and architectural protein exchange

In late gastrula/early prism stage embryos, turnover as judged simply from tubulin label appears to be minimal, mainly because tubulin synthesis has decreased (Stephens, 1991a), yet the architectural proteins continue to become labeled. This fact provides the opportunity to investigate further the behavior of newly synthesized proteins in steady-state cilia in terms of their compartmentation within the axoneme. A 4 hour pulse/4 hour chase protocol was used on duplicate cultures of late gastrula *S. droebachiensis* embryos, one at steady-state and one regenerating cilia. Cilia were isolated from each after 8 hours, as was a second crop of regenerated cilia. These were fractionated as above into membrane/matrix, soluble tubulin and insoluble remnant fractions, but the ciliary remnants were further fractionated into Sarkosyl/urea-soluble and insoluble tektin filament fractions (Linck and Langevin, 1982; Linck and Stephens, 1987). The results of SDS-PAGE and fluorographic analysis are illustrated in Fig. 5.

As in the case of early regeneration from pre-labeled embryos, illustrated above, the initial pattern of protein labeling in the solubilized microtubule and insoluble remnant

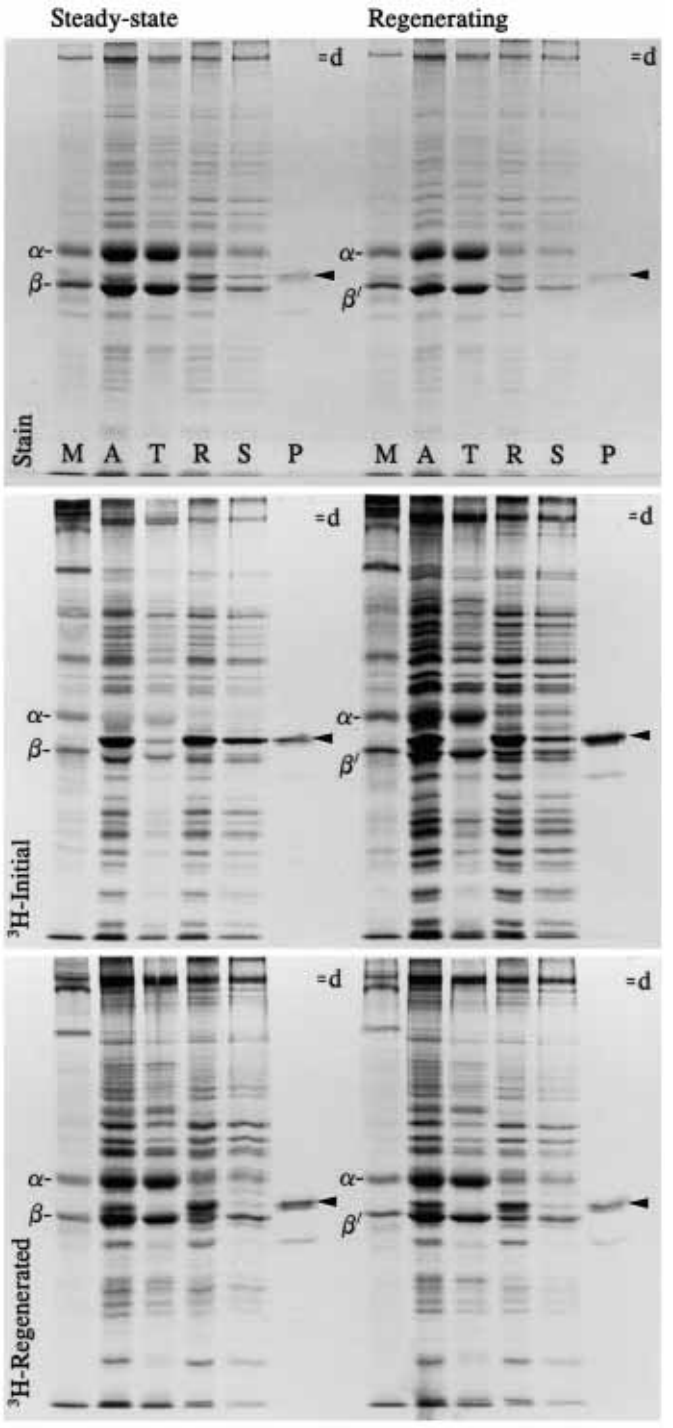

Fig. 5. Differences in proteins incorporated into membrane/matrix (M), axoneme (A), solubilized microtubule (T), ninefold remnant (R), and Sarkosyl/urea-soluble (S) or -insoluble (P) tektin-A containing fractions of steady-state and regenerating late gastrula cilia. Top panel, SDS-PAGE gel stained with Coomassie Blue; center panel, ^{3}H-fluorogram of same; bottom panel, ^{3}H-fluorogram of the equivalent fractions derived from a second crop of cilia regenerated from the two cultures. d, Dynein heavy chain region; α and β, α- and β-tubulin; arrowhead, tektin-A. As in short-term labeling (Fig. 4), proteins characteristic of the remnant fraction are readily detected in the solubilized microtubule fraction during steady-state turnover but this latter fraction more closely reflects its major protein composition after full regeneration. Newly synthesized tektin-A appears mainly in the Sarkosyl/urea-soluble (exchangeable) fraction in steady-state axonemes but it is incorporated into the insoluble (stable) filament fraction in fully regenerated cilia.

fractions from steady-state cilia are very similar (Fig. 5, ^{3}H-Initial, lane T vs R), as is the relative paucity of label in axonemal versus membrane/matrix α-tubulin (^{3}H-Initial, lane A or T vs M, compared with the stained counterpart). However, labeling of these fractions in the parallel regeneration case more closely reflects the protein composition of the individual fractions, with the membrane/matrix and axonemal α-tubulin having very similar degrees of labeling. The cilia further regenerated from these cultures are indistinguishable and show the expected fraction-specific labeling (Fig. 5, ^{3}H-Regenerated). Therefore, the same basic conclusion can be drawn, namely, that newly synthesized, non-tubulin axonemal proteins are associated with the axoneme before assembly (or exchange) at steady-state, since proteins characteristic of the ciliary remnant, most notably tektin-A, are released when the microtubules are solubilized. Virtually identical results were obtained when steady-state and regenerating *T. gratilla* cilia were fractionated in this manner (data not shown). This experimental approach of using late-stage, full-length cilia has one major advantage over that of using short, regenerating cilia: the observation of remnant components in the solubilized microtubule fraction cannot be explained away by assuming that the cilia are less stable simply because they are not yet fully grown.

Additional information supporting architectural protein exchange can be gleaned from investigating the distribution of the quantally synthesized, integral outer doublet protein component, tektin-A. Fractionation of the ciliary remnant with Sarkosyl/urea releases approximately half of the tektin-A from the structure, leaving the other half as highly insoluble filaments; the former is presumably more free to exchange than the latter. In steady-state, the majority of labeled tektin-A is found in the Sarkosyl/urea-soluble fraction while in regeneration, the reverse is true (Fig. 5, center panel, lanes S vs P). In the latter case, since synthesis and elongation are coincident (Stephens, 1989), much of the tektin-A found in the Sarkosyl/urea-soluble fraction should be unlabeled, i.e. 'chased' with newly synthesized but now-unlabeled protein, whereas the initially synthesized (i.e. labeled) tektin-A should be locked in stable filaments. This supposition is confirmed after regeneration of both cultures, where the (now much-reduced) label of the initially synthesized tektin-A is found mainly in the Sarkosyl/urea-insoluble (stable filament) fractions of each (Fig. 5, bottom panel, lanes S vs P). These observations of late-stage steady-state and regenerating cilia are consistent with a relatively rapid turnover of architectural proteins, as demonstrated with the easily detected tektin-A. These results also confirm, independently, the contention that outer doublets contain two distinct classes of tektin filament (Linck, 1976; Stephens and Prior, 1991) and suggest that one class is exchangeable while the other must co-assemble into the doublet as the axoneme grows.

DISCUSSION

At least in the post-hatch to mid-gastrula stages of sea urchin

embryos, the majority of the amino acid label found incorporated into virgin, steady-state cilia results from protein turnover, approaching two-thirds of that seen after the complete regeneration of cilia. Little of this incorporation can be due to the random regrowth of cilia resulting from resorption at cell division, loss by mechanical shear or spontaneous resorption. Consistent with turnover is the relatively high amount of axonemal precursor tubulin found in the detergent-solubilized membrane/matrix compartment.

Ciliary protein turnover is not unique to sea urchin embryos. Rosenbaum and Child (1967) estimated that non-regenerating *Ochromonas* flagella incorporated 25% as much amino acid label as regenerating flagella while the value was nearly 50% in the case of *Euglena*. Papers by Witman (1975), Rosenbaum et al. (1969), and Remillard and Witman (1982) all note significant protein turnover of *Chlamydomonas* flagella, although to a somewhat lesser degree. Nelson (1975) reported that steady-state *Tetrahymena* cilia incorporate outer doublet tubulin to half the level seen during a full regeneration. These authors all point out that elongation or regrowth were insignificant during the course of their measurements.

The relatively high protein turnover observed here may explain the relatively high constitutive tektin-A mRNA levels found in steady-state and zinc-animalized embryos versus their deciliated equivalents (Norrander et al., 1988, abstract, and unpublished data). The requirement for the quantal and coincident synthesis of tektin-A during ciliary regeneration may have its functional counterpart during steady-state turnover.

Protein turnover in fully functional cilia and flagella resembles that in other presumably static 'cytoskeletal' structures. Various myofibrillar proteins turn over at relatively rapid, characteristic rates in intact sarcomeres (Zak et al., 1977) and even synthetic thick filaments undergo uniform myosin exchange, taking less than an hour (Saad et al., 1991). The intestinal brush border microvillar cytoskeleton likewise undergoes significant in vivo turnover, measured in terms of hours (Stidwill et al., 1984). Perhaps most surprising is the uniform, dynamic exchange between subunits and polymer of vimentin intermediate filaments, measured in tens of minutes by fluorescence recovery after photobleaching of tissue culture cell IF networks (Vikstrom et al., 1992).

The coordinated synthesis and incorporation of tektin-A during ciliary regeneration and its constitutive synthesis during turnover would suggest that it, like tubulin, might be found in relative abundance in the membrane/matrix fraction, either in transit to the growing axonemal tip or in equilibrium with the dynamic axoneme. As described previously (Stephens, 1992) and above, neither it nor many other axonemal components have been detected in this compartment, even in partially regrown cilia.

There seems to be little question, however, that flagella do grow by the tip-wise addition of axonemal precursors. This was first demonstrated by light microscope autoradiography of pulse-labeled regenerating *Ochromonas* flagella by Rosenbaum and Child (1967), followed by similar studies on *Chlamydomonas* flagella by Rosenbaum et al. (1969), and later by electron microscope autoradiography of *Chlamydomonas* axonemes by Witman (1975). All three studies were seemingly complicated by a significant level of proximal incorporation, which was explained in terms of turnover. A more recent study with *Chlamydomonas* flagella has elegantly and unequivocally proven that epitope-tagged tubulin adds distally to the outer doublet and central pair microtubules and that radial spokes are similarly incorporated into a spoke-less mutant after dikaryon rescue (Johnson and Rosenbaum, 1992).

The above autoradiographic evidence from steady-state and regenerating sea urchin embryonic cilia parallels the results obtained for regenerating *Euglena* flagella by Rosenbaum and Child (1967). In both cases, the high degree of turnover (~50%) leads to the observation of a fairly uniform label distribution and, in the case of sea urchin embryos, provides no clear evidence for proximal addition or exchange, even at very early time points in steady-state. Therefore, based mainly on solid, distal incorporation evidence from other systems, plus the uniform labeling patterns obtained with sea urchin cilia, we can discard the physically unlikely hypothesis of proximal incorporation.

The early regeneration results presented here took advantage of a situation where nearly half of the total ciliary tubulin was present in the membrane/matrix fraction and yet no labeled tektin-A nor most other architectural components could be detected, even with over-exposure of the fluorogram. This would argue against the hypothesis that these components associate at the elongating tip of the cilium with such high affinities that their relative concentration in the membrane/matrix compartment is vanishingly small. These data do not totally negate the hypothesis, however, since we do not know the relative binding constants and hence we cannot place any lower limits on expected concentrations.

The fact that heavily labeled architectural proteins characteristic of the stable ciliary remnant, particularly tektin-A, were found associated with the solubilized microtubule fraction during both early regeneration and steady-state turnover argues in favor of the hypothesis that these proteins are transported along or within the ciliary shaft during both regeneration and turnover. The early regeneration experiment succeeds because very little labeled pool proteins are incorporated into the axoneme within the first 2 hours of regeneration. Therefore, by the time of the 3 hour deciliation, any label seen should be dominated by newly synthesized proteins yet to be assembled, even if those proteins represent only a very small fraction of the total axonemal protein, which is probably the case. The late stage steady-state experiment works for a similar reason: axonemal components on their way to incorporation or exchange are the dominant labeled proteins, since the axoneme proper was, by design, fully constructed from unlabeled proteins.

If the conclusion concerning the axonemal association and transport of the non-tubulin proteins is correct, this raises two critical questions: what mediates this association and why is the axonemal precursor tubulin conveyed by a separable membrane/matrix associated pathway? A potential answer to the first question may lie in the recent work of Kozminski et al. (1993), who have demonstrated a new form of motility in *Chlamydomonas* flagella, referred to as intraflagellar transport, wherein lollipop-like particles, positioned between the outer doublets and the membrane, shuttle bidirectionally along the flagellum. Preliminary results cited by these authors indicate the presence of myosin I and kinesin-like proteins, thus providing potential motors for this movement. Axonemal dynein, on its way to assembly, could also provide transport for itself and any associated proteins. Such a shuttle transport

mechanism for newly synthesized proteins could explain the consistent observations of proximal labeling reported in previous pulse-chase autoradiographic studies and the relatively uniform autoradiographic labeling reported here for steady-state and final-growth cilia.

As to the second question, concerning separate transport routes, one can speculate that several interdependent points of control may be involved in length regulation. The constant partitioning of tubulin from the cytoplasmic pool into the ciliary membrane/matrix compartment in conjunction with post-translational modification (Stephens, 1992), the postulated release of tubulin and lipids from the tubulin-lipid complex at the distal tip, and the subsequent co-assembly of this tubulin with the quantally regulated tektin-A and other architectural proteins are three obvious candidates for inter-related regulatory processes.

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