Image analysis shows that variations in actin crossover spacings are random, not compensatory

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ABSTRACT A recent paper by Bremer et al. (1991. *J. Cell Biol.* 115: 689–703) has argued that the random angular disorder model for actin is wrong, and that the variations in crossover spacing observed in electron micrographs of F-actin filaments can be best explained by a compensatory disorder caused by the lateral slipping of the twin (or two-start) strands which comprise the actin filament. We have analyzed the images of F-actin presented in Bremer et al. and show that their data argues against compensatory disorder and in favor of random disorder, independent of the cause of the disorder. We also revise our estimate of the angular component and show that the magnitude of this disorder is about 5–6° per subunit, which is less than the 10–12° that we originally proposed.

Observations in the electron microscope have shown that the axial periodicity in actin filaments arising from the crossovers of the two long-pitch helices is quite variable (Hanson, 1967), but the explanation of this variability has been controversial (Egelman et al., 1982; Erickson, 1989; Egelman and DeRosier, 1991). We developed a quantitative model for this disorder in actin. In a perfectly ordered actin filament, each successive subunit is translated 2.7 nm along the helix axis and rotated about the helix axis by 167° relative to its neighbor. In our model for disorder, the axial rise remains unchanged but a random deviation of ~10° (rms) exists in the angular position of nearest neighbors. Since the position of each subunit is only relative to its neighbors, i.e., there are no long-range forces that impose a helical lattice, the total angular deviation from the helically ideal position increases with increasing numbers of subunits. This random disorder is thus cumulative (Egelman et al., 1982; Egelman and DeRosier, 1982; Stokes and DeRosier, 1987). This model provided a formalism that expanded upon a qualitative conclusion drawn from observations of muscle x-ray patterns, that actin could be easily twisted but not stretched (Huxley, 1972).

A recent paper (Bremer et al., 1991) presents images of actin in which the twin strands that constitute the filament appear to be locally separated. They also present reconstructions of actin filaments that show variations in the radial separation of these strands. They call this variation "lateral slippage" and argue that this, rather than angular disorder, accounts for the variation in crossover spacings seen in populations of actin filaments. They leave the precise description of this disorder, in terms of the variations in subunit positions due to a radial and a coupled angular component, for future work. Thus, both models (lateral slippage and random angular disorder) have a variation in intersubunit angle. However, Bremer et al. conclude that the deviations are compensatory as opposed to cumulative. Since an understanding of actin's flexibility and dynamics provides new insight into a host of biological systems, from muscle (e.g., Vale and Oosawa, 1990) to stereocilia, it is important to be able to discriminate between compensatory and random disorder. It is this issue alone that we would like to address in this paper.

Bremer et al. (1991) state that "quantitative evaluation of successive crossover spacings along individual F-actin filaments revealed the deviations from the mean repeat to be compensatory, i.e., short crossovers frequently followed long ones and vice versa." The evidence presented in the paper is contained in Fig. 8 of Bremer et al. which plots variations in sequential crossover spacings along two filament segments. The oscillating crossover spacings of one of these plots (Fig. 8 *e* of Bremer et al.) has been reproduced in Fig. 1 *a* of this paper, and this constitutes the first part of the evidence given in their paper for the proposed compensatory nature of the helical disorder. Fig. 8 *f* in Bremer et al. also shows the pattern of crossover spacings from a different actin filament (reproduced in our Fig. 1 *b*), where there is no such oscillatory behavior.

These data do not necessarily allow one to distinguish between compensatory and random disorder. In particular, for random disorder, long crossovers would follow short ones half of the time, which, by chance, would often lead to runs of alternate long and short crossovers. To show this to be the case, we used a computer to generate ten filaments with random variations in their crossover spacings. To eliminate the question of the cause of the variation (i.e., lateral slipping vs. angular disorder), the program pieced together a filament by selecting, at random, crossovers from a population having the distribution given in Fig. 9 of Bremer et al. in which the mean length is 36.1 nm and the standard deviation is 3.36 nm. By this method, a long crossover is as likely to follow a short one as it is to follow a long one, and vice versa. This is in contrast to compensatory disorder in which a long crossover is more likely to follow a short crossover than it is to follow a long one. We plotted crossover spacings as a function of position along the filament for two of the ten model filaments, and this is shown in our Fig. 1, *d* and *e*. Further, we took the filament of Fig. 8, *a–d* of Bremer et al. and displayed the crossover spacings along

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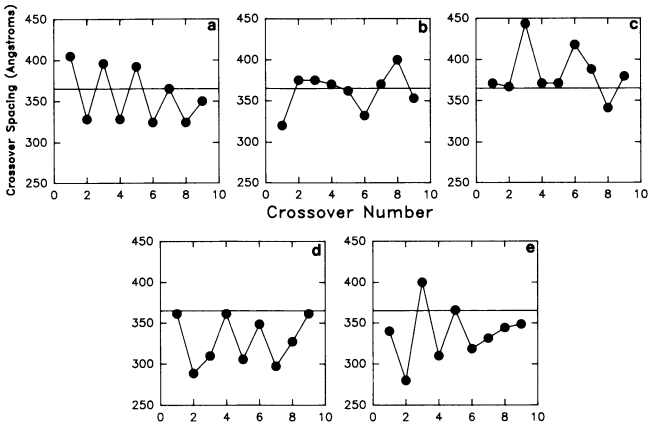

FIGURE 1 The data from Bremer et al. (1991), Fig., 8 *e* and *f* for the crossover spacings along single actin filaments are shown in *a* and *b*, respectively. We have also taken the filament shown in Fig. 8, *a* of Bremer et al., Fourier transformed the image, and back-transformed the meridian out to 1/300 Å (as described in Bremer et al.) to generate a plot similar to Fig. 8 *c* of Bremer et al. which shows the negative stain modulation arising from the crossovers. The spacing of these crossovers is then plotted in *c*. Model actin filaments were generated on a computer with random crossovers. Patterns of alternating short and long crossovers could frequently be generated by this random model. The two filaments that are shown in *d* and *e* were selected out of a set of only ten filaments.

this filament in Fig. 1 *c*. A comparison between the filament data from Bremer et al. in our Fig. 1, *a–c* and between model filaments with random disorder (i.e., randomly chosen crossover lengths) in Fig. 1, *d–e* does not resolve whether actin filaments are best described by a random disorder or a compensatory disorder. What is needed to test the authors' ideas is a simple statistic which combines data from many filaments and which better distinguishes between the two kinds of disorder.

The second part of their evidence comes from their Fig. 9, which we have reproduced in Fig. 2 *a*, which shows the running mean as a function of the number of crossovers. They say (on p. 701): "also our analysis of successive crossover spacings (see Fig. 9) has revealed that, unless the variations are small, usually after one or at most two long crossovers follows a short one and vice versa such that already after two or three crossovers the running mean of the crossover spacing becomes very stable, just as predicted by compensatory lateral slipping of the two long-pitch helical strands." This evidence does not distinguish between compensatory and random disorder because of the phenomenon of convergence to the mean: the variance of a sample average from a true mean, for a random population, decreases linearly with the number of measurements contained in the sample average. In other words, the standard error (the square root of the variance) of the mean of a sample is reciprocally related to the square root of the number of measurements in the sample (Bernoulli, 1713; Laplace, 1809).

To illustrate this point, consider a bag containing a large population of "crossovers" of random length, with a mean length of 36.1 nm and a standard deviation of 3.36 nm (corresponding to the mean and standard deviation, respectively, of actin crossover spacings given in Fig. 9 of Bremer et al.). Imagine drawing a single crossover from this bag, and then plotting the length. Next, a second crossover is drawn at random, and the length of the pair is averaged, and then plotted. Then a third crossover is drawn at random, and the average length of the three crossovers is plotted. Bremer et al. presented data

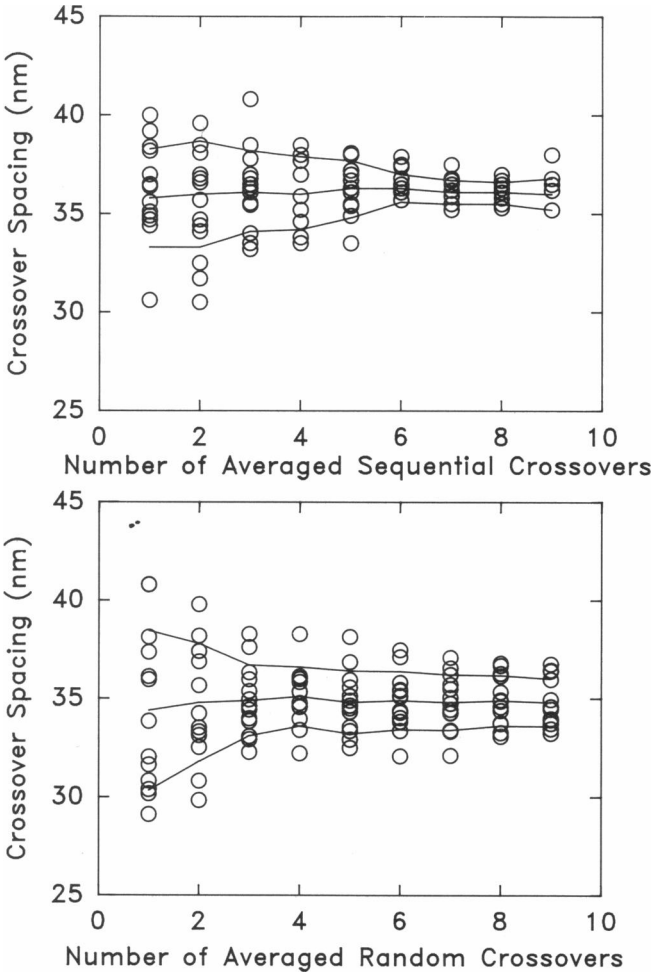

FIGURE 2 The data from Fig. 9 of Bremer et al. is reproduced (*top*), and for comparison, a computer simulation of an experiment involving picking crossovers from a large bag of crossovers of random length (*bottom*). The average length of the crossovers in the population was set at 36.1 nm, with a standard deviation of 3.36 nm, to match the parameters given for actin crossovers in Bremer et al. Nine crossovers were drawn at random, and the running mean was plotted as a function of the number of crossovers in the average. This was done for 13 different sets of nine crossovers. The mean of these 13 trials and the envelope determined by the mean ± the standard deviation are plotted with the solid lines. While Bremer et al. argue that the convergence of a running mean to a population mean supports their model of compensatory disorder, this behavior is indistinguishable from that exhibited by uncorrelated, random events, as shown by the random crossovers.

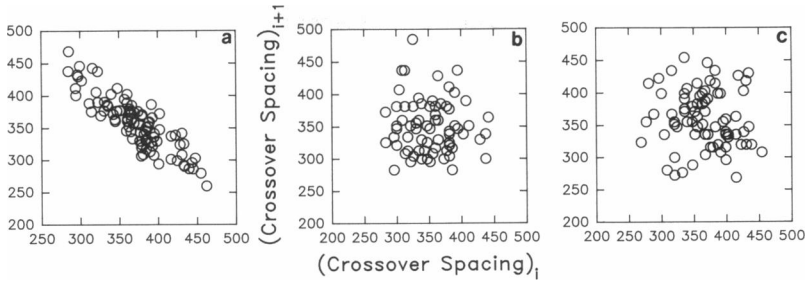

FIGURE 3 (*a*) The lateral slipping model of Bremer et al. states that crossover deviations are compensatory, i.e., short crossovers are bracketed by long crossovers and vice versa. The scatter plot between any crossover and its next neighbor would then be a straight line with a negative slope for such a model. In the presence of noise and additional forms of disorder one might expect to see the scatter plot shown above, where there is a strong negative coefficient of correlation (for the 90 fictitious data pairs shown, $r = -0.87$, with a standard error of 0.05). The random model that generates the data shown in Fig., 1 *d–e* generates the data shown in *b*. Eight filament sections, each containing about ten crossovers, were used to generate the 71 pairs shown. The coefficient of correlation is −0.06, with a standard error of 0.12, consistent with the coefficient of correlation of 0.0 expected for such a random model. Eleven actin filament sections from Fig. 1a of Bremer et al. were analyzed by Fourier transforming the images, zeroing all Fourier coefficients more than 1/175 Å away from the equator, and back-transforming. The crossover points were then determined from the filtered images. The absolute magnification was determined by the strong 1/59 Å layer line. The 77 crossover pairs generate a coefficient of correlation of −0.11, with a standard error of 0.12. For a sample of this size, and a population coefficient of correlation of 0.0, one would expect that 95% of the time one would measure a sample coefficient of correlation between about −0.24 and +0.24. Therefore, the observed sample coefficient of correlation is consistent with a population coefficient of correlation of 0.0 and inconsistent with any strong model of compensatory disorder.

for 13 running means that included 9 crossovers each. Therefore, our *gedanken* experiment is done until 9 crossovers are drawn from the bag, and the entire exercise is repeated 13 times. Would the results of this random experiment look different from the results of Bremer et al., shown in our Fig. 2 *a*? The answer is no, and we have used a computer simulation of this *gedanken* experiment in Fig. 2 *b* to illustrate that this is the case.

A test exists that can quantitatively distinguish between these two models. Compensatory disorder, according to Bremer et al., means that a deviation of a crossover from the mean in one direction (e.g., larger) would be followed by a crossover deviating in the opposite direction (i.e., shorter). There should also be a coupling in the magnitude of the deviations: very short crossovers should be bracketed by very long crossovers, and there should be a strong negative coefficient of correlation between the spacing of any crossover and its immediate neighbor. On the other hand, in our random disorder model there would be no correlation between the length of one crossover and the next.

To see what a compensatory model might look like, we modified the previous computer program so that if a longer than average crossover was selected at one step, the probability of selecting a shorter than average one at the next step was greatly increased. The program was compensatory such that the longer one crossover, the smaller its neighbor. Fig. 3 *a* shows a graph of crossover length plotted against the length of the adjacent crossover (crossover$_i$ versus crossover$_{i+1}$) for this model with compensatory disorder. It shows a significant negative coefficient of correlation. In contrast, Fig. 3 *b* shows the distribution that is generated by model filaments possessing random disorder, in which there is no correlation between successive crossovers, and in which the coefficient of correlation is consistent with 0.0. The key question, therefore, is whether the distribution from real actin filaments resembles Fig. 3 *a* or *b*. Since there may well be important differences in buffers, divalent cations and preparative procedures between the actin in our laboratories and that of Aebi, we have used the filaments shown in Fig. 1 *a* of Bremer et al. to generate the scatter plot shown in Fig. 3 *c*. The coefficient of correlation in this case is −0.113, with a standard error of 0.115, which means that there is no statistically significant correlation. Thus, while long crossovers may frequently follow short ones, they do so no more frequently than would be expected randomly. This test shows that the filaments do not behave in a statistically significant compensatory manner.

DEVIATIONS AS A FUNCTION OF DISTANCE

Although Fig. 3 *c* shows that it is unlikely that actin filaments have a variability of crossover spacings that is strongly compensatory, the data of Fig. 3 *c* might still be compatible with a model in which there is weak compensation that is distributed among a number of adjacent crossovers. In this weaker compensatory model, a long crossover would be bracketed by a number of shorter crossovers, such that the deviations from the average crossover spacing are minimized. To test this possibility, and further distinguish compensatory from random disorder, we have plotted in Fig. 4 the root mean square deviation of crossover spacings as a function of the number of crossovers examined. The difference between this plot and the previous is that in Fig. 2 the running average

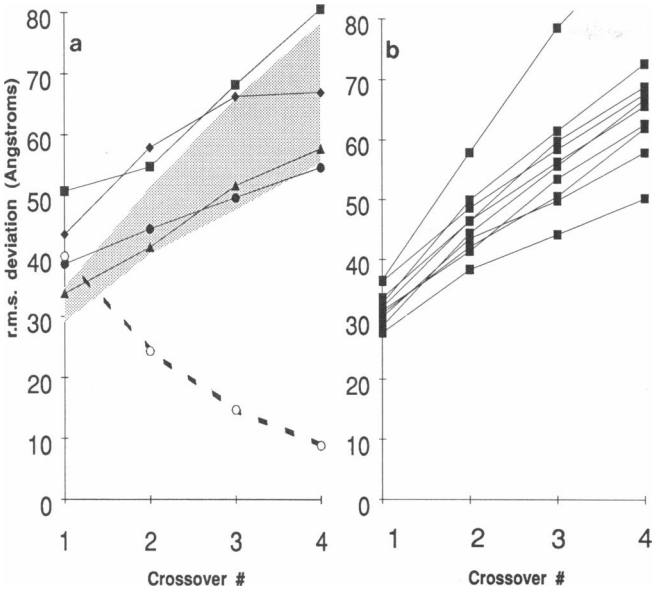

FIGURE 4 The root mean square deviation of crossover spacings from the average crossover spacing is shown as a function of the number of crossovers for a random model (*b*) and for real actin filaments (*a*). For "compensatory" disorder, as described in Bremer et al., the deviations should decrease with the number of crossovers from any given starting point, since the compensatory model holds that short crossovers are compensated for by long crossovers, and vice versa. A strong compensatory model, in which deviations are canceled by adjacent crossovers, is excluded by the data of Fig. 3, where it is shown that the coefficient of correlation between neighboring crossovers is consistent with zero. However, a weaker compensation might exist in which the deviations are canceled over a number of adjacent crossovers. The dashed curve in *a* illustrates what a weaker compensation might look like in this statistic. Model actin filaments containing 5° per subunit of cumulative random angular disorder, or torsional freedom, generate the points shown in *b*. Each curve in *b* represents the data from a set of eight filaments, where each filament contains about ten crossovers. This corresponds approximately to the amount of data contained within the real actin data sets shown in *a*. It can be seen that the spread of this data in *b* is rather large, and therefore that the different curves in *a* may all come from the same population. The rms envelope of the curves in *b* have been shown as a shaded region in *a*. The pure actin filaments from Bremer et al., Fig. 1 *a*, generate the points shown by the diamonds in *a*, while the actin-phalloidin filaments from Fig. 1 *b* generate the points represented by the triangles. Pure actin filaments from the Egelman laboratory generate the points shown by the circles, while the crossover spacings within three actin angle layered aggregates (Egelman et al., 1983) are shown by the squares. The rms deviation as a function of crossover distance is defined by:

$$d(k) = \sqrt{\frac{1}{n}\sum_{l=1}^{n}\frac{1}{t_l - k + 1}\sum_{j=0}^{t_l-k}\left[\left(\sum_{i=j+1}^{k+j} c_i^l\right) - k\cdot\langle c\rangle\right]^2},$$

where $\langle c\rangle$ is the average crossover spacing, c_i^l is the ith crossover spacing in filament section l, n is the total number of filament sections, and t_l is the number of crossovers in filament section l. Therefore, $d(1)$ is the rms deviation of a single crossover spacing from the average, while $d(2)$ is the rms deviation of any two sequential crossover spacings from twice the average crossover spacing, etc.

is plotted as a function of the number of crossovers, and, for both random or compensatory disorder, the running average approaches the mean (although not at the same rate). The plot that we propose looks at the total length, $l(n)$, of n crossovers, rather than the average, $l(n)/n$, and compares this to the expected value. Thus, we want to compare $l(n)$ to $\langle l(n)\rangle$, which is $n \cdot 36.1$. If the disorder is random, the mean deviations of $l(n)$ from $\langle l(n)\rangle$ will increase with n, the number of crossovers (i.e., random disorder is cumulative). The essence of compensatory disorder is that deviations are compensated, in distinction to cumulative disorder, and deviations should grow smaller with distance. A third alternative, equivalent to random fluctuations of crossovers about positions determined by a long-range lattice, would be that the mean deviations would be independent of distance from any particular crossover. Therefore, we must look at the root mean square deviations in summed sequential crossover spacings from the average, as a function of the number of crossovers in the interval, to see if the deviations grow (random disorder), remain constant (disorder about a lattice), or decline (compensatory disorder).

It can be seen in Fig. 4 *a* that both the pure actin and the actin-phalloidin filaments from Fig. 1 of Bremer et al. show growing deviations. We have also taken crossover data from actin filaments prepared in the Egelman laboratory and from angle layered aggregates (Egelman et al., 1983) and show them in this figure for purposes of comparison. Data from model filaments containing cumulative random disorder (Fig. 4 *b*) show the same behavior. To better see the similarity, we have shown the root mean square (rms) envelope of the model data superimposed on the real data in Fig. 4 *a* as a shaded area. It was found that a value of 5° per subunit gave the best fit to the deviations of crossover spacings in real actin filaments.

MAGNITUDE OF THE DISORDER

If the variation in crossover spacing is due to angular disorder, then 5° of disorder per subunit accounts for the observed variation in crossover spacing. We have previously stated (Egelman et al., 1982) that the angular disorder in actin was ~10° degrees per subunit, and a more rigorous analysis (Stokes and DeRosier, 1987) showed that the best estimate for this parameter was 12°. Why, therefore, does the present data suggest that this parameter is 5–6°? In the first place, an analysis of crossover spacings yielding only 5–6° per subunit is not new. In Egelman et al. (1982) we stated that we needed an rms deviation of 6° per subunit to fit the crossover data of Hanson (1967), but that 10° per subunit was needed to fit other observations. In an analysis of actin angle layered aggregates (Egelman et al., 1983) we stated that the distribution of crossover spacings in these structures was best fit with 6° of rms deviation per subunit. However, we stated that "while this amount is less than the 10° degrees we have taken as the best estimate for the disorder in free actin filaments, one would expect that

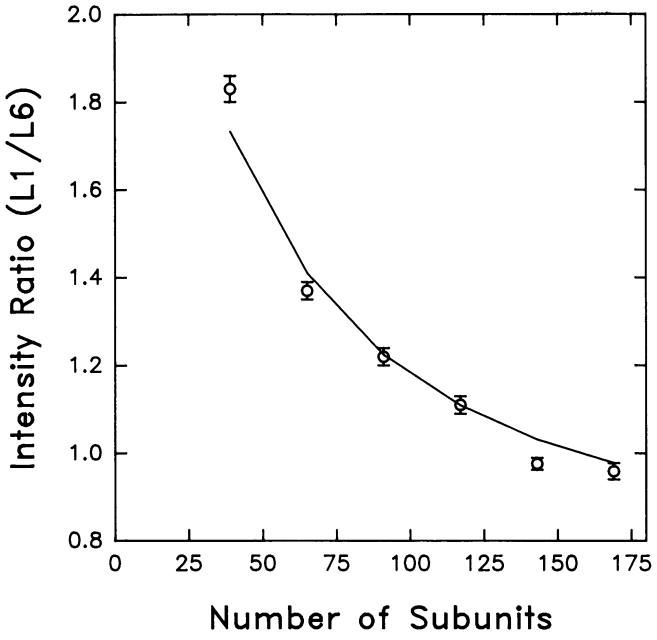

FIGURE 5 Model filaments, using the Holmes et al. (1990) model for F-actin, were generated with 5° rms cumulative angular disorder per subunit. The ratio of the projected first layer line intensity to the projected sixth layer line intensity was calculated according to Stokes and DeRosier (1987) as a function of the number of subunits in the filament. These ratios are shown by the discrete data points, with the error bars representing the standard error of the mean for the ratio. The data was obtained from 200 model filaments generated for each filament length examined. A χ^2 fit was then made to a theoretical model for the ratio, based upon the formalism of Egelman and DeRosier (1982), with two free parameters: the absolute intensity ratio of the two layer lines in the absence of disorder and the rms angular disorder per subunit. The solid line shows the best fit, which surprisingly corresponds to 11° of rms deviation per subunit, even though the model filaments contained 5° of rms deviation per subunit. The basis for this wrong fit is shown in Fig. 6.

aggregate interactions would tend to minimize the amount of variation." The most rigorous analysis of angular disorder was contained in Stokes and DeRosier (1987), where data on the ratio of layer line intensities as a function of filament length from individual actin filaments was fit to a theoretical curve (Egelman and DeRosier, 1982). The χ^2 analysis that was used yielded the value of 12° per subunit. Stokes and DeRosier also measured the variations in the positions of the first and sixth layer lines for long lengths of filaments (approximately seven crossovers). These data also gave higher estimates of the rms angular variation. Thus, there appears to be a paradox: all measurements in real space of crossover spacings yield values of 5–6° per subunit, while measurements in reciprocal space of layer line ratios yield a value of 10–12°. We have now reanalyzed the data and method used in Stokes and DeRosier and believe that this discrepancy can be explained by a rather subtle artefact, and that the data is consistent with 5–6° of disorder per subunit. Fig. 5 shows the method of Stokes and DeRosier (1987) applied to model actin filaments containing 5° of rms cumulative angular disorder per subunit. The choice of model is important to uncover the artefact. If one uses a single sphere model for actin the effect is greatly reduced. Here we use a realistic model derived from the actin filament presented by Holmes et al. (1991). We built the same filament as Holmes et al. from the atomic coordinates of G-actin but with angular disorder. A theoretical curve fit to this data yields an estimate of the disorder of 11°, and not the 5° employed in the model. The explanation for this artefact depends upon the relative strength of the first layer line to the equator. The position and strength of the peak intensity of the first layer line is shifted by the equatorial layer line due to layer line broadening and overlap. The effect is greater for short segments because layer line width varies inversely with segment length. The shorter the segment, the greater the overlap. The effect is also greater for structures with a weak first layer line which is more easily perturbed by the overlap from the strong equatorial layer line. In our model, we find that the overlap causes the intensity ratio of the first to sixth layer lines to climb higher than it should as one goes to shorter filament lengths (Fig. 5).

To test for this artefact, we returned to the original data of Stokes and DeRosier. They chose segment lengths so that the position of the first layer line should fall at a node of the broadened equator. If the equator is perturbing the first layer line it will shift its apparent position off of the node. We demonstrate this effect with the model filament of actin in Fig. 6, *a* and *b* for segments of five and three crossovers, respectively. The most probable position for the first layer line peak should be at 16 pixels. The equatorial layer line, whose relative intensity distribution is indicated by the solid line, has a node at exactly this position. If there is no effect of the equator, we should see a Gaussian distribution centered at position 16. The distributions, however, are bimodal with a minimum at position 16. Thus, the strong lobes of the equator are shifting the apparent position of the first layer line. We then determined the same distribution from the data derived from the real actin filaments measured by Stokes and DeRosier. The same bimodal peaks are seen with a node at the position of the node from the equatorial layer line. Since we have shown that the apparent position of the first layer line is perturbed by the equatorial layer line, we argue for real filaments that the intensities are also perturbed as they are for model filaments.

The perturbation of the position of the first layer line might also account for the variations in the layer line positions seen by Stokes and DeRosier for longer segments of filaments. While this is possible, we do not think it likely because the model data show almost no perturbation from the equatorial layer line by the time one gets to segments of seven crossovers. Thus, the increased variance in the position of the first layer line is

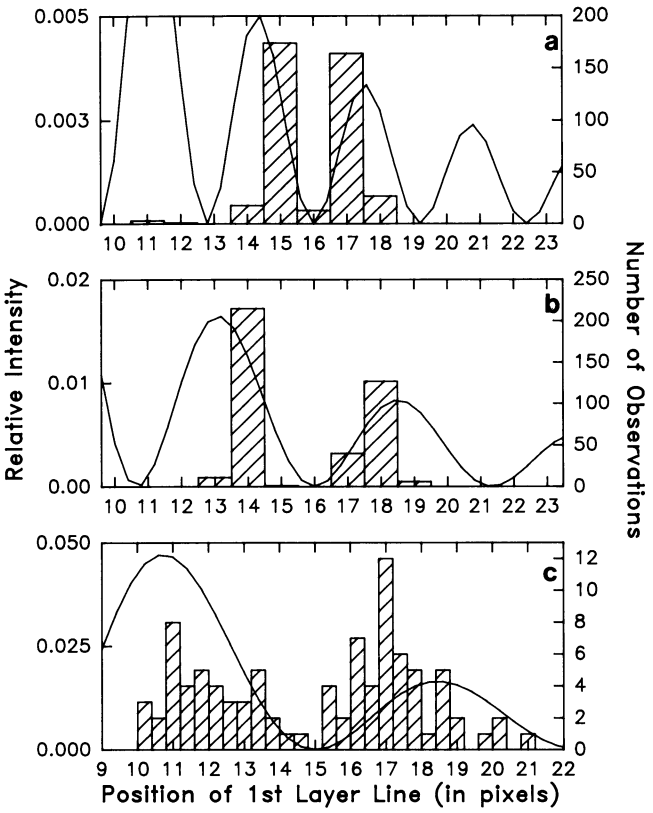

FIGURE 6 As the length of a filament is shortened, the layer line width in the Fourier transform will become broader, as the layer line is convoluted with a sinc function of the form:

$$2a^* \sin(2\pi aX)/2\pi aX,$$

where the filament length is equal to $2a$. For short filament lengths, one can therefore overlap the intensity from one layer line onto a region surrounding a neighboring layer line. The histogram in *a* shows the position of the first layer line (in pixels, on a 1024 pixel transform, with 5.5 Å/pixel in the image) that was found in 400 model filaments, generated as described in Fig. 5, where the filament length corresponded to 5 crossovers or 65 subunits. The scale on the right ordinate shows the number of times that the first layer line position was found at a given pixel value shown along the abscissa. In the absence of an artefact caused by the convolution of the equator, the peak of this distribution should fall on pixel 16. However, what is seen is that there are almost no peaks found here, and instead the peaks are shifted by the subsidiary maxima of the convolution of the equator with the sinc function, which is shown by the solid line. The scale on the left ordinate gives the intensity of the sinc function that is convoluted with the equator. In *b* we show the corresponding histogram and sinc function for a filament length corresponding to 3 crossovers or 39 subunits. In *c* we show the actual data from negatively stained actin filaments used in Stokes and DeRosier (1987) for filament sections containing about 26 subunits or 2 crossovers. The scale of these transforms is different (~10.7 Å/pixel in the images, with a transform that is 512 points) and the peak profiles were curve fit, so the peak values were not constrained to be integer, as they are in *a* and *b*. The most probable position for the first layer line position on this scale would be at 15, but it can be seen that no peaks were found at this position, which corresponds to a node of the sinc function that is convoluted with the equator, and which is shown by the solid line. Thus, the first layer line intensities that were used very likely contained a significant component due to the overlap of the equator with the first layer line.

most probably due to noise not arising from angular disorder. We therefore believe that the actin filaments analyzed by Stokes and DeRosier (1987) probably had only ~5–6° of rms disorder per subunit, but the artefact introduced by the equatorial convolution biased the answer given to ~12° per subunit.

CONCLUSION

The model for compensatory disorder in actin that is proposed as an alternative to cumulative random disorder is based upon a reported observation of compensatory deviations of crossover spacings. A statistical analysis of electron micrographs of actin filaments, including those filaments used by Bremer et al., fails to show this effect. The observed lack of statistical correlation between successive crossovers in actin is, in fact, consistent with cumulative random disorder. The formalism of cumulative random angular disorder, with 5–6° of rms freedom per actin subunit, remains an explanation that is consistent with observations of actin filaments. This formalism, when combined with high-resolution structural data (e.g., Kabsch et al., 1990; Holmes et al., 1990) will be important in eventually understanding the molecular basis for actin disorder and dynamics.

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