FINE STRUCTURE IN NEAR-FIELD AND FAR-FIELD LASER DIFFRACTION PATTERNS FROM SKELETAL MUSCLE FIBERS

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ABSTRACT Regions of muscle fibers that are many sarcomeres in length and uniform with regard to striation spacing, curvature, and tilt have been observed by light microscopy. We have investigated the possibility that these sarcomere domains can explain the fine structure in optical diffraction patterns of skeletal muscle fibers. We studied near-field and far-field diffraction patterns with respect to fiber translation and to masking of the laser beam. The position of diffracted light in the near-field pattern depends on sarcomere length and position of the diffracting regions within the laser beam. When a muscle fiber was translated longitudinally through a fixed laser beam, the fine structural lines in the near-field diffraction pattern moved in the same direction and by the same amount as the fiber movement. Translation of the muscle fiber did not result in fine structure movement in the far-field pattern. As the laser beam was incrementally masked from one side, some fine structural lines in both the near-field and far-field diffraction patterns changed in intensity while others remained the same. Eventually, all the fine structural lines broadened and decreased in intensity. Often a fine structural line increased in intensity or a dark area in the diffraction pattern became brighter as the laser beam was restricted. From these results we conclude that the fine structure in the laser diffraction pattern is due to localized and relatively uniform regions of sarcomeres (domains) and to cross interference among light rays scattered by different domains.

INTRODUCTION

In studies of muscle contraction, the method of optical diffraction has become a common technique for monitoring striation spacing (Ranvier, 1874; Sandow, 1936; Cleworth and Edman, 1972; for review see McCarter, 1981). The one dimensional grating equation, $d = k\lambda/\sin\theta_k$, is used to calculate striation spacing (d) from the angle (θ_k) of light in the diffraction order (k), and the wavelength of the incident light (λ). However, several authors have noted departures in the shape, position, and symmetry of the optical diffraction spectra from those expected of an ideal diffraction grating (Cleworth and Edman, 1972; Rüdel and Zite-Ferenczy, 1979; Baskin et al., 1981; Goldman and Simmons, 1984; Leung, 1984). The diffraction pattern of muscle illuminated with laser light shows asymmetries between left and right orders as well as a complex set of bright and dark patches termed "fine structure" within each diffraction order (Fig. 1).

Since the diffraction pattern is related to the muscle structure, explanations for these anomalies have been proposed in terms of corresponding structural features within the muscle fiber. Striations oriented more or less in the direction of the critical angle for volume diffraction (Bragg angle) may intensify diffraction by some groups of myofibrils (Rüdel and Zite-Ferenczy, 1979; Baskin et al., 1981). Striation inclination around an axis parallel to the laser beam should result in off-meridional diffraction intensities. In addition, subpopulations of sarcomeres of differing lengths have been implicated as contributing to fine structure in the diffracted orders (Judy et al., 1982; Leung, 1982, 1983).

Regions of skeletal muscle fibers that are many sarcomeres in length, many myofibrils wide, and are relatively uniform in striation spacing and inclination, have been observed by light and electron microscopy (Tiegs, 1934, 1954; Peachey and Eisenberg, 1978; Rüdel and Thaer, 1981). These domains are separated by dislocations and other irregularities in the sarcomere pattern (Fig. 2) and the presence of many such domains in the illuminated region of a fiber may form the basis of the observed diffraction fine structure. In the present experiments we have investigated the correlation between fine structure in the diffraction patterns and localized diffracting regions in the muscle fibers.

We have studied far-field and near-field diffraction patterns from portions of single muscle fibers. Far-field diffraction, often referred to as Fraunhofer diffraction, occurs whenever the pattern is observed in a plane that is

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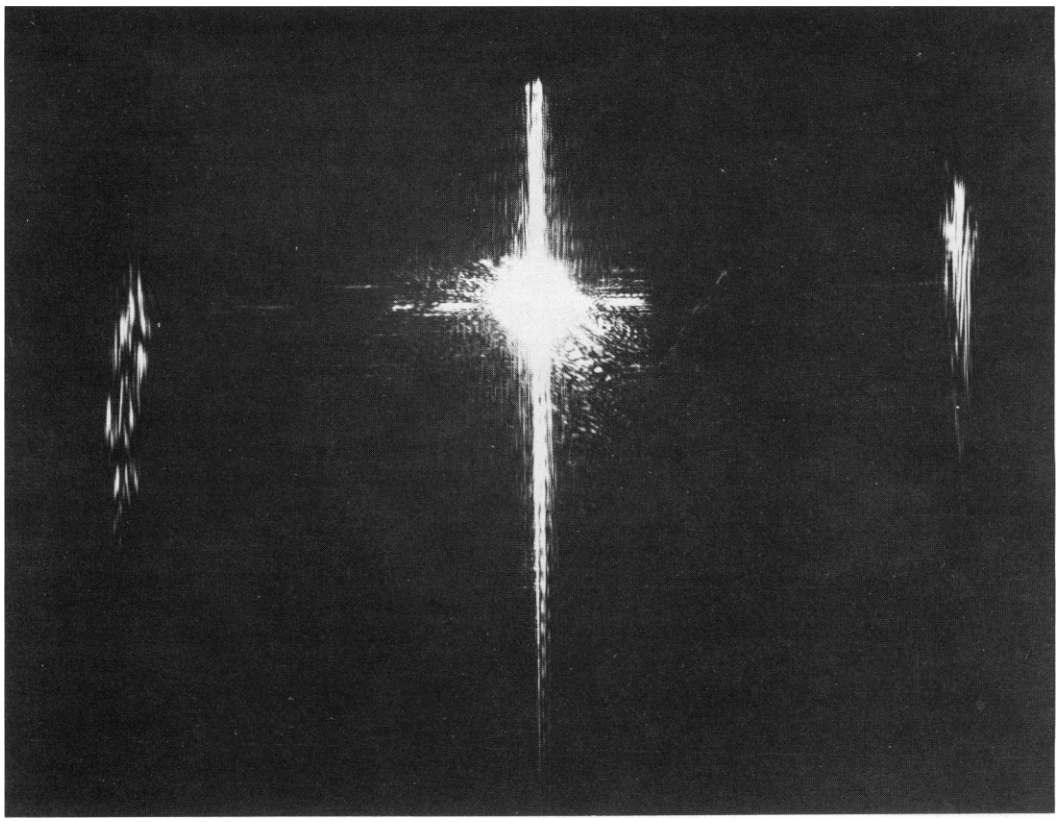

FIGURE 1 The HeNe laser diffraction pattern of a single glycerinated rabbit psoas fiber. The transmitted beam is in the center and the bright areas near the left and right sides are the first order diffracted beams. The intensities of the left and right diffracted orders are often not equal. Each diffracted order contains a number of fine discrete lines termed "fine structure," which are separated by dark areas.

conjugate to the light source (Hecht and Zajac, 1974; Born and Wolf, 1980). For the practical case of a muscle fiber illuminated with an approximate plane wave from a laser, the far-field pattern is observed at large distances from the fiber or near the focal plane of a lens that projects the diffraction pattern. The intensity distribution in a Fraunhofer diffraction pattern depends purely on the angle of the scattered light, and thus is independent of translation of the object through the illuminating beam (Cowley, 1981, p. 36).

When the diffraction pattern is observed close to the scattering object and no lens is used, near-field or Fresnel diffraction pertains. In this case the position of a diffracting array within the illuminated area, as well as its spacing and orientation, affect the distribution of intensities in the diffraction pattern.

The position sensitivity of near-field diffraction patterns allows us to separate position effects from other parameters, such as striation spacing and inclination that influence diffraction intensities, and thus to evaluate the contributions of localized and distributed groups of sarcomeres to fine structure in diffraction patterns. Our results support the hypothesis that structural domains, defined as localized regions of relatively uniform sarcomere length and striation orientation, are the primary basis for the observed diffraction fine structure, while cross interference of light scattered from multiple domains contributes to details of the diffraction pattern. Some of our results have been reported briefly to the Biophysical Society (Sundell et al., 1984, 1985).

METHODS

Rabbit psoas muscle fibers were chemically skinned according to the method of Eastwood et al. (1979), which was modified as indicated by Goldman et al. (1984). After extraction, muscle fiber bundles were kept at −22°C for up to three months in a storage solution containing in mM: ATP-2.5, $MgCl_2$-2.5, EGTA-5, Imidazole-10, K propionate-170, NaN_3-5, pH 7.0 and glycerol 50% (vol/vol). Electron microscopy of the fibers showed that the structural integrity and alignment of the sarcomeres were well maintained (Fig. 3). For light microscopy, muscle fiber bundles were fixed in gluteraldehyde and embedded in plastic. 2 μm thick sections were observed with a single-sideband edge enhancement microscope (Ellis, 1978, 1981).

For optical diffraction studies, segments of single muscle fibers, ~10 mm in length, were dissected in silicone oil. Clear segments, free of damaged opaque areas, were mounted horizontally in a glass chamber by means of steel hooks and aluminum foil T-clips (Goldman and Simmons, 1984). Fibers were not usually circular in cross-section and were mounted with the smallest diameter parallel to the laser beam axis at approximately slack length, corresponding to sarcomere lengths of 2.3–2.7 μm. The muscle fibers were immersed in a relaxing solution with the following composition in mM: TES-200, $MgCl_2$-2.4, EGTA-40, glutathione-1, Na_2ATP-5, pH 7.1 at 20°C and ionic strength 200 mM.

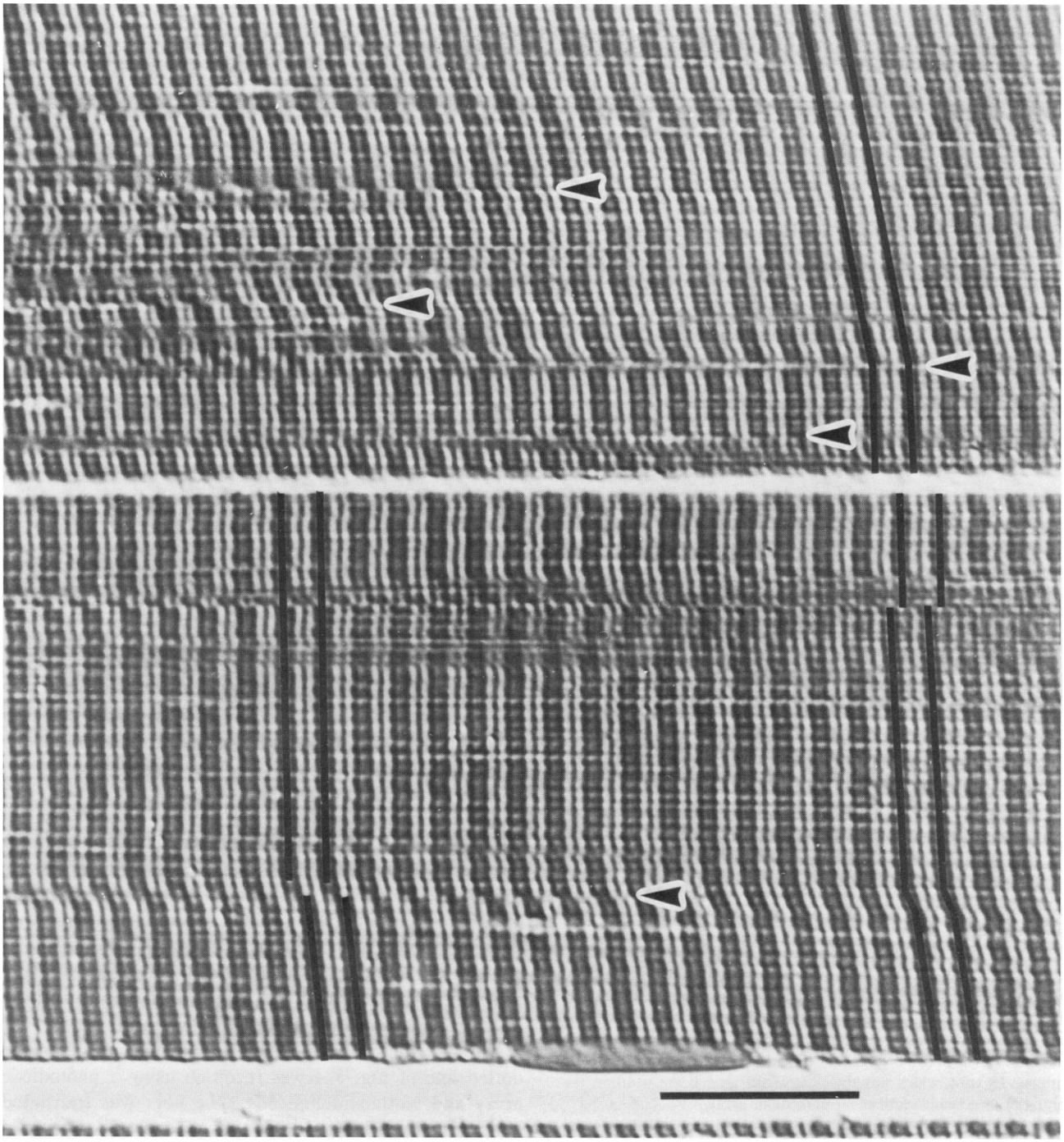

FIGURE 2 A single-sideband edge enhancement micrograph of a bundle of glycerol-extracted rabbit psoas fibers. Arrowheads delineate dislocations in the striation pattern that form the boundaries between domains of different sarcomere length. Solid lines are drawn along striations to emphasize regions that have different inclination angles with respect to the fiber axis. Relatively large areas of the striation pattern that are highly regular but differ significantly from other regions are separated by dislocations. It is the three-dimensional equivalents of these regularly arranged areas that we refer to as domains. The calibration bar corresponds to 16 μm.

The glass chamber containing the muscle fiber was mounted on the stage of a compound microscope. A HeNe laser (5 mW, Melles Griot Irvine, CA) was aligned horizontally and perpendicular to the long axis of the muscle fiber (Fig. 4). The intensity profile of the laser beam was Gaussian, with a width at half maximum intensity of 1.14 mm at the position of the fiber. The left first order diffraction line 50 cm from the fiber was recorded photographically on holographic film (SO253; Eastman Kodak Co., Rochester, NY) or with a photodiode array. This detector was a 1024 element scanning photodiode array 1.6 cm in length (model CCPD-1024; EG&G Reticon, Sunnyvale, CA) and oriented parallel to the muscle fiber axis. The output of the photodiode array was displayed on a digital oscilloscope (model 4094A; Nicolet Instrument Corp., Madison, WI) interfaced with a Z-80 based microcomputer. Intensity scans were recorded and stored on floppy diskettes and

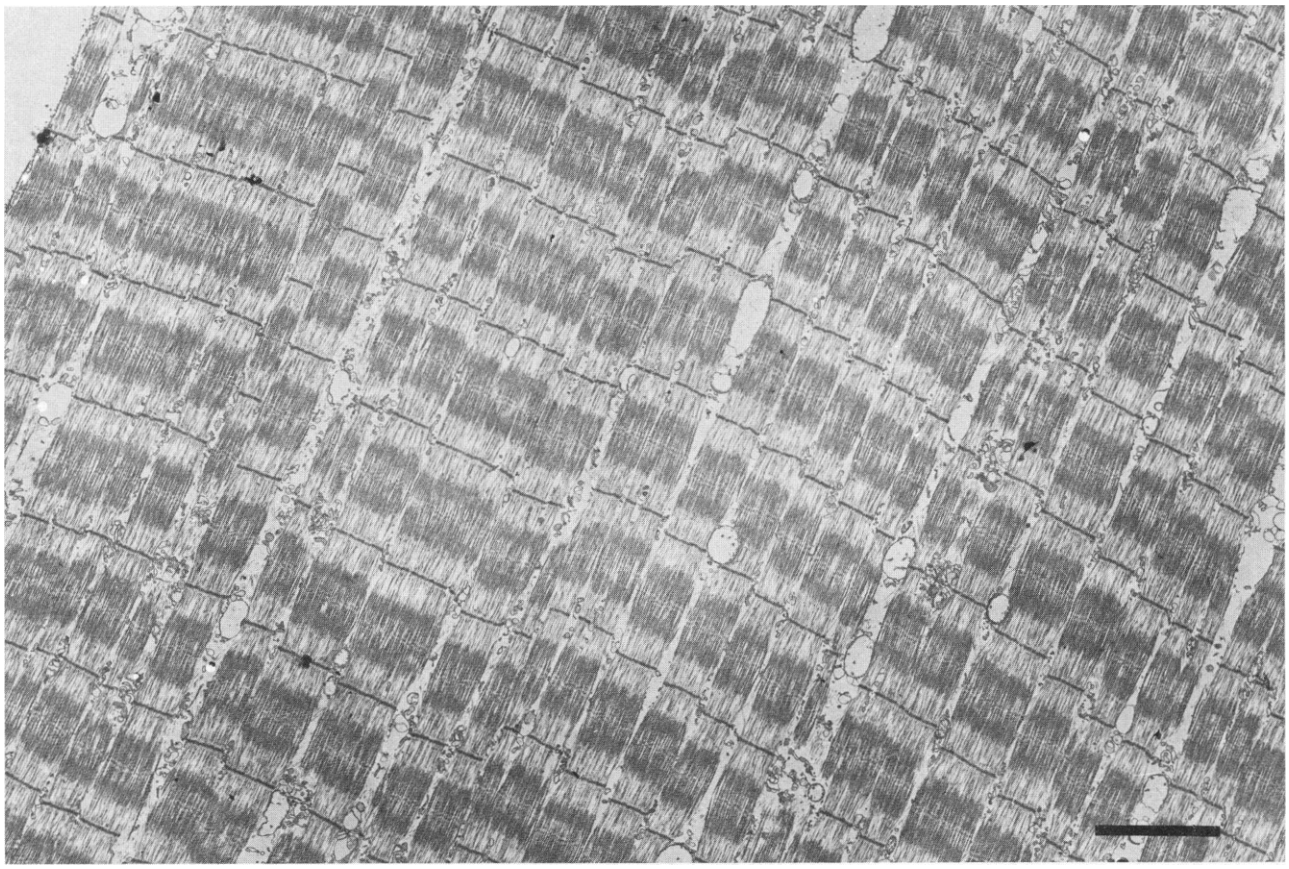

FIGURE 3 Electron micrograph of a bundle of glycerol-extracted rabbit psoas fibers from the same batch of fibers used for the optical experiments. The micrograph shows that the integrity and alignment of the sarcomeres was well maintained during skinning and storage. The calibration bar represents 2 μm.

displayed on a video screen or a digital plotter (model 7475A; Hewlett Packard, Palo Alto, CA). A lens with a 25 cm focal length could be placed between the fiber and the recording device with the optic axis of the lens parallel to the incident laser beam to approximate the far-field diffraction condition.

Successive diffraction patterns were recorded as the fiber was translated longitudinally in 100 μm increments using the microscope stage movement. Data from the near-field and far-field translation experiments were analyzed for the position of well-defined fine structure peaks that could be followed through several 100 μm fiber translation steps. To correct for laser beam divergence, the near-field position data were multiplied by 0.74, which was the ratio of the laser beam width at the fiber to the laser beam width at the photodiode array.

In a second type of experiment, the laser beam was masked close to and in front of the fiber by moving a razor blade edge (Fig. 4) across the beam in successive steps of 50 μm. Traces of the diffraction pattern intensity distributions corresponding to incremental translations of the muscle fiber or incremental masking of the laser beam are presented in the figures with individual traces offset vertically for clarity.

RESULTS

Translation Experiments

The first order line of a near-field diffraction pattern recorded on photographic film is shown in Fig. 5 *A*. As observed by other investigators (Cleworth and Edman, 1972; Leung, 1984), the diffraction pattern consisted of a number of intense fine lines separated by dark areas. As the muscle fiber was translated horizontally through the fixed laser beam, individual fine structural lines appeared in the first order, and moved parallel to the meridian and in the same direction as the fiber for variable distances before disappearing. Returning the fiber to its initial position restored the original pattern of fine structural lines.

The distribution of diffracted light intensity along the dotted line of Fig. 5 *A* was recorded using a photodiode array and plotted in Fig. 6 *A*. The fiber was translated longitudinally in 100 μm steps between successive recorded traces. Peaks in the intensity distribution, which correspond to fine structural lines in the diffraction pattern, shift horizontally from one trace to the next, indicating that the fine structure moved parallel to the meridian as the fiber was translated. As the muscle fiber moved through the laser beam, peaks in the distribution of diffracted light became narrower and increased in intensity to a maximum, and then decreased in intensity and became broader. This is most apparent in the peak marked *A* in Fig. 6 *A*. The fine structure in the diffraction pattern always moved in the same direction as the fiber. Individual fine structural lines remained visible in the diffraction

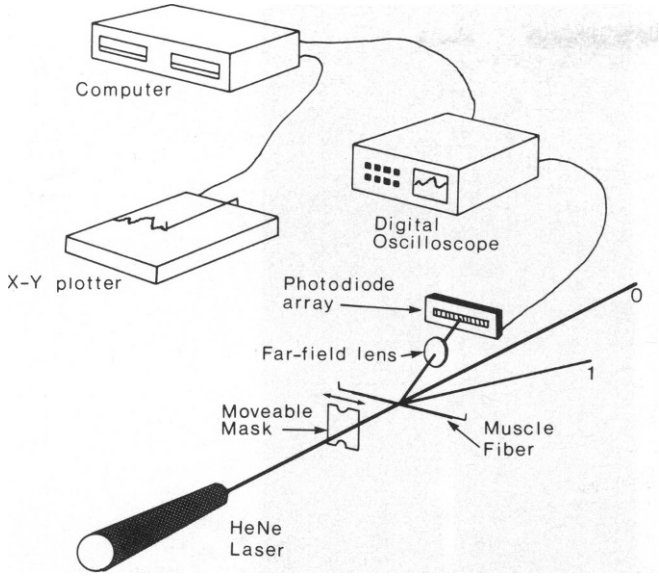

FIGURE 4 The optical and recording set-up. A HeNe laser was aligned perpendicularly to the long axis of the fiber. The left diffraction order was recorded 50 cm from the fiber photographically or with a photodiode array. A 25 cm lens could be placed between the muscle fiber and the photodiode array to approximate the far-field diffraction pattern. A razor blade edge placed close to and in front of the muscle fiber was used to mask the laser beam. The double headed arrow indicates movement of the muscle fiber during the translation experiment, or movement of the razor blade during the masking experiment.

pattern for 0.6–1.4 mm of fiber translation (movement between half-maximum intensity positions).

Fig. 5 *B* shows the far-field diffraction pattern from the same region of the fiber illustrated in Figs. 5 *A* and 6 *A*. The near-field and far-field first orders have a similar overall pattern of fine structural lines, although the individual intensity maxima were focused by the far-field lens into a smaller region at the recording plane. The muscle fiber was again translated through the laser beam in 100 μm steps, and a section of the far-field diffraction pattern was recorded with the photodiode array (Fig. 6 *B*). During translation the fine structural lines in the far-field pattern changed intensity, but moved very little.

A summary of fine structure movement vs. fiber translation for several experiments is presented in Fig. 7. These data are plotted as the means plus or minus standard error of the mean for 10 near-field (solid line) and 8 far-field (dotted line) experiments. Data from the near-field translation experiments were corrected for laser beam divergence as described in Methods. A line was fitted by least squares regression to the data from each near-field and each far-field experiment and lines corresponding to the mean slopes and intercepts are plotted in Fig. 7. The near-field data fit a straight line with a slope 0.98 ± 0.02 (SEM, n = 10) and intercept −2.8 ± 9.0 micrometers (SEM, n = 10). This slope does not differ significantly from 1.0 and the intercept is not significantly different from 0.0 (t-test). These results indicate that longitudinal movement of the fiber results in an equal movement of the near-field fine structure.

Movement of fine structural lines in the diffraction pattern was drastically reduced when the far-field condition was approximated.[1] The fine structure movement data in the far field (dotted line in Fig. 7) fall on a line with a slope (−0.04 ± 0.04 SEM, n = 8) and intercept (−1.1 ± 4.0 μm SEM, n = 8) not significantly different from zero (t-test), indicating that in the far-field diffraction pattern, the fine structure does not move appreciably when the fiber is translated.

Masking Experiments

The influence of position of the diffracting regions within the illuminated area was studied further by masking part of the incident laser beam (Fig. 4). As the mask was moved incrementally across the laser beam from one side, the intensities of some fine structural lines in both the near-field and far-field diffraction patterns changed while others remained the same. Eventually, all the fine structural lines decreased in intensity and broadened as the illuminated region of the fiber was restricted.

A section of the near-field diffraction pattern (dotted line in Fig. 8 *A*) was recorded with the photodiode array when the mask was moved in a series of 50 μm steps (Fig. 8 *B*). Peaks in the intensity distributions corresponding to individual fine structural lines had various responses to movement of the mask. The most common result was a decrease in intensity and broadening of individual fine structural lines. Often, the intensity of a peak increased as the illuminated region of the fiber was reduced (Fig. 8 *C*, traces *a–b*) or a trough in the intensity distribution disappeared to form a single peak from two peaks (asterisks in Fig. 8 *B* and Fig. 8 *D*, traces *c–e*). The amount of laser beam restriction required to change the intensity of an individual fine structural line was not correlated with the position of that line in the diffraction pattern. For example, the peak labeled *A* on the left side of the diffraction pattern in Fig. 8 *B* disappeared before the other peaks as the mask was moved across the laser beam from right to left.

[1]The degree to which the far-field condition was approximated was dependent on the position of the far-field lens (f = 25 cm) as well as by other factors, such as laser beam divergence and possible bowing or obliquity of the walls of the chamber. Because of these uncertainties, it was difficult to locate the lens to provide a precise approximation of the far-field condition. In four experiments, the photodiode array was at the focal point of the lens, i.e., the lens was 25 cm from the array. In three other experiments, the lens was 28 cm from the array, to allow for the laser beam divergence. In three of the former, the small movement of diffraction peaks observed was in the same direction as the fiber movement, and in all the others, it was in the opposite direction, suggesting that the lens sometimes was too close to the array and sometimes too far from it.

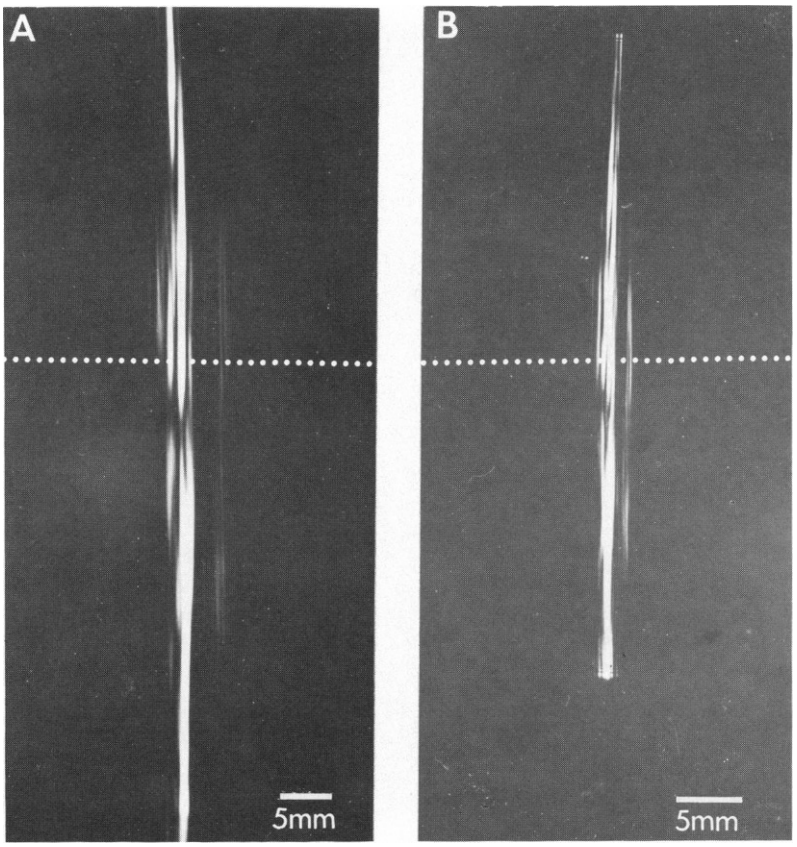

FIGURE 5 The near-field (*A*) and far-field (*B*) first order diffraction lines from a glycerol-extracted single fiber. The fiber was not moved when the far-field lens was put in place. The dotted lines represent the regions recorded by the photodiode array during the translation experiments shown in Figs. 6 *A* and 6 *B* respectively.

DISCUSSION

The movement of fine structural lines in the near-field diffraction pattern by the same amount and in the same direction as axial fiber translation (Figs. 6 *A* and 7) strongly suggests that the diffracting regions responsible for fine structural lines are longitudinally localized domains of striations rather than populations of sarcomeres with similar length spread along the length of the fiber. As the fiber translates, a domain of sarcomeres enters and traverses the laser beam. As more of the domain enters the laser beam, its fine structural line becomes narrower and more intense until the domain completely fills or lies completely within the illuminated region. As the domain starts to leave, the fine structural line decreases in intensity and broadens. These results are consistent with diffraction theory and the domain hypothesis.

An alternative explanation of the results on the basis of variable striation spacings within the fiber should be considered, since the position of scattered light in a near-field diffraction pattern depends on striation spacing as well as position of the diffracting structures. If the striations in some areas had smooth gradients of sarcomere length, then the diffraction pattern would also move with fiber translation. In that case the fine structural lines should move in either direction, depending on the direction of the sarcomere length gradient. However, the fine structural lines always moved in the direction of fiber movement. Additionally, gradients of sarcomere length along the fiber would be expected to produce movement of fine structure in the far-field diffraction pattern, which was not observed. Therefore, movement of fine structural lines is best interpreted in terms of localized well-ordered domains of constant sarcomere length.

A correspondence between regions of the muscle fiber and individual fine structural lines was further demonstrated by restricting the laser beam with a movable mask. When the laser beam was masked from one side, fine structural lines that arose from regions in the shadow of the mask dropped out of the diffraction pattern. If the fine structural lines arose from delocalized regions, masking would be expected to result in a simultaneous diminution and broadening of all the diffracted intensity peaks, which was not observed.

Often, a fine structural line increased in intensity or a dark area became brighter as the laser beam was masked. This is consistent with the mask eliminating scattered light from a region of the fiber that was destructively interfering with the observed peak. Removal of the destructive inter-

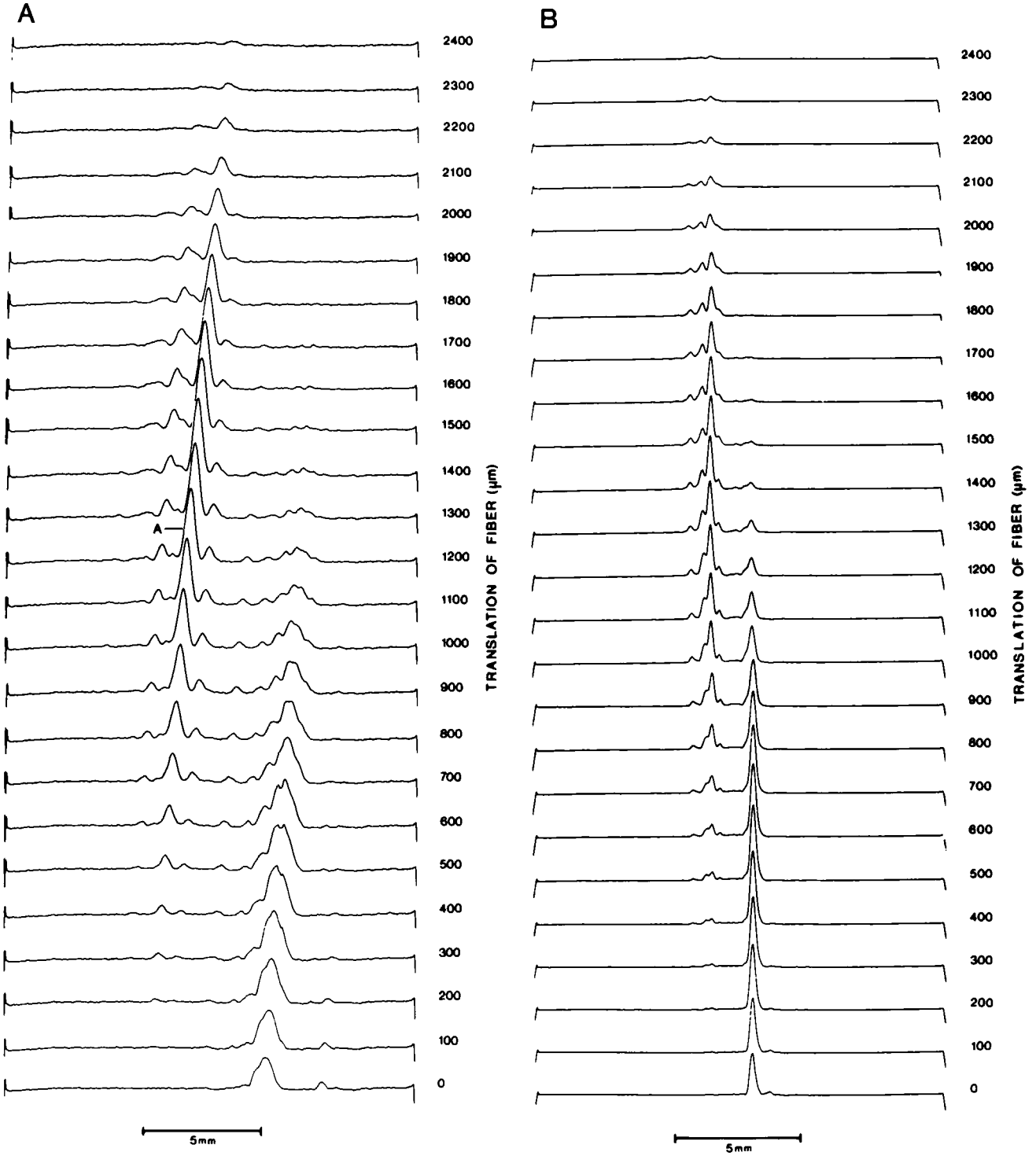

FIGURE 6 Light intensity profiles of the near-field (panel *A*) and far-field (*B*) first order diffraction lines recorded with a photodiode array and corresponding to the regions indicated by the dotted lines in Figs. 5 *A* and 5 *B*. In the traces of Fig. 6 *B*, the gain of the oscilloscope has been decreased by a factor of 2.5 with respect to *A*. Each trace represents the intensity distribution after a 100 μm translation of the muscle fiber. The length scale indicates 5 mm at the detector. Peaks in the intensity profiles correspond to individual fine structural lines. As the muscle fiber is moved horizontally through the laser beam, the fine structure in the near-field pattern (*A*) moves in the same direction. Very little movement of the fine structure in the far-field pattern (*B*) is observed.

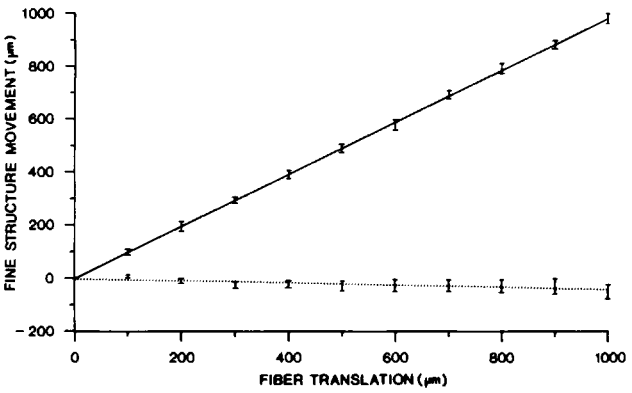

FIGURE 7 Graph of fine structure movement vs. fiber translation for 10 separate near-field (solid line) and eight far-field (dotted line) experiments. The brackets correspond to ±SEM from all near-field and far-field experiments at each translation increment. The average lines are plotted as described in the text. The near-field fine structure moves at the same rate as the fiber, whereas the far-field fine structure does not move significantly.

ference then resulted in an increased intensity in the diffraction pattern. These results suggest that there is not a one-to-one correspondence between the domains in the muscle fiber and fine structural lines in the diffraction pattern. Cross interference among several domains was less likely when the width of the laser beam was decreased.

As the mask was moved in from one side of the laser beam, the first fine structural lines to be affected were on either side of the diffraction order in different experiments. This suggests that in the near-field experiments, the location of a fine structural line within the diffraction pattern was controlled largely by sarcomere length and less by the position of the corresponding domain in the fiber. This conclusion is supported by the observation that similar fine structural features were often observed in near-field and far-field diffraction patterns (e.g. Figs. 5 *A* and 5 *B*).

Results of translation and masking experiments provide limited information concerning the dimensions of the relatively uniform domains. If regions responsible for intense, fine structural lines were considerably longer than the laser beam width, individual fine structural lines would remain in the diffraction pattern for correspondingly long fiber translations. While such a long domain completely filled the illuminated area, fiber translation would not cause movement of the fine structural line. These effects were not observed. If a domain was shorter than 50 μm, its corresponding fine structural line would be eliminated within one 50 μm increment of the mask, which was also not observed. Thus the results suggest that the regions of fibers responsible for the fine structural lines extend several hundreds of micrometers along the fiber. From microscopic observations and from the number of diffraction lines observed, it seems likely that domains exist side-by-side in the fiber, and therefore have widths less than the fiber diameter. Further details of the domain length and width distributions cannot be obtained from the present data because effects due to destructive interference of light scattered from different domains, and shape characteristics of the laser beam and domains such as taper, variable inclination, and fiber depth would strongly influence the intensity pattern.

Fine structure (microstructure) in the laser diffraction pattern from single muscle fibers was first described by Cleworth and Edman (1972), who suggested that the fine structural lines may correspond to localized regions in the fiber. They found that during fiber shortening associated with the onset of tetanic contraction, the fine structural lines moved in parallel away from the zeroth order. During relaxation the fine structural lines moved either toward or away from the zeroth order, resulting in an overall widening of the first order diffraction line. These results indicate that the domains responsible for the fine structural lines were shortening simultaneously and uniformly during the onset of contraction, whereas, during relaxation some domains shortened further while others elongated. The pattern of fine structure lines after relaxation remained constant after repeated contraction-relaxation cycles of the same fiber, suggesting that the domains that form the basis of the fine structure are relatively stable features of the muscle fiber.

Judy et al. (1982) and Leung (1982) have calculated theoretical far-field diffraction patterns based on various assumed spatial arrangements of the sarcomeres. To obtain fine structure, both calculations required that populations of striations relatively uniform in length be arranged contiguously along the myofibrils. Conversely, no fine structure was obtained if groups of relatively uniform striations were dispersed randomly within the diffracting region (Judy et al., 1982). Thus these calculations are consistent with our experimental results.

What characteristics determine the regions that contribute substantially to intense fine structural lines? Large, well-ordered domains are likely to make more significant contributions than small disordered domains. However, a muscle fiber is considerably thicker than the optical wavelength, so volume effects, such as tilt angle and arrangement of neighboring regions, will have important influence. Rüdel and Zite-Ferenczy (1979) explained changes in the intensity of the diffraction orders when fibers were rotated in the laser beam by alignment of striation tilt angles with the critical angle for volume diffraction (Bragg, 1933). With a particular incident angle, the Bragg-angle effect may dominate in the selection of which domains produce bright fine structural lines (Baskin et al., 1981).

During active and passive steady shortening, sarcomere length, calculated from the spacing of the diffraction pattern, often shows steps and pauses. These have been interpreted as indicating stepwise sarcomere shortening (Pollack et al., 1977; Lacktis et al., 1982). Recently, Goldman and Simmons (1984) found that such irregularities could be reduced by detecting the diffraction pattern

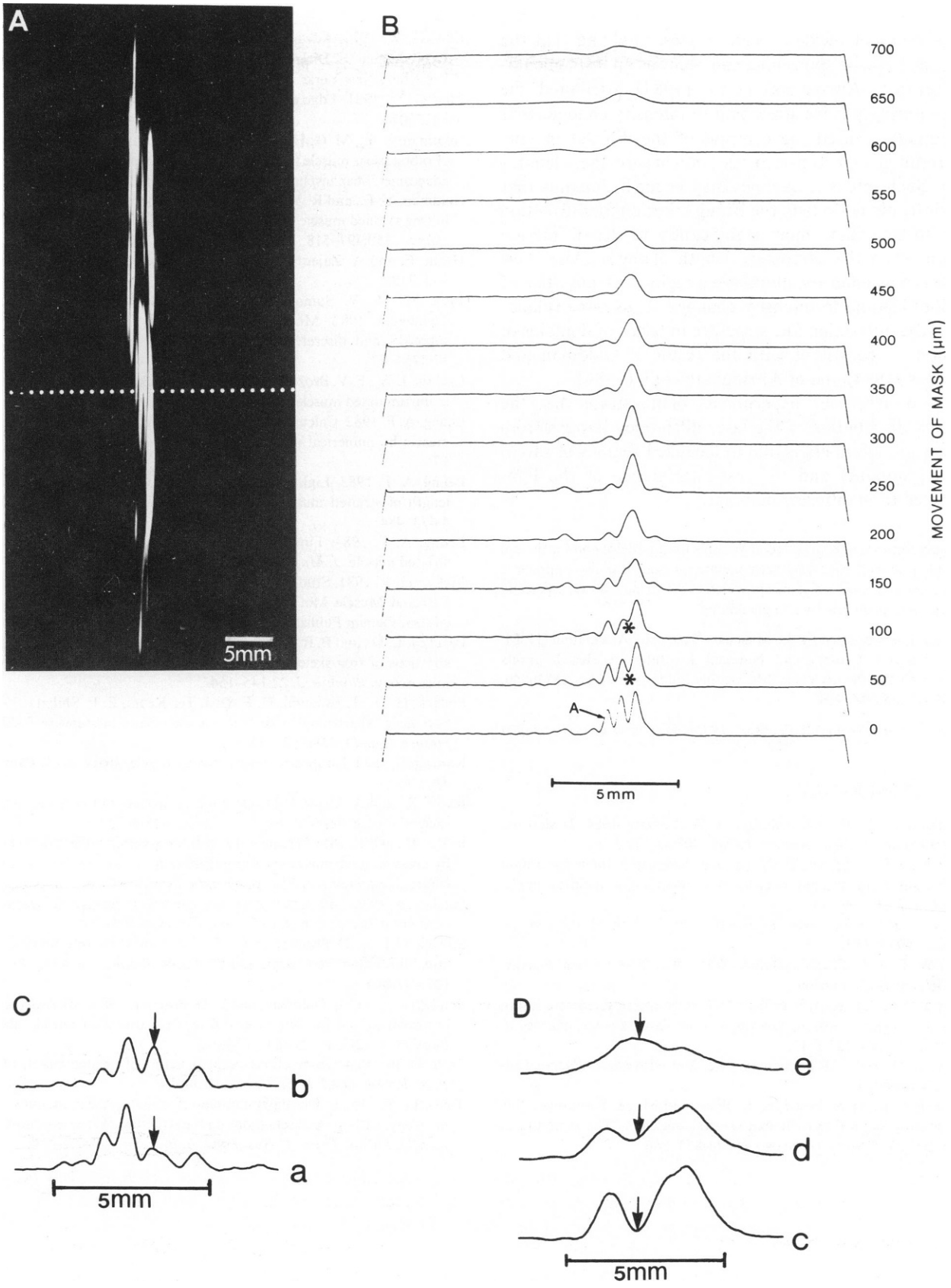

FIGURE 8 Masking experiment data. (*A*) near-field diffraction pattern recorded photographically. (*B*) Intensity profiles of the diffraction pattern corresponding to the region indicated by the dotted line in panel *A*. Successive traces show the intensity distribution as the laser beam was restricted from right to left in 50 μm increments. Panels *C* and *D* show examples from different fibers of various results obtained. The mask was moved 50 μm from right to left in *C*, traces *a–b*, and in 100 μm increments in *D*, traces *c–e*. The peaks in panel *D* are wide because the laser beam was nearly fully masked. The asterisks in *B* and the arrows in *C*, traces *a–b*, indicate positions at which the intensity of fine structure lines increased during masking. The arrows in *D*, traces *c–e* indicate disappearance of a trough as the laser beam was masked. These phenomena are typical cross-interference effects.

over a range of incident beam angles, implying that the apparent stepwise shortening can occur as an instrumentation artifact. Altringham et al. (1984) attributed the pauses during passive stretching to intensity changes that artifactually shifted the centroid of the diffraction line. This resulted in an apparent cessation of sarcomere lengthening. Such intensity changes may occur if domains that previously did not satisfy the Bragg criterion for diffraction begin to contribute more significantly to the diffraction pattern when the sarcomere length changes. Also, new domains may enter the illuminated region by translation of the fiber leading to intensity changes. Thus our explanation of the diffraction fine structure in terms of domains of striations is consistent with the result of Goldman and Simmons (1984) and of Altringham et al. (1984).

In summary, our experiments demonstrate that the intensity distribution of the laser diffraction pattern from skeletal muscle fibers is due to localized regions of sarcomeres (domains) and to cross-interference of the light diffracted from different domains.

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