Transformation by Rous sarcoma virus prevents acetylcholine receptor clustering on cultured chicken muscle fibers

(pp60^{src}/cytoskeleton/temperature-sensitive mutants/extracellular matrix)

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ABSTRACT   Acetylcholine receptors aggregate in the membrane of cultured chicken myotubes; the process of receptor clustering can be stimulated by exogenous factors that we, among others, have begun to characterize. Chicken myoblasts transformed by temperature-sensitive mutants of Rous sarcoma virus, such as tsNY68, fuse to form multinucleated myotubes at 42°C, the nonpermissive temperature for transformation. However, tsNY68-infected myotubes do not cluster acetylcholine receptors at 42°C, even in the presence of active clustering agents. This phenomenon is not merely a result of viral infection, since myotubes infected with a transformation-deficient viral mutant, td107A, behave like noninfected myotubes with respect to receptor clustering; thus, the effects of tsNY68 on the clustering process must be mediated by the *src* gene product. These experiments may provide a method of identifying essential elements of acetylcholine receptor clusters.

Motor innervation of embryonic skeletal muscle fibers causes uniformly distributed acetylcholine receptors (AcChoRs) to accumulate in high concentrations at synaptic sites (1, 2). This appears to result from secretion of a factor, distinct from acetylcholine, from the nerve terminals (3–5). Once an AcChoR cluster is formed, its maintenance as a stable unit, even in denervated muscle, seems to depend upon a component of the muscle fiber's basal lamina (6, 7). The postsynaptic region of muscle is also characterized by other structural features, such as secondary membrane folds and soleplate nuclei (8). The external AcChoR-localizing molecules have not yet been characterized in detail, and very little is known about other components of the muscle cell, such as elements of its cytoskeleton, which function in the generation of the postsynaptic specialization. Experimental methods for identifying these components would be very valuable.

Muscle fibers grown in tissue culture have been used as a convenient system to study events associated with the synthesis and localization of synaptic macromolecules (reviewed in ref. 9). These cells are able to synthesize AcChoRs and to aggregate them into high-density clusters. They also respond to a variety of exogenous factors by producing increases in AcChoR number, AcChoR cluster number, or both (3–5, 10, 11). It has been thought that studying the process of AcChoR aggregation on cultured muscle fibers will assist in understanding the mechanism of AcChoR localization at the neuromuscular junction.

Rous sarcoma virus (RSV) is an avian retrovirus whose ability to produce cell transformation is due to the activity of its M_r 60,000 tyrosine-specific protein kinase, pp60^{src}. Temperature-sensitive (ts) mutants of RSV have been used to study events occurring during cellular differentiation (12–

15). Cultured avian myoblasts transformed by ts mutants of RSV remain as replicating, mononucleated cells at the permissive temperature for viral transformation and express biochemical markers associated with the transformed state, such as plasminogen activator (16). When shifted to the nonpermissive temperature, they regain the capacity to fuse into myotubes and begin to synthesize molecules—such as myosin and creatine kinase—characteristic of normal embryonic myotubes (17, 18).

We report that myotubes formed by the fusion, at the nonpermissive temperature, of chicken myoblasts transformed by a temperature-sensitive mutant, tsNY68, of RSV (19) are unable to cluster AcChoRs, in distinct contrast to nontransformed myotubes maintained at the same temperature. Our results suggest that the *src* gene product is involved in the observed defect in AcChoR clustering and should eventually be important in determining the molecular basis of this process.

MATERIALS AND METHODS

Cell Culture. The hind-limb muscles of 11- to 12-day chicken embryos were enzymatically dissociated and preplated on plastic dishes to enrich for myoblasts (20). Cells were then plated on collagen-coated plastic dishes in a medium consisting of Eagle's minimal essential medium with 10% horse serum and 5% chicken embryo extract and infected at 37°C several hours later with 1–2 focus-forming units of virus per cell with the addition of DEAE-dextran to aid in the entry of virus (21). Transformation was assessed by the appearance, after 3–4 days, of rounded, refractile cells with diminished contact inhibition of growth; some myotubes were present before subsequent passage of the transformed cells. Transformed myoblasts were shifted to 42°C; substantial fusion occurred within 24–48 hr. Similar prefusion cultures were infected at 37°C with td107A, a transformation-deficient RSV mutant that lacks the *src* gene, and shifted to 42°C along with the transformed cultures. Noninfected cells were shifted to 42°C 1 day after their initial dissociation.

Receptor Labeling and Autoradiography. To assess the distribution of AcChoRs, transformed or normal cells were labeled with 5 nM ^{125}I-labeled α-bungarotoxin (^{125}I-α-bungarotoxin) (Amersham) at least 48 hr after the shift to 42°C in the case of the transformed cells or 5–6 days after the initial dissociation in the case of noninfected cells or cells infected with td107A. At the end of a 90-min labeling procedure, carried out at 42°C, cells were fixed in 1% paraformaldehyde/1% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.4, dipped in Kodak NTB2 emulsion, and processed for autoradiography.

Immunofluorescence. Myotubes were incubated with tetramethylrhodamine-conjugated α-bungarotoxin [prepared by the method of Ravdin and Axelrod (22)] for 120 min to

Abbreviations: AcChoR, acetylcholine receptor; RSV, Rous sarcoma virus; ts, temperature sensitive; td, transformation deficient.

label AcChoRs and fixed in absolute methanol at $-20°C$ for 10 min. They were labeled with rabbit anti-RSV virion antibody (obtained from James Krueger of The Rockefeller University) for 30 min at room temperature and then with fluorescein isothiocyanate-conjugated goat antiserum to rabbit IgG (Miles) for 30 min at room temperature.

$pp60^{src}$ Kinase Assays. $pp60^{src}$ kinase activity was determined by utilizing the ability of $pp60^{src}$ to phosphorylate the heavy chain of anti-$pp60^{src}$ IgG according to the method of Krueger *et al.* (23). tsNY68-infected myoblasts were allowed to fuse at 42°C and were then treated with 10 μM cytosine arabinonucleoside to eliminate fibroblasts. Chicken embryo fibroblasts transformed by tsNY68 were shifted to 42°C for at least 48 hr before kinase activity was assayed. Cultures of myotubes and fibroblasts were maintained at 42°C or shifted to 37°C for 10 hr before lysis of the cells on ice in buffer B + 1% Triton X-100 (buffer B consists of 10 mM KCl, 20 mM Tris·HCl at pH 7.0, trasylol at 100 kallikrein inhibitor units/ml, 0.1% 2-mercaptoethanol, and 1 mM EDTA). Then 2.5–5 μl of tumor-bearing rabbit serum (obtained from Allan Goldberg of The Rockefeller University) was added to an aliquot of the cell lysate and incubated on melting ice for 1.5–2.5 hr before precipitation of the immune complexes with staphylococcal protein A-Sepharose (Pharmacia) and reaction with [γ-^{32}P]ATP (Amersham) followed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Bands corresponding to the heavy chain of IgG were excised from the dried gel and their radioactivities were measured in Liquiscint (Amersham). Protein contents of cell lysates were determined by the method of Lowry *et al.* (24).

Electrophysiology. The apparent mean channel open time (τ) of AcChoRs on cultured myotubes was assayed by spectral analysis of acetylcholine-induced current fluctuations (25, 26). Acetylcholine-induced currents were recorded with a focal extracellular electrode filled with 10–20 μM acetylcholine. Muscle cells were bathed in well-oxygenated L-15 tissue culture medium (GIBCO) and observed with a Zeiss UEM microscope equipped with Normarski optics. Other details are as described previously (27).

RESULTS

Properties of tsNY68-Infected Myotubes. Although tsNY68-infected myotubes were not able to cluster Ac-ChoRs at 42°C (see below), other cellular properties were not significantly altered at this temperature. Myotubes had resting potentials of 50–60 mV and were capable of contracting. Total acetylcholinesterase levels were not substantially changed, nor was the sedimentation profile in sucrose density gradients of the different forms of acetylcholinesterase changed. AcChoR levels were slightly lower, which is in agreement with the alterations in AcChoR metabolism measured by Miskin *et al.* (28). AcChoR channels were functional and showed kinetics characteristic of embryonic chicken AcChoRs (27) (Fig. 1).

AcChoR Distribution on Infected and Uninfected Myotubes at 42°C. To study the distribution of AcChoRs on normal and transformed cells, we labeled muscle cultures with ^{125}I-α-bungarotoxin and processed them for autoradiography. Normal muscle cells grown at 42°C have a few AcChoR clusters (ref. 29; Fig. 2A). These might result from interactions between muscle cells and serum or embryo extract factors or might be related to interactions at sites of contact between the ventral myotube surface and the culture dish (9). In contrast, myotubes formed from transformed myoblasts had a uniform distribution of AcChoRs; that is, there were no Ac-ChoR clusters (Fig. 2C). This apparent defect in AcChoR-clustering in tsNY68-RSV infected myotubes was studied further by treating the cells with either of two types of crude extracts previously shown to increase the number of Ac-ChoR clusters in normal cells. The first was a high-salt ex-
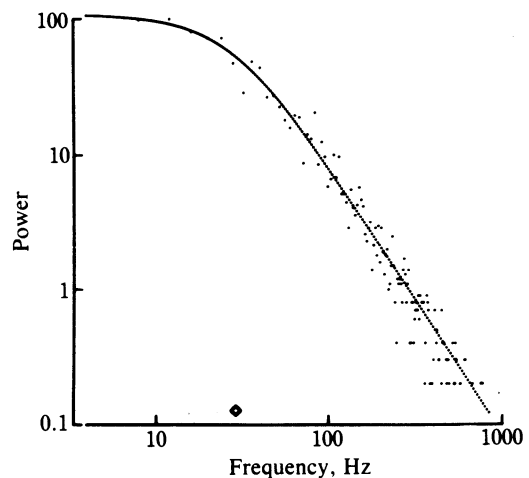

FIG. 1. Power spectrum of acetylcholine-induced current fluctuations recorded from a muscle cell infected with tsNY68. Power [$S(f)$] is expressed in relative units. The diamond indicates the half-power frequency. Temperature = 20°C. τ = 5.5 ms.

tract of *Torpedo californica* electric organ extracellular matrix containing a high molecular weight component that causes a rapid redistribution and aggregation of AcChoRs (refs. 10 and 11; Fig. 2B). The second was a low-salt, cell-free extract of embryonic chicken brain containing a small peptide factor (and possibly others) that also causes an increase in the number of AcChoR clusters (5). Neither treatment produced any AcChoR clusters in transformed myotubes at 42°C (Fig. 2D; data shown are for treatment with *Torpedo* extracellular matrix only).

AcChoR Distribution on Cells Infected with a Transformation-Deficient Mutant of RSV. These results suggested that some aspect of viral infection or transformation by a temperature-sensitive mutant markedly reduces the ability of muscle cells to cluster AcChoRs, even at 42°C. To confirm that this defect was related to the presence of the *src* gene product, we infected cells with a mutant of RSV (td107A) containing a deletion in *src* but having no alterations in the genes for viral structural proteins. We determined that cells were infected with td107A by immunofluorescence using an antiserum that recognizes viral structural proteins (Fig. 3A). Colabeling the cells with rhodamine-α-bungarotoxin clearly demonstrated that the infected myotubes had endogenous AcChoRs and increased their number of clusters in response to clustering stimuli (Fig. 3B). For comparison, Fig. 3 also shows normal uninfected cells that are not labeled by anti-virion antiserum (Fig. 3C) but do, of course, cluster Ac-ChoRs (Fig. 3D).

$pp60^{src}$ Kinase Activity in tsNY68-Infected Cells. Our experiments demonstrated that the failure of transformed cells to cluster AcChoRs was due to the presence of $pp60^{src}$. We measured $pp60^{src}$ kinase activity in transformed cells at 42°C and 37°C by the ability of cell lysates to phosphorylate the heavy chain of anti-$pp60^{src}$ IgG. When tsNY68-infected myotubes or chicken embryo fibroblasts were grown at 42°C and then shifted to 37°C for 10 hr, we found that the ratio of $pp60^{src}$ kinase activity in total cell lysates at 37°C was about 2 times that of the activity at 42°C for both cell types (Table 1). Uninfected cells had no measurable activity at either temperature.

DISCUSSION

Our experiments suggest that $pp60^{src}$ inhibits the activity of a molecule essential for the formation of AcChoR aggregates on cultured avian myotubes, even at 42°C when a temperature-sensitive mutant was used for transformation. It may
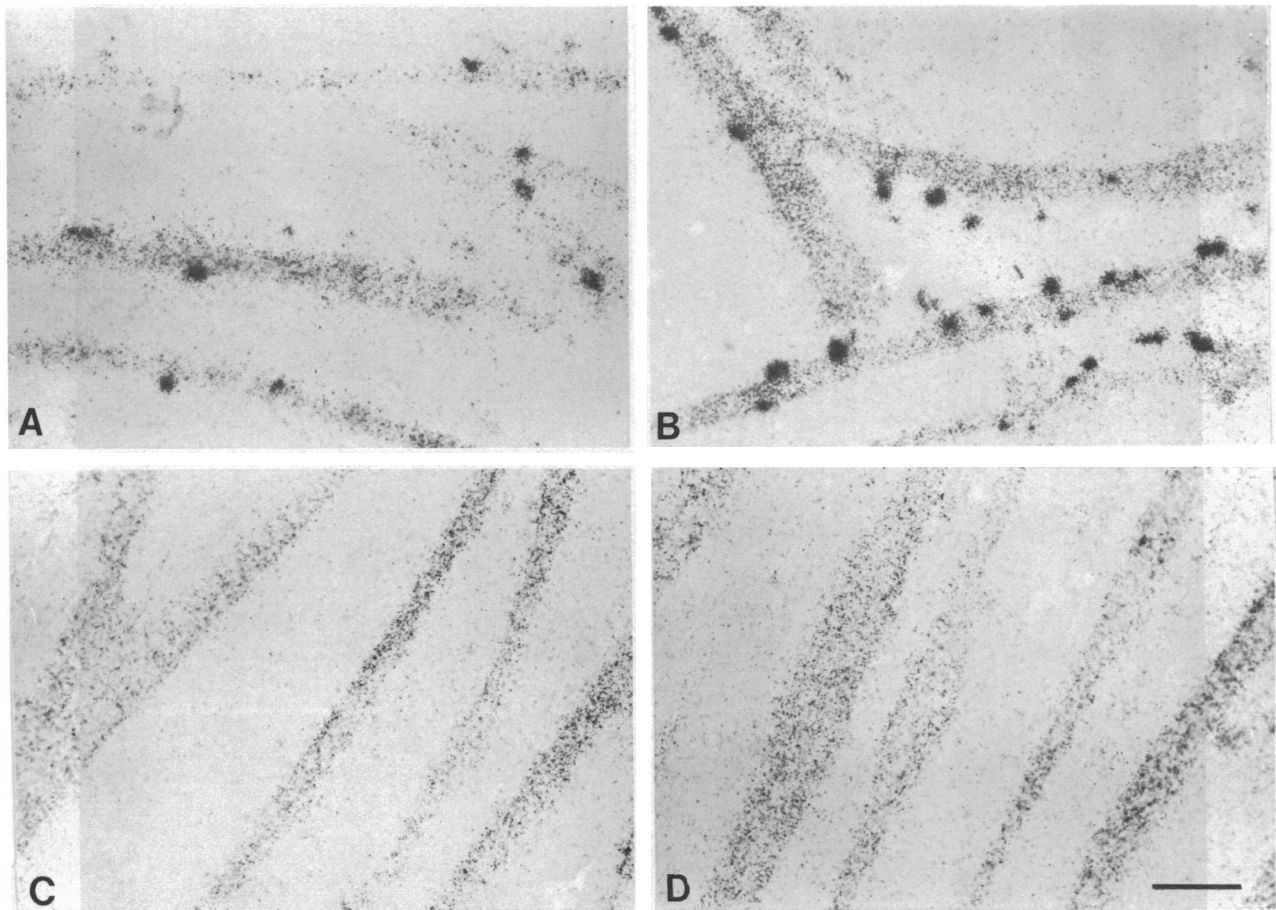
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FIG. 2.   Autoradiography of ^{125}I-α-bungarotoxin-labeled cells. (*A*) Uninfected myotubes grown at 42°C. These cells have a few AcChoR clusters. (*B*) Uninfected myotubes grown at 42°C and treated with *Torpedo* extracellular matrix clustering factor at 50 μg/ml. These cells now have numerous AcChoR clusters. (*C*) tsNY68-infected myotubes grown at 42°C. (*D*) tsNY68-infected myotubes treated as in *B*. There are no AcChoR clusters in either *C* or *D*. (Calibration bar = 50 μm.)

seem surprising that this deficit is observed at this temperature. One possibility is that clusters do form at 42°C, but then they are completely disrupted during brief temperature shifts while ^{125}I-α-bungarotoxin-labeled cells were rinsed prior to fixation; however, even after muscle cells were treated with metabolic inhibitors, clusters do not disperse for 4–6 hr (30). Also, cytoskeletal alterations, characteristic of RSV transformation, are observed in a majority of chicken embryo fibroblasts only 3 or more hours after temperature shift, with complete loss of cytoskeletal stress fibers only after 6–12 hrs (31). Our results, therefore, favor the explanation that AcChoRs are unable to form clusters at 42°C in cells transformed with tsNY68.

Much evidence indicates that pp60^{src} exerts its effects via its ability to phosphorylate tyrosine residues of one or more molecules (32, 33). Traditionally, it has been thought that pp60^{src} of RSV mutants temperature-sensitive for transformation is inactive at the nonpermissive temperature. Although several laboratories have demonstrated reduced pp60^{src} kinase activity at 42°C, the biological significance of the resid-

ual kinase activity has been disputed (34, 35). In addition, Miskin *et al.* (16) have reported that chicken myotubes infected with tsNY68 continue to produce higher levels of plasminogen activator at 42°C than do fibroblasts transformed by the same stock of virus, suggesting that some residual transforming function remained at 42°C in these myotubes. Our measurements of total cell pp60^{src} kinase activity for fibroblasts and myoblasts infected with tsNY68 do not reveal any marked differences between kinase levels in the two cell types. However, plasma membrane association of pp60^{src} is apparently crucial to its transforming activity in chicken embryo fibroblasts, and we have not determined the localization of pp60^{src} in tsNY68-infected myotubes at 42°C. Further, there are reports that the *src* gene product is still able to phosphorylate a putative 50-kilodalton protein substrate even at 42°C in chicken embryo fibroblasts infected with tsNY68, and the suggestion has been made that this molecule may be a high-affinity substrate (36).

Both extracellular and cytoskeletal alterations induced by viral transformation of fibroblasts have been documented (37, 38). For example, levels of extracellular fibronectin decrease with cellular transformation (39). It is possible, then, that transformed myotubes lack receptors for exogenous clustering stimuli. This seems unlikely since our transformed cells are unable to respond to a variety of presumably different stimuli (*Torpedo* extracellular matrix, chicken brain extracts, serum factors, cell–substrate interactions). A stronger possibility is that interactions of AcChoRs with myotube cytoskeletal or other cytoplasmic elements are affected by transformation. Dramatic rearrangement of the cytoskeleton

Table 1.   pp60^{src} kinase activity in tsNY68-infected cells

Infected cells	Kinase activity		
	cpm/μg of protein		Ratio,
	42°C	37°C for 10 hr	37°C/42°C
Fibroblasts	78	141	1.8
Myotubes	63	122	1.9

Activity is in terms of ^{32}P transferred to the heavy chain of IgG.
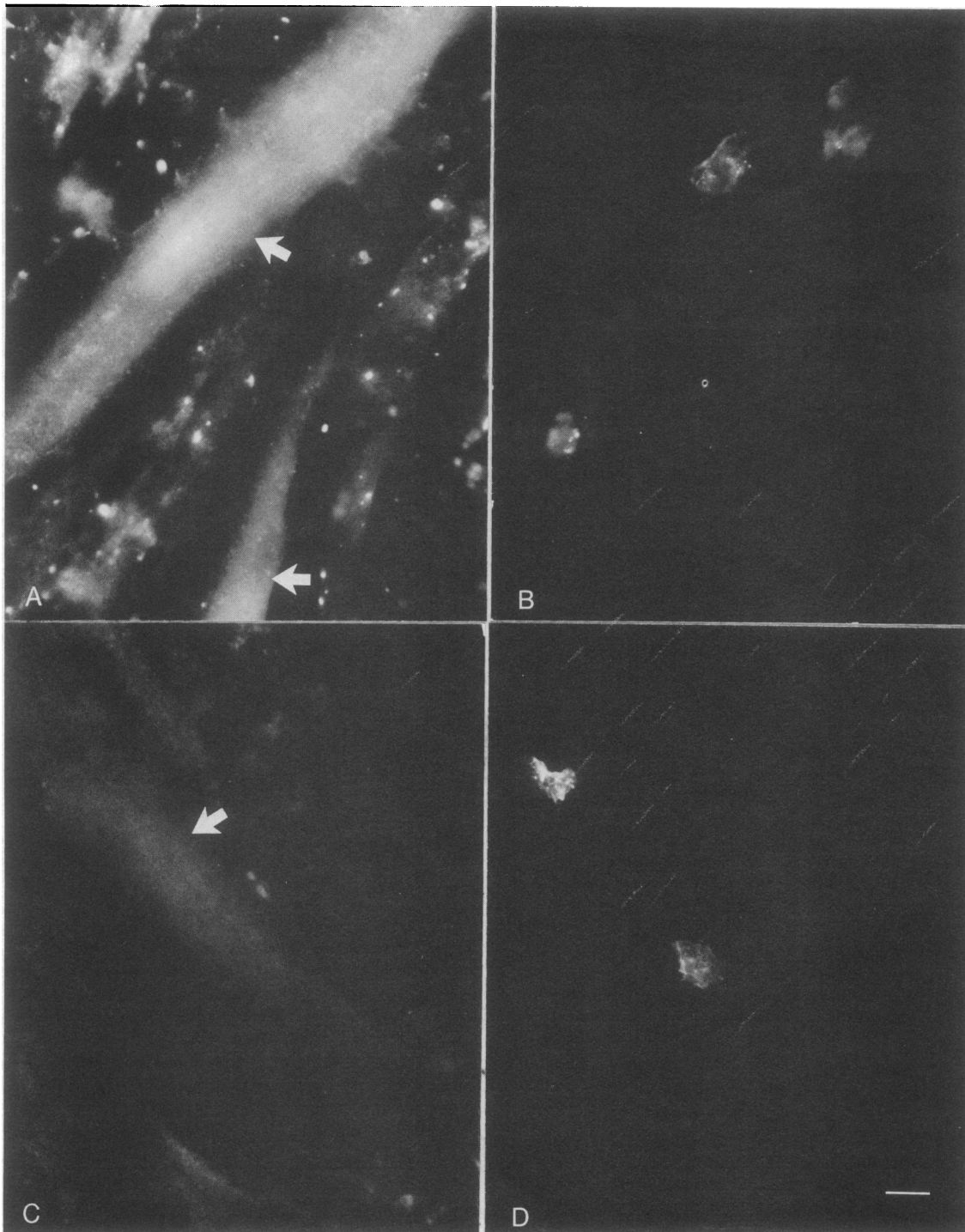
Fig. 3.   Immunofluorescence demonstration of AcChoR clusters on td107A-infected myotubes. Myotubes infected with td107A (*A* and *B*) and control cultures (*C* and *D*) were treated for 24 hr at 42°C with *Torpedo* clustering factor, incubated with tetramethylrhodamine-conjugated α-bungarotoxin (*B* and *D*), and fixed as described in *Materials and Methods*. They were then labeled with rabbit anti-RSV virion antibody and with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (*A* and *C*). Myotubes in *A* and *C* are indicated with arrows; fibroblast staining in *A* is bright and patchy. The infected myotubes that stain brightly with the anti-virion antibody also have AcChoR clusters; control cells do not stain with the anti-virion antibody, but, as usual, have AcChoR clusters. (Calibration bar = 10 μm.)

in chicken embryo fibroblasts is produced by transformation at 37°C but not at 42°C, so that the alterations in tsNY68-infected muscle cells at 42°C involved with the failure of these cells to cluster AcChoRs may be more discrete. This is reinforced by the observation (unpublished data) that even cytochalasin D or colchicine treatments of cultured chicken muscle fibers, which produce marked cell shape change, do not prevent AcChoRs from clustering.

Since we have found that transformed cells fail to produce any AcChoR clusters in response to diverse stimuli, even at 42°C, it seems likely that a high-affinity substrate for pp60^{src}, which becomes inactive when phosphorylated, plays some fundamental role in the process of AcChoR clustering. This observation may provide a molecular probe for an essential element of the AcChoR cluster complex. Exploring the actions of membrane-associated tyrosine-specific protein ki-

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nase in tsNY68-infected myotubes at the nonpermissive temperature should allow us to identify molecules involved in the localization of AcChoRs at the neuromuscular junction.

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