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CHARACTERIZATION OF A UNIQUE MUSCLE CELL LINE

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ABSTRACT

A clonal cell line derived from a mouse neoplasm is described which shares many properties with smooth muscle. The cells have electrically excitable membranes capable of generating overshooting action potentials, and they contract both spontaneously and with electrical stimulation. They respond to the iontophoretic application of acetylcholine with a depolarizing response, and to norepinephrine with a hyperpolarizing response. Electron microscopy reveals that the cells have a morphology similar in many, but not all, respects to that of smooth muscle cells in vivo. The cells secrete soluble collagen-like molecules in addition to several proteins of undefined function. Finally, there is an increase in the specific activities of creatine phosphokinase and myokinase associated with increased cell density and the cessation of cell division.

INTRODUCTION

It has previously been possible to maintain primary cultures of smooth muscle cells for a limited amount of time (1–4), but no permanent clonal cell lines have been described for this cell type. Such cell lines should facilitate the study of both the electrophysiological and biochemical properties of the smooth muscle cell, and its interaction with nerve cells. The following paragraphs describe a cell line derived from a mouse neoplasm which has many properties associated with smooth muscle.

METHODS

Cell Culture

The cell line to be described is designated BC_3H1, and was derived from a neoplasm induced with nitrosoethylurea (NEU) in the C_3H mouse strain. The induction procedure was similar to that described by Ivankovic and Druckrey for the transplacental induction of brain tumors in rats (5). 15-days pregnant mice were injected intraperitoneally with 0.8 mg of NEU per 10 g of body weight, a dose which reduced the average litter size about 50%. Of 36 offspring derived from treated mothers, two developed intracranial tumors. These neoplasms were removed and the cells adapted to cell culture in modified Eagle's medium (6) containing 20% fetal calf serum at 37°C in an atmosphere of 12% CO_2 and 88% air. Unless otherwise indicated, 60-mm Falcon plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.), were used. One tumor was of apparent glial origin and will not be discussed further. The other cell line, called BC_3H1, was initially heterogeneous with respect to cell morphology. During the early passages in culture, clusters of spontaneously contracting cells were seen on several distinct areas of the culture dishes. These were isolated with a glass cloning ring, dissociated with 0.25% Viokase (Grand Island Biological Co., Grand Island, N.Y.), and removed to other culture dishes. The cells were then allowed to become nearly confluent, dissociated to single cells with Viokase, and were cloned twice by isolating

single cells or colonies derived from single cells with cloning rings. Clone 9 was used exclusively in these studies. Several other clones were examined which had properties similar to those of clone 9.

Karyotyping was done according to Horibata and Harris (7). Growth curves were done by dissociating attached cells with 0.25% Viokase in modified Eagle's medium at 37°C for 30 min; samples of the resulting single cell suspension were diluted into 0.9% NaCl, and counted in a Coulter Counter standardized to visual cell counts (Coulter Electronics Inc., Fine Particle Group, Hialeah, Fla.).

Electrophysiology

For the electrophysiological studies, micropipettes for intracellular recording were filled with 3 M KCl and had resistances of 70–150 Mohm; those for iontophoresis were filled with either 2.7 M acetylcholine or 1 M L-norepinephrine and had resistances of 100–300 Mohm. In the case of norepinephrine-filled electrodes, care was taken to use electrodes that were less than 2 days old. The acetylcholine and norepinephrine were applied according to published procedures (8). To visualize the cells, a modified upright microscope (McBain Instruments, Chatsworth, Calif.) was used, with a ×40 water immersion phase-contrast objective. All electrophysiology was done in Eagle's modified medium plus 20% serum. The pH of the medium was maintained by blowing a stream of water-saturated CO_2 across its surface. The culture dish with attached cells was placed on the bottom of a chamber that was warmed by passing warm water through a surrounding jacket. The temperature of the fluid in the chamber varied from 35°C at the outside edge to 32°C in the center.

Isotopic Labeling

The radioactive isotope labeling of cells was carried out as described previously (9). To label with leucine for up to 8 h, the cells were washed twice *in situ* with 5 ml of serum-free modified Eagle's medium minus leucine. The plate was then flooded with 2.5 ml of the same medium containing 1×10^{-5} M leucine. The isotope was added, and the cells were incubated in a 12% CO_2, 88% air incubator at 37°C. For labeling cells with leucine between 8 and 24 h in serum-free media, 5×10^{-5} M leucine was used. [^{3}H]leucine (50,000 Ci/mol) or [^{14}C]leucine (165 Ci/mol) was added to a final concentration of 20 μCi/ml and 2 μCi/ml, respectively. Uptake of isotope into 10% tricholoroacetic acid (TCA)-precipitable material was linear for up to 24 h under these conditions. For isotopic labeling with proline, alanine, glycine, fucose, and in some cases, leucine, the radioactive isotope was added directly to the unchanged culture media or to cells in fresh serum-free media. [^{3}H]Proline (1,500 Ci/mol), [^{3}H]glycine (5,500 Ci/mol), [^{3}H]-alanine (2,300 Ci/mol), and [^{3}H]fucose (2,700 Ci/mol) were added to final concentrations of 10 μCi/ml and incubated as described above.

For the examination of secreted protein, the medium was removed from the cell culture dish and centrifuged at 30,000 *g* for 30 min. The supernatant material was then dialyzed exhaustively at 4°C against 0.9% NaCl in 0.01 M sodium phosphate buffer (PBS), pH 7.1. There was no detectable loss of TCA-precipitable isotope during this procedure.

For the electrophoretic examination of total cellular protein, cells were scraped from the tissue culture dishes with rubber policemen, centrifuged, washed once with modified Eagle's medium minus leucine, and resuspended in cold PBS. Cells were lysed by homogenization with 30 strokes of a Duall tissue grinder (Kontes Glass Co., Vineland, N. J.).

For acrylamide gel electrophoresis, the appropriate samples were mixed and lyophilized. They were then dissolved in 0.2 ml of a solution containing 9 M urea, 1% sodium dodecyl sulfate (SDS), 0.2 M 2-mercaptoethanol, and 0.5 M Tris-HCl, pH 8.5, at 37°C for 3 h. Iodoacetamide in 2 M Tris-HCl, pH 8.5, was then added to a concentration of 0.30 M. Incubation was continued for 30 min, a 10% molar excess of 2-mercaptoethanol was added, and the solution dialyzed overnight at 37°C against 0.1% SDS, 0.2 M 2-mercaptoethanol, 0.5 M urea, and 0.01 M sodium phosphate, pH 7.1. Samples were then electrophoresed on 6.0% acrylamide gels containing 0.1% SDS, 0.5 M urea, and 0.01 M sodium phosphate, pH 7.1. Electrophoresis and counting of the gels were carried out as previously described (9, 10). Fractions were numbered from the negative to the positive electrodes. Since this gel system contains SDS, separation of proteins is primarily on the basis of molecular weight, but the mobility of both glycoproteins and collagen is possibly anomalous (10, 11).

The digestion of secreted material with collagenase (Worthington Purified, Worthington Biochemical Corp., Freehold, N.J.), was done in 0.067 M phosphate buffer, pH 7.4, containing 0.45% sodium chloride at 37°C (12).

Enzyme and Hydroxyproline Assays

Creatine phosphokinase and myokinase were assayed by published procedures (13) after the cells were washed twice in PBS and then lysed by 1% Nonidet-P-40 (Shell Chemical Corp., New York), in 0.05 M glycylglycine buffer, pH 6.75. Protein concentration was determined using bovine serum albumin as a standard (14).

[4-^{14}C]Hydroxyproline and [^{14}C]proline were analyzed with a Beckman amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.), after hydrolysis of lyophilized supernatant material in 6 N

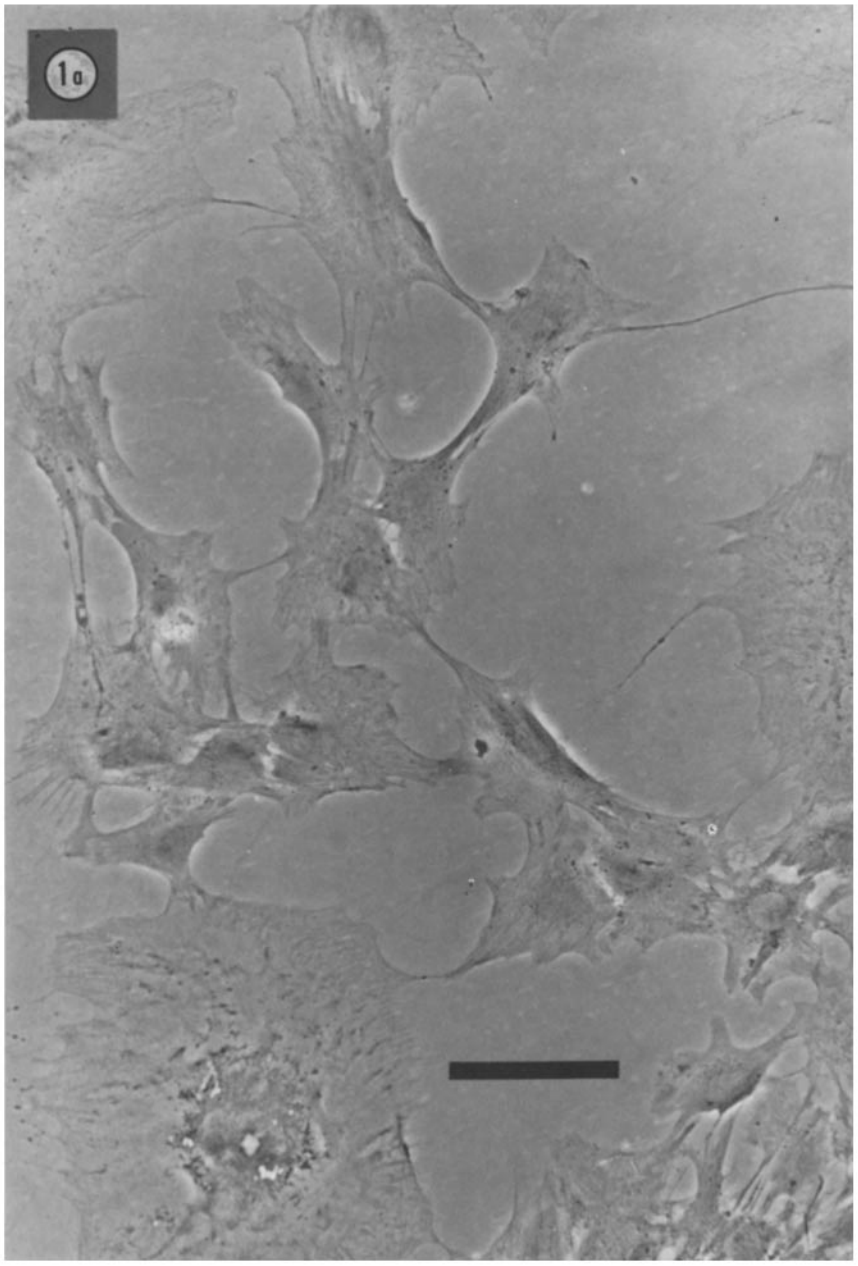
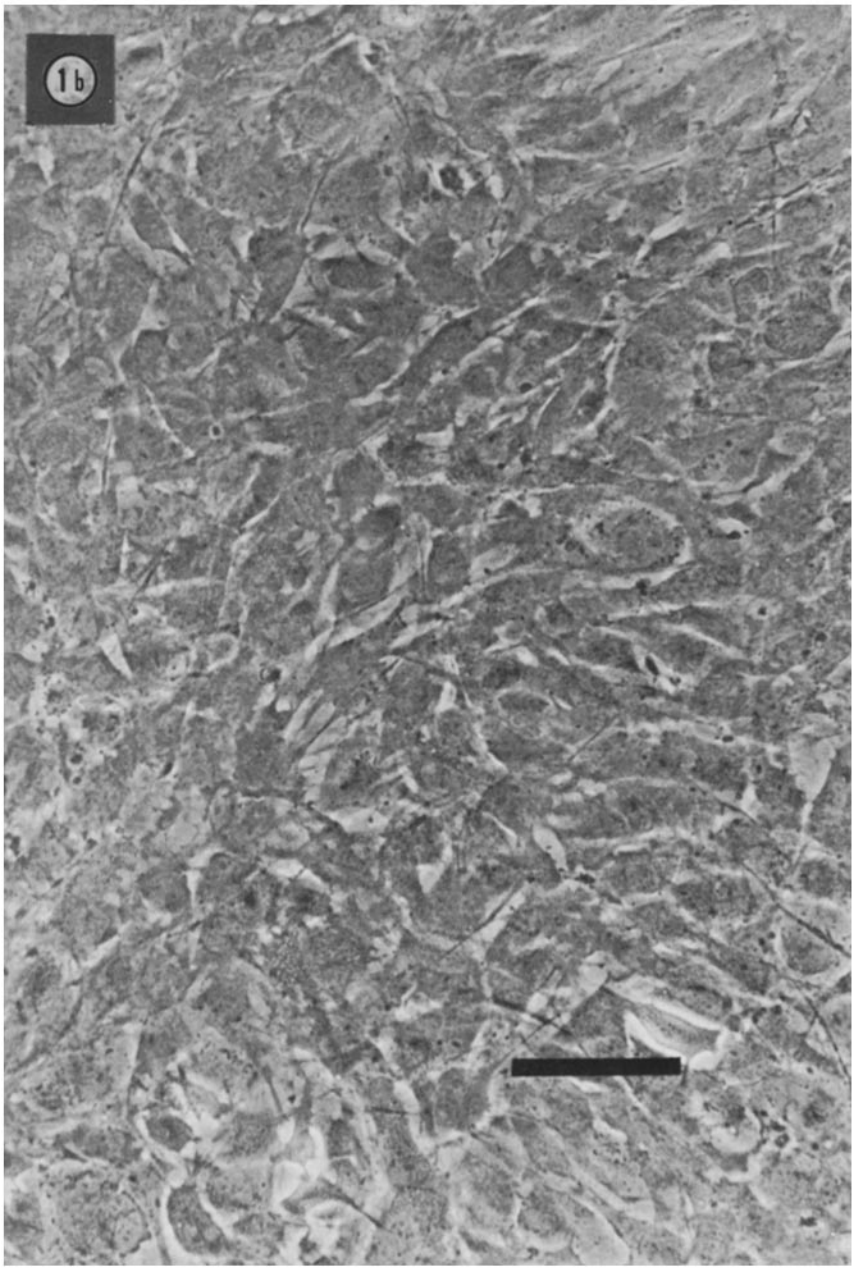


FIGURE 1 Morphological characteristics of BC_3H1. (A) Exponentially dividing cells; (B) confluent culture; (C) culture 1 wk after reaching confluency. The bar represents 50 μm.

HCl at 110°C for 24 h (15). The radioactivity in the column was monitored by scintillation counting, and the data were expressed as the percentage of the [^{14}C]isotope in the secreted protein which cochromatographs with 4-hydroxyproline. In all cases, only proline and 4-hydroxyproline contained detectable isotope.

Electron Microscopy

For electron microscopy, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) was added dropwise to the incubation medium containing cells growing in culture dishes, in which glass cover slips were placed. Once the concentration of glutaraldehyde reached approximately 1%, the dish was allowed to stand for

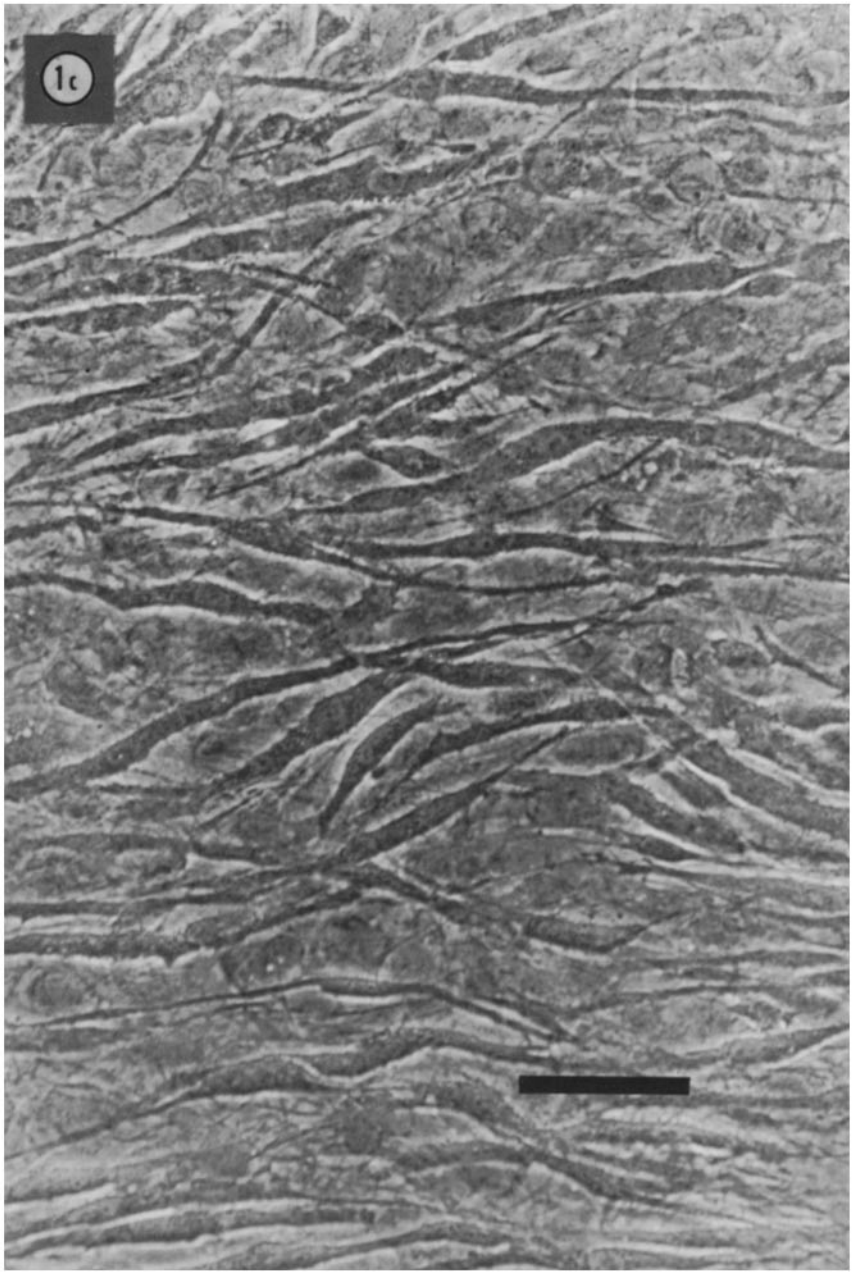


5 min, and the medium and fixative were replaced by cacodylate-buffered 2.5% glutaraldehyde, and fixed for 2 h. After a buffer rinse, the tissues were postfixed for 2 h in cacodylate-buffered 2% osmium tetroxide, stained with aqueous uranyl acetate for 30 min, dehydrated in ethanol and propylene oxide, and embedded in Epon. Some tissues examined were scraped off the culture dish and embedded (subsequent examination showed that little cell damage occurred). Ultrathin sections cut with a diamond knife were stained with alkaline lead citrate and examined in a Hitachi HU11A electron microscope.

RESULTS

Morphological Changes Accompanying Cell Growth

Exponentially dividing BC_3H1 cells adhere tightly to the surface of the culture dish and have



a flat morphology, with numerous small processes extending from the cell (Fig. 1 A). The karyotype of the clone described here was hypotetraploid with a modal chromosome number of 56. At confluency, the cells form a tightly packed monolayer (Fig. 1 B), and approximately 5 days later some of the cells appear to detach slightly from the surface of the culture dish and elongate (Fig. 1 C). The fraction of the stationary phase cells which makes this morphological change varies among cultures and is rarely greater than 50% of the total cell number. Since the elongated, but not the flattened cells were frequently observed to contract spontaneously at a rate of 1–3 times per second, it seemed possible that this cell line represented a type of muscle. Multinucleate fibers were never

observed, making it unlikely that the cells were of skeletal muscle origin. The following paragraphs describe several properties of BC_3H1 which indicate that it may be of smooth muscle origin.

Electron Microscopy

The major electron microscope characteristics of the elongated (Fig. 1 C) BC_3H1 cells suggest that they are of smooth muscle origin. However, it will be pointed out that not all of the ultrastructural features of this line resemble those of in vivo smooth muscle. This fact could be accounted for either by changes due to in vitro growth (4), or by a unique cell of origin, for several muscle types have been described which share some properties of smooth and skeletal muscle (see later). The elongated cells are approximately 5 μm in diameter, with myofilaments forming a cylinder around the central region containing the nucleus and mitochondria. In transversely sectioned cells, the myofilaments are present near the cell membrane as a ring composed of both thick (15–18 nm in diameter), and thin (6–8 nm) myofilaments (Fig. 2 C). In longitudinally sectioned cells, the myofilaments are oriented in the long axis of the cell and, depending on the plane of section, the cylinder of filaments is seen either as a narrow region of filaments near the cell membrane, or as a sheet of filaments occupying the major portion of the cell (Fig. 2 A). Individual filaments vary in length, small groups are parallel, and most filaments present a rather disorganized appearance (Fig. 2 A). Dense regions (Fig. 2 B) near the membrane were found resembling dense bodies seen in smooth muscle (16), and dense patches were also seen in the filament areas (Fig. 2 C). No sarcomeres with Z bands were found in any cells, and no apparent regular lattice of thick and thin filaments was seen in transverse sections. Filaments approximately 10 nm in diameter previously observed in primary cultures of smooth muscle cells (4) were also found in the BC_3H1 cell line.

Invaginations of the cell membrane were seen which apparently penetrated some distance into the cell. Surface vesicles were also present (Fig. 2 B and C), but they were not as common as in normal smooth muscle cells (see, for example, reference 17). This is consistent with the relative scarcity of surface vesicles noted in primary cultures of smooth muscle cells (4). The cell membrane and the sarcoplasmic reticulum (SR) formed close relationships with each other (Fig. 2 A inset), similar to couplings which are most pronounced in skeletal and cardiac muscle, but also present in smooth muscle. Membrane invaginations were often found associated with the SR (Fig. 2 A), but this type of relationship showed no apparent regularity. Ribosomes were found on parts of the sarcoplasmic reticulum and throughout the cytoplasm, together with mitochondria, vacuoles, and microtubules.

Collagen fibrils with the characteristic banded appearance were seen in the extracellular space (Fig. 2 C). A basement membrane was also present, but was not as well developed as has been described for normal smooth muscle.

Electrophysiological Properties

Exponentially dividing BC_3H1 cells are electrically coupled by nonrectifying electrotonic junctions, for if either hyperpolarizing or depolarizing current is passed into one cell via an intracellular electrode, a voltage deflection can be recorded in an adjacent cell (Fig. 3 A). When the cells are excited by anode break stimulation, overshooting action potentials are observed (Fig. 3 B). Depolarization of elongated cells in confluent cultures with electrical stimulation, or by the iontophoretic application of acetylcholine (Fig. 1 C), results in their contraction; this has not been observed in exponentially dividing cells, or in stationary phase cells which are not elongated.

The sensitivity of the cells to the iontophoretic application of acetylcholine and norepinephrine is shown in Figs. 3 C and 4. BC_3H1 cells respond to acetylcholine with a fast depolarizing response, and to norepinephrine with a slow hyperpolarizing response.

Enzymatic Changes during Growth

It has been shown that the specific activities of creatine phosphokinase and myokinase in skeletal muscle myoblast cultures increase as a function of cell fusion during differentiation (see, for example, reference 13). Since BC_3H1 has the ability to develop a contractile apparatus, it might be expected that the specific activities of these enzymes would increase as a function of differentiation in this cell line, also. The data in Fig. 5 show that the specific activities of both creatine phosphokinase and myokinase increase as a function of time in culture. The specific activity of creatine phosphokinase increases maximally at a point where cells are starting to elongate, while the

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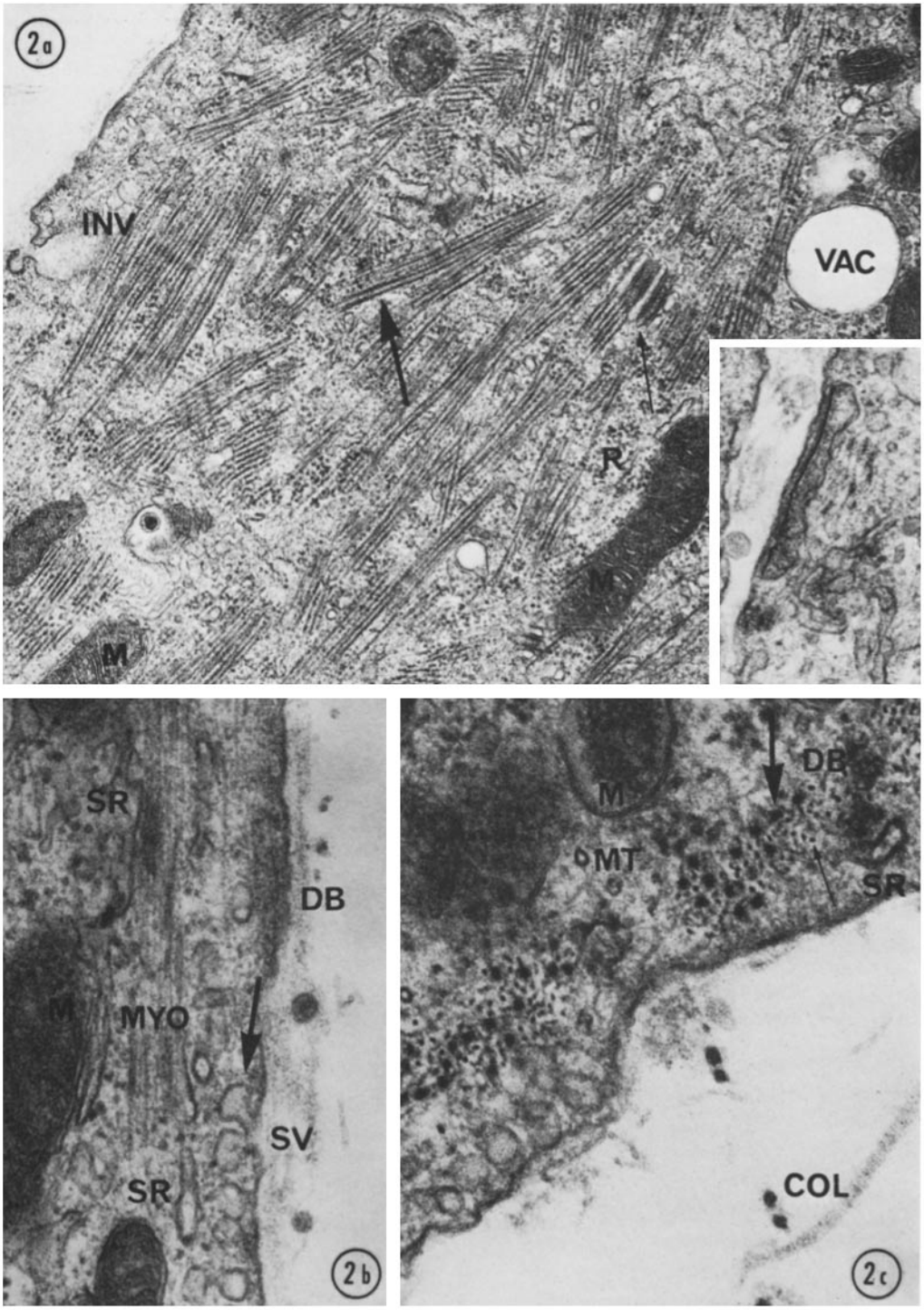

specific activity of myokinase increases approximately linearly throughout the growth curve. The specific activities of both enzymes are maximal at about 2 wk after plating the cells at 2×10^4 per 60-mm dish (Fig. 5 day 14) and remain constant at this level for up to 5 wk.

Secreted Protein

If a confluent culture of BC_3H1 cells is labeled for 24 h in serum-free medium with [^{14}C]leucine, and the cell supernate (tissue culture medium) exhaustively dialyzed against PBS, lyophilized, reduced, and alkylated, and electrophoresed on acrylamide gels, 11 reproducible peaks of radioactivity are observed. These are designated as secretory proteins A–K (Fig. 6). The uniqueness of these protein bands was verified by electrophoresis on a high resolution SDS acrylamide gel system followed by autoradiography (18).

One of the two processes could account for these observations. Either these proteins were secreted by the BC_3H1 cell line, or their appearance in the cell supernate was the result of the lysis of cells which had previously incorporated isotope into protein. The latter possibility was ruled out by the following experiments. Supernatant material containing secreted protein labeled with [^{14}C]leucine was mixed with identical cells which had been labeled for the same length of time with [^{3}H]leucine. The cells were then lysed by homogenization and dialyzed against PBS, and the material was electrophoresed on SDS acrylamide gels after reduction and alkylation. Fig. 7 demonstrates that although several of the major intracellular proteins comigrate with the presumptive secreted material, the majority of the intracellular proteins are either not resolved or migrate in the gel in a manner different from that for secreted proteins. Results identical to these were also obtained when the isotopes were reversed. In addition, there was nearly base-line resolution between some of the peaks in the secreted material, while gel electrophoresis of total intracellular protein resulted in a more continuous distribution of radioactivity across the gel.

Another argument against the contribution of cell lysis to the appearance of extracellular soluble protein can be derived from an examination of the rate of appearance of these proteins in the cell supernate. Cells were labeled with [^{3}H]leucine for various lengths of time and the TCA-precipitable material released into the medium was examined.

FIGURE 2 Electron microscopy of BC_3H1. (A) A longitudinal section through a myofilament-rich region showing irregularly oriented thick myofilaments (heavy arrow). Mitochondria, ribosomes, large vacuoles, and small vesicle-like elements of a sarcoplasmic reticulum are present throughout the cell. Occasionally, portions of the sarcoplasmic reticulum form regions of close apposition with apparent invaginations of the cell membrane seen as an electron-lucent region with densely-staining sarcoplasmic reticulum adjacent to it (light arrow). × 30,000. Inset: a region of close apposition of an element of sarcoplasmic reticulum to the cell membrane. × 60,000. (B) A longitudinal section at the region of the cell membrane showing several surface vesicles 70–100 nm in diameter. Some vesicles apparently open to the extracellular space (heavy arrow), while others are sectioned in such a way that they appear completely within the cytoplasm. The vesicles, while appearing irregular in shape when they are joined together, are different from the flattened elements of the sarcoplasmic reticulum. Ribosomes, myofilaments, and mitochondria are present in the cytoplasm. In the extracellular space, collagen, virus-like particles, and a lightly staining basement membrane are present. × 60,000. (C) A portion of a cell showing thick (heavy arrow) (15–18 nm in diameter) and thin (light arrow) (6–8 nm in diameter) myofilaments which do not have a regular lattice spacing. Surface vesicles are adjacent to a region of increased electron opacity. Some collagen fibrils, one with typical cross banding, lie in the extracellular space. × 90,000. *COL*, collagen; *DB*, dense body; *INV*, invagination; *M*, mitochondria; *MT*, microtubule; *MYO*, myofilaments; *R*, ribosomes; *SR*, sarcoplasmic reticulum; *SV*, surface vesicle.

FIGURE 3 Electrical coupling and membrane excitability in exponentially dividing BC_3H1 cells. Two adjacent exponentially dividing cells were impaled with microelectrodes. (A) Electrical coupling was demonstrated by recording from one cell (marked *R*, upper tracing) and passing both depolarizing and hyperpolarizing current into the other cell (marked *I*). (B) The cell at the left was excited by anode break stimulation, and an action potential was observed. (C) Acetylcholine was iontophoresed onto the surface of the cells at the points indicated by the arrows, and the electrical response was recorded in the cell with the recording electrode (marked *R*).

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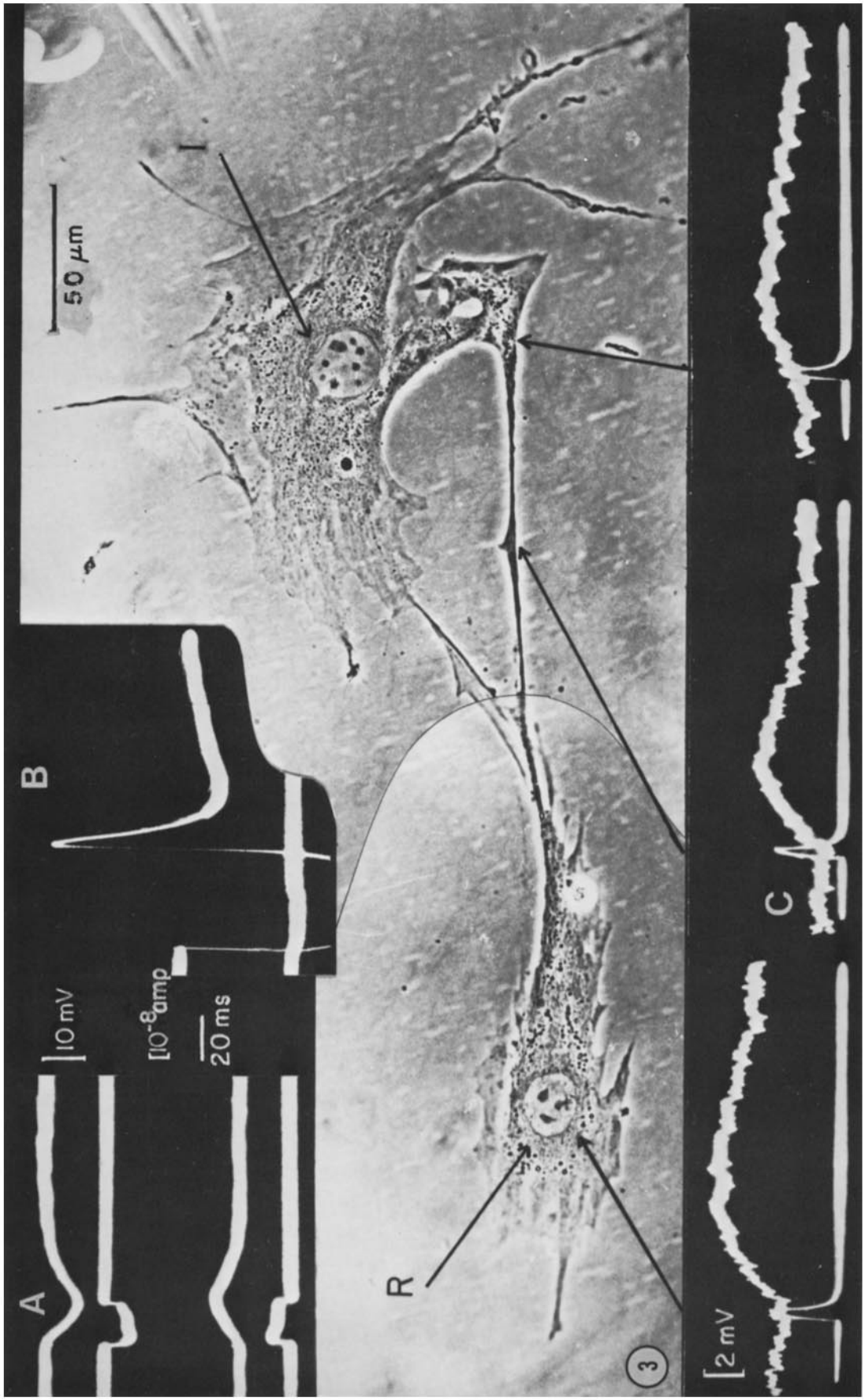

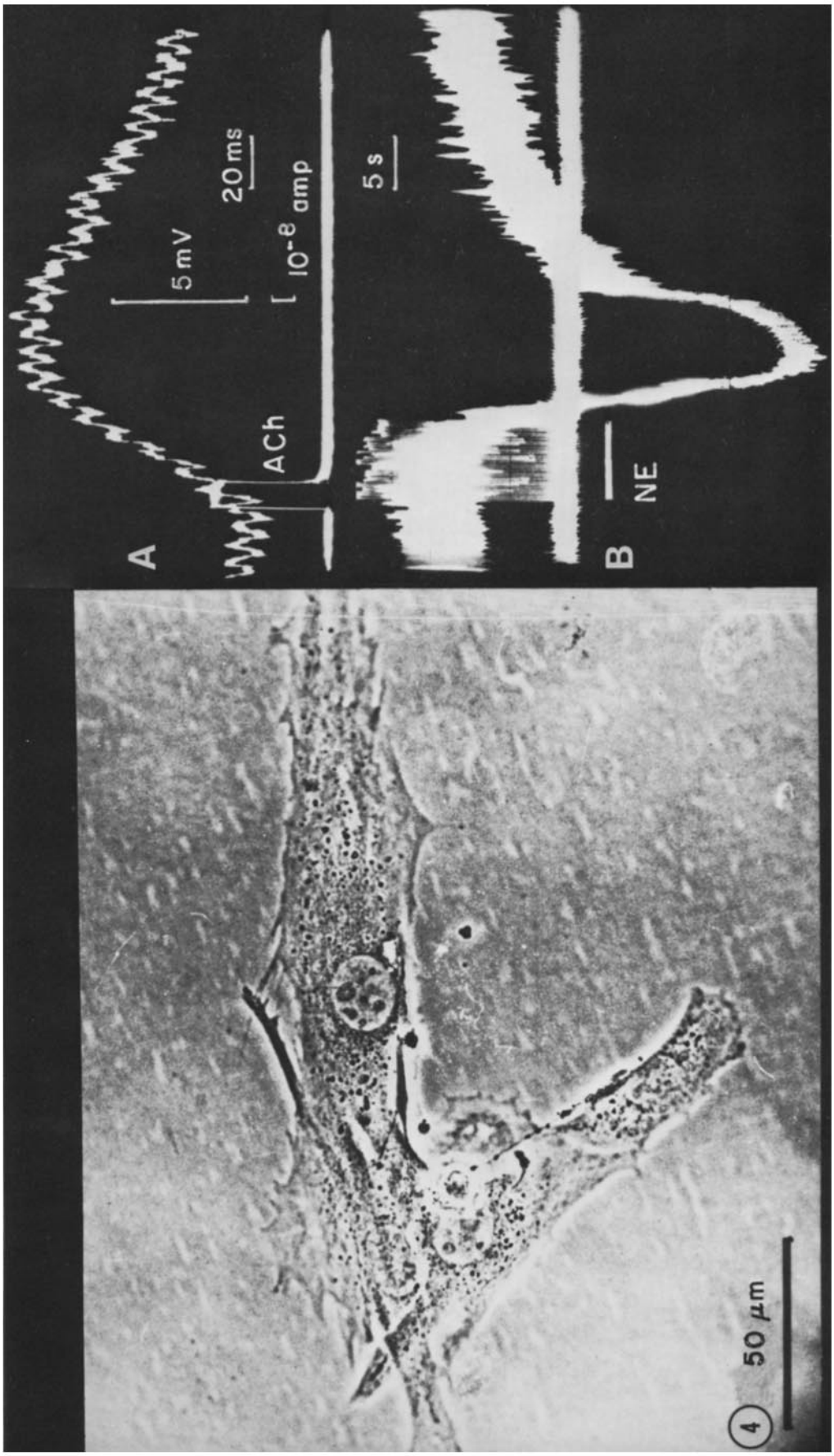

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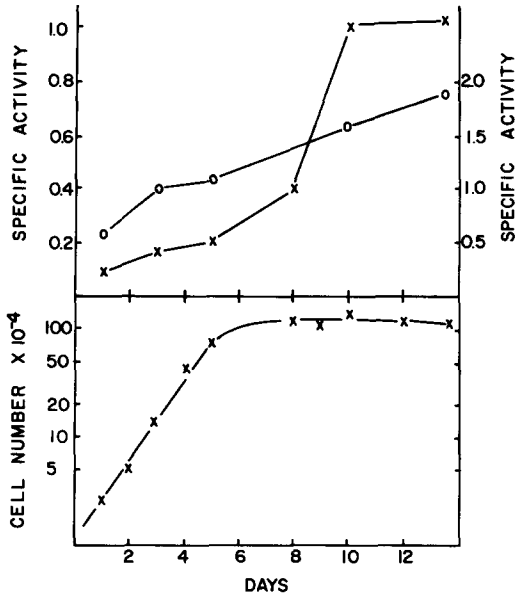

FIGURE 5 Myokinase, creatine phosphokinase, and cell number. Exponentially growing cells were dissociated with Viokase and plated in 60-mm tissue culture dishes at 2 × 10^4 cells per plate. Enzymatic activities and cell number were then followed as a function of time. The medium was not changed for the duration of the experiment. Enzymatic activities are expressed as ΔOD_{340} per minute per milligram protein. (A) O–O–O, specific activity of myokinase (right ordinate); X–X–X, specific activity of creatine phosphokinase (left ordinate). (B) Cell number per culture dish.

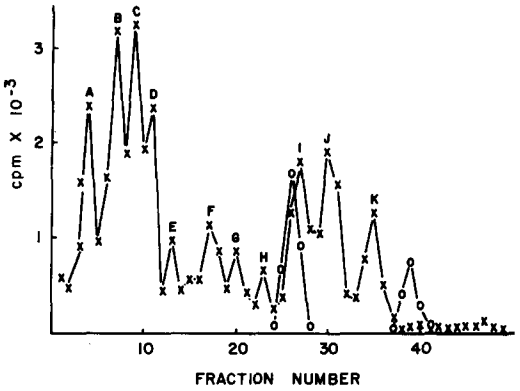

FIGURE 6 Supernatant proteins of BC_3H1. Exponentially growing cells were labeled for 24 h with [^{14}C]leucine and the tissue culture medium was exhaustively dialyzed against PBS. This material was then lyophilized, reduced and alkylated, and electrophoresed on 6% acrylamide gels containing SDS. IgG immunoglobulin labeled with [^{3}H]leucine was coelectrophoresed in the same gel as a marker. X–X–X, [^{14}C]leucine secreted BC_3H1 protein; O–O–O, [^{3}H]leucine immunoglobulin heavy (50,000 mol wt, fraction 26) and light (23,000 mol wt, fraction 39) chains.

Fig. 8 shows that isotope was linearly incorporated into intracellular TCA-precipitable material from the time of addition of the labeling reagent, while there was approximately an hour's delay before the onset of the linear appearance of extracellular material. Similar secretion kinetics have been observed for chick embryo lens collagen (19), for immunoglobulin secretion (10), and for protein secretion in skeletal muscle (20). The rate of appearance of extracellular soluble protein is about 4% of the rate of net protein synthesis by the cells. It can thus be concluded that the appearance of extracellular protein is a result of secretion, not cell lysis.

Although it can be shown that proteins are secreted, the major difficulty lies in assigning functions to these macromolecules. Since it has been argued that both smooth muscle (see, for example, references 3 and 21) and, more recently, skeletal muscle (20) are capable of collagen synthesis and secretion, the proteins secreted by BC_3H1 were examined for the presence of this protein. The following experiments show that at least three of the secreted proteins are soluble collagen molecules.

Collagen contains a high content of the amino acids proline, alanine, and glycine relative to most other proteins (see, for example, references 22–24). To determine if such proteins existed among the 11 secreted by BC_3H1, stationary phase cells were labeled for 24 h with either [^{3}H]proline, [^{3}H]-glycine, [^{3}H]alanine, or [^{14}C]leucine. The ^{3}H-labeled secreted proteins were then mixed separately with the [^{14}C]leucine-labeled secreted protein and electrophoresed on SDS acrylamide gels.

FIGURE 4 Electrical response to the iontophoretic application of acetylcholine and norepinephrine. Exponentially dividing cells were impaled with a microelectrode, and either acetylcholine or norepinephrine was iontophoresed onto the cell with another pipette. The release of the transmitters into the medium at points equidistant from the recording electrode elicited no response. (A) Rapid depolarizing response to acetylcholine. (B) Slow hyperpolarizing response to norepinephrine.

The percent of the total isotope in the gel was determined for each resolved protein, and then the ratio of the percent ^{3}H-labeled protein to the percent ^{14}C-labeled protein in each protein was calculated. From the data presented in Table I it can be concluded that secreted proteins B, C, and D contain higher concentrations of proline, alanine, and glycine relative to leucine than the other proteins in the gels. Also included in Table I are the results of labeling the cells with [^{3}H]fucose, a compound which specifically labels glycoproteins (25–28). Fucose was found associated with all of the secreted proteins.

While high concentrations of glycine, alanine, and proline are a necessary but not sufficient criterion for the identification of collagen, a more

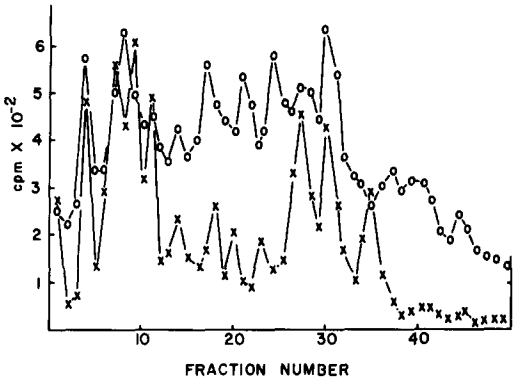

FIGURE 7 Acrylamide gel electrophoresis of supernatant protein and cell lysate. Exponentially growing cells were labeled for 24 h with either [^{3}H]- or [^{14}C]leucine. [^{14}C]leucine-labeled supernate and ^{3}H-labeled cells were cooled at 4°C, mixed, homogenized, and dialyzed against PBS. This material was then lyophilized, reduced and alkylated, and electrophoresed on 6% acrylamide gels containing SDS. X–X–X, [^{14}C]leucine-labeled supernatant material; O–O–O, [^{3}H]leucine-abeled cell lysate.

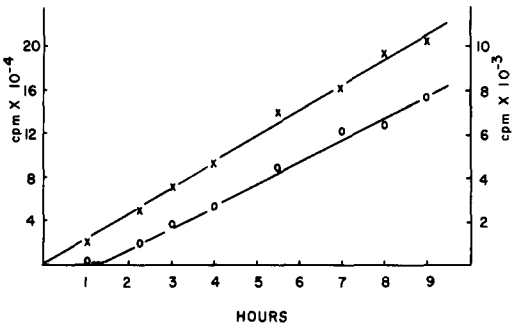

FIGURE 8 Rates of protein synthesis and secretion. Exponentially dividing cells were labeled with [^{3}H]leucine and the incorporation of isotope into TCA-precipitable cellular and extracellular protein was assayed as a function of time. O–O–O, extracellular material (right ordinate); X–X–X, cellular material (left ordinate).

TABLE I

*Amino Acids Incorporated into Secreted Protein**

Protein	Percent total	Alanine/leucine ratio	Glycine/leucine ratio	Proline/leucine ratio	Fucose/leucine ratio
A	12.5	0.48	1.10	0.78	1.16
B	16.0	1.48	3.83	3.00	0.81
C	15.3	1.54	5.26	3.38	0.74
D	10.4	1.92	4.53	2.28	0.76
E	4.2	0.68	1.68	0.89	1.00
F	6.3	0.44	1.12	0.79	1.27
G	3.5	0.45	1.13	0.62	1.29
H	2.1	0.33	2.00	0.78	0.91
I	11.1	0.52	0.65	0.67	1.11
J	11.8	0.38	0.56	0.79	0.74
K	6.9	0.55	0.75	0.67	0.73

* Secreted proteins from stationary phase cells were labeled with [^{3}H]proline, alanine, glycine, or fucose, and coelectrophoresed on SDS acrylamide gels with [^{14}C]leucine secreted protein. The percent of total ^{14}C-labeled secreted protein in each peak was determined and indicated above as percent. Similarly, the percent of total ^{3}H incorporated into each ^{3}H peak was calculated. Then the ratio of the percent ^{3}H-labeled protein to the percent [^{14}C]leucine-labeled secreted protein in each peak was determined and represented as Ratio.

TABLE II

*4-Hydroxyproline Content of Secreted Protein**

Cell line	4-Hydroxyproline
BC_3H1	3.81%
3T3‡	17.5%
P3§	<0.005%

* Stationary phase cells were labeled with [^{14}C]-proline for 24 h. The cell supernate (medium) was then dialyzed and assayed for the presence of 4-hydroxyproline. The results are expressed as the percent of total ^{14}C recovered in 4-hydroxyproline.

‡ 3T3 is a mouse fibroblast cell line. § P3 is an IgG immunoglobulin-secreting mouse plasmacytoma.

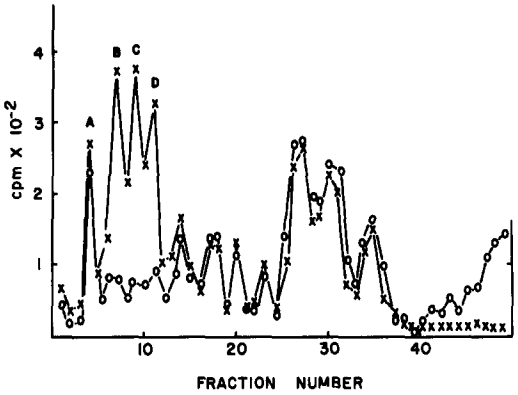

FIGURE 9 Collagenase treatment of secreted protein. ^{3}H-labeled secreted protein was dialyzed against 0.067 M phosphate, 0.45% NaCl, pH 7.5 and treated with 0.002% collagenase for 5 min. The enzymatic reaction was stopped by the addition of TCA to 5%, and [^{14}C]-leucine-labeled secreted protein, treated identically except for the omission of the enzyme, was added. The mixtures were then lyophilized, reduced and alkylated, and electrophoresed on SDS acrylamide gels. o–o–o, [^{3}H]leucine-labeled secreted protein treated with enzyme; x–x–x, [^{14}C]leucine-labeled secreted protein untreated.

rigorous marker is the relatively collagen-specific amino acid, 4-hydroxyproline (see, for example, reference 24). To assay for the presence of 4-hydroxyproline in the secreted material, cells were labeled with [^{14}C]proline and the supernatant material was dialyzed, lyophilized, hydrolyzed to amino acids, and assayed for the presence of radioactivity in 4-hydroxyproline. Table II shows that the secreted proteins contain hydroxyproline. Control cultures of mouse plasmacytoma and fibroblast cells labeled under identical conditions showed that the latter secreted large amounts of 4-hydroxyproline while none was detected in the former, although it secreted large amounts of proline-containing immunoglobulin (10).

Another criterion defining a protein as collagen is its susceptibility to the specific proteolytic enzyme collagenase. To strengthen the argument that secreted proteins B, C, and D are indeed soluble collagen molecules, the secreted proteins from cells were labeled with [^{3}H]leucine and treated with collagenase for various lengths of time. The kinetic analysis of collagenase digestion was required since the collagenase used contained small amounts of other proteolytic activities (see reference 20 for details). They were then coelectrophoresed on SDS acrylamide gels with [^{14}C]-leucine-labeled, untreated, secreted protein. Fig. 9 shows that after a 5-min exposure to collagenase, proteins B, C, and D were substantially reduced, while the other proteins were not affected.

DISCUSSION

On the basis of the following results, it can be concluded that the mouse cell line BC_3H1, clone 9, is of muscle origin, and possibly derived from a type of smooth muscle. (*a*) Elongated mononucleate cells in stationary phase cultures contract spontaneously (Fig. 1 C). (*b*) Both exponentially dividing and stationary phase cells are capable of generating an action potential when electrically stimulated (Fig. 3), and the elongated cells contract when artificially depolarized. (*c*) The cells respond to the iontophoretic application of acetylcholine with a depolarizing response, and to norepinephrine with a hyperpolarizing response (Figs. 3 and 4). (*d*) Electron microscopy shows that elongated cells (Fig. 1 C) have groups of filaments lacking Z bands and also show surface vesicles characteristic of smooth muscle (Fig. 2). (*e*) Cells secrete soluble, collagen-like molecules (Fig. 9 and Tables I and II) in addition to a minimum of eight other unidentified proteins (Fig. 6). Polymerized collagen is found extracellularly (Fig. 2C). (*f*) There are increases in the specific activities of both myokinase and creatine phosphokinase associated with increased cell number and morphological elongation of the cells, respectively (Fig. 5).

Morphological evidence for the secretion of both collagen and elastin has been presented for primary cultures of aorta smooth muscle (3, 21, 29). The results with BC_3H1 indicate that this

cell line is capable of collagen synthesis, and similar data have been obtained which indicate that a clonal skeletal muscle myoblast cell line also secretes collagen (20).

In primary and clonal cultures of skeletal muscle myoblasts, a rapid increase in the specific activities of myokinase and creatine phosphokinase apparently parallels cell fusion and myotube formation (see, for example, reference 13). In the case of BC_3H1, the most rapid increase in creatine phosphokinase occurs after the apparent cessation of cell division, while myokinase increases approximately as a function of cell density (Fig. 5).

The major ultrastructural characteristics of BC_3H1 are those of smooth muscle, although some features of skeletal muscle, such as the apposition of the sarcoplasmic reticulum with invaginations of the cell membrane (Fig. 2 A inset, reference 30) and the relatively low thin to thick filament ratio, are present.

Thick myofilaments are observed (Fig. 2), but no sarcomeres with Z bands are present. Both rough and smooth sarcoplasmic reticula are present, and their relationship to the limiting cell membrane is analogous to those observed in smooth muscle in vivo (30), although deeper invaginations than those normally seen in smooth muscle are present. Finally, dense bodies and surface vesicles are present; these structures are characteristic of smooth muscle, but are rarely found in striated muscle (see, for example, references 30–33). Thus the similarity of many aspects of this clonal line to smooth muscle in vivo, and also primary smooth muscle cell cultures (4), indicate a possible smooth muscle origin. Although exact ultrastructural correlations are not found, differences between the BC_3H1 line and smooth muscle in vivo may be expected, since even primary cultures of smooth muscle show differences when compared with smooth muscle in vivo (4). Striated muscle cells have been found in the rat pineal by electron microscopy (34), and striated muscle has also been observed by light microscopy in rat cerebellum (35) and human leptomeninges (36). The general arrangement of the sarcomeres with Z bands in the rat pineal (34) is more regular than the random arrangement of the filaments in the BC_3H1 line, although not as regular as in most striated muscles.

The electrophysiological characteristics of smooth muscle are quite variable (see, for example, reference 37). The results shown in Fig. 4 indicate that the same cell in the exponentially growing clonal BC_3H1 line responds to the iontophoretic application of acetylcholine with a rapid depolarizing response, and to norepinephrine with a slow hyperpolarizing response. The responses to these neurotransmitters are thus analogous to the smooth muscle cells of guinea pig *Taenia coli*, where acetylcholine depolarized, and adrenaline hyperpolarized the muscle (38). Similar results were also obtained with the cat jejunum (39) and rat uterine muscle fibers (40).

In addition to the ultrastructural and electrophysiological properties that BC_3H1 cells share with smooth muscle, there are a number of characteristics which they do not share with clonal skeletal muscle cell lines: (*a*) Mononucleate cells in clonal lines of skeletal muscle myoblasts have never been observed to contract spontaneously (8, 41, 42), while mononucleate BC_3H1 cells do. (*b*) Skeletal myoblasts fuse to form myotubes under identical growth conditions described for BC_3H1 (8, 41, 42). (*c*) Clonal mononucleate skeletal muscle myoblasts are incapable of generating an overshooting action potential and do not respond to acetylcholine with a depolarizing response (8, 41–43). (*d*) Mononucleate skeletal myoblasts do not bind α-neurotoxin, while the stationary phase mononucleate BC_3H1 cells do (43; J. Patrick, personal communication).

The above data show that BC_3H1 clone 9 possesses several of the structural and physiological properties of smooth muscle origin. It is clearly impossible to define the tissue of origin, but this fact should not limit the experimental usefulness of the cell line.

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