

Trichinella spiralis Infected Skeletal Muscle Cells Arrest In G₂/M and Cease Muscle Gene Expression

Douglas P. Jasmer

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040

Abstract. Infection by *Trichinella spiralis* causes a variety of changes in skeletal muscle cells including the hypertrophy of nuclei and decreased expression of muscle specific proteins. Potential cellular processes leading to these changes were investigated. In synchronized muscle infections, [³H]thymidine was incorporated into infected cell nuclei from 2–5 days post infection. Labeled nuclei were stably integrated into the infected cell up to 60 days post infection and appear to originate from differentiated skeletal muscle nuclei present at the time of infection. These nuclei were further shown to contain a mean DNA content of ~4N, indicating that the [³H]thymidine uptake reflects

DNA synthesis and subsequent long-term suspension of the infected cell in the cell cycle at G₂/M. Associated with these changes, muscle specific gene transcripts were reduced to <1–<0.1% in the infected cell compared to normal muscle. Transcript levels of the muscle transcriptional regulatory factors myogenin, MyoD1, and Id were reduced to <10, <1, and increased ~250%, respectively, in the infected cell compared to normal muscle, indicating transcriptional inactivation of muscle genes. DNA synthesis in the infected cell may represent the initiation event which leads to expression of this infected cell phenotype.

TRICHINELLA SPIRALIS is an intracellular nematode parasite of mammalian skeletal muscle cells. Invasion of muscle by first stage larvae (new born larvae) initiates dramatic changes in these cells. With the exception of its multinucleate nature, the infected cell has no morphological similarity to that of the original muscle cell (10, 36). Ultimate changes to the infected cell and the parasite appear to be achieved by 20 days post infection (dpi)¹ (10). After that time, the host/parasite relationship can remain stable for years.

Changes in the phenotype of the infected muscle cell have been demonstrated at the morphological, ultrastructural and biochemical levels (reviewed in 36). The best studied of these include the development of a collagen capsule (38) and a circulatory rete surrounding the infected cell (2), greatly enlarged nuclei (13), increased activity of lysosomal acid phosphatase (ACP) (24, 27) and decreased expression of myofibrillar proteins (27, 28). Some of these characteristics may be derived from the host inflammatory cells which are persistently associated with infected cells. However, the complete dissolution of myofibrillar organization (10), increased ACP activity and increased size of infected cell nuclei are clearly related to processes within the infected muscle cell. Infection initiates the dissolution of myofibrils beginning by about ~2 dpi and is essentially completed by

10 dpi (10). Several myofibrillar proteins were undetectable and reduced to <1% in isolated infected cells relative to normal muscle (28). Decreased abundance of myofibrillar proteins was demonstrated in the infected cell by 10 dpi in situ (27), although cellular processes responsible could have been initiated earlier. The level at which these changes are regulated is currently unknown and there are no characteristics that provide a clear link between the infected cell and any stage in the muscle cell lineage.

Host cellular processes involved in initiating expression of the infected cell phenotype have not been identified, but characteristics of infected cells may provide important insights into these processes. Infected cells can contain over 100 nuclei (13) which are greatly enlarged (up to 15 μm diam) and have well developed nucleoli. Enlargement of nuclei has been detected by 5 dpi which is temporally correlated with increased ACP activity. ACP activity is the earliest known biochemical marker for the infected cell phenotype and can be detected by 5 dpi (27). Thus, the enlargement of infected cell nuclei may signify changes which initiate expression of the infected cell phenotype. It was also demonstrated that [³H]thymidine (3HT) could be incorporated into infected cell nuclei during asynchronous muscle cell infections (19). It was postulated that these nuclei are acquired during a regeneration response induced by the infection, which implies that they originate from satellite cells (36). Alternatively, infected cell nuclei could be derived from differentiated adult skeletal muscle nuclei present at the time of infection. DNA synthesis can be induced in

1. Abbreviations used in this paper: ACP, lysosomal acid phosphatase; AChR-α, acetylcholine receptor subunit alpha; dpi, days post infection; 3HT, tritiated thymidine.

mammalian myotubes recently fused from myoblasts which are infected with SV40, polyoma or Rous sarcoma viruses (18, 20, 23, 40). Large T antigen is involved in inducing this DNA synthesis (15, 22). Interestingly, virus infected cells appear to be blocked in G₂/M and these nuclei are similar in size and morphology to *T. spiralis* infected cell nuclei. Clarifying fundamental changes such as these that occur in *T. spiralis* infected cells could identify host cellular processes involved in initiating expression of the infected cell phenotype, and contribute to the identification of parasite factors involved in inducing these processes.

Here, changes acquired by infected cell nuclei were investigated by determining both the origin of these nuclei in vivo and their DNA content. The biochemical level at which muscle gene expression is regulated in the infected cell was also investigated by transcript analysis of muscle specific genes. Expression of muscle transcriptional regulatory protein genes, which comprise both positive (MyoD1 and myogenin) and negative (Id) helix loop helix regulatory factors (3, 9, 14), was also investigated. It was shown that nuclei of the infected cell are derived from differentiated skeletal muscle nuclei extant at the time of infection, and that these nuclei are induced to stably maintain an approximate 4N complement of DNA. Further, it was shown that transcripts for muscle structural and positive regulatory genes are greatly reduced in the infected cell compared to normal muscle, indicating that the decrease in muscle gene expression is regulated at the level of transcription.

Materials and Methods

Synchronized Infections and [³H]thymidine Labeling

Synchronized *T. spiralis* infections were initiated with new born larvae released from adult female parasites in vitro (11). Approximately 50,000 new born larvae were injected into the thigh muscle of each mouse (6–8 wk old) on day 0. 3HT (2 μ Ci/gm/d) was injected intraperitoneally in three equivalent doses given at 4 h intervals, for the days indicated. One exception to this protocol was in short-term labeling experiments in which infected mice received 2 μ Ci/gm-mouse dispersed in three equivalent injections at 0, 1, and 2 h after initiation of labeling. Tissues for these mice were obtained 1 h after the last injection. All tissues were fixed in 10% formalin, embedded in wax and then cut in 5 μ m sections. Sections were overlaid with emulsion for autoradiography and exposed for 6–24 d before development. Developed slides were counterstained with Mayer's hematoxylin. Exposure times were identical for all tissue sections in a given experiment and in figures shown. Only infected cell nuclei, identified by their large size and prominent nucleoli, within the infected cell were counted in these studies. Care for animals used in all experiments was in accordance with institutional guidelines.

Muscle Regeneration and Irradiation Experiments

To induce regenerating muscle, mice were first anesthetized with methoxyflurane. Surgical clamps were then externally applied to the gastrocnemius muscle for 2 min to induce muscle trauma. Synchronized *T. spiralis* infections were initiated as described above. For irradiation, the right rear leg was x-irradiated (2 krad) immediately before infection or clamping of muscle, which occurred on day 0. Mice then received the same form of trauma (clamping or infection) to each of their rear gastrocnemius muscles. Mice were labeled with 3HT as in synchronized infection experiments.

In Utero Labeling of Adult Skeletal Muscle Nuclei

To label differentiated skeletal muscle cell nuclei, young mice were labeled with 3HT during in utero development and as neonates up to 5 d post-parturition (34) as described for labeling infected cell nuclei. The labeled nuclei of muscle precursor cells (satellite cells) were chased into myotubes

during normal muscle growth over a 4-wk period. Muscles of mice were then infected with newborn larvae or induced to undergo regeneration. Nuclei counts were pooled from three mice comprising each group. Muscle samples from contralateral legs of each mouse served as a control. Regenerating muscle was obtained 5 d post-trauma and infected muscle was obtained at 10 and 20 dpi.

Quantitation of DNA Content in Infected Cell Nuclei

Infected cell nuclei were obtained by mechanically lysing infected cells, allowing larvae to sediment and filtering out other debris through lens paper (28). The filtrate containing infected cell nuclei and infiltrating cells was pelleted at 300 g, resuspended in 0.5% non-idet P40, incubated on ice for 4 min, fixed in 10% buffered formalin and incubated in Hoechst 33258 DNA binding dye. Purified preparations of infiltrating cells were obtained as described (28), and their nuclei prepared similarly. Nuclei were then analyzed by microfluorimetry using a Nikon-Diaphot-TMD inverted, fluorescence microscope equipped with an f1 photometer. Infected cell nuclei were differentiated from infiltrating cell nuclei by their large size (>10 μ m) and well developed nucleoli. No nuclei with these characteristics were ever observed in the purified preparations of infiltrating cells. As an additional control, mouse astrocytes maintained in vitro were processed as described for infected cell nuclei and analyzed similarly. Astrocyte cultures were obtained from Timothy Baszler (Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA).

Transcript Analysis

RNA was obtained from all tissues and cells using an acid guanidinium thiocyanate method (6). Intact infected cells from 4–6 mo-old infections were isolated as described (27) and lysed immediately in the guanidinium thiocyanate solution, muscle larvae released during this procedure were immediately pelleted at 2,900 g. The larval pellet was discarded and the supernatant used in the extraction procedure. RNA obtained from isolated infiltrating cells associated with infected cells, mouse thigh muscle and mouse liver was extracted using a similar protocol as for infected cells.

For dot blots, RNA from muscle and infected cells, isolated in the presence or absence of 350 μ M cycloheximide, was dotted onto nylon filters (Gene Screen II plus) in 10-fold dilutions. Membranes were then vacuum dried. Filters were prehybridized in 6 \times SSPE (1 \times SSPE: 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 1% SDS, 200 μ g ml⁻¹ salmon testis DNA and 50% formamide for 30 min at room temperature. Filters, rinsed in hybridization solution (prehybridization solution without salmon testis DNA), were then hybridized with 2 \times 10⁶ cpm ml⁻¹ gamma-³²P-dATP labeled oligonucleotide probes (labeled with T4 nucleotide kinase as described [17]) for various gene sequences (Table I) at room temperature for 16 h. Filters were then washed twice in 2 \times SSPE, 1% SDS at room temperature, followed by two washes at 60°C for 20 min. Dried filters were then autoradiographed.

For northern blots, 20 μ g of RNA were separated on 1.2–1.5% agarose formaldehyde gels and transferred to nylon filters which were vacuum dried, as described (26). Filters were prehybridized and hybridized as with dot blots except that hybridizations were done at 42°C in the presence of salmon testis DNA and final washes were done at 65°C in 0.2 \times SSPE and 0.1% SDS. Probes used in northern blots were inserts derived from plasmids containing cDNAs for Id [pE:Id(S) (3)] and acetylcholine receptor subunit- α (AChR- α) (pAChRA [25]). Digests of plasmids with SmaI and EcoRI were used to excise inserts from pE:Id(S) and pAChRA, respectively. Inserts were electroeluted from gels and labeled with α -³²P-dATP by a random priming kit (Boehringer-Mannheim Corp., Indianapolis, IN) and used at 2 \times 10⁶ cpm ml⁻¹. Transcript sizes were estimated from RNA markers (GIBCO, BRL, Gaithersburg, MD) of 1.77, 1.62, 1.28, 0.78, 0.53, 0.4, 0.28, and 0.16 kb.

For PCR analysis, specific primers were made for β -actin, α -actin, myosin heavy chain, MyoD1 and myogenin (Table I). RNA used in reactions was from muscle at 1 μ g alone or 0.1, 0.01, and 0.001 μ g mixed with 1 μ g of liver RNA, from infected cells at 1 or 0.1 μ g (adjusted to 1 μ g with liver RNA) and from liver at 1 μ g. Samples containing RNA were first reverse transcribed in 20 μ l [50 mM KCl, 20 mM Tris-Cl (pH 8.4), 2.5 mM MgCl₂, 0.1 mg ml⁻¹ nuclease free BSA (GIBCO-BRL), 20 units RNasin (Promega Corp., Madison, WI), 200 U MLV reverse transcriptase (GIBCO-BRL), 0.1 μ g oligo-dT and 1 mM each dNTPs] at 42°C. After cDNA synthesis reaction mixes were heated to 65°C and adjusted to 100 μ l (50 mM KCl, 20 mM Tris-Cl [pH 8.4], 0.1 mg ml⁻¹ nuclease-free BSA, 50 pM forward and reverse primers and 2.5 U Taq DNA polymerase [Perkin Elmer

Table I. Summary of Oligonucleotide Probes and PCR Primers

Gene (reference)	Forward/Reverse primer	Probe
Dot blots		
actin* (1)	N/A‡	GAAGGTGTGGTGCCAGATCTTCTCCATGTCGTCCCA
α-actin (1)	N/A	GTCGTCCTGAGGAGAGAGAGCGCAACGCAGACGCG
MHC§ (39)	N/A	CCGTTCCGATGTTGCGCTTCTGTTTCATTTCCACCTC
MCK (4)	N/A	GCCGAAACCCAGAGGACAGAGCCCAATGATTGGACT
PCR		
α-actin (1)	GTCGACATCAGGAAGGACCT GAGAGAGCGCAACGCAGAC	AACGTCATGTCAGGGGGCACCACCATGTACCCTGGT
β-actin (1)	TTGGGTATGGAATCCTGTGG GGTGTAAAACGCAGCTCAGT	GAAACTACATTCAATCCATCATGAAGTGTGACGTT
MHC (39)	TTGGTGGACAACTACAGAC TTCTTCGCTTATGACTTTAG	AAAGTGAAAGCCTACAAGAGACAGGCTGAGGAGGCT
MyoD1 (9)	CCTGTCCAGCATAGTGGAGC AATCGCATTGGGGTTTGAGC	GTCGCTTAGGGATGCCCCCTCTGGCGGACCCGGAGG
Myogenin (14)	GGCTATGAGCGGACTGAGCT GAGCAAGGCCCTGTAGGCGCT	CTGCCGTGGGCATGTAAGGTGTGTAAGAGGAAGTCT

* Cyttoplasmic and α-actin.

‡ Not applicable.

§ Adult skeletal muscle myosin heavy chain.

|| Muscle creatine kinase.

Cetus Corp., Norwalk, CT]). Reactions cycled among the following temperatures: 94°C (1 min), 55°C (2 min) and 72°C (3 min). Sequences were amplified in 20–30 cycles depending on the sequence of interest. In control experiments 1 μg each of muscle and infected cell RNA were mixed and amplified. In no case was amplification of muscle specific transcript sequences inhibited by infected cell RNA. PCR products were analyzed on 6% polyacrylamide gels that were electroblotted (Novablot, Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) to nylon filters. Filters were hybridized to 36-nucleotide gene probes (Table I) which were labeled and hybridized under conditions used for dot blots. In control experiments, pretreatment of RNA with RNase (500 μg ml⁻¹) eliminated the generation of PCR products for each gene investigated, indicating RNA dependent amplification.

Results

During regeneration, satellite cells are induced to replicate and they then fuse with the damaged muscle cell, donating their nuclei (5, 34, 35). To investigate the origin of infected cell nuclei, groups of mice harboring synchronized muscle infections were labeled with 3HT for 4-d periods beginning

at several different times post-infection. Infected tissue was harvested at maturity (20 dpi) and autoradiographed. About 85% of infected cell nuclei could be labeled in these experiments, and labeling occurred over a well defined period of time (2–5 dpi) (Figs. 1 and 2). No labeling of nuclei was detected when chronically infected mice (harboring infections >40 d old) were injected with 3HT over a 3-d period (not shown).

To determine if labeled nuclei are stably integrated into the host cell, infected cell nuclei were labeled from 2–5 dpi and tissues harvested at 20, 40, or 60 dpi. Regardless of the time obtained post-infection, ~85% of nuclei were labeled for all infected tissues (Fig. 2), indicating stable integration into the infected cell without perceptible turnover.

The origin of 3HT labeled nuclei was investigated by comparing nuclei labeling and acquisition in regenerating (induced by clamping) or infected muscles which were irradiated (2 krad) immediately before inducing either form of trauma. In sections of clamped nonirradiated muscle, numer-

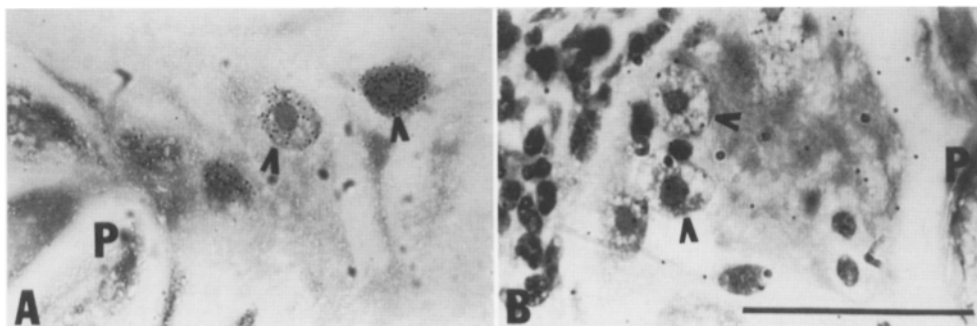


Figure 1. 3HT labeling of infected cell nuclei. Mice harboring synchronized infections of *T. spiralis* muscle larvae were injected three times daily with 3HT for 4-d intervals beginning at various dpi, tissue was harvested at 20 dpi and autoradiographed. Sections from infections labeled between 2–6 dpi (A) and 6–10 dpi (B) are shown. Results are representative of two similar experiments in which two mice per time interval were used and several other experiments with different designs. Complete results are summarized in Fig. 2 A. Bar, 75 μm.

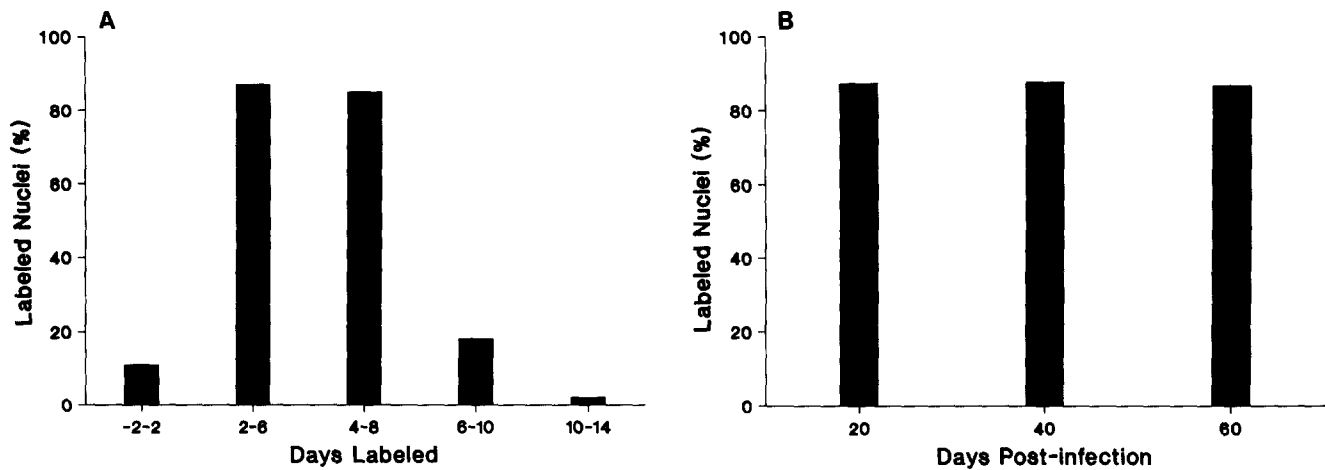


Figure 2. Timing of 3HT uptake by infected cell nuclei. (A) Graphic representation of experiments from Fig. 1. 100 nuclei from infected cells were counted for each of two mice labeled during the intervals shown. (B) Synchronized infections were initiated and mice labeled from 2-6 dpi, as in Fig. 1. Infected tissues were harvested at 20, 40, and 60 dpi and percentage of labeled nuclei determined for 100 infected cell nuclei from each of two mice at each time point. Results in B are from a single experiment.

ous tubular cells were observed with classic characteristics of regenerating muscle including centralized nuclei which were labeled with 3HT (Fig. 3). In many cases, the cytoplasm of these cells was continuous with other cytoplasmic regions in which changes were less pronounced and striations were evident. In contrast, prior irradiation of the traumatized contralateral muscle completely prevented regeneration (Fig. 3). Damaged, irradiated muscle was identified by its loss of myofibrillar structure, decreased level of staining, occurrence of associated infiltrating cells, and its degenerative appearance. For the irradiated limb, no labeled nuclei were detected in damaged muscle cells, nor was there any evidence of a regeneration response detected for cells in any tissue sections from these muscles. The results are in close agreement with those obtained by others using irradiated rat muscle damaged by cold (32, 33). In contrast, infected cell nuclei of pre-irradiated muscles were both present and labeled in infected muscle cells at 5 dpi (Fig. 3). Beyond the fact that *T. spiralis* only infects muscle cells, that these cells are infected muscle cells is indicated by the following: (a) only cells containing parasites were considered, (b) myofibrillar proteins can be detected in all infected cells at 5 dpi (27), and (c) regions of infected cell cytoplasm lacking striations were often continuous with regions which were less affected, but in which striations were present (not shown).

Because of the extensive tissue damage and inflammatory response it was not possible to quantitatively compare labeled nuclei in nonirradiated and irradiated infected muscle at 5 dpi. However, the percentage of nuclei labeled between 2-5 dpi and present at 20 dpi in nonirradiated (86%) and irradiated muscle cells (92%) was not statistically different (Chi square, $p > 0.05$). It was concluded from these experiments that infected cell nuclei are not derived from satellite cells during a normal regeneration response.

To further assess the origin of infected cell nuclei, satellite cells of baby mice were labeled with 3HT in utero and during 5 d after birth as described in Materials and Methods. Labeled muscle satellite cells were chased into myotubes during a 4-wk period in which mice received no 3HT. If muscle

is then induced to regenerate, labeled adult skeletal muscle nuclei are replaced by unlabeled satellite cells (34). Preliminary experiments demonstrated that nuclei labeled in this way remained after infection (data not shown). The experiment was repeated, including regenerating muscle as a control. As shown in Table II, <1% of nuclei in regenerating muscle were detectably labeled, indicating (a) that the label was effectively chased from the muscle precursor cell population, and (b) that reutilization of label by muscle precursor cells (21) is not an issue here. In contrast, labeled nuclei persisted in infected muscle cells at a similar level as that found in normal muscle.

It was also shown that nuclei within infected muscle cells could be labeled on day 5 pi in a 3-h period (Fig. 3). Although not all infected cell nuclei were labeled detectably, results demonstrate that many can be during the short time employed. Furthermore, these nuclei were located away from the membrane and well within the muscle cell cytoplasm. Little 3HT labeling was detected in nuclei of other cells in these sections. Hence, the results indicate that

Table II. Retention of Differentiated Skeletal Muscle Nuclei by Muscle Cells Infected with *Trichinella spiralis*

Cell type	Number unlabeled	Number labeled	Percentage labeled
Regenerating muscle	1,061	3	0.3
Control*	1,128	106	8.6
Infected muscle (10 dpi)†	940	168	15.1
Control	1,071	206	16.1
Infected muscle (20 dpi)	969	116	10.7
Control	1,046	92	8.1

* Normal muscle cell nuclei counted from contralateral legs served as controls, † days post-infection. Muscle from mice labeled with tritiated thymidine in utero and 3 and 5 d post-parturition was infected with 50,000 *T. spiralis* newborn larvae or induced to regenerate. Regenerating muscle was obtained at 5 d post-trauma. All tissues were exposed in autoradiography for 40 d. Counts were pooled from three mice in each treatment group.

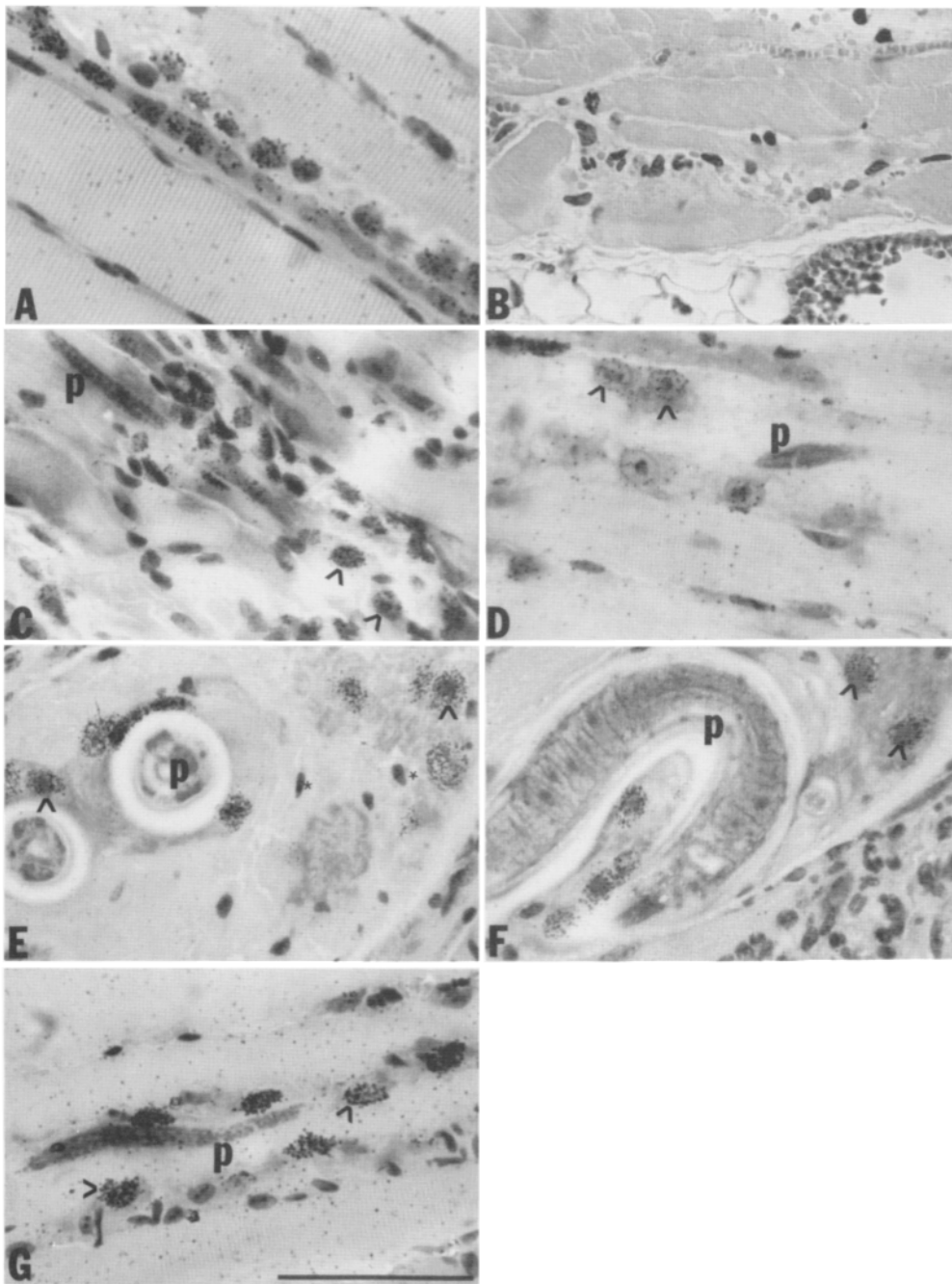


Figure 3. Comparison of 3HT nuclei labeling in regeneration-inhibited and *T. spiralis* infected skeletal muscle. (A) Nonirradiated, crushed muscle, 5 d post-trauma, labeled from days 0-4. Note centralized, labeled nuclei in cells running on the central diagonal from lower right to upper left. (B) Eosin and hematoxylin histological sections of pre-irradiated (2 krad) damaged muscle at 5 d post-trauma (crushing). Note absence of enlarged, centralized nuclei. (C and D) Nonirradiated and pre-irradiated, respectively, *T. spiralis* infected muscle cells, 5 dpi, labeled from 0-4 dpi. (E and F) Nonirradiated and pre-irradiated infected muscle cells, 20 dpi, labeled from 2-5 dpi. (G) Nonirradiated infected muscle cells, 5 dpi, labeled over a 3 h period on day 5 pi, as described in the methods. (Arrowheads) Examples of infected cell nuclei; (asterisks) examples of infiltrating cells; (P) parasite. The bar represents 75 μm for B-G and 50 μm for A. A-D, and G were derived from mice within the same experiment, similar results were obtained in two experiments for A-D. (E and F) Results are representative of three independent experiments. (G) Similar results were obtained in two additional experiments, except that an 8 h labeling period was used for these. Three mice were included for each treatment in all experiments represented here.

3HT uptake by infected cell nuclei occurs within the infected cell.

The 3HT incorporation could reflect DNA repair or replication. If replication occurs, then it is anticipated that these nuclei would contain a $>2N$ complement of DNA because karyokinesis has never been observed in the infected cell. This possibility was tested by quantitating staining of infected cell nuclei with the DNA binding dye Hoechst 33258. Infected cell nuclei preparations were contaminated by infiltrating cell nuclei, but could be distinguished from the latter by their large size and prominent nucleoli. Mean fluorescence intensity of infected cell nuclei was approximately twice that of infiltrating cell nuclei (Table III). The mean fluorescence of nuclei from in vitro grown-mouse as-

Table III. Mean Fluorescence Intensity of Nuclei Stained with the DNA Binding Dye Hoechst 33258

Cell source of nuclei	Experiment	N	Mean fluorescence (\pm SD)	P*
Infected	1	46	20.6 (4.3)	<0.00001
Infiltrating	1	46	11.5 (3.5)	
Infected	2	39	50.1 (10.5)	<0.00001
Infiltrating	2	36	26.9 (9.4)	
Infected	3	40	30.2 (4.9)	<0.00001
Infiltrating	3	40	16.6 (2.3)	
Astrocyte	3	40	19.9 (6.4)	

* Statistical probability level for differences between means of infected cell nuclei and nuclei from infiltrating and astrocyte cells.

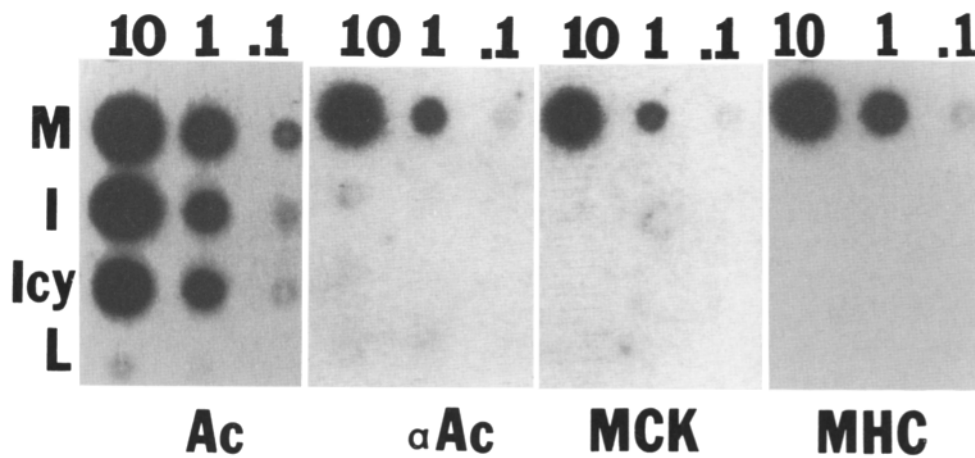


Figure 4. Muscle gene RNA transcripts are decreased in the infected cell. Oligonucleotide probes, *Ac* (cytoplasmic and α -actin), α *Ac*, (α -actin) *MCK* (muscle creatine kinase), and *MHC* (adult skeletal myosin heavy chain), were hybridized to RNA from muscle (*M*), infected cells (*I*), infected cells isolated in the presence of cycloheximide (*Icy*), and liver. Numbers at the top indicated micrograms of total RNA.

trocytes (range 14–35) was slightly higher than that for infiltrating cells, which is expected for an actively growing population of cells. Using experiment 3 as an example, fluorescence intensity for infected cell nuclei were unimodal, and 63% of nuclei had values in fluorescence units between 26 and 34. Fluorescence intensity for astrocyte nuclei extended over an approximate twofold range, the maximum of which was very near the mean for infected cell nuclei. These results indicate that infected cell nuclei have $\sim 4N$ complement of DNA.

To investigate muscle gene expression in the infected cell, quantitative dot blots were conducted on RNA from the infected cell using muscle gene probes (Fig. 4). Transcripts for myosin heavy chain and muscle creatine kinase were not detectable, while very weak signal for α -actin transcripts was observed and the signal using the generic actin probe was

comparable in muscle and the infected cell. The level of muscle specific transcripts investigated were reduced to $<1\%$ in isolated infected cells compared to normal muscle. Addition of cycloheximide during the isolation of infected cells had no effect on the outcome of these experiments.

Results of PCR assays of myofibrillar transcripts, similar to experiments shown in Fig. 5, were consistent with those obtained for dot blots (not shown). In this case, α -actin and myosin heavy chain transcripts were detected in RNA preparations from the infected cell but at levels between 1% and 0.1%, respectively, compared to normal muscle. The possibility that the assay detected contaminating transcripts from normal muscle cannot be excluded.

Using PCR analysis, it was further determined that *MyoD1* transcripts were reduced to $<0.1\%$, and myogenin transcripts were reduced to $<10\%$ (Fig. 5) compared to lev-

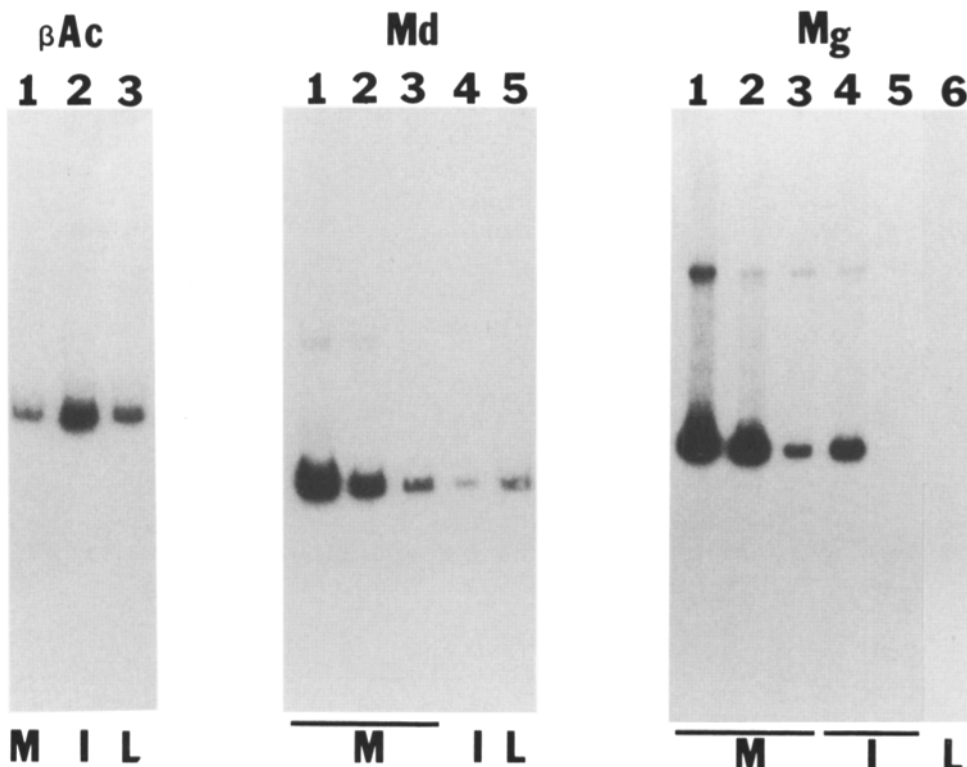


Figure 5. *MyoD1* and myogenin transcript levels are decreased in the infected cell. Gene specific primers (Table I) for β -actin (β *Ac*), *MyoD1* (*Md*) or myogenin (*Mg*) were used to amplify RNA sequences from muscle (*M*), infected cells (*I*) or liver (*L*). Gene-specific probes were then hybridized to Southern blots of the PCR products. Micrograms of tissue specific RNA used in each lane is as follows: β -*Ac*, 1 μ g each lane; *Md*, 1 μ g (lanes 1, 4, and 5), 0.1 and 0.01 μ g (lanes 2 and 3, respectively); *Mg*, 1 μ g (lanes 1, 4, and 6), 0.1 μ g (lanes 2 and 5), 0.01 μ g (lane 3). Reactions with <1 μ g of tissue specific RNA (total RNA) were adjusted to 1 μ g with RNA from liver. Dominant PCR products indicated by a hashmark occur at expected sizes of 360 (β -actin), 192 (*MyoD1*) and 309 bp (myogenin).

els in muscle. The low level detection of MyoD1 transcripts in liver cells (lane 5) does not appear to be from contamination, because this result was repeatable and specific for MyoD1 as compared to myogenin and other muscle specific transcripts. In contrast, transcript levels for a negative transcriptional regulatory protein (Id) were ~ 2.5 times higher in isolated infected cells compared to normal muscle, and equivalent transcript levels were detected in RNA from infiltrating cells associated with the infected cell (Fig. 6).

It was shown previously (29) that expression of AChR- α transcripts is independent of that for other muscle gene transcripts which are induced by MyoD1 or myogenin. In contrast to other muscle specific genes, transcripts for AChR- α were detected in the infected cell at levels similar to that in normal skeletal muscle (Fig. 6), but not detected in RNA from the infiltrating cell control. This result indicates that the integrity of infected cell RNA is maintained during isolation procedures and supports the findings that the decreased levels of other muscle transcripts are real. Using the PCR, transcripts for mouse myogenin, Id and AChR- α have not

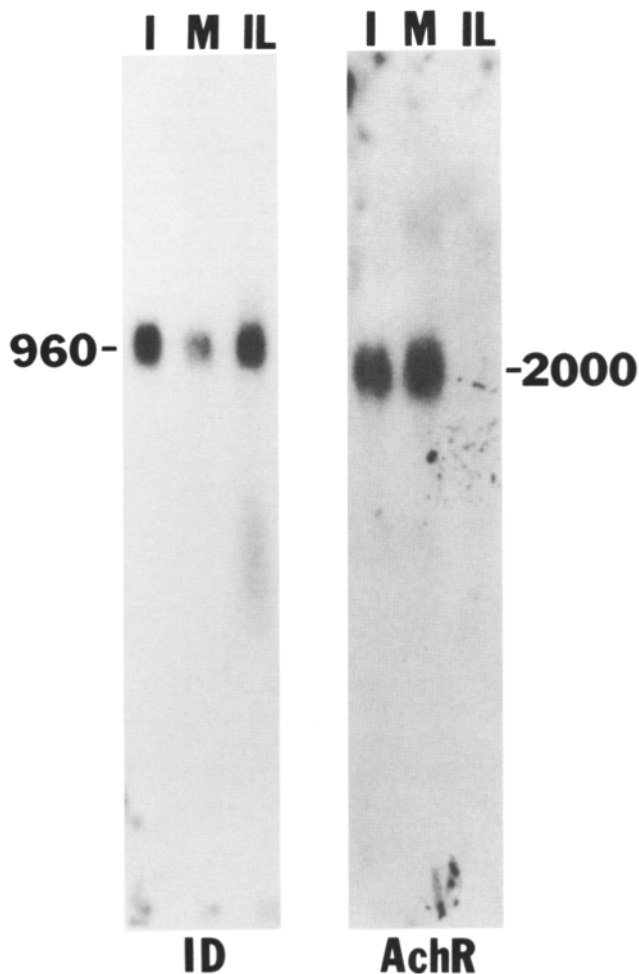


Figure 6. Id and acetylcholine receptor- α transcripts expression in the infected cell. Total RNA (20 μ g) from muscle (M), infected cell (I), or infiltrating cells associated with the infected cell (IL), was hybridized in Northern blots to probes specific for Id or acetylcholine receptor- α (AChR). Numbers on the sides refer to transcript sizes in nucleotides.

been detected in RNA from *T. spiralis* (not shown). In addition, based on RNA yields from isolated infiltrating cells and cell counts in infected cell preparations, it was estimated that the maximum contamination by infiltrating cells was 10% in infected cell RNA preparations. Thus, contamination by infiltrating cell RNA cannot account for transcripts detected in infected cell RNA.

Discussion

Results presented here provide strong evidence that nuclei of the infected cell are derived from adult skeletal muscle nuclei present at the time of infection. Prior irradiation of muscle completely inhibited regeneration in traumatized, uninfected muscle, but did not block 3HT labeling of infected cell nuclei or normal morphological development of *T. spiralis* infected muscle cells. 3HT incorporation by nuclei within the infected cell further indicates that infected cell nuclei are not derived from satellite cells. In addition, differentiated muscle cell nuclei labeled before infection were retained after infection. An alternative origin of infected cell nuclei is infiltrating cells which fuse with the infected muscle during the first 5 dpi. However, this scenario would require a process which resulted coincidentally in similar levels of prelabeled nuclei in infected and uninfected muscle cells, a possibility which seems very unlikely. The data most strongly support differentiated muscle cell nuclei as the source of infected cell nuclei.

Nuclei of infected cells were induced to incorporate 3HT predominately between 2–5 dpi. These nuclei were stably integrated into the infected cell for at least 60 dpi and presumably for the life of the cell. Thus, nuclei obtained from chronically infected cells represent those present at the time of infection. Because nuclei from chronically infected cells possess an approximate 4N complement of DNA, it follows that 3HT uptake represents DNA synthesis by infected muscle cells which are subsequently blocked in G₂/M. Furthermore, because 3HT incorporation was essentially completed by 5 dpi, suspension in the cell cycle appears to be a feature of these cells throughout the course of infection, which can last for years.

Although myoblasts withdraw from the cell cycle before fusion, there is precedence for induced DNA synthesis in nuclei of skeletal muscle cells and subsequent suspension in G₂/M. SV40, polyoma and Rous sarcoma viruses induce DNA replication in in vitro cultured skeletal muscle cells (18, 20, 23, 40). It has also been shown that large T antigen induces these changes (15, 22), which may occur via interactions with the retinoblastoma protein (16). While no data currently exist regarding factors involved, *T. spiralis* may be able to induce DNA synthesis in differentiated muscle cells via similar mechanisms. Once replication occurs, both virus and parasite infected cells apparently become suspended in G₂/M of the cell cycle (15, 18, 20, 40). The apparent suspension in G₂/M in both virus and parasite muscle infections could reflect either an inhibitory effect by each of these agents, or a characteristic of the host cell. For instance, centrioles tend to be lost from fused muscle cells (7, 37), and centrioles are required for progression through M phase in at least one mammalian cell system (31). It is possible that in the absence of centrioles, fused skeletal muscle cells once stimulated to enter S phase will inevitably become sus-

pended in the cell cycle before completing mitosis. Because mitotic figures were evident in the virus infected and large T antigen transformed cells, centrioles could be more persistent in the in vitro cultured cells, providing for occasional passage into M phase.

Changes in *T. spiralis* infected cell nuclei are associated with decreased muscle gene expression which appears to be regulated transcriptionally. This parasite effect on muscle gene expression appears to be unique because similar changes were not observed in SV40 virus infected muscle cells (22). These changes in muscle gene expression may represent dedifferentiation of skeletal muscle cells, since myogenin and AChR- α gene products are also expressed in myoblasts/satellite cells (8, 21). This similarity with proliferating muscle precursor cells could reflect identity, and analysis of other cellular products differentially expressed during myogenesis should help resolve this issue. Our results indicate that the altered expression of muscle transcriptional regulatory factors proximally regulate decreased muscle gene expression in the *T. spiralis* infected cell. In addition, other regulatory factors are likely to be involved. Both Fos and Jun have been implicated in inhibiting muscle gene expression (29, 30), and preliminary evidence indicates that *fos*, *c-jun*, and *jun B* gene expression are increased in the infected cell (unpublished data). Furthermore, the status of transcripts for myogenin and AChR- α is reminiscent of that for C2C12 myoblasts transfected with activated *ras* oncogene and grown in differentiation medium (29). Therefore, other regulatory factors may act distally on muscle gene expression in this infected cell. The order in which genes for these regulatory factors are expressed during development of the infected cell is not clear but will be important for elucidating cellular pathways leading to expression of the infected cell phenotype.

The results show that normal host cellular mechanisms might account for altered muscle gene expression in the infected cell. Therefore, muscle genes and other infected cell characteristics could be indirectly influenced by the parasite. The timing of 3HT uptake is temporally correlated with the occurrence of increased ACP activity in the infected cell (which can be detected by 5 dpi [27]). Thus, 3HT uptake may represent "the" host cellular event which initiates expression of some or all characteristics of the infected cell phenotype. Because withdrawal from the cell cycle is naturally concomitant with expression of the myogenic program, an inability to proceed through M phase could preclude the infected cell from expressing the myogenic program. If true, parasite factors affecting muscle gene expression may do so by inducing infected muscle cells to enter into S phase rather than by direct interaction with muscle structural genes or transcriptional regulatory factors. Detection of muscle gene transcripts in large T antigen expressing myotubes blocked in G₂/M (22) does not invalidate this proposal, because these cells are short-lived and muscle gene transcripts may be lost with additional time. In addition, since large T antigen is known to bind several different cellular proteins (16), it may interfere with the function of negative muscle regulatory factors in these cells. Because of the *T. spiralis*/muscle cell system is long-lived, it provides an important model to investigate these questions.

Finally, it is worth noting that parasite antigens have been identified in the nucleus of the infected cell (12). However,

they have not been detected before 7 dpi, and by this time host nuclei no longer incorporate 3HT. While the low level presence of these antigens earlier than 7 dpi cannot be excluded, other factors of host and/or parasite origin may induce the DNA synthesis, nuclear hypertrophy and increased ACP activity of the infected cell, which all occur at or before 5 dpi.

I would like to thank Richard Dixon, Stewart Bohnet and Sufang Yao for excellent technical assistance provided throughout this research. I also thank Donald P. Knowles and Mark M. Robinson for their critical reading and discussion of this manuscript, Stephen Tapscott for discussions regarding centrioles in muscle cells and Harold Weintraub for providing the pE:Id(S) plasmid.

This work was supported by the National Institutes of Health (R29 AI 26157).

Received for publication 1 October 1992 and in revised form 24 February 1993.

References

- Alonso, S., A. Minty, Y. Bourlet, and M. Buckingham. 1986. Comparison of three actin-coding sequences in the mouse; evolutionary relationships between actin genes of warm-blooded vertebrates. *J. Mol. Evol.* 23:11-22.
- Baruch, A. M., and D. D. Despommier. 1991. Blood vessels in *Trichinella spiralis* infections: a study using vascular casts. *J. Parasitol.* 77:99-103.
- Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell.* 61:49-59.
- Buskin, J. N., J. B. Jaynes, J. S. Chamberlain, and S. D. Hauschka. 1985. The mouse creatine kinase cDNA and deduced amino acid sequences: comparison to evolutionarily related enzymes. *J. Mol. Evol.* 22:334-341.
- Carlson, B. M., and J. A. Faulkner. 1983. The regeneration of skeletal muscle fibers following injury: a review. *Med. Sci. Sports Exerc.* 15: 187-198.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Connolly, A., B. W. Kiosses, and I. Kalnins. 1985. Centrioles are lost as embryonic myoblasts fuse into myotubes in vitro. *Eur. J. Cell Biol.* 39:341-345.
- Cossu, G., F. Eusebi, F. Grassi, and E. Wanke. 1987. Acetylcholine receptor channels are present in undifferentiated satellite cells but not in embryonic myoblasts in culture. *Dev. Biol.* 123:43-50.
- Davis, R. L., H. Weintraub, and A. B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell.* 51:987-1000.
- Despommier, D. D. 1975. Adaptive changes in muscle fibers infected with *Trichinella spiralis*. *Am. J. Pathol.* 78:477-496.
- Despommier, D. D., L. Aron, and L. Turgeon. 1975. *Trichinella spiralis*: growth of the intracellular (muscle) larvae. *Exp. Parasitol.* 37:108-116.
- Despommier, D. D., A. M. Gold, S. W. Buck, V. Capo, and D. Silverstein. 1990. *Trichinella spiralis*: secreted antigen of the infective L₁ larva localizes to the cytoplasm and nucleoplasm of infected host cells. *Exp. Parasitol.* 71:27-38.
- Despommier, D., W. F. Symmans, and R. Dell. 1991. Changes in nurse cell nuclei during synchronous infection with *Trichinella spiralis*. *J. Parasitol.* 77:290-295.
- Edmondson, D. G., and E. N. Olson. 1989. A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev.* 3:628-640.
- Endo, T., and B. Nidal-Ginard. 1988. SV40 large T antigen induces reentry of terminally differentiated myotubes into the cell cycle. In *Cellular and Molecular Biology of Muscle Development*. L. H. Kedes and F. E. Stockdale, editors. Alan R. Liss Inc., New York. 95-104.
- Fanning, E. 1992. Simian virus 40 large T antigen: the puzzle, the pieces, and the emerging picture. *J. Virol.* 66:1289-1293.
- Feagin, J. E., D. P. Jasmer, and K. Stuart. 1987. Developmentally regulated addition of nucleotides within apocytochrome *b* transcripts in *T. brucei*. *Cell.* 49:337-345.
- Fogel, M., and V. Defendi. 1967. Infections of muscle cultures from various species with oncogenic DNA viruses (SV40 and Polyoma). *Proc. Natl. Acad. Sci. USA.* 58:967-973.
- Gabryel, P., and L. Gustowska. 1967. Veranderungen der quegestreiften muskelfaseren im fruzen stadium since *Trichinella spiralis*-infektion. *Gegenbauers Morph. Jahrb.* 111:174-180.
- Graessmann, A., M. Graessman, and M. Fogel. 1973. The relationship of polyoma virus-induced tumor (T) antigen to activation of DNA synthesis

- in rat myotubes. *Dev. Biol.* 35:180-186.
21. Grounds, M. D., and J. K. McGeachie. 1987. Reutilisation of tritiated thymidine in studies of regenerating skeletal muscle. *Cell Tissue Res.* 250:141-148.
 22. Iujvidin, S., O. Fuchs, U. Nudel, and D. Yaffe. 1990. SV40 immortalizes myogenic cells: DNA synthesis and mitosis in differentiating myotubes. *Differentiation.* 43:192-203.
 23. Lee, H. H., M. E. Kaighn, and J. D. Ebert. 1968. Induction of Thymidine-³H-incorporation in multinucleated myotubes by Rous sarcoma virus. *Int. J. Cancer.* 3:126-136.
 24. Maeir, D. M., and H. Zaiman. 1966. The development of lysosomes in rat skeletal muscle in trichinosis myositis. *J. Histochem. Cytochem.* 14:396-400.
 25. Isenberg, K. F., J. Mudd, V. Shah, and J. P. Merlie. 1986. Nucleotide sequence of the mouse muscle nicotinic acetylcholine receptor α subunit. *Nucleic Acids Res.* 14:1511.
 26. Jasmer, D. P., J. E. Feagin, and K. Stuart. 1985. Diverse patterns of expression of the cytochrome c oxidase subunit I gene and unassigned reading frames 4 and 5 during the life cycle of *Trypanosoma brucei*. *Mol. Cell. Biol.* 5:3041-3047.
 27. Jasmer, D. P., S. Bohnet, and D. J. Prieur. 1990. *Trichinella spp.*: differential expression of acid phosphatase and myofibrillar proteins in infected muscle cells. *Exp. Parasitol.* 72:321-331.
 28. Jasmer, D. P. 1990. *Trichinella spiralis*: altered expression of muscle proteins in trichinosis. *Exp. Parasitol.* 70:452-465.
 29. Lasser, A. B., M. J. Thayer, R. W. Overell, and H. Weintraub. 1989. Transformation by activated *ras* or *fos* prevents myogenesis by inhibiting expression of MyoD1. *Cell.* 58:659-667.
 30. Li, L., J-S. Hu, and E. N. Olson. 1990. Different members of the *jun* protooncogene family exhibit distinct patterns of expression in response to type β Transforming growth factor. *J. Biol. Chem.* 265:1556-1562.
 31. Maniotis, M., and M. Schliwa. 1991. Microsurgical removal of centrosomes blocks cell reproduction and centriole generation in BSC-1 cells. *Cell.* 67:495-504.
 32. Reznik, M. 1967. Influence d'une irradiation locale unique sur le muscle strie' en regeneration. *Arch. Biol.* 78:91-106.
 33. Reznik, M., and E. H. Betz. 1967. Influence de l'irradiation local prealable sur les capacites regeneratrices du muscle strie' squelettique. *Pathol. Eur.* 2:69-80.
 34. Snow, M. H. 1978. An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscles in young rats. *Cell Tissue Res.* 186:535-540.
 35. Snow, M. H. 1977. Myogenic cell formation in regenerating rat skeletal muscle injured by mincing. *Anat. Rec.* 188:201-208.
 36. Stewart, G. L. 1983. Pathophysiology of the muscle phase. In *Trichinella* and trichinosis. W. C. Campbell, editor. Plenum Publishing Corp., New York. 241-264.
 37. Tassin, A.-M., B. Maro, and M. Bornens. 1985. Fate of microtubule-organizing centers during myogenesis in vitro. *J. Cell Biol.* 100:35-46.
 38. Teppema, J. S., J. E. Robinson, and E. J. Ruitenbergh. 1973. Ultrastructural aspects of capsule formation in *Trichinella spiralis* infection in the rat. *Parasitology.* 66:291-296.
 39. Weydert, A., P. Daubas, M. Caravatti, A. Minty, G. Bugaisky, A. Cohen, B. Robert, and M. Buckingham. 1983. Sequential accumulation of mRNAs encoding different myosin heavy chain isoforms during skeletal muscle development in vivo detected with a recombinant plasmid identified as coding for an adult fast myosin heavy chain from mouse skeletal muscle. *J. Biol. Chem.* 258:13867-13874.
 40. Yaffe, D., and D. Gershon. 1967. Multinucleated muscle fibers: induction of DNA synthesis and mitosis by polyoma virus infection. *Nature (Lond.)* 215:421-424.