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22. We ackowledge help and discussion with J. Habener and G. Heinrich, technical assistance from J. Evans, V. Stranov, and C. Pagonis, and assistance with the manuscript by J. Canniff. We are indebted to W. Schwartz for assistance with pilot studies. This work, from the Howard Hughes Medical Institute Neuroscience Laboratories, was also supported by the McKnight Foundation, Sloan Foundation, American Parkinson Disease Association, NIMH, NINCDS, and the American Heart Association (S.R., established investigator).

17 September 1985; accepted 6 January 1986

Existence of High Abundance Antiproliferative mRNA's in Senescent Human Diploid Fibroblasts

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Polyadenylated RNA isolated from senescent human diploid fibroblasts (HDF) inhibited DNA synthesis in proliferation-competent cells after microinjection, whereas polyadenylated RNA from young HDF had no inhibitory effect. Polyadenylated RNA from young cells made quiescent by removal of serum growth factors had a slight inhibitory effect on DNA synthesis. The abundance level of inhibitor messenger RNA (mRNA) from senescent cells was estimated at 0.8 and that of quiescent cells at 0.005 percent. These results demonstrate the existence of one or more antiproliferative mRNA's in nonproliferating normal human cells; these RNA's code for factors that either work antagonistically to initiators of DNA synthesis or regulate the expression of the initiators in some way. The abundance level of the inhibitory mRNA in senescent cells indicates the feasibility of developing a complementary DNA probe that will be useful in studying cell cycle control mechanisms.

LIMITED PROLIFERATIVE POTENTIAL is an inherent property of normal cells grown in culture (*1, 2*) and leads to cellular senescence. The mechanisms responsible for this phenomenon and the events that result in loss of proliferative control and cellular immortalization are not known. However, cell fusion studies have demonstrated that the phenotype of limited proliferation is dominant over the phenotype of immortality (*3–5*). We have found that senescent cells produce a membrane-associated protein that inhibits initiation of DNA synthesis (*6*). It is likely that production of this inhibitory activity is part of the mechanism resulting in cellular senescence. Conversely, modification in the protein itself or in regulation of its production would result in immortalization, which is necessary for tumor progression. Identification of the protein or the nucleic acid sequence coding for the protein would be important in understanding the regulation of cell division. As a first step in the identification of the RNA coding for this inhibitor protein, we microinjected polyadenylated [poly(A)$^+$] RNA isolated from senescent and from young cycling human diploid fibroblasts (HDF) into proliferation-competent cells.

Proliferation-competent cells were obtained by arresting young HDF in the G_1 phase of the cell cycle by maintenance in medium containing a low concentration of serum (0.5 percent) for at least 1 week (*7*). Polyadenylated RNA was isolated from young cells having at least 50 population doublings remaining (*8*) and from senescent cells that could not grow to confluence in 4 weeks after subculture and had a labeling index of <1 percent (after 24 hours of labeling). The poly(A)$^+$ RNA, at a concentration of 1 mg/ml, was microinjected into the cytoplasms (4×10^{-11} ml per cell) of the proliferation-competent cells (*8, 9*). Immediately after injection, the medium was replaced with medium containing 10 percent fetal bovine serum (FBS) and [^{3}H]thymidine (0.1 μCi/ml). The cells were incubated for approximately 16 hours, at which time most of the uninjected cells were labeled. They were then fixed in 95 percent ethanol and processed for autoradiography. The senescent cell poly(A)$^+$ RNA had a dramatic inhibitory effect while the young-cell poly(A)$^+$ RNA produced no significant inhibition (Table 1). To test whether the RNA preparation from senescent cells contained any nonspecific inhibitory activity, the material not bound to the oligo-deoxythymidylate column [non-poly(A)$^+$ RNA] was injected. This RNA did not inhibit DNA synthesis. The effect of the microinjection procedure itself on the incorporation of [^{3}H]thymidine was tested by microinjection of buffer. This resulted in a slight stimulation. The inhibitory effect of poly(A)$^+$ RNA from senescent cells was lost after incubation with ribonuclease A. Thus, intact poly(A)$^+$ RNA from senescent cells is responsible for inhibition of DNA synthesis. About 10 hours after the uninjected cells began to synthesize DNA, the cells injected with senescent cell poly(A)$^+$ RNA also entered S phase. We assume that this is due to intracellular degradation of the injected RNA.

Young cells prevented from proliferating for longer than 2 weeks by maintenance in medium containing only 0.5 percent serum (quiescent cells) also produce a membrane-associated protein that inhibits DNA syn-

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thesis (*6*). To determine if quiescent cells contained inhibitor messenger RNA (mRNA), we extracted poly(A)$^+$ RNA from young cells that had been made quiescent by maintenance in low serum for at least 2 weeks. Microinjection of this quiescent cell RNA, at a concentration of 1.7 mg/ml, resulted in a significant, though consistently low, inhibition of DNA synthesis (Table 1).

To determine the relative abundance of the senescent- and quiescent-cell inhibitor mRNA's, dilution experiments were done (Table 1). Senescent-cell poly(A)$^+$ RNA retained consistent inhibitory effect at a 1/30 dilution (0.03 mg/ml) but inhibition was lost or decreased at a 1/100 dilution. The quiescent-cell poly(A)$^+$ RNA achieved marked inhibition (57 percent) only at a concentration of 5 mg/ml, which was 160-fold greater than the concentration of senescent cell poly(A)$^+$ RNA that gave an equivalent inhibition. Control injections of young-cell poly(A)$^+$ RNA at 5 mg/ml showed a slight inhibitory effect. Whether this was due to competition for ribosomes or to the small percentage (1 to 2 percent) of senescent cells in young cell populations remains to be determined.

To calculate the relative abundance level of the inhibitor mRNA from quiescent and senescent cells, we make the following assumptions: (i) a microinjection volume of 4×10^{-11} ml per cytoplasm (*10*), (ii) a total RNA content per cell of 10^{-5} μg (*11*), (iii) poly(A)$^+$ RNA is 3 percent (3×10^{-7} μg) of total cellular RNA (*11*), (iv) a cell contains 3×10^5 mRNA molecules (*11*), and (v) at least ten copies of inhibitor mRNA are required for significant inhibition. With these assumptions, injection of 4×10^{-11} ml of senescent cell poly(A)$^+$ RNA at a concentration of 30 μg/ml (minimum concentration for consistent inhibition) results in the delivery of 1.2×10^{-9} μg of poly(A)$^+$ RNA per injected cell. Since 3×10^{-7} μg is the total poly(A)$^+$ RNA content per cell, this represents 1/250 of a cell equivalent of poly(A)$^+$ RNA injected. Thus, each senescent cell contains approximately 2500 copies of the inhibitor mRNA or an abundance of 0.8 percent. Similar calculations reveal that each quiescent cell contains approximately 15 copies of the inhibitor mRNA or a low abundance of 0.005 percent.

This marked difference between the copy numbers of inhibitory RNA in senescent and quiescent cells might be expected on the basis of recent results from cell fusion studies. The inhibitory activity of senescent cells is rapidly lost after cycloheximide treatment (*4, 5*), whereas the inhibitor activity of quiescent cells is stable in the presence of cycloheximide for periods of up to 24 hours (*6, 12*). Protein synthesis is required for the production of the inhibitor from quiescent cells, as demonstrated by the observation that when these cells are treated with trypsin (which eliminates the inhibitory activity) and then allowed to recover in the presence of cycloheximide, the inhibitory activity is not regained (*6*). These results indicate that the inhibitor protein from quiescent cells is very stable compared with the inhibitor from senescent cells. Our finding that inhibitor mRNA from senescent cells is 160-fold more abundant than that in quiescent cells conforms to the above data since the low turnover quiescence protein needs much less RNA to maintain an active level than the higher turnover senescence protein. These results provide strong evidence for the production of a high abundance mRNA coding for a protein that blocks initiation of DNA synthesis in senescent cells. It remains to be determined whether the production of this inhibitor is the primary cause of division cessation or occurs as a secondary result of some other event. We have not conclusively demonstrated that the injected mRNA is actually translated. However, since the effect is specific to poly(A)$^+$ RNA, sensitive to ribonuclease treatment, and present in dilute RNA solutions, the effect is most likely due to the messenger activity of the RNA.

The abundance of senescent cell inhibitor mRNA indicates the feasibility of screening a senescent cell complementary DNA (cDNA) library with cDNA from young cells to eliminate common sequences and then with senescent cell cDNA to identify sequences of 500 copies or more (*13*). It might then be possible to identify senescence-specific mRNA's by assaying cloned samples for inhibitory activity after microinjection. At this time we do not know if only one inhibitory mRNA or multiple mRNA's cause the inhibition of DNA synthesis we observe. However, if we consider the complex series of events that must occur to allow a cell to synthesize DNA, it is reasonable to suggest that inhibition of DNA synthesis will result if one of those events is inhibited. Therefore, we may indeed be dealing with a single species of inhibitory mRNA.

The dominance of the senescence-protein inhibitor was first demonstrated by the finding that DNA synthesis was inhibited in young and some immortal cells after fusion with senescent cells (*14*). This was confirmed by long-term studies of hybrid proliferation in which all hybrids obtained by fusion of normal cells with a variety of immortal cell types (including those used in the heterokaryon experiments) exhibited

Table 1. Differential inhibitory activity of poly(A)$^+$ RNA's isolated from senescent, quiescent, and young cells. For analysis of inhibition of DNA synthesis, the cells were divided into two categories: injected (within an etched circle) and uninjected (peripheral to the circle). Within each category the cells whose nuclei contained >10 silver grains were counted as labeled. The percent inhibition was calculated by the formula [(% labeled nuclei in uninjected cells − % labeled nuclei in injected cells)/% labeled nuclei in uninjected cells] × 100. The recipient cells had at least 50 population doublings remaining in their in vitro life-span. Approximately 12 to 16 hours after addition of medium containing high (10 percent) serum, a large percentage (72 to 90 percent) of these cells synchronously synthesize DNA and incorporate [^{3}H]thymidine. Any significant decrease in the percentage of labeled cells indicates an inhibitory effect (*19*). Experiments 1 and 2 were completely independent. The poly(A)$^+$ RNA's were obtained from different batches of cells at different times. Microinjection was performed at different times utilizing independently prepared recipient cells.

Material injected	Concentration (mg/ml)	Inhibition (%)*		Average inhibition (%)†
		Experiment 1	Experiment 2	
Buffer alone	0.0	−22.0 (164)	−2.3 (362)	−8.4
		Senescent cell RNA		
Poly(A)$^+$	1.0	52.6 (105)	80.5 (603)	76.4
	0.3	69.0 (286)		69.0
	0.1	72.5 (285)	87.2 (133)	77.2
	0.03	46.3 (185)	45.2 (234)	45.7
	0.01	4.0 (148)	39.0 (177)	23.1
Plus RNase	1.0	2.5 (345)		2.5
Non-poly(A)$^+$	2.0	2.9 (139)	0 (360)	0.8
		Quiescent cell RNA		
Poly(A)$^+$	5.0	79.0 (96)	35.0 (177)	50.5
	2.5	22.0 (209)		22.0
	1.7	9.0 (150)	11.2 (119)	10.0
		Young cell RNA		
Poly(A)$^+$	5.0	11.0 (159)		11.0
	2.5	1.8 (433)	2.2 (369)	2.0
	1.0	2.6 (182)	4.0 (120)	3.3

*Numbers in parentheses represent the number of cells injected. A negative number indicates stimulation of DNA synthesis. †Number-weighted average.

limited proliferative capacity (*3*). Recently, we have obtained more evidence (*15*) for the dominance of the senescence inhibitor in that microinjection of human c-H-*ras* DNA does not stimulate the entry of senescent cells into S phase. The c-H-*ras* DNA, when microinjected together with DNA coding for the E1A gene of adenovirus, is also unable to stimulate DNA synthesis in senescent cells. This combination of oncogene DNA's permits primary rodent cells to proceed to full transformation and tumorigenicity (*16*). These results support the observations of Sager *et al.* (*17*) that oncogene DNA, transfected into normal human cells, failed to endow them with tumorigenic potential. Sager (*18*) has proposed the existence of anti-oncogenes in normal cells that suppress the action of the oncogene protein products. We consider the senescent cell inhibitor gene a prime candidate for an anti-oncogene.

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19. It was possible that the high abundance inhibitor poly(A)$^+$ RNA found in senescent cells was simply a result of the presence of cells that are nonproliferating in high levels of serum growth factors (10 percent FBS). We tested this possibility by microinjecting poly(A)$^+$ RNA obtained from young cells that had been maintained in density-inhibited confluent culture (in medium containing 10 percent FBS) for 2 weeks after confluence was reached. When microinjected at a concentration of 1 mg/ml, about 20 percent inhibition was observed. Dilution to 0.1 mg/ml resulted in essentially no inhibition. Therefore, it seems that the high abundance of inhibitor RNA found in senescent cells is a property of in vitro cellular senescence rather than a non–age-related effect of serum concentration.

20. This work was supported by NIH grants AG-04749, AG-05333, and T32-CA-09197.

3 September 1985; accepted 21 February 1986

Androgens Regulate the Dendritic Length of Mammalian Motoneurons in Adulthood

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Sex steroid hormones have been thought to alter behaviors in adulthood by changing the activity of neural circuits rather than by inducing major structural changes in these pathways. In a group of androgen-sensitive motoneurons that mediate male copulatory functions, decreases in androgen levels after castration of adult rats produced dramatic structural changes, decreasing both the dendritic length and soma size of these motoneurons. These changes were reversed by androgen replacement. These results imply a surprising degree of synaptic plasticity in adult motoneurons and suggest that normal changes in androgen levels in adulthood are associated with significant alterations in the structure and function of these neurons.

DURING DEVELOPMENT, STEROID hormones produce permanent sexual dimorphisms in neuron number, morphology, and connectivity; in adulthood these neurons respond transiently to steroid hormones to produce sex-specific behaviors (*1*). In contrast to these dramatic developmental effects, steroid influences on neurons in adulthood are thought to be modest and impermanent (*2*). Until recently the actions of steroids in the adult were not thought to involve major structural changes, such as synaptic reorganization of steroid-sensitive neural circuits. However, such major alterations have been demonstrated in the avian brain; treatment of adult female canaries with androgens induces song behavior and stimulates dendritic growth and the formation of new synapses in at least one sexually dimorphic brain area involved in song (*3*). We now report that in a sexually dimorphic nucleus in the mammalian spinal cord, the dendritic length of motoneurons is dramatically affected by variations in androgen levels during adulthood. The reversibility of this androgenic effect on dendritic length indicates a remarkable androgen-regulated structural plasticity. The neurons involved are important for copulatory behaviors (*4*), which are sensitive to alterations in androgen levels (*5*). Our results therefore suggest that the reversible effects of androgen on sexual behavior in adulthood are mediated in part by alterations in dendritic morphology and, consequently, in the synaptic organization of specific neural pathways.

The spinal nucleus of the bulbocavernosus (SNB) is a cluster of motoneurons located in the fifth and sixth lumbar segments of the rat spinal cord, and consists of approximately 200 motoneurons in adult males but only 60 in adult females. In males, the majority of SNB motoneurons innervate the muscles bulbocavernosus (BC) and levator ani (LA) (*6*), which attach exclusively to the penis. In contrast, adult females lack the BC and LA musculature (*7*); their SNB motoneurons innervate the apparently nondimorphic anal sphincter, as do the remaining SNB motoneurons in males (*8*). Female SNB motoneurons are approximately 50 percent smaller than those of males (*9*). These sex differences in SNB motoneuron number, size, and projections, and differences in the presence of the SNB target musculature, are determined by the action of androgens during a critical perinatal period (*7, 10*). In adulthood, both the SNB motoneurons and their perineal target muscles accumulate androgens (*11*) and continue to be sensitive to androgenic effects. For example, after castration both SNB soma size and peripheral muscle mass are significantly reduced, but can be maintained at normal values by treatment with testosterone propionate (*9, 12*).

In our study we examined the length and distribution of the dendritic arbor of SNB motoneurons to determine if these aspects of dendritic morphology were also sensitive to androgen levels. Twenty-five adult male rats (Sprague-Dawley, Simonsen) were assigned to one of the following six groups: normal males 60 days old ($n = 4$); males that were either castrated ($n = 5$) or sham-

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